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Bacterial and fungal communities in the rhizosphere of field-grown genetically modified pine trees (*Pinus radiata* D.)

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This study assessed the impact of *Pinus radiata* (D. Don) genetically modified (GM) by biolistic insertion of the *LEAFY* and *npt*II genes on rhizosphere microbial communities of field grown trees. Rhizosphere soil was sampled quarterly for two consecutive years. A culture-independent approach was used to characterise the microbial communities based on PCR and denaturing gradient gel electrophoresis (DGGE) of 16S/18S rDNA gene fragments, and internal transcribed spacer (ITS) fragments amplified from total rhizosphere DNA. Trees from two independent transformation events were sampled, together with non-modified control trees of the same parental genotype. DGGE profiles of rhizosphere general *Bacteria* did not differ between GM and control trees with one exception (summer 2006 sample). For *Alphaproteo*- and *Actinobacteria*, significant differences between treatments were detected in one out of eight samplings. Small seasonal shifts could be detected in all bacterial communities. General fungal and ectomycorrhizal communities did not differ significantly between GM and control trees with the exception of summer 2006, when ectomycorrhizal communities associated with GM trees from one transformation event differed from those associated with control trees. Small seasonal shifts of general fungal and ectomycorrhizal communities were seen over the two-year sampling period. More detailed analysis of microbial communities at one sampling date (using amplified rDNA restriction analysis (ARDRA) and 16S/18S rDNA sequencing) revealed significant differences in four ARDRA groups between one GM treatment and the control (bacteria), and significant differences in one ARDRA group between the two GM treatments (fungi). When data from all sampling dates are considered together, the low incidence of statistical differences in the microbial communities associated with the genetically modified and control trees suggests that there was no significant impact of this genetic modification on rhizosphere microbial communities.

Keywords: Bacterial and fungal communities / ectomycorrhizal fungi / DGGE / ARDRA / diversity / genetic engineering (GE) / transgenic trees / biosafety

INTRODUCTION

Advances in technology for genetic transformation have accelerated the development of genetically modified (GM) trees in the last 15 years. More than 30 species of transformed forest trees have been successfully regenerated, amongst them several commercially important conifers, poplars and eucalypts (Henderson and Walter, 2006; van Frankenhuyzen and Beardmore, 2004). Major target traits include herbicide tolerance, pest resistance, abiotic stress tolerance, modified fibre quality and quantity, and altered growth and reproductive development (Bishop-Hurley et al., 2001; Grace et al., 2005; Wagner et al., 2007). Global commercialisation of GM agricultural crops has intensified the public debate around risks and benefits of genetic engineering. Although GM trees do not evoke immediate health concerns, the prospect of their commercialisation is raising concerns regarding potential ecological impacts (Valenzuela et al., 2006; Walter, 2004). The debate around risks of GM trees focuses on several issues, including the possibility that the transgene could spread *via* vertical or horizontal gene transfer. More economically motivated concerns include the potential instability of gene expression and possible negative effects on tree fitness which could lead to reduced resilience against pathogens or other environmental challenges. As with transgenic agricultural crops, there is also concern that GM trees may impact on soil ecosystem biota and functions, in particular because trees will grow for many years in one location, unlike short-lived annual crops.

Methodological advances in microbial ecology, especially molecular techniques, have facilitated detailed research on the impact of a range of GM plants on soil microbial community structure and function. Effects

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Table 1. Hotelling T^2 probabilities of sampling times with significant differences in DGGE pattern. Treatments A, B = genetically modified, treatment C = controls.

DGGE pattern	Season (sampling time)	Treatment	A	B
Bacteria	Summer 2006 (8)	B	**0.0380**	-
		C	**0.0009**	**0.0455**
Alphaproteobacteria	Winter 2005 (2)	B	0.5115	-
		C	0.0964	**0.0170**
Actinobacteria	Autumn 2005 (1)	B	**0.0228**	-
		C	0.3838	0.3118
Ectomycorrhiza	Summer 2006 (8)	B	0.1128	-
		C	**0.0457**	0.9572

detected to date have been minor when compared with common sources of variation such as agricultural practices, season, plant developmental stage, soil type or plant genotype (Batista et al., 2008; Dunfield and Germida, 2001, 2004; Gyamfi et al., 2002; Heuer et al., 2002; Lottmann et al., 1999; Lukow et al., 2000). Root-associated bacteria and fungi form close associations with tree roots and are the primary consumers of exudates and other plant-derived materials, so are the most likely group of non-target soil organisms to respond to any changes in plant activity and metabolism that result from genetic modification processes. Different tree species have been shown to harbour different microbial communities (Priha et al., 2001) but to date there has been only very limited research carried out of the impact of transgenic trees on microbial communities, despite more than 700 GM tree field trials that we have been able to find in publicly available databases.

The enormous biological complexity in soils demands that impact studies on soil ecosystems focus on specific and relevant microbial groups. Ectomycorrhizal (EM) fungi play a fundamental role in forest ecosystem processes, by influencing the uptake of minerals and carbon cycling, and alleviating stress to their associated hosts. For many trees it is clear that normal growth and survival is dependent on colonisation by EM fungi (Smith and Read, 1997). Kaldorf et al. (2002) conducted the first field study of mycorrhization of *rol*C-transgenic (modifies phytohormone balance) aspen trees in a four-year trial. Mycorrhizal colonisation, diversity and community structure did not differ between GM and control trees.

Monterey or radiata pine (*Pinus radiata* D. Don) is well known as a conifer species deployed in forest plantations, predominantly in New Zealand, Australia, Chile and Spain. The introduced *LEAFY* (*LFY*) gene from *Arabidopsis thaliana* is involved in the control of floral development. This was the first time that this gene had been introduced to conifers and given that *LEAFY* originates from an angiosperm and was introduced into a gymnosperm, it was not known whether the gene would be expressed, and for how long. The objective of this study was to characterise the structure of the bacterial, fungal and ectomycorrhizal communities associated with the roots of GM and unmodified field grown *P. radiata* over several seasons and to provide baseline data for future biosafety evaluations of a range of GM radiata pine currently under development in New Zealand. General bacterial, fungal and taxon-specific primers were used to PCR-amplify 16S/18S rRNA gene fragments and ITS fragments from community DNA followed by denaturing gradient gel electrophoresis (DGGE). To investigate the diversity of rhizosphere communities, clone libraries of the partial 16S rDNA (bacteria) and the partial 18S rDNA (fungi) were generated from whole community DNA. Clones were characterized by Amplified rDNA Restriction Analysis (ARDRA) and sequencing.

RESULTS

Rhizosphere bacterial community structure

DNA was successfully extracted from all 120 rhizosphere soil samples. Prior to bacterial community fingerprinting, the 16S rRNA gene fragments were amplified by PCR from whole community DNA with primers specific for general *Bacteria*, *Alpha-* and *Betaproteobacteria*, and *Actinobacteria*. DGGE profiles of general bacterial communities showed complex fingerprints composed of large numbers of bands with trees sharing many common bands. Consistent bands specific to GM or control trees were not observed but over the whole sampling period of two years, all trees showed minor temporal variations in their DGGE patterns. The principle coordinate analysis (PCO) of DGGE banding patterns for general *Bacteria* indicated that only at the last of eight sampling times (summer 2006) were significant differences found between GM and non-modified trees (Tab. 1, Fig. 1). Note that, as there were so few sampling dates in the study when significant differences between microbial communities were observed, only Hotelling T^2 probabilities, where there was a significant difference, are presented

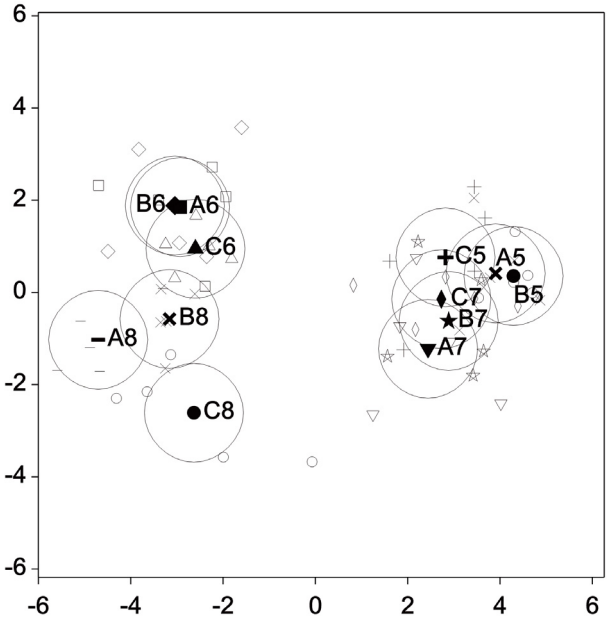

Figure 1. Principle coordinate analysis of bacterial DGGE profiles in 2006. The fingerprints were generated by separation of 16S rDNA fragments amplified with primers F968/R1378. A = GM treatment A, B = GM treatment B, C = control. Arabic numerals refer to sampling times: 5 = autumn 2006, 6 = winter 2006, 7 = spring 2006, 8 = summer 2006. Significance of difference between treatments should be read from Table 1.

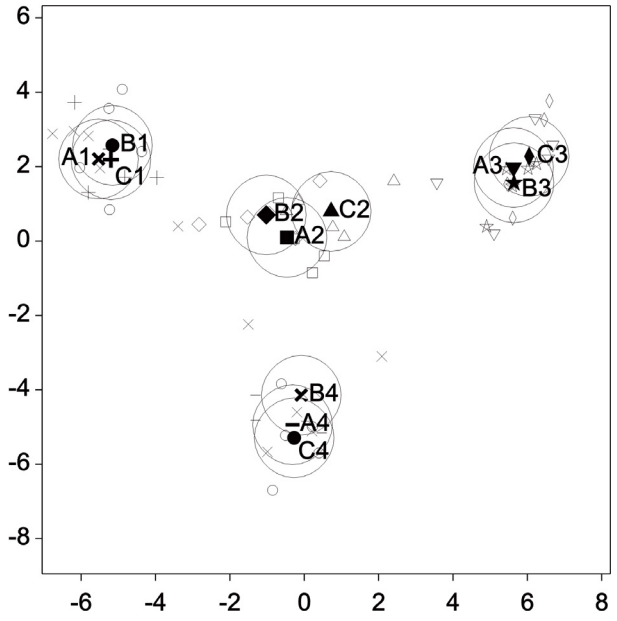

Figure 2. Principle coordinate analysis of alphaproteobacterial DGGE profiles in 2005. A, B = GM treatments, C = control. Arabic numerals refer to sampling times: 1 = autumn 2005, 2 = winter 2005, 3 = spring 2005, 4 = summer 2005. Significance of difference between treatments should be read from Table 1.

in Table 1. Significant seasonal shifts in bacterial communities were found over the two-year sampling period ($P < 0.05$) and were clearly visible for 2006 in Figure 1.

To reduce the complexity of bacterial fingerprints and to allow the analysis of minority populations, primers specific for three taxonomic groups were applied. The fingerprints of *Alphaproteobacteria* were similar for all trees but showed significant differences between the GM treatment B and the non-GM controls at one of the eight sampling times (winter 2005, Fig. 2, Tab. 1). DGGE profiles of *Betaproteobacteria* showed no differences between the three treatments but significant seasonal differences in both years ($P < 0.05$). Actinobacterial communities showed significant differences between the two GM treatments A and B at the first sampling time (autumn 2005) but at all other sampling times there were no differences between the three treatments (Fig. 3, Tab. 1). Significant seasonal effects were detected in both years ($P < 0.05$). Figure 4 shows the DGGE patterns of *Alphaproteobacteria*, *Betaproteobacteria* and *Actinobacteria* in 2006 and demonstrates the high similarity of bacterial communities between GM and control trees and minor seasonal changes.

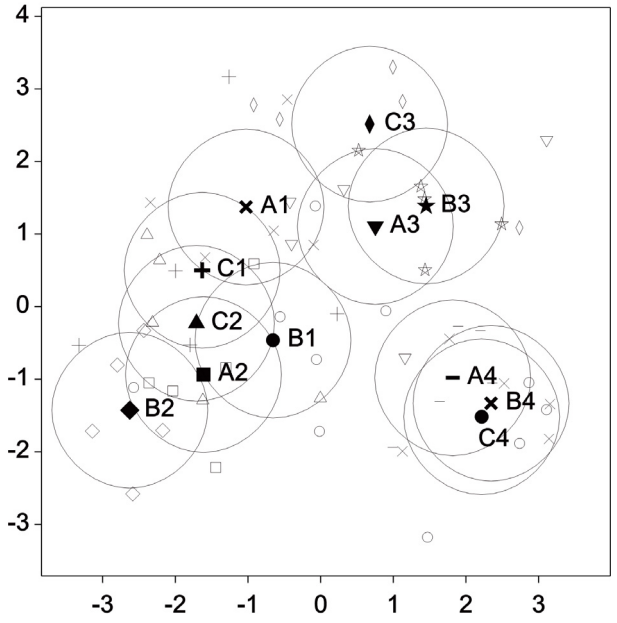

Figure 3. Principle coordinate analysis of actinobacterial DGGE profiles in 2005. A, B = GM treatments, C = control. Arabic numerals refer to sampling times: 1 = autumn 2005, 2 = winter 2005, 3 = spring 2005, 4 = summer 2005. Significance of difference between treatments should be read from Table 1.

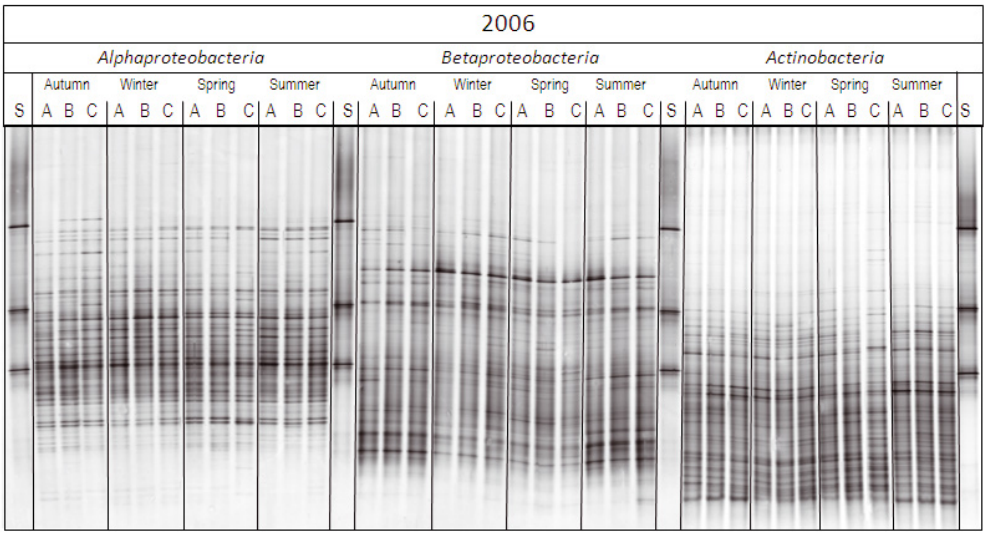

Figure 4. DGGE profiles representing the bacterial communities (*Alpha-*, *Betaproteobacteria*, *Actinobacteria*) of pooled samples of transgenic trees in 2006. A = 5× GM treatment A, B = 5× GM treatment B, and C = 5× control treatment. The fingerprints of bacterial communities were generated by separation of 16S rDNA fragments amplified with taxon-specific primers (Tab. 2) followed by a second PCR using primers F968-GC and R1378. The following bacterial isolates, from top to bottom, were used as standards (lanes S): *Pectobacterium carotovorum*, *Variovorax paradoxus*, and *Arthrobacter* sp.

Rhizosphere fungal community structure

The complexity of general fungal communities varied between individual trees, although some bands were present in all treatments and some were detected at certain time points in particular treatments. None of the differences were consistent throughout the two-year sampling period or greater than the variability between the seasons. Discriminant analysis showed significant seasonal shifts ($P < 0.05$) but the fungal communities did not differ significantly between GM and control trees (data not shown).

Specific ITS sequences, representing ectomycorrhizal fungi, were amplified by application of the primers ITS1F and ITS4B-GC (Tab. 2). The DGGE profiles revealed 2 to 7 bands per tree with some variation between individual trees. Only at the last sampling time (summer 2006) did the discriminant analysis reveal significant differences between the GM treatment A and non-transgenic controls (Fig. 5, Tab. 1) Significant seasonal shifts were detected in both years but were more pronounced in 2006. Characteristic bands were excised from DGGE gels and after re-amplification the ITS fragments were cloned and sequenced. The sequences of all bands were associated with ectomycorrhizal fungi such as *Polyporus tuberaster* (99% similarity), an uncultured ectomycorrhiza *Cantharellales* (98%), an uncultured *Tremellales* (98%), an uncultured *Clavulina* (97%) and an uncultured *Suillacea* (99%).

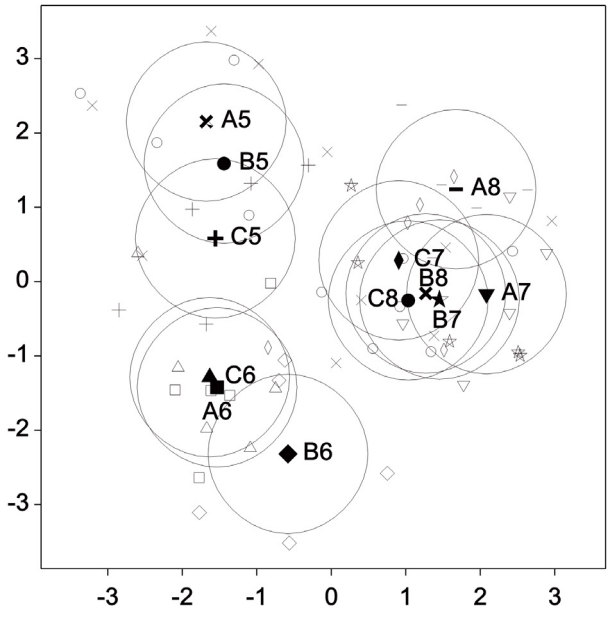

Figure 5. Principle coordinate analysis of ectomycorrhizal DGGE profiles in 2006. The fingerprints were generated by separation of the ITS region amplified with primers ITS1F/ITS4B-GC. A, B = GM treatments, C = control. Arabic numerals refer to sampling times: 5 = autumn 2006, 6 = winter 2006, 7 = spring 2006, 8 = summer 2006. Significance of difference between treatments should be read from Table 1.

Table 2. Primers used in this study targeting the 16S, 18S rRNA genes and ITS genes.

Primer	Sequence 5'-3'	Target region/ Specificity	Reference
F27	AGAGTTTGATCCTGGCTCAG	16S rRNA gene, *Bacteria*	Lane (1991)
F968	AACGCGAAGAACCTTAC	16S rRNA gene, *Bacteria*	Nübel et al. (1996)
F968-GC	**gc-**AACGCGAAGAACCTTAC	16S rRNA gene, *Bacteria*	Nübel et al. (1996)
F203α	CCGCATACGCCCTACGGG GGAAAGATTTAT	16S rRNA gene, *Alphaproteobacteria*	Gomes et al. (2001)
F948β	CGCACAAGCGGTGGATGA	16S rRNA gene, *Betaproteobacteria*	Gomes et al. (2001)
F243HGC	GGATGAGCCCGCGGCCTA	16S rRNA gene, *Actinobacteria*	Heuer et al. (1997)
R1378	CGGTGTGTACAAGGCCC GGGAACG	16S rRNA gene, *Bacteria*	Heuer et al. (1997)
R1494	CTACGGRTACCTTGTTACGAC	16S rRNA gene, *Bacteria*	Weisburg et al. (1991)
NS1	GTAGTCATATGCTTGTCTC	18S rRNA gene, Fungi	White et al. (1990)
FR1	AICCATTCAATCGGTAIT	18S rRNA gene, Fungi	Vainio and Hantula (2000)
FR1-GC	**gc-**AICCATTCAATCGGTAIT	18S rRNA gene, Fungi	Vainio and Hantula (2000)
ITS1-F	CTTGGTCATTTAGAGGAAGTAA	ITS region, Fungi	Gardes and Bruns (1993)
ITS4B	CAGGAGACTTGTACACGGTCCAG	ITS region, *Basidiomycetes*	Gardes and Bruns (1993)
ITS4B-GC	**gc-**CAGGAGACTTGTACACGGTCCAG	ITS region, *Basidiomycetes*	Landeweert et al. (2005)
GC-clamp (**gc**)	CGCCCGGGGCGCGCCCCGGGCG GGGCGGGGGCACGGGGGG	-	Nübel et al. (1996)

These genera all belong to the phylum *Basidiomycota* and are all well known as ectomycorrhizal fungi of conifers.

Diversity of bacterial and fungal communities

Nearly full-length bacterial 16S rDNA genes were amplified with primers F27/R1494 from community DNA from the summer sampling 2005 and used to generate a random clone library. For each of the three treatments more than 100 clones were randomly selected and checked for the correct insert. Prior to sequencing, ARDRA was used to characterise all clones. The restriction enzyme *Hinf*I was used to carry out ARDRA on 320 cloned 16S rRNA gene fragments. Data analysis using GelComparII software (Applied Maths, Belgium) revealed 130 *Hinf*I patterns. Each different restriction pattern was defined as an operational taxonomic unit (OTU). Seventy-six OTUs were recovered only once. The GM treatment B yielded 29, the control treatment 27, and the GM treatment A 20 unique OTUs. The statistical analysis of OTUs revealed significant differences only between the controls and GM treatment A for OTU2, OTU3, OTU4 and OTU8 with *P*-values of 0.0315, 0.0207, 0.0345 and 0.0341, respectively.

Based on the OTUs, the diversity indices according to Shannon and Weaver (1949) were calculated. The overall diversity was 4.44. The highest diversity for bacterial clones was found for the GM treatment B (4.05) whereas the diversity indices for controls and GM treatment A were 3.94 and 3.83, respectively. Inserts from each OTU with at least three clones were selected for sequence analysis. A total of 92 clones were sequenced. The results of the sequence analysis are shown in Table 3. The most frequently represented groups detected in all treatments were members of the *Proteobacteria*. *Beta-* and *Alphaproteobacteria* had the greatest representation followed by *Bacilli*. Nineteen clones grouped into OTU1 and were affiliated with the genus *Burkholderia*. Figure 6 shows the proportional occurrence of the most frequently

Table 3. BLAST search results of nucleotide-nucleotide comparison in GenBank and most frequent OTUs associated with the three treatments: non-modified controls (C), and two GM treatments (A and B).

Most frequent OTUs[1]	Number of occurrence among treatments				GenBank BLAST analysis			Accession no. of clones[2]	
	Total	C	A	B	Species[Class]	Accession no.	Identity [%]	Clone	Accession no.
OTU1	19	9	2	8	*Burkholderia phenazinium*[3]	AM502992	99	CB73	FM206295
					Burkholderia phytofirmans[3]	CP001053	99	2B25	FM206296
					Burkholderia sedimenticola[3]	EU035613	98	2B91	FM206297
OTU2	18	6	10	2	Uncultured bacterium	EF516003	94	1B83	FM206298
					Uncultured bacterium	EU335246	98	CB52	FM206299
					Alicyclobacillus sp.[4]	AB060164	96	1B29	FM206300
OTU3	10	1	5	4	*Bradyrhizobium japonicum*[5]	EU481826	97	2B22	FM206301
					Bacterium Ellin 332[5]	AF498714	92	1B82	FM206302
					Devosia sp.[5]	EF540511	91	1B112	FM206303
OTU4	10	2	5	3	*Collimonas fungivorans*[3]	AY593480	98	CB27	FM206304
					Zoogloea ramigera[3]	X74914	92	1B10	FM206305
OTU5	10	4	2	4	*Burkholderia* sp.[3]	AF247494	99	2B57	FM206306
					Burkholderia phenazinium[3]	AY154372	99	CB30	FM206307
OTU6	8	2	3	3	*Mycobacterium colombiense*[6]	AM062764	99	2B60	FM206308
					Mycobacterium riyadhense[6]	EU274642	99	CB36	FM206309
OTU7	7	4	1	2	*Mucilaginibacter paludis*[7]	AM490402	93	CB76	FM206310
OTU8	7	1	4	2	*Labrys monachus*[5]	AJ535707	99	2B26	FM206311
OTU9	7	4	3	0	Uncultured bacterium WD229	AJ292593	98	1B77	FM206312
OTU10	6	1	2	3	*Sphingoterrabacterium koreensis*[7]	AB267721	91	1B103	FM206313
OTU11	5	1	2	2	*Acidocella* sp.[5]	AF376021	96	2B18	FM206314
OTU12	5	2	2	1	*Burkholderia sedimenticola*[3]	EU035613	99	1B6	FM206315
OTU13	5	1	2	2	*Frankiaceae* bacterium[6]	DQ490442	97	1B12	FM206316
OTU14	5	1	3	1	*Methylobacterium* sp.[5]	AM910535	100	1B113	FM206317
OTU15	4	2	1	1	*Sphingomonas* sp. K101[5]	AJ009706	98	CB101	FM206318
OTU16	4	1	2	1	*Sphingomonas* sp. EC-K085[5]	AB264174	92	1B97	FM206319
OTU17	4	2	2	0	*Serratia proteamaculans*[8]	CP000826	99	CB113	FM206320
OTU18	4	2	0	2	*Methylocystis* sp.[5]	DQ852350	92	CB29	FM206321
OTU19	3	1	0	2	Uncultured bacterium	EF019697	94	2B45	FM206322
OTU20	3	1	1	1	*Paenibacillus ehimensis*[4]	EF025575	96	CB54	FM206323
OTU21	3	1	1	1	*Burkholderia* sp.[3]	EU184087	96	CB17	FM206324
OTU22	3	2	0	1	Uncultured bacterium	EU133311	96	CB10	FM206325
OTU23	3	1	0	2	*Burkholderia* sp.[3]	AB212228	99	2B109	FM206326
OTU24	3	0	2	1	*Alicyclobacillus herbarius*[4]	AB042055	99	1B45	FM206327
OTU25	3	3	0	0	*Sphingoterrabacterium pocheensis*[7]	AB267718	97	CB119	FM206328
OTU26	3	1	1	1	*Bradyrhizobium* sp.[5]	AF216780	99	2B23	FM206329
OTU27	3	0	1	2	Uncult. *Acidobacteria* bacterium[9]	EU359912	98	2B6	FM206330
OTU28	3	1	0	2	*Sphingomonas oligophenolica*[5]	AB018439	99	2B55	FM206331

[1] Operational taxonomic units. Defines different amplified rDNA restriction analysis (ARDRA) patterns.
[2] Partial sequences of the 16S rDNA gene fragment.
[3] *Betaproteobacteria.*
[4] *Bacilli.*
[5] *Alphaproteobacteria.*
[6] *Actinobacteria.*
[7] *Sphingobacteria.*
[8] *Gammaproteobacteria.*
[9] *Acidobacteria.*

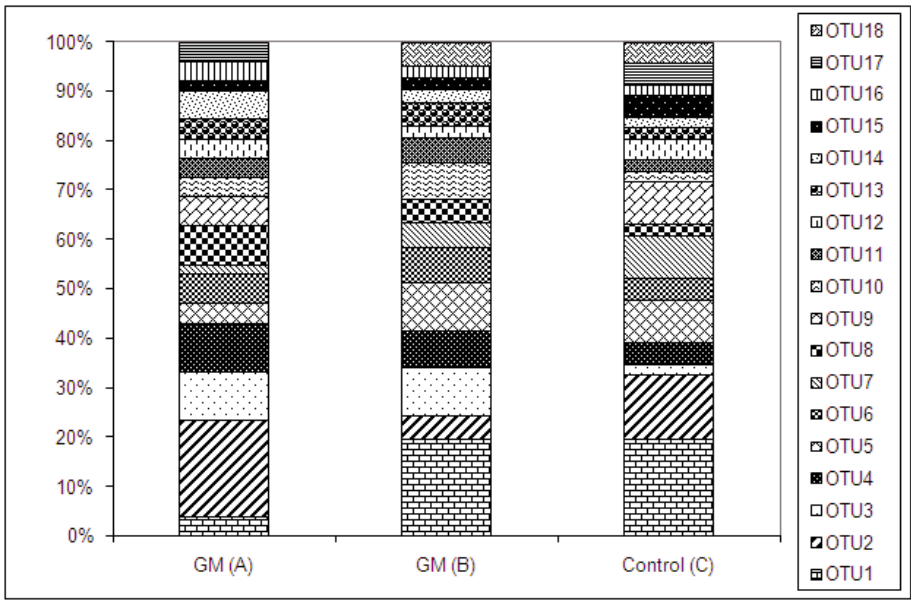

Figure 6. Relative distribution of the most frequent ARDRA pattern of cloned bacterial 16S rDNA fragments derived from the rhizosphere of transgenic (GM treatment A, GM treatment B) and control pine trees (C) from the summer sampling 2005. OTU = operational taxonomic unit.

found OTUs and their distribution between the GM and control treatments.

PCR products obtained with primers NS1/FR1 (fungi) and community DNA from the summer sampling 2005 were cloned and sequenced as described above. Data analysis revealed 92 *Hinf*I patterns (OTUs). Seventy-three OTUs were recovered only once. The highest occurrence of these unique OTUs was found for the two GM treatments. The control treatment revealed 12 unique OTUs whereas the two GM treatments revealed 33 (A) and 28 (B) unique OTUs.

The statistical analysis of OTUs revealed significant differences between the two GM treatments A and B for OTU6 ($P = 0.0211$). Based on the OTUs, the diversity indices according to Shannon and Weaver (1949) were calculated. The overall diversity was 3.51. The highest diversity for fungal clones was found for GM treatment A (3.45) whereas the diversity indices for control and GM treatment B were 2.75 and 3.14, respectively. Inserts from each OTU with at least three clones were selected for sequence analysis. A total of 88 clones were sequenced. Most of the 18S rDNA gene sequences shared $\geqslant$ 98% sequence similarity with GenBank entries. Phylogenetic analysis of the different OTUs and their closest relatives in the GenBank database was used to assign clones to a species or phylogenetic group. The results of the sequence analysis of selected clones, their phylogenetic affiliation, and their occurrence among GM and control trees are shown in Table 4. The highest proportion of fungal clones was classified as *Basidiomycota* (39%). *Ascomycota* represented 23% of clones in the library, *Mucormycotina* 5%, whereas the *Zygomycota* had low representation (1%). Numerous OTUs were recovered only once (32% of all clones). Forty-two clones grouped into OTU1 and were identified as *Bensingtonia yamatoana.* The second largest group (OTU2) was identified as *Resinicium bicolor*. Figure 7 shows the proportional occurrence of the most frequently found OTUs and their distribution between the GM and control treatments.

DISCUSSION

Understanding of the natural variability and fluctuations in microbial communities associated with trees is a necessary prerequisite to assessment of impacts of GM trees on their associated micro flora. As seen with annual plants, the structure of rhizosphere microbial communities of trees has been shown to vary significantly depending on tree species (Priha et al., 2001), tree health (Filion et al., 2004), or environmental conditions (Graff and Conrad, 2005). The present study investigated the impact of field-grown GM and control *P. radiata* trees on the structure and diversity of rhizosphere microbial communities over a period of two years. To our knowledge there have been no studies in which the microbial communities of trees were monitored over such a long period. Most studies used only a single sampling date

Table 4. BLAST search results of nucleotide-nucleotide comparison in GenBank and most frequent OTUs associated with the three treatments: non-modified controls (C), and two GM treatments (A and B).

Most frequent OTUs[1]	Number of occurrence among treatments				GenBank BLAST analysis			Accession no. of clones[2]	
	Total	C	A	B	Species[Phylogenetic group]	Accession no.	Identity [%]	Clone	Accession no.
OTU1	42	18	13	11	*Bensingtonia yamatoana*[B]	AF101826	98	2F67	FM200010
					Uncultured *Boletaceae*[B]	EF024790	99	CF77	FM200020
OTU2	38	11	9	18	*Resinicium bicolor*[B]	DQ834914	98	1F53	FM200025
OTU3	23	7	9	7	*Chloroscypha enterochroma*[A]	AY544700	98	CF28	FM200021
					Chalara fusidioides[A]	AF203463	99	1F27	FM200009
OTU4	19	9	3	7	*Lophium mytilinum*[A]	DQ678030	99	2F11	FM200012
OTU5	13	5	3	5	*Trechispora alnicola*[B]	AY657012	97	2F34	FM200013
					Phialiocephala fortinii[A]	EU434871	99	2F38	FM200014
OTU6	11	3	7	1	*Mortierella parvispora*[M]	AY129549	99	1F42	FM200026
OTU7	10	7	3	0	*Marasmius alliaceus*[B]	AY787214	98	1F30	FM200027
OTU8	9	6	1	2	*Suillus granulatus*[B]	DQ534691	99	2F61	FM200015
					Suillus variegatus[B]	DQ534693	98	1F94	FM200028
OTU9	9	4	4	1	*Penicillium italicum*[A]	AF548091	100	1F86	FM200029
					Penicillium expansum[A]	EU590663	99	2F119	FM200016
OTU10	8	3	4	1	Uncultured *Boletaceae*[B]	EF024981	98	1F82	FM200030
OTU11	4	0	2	2	*Satchmopsis brasiliensis*[A]	DQ195809	99	2F17	FM200017
OTU12	4	1	0	3	*Umbelopsis nana*[M]	AF157167	99	2F68	FM200018
OTU13	3	3	0	0	*Zygomycete* sp.[Z]	EU428773	99	CF47	FM200022
OTU14	3	0	2	1	*Elaphocordyceps subsessilis*[A]	EF469124	98	2F12	FM200019
					Oidiodendron tenuissimum[A]	AB015787	97	1F99	FM200031
OTU15	3	1	1	1	*Heteroconium chaetospira*[A]	DQ521604	99	CF2	FM200023
					Ascomycete sp.[A]	EF638692	99	1F62	FM200032
OTU16	2	0	1	1	Uncultured *Boletaceae*[B]	EF024619	98	1F60	FM200033

[1] Operational taxonomic unit. Defines different amplified rDNA restriction analysis (ARDRA) patterns.
[2] Partial sequences of the 18S rDNA gene fragment.
[A] *Ascomycota.*
[B] *Basidiomycota.*
[M] *Mucoromycotina.*
[Z] *Zygomycota.*

(Andreote et al., 2009; Izumi et al., 2008; Lamarche and Hamelin, 2007; LeBlanc et al., 2007). The results of the present study, in which significant seasonal shifts in various components of the microbial communities occurred, suggests that caution is needed when drawing conclusions about root-associated microbial communities based on results for a single time point.

Seasonal shifts in microbial communities were observed in this study but most dominant populations remained stable during the monitoring period. Smalla et al. (2001) found seasonal shifts in the abundance and composition of the bacterial rhizosphere populations in field-grown strawberry, oilseed rape and potato. Lottmann et al. (2000) also observed the appearance of additional dominant DGGE bands in the rhizosphere of field-grown potatoes at the time of flowering. Bacteria are considered as useful bio-indicators in environmental impact studies because they interact continuously with plants and most react quickly to changes in their environment. The DGGE profiles of 16S rRNA gene fragments obtained with *Bacteria*-specific primers generate a profile of the most dominant populations (Muyzer et al., 1993; Muyzer and Smalla, 1998), while the nested PCR approach of using taxon-specific primers allows the analysis of minority populations (Heuer et al., 1997, 2001, 2002). The sensitivity of taxon-specific primers has been clearly demonstrated in prior studies of bacterial communities associated with GM and non-GM crop plants (Andreote et al., 2008; O'Callaghan et al., 2008). The statistically significant differences between GM and non-GM treatments detected in this study were always related to only one sampling time (Tab. 1). This finding shows that multiple sampling over a longer period is essential to draw conclusions on the biological relevance of changes in bacterial communities. LeBlanc et al. (2007) found statistically significant differences between the bacterial communities inhabiting the rhizosphere of transgenic white spruce and control trees. Lamarche and Hamelin

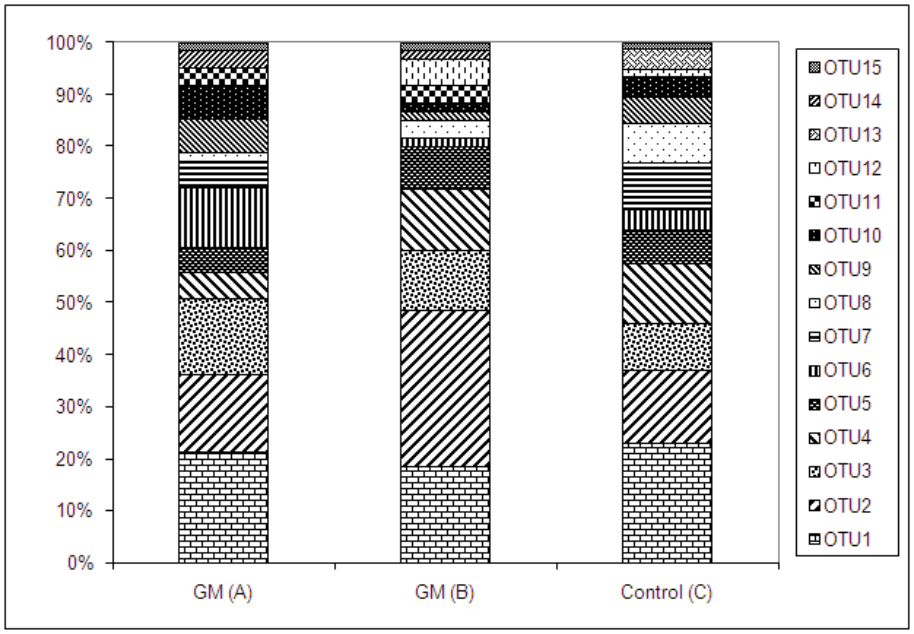

Figure 7. Relative distribution of the most frequent ARDRA pattern of cloned fungal 18S rDNA fragments derived from the rhizosphere of transgenic (GM treatment A, GM treatment B) and control pine trees (C) from the summer sampling 2005. OTU = operational taxonomic unit.

(2007) found no impact on soil nitrogen-fixing bacteria of GM white spruce (*Picea glauca*) but diazotroph diversity differed between white spruce trees from the plantation site and those from two natural stands near the plantation. Andreote et al. (2009) detected no differences in diversity of *Alphaproteobacteria* in the rhizosphere of GM and eucalyptus and control trees.

A clone library was generated using rhizosphere DNA collected in summer 2005, to examine rhizosphere bacterial diversity more closely. A summer sample was used because microbial growth and metabolic activity often increase in the spring and summer in conjunction with higher soil temperatures, mobilization of accumulated soil organic matter, and accelerated root growth (Grayston et al., 2001). The bacterial sequences amplified from the rhizosphere of *P. radiata* revealed a very high proportion of populations belonging to the *Alpha-* and *Betaproteobacteria. Alphaproteobacteria* is a diverse class of organisms within the phylum *Proteobacteria*. Environmentally important characteristics such as nitrogen fixation or ammonia oxidation are present in bacteria belonging to this order. *Methylobacterium* sp., which is ubiquitous in the environment and commonly found in association with plants, was found in association with all three treatments of radiata pine. These results are in accordance with other recent studies of bacterial rhizosphere diversity. Chow et al. (2002) reported that *Alpha-* and *Betaproteobacteria* and *Acidobacteria* dominated the rhizosphere of *Pinus contorta* grown under forest conditions. The main bacterial groups identified in a study conducted on Black spruce (*Pinus mariana*) seedlings by Filion et al. (2004) were also *Proteobacteria*, *Acidobacteria* and *Actinobacteria*. In our study *Burkholderia* spp. was the most frequently detected genus (OTU1, Tab. 3). It was found in all three treatments but showed significant differences between the control treatment C and GM treatment A. It is unlikely that this finding is of any biological significance as none of the detected shifts or changes found in this study were persistent. *Burkholderia* species are common residents of rhizosphere soil and beneficial attributes of some members include nitrogen fixation, plant growth promotion and biological disease control (Opelt et al., 2007; Parke and Gurian-Sherman, 2001). Timonen and Hurek (2006) reported that almost all of the bacteria isolated from the mycorrhizosphere of *Pinus sylvestris* were identified as *Burkholderia* spp. Bacteria associated with ectomycorrhiza of Slash pine (*Pinus elliotii*) were mainly affiliated to species of the genus *Burkholderia* (Izumi et al., 2008). Kataoka et al. (2008) analysed bacterial communities on *Pinus thunbergii* root tips and found *Burkholderia* spp. and *Bradyrhizobium* spp. were the dominating bacteria. Fluorescent pseudomonads are commonly found in association with plant roots, hence it was surprising that none

of the clones analysed in our study showed closer similarity to *Pseudomonas* spp.

To our knowledge this is the first study on the composition and dynamics of both bacterial and fungal (including ectomycorrhizal) communities of GM trees. Like the bacteria, general fungal communities also showed seasonal shifts but communities did not differ significantly between GM and control trees. Fungi are very important members of the microbial communities associated with plant roots and in the present study members of the *Basidiomycota* and *Ascomycota* were the most frequently identified fungi of *P. radiata*. The majority of identified OTUs were found in association with all three treatments (Tab. 3). Oliver et al. (2008) found members of the *Ascomycota* as the dominant group of fungi in the rhizosphere of transgenic polyphenol oxidase-overexpressing and non-GM control aspen but the abundance of the most dominant OTUs was the same in both the transgenic and control clone libraries. In our study the most frequently found OTU was affiliated with the basidiomycetous red yeast *Bensingtonia yamatoana,* formerly known as *Sporobolomyces yamatoanus.* This species is extensively distributed throughout the environment. A second phylogentic group associated with OTU1 was affiliated with an uncultured *Boletaceae,* a family of mushrooms found on every continent except Antarctica. The white-rot basidiomycete *Resinicium bicolor* (OTU2) is commonly associated with *Pinaceae* and was found in all three treatments but showed significant differences between the two GM treatments A and B. Our study suggests that such variations are most likely to be temporary. OTU7 was affiliated with the species *Marasmius alliaceus*, a basidiomycete found in forests growing on rotting wood. This OTU was only found in the control and GM treatment A. *Suillus* spp. (OTU8) was found in all three treatments and often grows in a mycorrhizal symbiosis with *Pinus* spp. OTU11 was affiliated with *Satchmopsis brasiliensis* which is frequently found on leaf litter (Plaingam et al., 2003) but the four clones were only associated with the GM treatments. However, OTUs with a low number of clones, *e.g.* OTU11, OTU14, or OTU16 showed some variation between transgenic and control pine trees. These variations were statistically not significant and are most likely caused by the discontinuous distribution of fungal populations rather than the influence of genetic modification.

In our study we used rhizosphere soil to amplify ectomycorrhizal fungi associated with GM and non-GM radiata pine. Landeweert et al. (2005) analysed soil and root tips and DGGE results revealed a slightly higher diversity of ectomycorrhizal fungi in soil compared to root tips. No fungal species were exclusively detected on root tips, indicating that the mycelia of most species occur in detectable quantities in soil. In our study ectomycorrhizal DGGE fingerprints showed a treatment effect at one sampling time (summer 2006) but no differences were detected at any other sampling time. However, seasonal effects were found in both years. Kaldorf et al. (2002) examined mycorrhizal colonisation of GM aspen in a field trial by PCR-RFLP. They found no differences in the mycorrhizal diversity between GM and control trees but observed differences in the abundance and development of one of the four common ectomycorrhizal morphotypes, which was rare and poorly developed on roots from one GM line compared to the parental line. The authors suggested the effect was most likely a somaclonal effect because the formation of this ectomycorrhiza type was not affected in the other GM line. To detect these possible clone specific effects, we have used trees from two different transformation events (GM treatment A and B) in this study. Hampp et al. (1996) showed that transgenic aspen expressing indole acetic acid-biosynthetic genes formed fully developed ectomycorrhiza *in vitro*. Seppänen et al. (2007) studied the effects of silver birch, genetically modified in lignin biosynthesis, on mycorrhizal fungi. The genetic transformation had a significant influence on root biomass and morphology but the GM lines formed normal ectomycorrhizal associations with the fungus *Paxillus involutus*.

Sequencing confirmed that all DGGE bands analysed in the ectomycorrhizal community analysis showed high similarities to typical ectomycorrhizal species of the phylum *Basidiomycota*. *Polyporus tuberaster* is a white-rot fungus found in Japan, Europe and North America. The order *Cantharellales* contains mycorrhizal species. The cosmopolitan order *Tremellales* contains taxa that occur on decaying wood. The family *Suillaceae* is well known for its symbiosis with pine trees. Because most *Suillus* ectomycorrhizas occur on *Pinus* spp. (Chu-Chou and Grace, 1988), and *P. radiata* is an exotic tree species in the southern hemisphere, it is likely that this fungal species was brought into New Zealand with the introduction of *P. radiata*.

The tight constraints associated with field trials of GM plants in New Zealand meant the trees could only be grown at a single field site, so effects of climatic variability and soil type could not be examined. A potential weakness of the study is that we generated a clone library from only one sampling time. However, the DGGE results from two consecutive years clearly indicated that differences between the GM and control trees were always smaller than seasonal effects.

Whether GM plants have any substantial influence on the soil microbial communities has never been an easy question to answer. Statistically significant changes might be transient and biologically insignificant. Future assessments of impacts of GM plants should focus on functional markers rather than monitoring the structure

and genetic diversity of microbial communities. New attempts must be made that combine the analysis of phylogenetic, functional and ecological marker genes for the study of soil microbial communities in their environments. Costa et al. (2007) developed a PCR-DGGE system to target *Pseudomonas* specific *gac*A gene fragments in environmental DNA. The *gac*A gene (gacA for global antibiotic and cyanide control) has been proposed to be a reliable phylogenetic marker within the genus *Pseudomonas* and it might be a suitable target for the simultaneous analysis of *Pseudomonas* community structure and function in soil.

The main strength of this study was that field-grown trees were monitored for two consecutive years and that both bacterial and fungal communities including ectomycorrhizal fungi were investigated. More long-term studies on field-grown GM trees at different locations are needed to provide the baseline data necessary to relate potential population shifts to natural variability caused by growth stage, soil type, climate, season, or tree species. The long life span of trees makes the evaluation of environmental risks difficult as current regulations in New Zealand restrict field releases of GM trees in both time and space. Full biosafety research on GM trees can only be carried out by permitting long-term and large-scale release of GM trees over a full rotation period.

MATERIALS AND METHODS

Plant material and experimental site

Transgenic radiata pines, containing the flower-meristem-identity gene *LEAFY* (*LFY*) and the *npt*II selective marker gene, were generated at the Scion Research Centre in Rotorua, New Zealand. Embryogenic tissue of *P. radiata* was genetically transformed using a biolistic technique (Walter et al., 1998). The plasmid vector used for bombardment contained the *npt*II antibiotic selection gene and the *LFY* gene, both under control of the CaMV 35S promoter which expresses constitutively in radiata pine. Bombarded tissue was selected on media containing 20 $mg.L^{-1}$ geneticin and after 5–6 weeks transgenic clones emerged on this medium. Putative transgenic clones were first confirmed by PCR and all positive clones were tested using *npt*II ELISA and Southern hybridisation. Only PCR- and ELISA-positive lines were further propagated and matured to form somatic embryos which were rooted and subsequently planted in soil according to Walter and Grace (2000). All plants were kept in a containment greenhouse from 1998 to 2003. Trees were then transferred to the field trial site in pots and after an adaptation period of five months they were planted into the field, spaced 3 m apart. The experimental field of approximately 360 m^2 was located at the Scion Research Centre in Rotorua, New Zealand. Fifteen trees were included in this study with five trees from each of two independent transformation events (GM treatment A and B) along with five non-transformed control trees (C) of the same genotype. The trial was carried out under the containment conditions specified by the New Zealand Environmental Risk Management Authority (Permit no. GMF99001). The soil type at the site was characterised as slightly gravelly sandy loam containing 13% clay, 65% sand, 23% silt, 9.6% organic matter with a pH of 5.3.

Rhizosphere soil sampling

From March 2005 to December 2006, root samples with adhering soil were collected from 15 trees (5 trees GM treatment A, 5 trees GM treatment B, 5 control trees) quarterly. The roots were dug out along the stem, in order to access fine root segments. Root ends (*ca.* 8 g) were cut off and placed in sterile plastic bags. Samples were kept at 4 °C until being processed within 24 h. A total of 120 samples were taken over two consecutive years.

Processing of rhizosphere soil and extraction of whole-community rhizosphere DNA

Microbial cells were dislodged from soil matrices and pellets were obtained prior to community DNA extraction. Five gram of tree roots with adhering soil were suspended in 25 mL of distilled water and treated in a Stomacher blender (BagMixer, Interscience, France) for 1 min at high speed. The rhizosphere soil/water solution was transferred into 50 mL Falcon tubes. This step was repeated with 20 mL of distilled water and the combined solutions were centrifuged for 30 min at 10 000× *g*. The supernatant was discarded and the pellet dissolved in 4 mL 0.85% NaCl. Aliquots of 2 mL were centrifuged for 30 min at 13 000× *g*. The supernatant was removed with a water-jet vacuum pump and the resulting microbial pellets were kept at –80 °C. Whole-community DNA was extracted from the microbial pellet using the FastDNA SPIN Kit (BIO 101 Systems, Q-Biogene) according to the manufacturer's protocol. The DNA was kept at –20 °C until PCR amplification.

PCR amplification of bacterial 16S rRNA gene fragments

PCR amplifications were performed with a thermal cycler (Perkin Elmer Gene Amp PCR System or Eppendorf Mastercycler). Prior to DGGE analysis of the bacterial profiles, the V6 to V8 variable regions of the 16S rRNA

Table 5. Conditions used in this study for DGGE analysis.

PCR products	Fragment length (bp)	Denaturing gradient	% Acrylamide	Voltage	Run time
Bacteria	433	40–65	7	90 V	16 h
Alpha- and *Betaproteobacteria, Actinobacteria*	433	35–60	7	90 V	16 h
Fungi (Basidiomycetes)	800	12–38	6	90 V	16 h
Fungi	1650	8–38	6	180 V	17 h

genes were amplified by PCR from rhizosphere DNA extracts with the primer pair F968-GC (Nübel et al., 1996) and R1378 (Heuer et al., 1997). All primers used in this study are summarised in Table 5. The reaction mixture (25 µL) was composed by 1 µL template DNA (whole-community DNA, 1:10 diluted), 2.5 µL 10 X PCR buffer IV (ABgene, UK), 0.5 µL 10 mM dNTPs, 1.5 µL 25 mM $MgCl_2$, 0.5 µL DMSO PCR reagent (Sigma-Aldrich), 0.125 µL T4 gene 32 protein (Roche, Germany), 0.5 µL of each primer (10 mM), and 1 U Thermoprime Plus DNA polymerase (ABgene, UK). For all PCR reactions DNase/RNase-free distilled water (Invitrogen) was used. After 5 min of denaturation at 94 °C, 35 cycles of 30 s at 94 °C, 30 s at 56 °C and 1 min at 72 °C were carried out. A final extension step of 10 min at 72 °C was used to finish the reaction. To emphasize effects of GM trees on microbial communities, the DNA extracts from individual trees were pooled according to the treatment, to give three samples per sampling time and 24 samples for the duration of the monitoring period. The DNA from pooled samples was PCR amplified and subjected to DGGE as described for individual trees. Statistical analysis was only carried out from DGGE data obtained from individual trees.

PCR amplification of taxon-specific 16S rRNA gene fragments

The amplification of actinobacterial, alpha- and betaproteobacterial 16S rRNA gene fragments was carried out in a nested-PCR approach according to Costa et al. (2005).

PCR amplification of fungal-specific 18S rRNA gene fragments

Fungal 18S rRNA genes were amplified from whole-community DNA using primers FR1-GC and NS1. The PCR mixtures (25 µL) contained 1 µL template DNA (whole-community DNA, 1:10 diluted), 2.5 µL 10 X PCR buffer IV (ABgene, UK), 0.5 µL 10 mM dNTPs, 3.75 µL 25 mM $MgCl_2$, 0.5 µL DMSO PCR reagent (Sigma-Aldrich), 0.125 µL T4 gene 32 protein (Roche, Germany), 0.5 µL of each primer (10 mM), and 1 U Thermoprime Plus DNA polymerase (ABgene, UK). For all PCR reactions DNase/RNase-free distilled water (Invitrogen) was used. After 5 min of denaturation at 95 °C, 35 cycles of 30 s at 95 °C, 45 s at 48 °C and 3 min at 72 °C were carried out. A final extension step of 10 min at 72 °C was used to finish the reaction.

PCR amplification of ectomycorrhizal-specific ITS fragments

Fungal ITS sequences were amplified from whole-community DNA using primers ITS1F and ITS4B-GC. The PCR mixtures (25 µL) contained 1 µL template DNA (whole-community DNA, 1:10 diluted), 2.5 µL 10 X PCR buffer IV (ABgene, UK), 0.5 µL 10 mM dNTPs, 3.75 µL 25 mM $MgCl_2$, 0.5 µL DMSO PCR reagent (Sigma-Aldrich), 0.125 µL T4 gene 32 protein (Roche, Germany), 0.5 µL of each primer (10 mM), and 1 U Thermoprime Plus DNA polymerase (ABgene, UK). After 5 min of denaturation at 94 °C, 30 cycles of 30 s at 94 °C, 1 min at 61 °C and 2 min at 72 °C were carried out. A final extension step of 10 min at 72 °C was used to finish the reaction.

Denaturing gradient gel electrophoresis (DGGE)

The DCode System (Bio-Rad Inc., Hercules, CA) was used to perform the DGGE analysis. The DGGE protocol was based on the initial protocol of Muyzer et al. (1993) and was performed by using different linear denaturing gradients (100% denaturant contained 7 M urea and 40% (vol/vol) formamide). The used gradients, voltages, and run times are summarised in Table 5. Aliquots of PCR products (3–5 µL) were loaded randomly on the gel. Electrophoresis was carried out in 0.5 X Tris-acetate-EDTA buffer at 58 °C. Gels were silver stained according to Heuer et al. (2001) and dried at 60 °C overnight.

16S rRNA gene random clone libraries

Bacterial 16S rRNA gene fragments of all individual tree samples (n = 15) from the first summer sampling (December 2005) were PCR amplified using primers F27

(Lane, 1991) and R1494. The PCR amplicons of trees of the same treatment (5× GM treatment A, 5× GM treatment B, 5× controls) were combined and separated by electrophoresis in 0.8% agarose gels. The bands were excised and the DNA purified using the Perfectprep® Gel Cleanup (Eppendorf). Purified fragments were ligated into the pGEM-T Easy vector (Promega) and ligations were transformed into competent *Escherichia coli* JM109 cells. Dilutions were plated onto LB agar plates with ampicillin^{100}/X-Gal and incubated overnight at 37 °C. For each treatment, 120 white colonies were randomly selected and cultured for 24 h at 37 °C on LB agar supplemented with ampicillin and X-Gal. Stable clones were sub cultured overnight in 96 square well storage plates containing 1.5 mL of LB broth and 100 μg.mL^{-1} ampicillin. Clones were checked for the correct size of inserts after PCR amplification with the primers SP6 and T7 (Promega) according to the manufacturer's instructions. Clones with the correct insert were grown overnight in LB broth supplemented with 100 μg.mL^{-1} ampicillin and 100 μL of the culture plus 20% glycerol was transferred into standard 96 well storage micro plates and stored at –80 °C.

18S rRNA gene random clone libraries

Fungal 18S rRNA gene fragments of all individual tree samples (n = 15) from sampling 4 (December 2005) were PCR amplified with primers NS1 and FR1. PCR amplicons of trees of the same treatment (5× GM treatment A, 5× GM treatment B, and 5× controls) were combined and separated by electrophoresis in 0.8% agarose gels. The bands were excised and the DNA purified using the Perfectprep® Gel Cleanup (Eppendorf). Purified fragments were ligated into the pGEM-T Easy vector (Promega) and ligations were transformed into competent *Escherichia coli* JM109 cells and treated as described above.

Amplified ribosomal DNA restriction analysis (ARDRA)

Inserts of the *Bacteria* clone library were PCR-amplified by using 2 μL of a liquid subculture of clones (LB^{Ap100}, 37 °C, overnight) as template, 2.5 μL 10 X PCR buffer IV (ABgene, UK), 0.5 μL 10 mM dNTPs, 1.5 μL 25 mM $MgCl_2$, 0.5 μL of each primer (T7/SP6, Promega), and 1 U Thermoprime Plus DNA polymerase (ABgene, UK). For all PCR reactions DNase/RNase-free distilled water (Invitrogen) was used. The PCR conditions were as follows: initial denaturation (94 °C for 10 min) followed by 35 cycles of denaturation (94 °C for 30 s), annealing (56 °C for 30 s), and extension (72 °C for 1 min) with a final extension step (72 °C for 10 min). For the restriction digestion 10 μL of positively amplified insert were incubated for 3 h at 37 °C with the restriction enzyme *Hinf*I (New England BioLabs) according to the manufacturer's specifications. The restriction fragments from each clone were separated by electrophoresis in a 2.5% agarose gel for 100 min at 80 V followed by ethidium bromide staining and UV transillumination. All restriction patterns were normalised and compared with the GelCompar II software (Applied Maths, Kortrijk, Belgium). Cluster analysis was performed by the Unweighted Pair Group Method with Arithmetic Mean (UPGMA), and patterns were compared by using the band-based Dice coefficient. The position tolerance and optimization for each set of ARDRA patterns (bacteria, fungi) was automatically calculated by the software. Clones with a 95% similarity of ARDRA patterns were grouped into the same operational taxonomic unit (OTU). Representatives of each cluster were selected for sequence analysis.

Inserts of the fungal clone library (partial 18S rDNA) were PCR-amplified by using 1 μL of a liquid subculture of clones (LB^{Ap100}, 37 °C, overnight) as template, 2.5 μL 10 X PCR buffer IV (ABgene, UK), 0.5 μL 10 mM dNTPs, 2.5 μL 25 mM $MgCl_2$, 0.5 μL of each primer (NS1/FR1), and 1 U Thermoprime Plus DNA polymerase (ABgene, UK). The PCR conditions were as follows: initial denaturation (94 °C for 10 min) followed by 35 cycles of denaturation (94 °C for 30 s), annealing (48 °C for 45 s), and extension (72 °C for 2 min) with a final extension step (72 °C for 8 min). After confirmation of amplicon size using gel electrophoresis, the restriction procedure was applied as described above.

Extraction and cloning of excised DGGE bands

Characteristic bands from gels displaying ectomycorrhizal fungi were excised and eluted for 24 h in 20 μL of PCR grade water at 4 °C. One micro litre of the resulting suspension was used in a DGGE-PCR to re-amplify the excised ITS fragments. After confirming the correct electrophoretic mobility of the excised band by DGGE, the PCR product (without GC-clamp) was ligated into a pGEM-T Easy vector and transformed into competent *E. coli* cells as described above. Clones containing inserts that shared the electrophoretic mobility of the original band were selected for ARDRA analysis. *Hinf*I patterns were used to select individual clones. Inserts that showed different ARDRA patterns were submitted for sequencing.

Sequencing of rDNA gene fragments from bacterial and fungal communities

DNA sequencing was performed with DNA from clones representing different ARDRA patterns. 16S/18S rDNA fragments were amplified with standard primers T7/SP6 as described for ARDRA. Column purified PCR products were then submitted for sequencing. The sequences were aligned with data from the NCBI GenBank using the megablast algorithm.

All sequenced clones were analysed for the presence of chimeras using the CHIMERA CHECK program from the Ribosomal Database Project (RDP, www.rdp.cme.msu.edu/html). Sequences suspected of being chimeras were not included in further analysis. Nucleotide sequences determined in this study were submitted to the EMBL Nucleotide Sequence Database. The accession numbers are FM200009 to FM200033 (fungal clones) and FM206295 to FM206331 (bacterial clones).

Data analysis

DGGE gels were scanned with the GS-800 densitometer (BioRad) using Quantity One software (version 4.4.1; Bio-Rad). The intensity and position of bands were saved using the Diversity Database software (version 2.2.0; Bio-Rad). DGGE gels were analysed based on the presence/absence of bands, using principle coordinate analysis (PCO). Lanes were aligned using a Viterbi algorithm for dynamic warping (Glasbey et al., 2005). Peaks were identified by moving a Gaussian model along the series, with the peak position taking all points on the curve, and at each position the amplitude and spread of the peak were estimated. The potential peak positions were then ranked by an index combining amplitude and quality of fit (percentage of variation explained by the model). The top ranked points on this index down to a given cut-off value were taken as the peak positions for each curve. The peaks were clustered into bands using a *k*-means cluster analysis. A similarity matrix between samples was created using the Jaccard similarity measure (Gower, 1985) for the presence and absence of bands. The similarity matrix was reduced to five dimensions using PCO, and linear discriminant analysis was used to evaluate the differences between the treatments. The 95% confidence regions around the group means depicted in Figures 1, 2, 3 and 5 were produced by the GenStat DISCRIMINATE procedure (Payne et al., 2007). The confidence regions in the graphs cannot be used to determine whether treatment means are significantly different to each other as these relate only to the variation of a single treatment in two dimensions, whereas the Hotelling T^2-test uses the joint variation of treatments in the full five dimensions of the discriminant space. The confidence regions are presented only to give an indication of the variability of the means. The significance of the treatment differences was assessed using a Hotelling T^2-test (Hotelling, 1947).

The analysis of ARDRA patterns (operational taxonomic units [OTUs]) used a log-linear modeling approach to compare the OTUs between the three treatments (Statistical software package R, version 2.8.1). Diversity indices (H') of the bacterial and fungal OTUs were calculated by the Shannon information theory function (Shannon and Weaver, 1949). According to the formula, the coefficient of the number of OTUs and the number of clones indicates the diversity for a treatment. The diversity index (H') is expressed on an unlimited scale where high numbers represent high diversity.

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