Structural and functional characterization of bacterial diversity in the rhizospheres of three grain legumes

Dissertation
zur Erlangung des Doktorgrades
der Fakultät für Biologie
der Ludwig-Maximilians-Universität Muenchen

Shilpi Sharma
Institut für Bodenökologie
GSF – Forschungszentrum für Umwelt und Gesundheit, Neuherberg

eingereicht am 16.12.2003

1. Gutachter: Prof. Dr. Anton Hartmann

2. Gutachter: Prof. Dr. Jörg Overmann

Tag der mündlichen Prüfung: 28.04.2004

Dedicated to My Parents

The following work has been performed at the Institute of Soil Ecology, GSF-National Research Center for Environment and Health, Neuherberg, under the guidance of Prof. Dr. Anton Hartmann and Dr. Michael Schloter.

My cordial thanks to:

Prof. Dr. A. Hartmann for giving me the opportunity to work on the project, his interest in progress of the research and constructive discussion for better understanding of the subject.
Prof. Dr. J. C. Munch for providing comfortable work environment in the institute and for his suggestions during the course of the work.
Dr. M. Schloter for the extensive technical and moral support and also for being there to discuss whenever needed.
Prof. Dr. J. Overmann for his willingness to review the work.
Dr. J. Mayer for the samples, physico-chemical data and fruitful discussions.
Ms. C. Galonska for her expert assistance with laboratory experiments.
Dr. A. Hagn for rendering her helping hand throughout the tenure.
All my colleagues for the friendly working environment, constructive scientific discussions and ever helping attitude.
Deutsche Forschungsgemeinschaft (DFG), Bonn, Germany, for their financial support for the present study.

My parents who have been the guiding force throughout my life and for their unconditional support. Mitu (sister) and Ratnesh (brother-in-law) for making dull moments bright and cheerful. My parents-in-law for having full faith in my performance and most of all to

Manish, my “best friend-colleague-husband”, for his unending support during the “low” phases, brain storming discussions both during and after lab timings and for creating the perfect environment at personal as well as professional front.

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ABBREVIATIONS

α	alpha (-subgroup of proteobacteria)
°C	degree centigrade
AP-PCR	arbitrarily primed PCR
APS	ammonium persulphate
β	beta (-subgroup of proteobacteria)
βGAM	β-glucosaminidase
BNF	biological nitrogen fixation
bp	base pairs
BSA	bovine serum albumin
CaL	calcium lactate
cDNA	complementary DNA
cm	centimetre
δ	delta (-subgroup of proteobacteria)
DEPC	diethylene pyrocarbonate
DGGE	denaturing gradient gel electrophoresis
dH_2O	distilled water
DMSO	dimethyl sulfoxide
DNA	deoxyribose nucleic acid
DNase	deoxyribonuclease
EDTA	ethylene diamine tetra acetic acid
e.g.	for example
et al.	et alteri
γ	gamma (-subgroup of proteobacteria)
g	gram
G+C	guanine and cytosine
h	hours
kg	kilogram
klx	kilolux
l	litre
LB	Luria Bertani (-medium)
lb	pound
μ	micron (10^{-6})
M	molar

m	milli (10^{-3})
min	minute
mRNA	messenger RNA
MUF	methlyumbelliferone
MUF-[GlcNAc]	MUF-*N*-acetyl-β-D-glucosaminide
N	nitrogen
n	nano (10^{-9})
P	phosphorus
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffer saline
PCR	polymerase chain reaction
pmol	pico moles (10^{-12})
RAP-PCR	RNA arbitrarily primed PCR
rDNA	ribosomal DNA
RFLP	restriction fragment length polymorphism
RNA	ribose nucleic acid
RNase	ribonuclease
rRNA	ribosomal RNA
RT	reverse transcription
SDS	sodium dodecyl sulphate
SSC	sodium chloride / sodium citrate
t	tonne
TAE	tris acetic acid EDTA buffer
TBE	tris boric acid EDTA buffer
TEMED	tetramethylethylenediamine
V	volt
viz.	videlicet (namely)
vol.	volume
vol/vol	volume / volume
w/v	weight / volume
yr	year

1. INTRODUCTION

1.1 Legumes: importance in agriculture

Chemical fertilizers have had a significant impact on food production in the recent past and today are an indispensable part of modern agriculture. They guarantee the production of food for a steadily growing population. However, the external costs of environmental degradation and human health pose a major limitation to their excessive use and urge for careful designing of its application. Input efficiency of N fertilizer is one of the lowest and, in turn, contributes substantially to environmental pollution. Nitrate in ground and surface water and the threat to the stability of ozone layer from gaseous oxides of nitrogen are major health and environmental concerns. Another concern is the decline in crop yields under continuous use of N fertilizers. These environmental and production considerations dictate that biological alternatives, which can augment and in some cases replace, N fertilizers, must be exploited. Long-term sustainability of agricultural systems relies on the use and effective management of internal resources.

Biological nitrogen fixation (BNF), a microbiological process that converts atmospheric nitrogen into plant-usable form, offers this alternative (Bohlool *et al*., 1992; Parr *et al*., 1992; Buyer and Kaufman, 1996). This process is mediated in nature only by bacteria. Nitrogen fixation by legumes is a partnership between a bacterium and a plant. In legumes, rhizobia live in small organelles on the roots called nodules. Within these nodules, the symbiotic forms of rhizobia (bacteroids) fix atmospheric nitrogen and the NH_4^+ produced is converted to organic nitrogen. Organic nitrogen produced in the nodules is then translocated to the plant. Other plants benefit from nitrogen fixing bacteria when the bacteria die and release N to the environment, or when the bacteria live in close association with the plant. BNF can be considered as “the” main entry of combined N in the N-cycle with estimates of 139 - 170 x 10^6 t N yr^{-1} for BNF as compared to 80 x 10^6 t N yr^{-1} for N-fertilizers (Peoples and Craswell, 1992; Anonymous, 1994).

Nitrogen fixation by legumes can be in the range of 25 - 75 lbs of nitrogen per acre per year in a natural ecosystem and several hundred pounds in a cropping system. Legumes may fix up to 250 lbs of nitrogen per acre and are not usually fertilized. Perennial and forage legumes such as alfalfa, sweet clover, true clovers and vetches may fix 250 - 500 lbs

of nitrogen per acre. Replacing it with fertilizer N would cost $7 to $10 billion annually (Graham and Vance, 2000). Some legumes are better at enriching soil nitrogen than others. Common beans are poor fixers (less than 50 lbs per acre) and fix less than their nitrogen needs. Other grain legumes such as peanuts, cowpeas, soyabeans, and faba beans are good nitrogen fixers, and will fix all of their nitrogen needs other than that absorbed from the soil.

Fertilizer N is frequently unavailable to subsistence farmers, leaving them dependent on N_2 fixation by legumes or other N_2-fixing organisms. The amount of nitrogen returned to the soil during or after a legume crop can be misleading. Almost all of the nitrogen fixed goes directly into the plant. Little leaks into the soil for a neighbouring non-legume plant. However, nitrogen eventually returns to the soil for a neighbouring plant when vegetation (roots, leaves, fruits) of the legume dies and decomposes. When the grain from a grain legume crop is harvested, little nitrogen is returned for the following crop. Most of the nitrogen fixed during the season is removed from the field. The stalks, leaves, and roots of grain legumes such as soyabeans and beans contain about the same concentration of nitrogen as found in non-legume crop residue. In fact, the residue from a corn crop contains more nitrogen than the residue from a bean crop, simply because the corn crop has more biomass. A perennial or forage legume crop only adds significant nitrogen for the following crop if the entire biomass (stems, leaves, roots) is incorporated into the soil (Lindemann and Glover, 1990). Now it is becoming quite clear that legumes have a major role to play in P sustainability. They have several mechanisms for solubilizing unavailable P, which results in enhanced P acquisition, particularly in low P containing soils. Incorporation of legumes into inter- and rotational-cropping systems provides a low cost alternative to adding P fertilizer for improved soil fertility (Uhde-Stone *et al.*, 2003).

The three most extensively used green manures in Central Europe in organic farming are *Vicia faba*, *Lupinus albus* and *Pisum sativum*. Besides their economic value as crops, legumes in agricultural systems contribute substantially to the overall N economy of the system by sequestering atmospheric N through symbiotic N_2-fixation and through subsoil N retrieval (Gathumbi *et al.*, 2002a), mainly in legume-crop rotations in sequential cropping systems comprising fast-growing fallow species that are capable of accumulating large amounts of foliage rich in N (Garland, 1996; Gathumbi *et al.*, 2002b). They possess all the other characteristic properties of green manures like improving soil by adding large

amounts of organic material and valuable nutrients to it, preventing soil erosion, preventing the leaching away of nutrients and improving soil's ability to hold water. Because of their unique capacity for symbiotic biological nitrogen fixation, legumes are critical to N sustainability through providing a low cost and renewable supply of N that is less prone to leaching and volatilisation.

1.2 Rhizosphere

The term rhizosphere, originally coined by Hiltner in 1904, was defined as the volume of soil adjacent to and directly influenced by plant roots. Over the years, however, the term has been redefined several times, mostly to incorporate parts of the root tissue. Rhizosphere can be divided into ecto and endo rhizosphere. The term endorhizosphere is used to describe the multi-layered microenvironment, which includes a mucoid layer on the root surface, the epidermal layer of the root tissue including the root hairs and the cortical cells. By comparison, the ectorhizosphere comprises the rhizosphere soil, which usually extends a few millimetres from the root surface. A usual separation of the rhizosphere and bulk soil is obtained by shaking the roots manually. Soil adhering to root is defined as the rhizosphere. In addition, plants may develop a dense "rhizosheath", which is a strongly adhering layer of root hairs, mucoid material, microorganisms and soil particles (Curl and Truelove, 1986; Sørensen, 1997).

Rhizosphere has been regarded as 'hot spot' for microbial colonization and activity (Bolton *et al*., 1993). Actively growing roots release organic compounds, such as sloughed off cells, secretions, lysates and exudates, into the rhizosphere (Lynch and Whipps, 1990; Bowen and Rovira, 1991). The greatest quantity of carbon loss from the root actually occurs at the root tips (corresponding to root cap sloughing), but a considerable amount is lost as diffusible substrates from the zone of root elongation and root hair zone. Another hot spot for exudation is the root branching points. These compounds support growth of the microbial community in the rhizosphere and result not only in an increased population density, but also in a community structure distinct from that in the bulk soil (Curl and Truelove, 1986; Bowen and Rovira, 1991; Kent and Triplett, 2002). Because microorganisms are not limited by the amount of C and energy sources in the rhizosphere, the number of microrganisms in the rhizosphere is 19- to 32- times larger than in root-free soil (Bodelier *et al*., 1997). The activity of microbes in the rhizosphere is also expected to be higher and qualitatively different in the rhizosphere as compared to microbes in bulk

soil. The amount and composition of organic materials released by the plants are important factors that determine the nature of this plant-microbe interaction (Griffiths *et al.*, 1999; Jaeger *et al.*, 1999). Since such root-released products can be highly specific for a given plant species or even a particular cultivar, plants are thought to selectively enrich their rhizospheres for microorganisms that are well adapted to the utilization of specific released organic compounds (Bowen and Rovira, 1991; Lynch and Whipps, 1990). Other mechanisms by which plant roots can affect microbial population in their vicinity are competition for nutrients and providing a solid surface for attachment.

Rhizosphere microoganisms exert strong effects on plant growth and health by nutrient solubilization, N_2 fixation or the production of plant hormones (Höflich *et al.*, 1994; Patten and Glick, 1996). Increased plant productivity also results from the suppression of deleterious microorganisms by antagonistic bacteria, while soil-borne pathogens can greatly reduce plant growth. As variation in microbial community structure may have effects on ecosystem processes (e.g. nutrient recycling, decomposition of organics) or plant-microbe interaction (e.g. growth of pathogens, release of plant-growth promoting rhizobacteria or genetically engineered microorganisms), understanding how community processes affect ecosystem processes is of central interest in ecology (Miethling *et al.*, 2000). Knowledge of rhizosphere microbial community also opens possibilities to promote disease suppressive microflora in the rhizosphere.

Numerous studies reveal differences in rhizodeposition by different grain legumes (Haynes *et al.*, 1993; Mayer *et al.*, 2003a) thereby most likely to influence microbial community structure and function in their respective rhizospheres. The establishment of superior or more effective strains of rhizobia for nitrogen fixation would be unsuccessful if introduced organisms do not compete well with efficient symbiotic organisms already in the soil. In addition, inoculation practices do not always result in a good distribution of microbes in the root zone. Therefore, studies on the ecology of nitrogen-fixing microbes in the rhizosphere are an essential component of BNF research. A better understanding of the microbial genes, plant genes, and other soil and plant factors influencing microbial ability to develop and function in the soil adjacent to (rhizosphere) and away from plant roots is needed. Investigations into the mechanisms involved in microbial responses to nutrient limitations and other environmental stresses should also be undertaken. Studies addressing this effect of legume rhizodeposition are to date limited. Development of convenient

genetic markers for the identification, enumeration, classification and tracking of microorganisms in the soil and rhizosphere will assist in the achievement of these goals. To study the functional aspects of rhizosphere microbial community, genes involved in specific anabolic and catabolic pathways can be targeted.

1.3 Tools to investigate community structure and function

The diversity of soil bacterial communities has been investigated for many years using methods based on isolating and culturing the bacteria. Such techniques are known for their selectivity and are not considered representative of the extent and diversity of the bacterial community. The proportion of cells, which can be cultured, is only a small fraction of the total population (Amann *et al*., 1995) and few data are available concerning how closely they reflect the actual composition of these communities. Use of culture-dependent techniques has the limitation that only 0.1% - 0.2% of soil bacteria are culturable. This figure increases to about 10% in rhizosphere soils (Sørensen, 1997). Our understanding of the total bacterial community and rhizosphere dynamics has been restricted due to the limitations of traditional pure culture techniques (Torsvik *et al*., 1990b; Sørensen, 1997).

The limitations of culture-dependent techniques have been a considerable handicap to microbial ecology. Ecological inferences based on the metabolic properties of cultivated bacteria are, by necessity, unrepresentative of the natural populations from which they were obtained (Brock, 1987). Although the biases of cultivation-based approaches were recognized by Winogradsky (1949), it is only recently that means have been developed to study the uncultivated majority. First Zuckerkandl and Pauling (1965), then Woese's advances in microbial phylogeny (Woese *et al*., 1985) coupled with developments in molecular biology provided the necessary methods to allow the identity of uncultivated bacteria to be determined.

To get a better insight into the bacterial community structure, methods independent of cultivation can be used. Methods that rely on direct amplification of 16S rRNA genes (rDNA) are rapidly replacing cultivation as an approach to compare the bacterial communities of various habitats (Ferris and Ward, 1997; Torsvik *et al*., 1998; Duineveld *et al*., 2001; Gremion *et al*., 2003). The development of techniques for the analysis of 16S rRNA sequences in natural samples has greatly enhanced our ability to detect and identify bacteria in nature (Olsen *et al*., 1986; Pace *et al*., 1986). This has confirmed earlier

estimates that only a small percentage of bacterial species have been isolated in culture (Ward *et al*., 1990; Barns *et al*., 1994; Choi *et al*., 1994). Structural and functional characterization of rhizosphere of legumes has been focussed primarily on symbionts, *Rhizobium* and *Bradyrhizobium* spp. (Hynes *et al*., 2001; Tan *et al*., 2001). Other bacterial genera present may play a very important, yet unidentified role in the rhizosphere of legumes.

rRNA content has been proposed as an appropriate target for assessing changes in active bacterial populations as its content represents a first approximation of bacterial activity (Wagner, 1994). Therefore, 16S rRNA and rDNA extracted directly from rhizosphere soil were targeted in reverse transcriptase PCR (RT-PCR) and PCR analysis respectively. Denaturing gradient gel electrophoresis (DGGE) can separate mixed PCR products recovered from environment. This offers a culture-independent tool for tracking dominant bacterial populations in space and time and to assess community changes (Muyzer *et al*., 1993). In addition, the PCR and RT-PCR products were cloned to construct libraries. These libraries were sequenced to identify the resident and active bacteria in the rhizospheres.

Molecular biological techniques are not free from limitations and biases, especially when applied to environmental samples. As with other techniques used by soil microbiologists, biases appear at the stage of soil sampling and storage before extraction of nucleic acids. Problems are encountered at the stage of DNA / RNA extraction, which includes the reliable and reproducible lysis of all bacterial cells as well as the extraction of intact nucleic acids, and the removal of substances, such as humic acids, bacterial exopolysaccharides and proteins, which may inhibit polymerase activity during PCR and DNA digestion with restriction enzymes (Trevors and van Elsas, 1995; Clegg *et al*., 1997; van Elsas *et al*., 1997; Cullen and Hirsch, 1998; Frostegård *et al*., 1999; Gelsomino *et al*., 1999). Generally, soil samples are recommended to be refrigerated or frozen if they are not analysed further. Miller *et al*. (1999) found that DNA yields decreased rapidly for refrigerated samples and decreased slowly over several weeks for frozen samples. Therefore, it is recommended to use freeze-dried samples (Miller *et al*., 1999). Impurities in the total DNA extracted significantly reduce the rate of DNA re-association, decreasing precision of the re-association estimates of the soil microbial diversity. Also, the source of the DNA that is originating not only from active bacterial cells but also from dead or

dormant cells and persistent DNA adsorbed onto soil particles, can affect the interpretation of the re-association kinetics, leading to an overestimation of detected genomes / species (Torsvik *et al*., 1990a; Stahl, 1997; Clegg *et al*., 1998).

Molecular techniques generate valuable information on microbial diversity and community structure in soil, taking into account both the culturable and unculturable fractions of microorganisms (van Elsas *et al*., 1998). Combining of different methods that work at a broad scale with those that can be used to identify particular groups or species of bacterial community seems to be a way of avoiding several limitations associated with analysis of complex communities in soils (Øvreås and Torsvik, 1997; Muyzer, 1998; Nüslein and Tiedje, 1998; Øvreås *et al*., 1998; Macnaughton *et al*., 1999; Sandaa *et al*., 1999).
These methods include:

- "Genetic fingerprinting", which provides a global picture of the genetic structure of the bacterial community;
- PCR fragment cloning followed by restriction and / or sequence analysis, which enable assessment of the diversity of the community in terms of the number of different species and, to a lesser extent, the relative abundance of these species.

1.3.1 Structural diversity

The gene coding for ribosomal RNA associated with the small ribosomal subunit (16S rDNA) has been used extensively to characterize bacterial communities. This gene is particularly suited for such studies for a number of reasons.

- All bacteria harbour this gene, which is essential for ribosome functioning in protein synthesis. Therefore, their evolutionary relationships can be deduced (molecular clock).
- A large number of 16S sequences of different organisms are stored in databases.
- PCR primers can be and have been designed using sequences in several highly conserved regions and
- Bacterial cells can be identified by *in situ* hybridisation targeting abundant ribosomes in cells.

Numerous studies have applied 16S as a molecular target to characterize soil bacterial communities (Ferris and Ward, 1997; Heuer and Smalla, 1997; Felske *et al*., 1998; Duineveld *et al*., 2001; Gremion *et al*., 2003). In nearly all of these studies, novel microbial linkages have been discovered, confirming our lack of understanding of the

microbial species that inhabit soil and their potentially important roles in ecosystem functions.

In recent years, a number of analyses have focussed on the characterization of soil microbial communities based on rRNA as opposed to rRNA genes encoded by rDNA (Felske *et al*., 1996; Sessitsch *et al*., 2002; Purdy *et al*., 2003). Like rDNA, rRNA has both conserved and highly variable regions that permit the discrimination of taxa at multiple taxonomic levels. In addition, use of rRNA offers three principle advantages over rDNA techniques:

- Because ribosomes are the sites of protein synthesis, cellular ribosome content (and thus rRNA content) is directly correlated with metabolic activity and growth rate (Wagner, 1994). Therefore, a high proportion of rRNA sequences detected in soil samples should correspond to metabolically active and growing microorganisms. Results with rRNA can be readily compared with those for simultaneously extracted DNA to estimate both the dormant and active community (Felske *et al*., 1996).
- Because rRNA sequences are typically present in cells in higher copy number than rDNA sequences, they should be easier to detect (Moran *et al*., 1993).
- When ribosomes are extracted directly from soil samples, free nucleic acids and many dormant microorganisms are excluded and only rRNA from active cells is detected (Felske *et al*., 1997).

Fingerprinting of amplified 16S gene can be performed using denaturing gradient gel electrophoresis (DGGE). The technique enables the separation of fragments of the same length depending on differences in sequences. Compared to RFLP / ARDRA that is appropriate for fine-structure analysis of specific components of microbial community structure, the patterns of DGGE or TGGE are more useful for a direct comparison of structural diversity between different microbial communities from different natural sites or environments perturbed by any way. Also, the possibility of hybridisation with group- or species-specific probes or excision of bands and its sequencing provides additional information of the microbial community composition that is impossible to obtain with RFLP / ARDRA (Kowalchuk *et al*., 1998; Muyzer and Smalla, 1998; Macnaughton *et al*., 1999; Yang and Crowley, 2000).

1.3.2 Functional diversity

Changes in the active microbial communities may lead to changes in the functions performed by the community. To study such changes, investigations have been made at mRNA and protein levels.

1.3.2.1 Arbitrarily Primed (AP) and RNA Arbitrarily Primed (RAP) PCR

For generating RNA profiles, Liang and Pardee (1992) introduced mRNA differential display RT-PCR (DDRT-PCR) in which an oligo (dT) primer is used in the reverse transcription reaction and an additional primer is used in the PCR step. The use of oligo (dT) renders this technique useful for eukaryotic gene expression where mRNA is polyadenylated. Fleming *et al*. (1998) optimised and applied the differential display technique to identify differentially expressed genes using pure cultures and soil microcosms to identify genes induced by toluene. The high percentage of false positives generated by DDRT-PCR strongly limits the usability of the method (Zegzouti *et al*., 1997). A derivative of DDRT-PCR, RNA arbitrarily primed-PCR (RAP-PCR) described by Welsh *et al*. (1992) has the potential to bypass these limitations. RAP-PCR uses an arbitrary primer or random hexanucleotide mixtures (Abu and Pederson, 1996) at a low annealing temperature for cDNA synthesis reactions. Due to low stringency temperatures the arbitrary primer is able to bind at random sites within the template that show limited, but not complete complementarity. Menke and Mueller-Roeber (2001) have described the optimisation of the protocol for fingerprinting plant cells.

1.3.2.2 mRNA analysis

Direct extraction of mRNA from soils (Sayler *et al*., 1989) and quantification of mRNA by an RNase protection assay (Fleming *et al*., 1993) have been used for naphthalene dioxygenase in soils and for soluble methane monooxygenase in aquifer sediments (Stapleton *et al*., 1998). RT-PCR of mRNA for soluble methane monooxygenase in aquifer sediments (Selvaratnam *et al*., 1995) and for lignin peroxidase in soils (Bogan *et al*., 1996) has also been performed. One of the major pre-requisites for such methods is the knowledge of the sequence flanking the target region for the design of amplification primers for PCR. There is a growing database of cultivable microbial genomes but less than 1% of soil bacteria can be cultured (Amann *et al*., 1991; Amann *et al*., 1995). This limits the choice of primers and thus methods that bypass the requirement of prior

sequence information are needed to investigate functional changes in the rhizosphere microbial communities in response to changes in the root exudates. In the present study, various genes important in the nitrogen cycle were targeted at the level of mRNA.

1.3.2.3 Enzyme assays

Soil quality is often linked to organic matter content and the activity of beneficial soil organisms. Soil enzymes are both mediators and catalysts of important soil functions such as organic matter degradation, mineralization and nutrient cycling, and have been used to measure the influence of natural processes and anthropogenic activities on soil quality (Dick, 1997). Soil enzyme activity can be used to evaluate plant productivity, nutrient cycling potential and improved soil chemical and physical status, especially in soils managed using long crop rotations, conservation tillage practices, and organic amendments (Dick *et al*., 1988; Martens *et al*., 1992; Jordan *et al*., 1995).

One of the inherent limitations of enzyme tests is that the actual microbial activity of a soil is not well reflected. Moreover, the tests show “historic” features of enzymes bound to soil organic matter or clay minerals. Enzyme activities in soil can be associated with active cells (animal, plant, microbial), entire dead cells and cell debris as well as being complexed with clay minerals and humic colloids (Burns, 1982). Therefore, Visser and Parkinson (1992) disputed the suitability of enzyme assays for microbial activity and soil quality assessments; with the exception of dehydrogenase because it’s biological properties make it unlikely to be present in soil in an extracellular state (Skujins, 1978).

Kandeler *et al*. (1996) suggested that the study of enzyme diversity and their associated activities provide an effective approach for examining functional diversity in soils. Furthermore, their responsiveness to environmental disturbances makes them a potential indicator of soil biological quality (Dick, 1994). *In situ* activities of hydrolytic ecto-enzymes of aquatic bacteria are routinely measured by assessing the rate of enzymatic cleavage of a fluorochrome-linked substrate (Hoppe, 1983; Chrost, 1991). The fluorochrome exhibits low fluorescence when bound to the substrate, and high fluorescence when released from it. Specific fluorogenic substrates are used to determine the activity of various hydrolytic enzymes, e.g. carbohydrases, proteinases and lipases (Hoppe, 1991; Martinez *et al*., 1996). Cleavage of MUF-[GlcNAc] is diagnostic for β–

glucosaminidases (βGAMs) and chitinases, enzymes that attack glycan or chitin chains from the free ends, cleaving off single aminosugar units (Gooday, 1990; Mulisch, 1993).

1.4 The Legume-Nitrogen Rhizodeposition Project

The present study was supported by a research grant MU 831/10-1 from the Deutsche Forschungsgemeinschaft (DFG), Bonn, Germany. This was a collaborative project between Department of Organic Farming and Cropping Systems, University of Kassel, Witzenhausen and Institute of Soil Ecology, GSF National Research Center for Environment and Health, Neuherberg. The group working at the University of Kassel aimed to quantify the rhizodeposits of three important grain legumes, *Vicia faba, Lupinus albus* and *Pisum sativum*. The group worked on estimating the amount of nitrogenous rhizodeposits released by the roots of these legumes and their subsequent take up by microbes in the rhizosphere. The N rhizodeposition constituted 13% of total plant N for *Vicia faba* and *Pisum sativum* and 16% for *Lupinus albus* at maturity respectively. Only 14 – 18% of the rhizodeposition N was found in the microbial biomass and 3 – 7% was found in mineral N fraction (Mayer *et al*., 2003a). This study indicated that N rhizodeposition from grain legumes represent a significant pool for N balance and N dynamics in crop rotations. In another study, the group investigated the residual N contribution from the legumes under comparison to subsequent crops of wheat and oilseed rape. The average total N uptake of the subsequent crops was influenced by the legume used as precrop and was determined by the residue N input and the N_2-fixation capacity of the legume species. The succeeding crops recovered 8.6 – 12.1% of the residue N at maturity. The absolute contribution of soil derived N to the subsequent crop was similar and averaged 149 mg N pot^{-1}.

1.5 Objectives

Goal of the study was to investigate the effect of differences in the rhizodeposition of *Vicia faba, Lupinus albus* and *Pisum sativum* on the structural and functional diversity of bacteria present in the rhizospheres of these economically important legumes.

The main objectives of the present study were:

- **(i)** to characterize and compare the resident and active bacterial community in the rhizospheres of the three grain legumes grown in the same soil,
- **(ii)** to identify rhizosphere effects of different legumes with respect to bacterial diversity,
- **(iii)** to identify the predominantly active bacterial groups as a result of rhizosphere effect,
- **(iv)** to generate and compare the metabolic profiles of the three rhizosphere soils and
- **(v)** to study the functional diversity of the rhizospheres in relation to various genes important in the nitrogen cycle.

2. MATERIALS AND METHODS

2.1 Experimental design and sampling

Soil samples from a farm near Osnabrück in northwest Germany (52.31° north; 8.13° east) were taken from the top 0 – 20 cm of a Eutric Cambisol. The field had been cultivated using organic farming management for the last ten years. Clover grass had been earlier grown in this soil as a green manure, which was followed by wheat cultivation. The field had been mulched once after the harvest of wheat. The soil was characterized as a sandy loam with 17.3% clay, 30.1% silt, 52.6% sand, pH (0.01 M $CaCl_2$) 6.0, 1.58% total C, 0.15% total N, 140 mg P kg^{-1} (CaL), 208 mg K kg^{-1} (CaL) and 100 mg Mg kg^{-1} (CaL). The maximum water holding capacity (10 mm sieved soil) was 309 g H_2O kg^{-1} dry soil. Fresh soil samples were sieved through a 10 mm mesh, prior to use. The soil was stored moist in a cool (6°C) and dark place until initiation of the experiment.

Ten rectangular polyethylene boxes per legume (60 x 45 x 40 cm) were filled with about 70 kg of soil per box. Seeds of faba beans (*Vicia faba* L., cv. Scirocco), peas (*Pisum sativum* L., cv. Duel) and white lupin (*Lupinus albus* L., cv. Amiga) were inoculated with legume specific *Rhizobium* inoculants (*R.leguminosarum* bv. *viciae* for *Vicia faba* and *Pisum sativum* and *R.lupini* for *Lupinus albus*) from Radicin, Radicin Institute, Iserlohn, Germany. Suspensions of the inoculants were prepared in PBS buffer (see section 2.2.6). Concentration was adjusted for both the inoculants to OD of 1.00 at 600 nm. Seeds of the legumes were incubated in this suspension for 1 h and then three seeds were sown in each pot (03.11.2000). All the thirty seeds germinated into plants. The plants were grown at 18°C with 70% humidity and a photoperiod consisting of 16 h of light and 8 h of darkness (luminance of ~14 klx) in a green house. The plants were watered equally every alternate day. The health of the plants was monitored during this period by the visual observation of the colour of their leaves. Sampling was performed six months after planting, in the fruiting stage (03.05.2001). Plants and soil were removed from the pots. Roots seemed intact and nodules could be observed at the time of sampling. Subsequently, excess bulk soil was removed from the roots by shaking, leaving firmly adhering soil, which was defined as rhizosphere soil. Samples were stored at –20°C.

2.2 Buffers and Media

2.2.1 CTAB Extraction Buffer

Solution A (10% CTAB – 0.7M NaCl)

CTAB	10 g
NaCl	4.09 g
DEPC treated distilled water	100 ml

Solution B (240 mM potassium phosphate buffer)

potassium phosphate buffer (pH 8.0)	3.26 g
DEPC treated distilled water	100 ml

Mixed equal volumes of Solutions A and B.

2.2.2 LB Medium

Peptone	10 g
NaCl	10 g
Yeast extract	5 g

Volume was adjusted to 1 l using distilled water. pH was adjusted to 7.5.

2.2.3 30% Polyethylene glycol – 1.6 M NaCl

Polyethylene glycol	30.00 g
NaCl	9.36 g
DEPC treated water	100 ml

2.2.4 50x TAE Buffer

Tris base	242.0 g
EDTA	18.6 g
Glacial Acetic Acid	57.1 ml

Volume was adjusted to 1 l using distilled water. pH was adjusted to 8.0.

2.2.5 5x TBE Buffer

Tris-base	54.0 g
Boric Acid	27.5 g
EDTA	2.92 g

Volume was adjusted to 1 l using distilled water. pH was adjusted to 8.0.

2.2.6 PBS Buffer

NaCl	8.0 g
Na_2HPO_4	1.4 g
KCl	0.2 g
KH_2PO_4	0.2 g

Volume was adjusted to 1 l using distilled water. pH was adjusted to 7.4.

2.3 Nucleic Acid Extraction

Nucleic acid extraction from the rhizosphere soil material was performed using the method of co-extraction of DNA and RNA described by Griffiths *et al.* (2000). This involved bead beating and solvent extraction of the nucleic acids (Figure 1). Precautions were taken to prevent degradation of RNA by RNases. All glassware were baked overnight at 200°C and rinsed with diethylene pyrocarbonate (DEPC) treated water. All solutions were prepared with DEPC treated water (2 h with 0.1% DEPC at 37°C, followed by autoclaving at 121°C for 20 min).

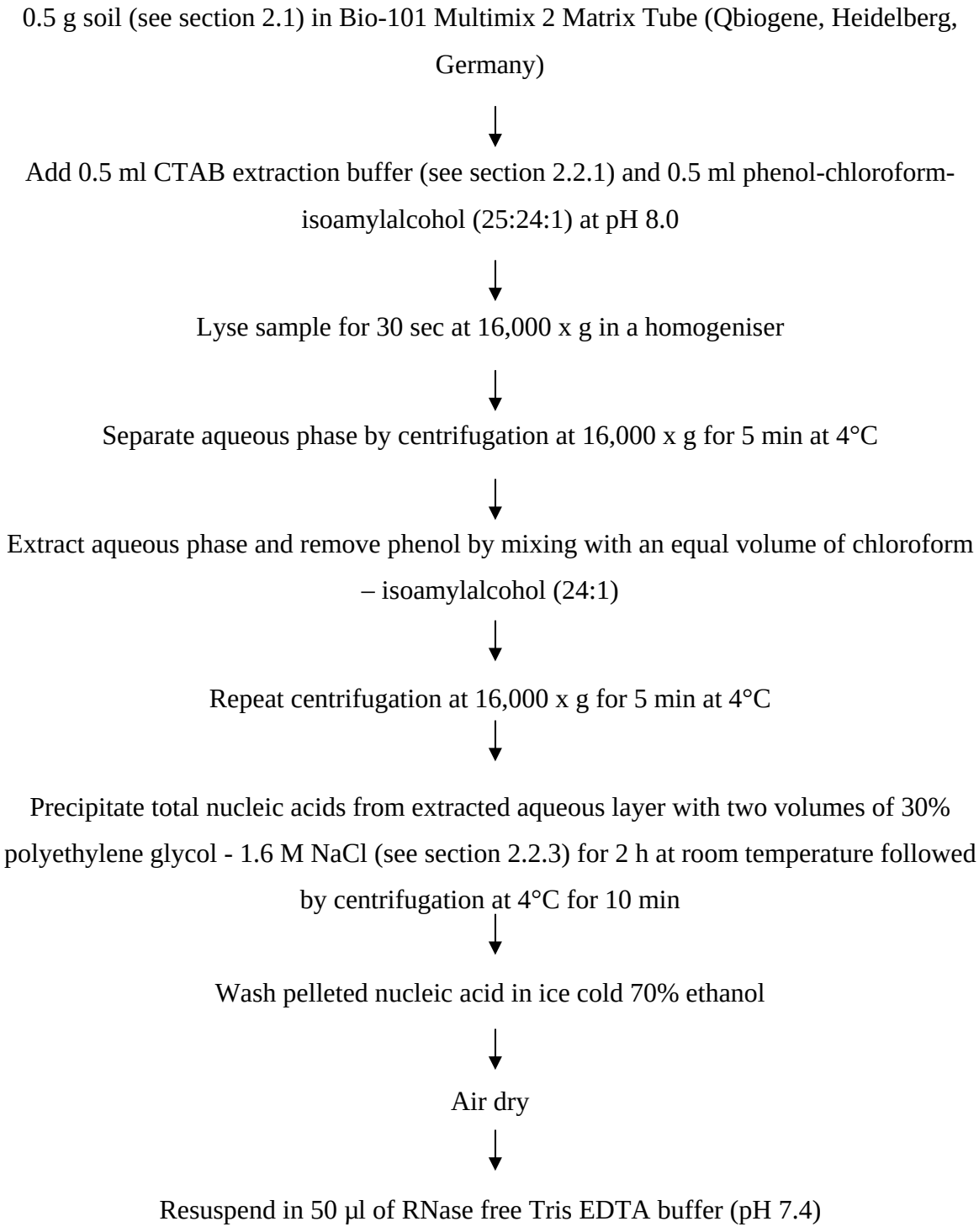


Figure 1. Flow diagram for extraction of nucleic acids from soil according to Griffiths *et al.*'s (2000) method of co-extraction of DNA and RNA.

To obtain pure DNA, RNA was removed by incubation with RNase A (Sigma, Munich, Germany) at a final concentration of 100 μg ml^{-1} at 37°C for 10 min. Prior to reverse-transcription, DNA was removed from RNA by treatment with DNase (1 U μl^{-1}; RNase free; Promega, Mannheim, Germany) according to the protocol described below.

- DNase digestion reaction:

Nucleic acid	24 μl
RQ1 RNase-Free DNase 10x Reaction Buffer	3 μl
RQ1 RNase-Free DNase	3 μl

- Incubated at 37°C for 30 min.
- 3 μl of RQ1 DNase Stop Solution was added to terminate the reaction.
- Incubated at 65°C for 10 min to inactivate the DNase.

2.4 cDNA Synthesis

Reverse transcription of RNA was performed in a final reaction mixture of 20 μl as described in following table using Omniscript RT Kit (Qiagen, Hilden, Germany).

Component	Volume / Reaction	Final concentration
10x Buffer RT	2.0 μl	1x
dNTP Mix (5 mM each dNTP)	2.0 μl	0.5 mM each dNTP
Primer* (10 μM)	2.0 μl	1 μM
RNase inhibitor (10 U μl^{-1})	1.0 μl	10 U 20 μl^{-1} reaction
Omniscript Reverse Transcriptase	1.0 μl	4 U 20 μl^{-1}reaction
RNase-free water	Variable	to make final vol. 20 μl
Template RNA (see section 2.3)	Variable	Up to 2 μg $reaction^{-1}$

*Random hexamer (NNNNNN) was used in the reaction.

The reaction mixture was incubated at 37°C for 90 min. The reaction was stopped by heating to 93°C for 5 min followed by rapid cooling on ice.

2.5 PCR and RT-PCR amplification

Amplifications were performed for different targets as described in the Table 2.

Target	Primers used (Reference)	Cycling Programme (denaturation / annealing / elongation)	No. of cycles
16S rDNA & rRNA	F984-GC & R1378 (Nübel *et al*., 1996)	94°C-1 min / 54°C-1 min / 72°C-1 min	35
Actinomycetes (nested)	F243 & R1378	94°C-1 min / 54°C-1 min / 72°C-1 min	35
	F984-GC & R1378 (Heuer *et al*., 1997)	94°C-1 min / 54°C-1 min / 72°C-1 min	35
Chitinase (nested)	GA1F & GA1R	94°C-1 min / 54°C-1 min / 72°C-1 min	30
	GASQF & GASQR (Williamson *et al*., 2000)	94°C-1 min / 54°C-1 min / 72°C-1 min	30
Serine peptidase	*sub* Ia & *sub* II (Bach *et al*., 2001)	94°C-30 sec / 55°C-30 sec / 72°C-30 sec	35
Neutral metallopeptidase	*npr* I & *npr* II (Bach *et al*., 2001)	94°C-30 sec / 55°C-30 sec / 72°C-30 sec	35
nirK	nirK1F & nirK5R (Braker *et al*., 1998)	94°C-30 sec / 48°C-40 sec / 72°C-40 sec	30
nirS	nirS1F & nirS6R (Braker *et al*., 1998)	94°C-30 sec / 48°C-40 sec / 72°C-40 sec	30

Table 2. Primers and cycling conditions used for various targets investigated in the present study.

Prior to the cycling conditions, a hot start of 94°C for 5 min was performed for all samples. Enzyme was added to the reaction mix during the pause. Cycles were followed by a final extension at 72°C for 10 min. All PCR and RT-PCR products were stored at 4°C for further analysis. Table 3 shows the volumes (in μl) of different components used in PCR and RT-PCR mix in 50 μl reactions. The primer stocks used were 100 pmol μl^{-1} unless otherwise specified.

Component \ Target	16S	Actinomycetes	Chitinase	*sub*	*npr*	*nirK*	*nirS*
Buffer (10x)	5	5	5	5	5	5	5
$MgCl_2$ (25mM)	-	-	3	6	6	-	-
dNTP (2mM)	5	5	2.5	2.5	2.5	5	5
Primer (Forward)	1[c]	1[c]	0.25	0.5	0.5	1	1
Primer (Reverse)	1[c]	1[c]	0.25	0.5	0.5	1	1
Bovine serum albumin (3%)	5	5	5	5	5	2	2
Dimethyl sulfoxide (DMSO)	2.5	2.5	2.5	-	-	1	1
Template (DNA / cDNA)[d]	1	1	1	1	1	1	1
Nuclease free water	28.5	28.5	30	29	29	33	33
Enzyme	1[a]	1[a]	0.5[b]	0.5[b]	0.5[b]	1[a]	1[a]

[a] Cloned *Pfu* DNA Polymerase (2.5 U μl^{-1}, Stratagene, Amsterdam, The Netherlands)
[b] AmpliTaq® DNA Polymerase, Stoffel Fragment (10 U μl^{-1}, Applied Biosystems, Foster City, CA).
[c] Stock of 10 pmol μl^{-1} of primers used.
[d] See section 2.3

Table 3. Amounts of various components in the amplification reactions.

2.6 AP-PCR and RAP-PCR

Fingerprinting of nucleic acids were performed using AP-PCR and RAP-PCR for DNA and RNA respectively. Table 4 shows the volumes of different components used in AP-PCR and RAP-PCR mix in a 25 µl reaction. Thermal cycler was programmed as: 94°C for 5 min to denature, 37°C for 5 min for low stringency annealing of the primer and 72°C for 5 min for extension for 2 cycles. This was followed by 45 cycles of 1 min at 94°C, 1 min at 37°C, 2 min at 72°C. Final extension was programmed at 72°C for 10 min followed by cooling at 4°C. The same protocol was used with two different primers: M13 reverse (5'GGAAACAGCTATGACCATG3') and 10 mer (5'TCACGATGCA3') primers.

Component	Vol. in µl
Buffer (10x)	2.5
$MgCl_2$ (50 mM)	1.0
dNTP (2 mM)	1.25
Primer (100 pmol)	0.5
BSA (3%)	2.5
DMSO	1.25
Template*	1.0
Nuclease free water	14.75
Taq DNA Polymerase	0.25

*Template: DNA for AP-PCR and RNA for RAP-PCR (see section 2.3 for nucleic acid extraction).

Table 4. PCR set up for AP-PCR and RAP-PCR.

2.7 Dot Blot Hybridisation

For dot blot hybridisation on positively charged nylon membranes (Roche Diagnostics, Mannheim, Germany), 10 µl of RAP-PCR product (see section 2.6) was denatured in 240 µl of 0.4 N NaOH for 20 min and vacuum blotted. DNA was fixed to the membrane by UV-cross-linking. Hybridisation with DIG labelled probes for neutral metallopeptidase and serine peptidase (Bach *et al.*, 2001) was performed as follows: formamide concentrations in the pre-hybridisation solution were 5% for neutral metallopeptidase and 0% for serine peptidase. Pre-hybridisation was performed in 5x SSC, 0.1% N-lauroylsarcosine, 0.02% SDS, 1% blocking reagent and formamide for 1.5 h at 45°C. Hybridisation with 10 pmol ml^{-1} of 5' DIG-labelled probe in pre-hybridisation solution was done for 2.5 h at 45°C. Washings were performed 2 × 5 min with 2 × SSC, 0.1% SDS at room temperature and 2 × 15 min in 0.5 × SSC, 0.1% SDS at 45°C. Detection was performed by using the DIG Luminescent Detection Kit (Roche Diagnostics, Mannheim, Germany) as recommended by the manufacturer. PCR products for *sub* (serine peptidase) and *npr* (neutral metallopeptidase) genes of *Bacillus cereus* were generated using the protocol described by Bach *et al.* (2001) and used as controls for specificity of the probes.

2.8 Gel Electrophoresis

2.8.1 Agarose Gel Electrophoresis

PCR and RT-PCR reactions (see section 2.5) were checked for products and their approximate concentrations on 1.5% agarose (Biozym, Oldendorf, Germany) gels prepared in 1x TAE buffer (see section 2.2.4) and run at 100 V for 1.5 h in 1x TAE buffer followed by 15 min staining with ethidium bromide (0.5 mg l^{-1}).

2.8.2 Polyacrylamide Gel Electrophoresis (PAGE)

8% non-denaturing polyacrylamide gels (ratio of acrylamide to bisacrylamide, 29:1) were prepared with the following constituents as described by Sambrook *et al*. (1989).

30% Acrylamide solution (Bio-Rad Laboratories, Munich, Germany)	10.64 ml
dH_2O	21.08 ml
5x TBE (see section 2.2.5)	8.00 ml
10% ammonium persulphate (APS)	250 µl
TEMED	17 µl

Appropriate volumes containing about 2 µg of AP-PCR and RAP-PCR products (see section 2.6) were loaded. The gels were electrophoresed at 50 V for 17 h in 1x TBE (see section 2.2.5) using D-Gene system (Bio-Rad Laboratories, Munich, Germany).

2.8.3 Denaturing Gradient Gel Electrophoresis (DGGE)

DGGE was performed using 6% polyacrylamide gels (ratio of acrylamide to bisacrylamide, 37:1) with a gradient of 54 - 60% denaturant as in Table 5. 100% denaturant is defined as 7 M urea plus 40% formamide (Abrams and Stanton, 1992). Appropriate volumes containing about 2 µg of the purified PCR and RT-PCR products, measured by absorbance at 260 nm, and were loaded. The gels were electrophoresed at 60°C at 50 V for 17 h using D-Gene system (Bio-Rad Laboratories, Munich, Germany).

Percentage of denaturant	Component	Volume of component
54%	0%	5.5 ml
	100%	6.5 ml
	APS (10%)	50 µl
	TEMED	10 µl
64%	0%	4.4 ml
	100%	7.6 ml
	APS (10%)	50 µl
	TEMED	10 µl
Stacking Solution	0%	8 ml
	APS (10%)	35 µl
	TEMED	8 µl

Table 5. Amounts of various components used in DGGE.

2.9 Silver staining

Polyacrylamide (PAGE) and denaturing gradient gels (DGGE) were silver stained using a modified version of the protocol described by Heukeshoven and Dernick, 1986 (see Table 6).

2.10 Image analysis

Dried gels were scanned using HP Scanjet 7400c. The AP-, RAP-PCR and DGGE profiles (see sections 2.8.2 and 2.8.3) obtained were analysed by clustering via the unweighted pair group method with mathematical averages (UPGMA; Dice coefficient of similarity) using GelCompar II Software (Applied Maths, Kortrijk, Belgium). The position tolerance was set at 1% and background subtraction was applied. Both strong and weak bands were included in the analysis, thus taking into account only the presence and absence of bands at specific positions. Cophenetic correlations were calculated using the same software.

Solution	Components	Duration
Fixing		>30 min
Glacial acetic acid	25 ml	
Milli Q water	225 ml	
Washing		3 x 2 min
Milli Q water	250 ml	
Silver reaction		25 min
$AgNO_3$	0.37 g	
Formaldehyde (37% w/v)	0.25 ml	
Milli Q water	250 ml	
Washing		2 x 1 min
Milli Q water	250 ml	
Developing		5 min or more
Na_2CO_3, anhydrous	6.25 g	
Formaldehyde (37% w/v)	0.25 ml	
Sodium thiosulphate (2% w/v)*	0.25 ml	
Milli Q water	adjust vol. to 250 ml	
Stopping		10 min
EDTA-Na_2 x 2H2O	3.65 g	
Milli Q water	adjust vol. to 250 ml	
Preserving		>30 min
Glycerol (100%)	30 ml	
Ethanol	75 ml	
Milli Q water	195 ml	

*Sodium thiosulphate stock solution (2% w/v):
$Na_2S_2O_3$ x $5H_2O$ 3.14 g
Milli Q water adjust vol. to 100 ml
Filter sterilize

Table 6. Protocol for silver staining.

2.11 Cloning

Purified amplification products were cloned into pCR®-Blunt II-TOPO® vector of Zero Blunt® TOPO® PCR Cloning Kit (Invitrogen, Karlsruhe, Germany) as described below.

Purified PCR / RT-PCR product	3 µl (approx. 0.2 µg)
Salt Solution	1 µl
Sterile water	1 µl
Topo Cloning Vector	1 µl

This ligation mix was incubated for 30 min at room temperature. 2 µl of ligation mix was transformed into chemically competent One Shot® DH5α^{TM}-T1^{R} cells provided in the kit following manufacturer's instructions. Colonies were inoculated in LB medium (see section 2.2.2; supplemented with 50 µg ml^{-1} kanamycin). Plasmids were isolated using Qiagen Plasmid Mini Kit (Qiagen, Hilden, Germany). Purified plasmids were tested for inserts by *Eco*RI digestion (MBI Fermentas, Heidelberg, Germany). Digestion was set using the following protocol.

10x Buffer *Eco*RI	2 µl
Plasmid	5 µl
Restriction enzyme (10 U µl^{-1})	0.1 µl
Nuclease-free water	12.9 µl

Digestion was incubated at 37°C for 1 h. This was followed by inactivation of the restriction enzyme at 65°C for 10 min. The digested products were checked on 1.5% agarose at 100 V for 1 h.

2.12 Restriction Fragment Length Polymorphism (RFLP)

Plasmids were digested in a final volume of 20 µl using two different restriction enzymes: *Msp*I and *Hin*6I (MBI Fermentas, Heidelberg, Germany) using the following protocol.

10x Buffer Y^{+}/Tango	2 µl
Plasmid (see section 2.11)	5 µl
Restriction enzyme (10 U µl^{-1})	0.1 µl
Nuclease-free water	12.9 µl

Digestions were incubated at 37°C for 4 h. This was followed by inactivation of the restriction enzyme at 65°C for 10 min. The digested products were checked on 4% high resolution agarose (Qbiogene, Heidelberg, Germany) at 100 V for 2 h.

2.13 Sequencing and sequence analysis

Inserts were sequenced on ABI PRISM® 310 Genetic Analyzer (Applied Biosystems, Foster City, USA) using CEQ 2000 Dye Terminator Cycle Sequencing with Quick Start Kit (Beckman Coulter, CA). The following protocol was followed.

DTCS Quick Start Master Mix	8 µl
-47 Sequencing Primer (1.6 pmol μl^{-1}; included in kit)	2 µl
Template	135-150 ng
dH_2O	adjust vol. to 20 µl

Thermal cycling programme followed was: 96°C for 20 sec, 50°C for 20 sec, 60°C for 4 min for 30 cycles. Ethanol precipitation was performed as described below.

- 4 µl of Stop Solution (1.5 M NaOAc + 50 mM EDTA prepared fresh by mixing equal volumes of 3 M NaOAc and 100 mM EDTA) and 1 µl of 20 mg ml^{-1} glycogen was added to each reaction.
- 60 µl cold 95% (vol/vol) ethanol / dH_2O was added and centrifuged at 14,500 x g at 4°C for 15 min. The supernatant was discarded and the pellet rinsed twice with 200 µl of 70% (vol/vol) ethanol / dH_2O and centrifuged at 14,500 x g at 4°C for 2 min.
- Pellets were vacuum dried for 40 min and resuspended in 40 µl of Sample Loading Solution (provided in the kit) before loading on to the sequencer.

Sequences were compared with NCBI BLAST and aligned using the CLUSTAL W programme of EMBL. Phylogenetic trees were viewed using TreeView software (Page, 1996). Only one of each set of repetitive sequences was used to construct phylogenetic trees.

2.14 Nucleotide sequence accession numbers

The clone sequences determined in this study have been submitted to GenBank under accession numbers AY143694 to AY143793 and AY144121 to AY144124 for 16S clones and AY386223 to AY386234 for *nirK* clones.

2.15 Enzyme assay

Reactions for determining the activity of chitinase were carried out in 96 well flat bottom micro-titre plates (NeoLab, Heidelberg, Germany). The protocol described in Table 7 enlists the different components and their amounts (in µl) for each well.

Components	**Sample**	**Autofluorescence control**	**Quenching control**	**Negative control**	**Calibration**
Soil suspension	50	50	50	-	-
Milli Q water	50	100	50	100	50
MUF-glucosaminide (200 µM)	50	-	-	50	-
H_2O + 2 µM MUF	-	-	50	-	100
Tris Ethanol* (after incubation)	100	100	100	100	100

*2.5 M Tris (pH 10-11) – 25 µl
Ethanol – 75 µl

Table 7. Set up for chitinase assay.

To obtain the calibration curve, 25 µM MUF was diluted according to Table 8.

Calibration solution well^{-1}	**0.5 µM**	**1.0 µM**	**1.5 µM**	**2.0 µM**	**2.5 µM**	**3.0 µM**	**3.5 µM**
25 µM MUF (µl)	20	40	60	80	100	120	140
Milli Q water (µl)	980	960	940	920	900	880	860

Table 8. Set up of calibration for chitinase assay.

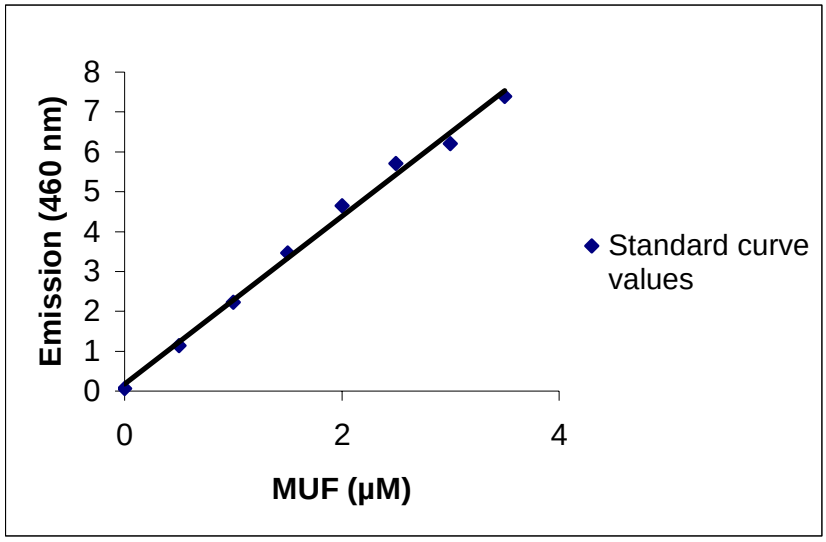


Figure 2. Standard curve for MUF.

Chitinase assay was performed in triplicate on the water-soluble fraction of the rhizosphere soil. To prepare water-soluble fraction 10 ml of MilliQ water was added to 100 mg of rhizosphere soil sample (see section 2.1) in a 50 ml falcon tube. The tube was shaken for 30 min and the supernatant passed through a nylon mesh of pore size 60 µm. The filtrate was then used in further reactions. Reactions were started by addition of the substrate and stopped with a mixture of 2.5 M Tris-ethanol. The plates were then centrifuged for 10 min. Readings were made in a spectrophotometer at excitation and emission wavelengths of 390 and 460 nm respectively. A standard curve was established by using MUF (Sigma, Munich, Germany) in the presence of chitin in order to relate relative fluorescence units to milli moles of MUF hydrolysed per gram (dry weight) of chitin (Figure 2).

3. RESULTS

3.1 STRUCTURAL DIVERSITY OF THE BACTERIAL POPULATION

3.1.1 Analysis of 16S rDNA by PCR and DGGE

Universal bacterial primers, F984-GC and R1378, were used to amplify the hypervariable V3-V6 region of 16S rDNA. The three rhizosphere soil samples yielded discrete bands with the predicted size of 473 bp (433 bp insert + 40 bp GC-clamp) after adaptation of the protocol to the requirement of the samples (Figure 3). BSA and DMSO had to be added to the amplification reaction during optimisation to prevent inhibitory effects of humic substances present in soil. The PCR products were purified using QIAquick PCR purification kit (Qiagen, Hilden) to get rid of single stranded nucleic acids and primer artefacts.

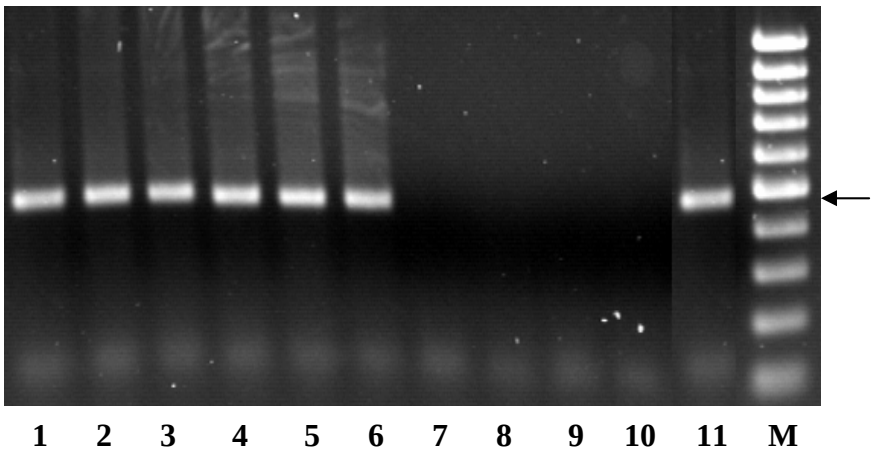


Figure 3. Agarose gel of PCR and RT-PCR fragments for 16S rDNA and rRNA. Lanes 1, 2, 3: PCR products; lanes 4, 5, 6: RT-PCR products; lanes 7, 8, 9: $DNase^{+}RT^{-}$ PCR products for *Vicia*, *Lupinus* and *Pisum* respectively; lane 10: negative control; lane 11: PCR with *E.coli* as positive control; lane M: 100 bp marker. Arrow marks the expected band of 473 bp.

The purified products were subsequently resolved by DGGE. Initially, the gradient used for resolution was 45 - 62%. High density of bands was obtained in the lower half of the gel. The gradient for the subsequent DGGE gels was then altered accordingly to 54 - 60% of urea and formamide, which resulted in better resolution of amplicons. DGGE profiles of thirty plants for each rhizosphere soil type (ten pots per legume and three plants per pot) were compared to look for sampling variations. High reproducibility of these patterns was found in duplicate performances of the PCR as well as multiple nucleic acid extractions of the same sample and subsequent PCR and DGGE analysis. The patterns for different pots

for the same legume were similar to each other as evident on comparison using GelCompar II (Figures 4a, b, c). In order to compare DGGE patterns, Dice coefficient was determined and UPGMA was used to create a dendrogram describing pattern similarities. The similarity level between the lanes for each legume was observed to be greater than 90%. Hence, only one representative sample from each rhizosphere soil type was used for further analysis.

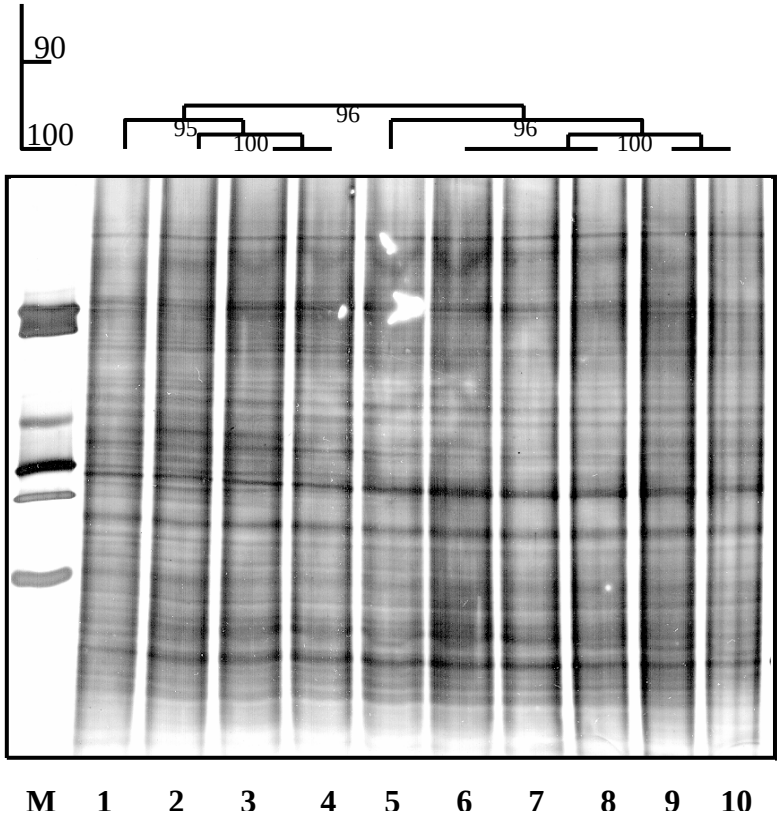


Figure 4a. DGGE profiles and UPGMA tree of PCR products of rhizosphere soil samples of ten different pots of *Vicia*. Lane M: marker, lanes 1 - 10: profiles from pots V1 - V10 respectively. Scale represents percent similarity. Values of cophenetic correlations are mentioned at the branches.

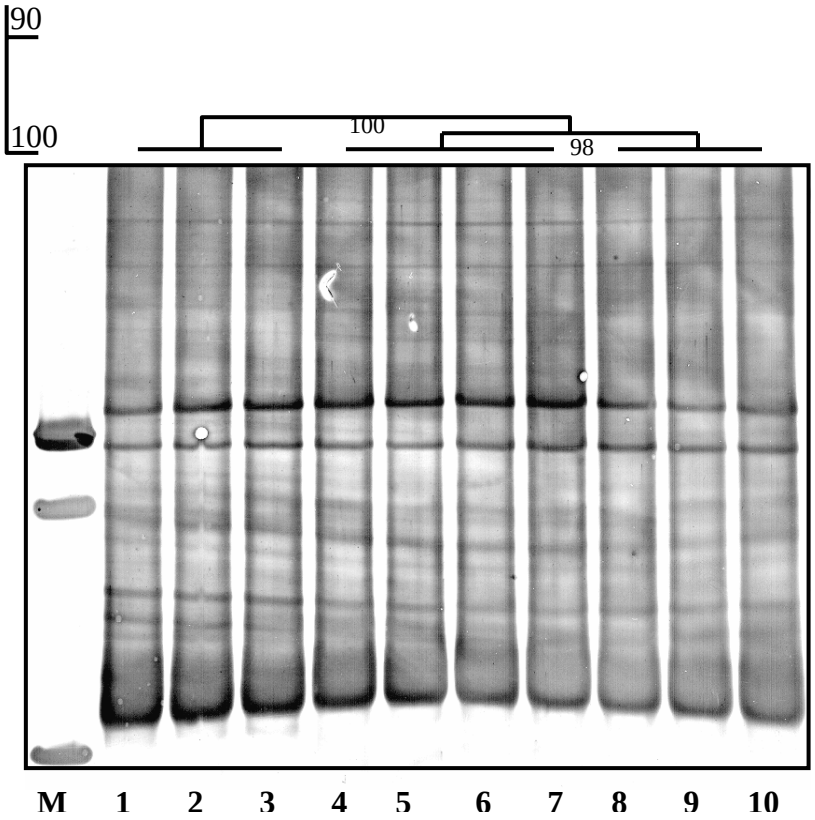


Figure 4b. DGGE profiles and UPGMA tree of PCR products of rhizosphere soil samples of ten different pots of *Lupinus*. Lane M: marker, lanes 1 - 10: profiles from pots Lu1 - Lu10 respectively.

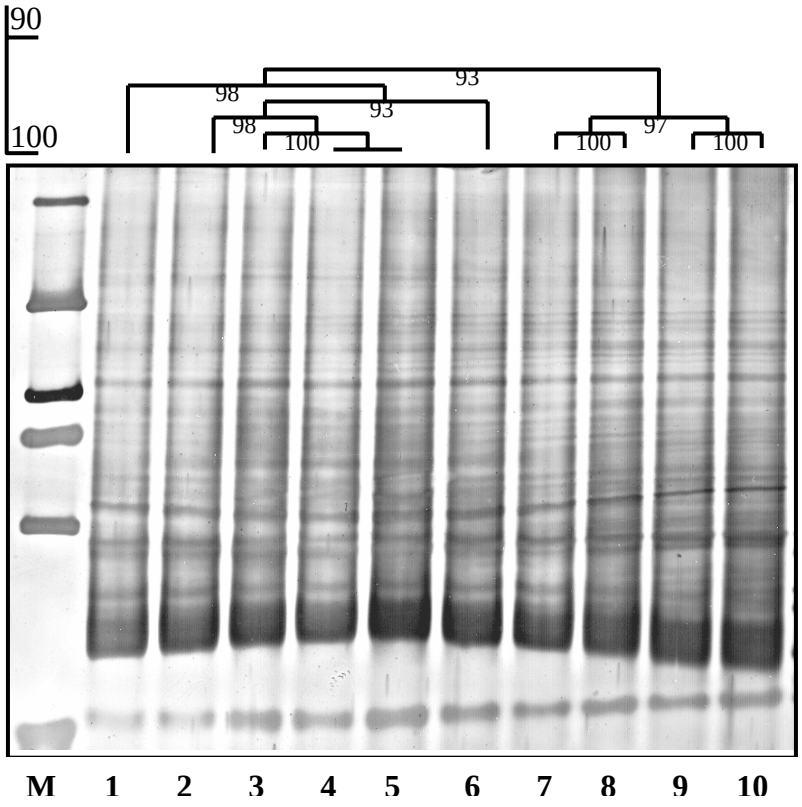


Figure 4c. DGGE profiles and UPGMA tree of PCR products of rhizosphere soil samples of ten different pots of *Pisum*. Lane M: marker, lanes 1 - 10: profiles from pots P1 - P10 respectively.

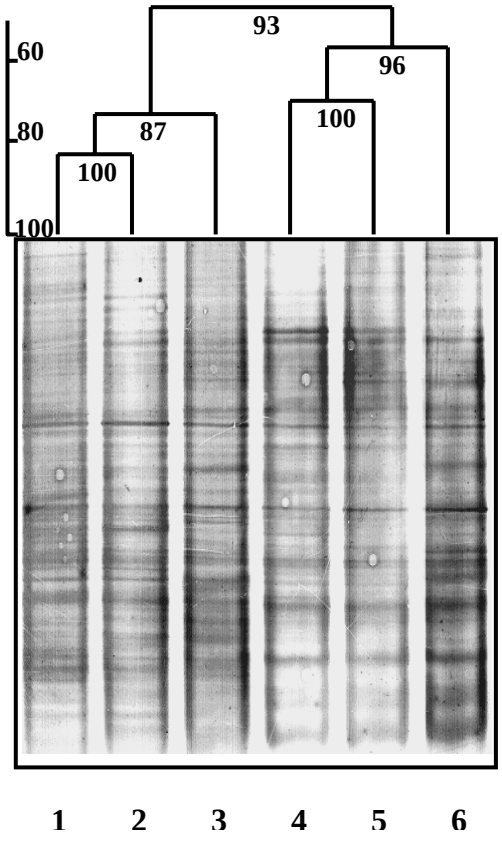


Figure 5. DGGE profiles and UPGMA tree of 16S rDNA and rRNA fingerprints of rhizosphere soil samples of the three legumes. Lanes 1, 2, 3: 16S rDNA fragments of *Lupinus*, *Vicia* and *Pisum* rhizospheres respectively; lanes 4, 5, 6: 16S rRNA fragments of *Lupinus*, *Vicia* and *Pisum* rhizospheres respectively. UPGMA tree represents the similarity of the bacterial community profile obtained by PCR-DGGE and RT-PCR-DGGE from the three rhizospheres. Scale represents percent similarity. Values of cophenetic correlations are mentioned at the branches.

Each of the three legume rhizosphere soil samples compared produced a distinct molecular profile, which was largely, but not completely, different from the profile generated by the other two plant rhizosphere samples (Figure 5). About 25 - 30 distinct bands could be observed in each profile. Bacterial communities of *Lupinus* and *Vicia* seemed to be more similar to each other with a similarity value of about 80%. The profile generated by the PCR product of *Pisum* was only about 75% similar to the other two rhizospheres. Cophenetic correlation was used as a parameter to express the consistence of a cluster.

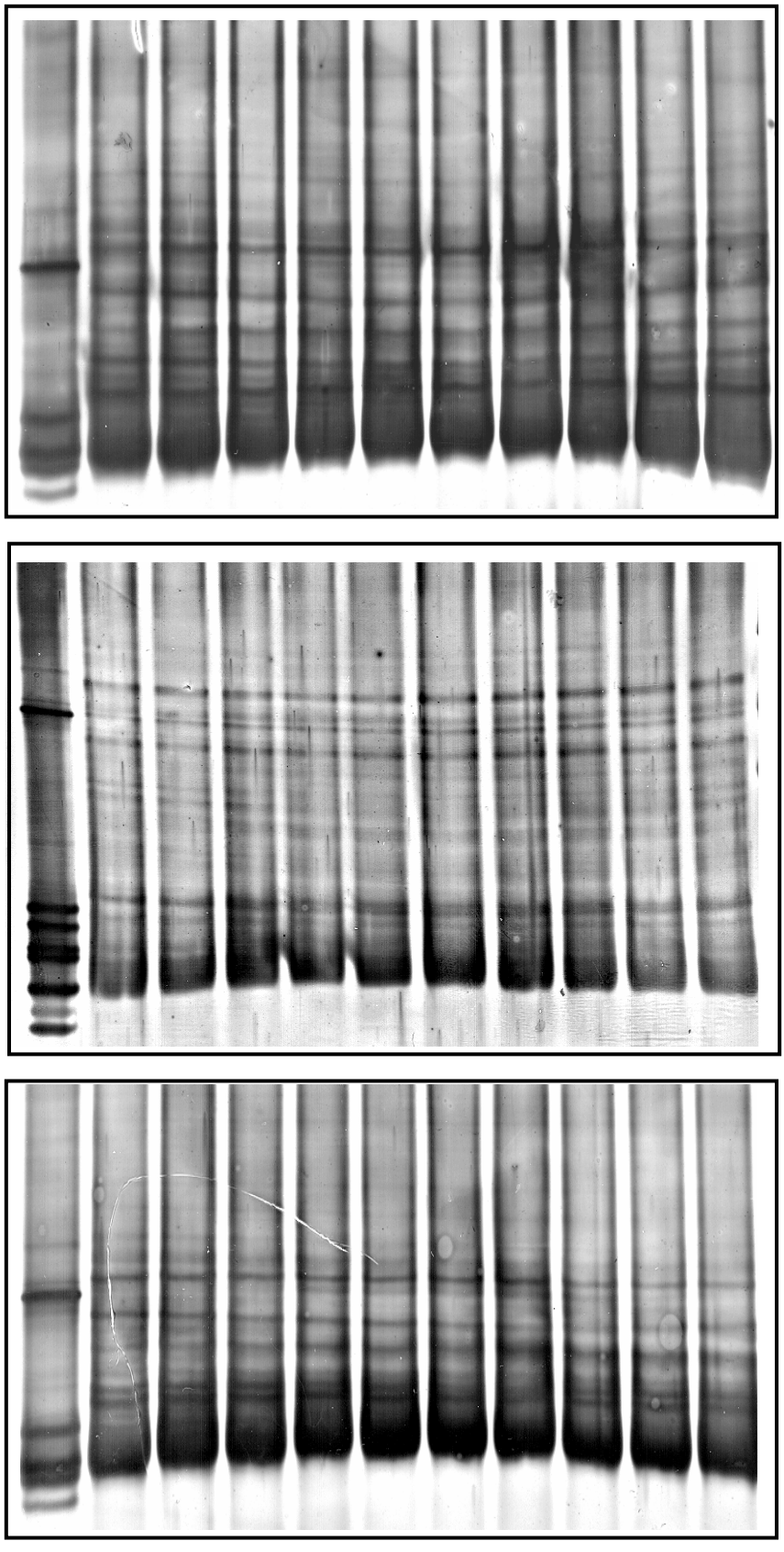


Figure 6a, b, c (top to bottom). DGGE profiles of 16S rRNA RT-PCR products of ten different pots of *Vicia* (a), *Lupinus* (b) and *Pisum* (c). Lane M: marker, lanes 1 - 10: profiles from pots 1 – 10 respectively.

3.1.2 Analysis of 16S rRNA by RT-PCR and DGGE

Reverse transcription of RNA was performed using random hexamers. Subsequent PCR (RT-PCR) using the same primers as used for 16S rDNA amplification, yielded the expected product of 473 bp. DNase treated nucleic acids, not subjected to RT ($DNase^{+}RT^{-}$), yielded no PCR products indicating no residual DNA in the RNA preparations (Figure 3). Similar to 16S rDNA analysis, the RT-PCR products were also resolved by DGGE on a gradient of 54 - 60% of urea and formamide. About 15 - 20 distinct bands could be observed in the fingerprints generated by the three plants. High reproducibility of the patterns generated by thirty plants for each rhizosphere soil type (ten pots per legume and three plants per pot) could be observed with duplicate PCR as well as multiple nucleic acid extractions and subsequent PCR and DGGE for same sample (Figures 6a, b, c). Similar patterns were observed for all the replicates. Hence, one representative sample from each rhizosphere soil type was used for further analysis.

The trend of similarity between the profiles was the same as observed in the profiles generated by 16S rRNA PCR products (Figure 5). *Vicia* and *Lupinus* profiles were more similar to each other than to *Pisum* but the values of similarity decreased to about 70% between *Vicia* and *Lupinus* and 60% between *Pisum* and the other two legume rhizosphere soil bacterial communities. This suggested that there were more differences between the "active" community present in these rhizospheres as compared to the total bacterial community. Cophenetic correlation values of more than 95% expressed the consistence of the cluster.

3.1.3 Relatedness between 16S rDNA and 16S rRNA profiles generated by DGGE

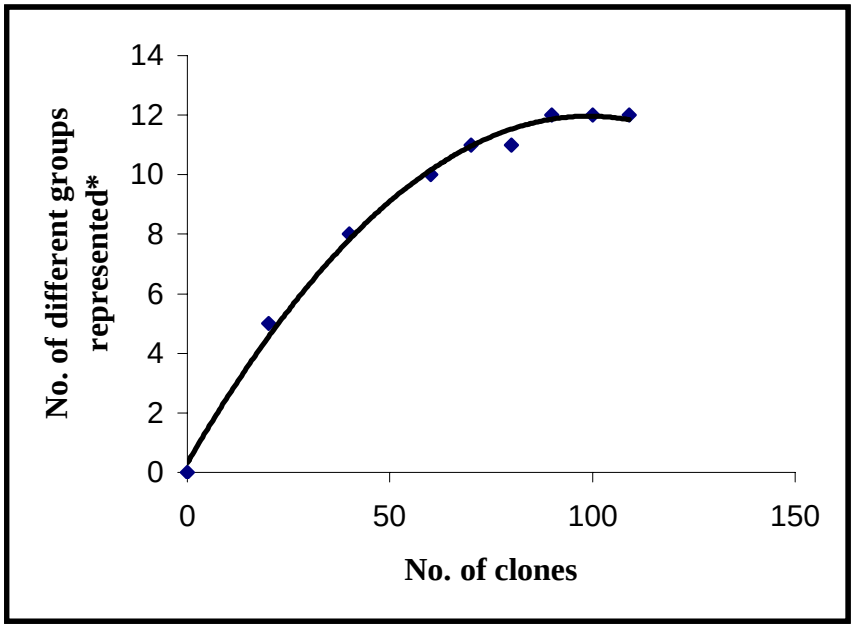
The number of distinct bands dropped from about 30 to only about 15 - 20 in the RT-PCR profiles. This indicated a lower diversity within the active component of the bacterial community. Comparison of the patterns generated by the PCR and RT-PCR products revealed that most of the bands (about 10 - 12) were common in the two profiles for each of the three different plant species under study. On analysing the profiles by GelCompar II, two distinct clusters were obtained (Figure 5). The PCR profiles clustered together and were only about 50% similar to the cluster formed by the RT-PCR profiles. This analysis clearly distinguished between the DNA and RNA derived DGGE patterns with two distinct clusters one each for DNA and RNA samples.

3.1.4 Cloning of 16S rDNA and rRNA products

Clone libraries were made for representative samples of each rhizosphere type with their respective 16S PCR and RT-PCR products to gain insight into the resident and active bacterial population. Varying amounts of amplified products were cloned during optimisation of the protocol. Approximately 0.2 µg of purified product resulted in the highest number of colonies per plate.

3.1.4.1 Collector's curve

To assess the number of clones sufficient enough to encompass the bacterial diversity, increasing numbers of clones were randomly picked and sequenced from 16S rDNA clone library of *Lupinus* rhizosphere. Collectors's curve or species abundance curve (the number of different groups detected plotted versus the number of clones analysed) was constructed (Kaiser *et al*., 2001). A plateau, as expected for full coverage of library, was obtained after screening 90 clones (Figure 7). No new group was observed even when the number of clones analysed was increased. Therefore, 100 clones were randomly picked from each of the remaining five libraries.



*Groups as defined in Figure 8, following NCBI nomenclature.

Figure 7. Collector's curve showing the number of different groups plotted as a function of number of clones for *Lupinus* rhizosphere 16S rDNA library.

3.1.4.2 Identification of clones and phylogenetic analysis

The amplified 433 bp sequences were used to assign the clones to bacterial groups using NCBI BLAST programme. A total of 600 clones were analysed (100 each from *Lupinus*, *Pisum* and *Vicia* 16S rDNA and rRNA libraries). After analysis for correct insert, five clones (1 each from the DNA library of *Lupinus* and *Pisum* and 3 from the cDNA library of *Lupinus*) had to be discarded due to lack of the expected insert. Figure 8 shows the broad phylogenetic distribution of clones within each library. Many clones belonged to previously characterized major groups including actinomycetes and proteobacteria. Recently recognized groups such as *Acidobacter* and *Verrucomicrobia* were also represented.

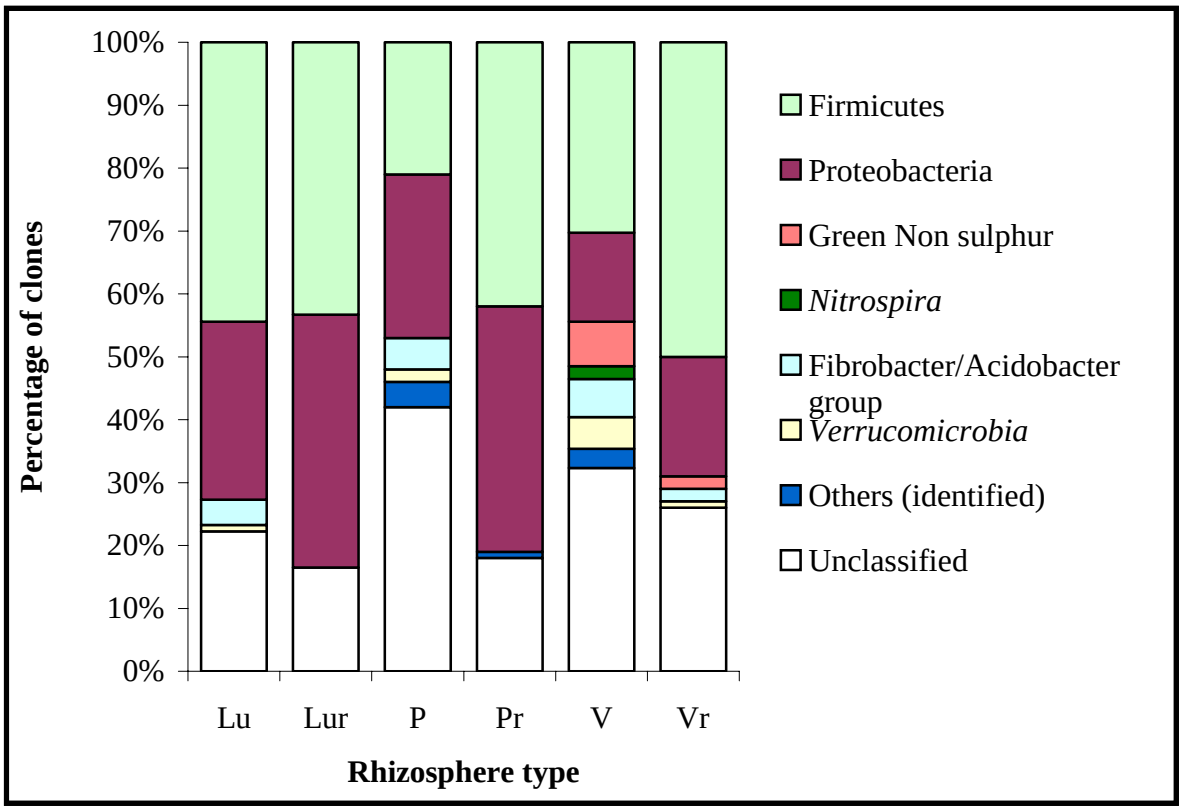


Figure 8. Relative distribution of clones to different phylogenetic groups. Lu, P and V stand for 16S rDNA libraries of *Lupinus*, *Pisum* and *Vicia* and Lur, Pr and Vr stand for 16S rRNA libraries of *Lupinus*, *Pisum* and *Vicia* respectively.

Firmicutes

Firmicutes constituted the most abundant group in both 16S rDNA and rRNA libraries for all the rhizosphere soil samples with more than 30% of the clones in *Vicia* and *Lupinus* libraries and 21% of the clones in *Pisum* DNA library (Figure 8). On the other hand, firmicutes in 16S rRNA library of *Pisum* constituted 42% of the clones, half of which were similar to *Actinobacteridae* members (Figure 9). Percentage of firmicutes increased considerably from 30% in rDNA libraries to 50% in *Vicia* rRNA library. In contrast, almost no change was observed between the values for *Lupinus* rhizospheres (44% in rDNA and 42% in rRNA libraries). Other groups like *Sphaerobacteridae*, *Rubrobacteridae*, *Bacillus-Clostridium* and unclassified firmicutes were present in variable proportions in the libraries with *Pisum* 16S rDNA and rRNA libraries containing all listed.

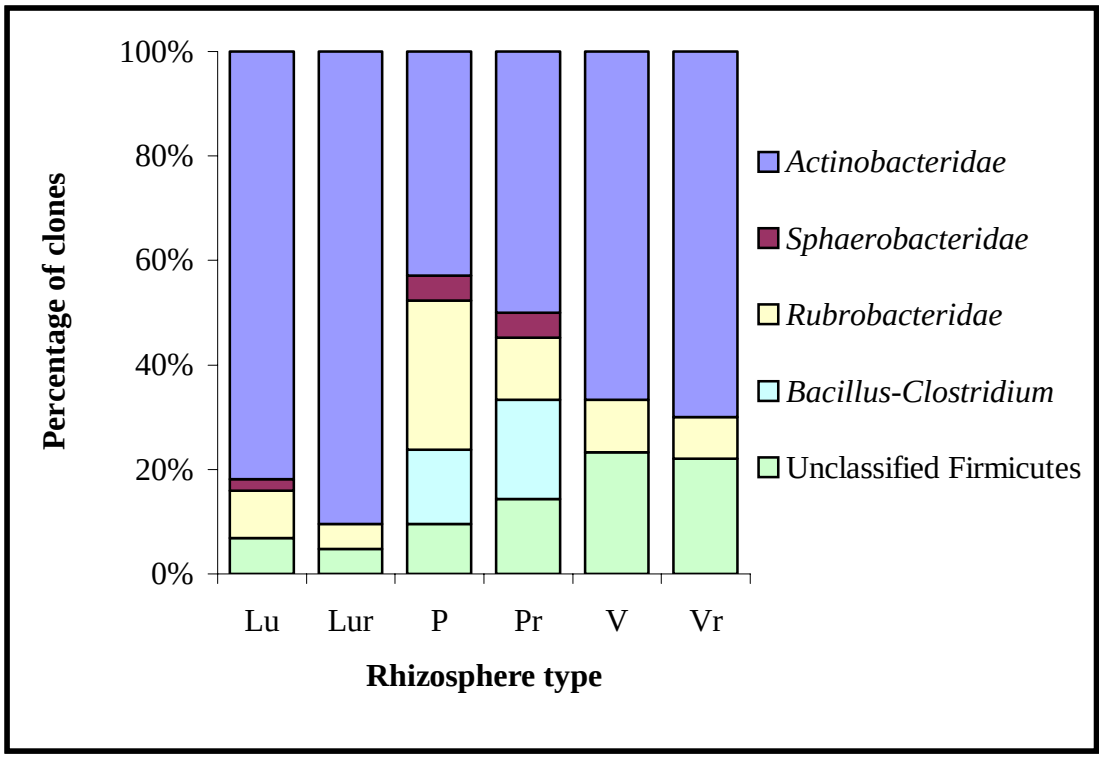


Figure 9. Relative distribution of clones to different groups of firmicutes. Lu, P and V stand for 16S rDNA libraries of *Lupinus*, *Pisum* and *Vicia* and Lur, Pr and Vr stand for 16S rRNA libraries of *Lupinus*, *Pisum* and *Vicia* respectively.

Firmicute clones in 16S rRNA libraries were observed to be a sub-set of clones of respective DNA libraries, with almost each clone being represented more than once in rRNA libraries. However, some clones were found exclusively in rRNA libraries. Phylogenetic correlation between selected reference bacteria and the firmicute clones displayed one prominent cluster including the majority of the DNA and RNA library clones clustering together with *Arthrobacter* sp. [Accession number AB070602] (Figure 10). A few clones were interspersed throughout the tree clustering closely with some of the genera like *Bacillus*, *Micromonospora*, *Nocardioides*, *Mycobacterium* and *Streptomyces*.

Figures 10. & 11. [Pages 38 & 39] Phylogenetic correlation between reference bacteria and clones (Figure 10. firmicutes; Figure 11. proteobacteria) found in this study. *Lupinus*, *Pisum* and *Vicia* rhizosphere clones are represented as Lu, P and V for rDNA clones and Lur, Pr and Vr for rRNA clones respectively, followed by the clone number and GenBank accession numbers. For environmental clones NCBI accession numbers have been mentioned. Accession numbers of reference organisms in trees have been mentioned in brackets besides the organism's name. For convenience, the tree was pruned from a larger tree containing additional sequences from reference bacteria. Clones in bold have band positions marked in Figure 13. The scale bar indicates the expected number of changes per sequence position.

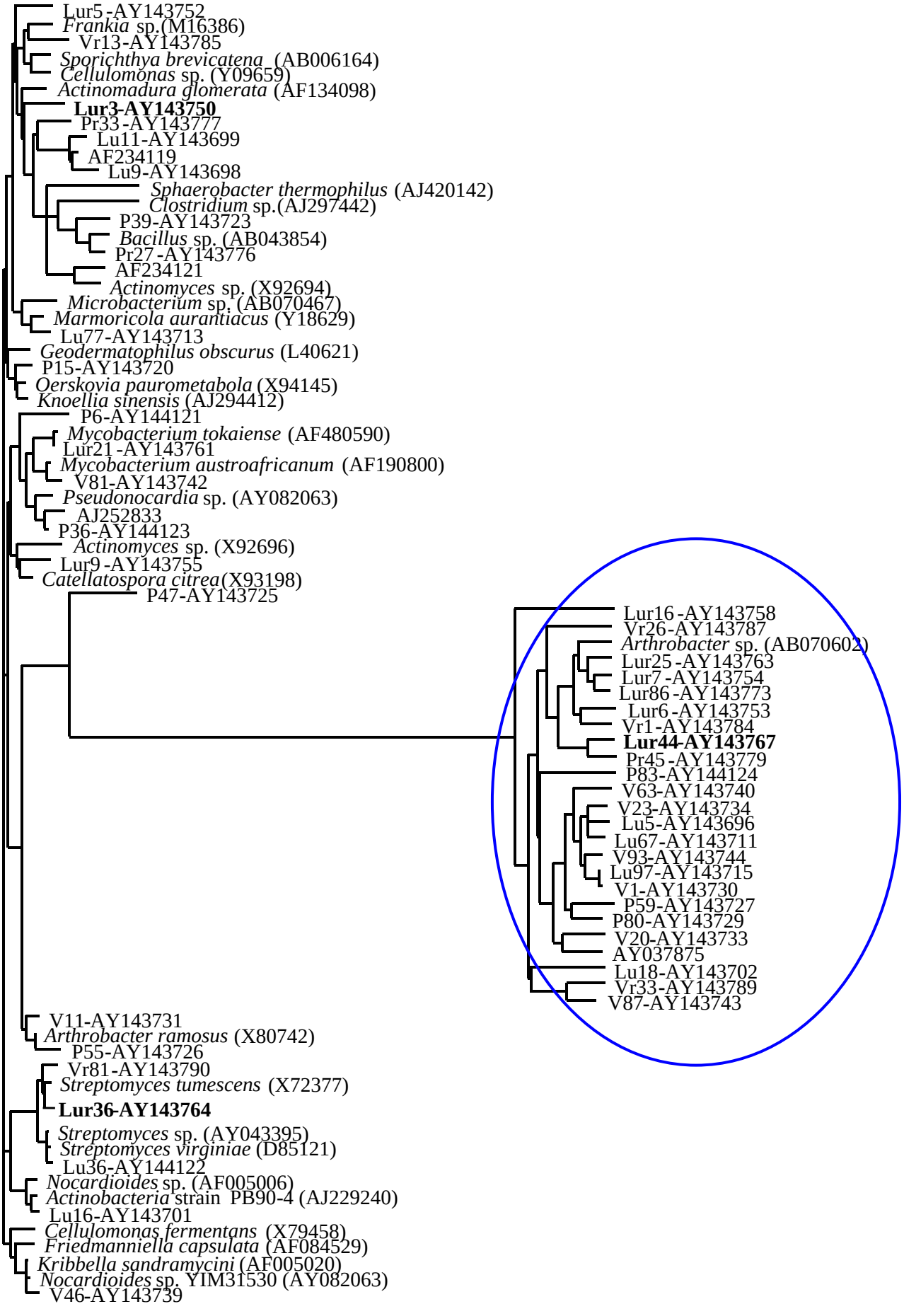


Figure 10. Phylogram showing relationship of clones of the three rhizosphere soils to reference members of firmicutes based on analysis of 433 bp of aligned 16S rDNA and rRNA sequences.

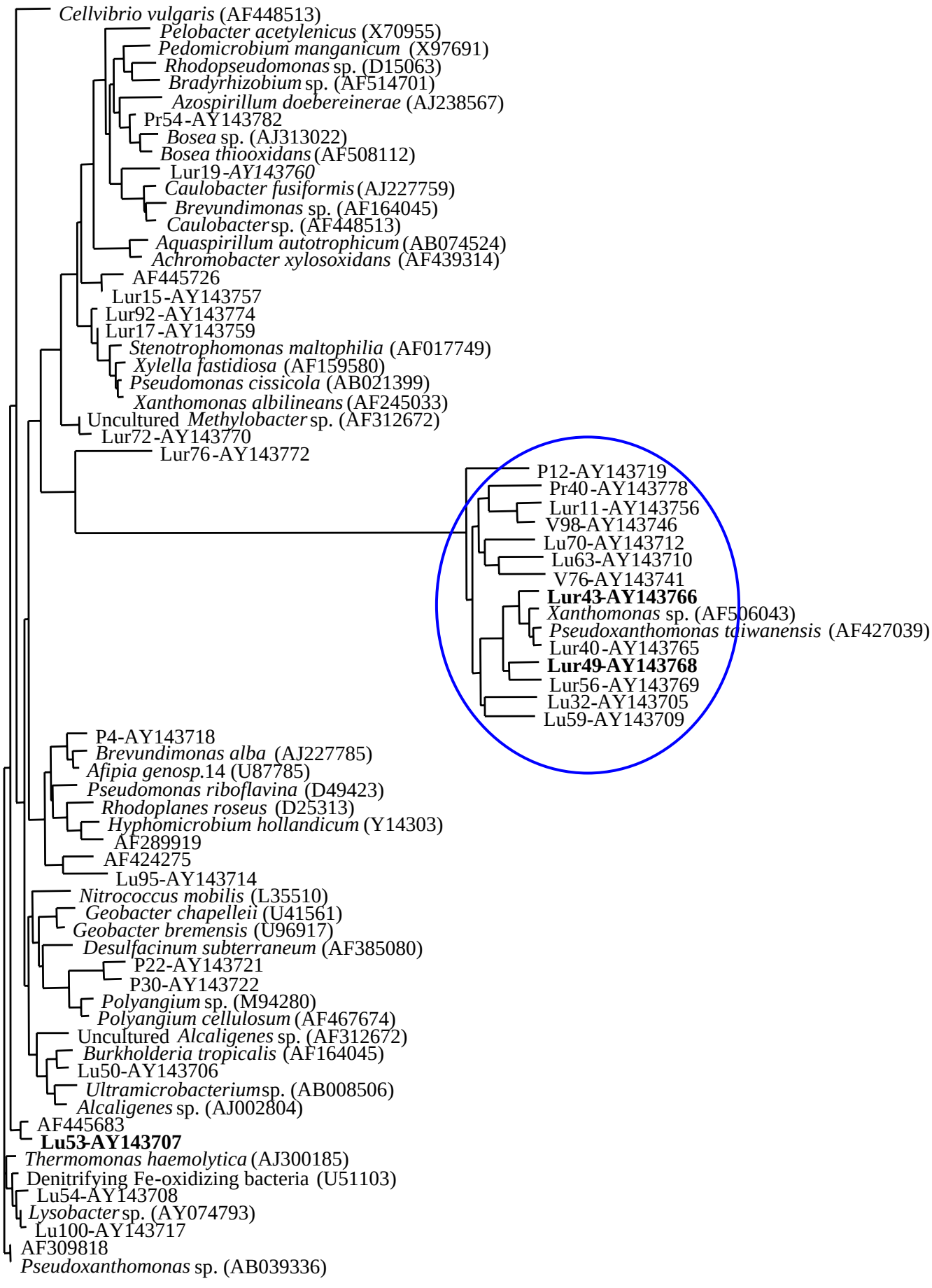


Figure 11. Phylogram showing relationship of clones of the three rhizosphere soils to reference members of proteobacteria based on analysis of 433 bp of aligned 16S rDNA and rRNA.

Proteobacteria

These constituted the second major group in all the six libraries with more than 20% representation in *Lupinus* and *Pisum* and about 15% in *Vicia* libraries (Figure 8).

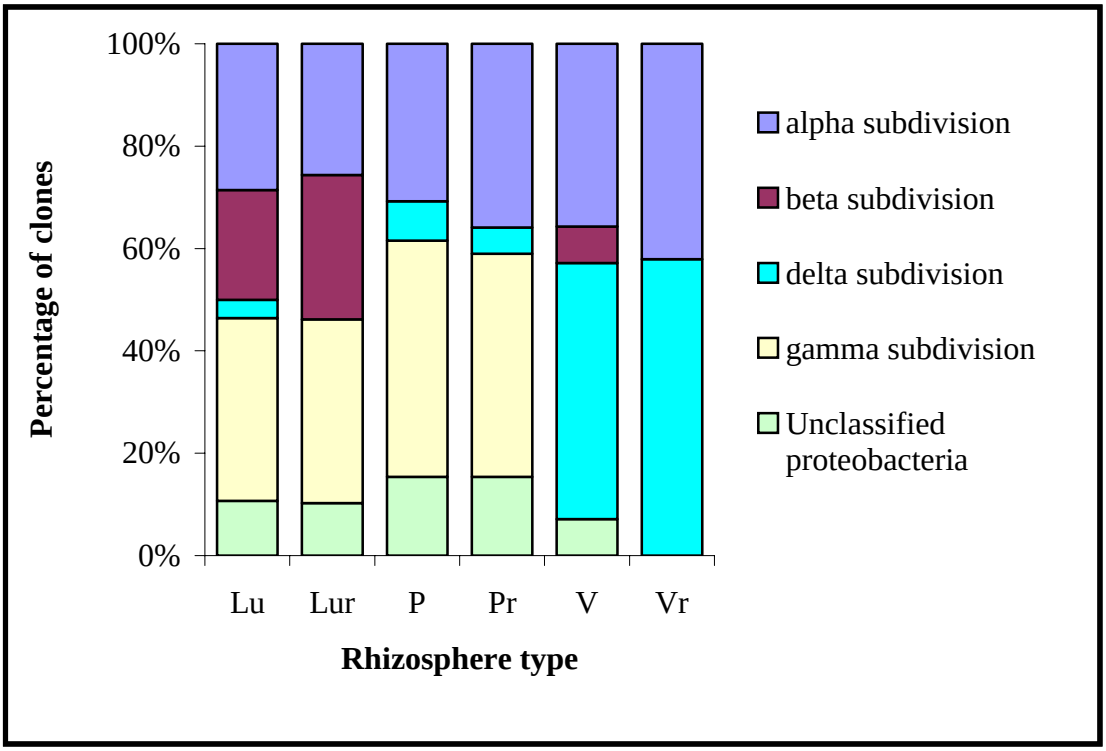


Figure 12. Relative distribution of clones to different groups of proteobacteria. Lu, P and V stand for 16S rDNA libraries of *Lupinus*, *Pisum* and *Vicia* and Lur, Pr and Vr stand for 16S rRNA libraries of *Lupinus*, *Pisum* and *Vicia* respectively.

Clones similar to the γ-subdivision predominated with more than 40% of the proteobacterial clones in 16S rDNA and rRNA libraries of *Lupinus* and *Pisum* (Figure 12). Striking absence of the γ-subdivision from *Vicia* libraries could be observed in which instead δ-subdivision constituted the major proportion (more than 50%) of proteobacterial clones. Clones related to β-subdivision could only be observed in *Lupinus* libraries with one exception in *Vicia* rDNA library. α-subdivision was the second most abundant group with more than 30% of proteobacterial clones in all samples. In addition, unclassified proteobacterial clones not yet assigned to any of the above mentioned groups were also present. Comparison of 16S rRNA and rDNA proteobacterial clones showed a trend similar to that of firmicutes. The percentages of proteobacteria were 28, 26 and 14% in rDNA libraries but their numbers increased to 39, 39 and 19% in rRNA libraries of *Lupinus*, *Pisum* and *Vicia* respectively. A distinct cluster comprising of majority of the

proteobacterial clones was observed, which was closely related to *Xanthomonas* sp. [AF506043] and *Pseudoxanthomonas taiwanensis* [AF427039] (Figure 11). Other clones were widely distributed throughout the phylogenetic tree.

Other groups

Highly variable proportions (18 - 42%) of clones in all the libraries were most closely related to uncultured / unidentified bacteria described in NCBI database and also identified in other studies (McCaig *et al*., 1999; Miethling *et al*., 2000). Such clones have been included under the group Unclassified environmental clones in Figure 8. Clones belonging to *Verrucomicrobia* and *Fibrobacter/Acidobacter* groups present in 16S rDNA libraries were not found in the corresponding 16S rRNA libraries, with *Vicia* being the only exception. Few clones related to Green Non Sulphur and *Nitrospira* groups were also observed among *Vicia* clones.

3.1.5 Correlation between DGGE profiles and analysis of clone libraries

To find correlation, if any, between DGGE and cloning, selected clones that were dominant in *Lupinus* clone libraries were PCR amplified as described before and checked on DGGE. All the six clones selected had corresponding bands in the DGGE profiles (Figure 13). Nevertheless, not all the dominant clones selected from clone libraries were represented by dominant bands in the profiles. These differences could be attributed to the two different techniques, with different sensitivities, employed in the study.

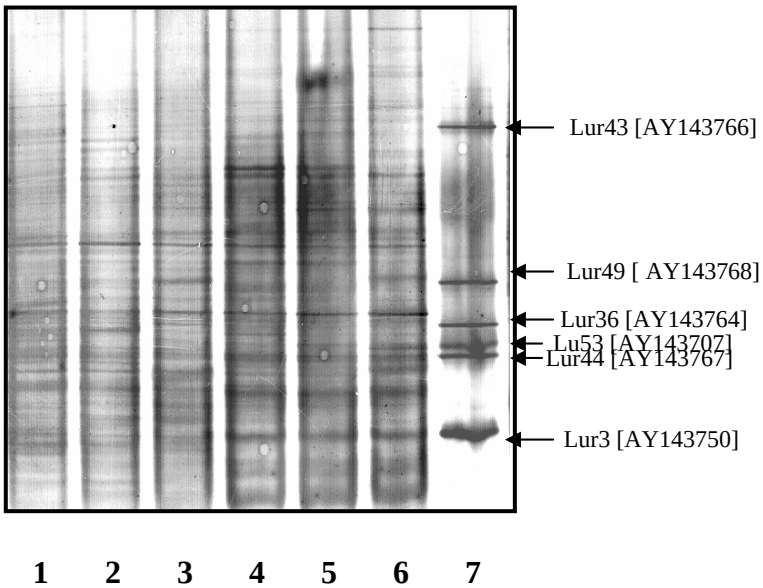


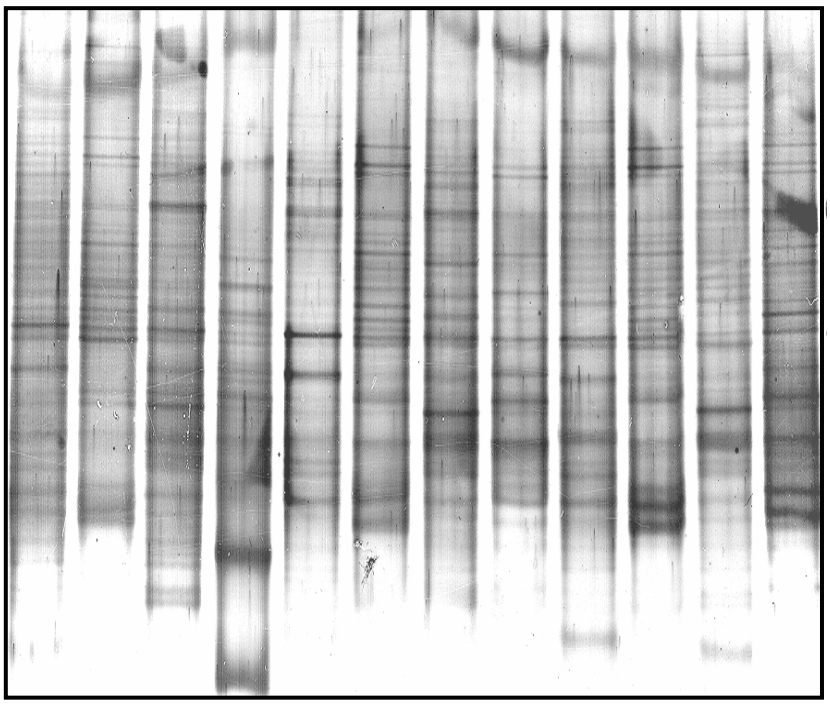
Figure 13. Relationship between DGGE profiles and dominant clones from *Lupinus* libraries. Lanes 1, 2, 3: 16S rDNA fragments of *Lupinus, Vicia* and *Pisum* rhizospheres; lanes 4, 5, 6: 16S rRNA fragments of *Lupinus, Vicia* and *Pisum* rhizospheres respectively; lane 7: marker constituting of dominant clones from *Lupinus* libraries.

These clones have been marked in bold letters in Figures 10 and 11.

3.1.6 DGGE profiles generated by group specific primers

The high abundance of firmicutes in both rDNA and rRNA libraries of the three legume rhizospheres under study prompted us to investigate further into the diversity of this group. Actinomycete specific primers, F243 and R1378 were used in the first PCR and universal bacterial primers F984-GC and R1378 were used subsequently in the nested PCR to compare the actinomycete population with total bacterial population. The three rhizosphere soil samples yielded positive PCR and RT-PCR results using these primers.

The products were subsequently resolved by DGGE. As performed for bacterial DGGE, initially a gradient of 45 – 62% was employed for resolving the products. The best resolution was achieved using a gradient of 54 - 60%. On comparing the DGGE profiles of total bacteria with the corresponding actinomycetes, it was observed that for all the legumes, most of the bands present in bacteria were also present in the profiles for actinomycetes, thus indicating a dominance of actinomycetes in the bacterial populations (Figure 14).



1 2 3 4 5 6 7 8 9 10 11 12

Figure 14. DGGE profiles of 16S rDNA and 16S rRNA fingerprints of total bacteria compared with actinomycetes. Lanes 1, 5, 9: 16S rDNA fragments of total bacteria from *Lupinus, Vicia* and *Pisum rhizospheres*; lanes 2, 6, 10: 16S rDNA fragments of actinomycetes from *Lupinus, Vicia* and *Pisum* rhizospheres; lanes 3, 7, 11: 16S rRNA fragments of total bacteria from *Lupinus, Vicia* and *Pisum rhizospheres*; lanes 4, 8, 12: 16S rRNA fragments of actinomycetes from *Lupinus, Vicia* and *Pisum* rhizospheres respectively.

However, there were some bands that were unique to either the bacterial or the actinomycete profiles. Many bands were observed in the RNA profiles of actinomycetes in all the three legumes indicating their activity in the rhizosphere. The rDNA and rRNA profiles of actinomycetes clustered together with their bacterial counterpart forming three distinct clusters for the three different legumes under study. Unlike comparison of only bacterial profiles, *Vicia* and *Pisum* were more closely related to each other (about 78% similarity), than to *Lupinus* (Figure 15).

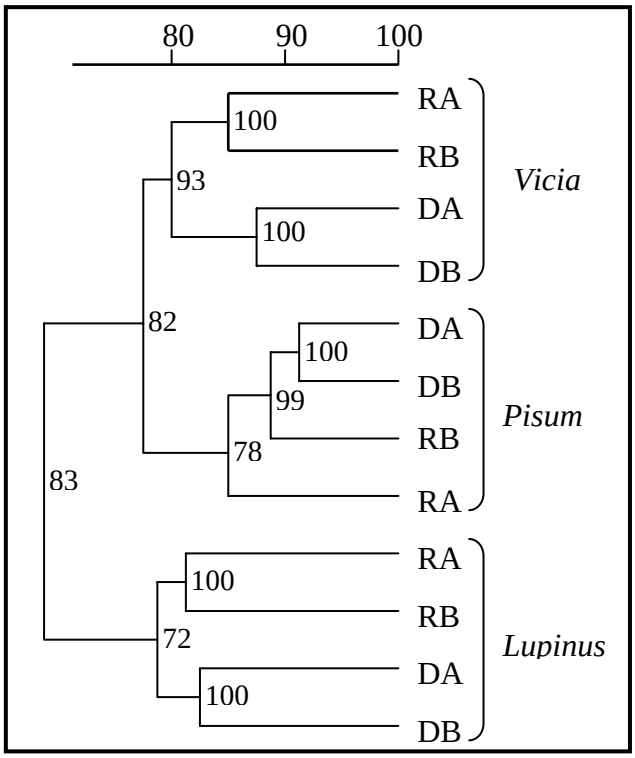


Figure 15. UPGMA tree representing the similarity of the total bacterial community profiles and actinomycetes profiles obtained by PCR-DGGE and RT-PCR-DGGE from the three rhizospheres. Scale represents percent similarity. Values of cophenetic correlations are mentioned at the branches. D refers to amplification from rDNA and R for amplification from rRNA of total bacteria (B) and actinomycetes (A).

3.2 FUNCTIONAL DIVERSITY OF BACTERIAL POPULATION

While structural diversity gives an idea of the total community present in the system, which includes dormant or dead cells and spores, functional diversity includes only the active component of the community. To get an idea of active members in the rhizospheres under comparison, total RNA was analysed.

3.2.1.1 AP-PCR with M13 reverse primer

M13 reverse primer was used to generate fingerprints with rhizosphere soil DNA of the three legumes. 10 - 15 distinct bands could be observed in the profiles of the three rhizosphere samples (Figure 16). PCR from different pots of the same legume produced a very identical profile (Figure 17). High density of indistinguishable bands was observed in the upper part of the gel indicating greater number of bands with high molecular weight. The profiles of the three rhizospheres were very specific for each type, both with respect to number and position of bands. On comparison of the fingerprints by GelCompar II using Dice coefficient, *Lupinus* and *Pisum* were found to be 95% similar to each other. *Vicia* was 75% similar to the cluster of *Lupinus* and *Pisum*.

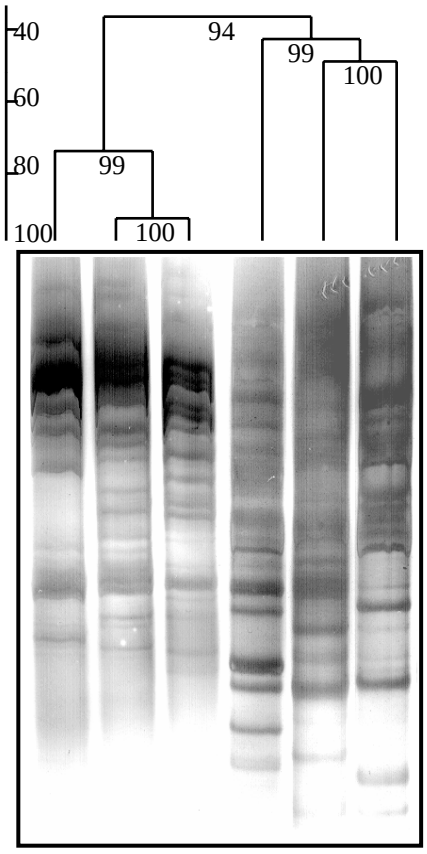


Figure 16. AP-PCR and RAP-PCR products with M13 reverse primer resolved by PAGE. Lanes 1, 2, 3: *Vicia*, *Lupinus* and *Pisum* DNA fingerprints; lanes 4, 5, 6: *Vicia*, *Lupinus* and *Pisum* cDNA fingerprints respectively. UPGMA tree representing the similarity of microbial community structural and expression profiles obtained by AP-PCR and RAP-PCR using M13 reverse primer for the three rhizospheres. Scale represents percent similarity. Values given at the branch points represent cophenetic correlation that is a parameter to express the consistence of a cluster.

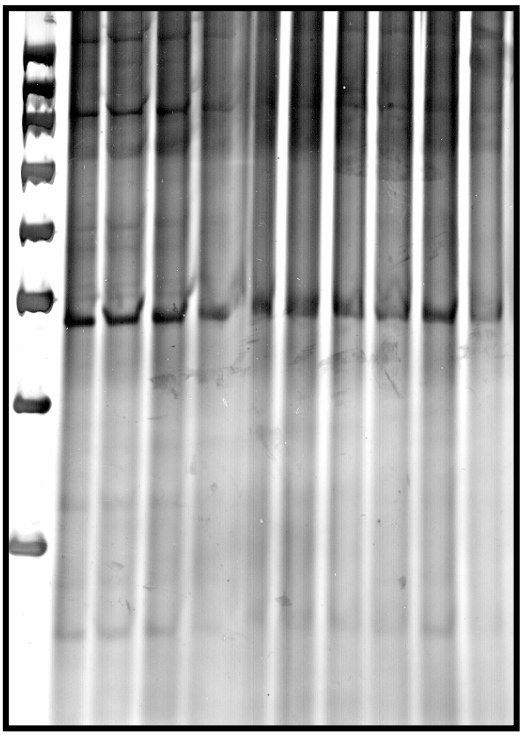


Figure 17. AP-PCR products of rhizosphere soil samples of ten different pots of *Vicia* with M13 reverse primer resolved by PAGE. Lane M: marker, lanes 1 - 10: profiles from pots V1 - V10 respectively.

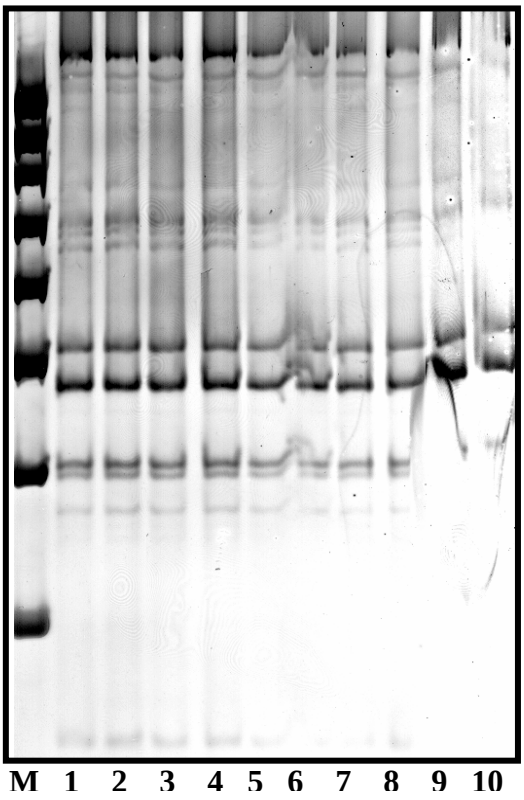


Figure 18. RAP-PCR products of rhizosphere soil samples of ten different pots of *Vicia* with M13 reverse primer resolved by PAGE. Lane M: marker, lanes 1 - 10: profiles from pots V1 - V10 respectively.

3.2.1.2 RAP-PCR with M13 reverse primer

Fingerprints were also generated with M13 reverse primer using cDNA (RAP-PCR) of the three rhizosphere soils. Increased number (approximately 20) of bands was obtained in the three soils (Figure 16). PCR from different pots of the same legume produced a very similar profile (Figure 18). Unlike the profiles generated using DNA, bands were also observed in the low molecular weight range. The trend of similarity between the three rhizospheres was the same as observed for DNA profiles but there was a remarkable difference in the similarity values obtained. The value of similarity decreased to 50% between the profiles of *Lupinus* and *Pisum* and this cluster was only 45% similar to the fingerprint generated with *Vicia* cDNA. The clusters were consistent as the values of cophenetic correlation were 100 for *Lupinus* and *Pisum* cluster and 99% for the cluster with *Vicia*.

3.2.1.3 Comparison of AP and RAP-PCR with M13 reverse primer

Comparison of the profiles generated by AP and RAP-PCR revealed that the profiles were distinct for the DNA and cDNA for each sample. Very few common bands were present between the fingerprints of the same rhizosphere type when comparing the DNA profiles with their respective cDNA profiles. Furthermore, clear plant-dependent effects on the microbial DNA and cDNA profiles were visible. The similarities between the DNA fingerprints of the three rhizosphere soil samples were high. The biggest differences obtained were between cDNA and DNA profiles. The profiles generated using DNA samples clustered together and the second cluster comprised of the fingerprints generated with cDNA of the three rhizosphere soils. The level of similarity between these two clusters was only 35%, with the value of cophenetic correlation being 94.

3.2.2.1 AP-PCR with 10 mer primer

Fingerprints were also generated using a smaller primer, 10 mer. Legume rhizosphere DNA produced 10 - 15 distinct bands when resolved on acrylamide gel (Figure 19). As in the profiles generated using M13 reverse primer, a number of indistinguishable high molecular weight bands were observed in the upper part of the gel. The profiles generated by plants from different pots of the same legume were identical to each other (Figure 20). Each rhizosphere type produced a distinct profile with respect to number and position of bands.

Lupinus and *Pisum* were about 75% similar to each other while *Vicia* was 60% similar to the cluster of *Lupinus* and *Pisum*. This trend of similarity was the same as obtained with M13 reverse primer.

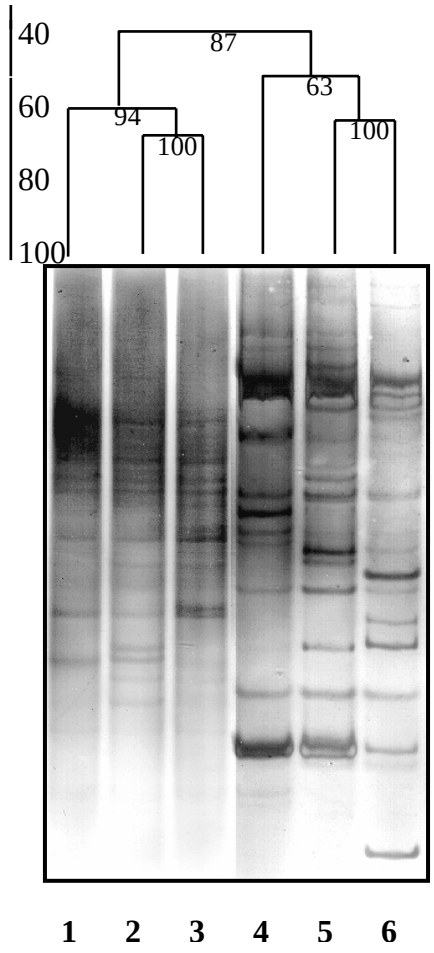


Figure 19. AP-PCR and RAP-PCR products with 10 mer primer resolved by PAGE. Lanes 1, 2, 3: *Vicia*, *Lupinus* and *Pisum* DNA fingerprints; lanes 4, 5, 6: *Vicia*, *Lupinus* and *Pisum* cDNA fingerprints respectively. UPGMA tree representing the similarity of microbial community structural and expression profiles obtained by AP-PCR and RAP-PCR using 10 mer primer for the three rhizosphere. Scale represents percent similarity. Values given at the branch points represent cophenetic correlation that is a parameter to express the consistence of a cluster.

3.2.2.2 RAP-PCR with 10 mer primer

10 mer primer was also used to generate fingerprints with cDNA (RAP-PCR) of the three rhizosphere soils. The number of distinct bands in this case increased to about 20 for three soils (Figure 19). The profiles generated by plants from different pots of the same legume were identical to each other (Figure 21). Unlike the profiles generated using DNA, bands were also observed in the low molecular weight range. The density of bands was equal throughout the gel. On comparing with GelCompar II, it was observed that *Lupinus* and

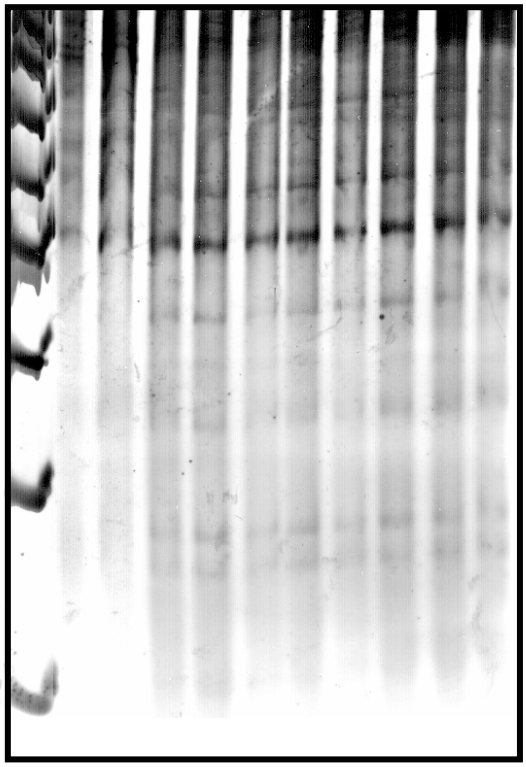


Figure 20. AP-PCR products of rhizosphere soil samples of ten different pots of *Vicia* with 10 mer primer resolved by PAGE. Lane M: marker, lanes 1 - 10: profiles from pots V1 - V10 respectively.

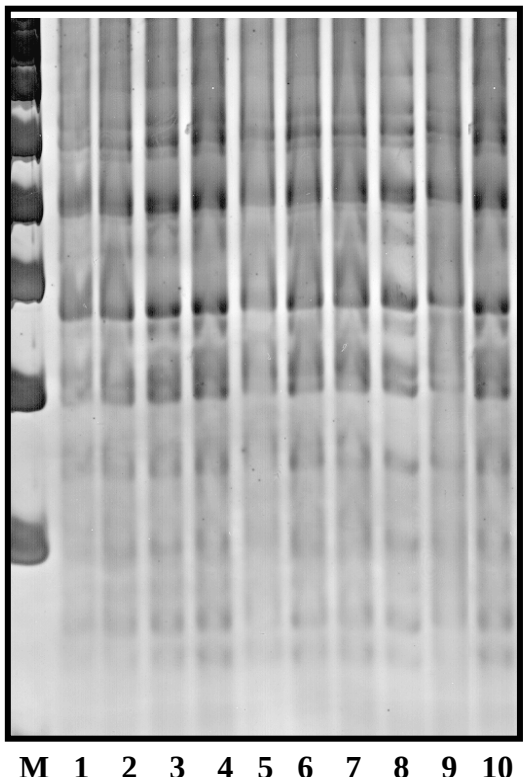


Figure 21. RAP-PCR products of rhizosphere soil samples of ten different pots of *Vicia* with 10 mer primer resolved by PAGE. Lane M: marker, lanes 1 - 10: profiles from pots V1 - V10 respectively.

Pisum were more similar to each other than to *Vicia*. The value of similarity was 65% between the profiles of *Lupinus* and *Pisum* and this cluster was only 50% similar to the fingerprint generated with *Vicia* cDNA. The cophenetic correlation value was only 63 between *Vicia* and the cluster formed by *Lupinus* and *Pisum* and 100 for the cluster formed by *Lupinus* and *Pisum*.

3.2.3 Chitinase detection as a part of Carbon cycle

Nested PCR approach was applied to amplify Group A bacterial chitinases. Primer pair GA1F and GA1R was used in the first PCR, while primers GASQF (with the GC clamp) and GASQR were used for the second PCR. Predicted band could be obtained only after optimisation with addition of BSA and DMSO in the reaction mixture. Amplification products were obtained with the DNA of the three rhizospheres but not with the cDNA indicating its presence in the gene pool but no or very low activity in the rhizospheres (Figure 22).

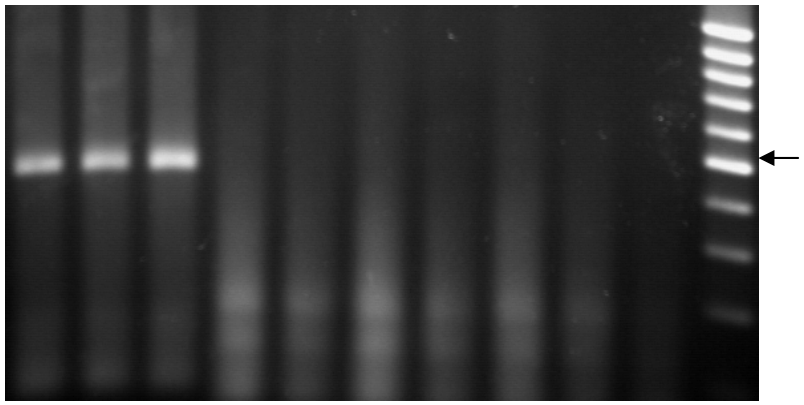


Figure 22. Agarose gel of PCR and RT-PCR products for chitinase. Lanes 1, 2, 3: PCR products; lanes 4, 5, 6: RT-PCR products; lanes 7, 8, 9: $DNase^{+}RT^{-}$ PCR products for *Vicia*, *Lupinus* and *Pisum* respectively; lane 10: negative control; lane M: 100 bp marker. Arrow marks the expected band.

The three rhizosphere soil samples were screened for activity of chitinase using MUF labelled β-glucosaminidase. Optimal activity curve showed that highest activity of the enzyme was at the concentration of 200 μM of substrate. Therefore, this concentration was used further for all samples. Values in negative range for *Lupinus* and *Pisum* and a very low value for *Vicia* rhizospheres indicated no or very low chitinase activity in these rhizosphere soil samples (Figure 23).

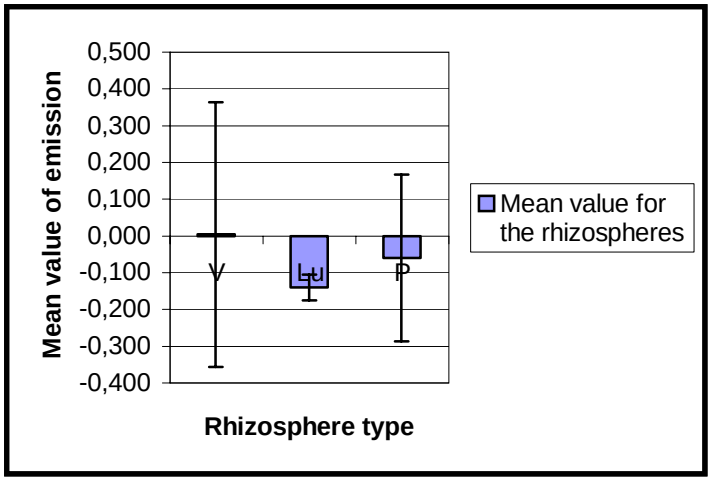


Figure 23. Determination of chitinase activity in the rhizospheres of three legumes. Mean value for each legume has been plotted to compare the values for the three legumes.

3.2.4 Nitrogen cycle

3.2.4.1 Proteolytic enzymes

Primers FP *sub* Ia and RP *sub* II were used for amplification of a 319 bp fragment of serine peptidase. For amplification of neutral metallopeptidases, primers FP *npr* I and RP *npr* II, which encompass a region of 233 bp, were used. Serine peptidase transcripts could be amplified from cDNA of the three rhizospheres indicating its activity in the system (Figure 24). Neutral metallopeptidase gene (*npr*) could be amplified from the DNA of the three rhizospheres but RT-PCR yielded products only for the rhizospheres of *Vicia* and *Pisum* (Figure 25). The result was consistent for all the replicates.

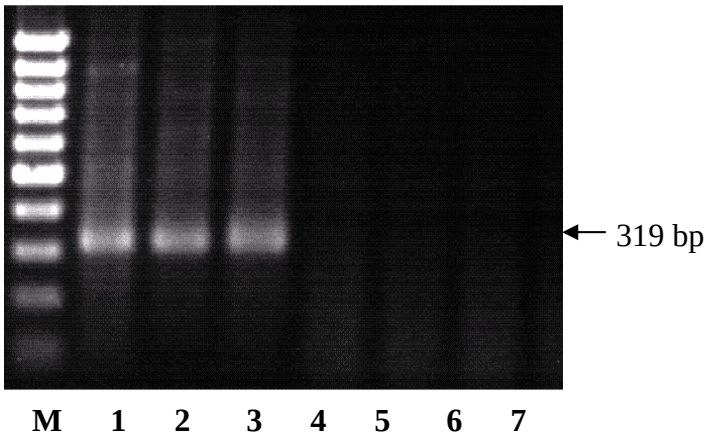


Figure 24. Agarose gel of RT-PCR products for serine peptidase gene (*sub*). Lane M: 100 bp marker; lanes 1, 2, 3: RT-PCR products; lanes 4, 5, 6: $DNase^{+}RT^{-}$ PCR products for *Vicia*, *Lupinus* and *Pisum* respectively. Arrow marks the expected band of 319 bp.

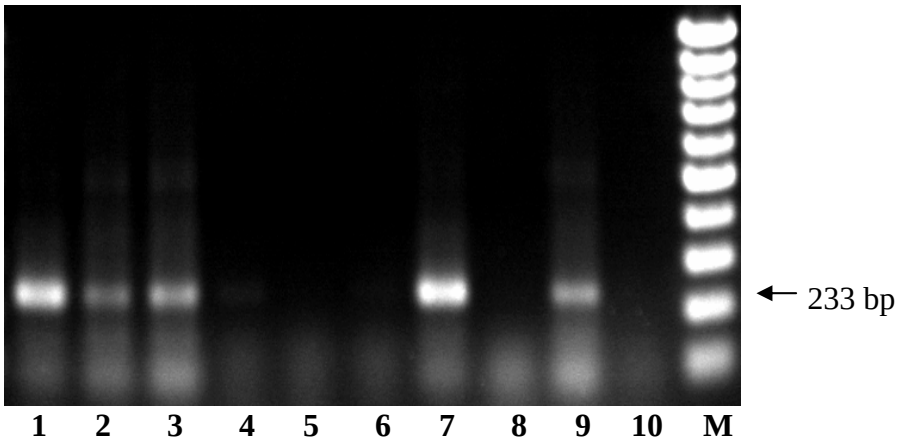


Figure 25. Agarose gel of PCR and RT-PCR products for neutral metallopeptidase gene (*npr*). Lanes 1, 2, 3: PCR products; lanes 4, 5, 6: $DNase^{+}RT^{-}$ PCR products; lanes 7, 8, 9: RT-PCR products for *Vicia*, *Lupinus* and *Pisum* respectively; lane 10: negative control; lane M: 100 bp marker. Arrow marks the expected band of 233 bp.

To verify that RAP-PCR profiles also contained mRNA transcripts, RAP-PCR products with 10 mer primer and M13 reverse primer were transferred to positively charged nylon membranes and then probed for bacterial peptidase (serine peptidase and neutral metallopeptidase) transcripts using DIG labelled probes. It was observed that while serine peptidase was expressed in all the three rhizosphere soils (Figure 26), neutral metallopeptidase could be detected in *Vicia* and *Pisum* rhizospheres only (Figure 27).

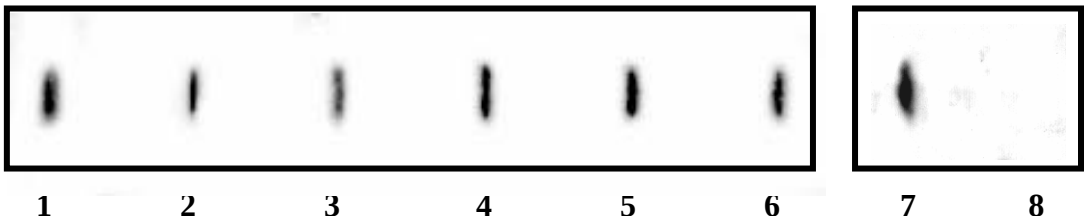


Figure 26. Dot blot for serine peptidase. Lanes 1, 2, 3: RAP-PCR products of *Vicia*, *Lupinus* and *Pisum* rhizosphere RNA with M13 reverse primer; lanes 4, 5, 6: RAP-PCR products of *Vicia*, *Lupinus* and *Pisum* rhizosphere RNA with 10 mer primer respectively, lane 7: PCR product for *sub* gene amplified from *Bacillus cereus*; lane 8: PCR product for *npr* gene amplified from *Bacillus cereus*.

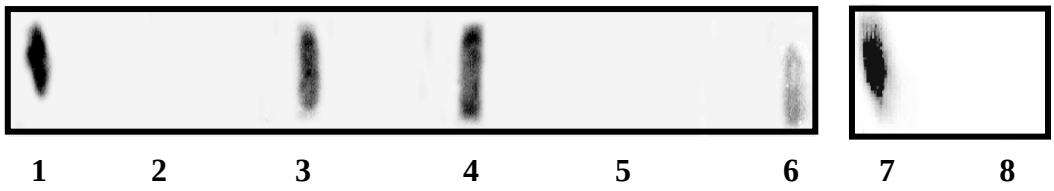


Figure 27. Dot blot for neutral metallopeptidase. Lanes 1, 2, 3: RAP-PCR products of *Vicia*, *Lupinus* and *Pisum* rhizosphere RNA with M13 reverse primer; lanes 4, 5, 6: RAP-PCR products of *Vicia*, *Lupinus* and *Pisum* rhizosphere RNA with 10 mer primer respectively; lane 7: PCR product for *npr* gene amplified from *Bacillus cereus*; lane 8: PCR product for *sub* gene amplified from *Bacillus cereus*.

3.2.4.2 Nitrite reductases

Amplification of *nir* genes and transcripts

Presence and expression of *nir* (*nirK* and *nirS*) genes was investigated in triplicates from the same pot as well as for the ten different pots studied for each legume. Successful amplification required optimisation of the protocol by varying the concentration of primers and addition of BSA and DMSO to the PCR reaction mix. *nirK* gene fragments of 514 bp were amplified from the rhizosphere soil samples of all three legumes (Figure 28).

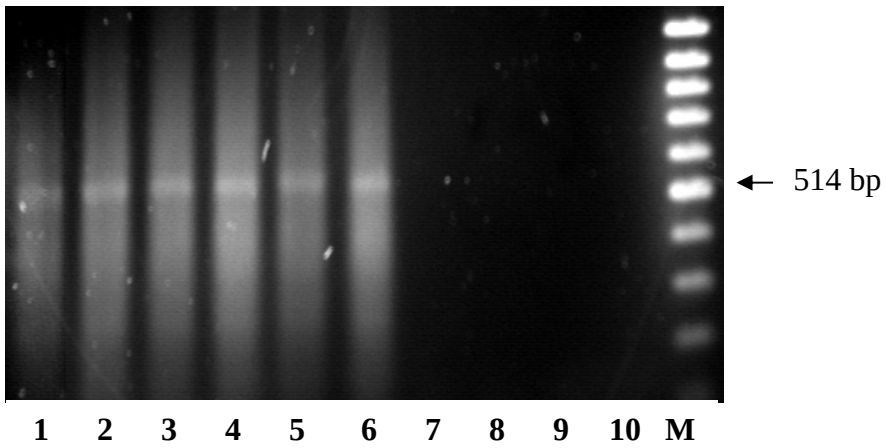


Figure 28. Agarose gel of PCR and RT-PCR fragments for nitrite reductase gene (*nirK*). Lanes 1, 2, 3: PCR products; lanes 4, 5, 6: RT-PCR products; lanes 7, 8, 9: $DNase^{+}RT^{-}$ PCR products for *Vicia*, *Lupinus* and *Pisum* respectively; lane 10: negative control; lane M: 100 bp marker. Arrow marks the expected band.

Presence (by DNA) and activity (by cDNA) of *nirK* was confirmed in all the thirty replicates. In contrast, no amplification of *nirS* gene could be achieved with DNA and cDNA for any of the thirty replicates in the three legumes. However, the predicted band of 890 bp for *nirS* fragment could be obtained for PCR with *Azospirillum brasilense* Sp7 that was used as positive control (Figure 29).

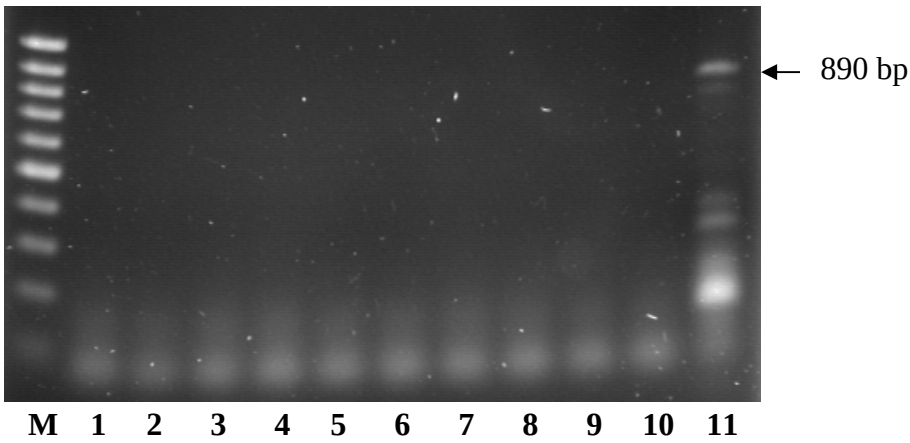


Figure 29. Agarose gel of PCR and RT-PCR products for nitrite reductase gene (*nirS*). Lane M: 100 bp marker; lanes 1, 2, 3: PCR products; lanes 4, 5, 6: RT-PCR products; lanes 7, 8, 9: $DNase^{+}RT^{-}$ PCR products for *Vicia*, *Lupinus* and *Pisum* respectively; lane 10: negative control; lane 11: *Azospirillum brasilense* Sp7 used as positive control. Arrow marks the expected band.

RFLP analysis of clones

RT-PCR products of *nirK* from one sample of each legume were cloned and 100 clones were screened from each rhizosphere soil type. A total of 266 clones (83, 92 and 91 from libraries of *Vicia*, *Lupinus* and *Pisum* respectively) with inserts of the correct size were used for further studies. Plasmids extracted from the clones were subjected to restriction digestion using *Msp*I. The digested products were resolved on 4% (wt/vol) high resolution agarose (Qbiogene, Heidelberg, Germany). The patterns could be grouped into twelve different types (Figure 30).

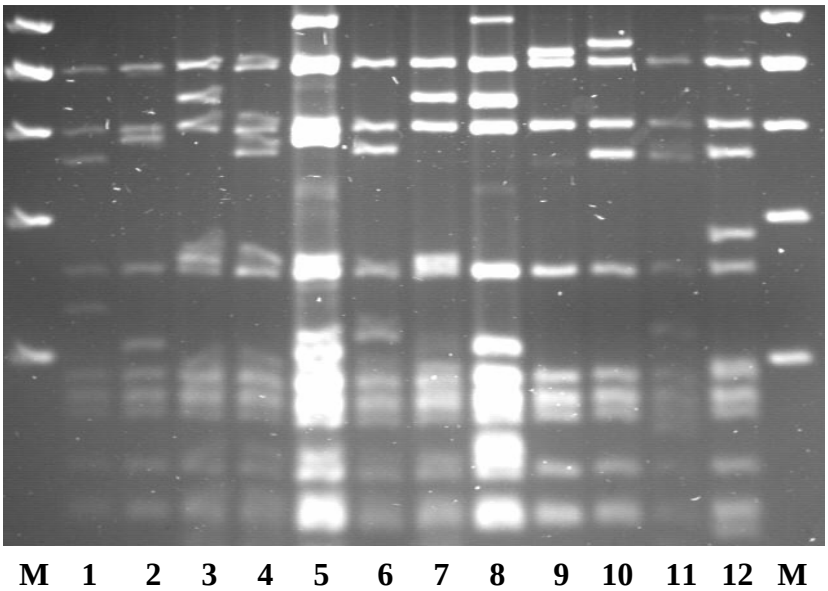
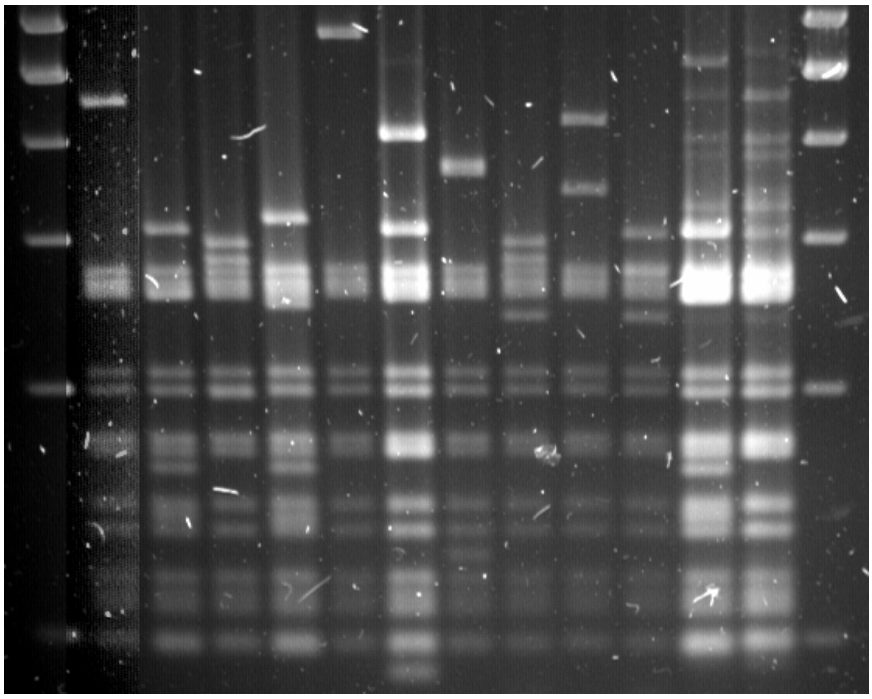


Figure 30. RFLP patterns of plasmids containing *nirK* fragment as insert, generated using *Msp*I. Lane M: 100 bp marker; lanes 1 - 12: twelve different patterns into which all the clones could be grouped.

Groupings were confirmed by digesting the plasmids with *Hin*6I as well (Figure 31).



M 1 2 3 4 5 6 7 8 9 10 11 12 M

Figure 31. RFLP patterns of plasmids containing *nirK* fragment as insert, generated using *Hin*6I. Lane M: 100 bp marker; lanes 1 - 12: twelve different patterns into which all the clones could be grouped.

Patterns 1, 3, 4, 5 and 7 were found in the clone libraries of all three rhizosphere types under study (Figure 32). Patterns 2, 9, 10 and 11 were observed only in *Vicia* and *Lupinus* rhizospheres in different proportions. Patterns 6, 8 and 12 were restricted to *Lupinus* and *Pisum* libraries. Pattern 1, which was the most dominant in *Vicia* and *Lupinus* libraries, constituted about 25% of all clones. Pattern 3, which was the most dominant in *Pisum* was represented only once in *Vicia* library. Patterns 2 and 4 together constituted about 20% of all clones. All the patterns were found in *Lupinus* libraries indicating it to be the most diverse with respect to *nirK* transcripts. *Vicia* and *Pisum* had nine and eight different RFLP patterns respectively. Each pattern was considered as one phylotype.

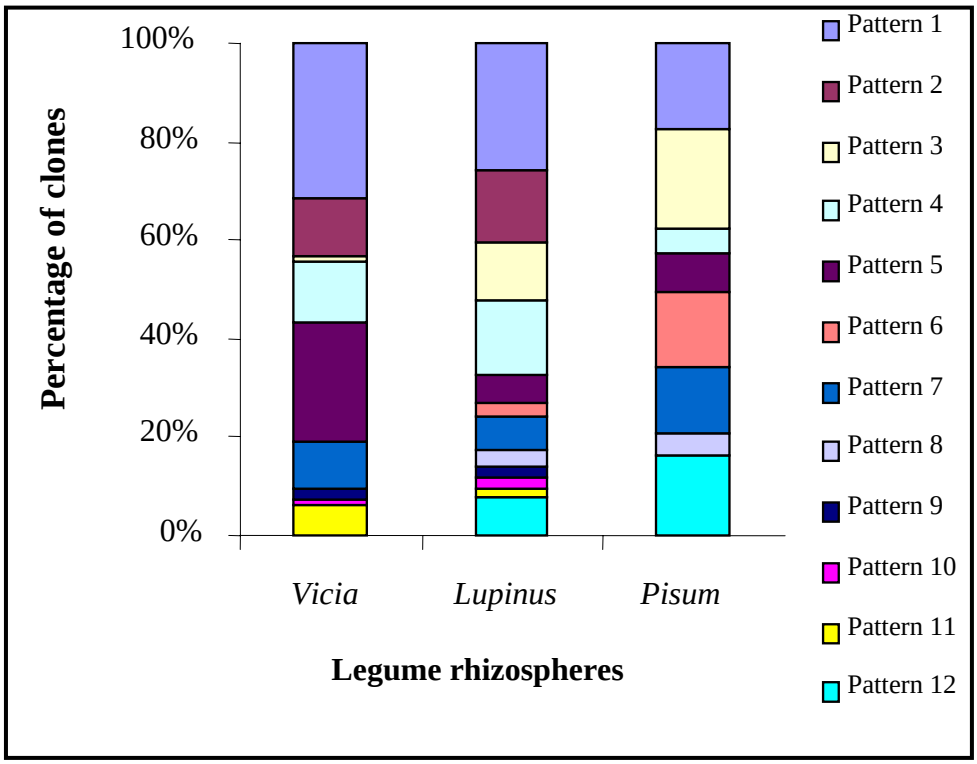


Figure 32. Relative distribution of phylotypes (RFLP patterns) in the rhizospheres of *Vicia*, *Lupinus* and *Pisum*.

Sequence analysis of representative patterns

nirK gene fragments (514 bp) of representative clones of each pattern were sequenced. Phylogenetic analysis of these sequences grouped all the patterns in one major group, which was similar to *nirK* sequence of *Mesorhizobium* sp. (accession number AY078254). Three sub-clusters could be identified within the major cluster (Figure 33). Patterns 1, 2, 4, 5, 6, 11 and 12 formed one cluster and patterns 3, 7, 9 and 10 formed the second cluster. Pattern 8 branched separately from these two clusters. Patterns 3, 5, 7, 8, 9 and 10 had no matching sequence in the NCBI database.

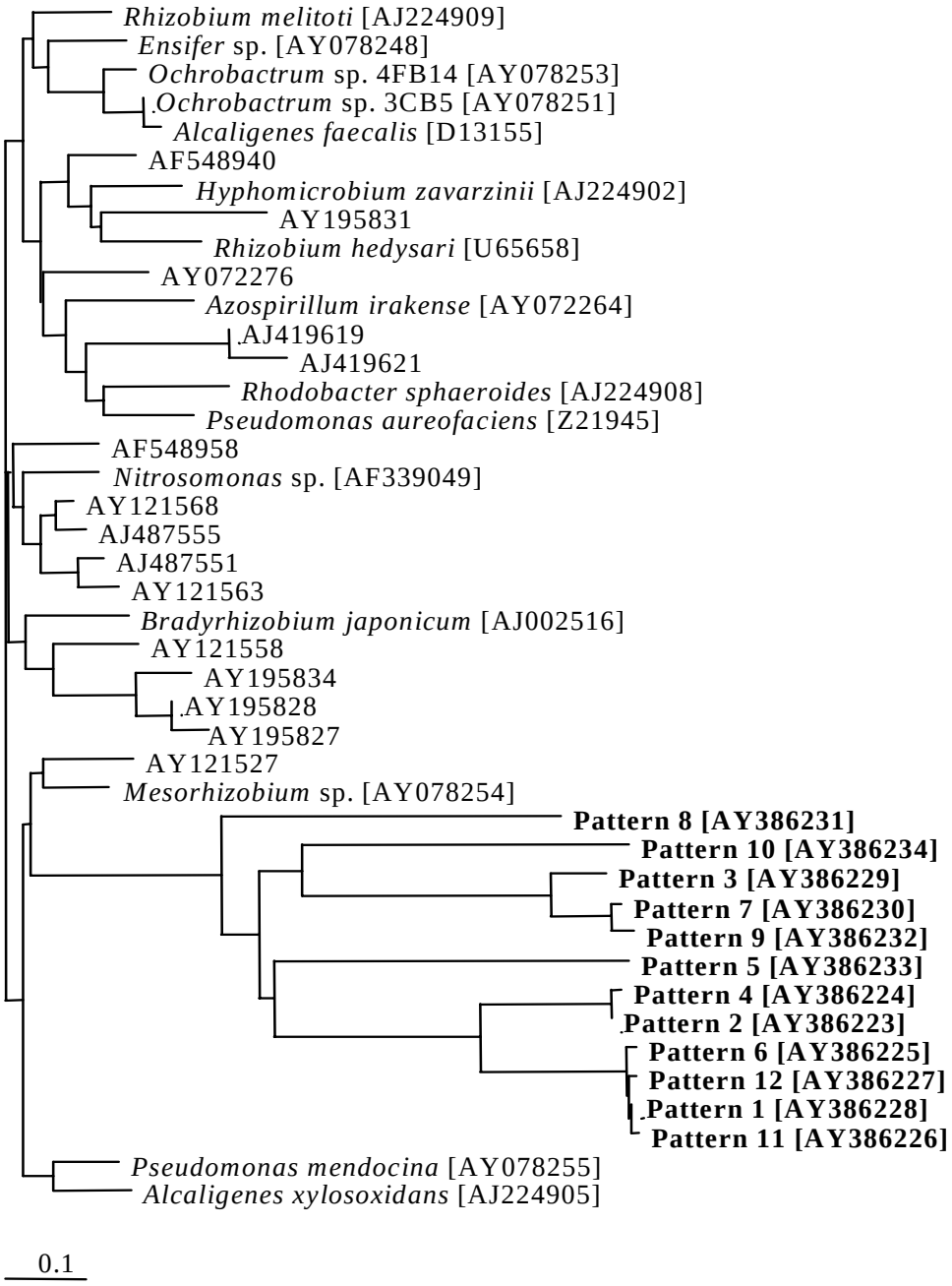


Figure 33. Phylogram showing relationship of *nirK* clones obtained from the three rhizosphere soils to *nirK* sequences of reference members. The GenBank accession number follows pattern number. For environmental clones NCBI accession numbers have been mentioned. Accession numbers of reference organisms in trees have been mentioned in brackets besides the organism's name. For convenience, the tree was pruned from a larger tree containing additional sequences from reference bacteria. The scale bar indicates the expected number of changes per sequence position.

4. DISCUSSION

A large number of environmental factors influence selection of specific bacterial communities in the rhizosphere. Crop species is a crucial factor for the supply of energy and carbon to the heterotrophic microbial community by producing root exudates (Miethling *et al*., 2000; Smalla *et al*., 2001). Miethling *et al*. (2000) reported crop species to be the major determinant of microbial community characteristics on the basis of community level physiological profiles, fatty acid methyl ester analysis and also by studying the diversity of PCR amplified 16S rRNA target sequences from directly extracted ribosomes.

The study by Mayer *et al*. (2003a) revealed differences in the quality and quantity of rhizodeposits from the legumes chosen for this study. The present study aimed at comparing the structural and functional diversity of bacterial community in the rhizosphere of three commercially important grain legumes and identifying plant-specific rhizosphere effects. To avoid the possibility of heterogeneity in the original soil, the soil was initially sieved, mixed and then used in the pot experiment.

4.1 Analysis of structural diversity of rhizosphere bacterial communities

16S rRNA has been commonly used to study the bacterial diversity in various ecosystems (Borneman *et al.*, 1996; Lee *et al*., 1996; Rheims *et al*., 1996; Kuske *et al*., 1997; Felske *et al*., 1998) because it possesses all those characteristics that define a phylogenetic marker. 16S rRNA has an essential function in protein synthesis in all bacteria. It is therefore ubiquitously distributed and hence it covers the phylogeny of the complete range of bacteria. 16S rRNA is derived from a common ancestor and hence is homologous (Stackebrandt and Rainey, 1995). 16S rRNA sequence analysis adds valuable information to culture-based methods for studying the composition and dynamics of microbial communities (Smalla *et al*., 2001). Universal bacterial primers, F984-GC and R1378, which amplify V3 - V6 region of 16S rDNA, have been commonly used for bacterial community analysis (Duineveld *et al*., 2001; Smalla *et al*., 2001).

4.1.1 Analysis of DGGE profiles obtained by PCR

DGGE allows the analysis of a large number of samples and is a good tool for initial screening of similarities or differences between different communities (Heuer and Smalla, 1997). In the present study, the method was employed to investigate experimental variations between the rhizosphere communities of the same legume species in ten different pots and also between the three different legume species in the same soil. Each DGGE profile of total bacterial community revealed about 25 - 30 distinct bands when PCR products were resolved. Visual observation revealed no differences between the fingerprints of the replicates from different pots of the same legume species (Figure 4). The similarity values as obtained by GelCompar II were greater than 90%. This suggests a low degree of variability caused by sampling, DNA extraction, PCR amplification and DGGE analysis. Another reason for not achieving 100% similarity between the lanes for the replicates can be due to the limitations of the normalizing features of GelCompar II software. However, occurrence of minor differences that go undetected by DGGE cannot be overruled. Similar numbers of bands using the same primer pair in studies of rhizosphere soil have earlier been reported (Miethling *et al*., 2000; Duineveld *et al*., 1998; Smalla *et al*., 2001). The method was also employed to get first hand information about similarities or differences between the profiles of the three legumes. Although several common bands occurred in the DGGE patterns of the three legume rhizospheres, a plant-dependent diversity in the rhizosphere bacterial communities could be demonstrated (Figure 5). There were differences in the profiles with respect to the number and position of bands, highlighting the dominance of different bacterial groups in different plant rhizospheres. This is in accordance with a previous study by Smalla *et al*. (2001), in which rhizospheres of strawberry, oilseed rape and potato were compared and a distinct plant-dependent rhizosphere effect was reported for the three plants. Patterns consisted of a number of strong bands and a lower number of weak bands. This is unlike the patterns obtained for bulk soil wherein fewer numbers of strong bands are obtained together with many weak bands (Smalla *et al*., 2001). The relative abundance of several bacterial populations is enhanced in the surroundings of roots, this being termed as “rhizosphere effect”. As a result, there are more strong bands in the profiles obtained in the present study.

Dice coefficient was used to measure the similarity between patterns. This measure takes into account the matches between the profiles based on presence or absence of bands. The

closer similarity between the rhizosphere bacterial profiles of *Vicia* and *Lupinus*, as compared to *Pisum*, may be attributed to similarities with respect to rhizodeposition as reported in the former two by Mayer *et al*. (2003a). They showed that 20 and 22% of the nitrogen derived from rhizodeposition could be traced in microbial biomass in *Vicia* and *Lupinus* rhizospheres respectively. In the case of *Pisum*, this value reduced to only 8%. This indicates that rhizodeposits are a major factor in the selection of bacterial community.

4.1.2 Analysis of DGGE profiles obtained by RT-PCR

DNA-based detection assays do not discriminate between dormant and active populations. DNA obtained from environmental samples could originate from dormant cells, dead cells, or even from free DNA. Adsorption of DNA at mineral surfaces, especially in soil, could harbour more or less intact nucleic acids a long time after lysis of the source organism (Lorenz and Wackernagel, 1987). To obtain information about the presence of metabolically active bacteria in the environment, 16S rRNA was targeted in RT-PCR using universal bacterial primers, F984-GC and R1378. The resulting DGGE profiles were compared with those obtained by DNA target analysis. Detection of RNA, which typically has a short half-life, provides evidence of activity that can be correlated with the physiochemical conditions. As the ribosome per cell ratio is roughly proportional to growth rate of bacteria, with few exceptions (Wagner, 1994), rRNA has been regarded as an indicator of total bacterial activity.

RNA derived bands were essentially a subset of the bands detected in the DNA-based analysis, suggesting that bacterial populations that were responsible for the most activity were also populations that were numerically dominant. Comparison of DNA and RNA profiles of the three legume rhizosphere communities revealed marked differences between the total and metabolically active bacterial communities. Unlike previous report by Duineveld *et al*. (2001), wherein a reduced number of bands in RNA generated profiles was reported from the rhizospheres of chrysanthemum as compared to DNA-based profiles, this study shows an equally complex RNA profile when compared to the respective DGGE profile generated by DNA. However, it has to be considered that the number of bands does not exactly represent the number and abundance of active species within the microbial community, as one organism may produce more than one DGGE band because of sequence heterogeneity in the ribosomal genes (Nübel *et al*., 1996). In contrast, it has been demonstrated that it is not always possible to separate 16S rDNA fragments that

have a certain amount of sequence variation (Vallaeys *et al*., 1997). Only the most dominant species of very complex communities will contribute to the fingerprint (Muyzer *et al*., 1993; Ferris and Ward, 1997). Higher number of bands in RNA profiles can also be accounted for by the high sensitivity of RT-PCR and also by the high level of expression of rRNA transcripts in highly active organisms (Wagner, 1994; Farrelly *et al*., 1995). Cluster analysis grouped the DNA profiles of each of the three legumes into one cluster while RNA fingerprints formed a separate cluster (Figure 5). Similarity levels in the DNA clusters were higher than that in the RNA cluster. The responses at rRNA level are more rapid and have greater amplitude than those at rDNA level, thus resulting in greater variation among rRNA-derived profiles (Duineveld *et al*., 2001). Presence of large number of bands in the lower part of the gel corresponding to higher gradient indicated the prevalence of high G+C Gram positive bacteria in the communities under study.

Inherent limitations of PCR amplification of 16S rDNA / rRNA from complex microbiota, like differential PCR amplification, formation of chimeric molecules, formation of deletion mutants (von Wintzingerode *et al*., 1997), cannot be completely ruled out. Such errors were minimized using replicate soil samples, multiple nucleic acid extractions and subsequent PCR. Although analysis of rRNA by RT-PCR is potentially a powerful approach for studying active populations in the environment, the technique suffers from inherent biases and limitations. Some of these are related to the RT-PCR itself, such as the requirement of primers that amplify a broad range of sequence types, the inhibition of reverse transcriptase or DNA polymerase (or both) by substances present in the RNA extracts, and the preferential amplification of certain templates during the PCR (Nogales *et al*., 2002).

4.1.3 Cloning and phylogenetic analysis

To rule out possible limitations of DGGE and also to identify the organisms, cloning of rDNA and rRNA PCR products was performed and the obtained clones analysed. Different groups of bacteria, considered as operational taxonomic units (OTU), when plotted to form Collector's curve, formed a plateau shaped graph after analysing 90 clones. In several studies analysing bulk soil microbial communities, no repetitive sequences have been observed even when higher number of sequences were screened (Zhou *et al*., 1997; Felske *et al*., 1998; Nüsslein and Tiedje, 1998). This is in contrast to the rhizosphere soil where plant roots directly influence the survival, growth and activity of bacteria in their vicinity

(Duineveld *et al*., 2001). Rhizosphere effect results in selection of specific bacterial population in the soil surrounding the plant root and this selection is plant species specific (Smalla *et al*., 2001). In fact, the rhizosphere has been recognized as an oligotrophic environment that contains minute cells whose growth is limited by lack of substrates (Foster, 1988). Collector's curve reaching a stationary phase after 90 clones indicates the ongoing selection process in the plant rhizosphere.

Diversity studies based on clone libraries from both 16S rDNA and 16S rRNA templates are considered to represent the bacterial community better, as 16S rRNA libraries in principle include metabolically active members. Yet, only a small number of studies have analysed soil bacterial communities using 16S rRNA clone libraries or a combination of libraries derived from rDNA and rRNA (Felske *et al*., 1997; Nogales *et al*., 2002; Gremion *et al*., 2003).

Sequencing of libraries revealed a major proportion of dominant population in the rhizosphere belonging to high G+C Gram-positive bacteria (Figure 8), as earlier indicated in the DGGE profiles. This group included clones similar to some ecologically important members like nitrogen fixers *Frankia* and *Clostridium* and anti-fungal actinomycetes like *Actinomadura* and *Geodermatophilus*. Clones similar to *Arthrobacter* and *Microbacterium*, which are known to possess biodegradation properties, were also present as active members. In addition, occurrence of clones related to *Mycobacterium*, *Streptomyces*, *Bacillus*, *Actinomyces* and *Nocardioides* species are typical of rhizosphere soil communities (Felske *et al*., 1998; Kaiser *et al*., 2001). The presence of actinomycetes in the rhizosphere soil has earlier been reported by Duineveld *et al*. (2001) where they were detected only at DNA level. Dominance of actinomycetes in rRNA clone libraries in the present study provides strong evidence for their presence and activity in the system under study. There is increasing evidence that Gram-positive bacteria may be more dominant in the rhizosphere soil than previously supposed. McCaig *et al*. (1999) reported that in a clone library obtained from grass rhizospheres, *Actinomyces* species were the second most abundant group after the most frequently found α–proteobacteria. *Arthrobacter* species were also found as dominant population in the molecular fingerprints of 16S rDNA fragments amplified from the rhizosphere of maize (Gomes *et al*., 2001), *Medicago sativa* and *Chenopodium album* (Schwieger and Tebbe, 1998). Such a high proportion of dominant population belonging to a diverse range of high G+C Gram-positive bacteria, as

found in present study, has not yet been reported from legumes. During the plant growth season, microorganisms become increasingly dependent on mobilization of autochtonous organic matter. Gram-positive bacteria, including coryneforms (e.g. *Arthrobacter* sp.), and true actinomycetes (e.g. *Streptomyces* sp.) become increasingly abundant in the rhizospheres of maturing plants (Sørensen, 1997). Similar observations have been reported earlier by Smalla *et al*. (2001) in the rhizospheres of strawberry, oilseed rape and potato. This coincides with the present study, which investigated the rhizosphere soil communities of six months old plants. In an earlier cultivation dependent study by Scott and Knudsen (1999) on pea rhizosphere, presence of actinomycetes and gram-negative bacteria was reported. No further characterization was attempted. Employing the technique of cloning, Gremion *et al*. (2003) also reported a dominance of *Actinobacteria*, both in bulk and rhizosphere soil contaminated by heavy metal.

No sequences similar to *Rhizobium* were obtained in any of the clone libraries despite the legume seeds being inoculated prior to potting. This reflects the endosymbiotic nature of *Rhizobium*. Nodules were excluded from the extraction of nucleic acids due to the fact that they have a very high density of bacteroids (*Rhizobium*) and its inclusion in the sample would lead to a dominance of *Rhizobium* sequences both as bands in DGGE patterns and as clones in the clone libraries. Rhizosphere soil samples were taken at late stages of plant development with possibly lower amounts of root exudates, reflecting a kind of rhizosphere climax. This would in turn promote the growth of K-strategic like bacteria and reduce competence of typical r-strategic like bacteria (like *Rhizobia*), which are abundant at the rhizoplane and in rhizosphere soil at times of higher root exudation (Hartmann *et al*., 2004). This hypothesis is also confirmed by the high abundance of Gram-positive bacteria (K-strategic like) in this study. The presence of *Rhizobium* in rhizosphere during earlier plant growth stages cannot be excluded as only the rhizosphere soil at maturity was investigated. Small number of *Rhizobia*, however, would go undetected within the complex bacterial community due to limits of detection by PCR.

The rhizosphere effect was very pronounced when the distribution of different proteobacterial subdivisions in the six libraries were compared (Figure 12). *Vicia* libraries lacked γ-subdivision members, while β-subdivision members were absent from *Pisum* libraries. *Lupinus* rhizosphere was the most diverse of the three with respect to distribution across the different subdivisions of the proteobacteria. Sequence analysis of *Lupinus* clones

confirmed our preliminary observations by DGGE fingerprinting that *Lupinus* could be the most diverse of the three plant rhizospheres compared.

Detection of sequences similar to *Stenotrophomonas maltophila* and *Polyangium* species were in accordance with the results of Yang *et al*. (2001). *Fibrobacter/Acidobacter* and *Verrucomicrobia* groups were also detected in this study and have been shown to be active also in other systems (van Veen *et al*., 1997; Felske *et al*., 1998). Clones similar to sequences from the group *Verrucomicrobia* were present in all DNA-derived libraries but only in *Vicia* RNA-derived library. Clone sequences similar to *Nitrospira*, which was assumed to be aquatic bacteria, were also obtained in low numbers. Recent studies have shown its occurrence in rhizosphere as well (Bartosch *et al*., 2002). A major proportion of clones displayed relationships with a wide range of environmental sequences from various as yet uncultured bacteria (Figure 8). The proportion was more than 40% for the DNA-derived clone library of *Pisum*. The representation of such sequences in RNA-derived libraries was considerably lower. Such high numbers of sequences related to uncultured bacteria have been reported earlier from rhizospheres (Gremion *et al*., 2003), which demonstrates the significance of the molecular methods employed in the present study over traditional culture dependent methods.

The cutting and sequencing of characteristic bands from the gels was not possible due to the high density of bands in the DGGE gels. Clones with sequences that occurred repetitively in the libraries, were PCR amplified and run on DGGE. This was an attempt to find correlation between the clones with repetitive occurrence and bands in DGGE profiles. Bands similar in position to the reference clones were present in the profiles generated by DGGE highlighting the concurrence of the two techniques viz. DGGE and cloning.

Identification of bacterial populations that are present and active in rhizospheres of legumes might help focus future biocontrol efforts. Actinomycetes, like *Streptomyces*, act as antagonists to many different phytopathogenic fungi, including different pathogenic races of the *Fusarium* wilt pathogen (Getha and Vikineswary, 2002), *Verticillium* wilt (Abd-Allah, 2001) and potato scab (Neeno-Eckwall *et al*., 2001). Detection of chitinolytic activity of actinomycetes (Gooday, 1990; De Boer *et al*., 1999; Gomes *et al*., 2000) also makes them promising candidates as biocontrol agents for diverse fungal diseases. The

apparent dominance of actinomycetes in the three rhizospheres opens the possibility of exploring them to identify members with antagonistic properties towards some economically hazardous legume pathogens, e.g. rust fungus (*Uromyces fabae*) and common root rot (*Aphanomyces euteiches*).

4.1.4 Population of actinomycetes in rhizospheres

Clone libraries showed a dominance of firmicutes in all the rhizospheres under study. Primers specific for actinomycetes were used to target only this fraction of the total population to check for variations between the actinomycete populations in the three rhizospheres. In addition, the relatively high G+C content of the actinomycete DNA might reduce the competitiveness of their DNA targets in PCR (Heuer *et al*., 1997). Studies on the distribution of actinomycetes in plant rhizospheres have been reported by only a few authors (Crawford *et al*., 1993; Toussaint *et al*., 1997; Trejo-Estrada *et al*., 1998). Nested PCR approach for actinomycetes, as introduced by Heuer *et al*. (1997), permitted comparison between the actinomycete population and the total bacterial population.

Comparison of profiles of total bacteria with their respective actinomycete profile showed a dominance of actinomycetes in the rhizospheres. The actinomycete profiles had many dominant bands that were common to the bands in the total bacterial profiles. Some new bands in actinomycete profiles, when compared to the bacterial profiles, were observed due to the selective amplification of actinomycetes in the first PCR. The number of bands in the actinomycete profiles could be severely limited by the high diversity of actinomycetes giving rise to only very little amounts of specific PCR products, which finally produce visible bands (Heuer *et al*., 1997). The RNA-derived profiles of actinomycetes were equally complex when compared to their respective DNA-derived profiles. This confirms actinomycetes to be the most dominant active group of the bacterial population in the rhizosphere soil of mature legumes. Dominance of actinomycetes in the rhizosphere could be attributed to the stage of sampling of the plants (Sørensen, 1997). Cloning results are in good accordance with DGGE analysis as actinomycetes constituted the major fraction of clones in RNA-derived libraries of the three rhizospheres as well.

Actinomycetes have been found to constitute a dominant fraction of the bacterial population in a study by Heuer *et al*. (1997) when rhizosphere soil of transgenic and wild type potato plants were compared. Smalla *et al*. (2001) obtained an astonishingly high

proportion of actinomycete populations in the rhizosphere soil of strawberry, potato and oilseed rape when they sequenced bands from DGGE fingerprints.

4.2 Analysis of functional diversity of rhizosphere bacterial communities

Rapid developments in reverse transcription-PCR (RT-PCR) have provided opportunities for the evaluation of active communities inferred from analysis of rRNA and mRNA. The detection of mRNA is a definitive indicator of activity but it may not always equate with phenotypic expression of the targeted gene because of the possibility of post-translational modifications of the enzyme. Plant-dependent rhizosphere effects on the production and activity of enzymes have also been previously reported, e.g. higher levels of both alkaline and acid phosphatase in the rhizosphere as compared to the bulk soil have been reported for various crop species by Tarafdar and Jungk (1987). Knauff *et al*. (2003) reported higher activity of arylsulfatase in rhizospheres as compared to bulk soil of *Sinapis album, Lolium perenne, Triticum aestivum* and *Brassica napus*. Their expression was shown to be influenced by crop species but not much was described about the source of enzymes.

4.2.1 Analysis of expression fingerprints obtained by M13 reverse and 10 mer primers

In the present study, non-radioactively labelled application of the protocol (Welsh *et al*., 1992) was successfully applied to investigate the metabolic profiles of rhizosphere soil samples. Besides RNA, DNA was also analysed in the present study to observe the extent of relatedness or differences between the genome and transcriptome of the three rhizospheres under investigation. The analysis was performed using two different primers, M13 reverse primer and also a 10 mer primer. With both primers, relatedness between the three legume rhizospheres was observed to be the same with variations only in the percentage similarity values. The application of RAP-PCR to describe microbial gene transcription from environmental samples has been restricted so far mainly to pure cultures. Fleming *et al*. (1998), for example, could show the induction of genes by toluene using *P. putida* and by salicylate using *B. cepacia* as model organisms. This has now been extended for the first time to environmental soil samples in the present study.

On comparing the DNA and RNA profiles generated using M13 reverse and 10 mer primers (figures 16 and 19), it was observed that there were major differences between the total DNA and total RNA profiles of the same rhizosphere. More bands were observed in the profiles generated by 10 mer primer, as compared to those generated by M13 reverse

primer. Similar to the results obtained with M13 reverse primer, the profiles for AP- and RAP-PCR were distinct for each rhizosphere soil sample. When comparing the DNA profiles with their respective cDNA profiles of the same rhizosphere type very few common bands were present in the fingerprints. Interestingly, the differences in the DNA and RNA profiles of the three legumes rhizospheres were not as clear using M13 reverse primer, as compared to the use of 10 mer primer. However, the overall patterns of similarity were similar showing high homologies between *Lupinus* and *Pisum* patterns and increased differences between the two and *Vicia*.

Differences between DNA and RNA based profiling have earlier been reported (Duineveld *et al*., 2001; Gremion *et al*., 2003). However, most of the studies have been based on ribosomal RNA and their corresponding genes. Differences between the active and resident bacterial populations has already been observed using 16S as a marker in the same rhizosphere soil. When the three rhizospheres were compared, more differences were observed with respect to their transcription profiles than to the DNA based structural profiles. This observation highlights the fact that the three different rhizospheres are more similar in their genetic potential. *Lupinus* and *Pisum* microbial communities are more than 90% similar to each other and these two are more than 75% similar to the community of *Vicia.* However, as revealed by their RNA based profiles with M13 reverse primer, they differ considerably in their metabolic profiles. *Lupinus* and *Pisum* microbial communities are only about 50% similar to each other and these two are about 45% similar to the community of *Vicia*. These observations can be attributed to qualitative and quantitative differences between the rhizodeposits of the three legumes. Similar trend is observed with AP-PCR and RAP-PCR profiles generated using the 10 mer primer though the proportion of differences and similarities vary. This study provides evidence that differences in rhizodeposition may not necessarily translate to differences in microbial community structure but rather affect the transcription profiles. In contrast, study by Miethling *et al*. (2003) on rhizosphere communities of alfalfa, common bean and clover reported that structural differences in rhizosphere might not always translate to physiological differences. Though differences were observed in the structural diversity of the three legumes, very similar physiological patterns, as measured by BIOLOG, were obtained for the rhizospheres investigated by Miethling *et al*. (2003). Several limitations are known for the assessment of functional diversity with the BIOLOG assay. Besides the statistical treatment of the data that can influence the results, a major question is whether the entire

population or only parts contribute to the observed carbon source utilization patterns. Therefore, community level physiological profiles do not necessarily reflect the functional potential of the dominant members of the communitues (Miethling *et al*., 2003).

Using M13 reverse and 10 mer primer, two different clusters were obtained, one comprising of profiles generated by DNA and the other by RNA. In each cluster, *Lupinus* and *Pisum* rhizosphere seemed to be more similar to each other than to *Vicia* rhizosphere though the proportion of similarities and differences varied with the primer used. Cophenetic correlation values of 94 in M13 reverse and 87 in 10 mer profiles between the DNA and RNA clusters suggest the clusters to be stable. As with cluster analysis for 16S DGGE profiles, the similarity levels decreased in RNA-derived bands as compared to DNA-derived ones because the amplitude of response is higher at RNA level, thus resulting in greater variation among RNA-derived profiles.

It is known that a large number of factors can influence the selection of specific microbial communities in the rhizosphere. 16S genes target only the bacterial population excluding other members of the rhizosphere (e.g. fungi, archeabacteria) which may be equally or more important. Moreover, studies dealing with structural diversity do not necessarily lead to an improved functional understanding. Using total RNA based approaches, both prokaryotic and eukaryotic members of the rhizosphere microbial community were included.

4.2.2 Chitinase detection

In soil, filamentous fungi and actinomycetes appear to be the dominant chitin degraders (Gooday, 1990; De Boer *et al*., 1999). Dominance and high activity of actinomycetes in the rhizosphere soil of the legumes under study, as revealed by 16S analysis, prompted the investigation of the presence and diversity of chitinase gene in the same. Using primers specific for group A bacterial chitinases (Williamson *et al*., 2000), the presence of respective gene in gene pool could be confirmed (Figure 22). Despite its presence, no chitinase expression was detected in RT-PCR. No previous attempts have been reported wherein chitinase gene has been targeted at RNA level. Confirmation of no detectable chitinase activity in the rhizospheres under study was obtained using enzyme assays as well. With the exception of *Vicia*, for which very low levels of chitinase were detected, negative values were obtained when enzyme assay was performed for chitinase. The

possibility of very low activity that is below the detection limits of the techniques employed can, however, not be overruled. Chitinase detection in upland pasture soil by enzyme assay has been reported by Metcalfe *et al*. (2002). They reported higher values of chitinase in litter bags buried in sludge-amended soil. The levels of actinobacteria were also found to be higher for the same treatment.

Chitinolytic bacteria may have negative effect on nodule formation of legume roots. Nod factors, which induce root nodule formation, are lipo-chito oligosaccharides that are excreted by rhizobia. It has been shown that Nod factors can be hydrolysed by a chitinase from the biocontrol strain *Serratia marcescens* (Krishnan *et al*., 1999). Hence, production of bacterial chitinase in the rhizosphere may reduce nodule formation. In the system under study, an extensive nodulation had been observed which could be an effect of absence of chitinase activity from the system.

4.2.3 Nitrogen cycle

4.2.3.1 Proteolytic enzymes

Hydrolysis of peptidic compounds by extra cellular microbial peptidases is of key importance for the release of ammonium from amino acids and subsequent nitrogen cycling processes in soil. Soil peptidases were targeted to gain information about the presence and activity of proteolytic bacteria in an environment that enriches soil nitrogen. While serine peptidase was detected at mRNA levels, rhizosphere effect was evident when the expression of bacterial neutral metallopeptidase in the three rhizospheres was investigated. It was observed that though transcripts for serine peptidase could be detected for all the three samples, no neutral metallopeptidase transcripts could be detected in *Lupinus* rhizosphere. Functional implications of the absence of neutral metallopeptidase expression in *Lupinus* rhizosphere warrant attention. In a study aimed at estimating the nitrogen rhizodeposition of grain legumes, Mayer *et al*. (2003a) using the same soil as described in the present study, reported that *Pisum* and *Lupinus* did not differ significantly in the amounts of nitrogen derived from rhizodeposition (Ndfr). In a related study by Mayer *et al*. (2003b) investigating the turnover of Ndfr, it was observed that the value was 21% in *Lupinus* whereas this value was 26 and 27% for *Vicia* and *Pisum*. Lack of neutral metallopeptidase expression could be one of the factors for the observed reduced mineralization. Using 16S rRNA as a phylogenetic marker, differences in the rhizosphere

bacterial communities of these three legumes were observed. *Lupinus* rhizosphere was shown to lack members of δ-subdivison of proteobacteria, *Bacillus-Clostridium* group and members of *Sphaerobacteridae*. By selective inhibition of specific microorganisms, it has been shown that soil peptidases are mainly of bacterial origin in a paddy soil (Hayano and Watanabe, 1990; Watanabe and Hayano, 1993a and b). Asmar *et al*. (1992) showed a high correlation between proteolytic activities and total counts of bacteria in glucose-amended soils.

4.2.3.2 Nitrite reductases

In organic farming systems, biological nitrogen fixation and the amendment with organic bound nitrogen in manures are the main source of nitrogen for the crop. It is especially interesting to study the process of denitrification that leads to loss of nitrogen from agricultural soil in such agricultural system. Several studies have targeted *nir* genes as a measure of the diversity of denitrifying bacteria. Denitrifiers are polyphyletic and widely dispersed in 16S rRNA phylogeny. Denitrifying community have been analysed from various habitats, e.g. marine sediments (Braker *et al*., 2000), estuarine sediments (Nogales *et al*., 2002), wastewater treatment plants and activated sludge (Hallin and Lindgren, 1999).

Successful amplification of *nirK* (coding for Cu containing nitrite reductase) from both DNA and mRNA revealed not only its presence in the gene pool but also its expression in the rhizospheres of the three legumes under investigation. *nirS* gene fragment (coding for heme *c* and heme d_1 containing nitrite reductase) could not be detected even with rhizosphere DNA thus indicating the absence of *nirS* containing denitrifiers. Their presence in very low numbers, which go undetected by PCR, can however, not be overruled. Using the same primer pair as in this study, Prieme *et al*. (2002) could detect *nirK* gene in both forest upland and wetland soils, but *nirS* could only be detected in wetland soils. *nirS* and *nirK* have previously been reported from various systems like sediments (Braker *et al*., 2000; Braker *et al*., 2001; Nogales *et al*., 2002), ground water (Yan *et al*., 2003) and acid forest soil (Roesch *et al*., 2002). It is not possible to explain the non-detectable level of *nirS* in the rhizosphere in an ecological context, as virtually nothing is known about the environmental preferences of *nirK* and *nirS* containing denitrifiers. Oxygen threshold, carbon requirement and kinetic parameters vary between different denitrifiers (Tiedje, 1988) and between different denitrifying communities (Cavigelli and

Robertson, 2000; Holtan-Hartwig *et al*., 2000) and the Cu-Nir and cd_1-Nir enzymes may also have unknown differences related to various factors, e.g. pH, kinetic parameters or oxygen tolerance. The apparent predominance of either *nirK* or *nirS* in rhizosphere soils may reflect differences in denitrifier enzyme properties in rhizosphere where this process is strongly selected. Alternatively, this diversity may reflect traits of the microbes, which are not connected to denitrification (Prieme *et al*., 2002).

RFLP analysis

A total of 266 clones (83, 92 and 91 from libraries of *Vicia, Lupinus* and *Pisum* respectively) were subjected to RFLP analysis. RFLP analysis revealed that all the clones could be grouped into twelve different patterns. The *Lupinus* library was the most diverse one with all the 12 phylotypes being represented. When using 16S rDNA as a molecular marker, the *Lupinus* rhizosphere was also found to be the most diverse one among the three rhizosphere communities with respect to distribution across the different subdivisions of proteobacteria. Major denitrifiers belong to various sub-groups of proteobacteria. Only α- and δ- subdivisions were found in the RNA clone library of *Vicia* that was the least diverse among the three rhizospheres under study. Metabolic fingerprinting of the total RNA extracted from the same samples using M13 reverse and also a 10 mer primer revealed that *Lupinus* and *Pisum* had profiles more closely related to each other than to *Vicia*.

Mayer *et al*. (2003b) reported different nitrogen rhizodeposition value with the lowest of 21 mg kg^{-1} soil in *Pisum* rhizosphere when compared to the other two (61 and 52 for *Vicia* and *Lupinus* respectively). High evenness in *Pisum* rhizosphere could be attributed to this low value, which in turn would reduce the selection effect of rhizodeposits on denitrifiers in the community. Least number of clones were analysed for the *Vicia* library and so the possibility to observe new phylotypes were reduced in this case as compared to the other two legumes.

Prieme *et al*. (2002) reported a higher diversity of *nir* genes from upland and marsh soils. In upland soil, 18 different phylotypes could be observed for 50 clones analysed. In the marsh habitat, the number of phylotypes increased to 37 when 93 clones were screened. This study was based on DNA analysis from bulk soil. Rhizosphere effect results in selection of specific bacterial population in the surrounding of the plant root and this selection is plant species specific (Smalla *et al*., 2001). Since only the mRNA transcripts

for clone libraries were analysed, the relatively low number of phylotypes observed in the present study can be explained.

Sequence Analysis

Sequences of *nirK* clones belonging to RFLP patterns 1, 2, 4, 6, 11 and 12 were very similar to the *nirK* sequence of *Mesorhizobium* sp. (accession number AY078254). The other six patterns had no match with *nirK* sequence of any cultured or uncultured bacteria in the database. The deduced amino acid sequences for these patterns revealed conserved copper ligands His-145, Met-150 and His-306 (*Achromobacter cycloclastes* [accession number Z48635] numbering) and His-255, which is not a copper ligand in the crystal form of the enzyme but is close to the active site of the enzyme (Adman *et al*., 1995), indicating that the sequences could produce functional enzymes. A study by Casciotti and Ward (2001) on several ammonia oxidizers had shown that despite sequence divergence in areas outside the copper-binding regions, the copper-binding residues are highly conserved in *nirK*. This suggests that there has been selective pressure for this type of nitrite reductase to remain functional during the course of its evolution. Sequences very divergent from those described for cultured denitrifiers have previously been reported from various environmental samples (Braker *et al*., 2000; Prieme *et al*., 2002). Since there is a lack of *nirK* gene sequence data for most of the denitrifying bacteria described so far, which include members of a wide variety of phylogenetically and metabolically diverse bacterial and archaeal genera (Zumft, 1997), it is not known whether new *nirK* sequences retrieved from environmental samples are sequences of unknown, uncultured bacteria or whether they are related to undetermined *nirK* gene sequences from known denitrifiers.

The *nirK* tree revealed three important patterns. First, all the patterns clustered together indicating them to be more closer to each other than to other reference sequences. Second, the cluster containing the patterns grouped together with the *nirK* sequences of *Mesorhizobium* sp. Third, three subclusters could be observed within the major cluster.

Any given RFLP pattern may represent sequences from multiple phylogenetic groups and may therefore not represent a true phylotype in the traditional sense (Prieme *et al*., 2002). RFLP analysis indicated widely different *nirK* communities in the three rhizosphere soils, with only five RFLP patterns are common in all three rhizosphere soils. This analysis alone could have led to the conclusion that widely different denitrifying communities inhabit the

three rhizosphere soils while sequence analysis showed that besides the five patterns that were common in *Vicia*, *Lupinus* and *Pisum* rhizospheres, there were high similarities between patterns 1, 6, 11 and 12 and also between 2 and 4. Similar observations have been reported by Prieme *et al*. (2002) wherein RFLP analysis indicated two soils under study to be distinctly different, while the sequence analysis showed that the two communities were overlapping.

4.3 Conclusions and perspectives

Plant roots selectively activate a specific microbial community in their vicinity. This effect is caused by the rhizodeposition from the roots. Using two different techniques, DGGE and cloning, marked differences between both structural and functional diversity of microbial communities present in the rhizospheres of the three legumes were shown. These differences can be related to differences in nitrogen rhizodeposition from the roots of these legumes.

The remarkably high number of actinomycete population observed in the rhizosphere suggests that actinomycetes play a major role in legume rhizospheres during the fruiting stage and some of these may have relevance in the biocontrol of pathogens. This stimulates new efforts to isolate actinomycetes from these rhizosphere soils to be able to test their properties in biocontrol in detail. Expression of other functional genes specific to the group needs to be investigated for a better understanding of their function in legume rhizosphere. Absence of neutral metallopeptidase gene (*npr*) from *Lupinus* rhizosphere warrants attention because reduced N-mineralization has been reported in the plant rhizosphere. Among the three legume rhizopheres under comparison, the *Lupinus* rhizosphere was the most diverse with respect to the bacterial community and nitrite reductase gene (*nirK*) composition. Further analysis of this increased diversity in *Lupinus* rhizosphere needs to be conducted to identify its potential as a better green manure.

5. SUMMARY

The aim of this study was to increase the understanding of diversity and activity of dominant bacterial populations in the rhizospheres of three economically important grain legumes (*Vicia faba*, *Lupinus albus* and *Pisum sativum*). A cultivation-independent approach was employed to achieve this aim bearing in mind the limitation of cultivation-dependent technique that only 10% of bacteria present in rhizosphere can be cultured. PCR amplification of 16S rDNA and subsequent separation of the amplicons by DGGE was used in an initial screening of replicates for experimental variation and for the first characterization of bacterial community composition of the three rhizospheres under study. Specific profiles generated by the three legumes, derived by both 16S rDNA and rRNA, emphasized the need to perform detailed analysis of the communities present in these rhizospheres. Clone libraries for PCR and RT-PCR products were generated for representative samples of all the three legumes. Firmicutes were found to be the most dominant in all the legumes, both in DNA- and RNA-derived libraries, indicating them to be the most active group as well. A plant-dependent rhizosphere effect was reflected by the absence of β-subdivision members in *Pisum* and γ-subdivision members of proteobacteria in *Vicia* rhizosphere. High numbers of as yet unclassified bacteria were also obtained. With this experimental set-up, using the same soil material but three different legumes and a uniform inoculation with *Rhizobium* sp., it became evident that plant roots influence the development of bacterial communities in the rhizosphere in a plant-specific manner. The extent of the rhizosphere effect could vary in natural field conditions as the present study was performed under controlled conditions in green house using soil from agricultural site.

Extraction and analysis of rRNA has enabled identification of active taxa in the present study. Fingerprints were obtained for total RNA using two different primers. The profiles generated revealed marked differences between the three rhizospheres of the three legumes under study, indicating differences between the metabolic status of the bacterial communities present in the rhizospheres of these three legumes. To address the question of functional diversity, mRNA extraction and subsequent RT-PCR were performed for various genes important in nutrient cycling. The presence of chitinase genes could be established by specific PCR amplification using DNA extracted from the three rhizospheres. However, no expression of the gene could be detected by RT-PCR. Enzyme assays confirmed no or very low levels of the chitinase protein in the rhizospheres.

Analysis of proteolytic enzymes (serine and neutral metallopeptidases) showed the presence and activity of serine peptidase in the three rhizospheres. Neutral metallopeptidase gene was also present in the three rhizospheres but no expression could be detected in the *Lupinus* rhizosphere. This was a confirmation of plant-dependent effect at the level of functioning of the bacterial communities. Genes for nitrite reductase (*nirK* and *nirS*), which may lead to removal of nitrogen from the system by denitrification, were targeted to gain an understanding of the importance of this enzyme in a nitrogen-enriching environment. The presence of *nirS* was not detected in any of the legume rhizospheres, but both the presence and activity of *nirK* was established for the three rhizospheres. The diversity of this gene was investigated by generating clone libraries with the RT-PCR products from the three plant rhizospheres. The observation of distinct differences in the distribution of phylotypes of expressed *nirK* gene in the three legume rhizospheres confirmed a plant specific effect on the functions of the rhizosphere bacterial communities.

The present study revealed a hitherto unknown diversity of rhizospheric bacteria associated with grain legumes. Entirely cultivation-independent approaches to characterize the structure and function of the bacterial community of the rhizosphere of the three grain legumes clearly revealed plant-dependent rhizosphere effect on bacterial community structure and function.

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7. APPENDIX

7.1 Figure legends

7.2 Table legends

7.3 Curriculum Vitae

Name: Shilpi Sharma

Date of birth: 16th March 1977

Place of birth: Varanasi, India

Parents: Prem Kant Sharma

Usha Sharma

Marital status: Married

Husband: Manish Kumar Aneja

Educational qualifications:

1991-1992 All India Secondary School Examination, Central Board of Secondary Education), India (Class 10th).

1992-1994 All India Senior School Certificate Examination, Central Board of Secondary Education), India (Class 12th).

1994-1997 Bachelor of Science (Botany), Mahila Mahavidyalaya, Banaras Hindu University, Varanasi, India.

1997-1999 Master of Science (Botany), Banaras Hindu University, Varanasi, India.
Dissertation Title: Transgenic Plants.

2000-2003 Ph.D under the guidance of Prof. Dr. Anton Hartmann and Dr. Michael Schloter at Institute of Soil Ecology, GSF-National Research Center for Environment and Health, Neuherberg, Germany.
Title: Structural and functional characterization of bacterial diversity in the rhizospheres of three grain legumes.