Ann Microbiol (2011) 61:765–772
DOI 10.1007/s13213-010-0193-2

ORIGINAL ARTICLE

Bacterial diversity and soil enzyme activity in diseased and disease free apple rhizosphere soils

Veerubommu Shanmugam · Rajni Verma · Subramani Rajkumar · Digvijay Singh Naruka

Received: 25 February 2010 / Accepted: 22 December 2010 / Published online: 6 March 2011

Abstract A culture-independent survey of the bacterial diversity in rhizosphere soils of diseased (scab) and disease-free apple trees was conducted to assess the role of bacteria in disease suppression. Community DNA was extracted from soil samples and amplified by PCR using primers specific for bacterial 16S rRNA gene sequences. Clone libraries were constructed with the PCR products and analysed based on amplified rDNA restriction analysis (ARDRA) patterns. The phylotypes and their frequency distribution in both libraries indicated that the phylotype did not represent a single group. Rarefaction curve, and Shannon and Simpson diversity indices exhibited insignificant diversity differences between the samples in terms of bacterial community composition, whereas more chitinase and β-1,3 glucanase activities were recorded in samples from disease-free trees than from diseased trees. Based on the operational taxonomic units identified in ARDRA, 80 representative clones were selected from the libraries and partially sequenced. Sequence similarity searches with the resulting sequences identified the dominance of uncultured bacteria to the extent of 70% and 72.5% in disease free and diseased rhizospheres, respectively. The foregoing studies conclude a possible role for enhanced microbial activity in terms of enzyme production in tree (apple) health, although no distinct partitioning of composition or significant diversity of bacterial communities inhabiting diseased and disease-free rhizosphere soils was observed at any given time.

V. Shanmugam (✉) · R. Verma · S. Rajkumar · D. Singh Naruka
Institute of Himalayan Bioresource Technology, Council of Scientific and Industrial Research,
Palampur,
176 061, Himachal Pradesh, India
e-mail: shanpatho@yahoo.com

Keywords Microbial diversity · 16S rRNA · Amplified rDNA restriction analysis · Rhizosphere

Introduction

Soil-borne microbes of the rhizosphere, the biologically active zone of soil around plant roots, are affected by rhizodeposition—the substances released from roots to soil. Bacteria are an important part of this soil microflora due to their abundance, diversity and the multiplicity of their metabolic activities. The extent of the diversity of micro-organisms in soil is seen to be critical to the maintenance of soil health and quality, as a wide range of microorganisms is involved in important soil functions. Efforts to identify their diversity remain futile mainly because most microbes from natural environments cannot be cultured with current techniques (Ward et al. 1990; Amann et al. 1995). Metagenomic analysis of the rhizosphere allows insight into the genomes of such microbes, including the unculturable fraction, and their possible effect on plant health. The recognition of microbial diversity and the importance of unculturable bacteria in microbial ecosystems have been analysed in agricultural soils (Lukow et al. 2000; Sessitsch et al. 2001; Filion et al. 2004; Garbeva 2005).

It is well known that pathogens are studied most effectively within an ecological context of interactions among microbes, their hosts, and the environmental conditions in which they live (Harvell et al. 1999). A basic knowledge of the composition and distribution of the microbial communities associated with healthy as well as diseased organisms is essential for the diagnosis and eventual treatment and prevention of plant diseases by using disease suppressive microbial communities. Regulation and exploitation of microbial diversity is widely recognized as

a component of sustainable and integrated pest management approaches for plant disease control.

Apple scab, also known as black spot, caused by the fungus *Venturia inaequalis* (Cooke) G. Wint., is the most widespread disease in apple orchards worldwide (Machardy 1996; Carisse et al. 2000). The disease manifests as brown or black lesions on leaves and fruits, resulting in reduced fruit yield and quality, without killing the fruit. However, in severe infestation, it defoliates and weakens the trees, making it prone to other pest attacks. If not controlled, the disease can cause extensive losses (70% or more) where humid, cool weather occurs during the spring months. Losses result directly from fruit or pedicel infections, or indirectly from repeated defoliation, which can reduce tree growth and yield. Himachal Pradesh (HP) is the second largest producer of apples in India, and provides a livelihood to a large population of the state. Since 1977, scab disease exhibited various epidemic levels of infection and disease outbreaks under field conditions in all apple growing regions. Subsequently, disease spread was so rapid that entire apple growing areas of HP were affected, resulting in a severe epiphytotic in 1983 (Gupta 1989). Intensive cultivation of a susceptible genotype of Royal Delicious apple over larger and under diverse growing conditions in HP has attracted a number of pathogens and insect pests, significantly affecting fruit yield and quality (Sharma and Bhardwaj 2003; Thakur et al. 2005). Assessment of the microbial community structure and diversity in healthy tree rhizosphere soils among scab-affected trees in a diseased field by contrasting the genetic diversity of the two populations would help elucidate the role played by such communities in disease incidence.

The aim of this study was to use 16S rDNA sequence data to assess and compare the diversity and community structure of rhizobacteria and soil enzyme activity to assess microbial activity (at a given time) from soil samples of disease-free and diseased (scab) apple trees.

Materials and methods

Experimental site and sample collection

An apple (cv. Royal Delicious) orchard (1 ha) showing natural scab infection of>50% was identified in April 2004, corresponding with the spring season, by visually assessing 50 leaves at random from each tree for the presence or absence of scab lesions in the Kinnaur district (78°16′10″E, 31°37′17″ N; 2,946 m a.s.l.) of HP, India, and used for sample collection. The disease occurred despite adoption of orchard sanitation practices such as disposal of diseased overwintered fallen leaves and fungicidal applications in the preceding autumn or winter. The climate was wet temperate with an average annual rainfall of 2,491 mm. The soil texture was silty clay loam (30% clay, 53% silt and 17% sand) with an average pH of 5.2 in both field sites. Immediately after assessing disease incidence, two composite rhizosphere soil samples, each comprising equal quantities of 12, uniformly mixed, samples from four trees (representing the orchard area) i.e. three samples of about 500 g per tree were collected from diseased (S_2) and disease-free (S_{14}) rhizospheres. Each of the sub-samples constituting the composite samples was collected with a sterilised spatula at a depth of about 40 cm. Samples of the same rhizospheres (i.e. healthy and diseased) were mixed well, placed immediately on dry ice for transport, and frozen at −20°C until DNA extraction was performed.

Extraction, purification and quantification of DNA from soil

Soil DNA was extracted from 1 g of each of the sieved composite samples using UltraClean soil DNA isolation kit (MoBio Laboratories, http://www.mobio.com) following the manufacturer's instructions. Small portions of crude extracts (40 μl) were then subjected to electrophoresis and the DNA band was excised and eluted using QIAEX II Gel extraction kit (Qiagen, Valencia, CA). The quantity and quality of DNA was estimated using agarose gel electrophoresis and spectrophotometric methods.

SSU rDNA library construction

Rhizosphere soil DNA was amplified by PCR by targeting 16S rRNA sequences conserved among all known bacteria (Kuske et al. 1997). Amplifications were carried out in a 50-μl reaction volume consisting of 10×buffer, 5.0 μl (Genei, India); 2 mM dNTPs, 5.0 μl; 3 U/μl *Taq* DNA polymerase, 0.33 μl (Genei, India); 100 ng/μl primer, 2 μl; 35–45 ng template DNA, 1 μl and H_2O, 34.67 μl in a Bio-Rad (Richmond, CA) thermocycler using the PCR conditions 95°C for 5 min, 94°C for 30 s, 50°C for 1 min, and 72°C for 2 min. The total number of cycles was 35, with a final extension of 72°C for 10 min. The reaction control consisted of all PCR components, except genomic DNA. The PCR products (10 μl) were electrophoresed and a 1.5 kb amplicon was gel eluted and purified with a Qiaquick gel extraction kit (Qiagen). The amplicon was cloned in pGEM-T easy vector (Promega, Madison, WI). Two hundred clones of the correct size for each of the samples were stored as glycerol stocks at −70°C. The clones from rhizospheres of diseased and disease-free trees were designated as EB (S_2) and EB (S_{14}), respectively, followed by the clone number (1–200).

Genetic fingerprinting of 16S rDNA clones by amplified rDNA restriction analysis

To sort the clones into groups or operational taxonomic units (OTUs), the recombinant plasmids were extracted and amplified using SP6 and T7 primers, and the single amplicon for total bacteria was individually digested with restriction endonucleases (*Rsa*I, *Mbo*I and *Alu*I) and resolved on a 1.5% agarose gel. The GeneRuler 100 bp DNA ladder plus (MBI Fermentas, Glen Burnie, MD) was used as a DNA marker. Restriction fragments shorter than 50 bp were not considered in the analysis. An unweighted pair-group method with arithmetical averages (UPGMA) dendrogram for both samples was constructed based on the developed similarity matrix using NTSYS.pc (Numerical Taxonomy System Applied Biostatistics, Setauket, NY) software. The clones were then grouped on the basis of the amplified rDNA restriction analysis (ARDRA) pattern, and representative clones from each group were selected for sequencing. The number of phylotypes and frequency distribution of types in libraries were evaluated by using two diversity indices (Shannon-Weiner H′=−Sum Pi Ln(Pi) and Simpson index λ=Sum (Pi^2). Since the libraries differed in size, estimated type richness was calculated by rarefaction, using the software Analytical Rarefaction 1.3 (http://www.uga.edu/strata/software/). The mean heterozygosity and genetic distance between these two populations were calculated using GenAlex ver.6 (Peakall and Smouse 2006).

Nucleotide sequencing and analysis of the amplicon

Both strands of the cloned DNA were sequenced by the dideoxy chain termination method (Sanger et al. 1977) using an automated sequencer (ABI PRISM 310, Applied Biosystems, Foster City, CA). The sequences were submitted to the CHECK_CHIMERA program of the Ribosomal Database Project (RDP) (http://rdp.cme.msu.edu ; Maidak et al. 1999) to detect the presence of possible chimeric artifacts and excluded these from analysis. Sequences were aligned with reference sequences obtained from the National Centre for Biotechnological Information (NCBI) nucleotide database using the BLASTN program (http://www.ncbi.nlm.nih.gov:80/BLAST/). The partial clone sequences determined in this study have been deposited with the GenBank database (see Table 1).

Enzyme assays

Chitinase and β-1,3-glucanase assays were carried out in soil suspensions in triplicate as described by Rodriguez-Kabana et al. (1983) and Sotolova and Jandera (1985), respectively. Chitinase activity was expressed as pkat (pmol/s) of *N*-acetylglucosamine GlcNAc equivalents/millilitre soil suspension. For β-1,3-glucanase, the amount of reducing sugars released was calculated from standard curves recorded for glucose, and the enzyme activity was expressed as nkat (nmol/s) of glucose released/millilitre of suspension. Soil autoclaved twice served as the control for both assays.

Results and discussion

In HP, the low productivity of apple is attributed mainly to scab (Sharma and Bhardwaj 2003). Since soil microbial diversity is an important index of agricultural productivity, the diversity within an ecosystem is suggested to determine its role in a particular environment. As the rhizosphere is a versatile and dynamic ecological environment of intense microbe–plant interactions (Jeffries et al. 2003), a basic knowledge of the composition and distribution of the microbial communities present is (highly) essential. Hence, as an approach to assess the role of microbial communities in suppression of apple scab, this study aimed to characterise the rhizobacteria associated with healthy and diseased rhizospheres at the same site using 16S rRNA gene clone library construction and analysis. Soil sampling coincided with the pink to petal fall phenological stages, where maximum infection is expected. The libraries were constructed primarily in an attempt to explore resident bacterial phylotype diversity, rather than to assess the ongoing community shifts that may occur under conditions of changing agronomic practices.

The DNA extracted from both soil samples was predominantly of high molecular weight, forming quite a strong sharp band of 23 kb in size on 0.7% agarose gels in routine electrophoresis unit (Fig. 1a), and was suitable for use as a PCR template, with minimal chance of introducing chimeric fragments due to sheared DNA. The amount of DNA extracted from the samples differed significantly ($P=0.05$) with S_2 yielding more DNA (1.6 μg/g soil) than S_{14} (1.2 μg/g soil). The concentration ranged from 65 to 100 ng/μl, with a purity range of 1.5–1.9. In optimising the template DNA concentration for PCR, a concentration range of 35–45 ng gave better amplification and was used for subsequent studies.

The universal eubacterial primers amplified a single DNA fragment of 1,500 bp from both samples (Fig. 1b). Although no single set of primers or community profiling technique is optimal for the assessment of bacterial diversity in all instances, the primers were chosen taking into account the target sites. Genetic fingerprinting of clones by ARDRA with *Rsa*I digestion resulted in the highest number of pattern types. Since each species represented more than one phylotype, in order to evaluate the phylotype richness of the populations, the frequency and distribution of phylotypes in each of the soil samples were analysed using rarefaction for comparison. Insignifi-

Table 1 Nearest neighbour of partially sequenced clones (16S rRNA) identified by BLAST analysis

S_2				S_{14}			
Clone	Best match	Identity (%)	Accession no.	Clone	Identity (%)	Identity (%)	Accession no.
EB01	Uncultured Acidobacteria bacterium	98%	AM168116	EB01	*Rhodoferax ferrireducens*	96%	AM265401
EB8	Uncultured alpha proteobacterium	94%	AM156955	EB06	Uncultured bacterium clone J06K	96%	AM265419
EB 07	Uncultured bacterium	96%	AM265538	EB07	Uncultured forest soil bacterium clone	96%	AM265402
EB9	Uncultured bacterium clone	94%	AM156954	EB09	Uncultured bacterium clone ci52	95%	AM265414
EB10	Uncultured bacterium	96%	AM265543	EB 10	Unidentified eubacterium.	90%	AM229071
EB 11	Uncultured bacterium	98%	AM168135	EB 11	Cyanobacteria sp.	92%	AM229072
EB13	Uncultured bacterium isolate PG-91	94%	AM265539	EB 12	Uncultured Acidobacterium	96%	AM229073
EB14	Uncultured bacterium	97%	AM265544	EB 13	Uncultured Chloroflexi bacterium	99%	AM265397
EB16	Uncultured Acidobacterium	97%	AM117498	EB 14	Uncultured soil bacterium	99%	AM265395
EB17	Uncultured bacterium	96%	AM265545	EB 15	Uncultured soil bacterium	99%	AM265396
EB19	Uncultured Bacteroidetes bacterium	98%	AM168117	EB17	Uncultured soil bacterium clone 6-1	97%	AM265403
EB20	Uncultured bacterium	98%	AM168118	EB 21	Uncultured soil bacterium	96%	AM229077
EB24	Uncultured bacterium	95%	AM265546	EB 23	Uncultured soil bacterium	97%	AM229078
EB28	Uncultured bacterium	98%	AM168132	EB 24	Uncultured Antaractic bacterium	95%	AM229074
EB 36	Uncultured soil bacterium clone DS-249	98%	AM265540	EB26	Uncultured bacterium clone	93%	AM265415
EB40	Uncultured soil bacterium clone	98%	AM168131	EB35	*Herbaspirillum magnetovibrio* TK-2	98%	AM265416
EB45	Uncultured bacterium	96%	AM265547	EB42	Acidobacteria bacterium Ellin7137	96%	AM265398
EB54	*Taxeobacter* sp. SAFR-033	92%	AM265541	EB64	*Flavobacterium* sp.	96%	AM265399
EB55	Uncultured Acidobacteria bacterium clone	96%	AM265548	EB70	Uncultured Bacteroidetes bacterium clone	94%	AM397663
EB56	Uncultured soil bacterium clone	98%	AM397662	EB 78	Uncultured soil bacterium	93%	AM229075
EB62	Uncultured bacterium clone BA046	96%	AM168133	EB 79	Uncultured soil bacterium	100%	AM229076
EB66	Uncultured Bacteroidetes bacterium	88%	AM265542	EB82	Uncultured eubacterium WD244	96%	AM265400
EB73	Alpha proteobacterium	90%	AM265549	EB88	Uncultured soil bacterium clone	95%	AM265417
EB89	Uncultured bacterium	98%	AM168136	EB90	Uncultured hydrocarbon seep bacterium	92%	AM265418
EB99	Uncultured soil bacterium clone	98%	AM265550	EB 135	Uncultured bacterium clone JE25	98%	AM265404
EB 111	Uncultured bacterium clone	97%	AM168119	EB136	Uncultured Acidobacteria bacterium clone	98%	AM265405
EB 112	Uncultured bacterium	97%	AM168120	EB 133	Uncultured bacterium isolate	94%	AM262313
EB 120	Uncultured bacterium clone	97%	AM168121	EB 139	Uncultured soil bacterium clone 27-1	94%	AM265407
EB126	Uncultured Acidobacteria bacterium	98%	AM168122	EB 141	Uncultured Acidobacteria bacterium	99%	AM265408
EB142	Uncultured Acidobacteria bacterium	97%	AM168123	EB 145	Uncultured Acidobacterium group bacterium	91%	AM265409
EB 143	Uncultured bacterium	98%	AM168124	EB146	Uncultured soil bacterium clone	98%	AM265410
EB 148	Uncultured bacterium	97%	AM168125	EB 147	Uncultured soil bacterium clone UA4	95%	AM265411
EB162	Uncultured bacterium	94%	AM156956	EB149	Uncultured soil bacterium clone	94%	AM265412
EB163	Uncultured alpha proteobacterium	93%	AM168129	EB150	Uncultured bacterium clone	94%	AM265413
EB167	Uncultured bacteroidetes	96%	AM168126	EB137	Uncultured bacterium	97%	AM265406
EB170	Uncultured bacterium	90%	AM168130	EB155	Uncultured soil bacterium clone UE9	98%	AM265561
EB172	Uncultured bacteria clone	96%	AM168136	EB165	Uncultured bacterium clone B133	92%	AM265562
EB173	Uncultured bacterium clone D112	88%	AM159180	EB171	Uncultured bacterium clone D118	95%	AM265563
EB174	Uncultured gold mine bacterium	97%	AM168128	EB199	Uncultured bacterium clone 1700b-15	94%	AM265565
EB 179	Uncultured bacterium partial	97%	AM168127	EB188	Uncultured soil bacterium clone	96%	AM265564

cant differences were observed for diversity between the populations (Fig. 2). This was substantiated by analysing the diversity indices between the populations. The phylotype richness was measured by Shannon and Simpson diversity indices. While the Shannon diversity indices of S_2 and S_{14} were 3.64 and 3.65, the Simpson diversity indices were 0.99 and 0.88, respectively. Although the mean heterozygosity based on the ARDRA profile for each population was found to be higher (He=0.058) for S_{14} as compared to S_2 (He=0.051), the overall genetic distance

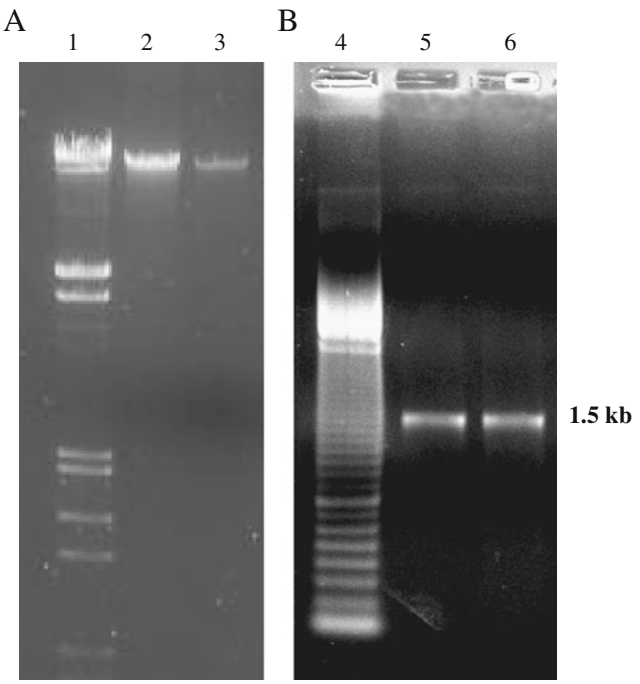

Fig. 1 **a** Isolated and purified soil DNA. **b** PCR amplification of 16S rRNA from soil DNA. Lanes: *1* *Hin*dIII-digested DNA ladder; *2*, *3* soil DNA of S_2 and S_{14}, respectively; *4* 100 bp ladder; *5*, *6* PCR products of S_2 and S_{14}, respectively

between these two populations was small (1.2%). Moreover, the frequency distribution of RFLP types were measured using evenness tests, in which both soil samples showed similar values (S_2=0.58 and S_{14}=0.60).

Partial sequencing of the 16S rRNA clones generated nucleotides sequences of 348 to 1,169 bp with a mean size of 751 bp for S_2, and 304 to 910 bp with 656 bp mean size for S_{14}. The results of BLASTN analysis of 80 representative clones are detailed in Table 1. All the sequences had more than 88% nucleotide similarity to rDNA sequences available in the database. No clones except two from S_2 and five from S_{14}, were found to be identical to any known 16S rRNA sequences from cultured organisms. Sequenced clones for S_2 fell into five major groups, viz. uncultured Acidobacteria, *Alpha proteobacteria*, uncultured bacteria, *Bacteroidetes* and *Taxeobacter*. Among the clones from sample S_{14}, four major taxa viz. Acidobacteria, uncultured soil bacteria, *Cyanobacteria-Flavobacterium-Bacteriodetes* group and Gram negative bacteria were mainly represented. The bacterial groups identified in this study were the same as those identified in other studies on different plant species and ecosystems. In investigating the microbial diversity of an agricultural soil in Wisconsin, in the United States, it was demonstrated that diversity was not distributed randomly among the major taxa, the *Proteobacteria* (16.1%), the *Cytophaga-Flexibacter-Bacteroides* group (21.8%), and the low GC-content Gram-positive group (21.8%) (Borneman et al. 1996). Predominance of proteobacteria and acidobacteria in rhizospheres of lodgepole pine (*Pinus contorta*) under forest conditions was reported (Chow et al. 2002). Interestingly, uncultured microbes were found to be predominant in both soil samples. Whilst S_2 was represented predominantly by uncultured bacteria (57%), S_{14} was dominated by uncultured soil bacteria (35%) (Fig. 3a,b). In view of this, the possibility of making reliable comparisons between the microbial communities appeared to be limited. Such complexity is clearly evident from earlier reports. A largely homogenous population of fluorescent pseudomonads from crop rhizospheres was reported (Ward et al. 1990). No difference was observed between the composition of bacterial communities of rhizosphere and that of rhizoplane in grassland soils, where the effect of rhizodepositon should be more pronounced (Nunan et al. 2005).

Enzyme activities have previously been used to document ecological effects on soil microbes (Nannipieri et al. 2007). Chitinase and β-1,3-glucanase are lytic enzymes elaborated by microbes for mycoparasitism (Hong and Meng 2003; Huang et al. 2005). This study, although not providing convincing evidence that the bacterial community structure and diversity influences disease incidence, at least at a given

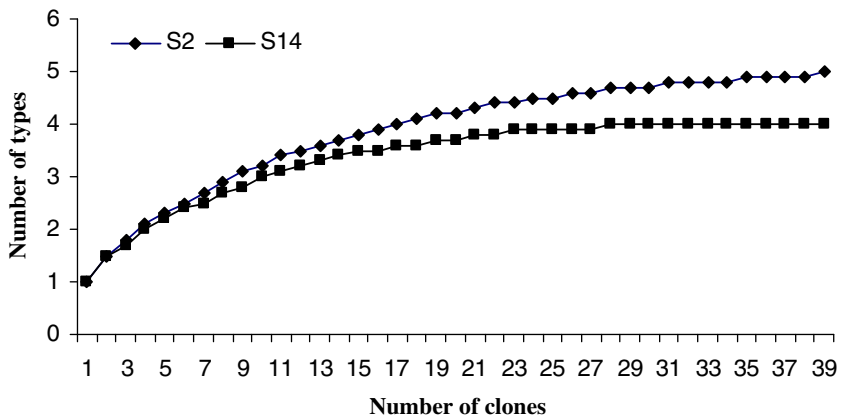

Fig. 2 Rarefaction curve showing difference in type richness of the libraries from rhizospheres S_2 and S_{14}

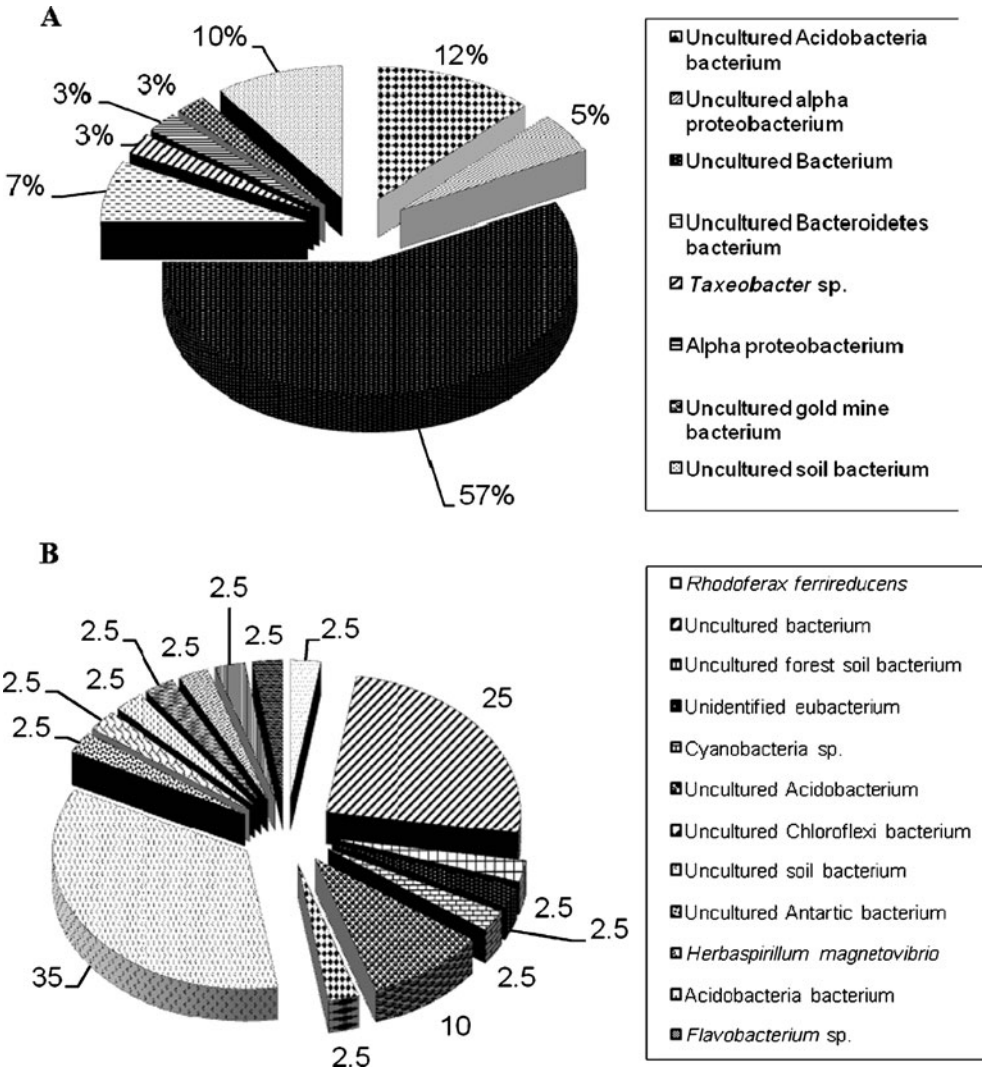

Fig. 3 Pie diagrams illustrating the division-level diversity of the partial 16S rRNA bacterial sequences comprising clones from (**A**) S_2 and (**B**) S_{14}

time, revealed that samples from disease-free (S_{14}) trees showed more lytic enzyme production than those from diseased (S_2) trees (Fig. 4), indicating the possibility that enhanced microbial activity contributes to plant health. While the maximum chitinase activity observed was 274 pkat GlcNAc/ml soil suspension, β-1,3-glucanase was recorded up to 4.5 nkat (nmol/s) glucose/ml. Such variation in enzyme activity between the samples, with S_{14} accounting for higher activity, might also be accounted for by release from roots, since the effect of rhizodeposition influencing microbial communities of the samples could be affected by root architecture, root age, plant age, and the complex interaction between soil type, plant species and root zone. Studies on several plant species have revealed that defence enzyme activities like chitinase and β-1,3-glucanase are increased by environment factors such as fungal infection (Mohammadi and Kazemi 2002; Jetiyanon 2007). The low enzyme activity of S_2 despite the trees being diseased indicated the possibility of lower enzyme release by the roots. Hence, the high enzyme activity for S_{14} could be due to enhanced microbial activity and might have contributed to tree health. However, further studies on the role of enhanced microbial activity on pathogen survival are required, to further confirm the cause of the healthiness of these trees. Contrary to this study, distinct partitioning in the composition and diversity of microbial communities in rhizospheres of healthy and diseased trees, and how these changes may influence disease development and/or resistance, have been reported. However, the role of these communities associated with healthy rhizospheres or roots in promoting disease suppression, or whether their presence is simply a direct consequence of the absence of the pathogen could only be ascertained partially if at all (Yang et al. 2001; Filion et al. 2004). Interestingly, these studies were based on soil-borne diseases, where the pathogen lives in close association with soil microbial communities. To our knowledge, this study on

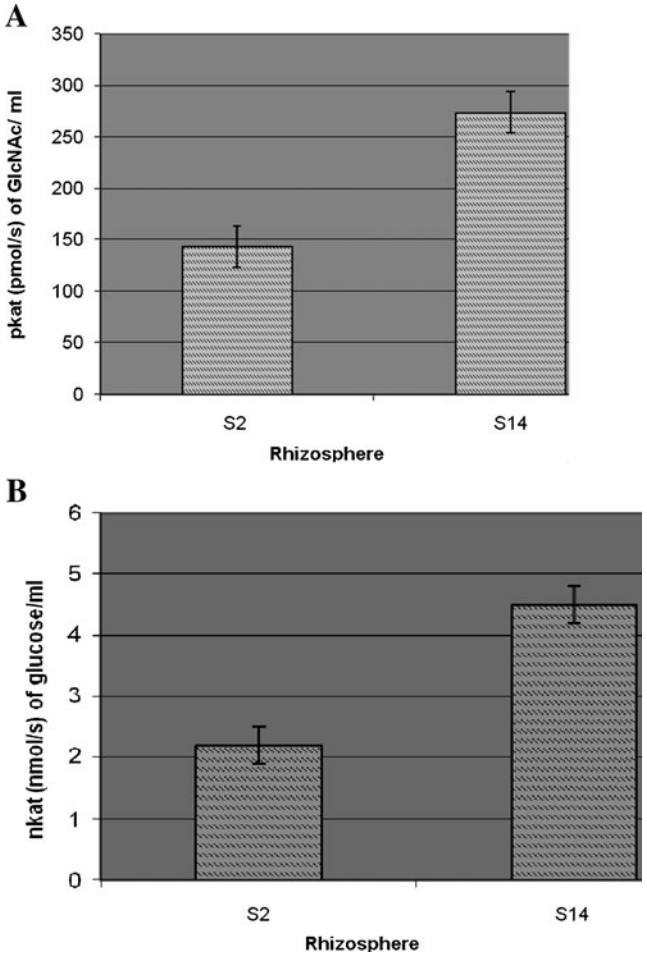

Fig. 4 **a**,**b** Antifungal enzyme activities in soil suspensions. **a** Chitinase, **b** β-1,3-glucanase. Values are the mean of three replications; *error bars* are standard deviation for the experiment; CD (P=0.05): chitinase −3.5; β-1,3-glucanase −0.5

the community-based comparative analysis of the micro-organisms associated with a disease infecting above-ground parts by culture independent studies is the first of its kind and serves as an avenue for further investigations.

Since the two main drivers of soil microbial community structure, i.e., plant type and soil type, are thought to exert their function in a complex manner (Garbeva et al. 2004), monitoring such changes in the bacterial community structure and diversity over time would affirm their role in plant health. Also, narrow searches using primer sets targeting smaller bacterial and fungal groups will be involved in further studies. Further studies on gene expression in the rhizosphere soils, supplementing measurements of microbial activities by classical enzyme assays, have been initiated to improve our knowledge of the microbial activity in these soils.

The results of this study conclude the absence of distinct partitioning in the composition, as well as the significant diversity, of bacterial communities inhabiting diseased and disease-free apple rhizosphere soils at a given time, even though enhanced microbial activity in terms of lytic enzyme production was observed in disease-free soils. It is likely that much greater sampling effort will be needed at different intervals over a period of time to explore the ongoing community shifts and to make any meaningful comparison of species diversity among the samples. Nevertheless, the results have also revealed a remarkable variety of rDNA sequences, most being only distantly related to those of cultivated species. Most of the sequences clustered into several large, phylogenetically diverse groups that are distinct from previously recognised bacterial divisions.

Acknowledgements The authors are thankful to the Director, Institute of Himalayan Bioresource Technology (IHBT, Palampur, India), for support and encouragement during the course of this investigation. This work was supported by the Council of Scientific and Industrial Research, Government of India through co-ordinated network program (SMM 002). IHBT Publication No. 0951.

References

Amann RL, Ludwig W, Schleifer KH (1995) Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol Rev 59:143–169

Borneman J, Skroch PW, O'Sullivan KM, Palus JA, Rumjanek NG, Jansen JL, Niehnuis J, Triplett EW (1996) Molecular microbial diversity of an agricultural soil in Wisconsin. Appl Environ Microbiol 62(6):1935–1943

Carisse O, Phillion V, Rolland D, Bernier J (2000) Effect of fall application of fungal antagonists on spring ascospore production of the apple scab pathogen *Venturia inaequalis*. Phytopathology 90:31–37

Chow ML, Radomski CC, Mcdermott JM, Davies J, Axelrood PE (2002) Molecular characterization of bacterial diversity in lodge pole pine (*Pinus contorta*) rhizosphere soils from British Columbia forest soils differing in disturbance and geographic source. FEMS Microbiol Ecol 42:347–357

Filion M, Hamelin RC, Bernier L, St-Arnaud M (2004) Molecular profiling of rhizosphere microbial communities associated with healthy and diseased black spruce (*Picea mariana*) seedlings grown in a nursery. Appl Environ Microbiol 70(6):3541–3551

Garbeva P (2005) The significance of microbial diversity in agricultural soil for disease suppressiveness. PhD Thesis, Leiden University, Leiden, The Netherlands

Garbeva P, van Veen JA, van Elsas JD (2004) Microbial diversity in soil: selection of microbial populations by plant and soil type and implications for disease suppressiveness. Annu Rev Phytopathol 42:243–270

Gupta GK (1989) Apple scab-economics and technology. Pesticides 23:33–39

Harvell CD, Kim K, Burkholder JM, Colwell RR, Epstein PR, Grimes DJ, Hofmann EE, Lipp EK, Osterhause ADME, Overstreet RM, Porter JW, Smith GW, Vasta GR (1999) Emerging marine diseases-climate links and anthropomorphic factors. Science 285:1505–1510

Hong TY, Meng M (2003) Biochemical characterization and antifungal activity of an endo-1,3-β-glucanase of *Paenibacillus* sp. isolated from garden soil. Appl Microbiol Biotechnol 61:472–478

Huang CJ, Wang TK, Chung SC, Chen CY (2005) Identification of an antifungal chitinase from a potential biocontrol agent, *Bacillus cereus* 29-9. J Biochem Mol Biol 38:82–88

Jeffries P, Gianinazzi S, Perotto S, Turnau K, Barea JM (2003) The contribution of arbuscular mycorrhizal fungi in sustainable maintenance of plant health and soil fertility. Biol Fertil Soils 37:1–16

Jetiyanon K (2007) Defensive-related enzyme response in plants treated with a mixture of *Bacillus* strains (IN937a and IN937b) against different pathogens. Biol Control 42:178–185

Kuske CR, Barns SM, Busch JD (1997) Diverse uncultivated bacterial groups from soils of the arid southwestern United States that are present in many geographic regions. Appl Environ Microbiol 63:3614–3621

Lukow T, Dunfield PF, Liesack W (2000) Use of the T-RFLP technique to assess spatial and temporal changes in the bacterial community structure within an agricultural soil planted with transgenic and non-transgenic potato plants. FEMS Microbiol Ecol 32:241–247

Machardy WE (1996) Apple scab: biology, epidemiology, and management. American Phytopathological Society, St. Paul, MN

Maidak BL, Cole JR, Parker CTJR, Garrity GM, Larsen N, Li B, Lilburn TG, Mccaughey MJ, Olsen GJ, Overbeek R, Pramanik S, Schmidt TM, Tiedje JM, Woese CR (1999) A new version of the RDP (Ribosomal Database Project). Nucleic Acids Res 27(1):171–173

Mohammadi M, Kazemi H (2002) Changes in peroxidase and polyphenol oxidase activities in susceptible and resistant wheat heads inoculated with *Fusarium graminearum* and induced resistance. Plant Sci 162:491–498

Nannipieri P, Ascher J, Ceccherini MT, Landi L, Pietramellara G, Renella G, Valori F (2007) Microbial diversity and microbial activity in the rhizosphere. Ci Suelo 25(1):89–97

Nunan N, Daniell TJ, Singh BK, Papert A, McNicol JW, Prosser JI (2005) Links between plant and rhizoplane bacterial communities in grassland soils, characterized using molecular techniques. Appl Environ Microbiol 71:6784–6792

Peakall R, Smouse PE (2006) GENALEX 6: genetic analysis in excel. Population genetic software for teaching and research. Mol Ecol 6:288–295, Notes

Rodriguez-Kabana R, Godoy G, Morgan-Jones G, Shelby RA (1983) The determination of soil chitinase activity: conditions for assay and ecological studies. Plant Soil 75:95–106

Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 74(12):5463–5467

Sessitsch A, Weilharter A, Gerzabek MH, Kirchmann H, Kandeler E (2001) Microbial population structures in soil particle size fractions of a long-term fertilizer field experiment. Appl Environ Microbiol 67:4215–4224

Sharma IM, Bhardwaj SS (2003) Efficacy and economics of different fungicide spray schedules in controlling premature defoliation disease in apple. Plant Dis Res 18(1):21–24

Sotolova I, Jandera A (1985) Activity of l, 3-β-D-glucanases in soil. Folia Microbiol 30:521–524

Thakur VS, Sharma N, Verma S (2005) Present scenario of apple scab in various apple orchards of Himachal Pradesh. Hortic J 18 (3):134–136

Ward DM, Weller R, Bateson MM (1990) 16S rRNA sequences reveal numerous uncultured microorganisms in a natural community. Nature 345:63–65

Yang CH, Crowley DE, Menge JA (2001) 16S rDNA fingerprinting of rhizosphere bacterial communities associated with healthy and *Phytophthora* infected avocado roots. FEMS Microbiol Ecol 35:129–136