

UNIVERSIDADE ESTADUAL DE CAMPINAS

Valéria Maia de Oliveira

**Diversidade Genética de Rizóbios em Amostras Ambientais
Analisada Através do Uso de Sondas Moleculares e *Primers* Específicos**

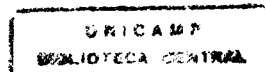
Este exemplar corresponde à redação final da tese defendida pelo(a) candidato(a) <u>Valéria Maia de Oliveira</u> e aprovada pela Comissão Julgadora.
--

04/11/99 *Gilson Paulo Manfio*

Tese apresentada ao Instituto de Biologia para obtenção do Título de Doutor em Genética e Biologia Molecular na Área de Genética de Microrganismos.

Orientador: Dr. Gilson Paulo Manfio

- 1999 -



UNIDADE	IB
INSTITUIÇÃO:	UNICAMP
V.	OL4d
TOMADA	278/00
PROD	278/00
C	☐
D	☒
PREC	278/00
DATA	27/02/00
Nº CDE	

CM-00137783-1

**FICHA CATALOGRÁFICA ELABORADA PELA
BIBLIOTECA DO INSTITUTO DE BIOLOGIA - UNICAMP**

Oliveira, Valéria Maia de
OL4d Diversidade genética de rizóbios em amostras ambientais analisada através do uso de sondas moleculares e *Primers* específicos /Valéria Maia de Oliveira. -- Campinas, SP: [s.n.],1999.
 175f.: illus.

Orientador: Gilson Paulo Manfio
 Tese (doutorado) - Universidade Estadual de Campinas, Instituto de Biologia.

1. Sondas moleculares. 2. Ácido desoxirribonucleico ribosomal.
 3. DNAr. I. Manfio, Gilson Paulo. II. Universidade Estadual de Campinas.Instituto de Biologia. III. Título.

Defesa Pública: Campinas, 04 de novembro de 1999

Banca Examinadora

Titulares:

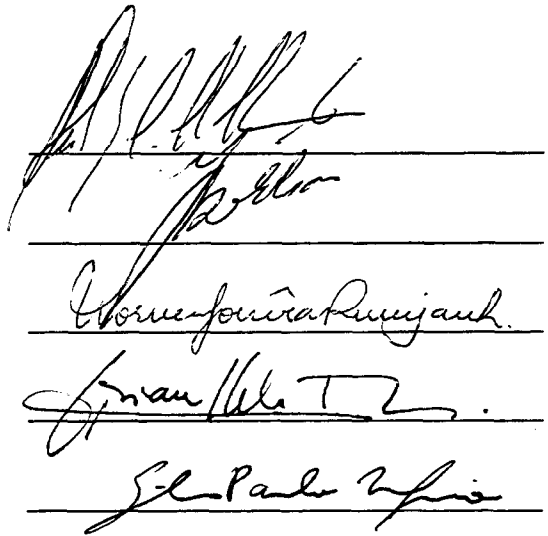
Dr. Heitor Luiz da Costa Coutinho

Dr. Jan Dirk van Elsas

Dra. Norma Gouvea Rumjanek

Dra. Vivian Helena Pellizari

Dr. Gilson Paulo Manfio (orientador)



Handwritten signatures of the five titular members of the exam board, each on a horizontal line.

Suplentes:

Dra. Fátima Maria de Souza Moreira

Dra. Suzete Aparecida Lanza Destefano



Two horizontal lines for the signatures of the substitute members of the exam board.

*Dedico esta tese a meus pais, Walter e Teresinha,
que me acompanharam e me apoiaram em todas
as etapas de minha vida, e a quem devo tudo.*

“...Queira
Basta ser sincero e desejar profundo
Você será capaz de sacudir o mundo
Vai, tente outra vez...”
Raul Seixas

Agradecimentos:

Ao Gilson, meu orientador e grande amigo, pela excelente orientação, apoio, confiança, companheirismo e inestimável amizade. Ainda, por todos os momentos excepcionais, que foram muitos, dentro e fora do laboratório.

Ao Heitor, pela orientação e apoio em muitos momentos durante o desenvolvimento do trabalho, e por sua sincera amizade.

Ao Dick, pela orientação, atenção dispensada e disponibilização de seu laboratório para desenvolvimento de parte do trabalho de tese. Também à Anneke e Ludwina, pelo excelente suporte técnico e convivência durante este período.

Aos amigos, Eliane, Luciano e Pedro, hoje distantes mas não esquecidos, cuja amizade, carinho e solidariedade me permitiram superar momentos difíceis.

Ao Cor e à Sonia, também distantes, pela companhia, pelos longos e informativos diálogos durante a hora do jantar, pela amizade simples e sincera que ficou.

À Yoko, pelo apoio e amizade de sempre, e pela pronta ajuda sempre que solicitada.

À Fundação André Tosello, pelo apoio e disponibilização de sua infra-estrutura para desenvolvimento do trabalho de tese, e pelas oportunidades oferecidas.

A todos os amigos da Fundação André Tosello, pela agradável convivência no dia-a-dia, pelos divertidos momentos nas nossas confraternizações e por toda a compreensão e ajuda nos momentos em que precisei.

À Lyriam, minha querida “amiuga”, pelo carinho, identidade, companheirismo e apoio em todos os momentos. Por estar sempre presente. Por tudo.

Ao Eduardo, pela sua doce amizade e pelos maravilhosos jantares.

Às amigas Fabiana e Susi, por todos os ótimos momentos que passamos juntas, pela ajuda em muitas etapas do trabalho, pela troca de idéias, pelo apoio de sempre, e, finalmente, pela eterna amizade.

À Silvana, minha “irmã”, pela nossa longa e sincera amizade.

À Regina, pelo conforto da nossa amizade, e por todos os momentos especiais.

Ao Marcos, pela doçura, carinho e companheirismo. Por me fazer feliz.

Aos meus pais, por sempre acreditarem e confiarem em mim. Pelo amor incondicional. Por tudo que sou.

Ao CNPq, FAEP e FAPESP, pelo apoio financeiro.

ÍNDICE

Resumo	vii
Abstract	viii
1. Introdução	1
2. Revisão de Literatura	4
2.1. Sistemática de rizóbios: “ <i>Systematics of Legume Nodule Nitrogen Fixing Bacteria: Agronomic and Ecological Applications</i> ”	4
<i>Introduction</i>	5
<i>Current knowledge: classification and taxonomy</i>	6
<i>The application of molecular probe technology</i>	13
<i>Agronomic and ecological applications</i>	17
<i>Concluding remarks</i>	24
<i>References</i>	24
2.2. Métodos moleculares no estudo da diversidade microbiana do solo: “ <i>Evaluating the Microbial Diversity of Soil Samples: Methodological Innovations</i> ”	32
<i>Abstract</i>	33
<i>Introduction</i>	33
<i>Molecular probes</i>	34
<i>Use of ribosomal RNA as phylogenetic markers</i>	35
<i>The extraction of nucleic acid from soils and sediments</i>	37
<i>rDNA sequence analysis</i>	38
<i>Sequencing and sequence analysis</i>	38
<i>Rapid methods for the comparison of DNA fragments (RFLP and T-tracking)</i>	39
ARDRA	39
DGGE	40
<i>Methodological limitations</i>	42
<i>General conclusions</i>	42
<i>References</i>	42
2.3. Diversidade de rizóbios em solos tropicais	46
2.4. Efeitos do manejo agrícola sobre a microbiota do solo	47
3. Objetivos Gerais	50
4. Objetivos Específicos	50

Artigo 1: “Discrimination of <i>Rhizobium tropici</i> and <i>R. leguminosarum</i> strains by PCR-specific amplification of 16S-23S rDNA spacer region fragments and denaturing gradient gel electrophoresis (DGGE)”	52
<i>Introduction</i>	52
<i>Materials and Methods</i>	53
<i>Results and Discussion</i>	54
<i>References</i>	55
Artigo 2: “Genetic diversity of <i>Rhizobium tropici</i> and <i>R. leguminosarum</i> in soil samples under different agricultural practices analyzed by DGGE of 16S-23S rDNA spacer sequences”	57
<i>Abstract</i>	58
<i>Introduction</i>	59
<i>Materials and Methods</i>	60
<i>Results</i>	66
<i>Discussion</i>	81
<i>References</i>	85
Artigo 3: “Evaluation of the diversity of rhizobia in Brazilian agricultural soils cultivated with soybeans”	89
<i>Abstract</i>	89
<i>Introduction</i>	89
<i>Materials and Methods</i>	90
<i>Results</i>	91
<i>Discussion</i>	94
<i>Conclusions</i>	96
<i>References</i>	96
Capítulo avulso: Abordagem Molecular Direta no Estudo da Diversidade de <i>Bradyrhizobium</i> sp.	98
<i>Introdução</i>	98
<i>Material e Métodos</i>	99
<i>Resultados e Discussão</i>	107
5. <i>Discussão Geral</i>	123
6. <i>Conclusões</i>	128
<i>Referências Gerais</i>	130

RESUMO

Uma estratégia molecular direta e rápida, baseada no uso de sondas e *primers* específicos, foi desenvolvida com a finalidade de se detectar e avaliar a diversidade de rizóbios em amostras de solo sujeitas a diferentes práticas agrícolas.

Primers específicos para *Rhizobium leguminosarum* e *Rhizobium tropici* foram desenhados através da análise de sequência de regiões espaçadoras 16S-23S do operon RNAr e testados em reações de PCR com linhagens-referência (**Artigo 1**). Estes *primers* foram a seguir empregados na amplificação de fragmentos de DNAr utilizando DNA extraído diretamente da comunidade microbiana do solo. Um passo intermediário de amplificação da região espaçadora 16S-23S, utilizando *primers* para regiões conservadas dentro do Domínio Bacteria, foi necessário para enriquecimento do número de moléculas-alvo de *R. leguminosarum* e *R. tropici* antes de se proceder ao PCR específico. Fragmentos de DNAr de *R. tropici* e *R. leguminosarum* foram posteriormente submetidos à eletroforese em gel gradiente desnaturante (DGGE) para separação de moléculas com sequências distintas. Este método permitiu a análise direta da riqueza e abundância relativas de *R. tropici*/*R. leguminosarum* em amostras de solo sob diferentes práticas agrícolas em função do número e intensidade de bandas visualizadas no gel DGGE (**Artigo 2**).

A estratégia molecular empregada na avaliação da diversidade do grupo *Bradyrhizobium* sp. envolveu o desenho de uma sonda de oligonucleotídeo, baseado na análise de sequências de RNAr 16S disponíveis em bases de dados, específica para *Bradyrhizobium* sp. e *B. elkanii*. A sonda foi utilizada na seleção de fragmentos de DNAr, amplificados a partir do DNA ambiental e clonados em plasmídeos, visando experimentos posteriores de PCR-RFLP para análise de diversidade. Entretanto, os resultados encontrados para o grupo de *Bradyrhizobium* sp. sugerem o desenvolvimento de *primers* específicos, baseados na sequência variável da região espaçadora 16S-23S, e o emprego de PCR e análises de DGGE, como estratégia alternativa a ser utilizada em estudos futuros, numa tentativa de aumentar a sensibilidade na detecção de organismos presentes em baixo número no solo, como no caso dos rizóbios (**Capítulo avulso**, não publicado).

Um estudo paralelo, baseado na caracterização genômica de rizóbios isolados das amostras de solo utilizando-se planta-isca e plaqueamento em meios de cultivo seletivos, foi realizado visando a comparação com a metodologia direta descrita anteriormente para *R. leguminosarum* e *R. tropici* (**Artigo 2**). Neste estudo, os microrganismos foram caracterizados utilizando RAPD e os padrões de bandas analisados pelo índice de diversidade de Shannon-Weaver (**Artigo 3**). A aplicação deste índice demonstrou que a diversidade de rizóbios foi significativamente reduzida nas amostras de solo originadas das parcelas cultivadas com soja, em comparação àquelas sob pastagem.

ABSTRACT

A direct and rapid molecular approach, based on the use of specific DNA probes and primers, was developed to detect and evaluate the diversity of rhizobia in soil samples under different agricultural practices.

Primers targeting *Rhizobium leguminosarum* and *Rhizobium tropici* 16S-23S rDNA spacer fragments were designed based on sequence data derived from representative strains and tested in PCR reactions using DNA from a range of phylogenetically related organisms (**Article 1**). A nested PCR approach was then used to amplify *R. leguminosarum*/*R. tropici* soil community rDNA, comprised by an initial enrichment amplification step using primer sets flanking conserved regions of bacterial 16S-23S rDNA spacer sequences, followed by subsequent specific amplification using the primers targeting *R. leguminosarum*/*R. tropici*. Rhizobia-specific rDNA fragments were separated using denaturing gradient gel electrophoresis (DGGE), generating complex fingerprints. The resulting banding patterns enabled the direct analysis of relative richness (number of bands) and abundance (intensity of bands) of *R. leguminosarum* and *R. tropici* strains in the soils under different agricultural treatments (**Article 2**).

The approach used to evaluate the diversity of *Bradyrhizobium* spp. comprised the design of a *Bradyrhizobium* sp./*B. elkanii* oligonucleotide probe, based on sequence analysis of 16S rRNA data recovered from databases. The specific probe was used to screen plasmid-cloned ribosomal sequences amplified from soil community DNA, aiming at further PCR-RFLP characterization for *Bradyrhizobium* diversity studies. However, the results obtained suggest that specific primers for *Bradyrhizobium* spp. should be developed, targeting the highly variable 16S-23S rDNA spacer region, and used in PCR/DGGE analyses. This latter approach might be more adequate for the detection of organisms present in very low numbers in soil (**Unpublished chapter**).

A parallel study, based on the genomic characterization of rhizobial strains isolated from soil samples using a legume as trap-host and selective media, was also carried out to enable a comparison with the direct molecular approach previously described for *R. leguminosarum*/*R. tropici* (**Article 2**). In this study, strains were characterized through the RAPD technique and fingerprint data analyzed by using the Shannon-Weaver diversity index (**Article 3**). The application of this index demonstrated that the diversity of rhizobia was significantly reduced in soil samples from plots cultivated with soybean compared with uncultivated pasture plots.

Nota preliminar: Todas as referências citadas em ítems que não se constituem em artigos publicados ou submetidos para publicação, estão listadas no ítem denominado **Referências Gerais**.

1. INTRODUÇÃO

Rizóbios são bactérias pertencentes à família Rhizobiaceae capazes de formar nódulos em raízes e caules de espécies de leguminosas, onde realizam a fixação biológica de nitrogênio (FBN) (Nutman, 1987; Hirsh, 1992).

Recentemente, a taxonomia da família Rhizobiaceae vem sofrendo várias mudanças, mostrando-se bastante complexa e tornando evidente a necessidade de uma revisão baseada numa abordagem polifásica (Martinez-Romero & Caballero, 1996; de Lajudie *et al.*, 1998; Ludwig *et al.*, 1998; Terefework *et al.*, 1998). Atualmente, a família Rhizobiaceae inclui os gêneros *Agrobacterium*, *Phyllobacterium*, *Allorhizobium*, *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium* e *Sinorhizobium*. Entretanto, as bactérias fixadoras de nitrogênio estão distribuídas apenas entre os 6 últimos gêneros. O gênero *Bradyrhizobium*, que continha uma única espécie válida, *B. japonicum* (Jordan, 1984), inclui agora duas novas espécies: *B. elkanii* (Kuykendall *et al.*, 1992) e *B. liaoningense* (Xu *et al.*, 1995). O gênero *Azorhizobium*, descrito para um grupo de bactérias capazes de nodular raízes e caule de *Sesbania rostrata*, contém apenas a espécie *A. caulinodans* (Dreyfus *et al.*, 1988b). O gênero *Rhizobium* foi reestruturado e divide-se em 9 espécies: *R. leguminosarum*, contendo os biovars *trifolii*, *phaseoli* e *viceae*, *R. galegae*, *R. tropici*, *R. etli*, *R. gallicum*, *R. giardinii*, *R. hainanensis* (Ludwig *et al.*, 1998), *R. huautlense* (Wang *et al.*, 1998) e *R. mongolense* (van Berkum *et al.*, 1998); o gênero *Sinorhizobium* em 6 espécies: *S. meliloti*, *S. fredii*, *S. xinjiangensis*, *S. saheli*, *S. teranga* e *S. medicae* (Ludwig *et al.*, 1998); e o gênero *Mesorhizobium* em 7 espécies: *M. loti*, *M. huakuii*, *M. ciceri*, *M. tianshanense*, *M. mediterraneum* (Coutinho *et al.*, 1999) e, recentemente, *M. plurifarium* (de Lajudie *et al.*, 1998) e *M. amorphae* (Wang *et al.*, 1999). O gênero *Allorhizobium* inclui apenas uma espécie válida, *A. undicola*, descrita recentemente para um grupo de isolados que nodulam caule de *Neptunia natans* (de Lajudie *et al.*, 1998).

A contribuição da FBN à economia da produção de leguminosas comerciais é muito significativa, uma vez que as aplicações de fertilizantes nitrogenados podem ser em grande parte reduzidas através da inoculação de linhagens de rizóbios eficientes na fixação de nitrogênio (Döbereiner & Duque, 1980; Boddey *et al.*, 1984). Além dos benefícios

econômicos, a redução na aplicação de fertilizantes aumenta a qualidade ambiental da produção agrícola, contribuindo para a sustentabilidade na agricultura.

A eficiência dos microrganismos diazotróficos em fixar nitrogênio e a capacidade destes em sobreviver no solo e formar nódulos dependem, além dos fatores genéticos inerentes aos simbiossiontes, da interação destes com o ecossistema. Os sistemas fixadores de nitrogênio que foram melhorados em laboratório, estufa ou viveiro não são necessariamente capazes de alcançar o seu potencial máximo no campo sem antes passarem por um processo de adaptação à situação de campo (Dreyfus *et al.*, 1988a). A introdução de linhagens fixadoras mais eficientes no solo contendo populações nativas ou naturalizadas de rizóbios pode, por exemplo, ser prejudicada devido à baixa competitividade ou falta de adaptação das linhagens inoculadas às condições ambientais locais (Anyango *et al.*, 1995; Neves & Rumjanek, 1997). O emprego de fungicidas e herbicidas em sistemas de agricultura intensiva é também um fator que afeta a nodulação de leguminosas quando aplicados em altas concentrações, reduzindo a fixação de nitrogênio (Fisher, 1976; Graham *et al.*, 1980; De-Polli *et al.*, 1986).

Torna-se evidente, portanto, a necessidade de avaliar, quantificar e monitorar a diversidade de rizóbios no solo, assim como a extensão do impacto de práticas agrícolas sobre comunidades naturais destes microrganismos. A alteração ou perda desta biodiversidade pode acarretar o desaparecimento de linhagens inoculantes potenciais, assim como a diminuição do potencial de fixação de nitrogênio dos solos, prejudicando, por exemplo, o desenvolvimento de práticas de recuperação e conservação de solos.

O estudo da diversidade de rizóbios tem se baseado normalmente na caracterização fenotípica e/ou genotípica de linhagens isoladas do ambiente (Geniaux *et al.*, 1993; Moreira *et al.*, 1993; Paffetti *et al.*, 1996; Sullivan *et al.*, 1996). Em geral, o isolamento dos rizóbios presentes no solo depende do uso de leguminosas-isca para a captura destes. Este procedimento pode levar a uma avaliação subestimada da diversidade destes microrganismos, uma vez que os rizóbios competitivamente menos eficientes na nodulação das espécies de leguminosas utilizadas para a captura não serão igualmente representados no estudo.

Métodos de extração direta de DNA do solo (Ogram *et al.*, 1988; Steffan *et al.*, 1988; Smalla *et al.*, 1993) e o uso de sondas moleculares e *primers* adequados para a detecção específica de microrganismos, ou de genes, vêm sendo extensivamente empregados em estudos de composição e monitoramento de populações microbianas naturais ou introduzidas no ambiente, na tentativa de superar as limitações impostas pelos métodos clássicos de isolamento e cultivo (Ford & Olson, 1988; Holben *et al.*, 1988; Pickup, 1991; Steffan & Atlas, 1991; Ludwig *et al.*, 1998).

Recentemente, as regiões variáveis dos genes codificadores dos RNAs ribossômicos vêm sendo exploradas como alvos para a detecção molecular de organismos no solo, dada a universalidade e alto número de cópias do RNAr (Ward *et al.*, 1990; Stackebrandt *et al.*, 1993; Felske *et al.*, 1998; Nüsslein & Tiedje, 1998). Um grande número de sequências está disponível em bases de dados e o alinhamento destas é facilitado por *softwares* de livre acesso, permitindo a identificação de regiões com sequências únicas para grupos taxonômicos ou organismos específicos. Entretanto, a variação genética do DNAr 16S pode não ser, muitas vezes, suficiente para diferenciar linhagens dentro de uma mesma espécie (Vallaeys *et al.*, 1997).

Em contraste, análises da região espaçadora entre o DNAr 16S e o 23S têm mostrado que esta pode apresentar relativamente maior variação quanto ao tamanho e sequência entre linhagens e/ou espécies relacionadas quando comparada com o DNAr 16S (Gürtler & Stanisich, 1996). Com o uso de *primers* para sequências conservadas que flanqueiam as regiões espaçadoras, as variações que ocorrem nestas últimas podem gerar produtos de PCR de tamanho variável capazes de diferenciar organismos em nível intra-específico (Barry *et al.*, 1991; Jensen *et al.*, 1993).

A técnica de eletroforese em gel gradiente desnaturante (DGGE) é uma estratégia que tem sido empregada recentemente na determinação direta da diversidade genética de populações microbianas complexas (Muyzer *et al.*, 1993; Wawer & Muyzer, 1995; Felske *et al.*, 1997). Esta técnica permite que fragmentos de DNA com mesmo tamanho, mas com sequências de nucleotídeos diferentes, sejam separados através de eletroforese em gel de poliacrilamida contendo um gradiente linear de agente desnaturante (Fischer & Lerman, 1983). Assim, fragmentos de um gene específico, como o gene para o RNAr 16S ou a região espaçadora 16S-23S, podem ser amplificados por PCR a partir de amostras mistas e separados por DGGE, constituindo um perfil de bandas que reflete a diversidade e/ou composição da população microbiana.

A aplicação de metodologias moleculares diretas, ou seja, que não dependam do isolamento e cultivo dos organismos, no estudo de populações naturais de rizóbios permitirá uma avaliação rápida e precisa da sua diversidade no ambiente.

2. REVISÃO DE LITERATURA

2.1. Sistemática de rizóbios

Obs.: O material apresentado a seguir será publicado como capítulo de livro: Systematics of Legume Nodule Nitrogen Fixing Bacteria: Agronomic and Ecological Applications *In*: F. Priest and M. Goodfellow (ed.), **Applied Microbial Systematics** , 105-131, 1999 Kluwer Academic Publishers. (no prelo)

5 SYSTEMATICS OF LEGUME NODULE NITROGEN FIXING BACTERIA

AGRONOMIC AND ECOLOGICAL APPLICATIONS

HEITOR L. C. COUTINHO ¹, VALÉRIA M. DE OLIVEIRA ²
and FÁTIMA M. S. MOREIRA ³

¹ *Embrapa Solos, Rua Jardim Botânico 1024, Rio de Janeiro -
RJ, 22460-000, Brazil;*

² *Fundação Tropical de Pesquisas e Tecnologia André Tosello,
R. Latino Coelho 1301, Campinas - SP, 13087-010, Brazil;*

³ *Departamento de Ciências do Solo, Universidade Federal de Lavras,
Caixa Postal 37, Lavras - MG, 37200-000, Brazil.*

1 Introduction

Biological nitrogen fixation (BNF) is easily the most studied microbial process applied to agriculture. It consists of the reduction of atmospheric dinitrogen (N_2), unavailable to higher plants, into ammonium (NH_4^+), an assimilable form of this nutrient. The BNF process is performed solely by microorganisms, the majority inhabiting the soil ecosystem, and is considered to be the most relevant component of the global nitrogen cycle (Ishizuka, 1992). The best known diazotrophs (dinitrogen fixing bacteria) are rhizobia, which are able to establish a symbiotic relationship with plants of the family Leguminosae, hereby called legumes. This symbiosis is characterized by a highly specific association between plant and bacteria. Particular varieties of legume species recognize specific strains of rhizobia, which are able to infect the legume roots. This process triggers the expression of certain plant genes, resulting in the development of nodules around the site of invasion. In the interior of the nodules the bacteria undergo morphological and physiological transformations, becoming nitrogen fixing bacteroides and supplying the plant with nutrient in exchange for carbon-rich material derived from plant photosynthesis.

BNF contributions to the economics of legume crop production are very significant, since the need for fertilizer applications can be greatly reduced. The inoculation of legume seeds with diazotrophs is a common practice in several countries. In Brazil, soybean inoculation with strains of *Bradyrhizobium elkanii* and *Bradyrhizobium japonicum* is a great success, and is responsible for a reduction in production costs of approximately 1.3 billion US\$ per year (Siqueira & Franco, 1988). Apart from the economic benefits, reductions in fertilizer applications may enhance the environmental quality of agricultural production, since there are lower rates of leaching of nitrates to both surface and ground waters (Boddey *et al.*, 1984).

The last decade has witnessed a redirection of global policies of development. This is characterized by assimilation of the concept of sustainability into projects funded by governmental and non-governmental agencies. This has led to the establishment of a common goal to all of those involved in the agricultural business, namely the achievement of sustainable production systems with minimal environmental impact and absence of the degradation of natural resources. Sustainable agricultural systems can benefit from BNF by using legumes at any stage of the production line. Agroforestry systems may contain tree legume species, which can supply fodder, fuelwood or shade, in consortia with annual crops, such as maize or sorghum. Tree legumes may also be used as wind barriers or live fences with high efficiency and low cost. In crop rotation systems, grasses like maize, wheat, sugarcane, or pastures, are followed by legume crops such as soybeans, common beans or peanuts, in order to fertilize the soil with the nitrogen fixed by rhizobia as well as producing an extra income. Legumes may also be used as green manure whereby fixed nitrogen transported to the leaves enriches the soil after decomposition of the plant residues and mineralization of the organic nitrogen.

In order to maximize the benefits rendered by biological nitrogen fixation, research efforts originally focused on the isolation and screening of diazotrophic bacteria that could be used as inoculants in agricultural systems. This initial stage was very successful with the development of commercial inoculants for legumes worldwide. Later, the introduction of more efficient nitrogen fixing strains to soil containing native or naturalized populations of rhizobia was hampered by low competitiveness or lack of adaptation of the inoculant strains to the soil environment (Neves & Rumjanek, 1997). This led to an increased interest in the ecology of nitrogen fixing microorganisms. More recently, global concerns regarding the potential loss of biodiversity due to human activities have boosted initiatives to assess, catalogue, preserve and monitor the diversity of nitrogen fixing microorganisms in soils of diverse ecosystems.

Microbial systematics tools are required for the development of research projects in all of the areas mentioned in the previous paragraph. Therefore, advances in rhizobial research have followed closely the development of methodologies to classify, identify, differentiate, and detect specific microorganisms in the environment. In this chapter we review the application of microbial systematics to elucidate agronomic and ecological aspects of legume nodule, nitrogen fixing bacteria and the symbiotic process. Initially, the taxonomy of rhizobia will be placed in a historical perspective. This will be followed by a consideration of the application of DNA probe technology to the study of rhizobia and, finally, we conclude with remarks on future prospects for solving agronomic and ecological problems with the aid of modern techniques used in microbial systematics.

2 Current Knowledge: Classification

2.1 HISTORICAL PERSPECTIVE

A comprehensive review of the early history of *Rhizobium* taxonomy, which is summarized here, was brilliantly presented by Nutman (1987). The occurrence of nitrogen fixation in legumes was first hypothesized by Boussingault in 1837 (cited by Nutman, 1987), who was

followed by others, who failed to consider nodules as the actual sites of the nitrogen fixation process. They were criticized by Liebig, who stated that only manure could provide mineral elements found in plant ashes (Nutman, 1987). The strong personality of Liebig discouraged others from trying to clarify something that had been evident since ancient times, namely, why had legumes more nitrogen content than other plants? Finally, Hellriegel and Wilfarth (1888) demonstrated the occurrence of nitrogen fixation in legumes. Their only opponent was Frank, who argued against nitrogen fixation at scientific meetings. Ironically, Frank (1889) was the one who proposed *Rhizobium leguminosarum*, the bacterium which had been isolated from legume nodules by Beijerinck (1888), who had named it "Bacillus radicicola".

For many years, the study of legume-*Rhizobium* symbiosis was mainly restricted to a few legume species of the sub-family Papilionoideae, most of them agricultural crops from temperate regions. The taxonomy of rhizobia was based on the cross-inoculation concept, which assumed that members of each *Rhizobium* species were able to nodulate a specific group of host legumes (Jordan & Allen, 1974; see Table 1). There are many exceptions to this "rule" and, as a result, it failed as a criterion for species classification (Graham, 1976). The cross-inoculation criterion has not been considered since the first edition of *Bergey's Manual of Systematic Bacteriology* (Jordan, 1984).

TABLE 1. Classification of *Rhizobium* species according to cross-inoculation groups (Jordan & Allen, 1974)

Group	<i>Rhizobium</i> species	Hosts
I	<i>R. leguminosarum</i>	<i>Lens</i> , <i>Pisum</i> , <i>Vicia</i>
I	<i>R. trifolii</i>	<i>Trifolium</i>
I	<i>R. phaseoli</i>	<i>Phaseolus angustifolius</i> , <i>P. multiflorus</i> <i>P. vulgaris</i> ,
I	<i>R. meliloti</i>	<i>Medicago</i> , <i>Melilotus</i> , <i>Trigonella</i>
II	<i>R. lupini</i>	<i>Lupinus</i> , <i>Ornithopus</i>
II	<i>R. japonicum</i>	<i>Glycine</i>
II	Unclassified	Cowpea group

2.2 THE VALUE OF PHENOTYPIC TESTS

Molecular characterization tools, particularly nucleic sequence analysis, are essential for the classification of microorganisms (see next section). However, many microbiology laboratories do not yet have access to the infrastructure required for routine molecular biological practices and some methods are expensive and time consuming when applied to large numbers of strains. Thus, a first approach in *Rhizobium* classification and identification requires the analysis of characteristics that are easily assessed. Rhizobial cells are Gram-negative, aerobic rods lacking endospores, usually containing granules of poly- β -hydroxybutyrate, which are refractile under phase-contrast microscopy.

Graham (1976) pointed out that three characteristics were useful for rhizobial classification: growth rate, pH reaction on yeast mannitol agar (YMA) medium, and the type of flagella.

Generation times vary a great deal among *Rhizobium* strains, namely from 1.4 to 44.1 hours (Martinez-Dretz & Arias, 1972; Tan & Broughton, 1981; Kennedy & Greenwood, 1982; Hernandez & Focht, 1984). The same is true with regards to the growth rates of rhizobia (Lim & Ng, 1977; Gross *et al.*, 1979; Moreira, 1991; Moreira *et al.*, 1993). Recently, growth rates and generation times proved to be useful characteristics for the description of new rhizobia taxa. Members of the genus *Bradyrhizobium*, the species *Bradyrhizobium liaoningense*, and the genus *Mesorhizobium* are slow, very slow, and intermediary growers, respectively. SDS-PAGE analysis of total proteins grouped the majority of the very slow growing strains isolated from Brazilian native forest species with known *Bradyrhizobium* strains, except for three strains which did not cluster with any other rhizobial species (Moreira *et al.*, 1993). Further characterization is necessary to verify if these very slow growing strains belong to *B. liaoningense* or to other species of this genus.

The type of flagella is a conserved characteristic within each genus (Graham, 1976). It is important that all strains used in comparative analysis of flagella types are grown on the same growth media (Table 2). The type of flagella is consistent with the phylogenetic divisions of rhizobia (see Fig. 1), which makes this characteristic an important tool for preliminary analyses of collections of strains. The pH reaction resulting from growth on YMA is also usually consistent within genera: *Mesorhizobium*, *Rhizobium*, and *Sinorhizobium* species have an acid reaction or in some cases the pH is not modified, whereas *Azorhizobium* and *Bradyrhizobium* species produce alkaline reactions. Other cultural characteristics of members of each of these genera are shown in Table 3.

TABLE 2. Types of flagella found in members of the different genera of rhizobia (Graham, 1976)

Type of flagella	<i>Azorhizobium</i>	<i>Bradyrhizobium</i>	<i>Mesorhizobium</i>	<i>Rhizobium</i>	<i>Sinorhizobium</i>
One lateral	+	-	-	-	-
One subpolar	-	+	+	-	+
Peritrichous	+	-	+	+	+

Patterns of utilization of carbon sources do not seem to distinguish between members of rhizobial taxa, not even at the genus level, except for *Azorhizobium* (Table 5). The ability to metabolize sugars is not a conserved trait among rhizobia. A significant proportion of the carbon sources tested can give variable results at the intrageneric level (Dreyfus *et al.*, 1988; de Lajudie *et al.*, 1994; Jarvis *et al.*, 1997). It is not possible, therefore, to classify rhizobia on the basis of carbon utilization patterns. *Bradyrhizobium* and *Mesorhizobium* strains are able to utilize the greatest number of carbon sources, while *Azorhizobium* strains utilize only 52% of the sources tested in the studies summarized in Table 4. Utilization of mannitol and sucrose, except for *Azorhizobium* strains, is widespread among rhizobial genera. However, it must be considered that these are the common components of the usual isolation media and can act as a selective pressure for strains utilizing these sources. The same is true for pH, since common isolation media have a pH around 7.0. It is important to make slight modifications in pH, carbohydrate source or other medium components in order to isolate strains that do not grow on conventional YMA medium.

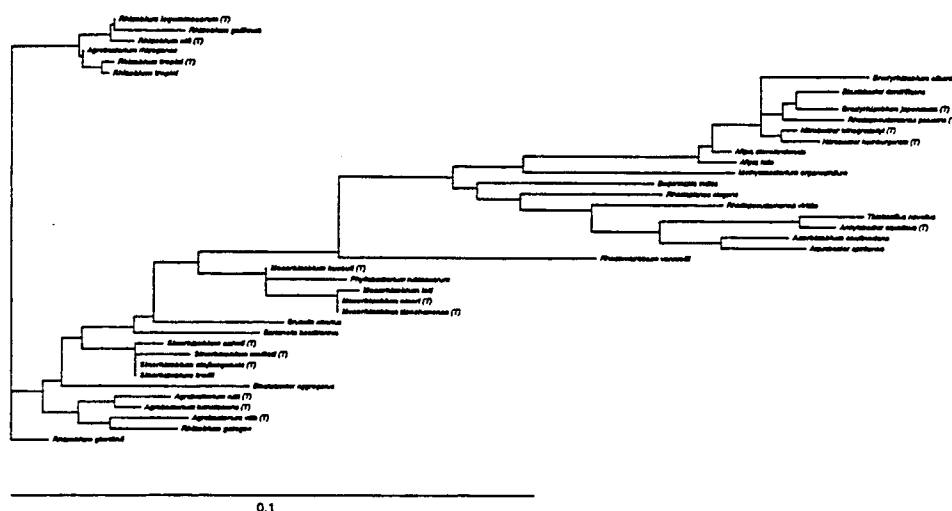


Figure 1. Phylogenetic tree of rhizobia species and their closest relatives derived by maximum likelihood analysis of small-subunit rRNA sequences. The tree is abstracted from that provided by the Ribosomal Database Project (Maidak *et al.*, 1997) by submitting the 16S rRNA sequences of *R. giardinii* and *R. gallicum* (GenBank accession numbers U86344 and AF008130, respectively) against the following RDP sequences: Afp.cleve, Ntb.winog2, Ntb.hambu2, Rps.palus5, Bdr.japon4, Bdr.jpj_76, Bld.denitr, Afp.felis, Mlb.orgn2, Bei.indica, Rm.vanniel, Rps.viridi, Thb.novell, Anc.aquati, Azr.cauli2, Aqb.spirit, Rhp.elegan, Rhb.etli42, Rhb.legu11, Ag.rhizog2, Rhb.tropi8, Rhb.tropi6, Srh.saheli, Srh.meli17, Srh.fredi2, Srh.xinjia, Bld.aggreg, Ag.vitis3, Rhb.galega, Ag.rubi3, Ag.tumefa6, Bar.bacill, Bru.aborts, Rhb.lot, Rhb.tiansh, Rhb.ciceri, Plb.rubi3, Rhb.huaku4. The scale indicates the evolutionary distance as the number of changes expected per sequence position (Olsen *et al.*, 1994). The species *Sinorhizobium medicae*, *S. teranga*, *Mesorhizobium mediterraneum*, and *Bradyrhizobium liaoningense* are not represented in the tree.

TABLE 3. Cultural characteristics of members of rhizobial genera grown on yeast-mannitol-agar (YMA) media (Vincent, 1970)

Azorhizobium - circular, cream coloured colonies, 0.5 mm diameter after 2 to 5 days of incubation at 28°C. Very little extracellular polysaccharide is produced (much less than *Bradyrhizobium*). The organism produces an alkaline reaction and usually grows within 3 to 5 days (fast to intermediate growers).

Bradyrhizobium - circular colonies, not exceeding 1 mm diameter after 6 or more days of incubation at 28°C. Extracellular polysaccharide production may be little to abundant and is usually observed in very slow growing strains (after 10 days). Colonies are opaque, rarely translucent, white and convex, granular in texture. The organism produces an alkaline reaction. Isolated colonies can usually be observed after 6 or more days (slow to very slow growers).

Mesorhizobium - circular colonies, 0.5 to 4 mm diameter after 2 to 7 days of incubation at 28°C. Colonies usually coalesce due to copious extracellular polysaccharide production. Colonies are convex, semi-translucent, raised, most with a yellowish centre because of the absorption of bromothimol blue (pH indicator). The organisms usually produce either an acid or neutral pH reaction. Isolated colonies can usually be observed after 4 to 5 days (fast to intermediate growers).

Rhizobium and *Sinorhizobium* - circular colonies, 2 to 4 mm diameter after 2 to 5 days of incubation at 28°C. Colonies usually coalesce due to copious extracellular polysaccharide production. Colonies are convex, semi-translucent, raised, most with a yellowish centre because of the absorption of bromothimol blue (pH indicator). The organisms usually produce either an acid or neutral pH reaction. Isolated colonies usually appear within 2 to 3 days (fast growers).

TABLE 4. Carbon source utilization by rhizobia strains according to their genera

	<i>Azorhizobium</i> (20)	<i>Bradyrhizobium</i> (17)	<i>Mesorhizobium</i>	<i>Rhizobium</i> * (29)**	<i>Sinorhizobium</i> (29)
Mannitol	-	88%	+	+	96%
Sucrose	-	82%	***	+	+
Number of other C sources tested	90	90	9	90	103
% of C sources on which no strain is able to grow	48	22	0	33	23
% of C sources on which at least one strain is able to grow	1	52	n.d.	42	56
% of C sources on which all strains are able to grow	51	26	n.d.	25	21

Data taken from Dreyfus *et al.* (1988); de Lajudie *et al.* (1994); Jarvis *et al.* (1997)

* *Sinorhizobium* strains included.

** Number of strains tested.

*** Some strains of *Mesorhizobium tianshanense* are not able to grow on sucrose.

2.3 PHYLOGENETICS

The development of molecular genetic tools, represented by the polymerase chain reaction (PCR) (Mullis & Faloona, 1987), along with the concept of evolutionary clocks (Kimura, 1983; Woese, 1987), provided the means to base microbial taxonomy on phylogenetic relationships. *Rhizobium* phylogeny, based on sequences of genes coding for the small subunit ribosomal RNA (16S rRNA), confirmed the close relationship between the genera *Agrobacterium* and *Rhizobium* suggested by Fred *et al.* (1932), and showed them to be distant relatives of *Azorhizobium* and *Bradyrhizobium*, which are closely related to *Afpia* (responsible for human cat-scratch infections), *Blastobacter* (a denitrifier), and *Rhodopseudomonas* (a purple phototroph), all in the alpha subdivision of *Proteobacteria* (Fig. 1). Molecular systematics also promoted a breakthrough in the taxonomy of rhizobia. The results of successive reclassifications and introduction of new taxa led to the classification of all known rhizobia into 5 genera and 21 species (Fig. 1; Table 5), contrasting with the 4 species previously described in *Bergey's Manual of Systematic Bacteriology* (Jordan, 1984). The evidence provided by the phylogenetic analyses points to the need for a review of the current suprageneric classification of rhizobia.

Only a few of the recently-described rhizobial species, for example *Rhizobium etli*, *R. tropici*, *Sinorhizobium saheli*, and *S. teranga* were based on the characterization of isolates

TABLE 5. Current classification of validly described bacteria able to fix nitrogen in association with leguminous plants

Genera	Species
<i>Rhizobium</i> (Frank, 1889)	* <i>R. leguminosarum</i> (Frank, 1889) with biovars <i>phaseoli</i> , <i>trifolii</i> and <i>viceae</i> (Jordan, 1984) <i>R. tropici</i> (Martinez-Romero <i>et al.</i> , 1991) <i>R. etli</i> (Segovia <i>et al.</i> , 1993) <i>R. gallicum</i> (Amarger <i>et al.</i> , 1997) <i>R. giardinii</i> (Amarger <i>et al.</i> , 1997) <i>R. hainanense</i> (Chen <i>et al.</i> , 1997)
<i>Sinorhizobium</i> (de Lajudie <i>et al.</i> , 1994)	* <i>S. meliloti</i> (Dangeard, 1926; de Lajudie <i>et al.</i> , 1994) <i>S. fredii</i> (Scholla & Elkan, 1984; de Lajudie <i>et al.</i> , 1994) <i>S. saheli</i> (de Lajudie <i>et al.</i> , 1994) <i>S. teranga</i> (de Lajudie <i>et al.</i> , 1994) <i>S. medicae</i> (Rome <i>et al.</i> , 1996b)
<i>Mesorhizobium</i> (Lindström <i>et al.</i> , 1995; Jarvis <i>et al.</i> , 1997)	* <i>M. loti</i> (Jarvis <i>et al.</i> , 1982) <i>M. huakuii</i> (Chen <i>et al.</i> , 1991) <i>M. ciceri</i> (Nour <i>et al.</i> , 1994a) <i>M. tianshanense</i> (Chen <i>et al.</i> , 1995) <i>M. mediterraneum</i> (Nour <i>et al.</i> , 1995)
Undefined	<i>Rhizobium galegae</i> (Lindström, 1989)
<i>Bradyrhizobium</i> (Jordan, 1982)	* <i>B. japonicum</i> (Jordan, 1982) <i>B. elkanii</i> (Kuykendall <i>et al.</i> , 1992) <i>B. liaoningense</i> (Xu <i>et al.</i> , 1995)
<i>Azorhizobium</i> (Dreyfus <i>et al.</i> , 1988)	* <i>A. caulinodans</i> (Dreyfus <i>et al.</i> , 1988)

* type species.

from nodules of tropical legume species (Martinez-Romero *et al.*, 1991; Segovia *et al.*, 1993; de Lajudie *et al.*, 1994), thereby underscoring the point that taxonomic knowledge of the micro-symbionts of tropical forest species is scarce, as is that of their hosts. It is probable that tropical rhizobia represent an untapped, and largely unexplored, source of microbial diversity with clear, and measurable, economic and ecological value.

2.4 TROPICAL RHIZOBIAL DIVERSITY

Almost a century after the first *Rhizobium* was isolated, Graham (1976) and Allen & Allen (1981) noted that only about 15% of Leguminosae species - the third largest family of flowering plants with about 19,000 species - were known to have the ability to form nodules. This information deficit was mainly attributed to species of tropical origin.

Extensive surveys of the nodulation capacity of tropical Leguminosae species were made recently in the Brazilian Amazonian and Atlantic forests (Faria *et al.*, 1989; Moreira *et al.*, 1992). This work led to the isolation of a large collection of Brazilian rhizobial strains, which have been studied by many workers (Moreira, 1991; Moreira *et al.*, 1993, 1995; Loureiro *et al.*, 1994, 1995; Coutinho *et al.*, 1995; Hollanda *et al.*, 1996; Moreira *et al.*, submitted). Comparative analyses of some of these Brazilian strains with others from Africa have revealed similarity among certain groups, as well as potentially new species (de Lajudie *et al.*, 1994; Dupuy *et al.*, 1994). Other geographical regions have also been explored, and the results show a high degree of diversity among rhizobia isolated from nodules of both woody and non-woody legumes (Rinaudo *et al.*, 1991; Zhang *et al.*, 1991; Oyaizu *et al.*, 1992; Haukka & Lindstrom, 1994; Novikova *et al.*, 1994; van Rossum *et al.*, 1995). Additional new genera and species of rhizobia can be expected as more strains are isolated from legume species in other regions of the world and examined by microbial taxonomists. Indeed, advances in molecular ecology and systematics may allow the discovery of new taxa in natural habitats without the need to cultivate the microorganisms (Stephen *et al.*, 1996).

2.5 THE NEED FOR MINIMAL STANDARDS

Rhizobial diversity is still a "black box" due to the scarce knowledge available on the vast majority of extant leguminous species and their microsymbionts. The ability of Leguminosae species to form effective symbiotic relationships with rhizobia is estimated to be unknown in about 80% of that plant family. Consequently, the characteristics of *Rhizobium* species nodulating hosts belonging to this group is not known. However, the last decade has been extremely important as studies revealed a huge diversity of previously unknown rhizobia, which are now well documented and preserved in appropriate microbial culture collections.

In order to avoid the introduction of poorly described new taxa, minimal standards for the description of new rhizobial genera and species were proposed by Graham *et al.* (1991). It was recommended that phylogenetic as well as phenotypic (symbiotic, cultural, morphological) traits are needed for the valid description of rhizobial species. The authors also recommended that relatively large numbers of strains should be represented in studies designed to describe new taxa. In addition, it is important that collaborative studies are undertaken so as to reach adequate standards for the description of new rhizobial taxa. One way to achieve this is by integrating the efforts of strain isolation and phenetic characterization carried out by workers in less well equipped laboratories with the phylogenetic analyses possible in institutes which have more developed microbiological facilities. This kind of collaboratum can be very effective provided all stages of the process are planned jointly by the partners involved, and training of students and personnel, as well as technological transfer is accounted for.

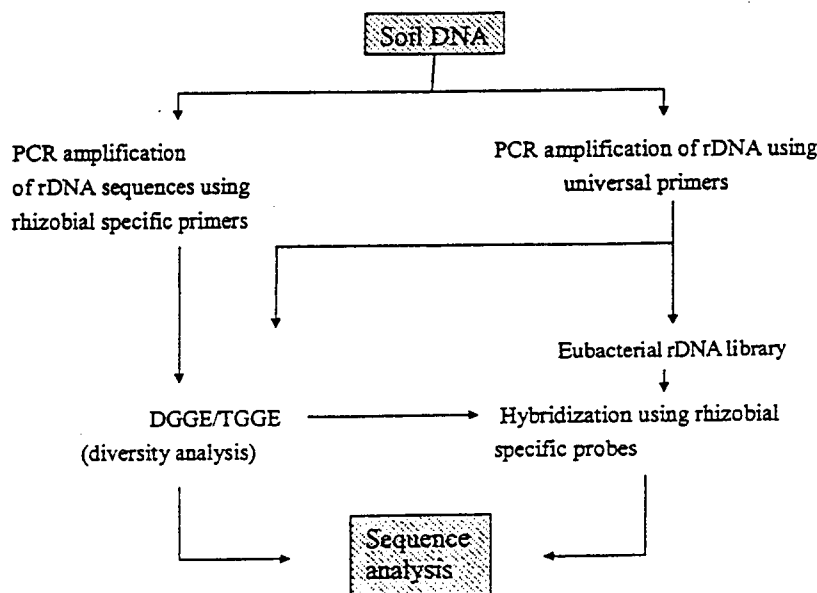


Figure 2. Methodological framework for analysing the molecular diversity of rhizobia in soil without the need for isolation or growth of bacteria in the laboratory.

3 The Application of Molecular Probe Technology

The need to study the genetics and regulation of important biochemical cycles mediated by microorganisms initiated the development of numerous molecular techniques, some of which turned out to be of great value in microbial systematics. Nucleic acid probes may be designed for the detection of bacteria that carry particular genes, such as *nif* or *nod* genes, or unique, taxon signature sequences. The latter can be transposons, insertion sequences, or oligonucleotide sequences of rRNA genes (Barkay *et al.*, 1985; Fredrickson *et al.*, 1988). The application of DNA probes to study natural microbial populations became even more practical with the development of PCR technology (Mullis & Faloona, 1987). This technique promotes a significant increase in the probability of the detection of rare sequences in a mixture of heterogeneous DNA. This is achieved through the exponential amplification of target sequences.

Initially, the genes involved in the symbiotic process were seen as the best candidates for the design of probes or PCR primers (Hahn & Hennecke, 1987). Technological advances enabled the development of rapid DNA sequencing procedures as well as facilitating phylogenetic studies. The ribosomal gene regions turned out to be sites of great interest for probe designers aiming to detect specific rhizobial taxa in the environment (Oliveira *et al.*, 1997).

3.1 SYMBIOTIC GENES

The obvious need to understand the regulatory mechanisms of the nitrogen fixation process of the legume-*Rhizobium* symbiosis, which involves bacteria-plant recognition events, bacterial invasion, nodule formation, and nitrogen fixation *per se*, led to the mapping and sequencing of the major genes involved in the symbiotic process, namely the *nod* (nodulation) and *nif* (nitrogen fixation) genes. The conservation, number of copies, and occurrence of these genes have been evaluated in different strains, species, and genera of rhizobia. The symbiotic genes were later found to be useful for research into the systematics of rhizobia (Masterson *et al.*, 1985; Cadahia *et al.*, 1986; Harrison *et al.*, 1988; Young, 1993; Dobert *et al.*, 1994; Lindström *et al.*, 1995).

Analyses of *nod* and *nif* gene regions pointed to the need for reclassifications of rhizobia taxa. The number of copies of the *nif* genes was the basis for the assignment of *Rhizobium leguminosarum* biovar *phaseoli* group II to the species *Rhizobium etli* (Segovia *et al.*, 1993). Data from restriction fragment length polymorphism (RFLP) using *nod* and *nif* genes as probes provided supporting evidence that *Bradyrhizobium japonicum* should be split into two groups (Stanley *et al.*, 1985; Hahn & Hennecke, 1987; Minamisawa *et al.*, 1992). Further corroborating molecular data resulted in strains belonging to DNA homology group II being reclassified as *Bradyrhizobium elkanii* (Kuykendall *et al.*, 1992). Similarly, most of the fast growing rhizobial strains that nodulate soybeans were found to contain their *nod* and *nif* genes on plasmids; these organisms differ from the slow growing strains, which carry their symbiotic genes in the chromosome (Masterson *et al.*, 1985). The fast growing soybean rhizobia are now classified as a separate species in a different genus, namely *Sinorhizobium fredii* (de Lajudie *et al.*, 1994).

Specific taxonomic problems, some of which were rather difficult to elucidate, have been resolved by sequencing symbiosis genes. Strain Or191, classified as *Rhizobium* sp., nodulates both alfalfa (*Medicago sativa*) and bean plants (*Phaseolus vulgaris*). In an attempt to assign this strain to a species, Eardly *et al.* (1992) sequenced its *nifH* gene and compared it with those of reference rhizobia species. The *nifH* sequence of strain Or191 differed significantly from those of the other test strains. However, its 16S rDNA sequence resembled those of a group of strains of *R. leguminosarum* bv. *phaseoli*, although differing from them by possessing only a single copy of the gene. This example clearly illustrates the need to characterize strains using a variety of genotypic and phenotypic tools in order to achieve a polyphasic taxonomic approach of the rhizobia.

The degree of conservation of *nif* and *nod* genes varies according to the species and genus that carries them, reflecting the evolutionary status of the different taxa. The divergence of *nod* genes among rhizobia has been studied (Young & Johnston, 1989; Lindström *et al.*, 1995). The degree of sequence conservation may determine the usefulness of symbiotic genes as sites for annealing oligonucleotide probes or PCR primers specific to different taxonomic levels from strain to genus. The more conserved a gene is within a species the more likely it is that a species-specific probe can be generated. However, it is more difficult to find a probe site specific to the strain level. It is important to determine to what extent genes are conserved among members of different taxa before spending time and effort in the pursuit of highly specific probing sites.

Analyses of RFLP using gene fragments as probes is a simpler and more affordable way to assess the distribution and conservation of a particular gene within a taxon than sequencing the whole gene of several strains or isolates. This was the method used to demonstrate the high conservation of *nif* genes in chickpea (*Cicer arietinum* L.) rhizobia, both fast and slow growers, and their absence in the isolated plasmids from the 27 strains analysed (Cadahia *et al.*, 1986). The *nif* genes are highly conserved among diazotrophs, as confirmed by sequence alignment and cluster analysis of *nifH* genes from divergent species, including ancient archaeal and clostridial groups (Manjula & Rakesh, 1990). These workers found two related but discrete clusters of *nifH* sequences from rhizobia, which were grouped according to their location in the genome, whether chromosomal or plasmidial.

Probes specific to *nif* or *nod* genes can be used to detect and quantify symbiotic plasmids in *Rhizobium* strains. This was done for *R. leguminosarum* biovar *trifolii* by using a repeated sequence incorporating the *nifH* promoter as a symbiotic plasmid specific probe (Harrison *et al.*, 1988). The investigators found a negative correlation between the number and size of Sym plasmids and the nitrogen fixation effectiveness of the strains carrying them.

Molecular probe technology is also helping in the development of methods for the selective isolation of rhizobia from soil. Laguerre *et al.* (1993 a, b) used a chromosomal probe to identify soil isolates of *R. leguminosarum*, and *nod* probes to assign isolates to biovars *viciae* or *trifolii*. A more refined and specific medium has also been developed for isolating *R. meliloti* from soil (Bromfield *et al.*, 1994). The authors used a *nodH* species-specific probe to evaluate the usefulness of the selective medium.

3.2 RIBOSOMAL GENES

Ribosomal nucleic acids are considered to be the most useful biopolymers for comparative studies in microbial molecular ecology. The rRNA genes (rDNA) are universally distributed, have a high degree of conservation, and can accumulate more or less variability in different sections of the molecule (Lane *et al.*, 1985). Bacterial rRNAs are classified, according to their centrifugation sedimentation rates, as 5S, 16S, or 23S. The 16S rRNA is associated with the smaller subunit of the ribosome, and is the most well studied molecule. One of the greatest advantages of rRNA sequence information is its cumulative nature, i.e., once obtained, it can be deposited in specialized databases, eg. RDP, GenBank, EMBL databases, thereby becoming available for future studies.

The automation of nucleic acid sequencing procedures resulted in a large number of microbial rRNA sequences becoming available in databases. These data can be used to build phylogenetic trees, depicting relationships between test organisms. The alignment of sequences from groups of microorganisms of any particular interest may allow one to find regions of the molecule that are valuable for designing oligonucleotide probes specific to different taxonomic levels, from universal probes, which can detect the domains Archaea, Bacteria, or Eucarya, to family, species or strain-specific probes. A great variety of ribosomal probes of different levels of specificity have been developed and used in microbial ecology (Pace *et al.*, 1986; Laguerre *et al.*, 1993 a, b; Louvrier *et al.*, 1995; Oliveira *et al.*, 1997).

Finding adequate sites in the 16S rRNA molecule that can be used to design oligonucleotide probes or PCR primers specific to rhizobial taxa is not an easy task, primarily because of the high similarity shown between rhizobial species and their phylogenetic

close relatives. Species of *Azorhizobium caulinodans*, *Bradyrhizobium japonicum*, *Mesorhizobium (ciceri, huakuii, and loti)*, and *Rhizobium (etli, leguminosarum, and tropici)* are closely related to *Aquabacter spiritensis*, *Rhodopseudomonas palustris*, *Phyllobacterium rubiacearum*, and *Agrobacterium rhizogenes*, respectively (Young, 1996). Members of the different taxa of a phylogenetic group may differ only in a few 16S rDNA nucleotides, and this makes it difficult to design an effective probe or primer specific to the species or genus level as such probes must be at least 10 base pairs long. In the case of PCR primers, the nucleotide bases that confer specificity to the primer should be concentrated at the 3'-end of the oligonucleotide, as this is the binding site of the enzyme DNA polymerase. These difficulties probably explain the low number of ribosomal probes or primers that are specific to rhizobial species or genera compared to the wealth of data on *nod* and *nif* probes.

An alignment of 16S rRNA sequences provided only one potential probing site specific for *R. tropici* and *R. leguminosarum*, but this site was unable to exclude *Agrobacterium rhizogenes* (Oliveira *et al.*, unpublished results). For the same reason, a 16S rDNA-directed probe specific to *Bradyrhizobium* spp. could not be designed due to the high similarity of this species with *Rhodopseudomonas palustris*. However, a potential probing site specific to *Bradyrhizobium elkanii* was detected and the probe designed was shown to be specific to that species and to several other *Bradyrhizobium* spp. Isolated from tropical tree and shrub legumes (Oliveira *et al.*, 1997).

The large subunit of ribosomal RNA, that is 23S rRNA, contains a higher degree of sequence variation than the corresponding 16S rRNA subunit and, hence, can be a potential target for probes. A probe specific to the 23S rDNA of *Bradyrhizobium japonicum* was designed and found to be effective (Springer *et al.*, 1993). These authors not only sequenced this molecule from *Bradyrhizobium japonicum* but also from representatives of closely related species. A highly variable region at the 5'-end of the 23S rRNA gene of rhizobia and agrobacterial strains has been identified as a potential target for the design of specific probes and PCR primers (Evgenieva-Hackenberg & Selenska-Pobell, 1995).

Similarly, a phylogenetic analysis using 16S rDNA sequences of five strains of bacteriochlorophyll-synthesizing rhizobia isolated from stem nodules of members of *Aeschynomene* species showed them to be homogeneous and members of the same line of descent as *Bradyrhizobium japonicum* (Wong *et al.*, 1994). These workers determined potential target sites for the design of probes specific to the photosynthetic rhizobia.

A promising genomic region for probing and priming sites is the spacer region between the 16S and 23S rRNA genes. This region sometimes contains tRNAs, as well as DNA segments with variable nucleotide sequences. Considerable variation can occur in the size and sequence of this region between strains or species (Honeycutt *et al.*, 1995; Gürtler & Stanisich, 1996). It seems likely that PCR amplifications with primers directed to conserved sequences flanking the intergenic spacer region (IGS) may reveal size variations. If these are not resolved, the amplified fragment can be digested by endonucleases generating polymorphisms among analysed strains to reflect sequence variations. The technique of amplifying a part of the genome and digesting the product with restriction enzymes is called PCR-RFLP. This technique has been used to identify species or strains of bacteria (Gürtler & Stanisich, 1996).

4 Agronomic and Ecological Applications

The development of molecular systematics opened up an array of possibilities for the resolution of current problems found in the application of *Rhizobium* technology in agriculture, as well as in understanding the ecology of these microorganisms and the role that different rhizobial taxa play in nitrogen cycling. Not all of these aspects are currently being investigated, since they require further development or simplification of techniques for *in situ* detection and identification of members of specific taxa. However, a great deal of ecological work has been carried out, particularly diversity studies in different biomes. There has been some progress in the application of systematics tools for the quality control of rhizobial inoculants and on the selection of improved inoculant strains. A few of the most recent advances on the application of modern molecular systematics techniques to improve inoculant quality and to evaluate rhizobia diversity in different environments will now be considered.

4.1 IMPROVING THE QUALITY OF INOCULANTS

One of the major challenges of *Rhizobium* technology is the development of improved inoculants, that is, ones containing strains able to fix nitrogen with greater efficiency, to compete well with native rhizobia for nodule occupancy, and to survive and persist under a wide range of environmental conditions.

The first step in the development of a good inoculant involves screening good quality material. This necessitates access to a suitable culture collection. A good culture collection requires personnel and a suitable infra-structure to promote sound and reliable systematics research. This involves thorough characterization of strains by both-conventional techniques and advanced methods, including chemosystematic and molecular tools.

Examples of the application of advanced systematics methods to investigate the performance and composition of rhizobial inoculants can be found in the literature. For example, two of the strains that are used in the formulation of the Brazilian commercial soybean inoculant were identified as members of the species *Bradyrhizobium elkanii* through analysis of their 16S rDNA sequences (Rumjanek *et al.*, 1993). DNA fingerprinting (RAPD) coupled with pyrolysis mass spectrometry (Py-MS) enabled the identification of Italian soybean nodule isolates as strains of different European inoculants (Kay *et al.*, 1994). The same approach was used to identify soybean nodule isolates from the Brazilian cerrados. Some of these isolates showed genetic drift, which was detected by Py-MS and correlated with their superior adaptation to the cerrado soils (Coutinho, 1993).

A major problem in the use of inoculants containing strains with greater nitrogen fixation efficiency is their generally low competitiveness compared to native or naturalized rhizobia. In this case, inoculations yield no response, since the majority of the nodules will be occupied by rhizobial strains already present in the soil. In some areas of the USA, strains of *Bradyrhizobium japonicum* serogroup USDA 123 that were introduced in the past, prevail in soybean nodules. They are so competitive that the attempts to use more efficient strains were unsuccessful (Kramicker & Brill, 1986).

Competition experiments with varying numbers of indigenous, ineffective rhizobia in the soil and different seed inoculum levels were performed by Tas *et al.* (1996).

These workers developed *R. galegae* species-specific primers that were used in competition experiments along with strain-specific primers developed earlier by Tas *et al.* (1994). Crushed nodules were used as DNA templates for PCR reactions. It was demonstrated that the ineffective strains present in the soil were very competitive and impaired growth and nodulation of the plants inoculated with the effective strain. Tas and his colleagues suggested that the use of PCR with specific primers is the most powerful technique available for ecological studies, since it may discriminate between closely related strains of the same species and hence can be used to amplify specific fragments from soil or nodule samples containing mixed populations. However, although analysis of PCR-amplified 16S rRNA genes is effective for identifying species, it is not so useful for typing at the infra-specific level, that is, at the level usually required for competition studies.

The success of rhizobial inoculation procedures also depends on how the inoculant is produced, stored, and distributed. Quality standards need to be followed, such as the minimum number of cells of the recommended strain per gram or millilitre of the carrier, and the degree of purity with an established limit for contaminant cells. Furthermore, the growing concern over the quality of rhizobial inoculants led to a restriction in the degree of tolerance regarding contaminant cells and standards of minimum rhizobial cell numbers in commercial inoculants (Olsen P. E. *et al.*, 1994).

It is clear that methods are needed for the detection of specific strains of rhizobia not only for ecological studies but also for the quality control of inoculants. Methods that enable the typing of a rhizobial strain without affecting its physiology in nature can be used to detect and help to enumerate specific strains in an inoculant. DNA fingerprinting techniques, such as RAPD, REP-PCR, or RFLP, can be employed for this purpose, provided the generated fingerprints are unique for each strain. For example, primers homologous to the extremities of a repeated sequence (RS α) of *Bradyrhizobium japonicum* were designed and shown to amplify fragments of the expected size exclusively from DNA of *Bradyrhizobium elkanii* and *B. japonicum* strains (Hartmann *et al.*, 1996). These workers recommend the use of PCR with these primers as a tool to assess the quality of soybean inoculants. Their results also showed that the amplified RS α fragment is largely conserved among *B. japonicum* strains, and hence may be used as a screening method to identify them among field isolates. They adapted the PCR method in order to quantify the cell numbers of *B. japonicum* in liquid inoculants as well as in growth media. By increasing the number of PCR cycles employed, the detection limit was of the order of 16 cells per gram, which is quite promising.

4.2 EXPLORING RHIZOBIAL DIVERSITY

Perhaps the most relevant consequence of the development of modern molecular systematics has been the possibility to investigate rhizobial diversity in soils of different biomes of the world. The use of DNA probes and fingerprinting techniques allow a better understanding of the taxonomic diversity of nodule isolates from different legume species growing in distinct environments. Strains that were once indistinguishable can now be discriminated and typed, and this allows their fate, either when introduced or following environmental perturbation, to be followed. Nodule occupancy can now be easily and unequivocally established, and competition studies facilitated.

Some of the advances in research on rhizobial diversity are reviewed below. The discovery of new species and genera can be considered to be a feedback from applied sciences to pure taxonomy.

4.2.1 Indirect evaluation of rhizobial diversity.

Indirect evaluations of microbial diversity are defined here as those that rely on characterization data of isolated and cultured microorganisms.

Genotypic differences and relatedness among members of a taxon can be assessed by sequence polymorphisms of their common genes. Hence, RFLP analyses of *nod* and *nif* genes have been widely used in studies of the diversity of different rhizobial species. Different probes directed towards the *nod* gene region have been applied to study the diversity of *Rhizobium leguminosarum* bv. *phaseoli*, bv. *trifolii*, and bv. *viciae* (Laguerre *et al.*, 1993a). It was possible to group the restriction patterns of the *nod* probe according to the main nodulation host, and this enabled the assignment of strains to the biovar level. Biovars *trifolii* and *viciae*, with legume hosts belonging to the genera *Trifolium* and *Vicia*, respectively, showed greater diversity of their *nod* regions than biovar *phaseoli*, which inoculates legumes belonging to the genus *Phaseolus*. The diversity of *Rhizobium meliloti* strains isolated from Italian soils was also evaluated by RFLP analysis of a 25-kb *nod* gene region located in the symbiotic plasmid: these data were complemented by the results of RAPD and RFLPs analyses of the ribosomal intergenic spacer region (Paffetti *et al.*, 1996).

Rhizobial sequence diversity and the distribution of symbiotic genes may vary according to the soil environment the rhizobia inhabitants. The restriction patterns of *nodDABC* genes of *Bradyrhizobium japonicum* strains isolated from nodules of soybeans grown in tropical soils of Thailand were found to be different from those isolated in Japan or the USA (Yokoyama *et al.*, 1996). A *nifH* probe was used to discriminate between rhizobia isolated from nodules of bean plants (*Phaseolus vulgaris*) grown in Kenyan soils showing different bulk pH values. The majority of rhizobia from a neutral soil (pH 6.8) had multiple copies of the *nifH* gene and were specific nodulants of *P. vulgaris* whereas most of the strains from an acid soil (pH 4.5) possessed a single copy of *nifH* and were characterized by forming effective nodules on a broad range of host legumes (Anyango *et al.*, 1995).

The use of probes for the characterization of strains can be greatly facilitated and improved by the application of PCR rather than by the use of conventional Southern hybridizations methods. The evolution of probe technology applied to rhizobial research was discussed by Laguerre *et al.* (1996). Instead of using probes and a conventional RFLP procedure, these investigators designed oligonucleotide primers directed towards the extremities of the *nifDK* and *nodD* gene regions and digested the resultant PCR products with restriction endonucleases. Primers were designed that were specific to the *nif* genes of all rhizobia, as well as *nod* primers specific to *Rhizobium etli* and the different biovars of *R. leguminosarum*. These investigators advocate the use of PCR-based methods rather than Southern hybridizations which are somewhat more laborious and time-consuming. A primer specific to the *nif* gene has been used in single primer PCR reactions (similar to RAPD) to obtain reproducible, unique amplification profiles for each rhizobial strain; this procedure is very useful for rhizobial diversity and for inoculant quality control studies (Richardson *et al.*, 1995).

The diversity of rhizobia isolated from root and stem nodules of legume species growing in the Philippines was analysed by sequencing of their 16S rRNA and six new species were proposed (Gamo *et al.*, 1991). The genetic diversity of *Bradyrhizobium* strains isolated from soybean nodules in Thailand was also evaluated by their *nod* genes, and compared with those of *Bradyrhizobium elkanii* and *B. japonicum* strains isolated in Japan and the USA (Yokoyama *et al.*, 1996). The workers were of the view that the Thai isolates belonged to new species of *Bradyrhizobium*.

Early classifications of rhizobia were dependent on host range studies. One of the advantages of using molecular techniques is that they can be used to unravel the taxonomic status of strains which nodulate diverse legume species. It has been shown that eight *Rhizobium* strains isolated from nodules of lucerne (*Medicago sativa*) also nodulate bean plants (*Phaseolus vulgaris*) (Eardly *et al.*, 1992). When a 260-bp segment of the 16S rDNA of these strains was sequenced, it was shown that they were different from the *Rhizobium leguminosarum*, *R. meliloti*, and *R. tropici* strains which commonly nodulate lucerne and beans. It seems likely that these eight strains are members of a new *Rhizobium* species.

A study of the diversity and ecology of bradyrhizobia isolated from surface and deep soil under *Acacia albida* trees in Senegal was carried out using SDS-PAGE analyses (Dupuy *et al.*, 1994). The strains belonged to the *Bradyrhizobium-Rhodopseudomonas* complex. This assignment was confirmed by hybridization of their genomic DNA using total rRNA from the *Bradyrhizobium japonicum* type strain as a probe. However, some of the isolates belonged to a separate lineage as far removed from *B. japonicum* as the latter is from the genus *Azospira*. Consequently, these isolates also seem to belong to a new species.

The 5'-half of the 23S rDNA gene is highly variable. Restriction analysis of this region enabled the clustering of strains currently referred to as *Rhizobium* "hedysari". These organisms were well discriminated from other species of the family *Rhizobiaceae* (*Agrobacterium tumefaciens*, *Rhizobium galegae*, *R. leguminosarum*, and *R. meliloti*). However, these strains, when PCR fingerprinting methods were applied, gave unique patterns (Selenska-Pobell *et al.*, 1996). Consequently, it can be concluded that analysis of rhizobial 23S rDNA provides useful taxonomic information.

Some results demonstrate the enormous unknown diversity of rhizobia. An extensive biodiversity survey was performed by Oyaizu *et al.* (1992) on 117 rhizobial strains isolated from 91 legume species and 28 reference strains of *Rhizobium* and *Bradyrhizobium* obtained from bacterial culture collections. These workers sequenced a 157-bp segment of the 16S rDNA of the test strains and assigned them to sixteen discrete groups, which correlated with DNA-DNA reassociation data thereby suggesting the existence of at least 16 species in this sample of microorganisms. DNA-DNA reassociation was also used to demonstrate that three genomic groups are present among rhizobia isolated from bean plants grown in French soils (Laguerre *et al.*, 1993b). One group was equated with *Rhizobium leguminosarum* whereas the others were considered to be new genomic species encompassing strains that nodulate both *Leucaena leucocephala* and *Phaseolus vulgaris*. Similarly, Amarger *et al.* (1994) examined 287 bean-nodulating rhizobia from four different geographical locations in France and concluded that they belonged to either *Rhizobium leguminosarum* or to *R. tropici*. The latter contains strains that nodulate both *Leucaena leucocephala* and *Phaseolus vulgaris*. These studies suggest that *Rhizobium tropici* is widely distributed in

French soils. Phylogenetic analysis of 16S rRNA gene sequences of *Rhizobium* strains that nodulate bean plants highlighted four clusters which corresponded to *Rhizobium etli*, *R. leguminosarum*, *R. tropici*, and to a single membered cluster (van Berkum *et al.*, 1996).

The highly variable intergenic spacer regions (IGS) between ribosomal genes have also been considered as adequate DNA regions to be analysed by those willing to investigate the diversity of bacteria (Barry *et al.*, 1991). Consensus primers directed to IGS-located tRNA genes have been used to analyse the genetic variability and phylogenetic relationships among isolates of *Pseudomonas solanacearum* (Seal *et al.*, 1992), *Streptococcus* spp. (McClelland *et al.*, 1992), and *Xanthomonas albilineans* (Honeycutt *et al.*, 1995). Variation has also been observed in the number and size of the IGS regions in diverse microorganisms, leading some workers to advocate the use of IGS analysis as a general and quick method for typing bacteria (Barry *et al.*, 1991; Jensen *et al.*, 1993).

Restriction analysis of the ribosomal IGS regions have been applied recently to characterize rhizobia, and rDNA intergenic spacer primers universal to all rhizobia designed (Laguerre *et al.*, 1996). Several polymorphisms have been observed at both the intra-generic and the intra-specific levels (Nour *et al.*, 1994b; Laguerre *et al.*, 1996; Selenska-Pobell *et al.*, 1996). This method was compared with other DNA fingerprinting techniques, such as REP-PCR and RAPD, in the characterization of 43 strains of *Rhizobium leguminosarum*. Intraspecific polymorphisms, independent of the biovar status of the strains, were found with all of the methods (Laguerre *et al.*, 1996). However, IGS PCR-RFLP data are easier to analyse than complex banding patterns obtained using other fingerprinting techniques; the method is also useful for the analysis of a large number of strains. The diversity of chickpea rhizobia was also studied using this method in association with phenotypic tests; these studies showed that two distantly related groups of strains were present amongst the isolates (Nour *et al.*, 1994b).

Analyses which include the 16S rRNA gene along with the spacer region increase the number of restriction sites and, consequently, the discriminative power of the method. Seventy-three isolates of rhizobia isolated from *Medicago trunculata* were analysed by this procedure and led to the discovery of two genotypic groups, which were confirmed by DNA-DNA hybridization data suggesting that the strains represent different genomic species (Rome *et al.*, 1996a).

The use of symbiotic plasmid-located *nod* probes allowed the assignment of *Rhizobium leguminosarum* strains to the biovar level, with biovars *trifolii* and *viciae* showing greater diversity in their *nod* regions than biovar *phaseoli* (Laguerre *et al.*, 1993a). Identification of this species to the biovar level is not possible when the target of the analyses are chromosomal regions or when DNA fingerprinting methods are employed (Laguerre *et al.*, 1996). Molecular assessments of rhizobial diversity should encompass analyses on both the chromosome and the symbiotic plasmids.

4.2.2 Direct evaluation of rhizobial diversity.

The indirect diversity assessments reported above were based on rhizobia isolated from legume nodules. The obvious limitation of this approach is that, even for the so called "promiscuous" legume species, the plant will select the *Rhizobium* strains that will occupy its nodules. Consequently, biodiversity analyses based on data from isolate characterizations probably underestimate the extent of rhizobial diversity. Similarly, there is no guarantee

that representative strains of rhizobia will be isolated directly from soil on selective or semi-selective media (Bromfield *et al.*, 1994; Louvrier *et al.*, 1995), which means that this approach is also likely to underestimate rhizobial diversity.

The limitations outlined above are particularly serious in studies designed to assess the impact of anthropic activities (agricultural, industrial, and mining) on the extent of rhizobial diversity. In such studies it is essential to monitor all rhizobial types in soils under different intensities of management. Assessments of the extent to which different human activities cause impact on biodiversity are top priorities to nations that have signed the Convention of Biological Diversity (UNEP, 1992). It is, therefore necessary to develop methods which will overcome the limitations presently imposed upon indirect evaluations of the diversity of soil microorganisms.

The development and application of nucleic acid probes offer a fast and reliable method to study environmental samples, such as soil samples. Probes can be used to study culturable and non-culturable organisms, and those which do not grow on isolation media (Pickup, 1991). Direct evaluations of microbial diversity are defined here as those which do not require the isolation and culture of the microorganisms under study.

Soil is a complex and difficult environment. Microbial ecologists have been applying molecular systematics techniques intensively to detect bacteria in soil samples in an attempt to overcome the difficulties associated with the isolation and growth of microorganisms. Detection methods which do not depend on *in vitro* microbial growth are usually based on the amplification of target DNA fragments followed by nucleic acid hybridization. The first step is to obtain the microbial DNA from the environmental sample. Strategies commonly involve the separation of microbial cells from soil particles, followed by lysis and recovery of their DNA (indirect lysis method) or the disruption of cells directly in the soil samples prior to alkaline extraction of DNA (Pickup, 1991). A rapid method for the direct extraction of DNA from soil involves mechanical lysis by bead beating aided by sodium-dodecyl-sulphate (SDS), lysozyme and low temperature treatment, followed by cold phenol extraction, precipitation of the DNA, and purification with caesium chloride and potassium acetate, spermidine-HCl or glass milk. The resulting DNA is usually amplifiable by PCR (Smalla *et al.*, 1993).

A molecular ecological study on Australian soil samples, using direct lysis of the microbial cells present in the soil matrix, 16S rRNA amplification, sequencing, and hybridization with taxon specific probes, revealed the occurrence of phylogenetically distinct groups associated with new, unknown lineages within the bacteria domain (Liesack & Stackebrandt, 1992). This approach was also used to investigate the genetic diversity of streptomycetes in soil samples taken from a subtropical forest in Australia. These results also revealed extensive microbial diversity with the majority of the 113 analysed 16S rRNA sequences belonging to new, unknown taxa, which were phylogenetically distinct from known, culturable bacteria (Stackebrandt *et al.*, 1993).

The molecular methodology for direct, specific microbial detection, which is qualitative in nature, can also be adapted to enumerate bacteria in soil. Rosado *et al.* (1996) developed a quantitative PCR method to detect and enumerate *Paenibacillus azotofixans* in soil and rhizosphere of wheat (*Triticum aestivum* L.). Following DNA extraction from soil, the 16S rRNA genes were amplified by using specific primers, and hybridized with an internal probe to the amplified fragment. The method was used to evaluate the impact of water

stress on the population of *P. azotofixans* in the environment, and to overcome the need to culture the bacteria.

The ribosomal IGS may also provide probing sites for rhizobial species. Oliveira *et al.* (pers. comm.) sequenced and aligned the IGS of five rhizobial strains belonging to two different species. The resultant data allowed the design of PCR primers specific to *Rhizobium leguminosarum* and/or *R. tropici*. Direct detection of rhizobia has been accomplished using these primers to amplify their IGS from DNA extracted from agricultural soils under different management practices. The PCR products were separated using denaturing gradient gel electrophoresis (DGGE), which has been used to reveal sequence variability among rhizobial strains (Vallaeys *et al.*, 1997). This procedure allows the separation of DNA fragments that are of the same size but different in their nucleotide sequences. The separation is achieved because differences in sequence composition alter the electrophoretic mobility of a DNA molecule when subjected to a denaturing gradient (Fischer & Lerman, 1983). The resultant sequence heterogeneity is directly related to the genetic diversity of the populations of *Rhizobium leguminosarum* and *R. tropici* in the soil. This procedure is being used to evaluate the applicability of rhizobial diversity as an indicator of the environmental impact of different agricultural management schemes.

This procedure was also used in a molecular microbial ecology study designed to analyse the genetic diversity of microbial populations. DNA fragments derived from the amplification of a variable region of the 16S rDNA of complex microbial populations were analysed, and the occurrence of up to 10 different fragments of rDNA detected in microbial communities of different origins (Muyzer *et al.*, 1993). Thus, the use of DGGE to analyse a mixture of PCR products enables the direct detection of different genomic species in environmental samples.

A methodological framework for the molecular evaluation of rhizobial diversity in soil is presented in Figure 2. The starting point of the methodology is soil-extracted total DNA. The initial approach will depend on the availability of oligonucleotide primers to amplify rhizobial ribosomal gene sequences from soil DNA. If one is interested in the diversity of a particular rhizobial species for which specific primers are available, their rDNA can be amplified and the PCR products separated by DGGE (or TGGE, i.e., temperature gradient gel electrophoresis). This will produce fingerprints that will reflect the diversity of genotypes or strains that are present in sufficient numbers to be detected in the soil samples analysed. A refinement of the analyses may be accomplished by sequence analysis of DNA fragments purified from bands excised from the DGGE gels. A different approach would be to use universal primers to amplify eubacterial ribosomal gene sequences from soil DNA. This procedure would require an extra step in order to identify among the PCR products those that derive from rhizobia. DGGE (or TGGE) analyses followed by hybridization with rhizobial specific probes would enable the evaluation of rhizobial diversity. Again, DGGE bands which hybridise to rhizobial-specific probes could have their DNA sequences analysed. If access to DGGE technology is a handicap, one could construct an eubacterial rDNA clone library. Detection of rhizobial rDNA containing clones could be achieved by hybridization with specific probes, while analysis of their DNA sequences would enable diversity evaluation. Methods for sequence analysis may vary from full or partial sequencing to more simple evaluations such as analysis of rDNA restricted fragments polymorphisms.

5 Concluding Remarks

The systematics of rhizobia has undergone revolutionary changes in the past fifteen years, that is, from the moment the definition of rhizobial species was no longer based solely on host range. Legume nodule, nitrogen fixing bacteria, can now be classified in up to five different genera, belonging to at least three divergent lineages in the α subgroup of the *Proteobacteria* (Young, 1996). The molecular evidence also shows that changes are needed at the suprageneric level. The family *Rhizobiaceae*, for instance, contains phylogenetically diverse rhizobial genera such as *Rhizobium* and *Bradyrhizobium*, and excludes *Rhodopseudomonas*, a genus closely related to *Bradyrhizobium*.

Rhizobial strains previously indistinguishable may now be recognized and assigned to different species. The methods responsible for this taxonomic advance open up an array of possibilities in the fields of ecology, environmental impact assessment, and development of inoculants. More innovations are expected in the near future, with automation of *in situ* hybridization techniques coupled with advanced electron microscopy. This will allow the investigation of subjects such as the chemical signalling mechanisms between symbiotic partners, which may shed more light on the processes and environmental factors involved in competition for nodulation and survival, and for the persistence as saprophytes of particular strains, species, or even genera of rhizobia. Succession and dynamics of rhizobial populations in soils undergoing environmental stress are also a field of interest aimed at better management and optimization of the ecological and agronomic benefits of biological nitrogen fixation. Last, but definitely not least, advances in rhizobial systematics will unravel even further the extent of the diversity of diazotrophic microorganisms and how much of this variatum is related to the actual process of nitrogen fixation of soils in ecosystems under different environmental pressures.

6 References

- Allen, O.N. & Allen, E.K. (1981). *The Leguminosae. A Source Book of Characteristics, Uses and Nodulation*. Madison, University of Wisconsin Press.
- Amarger, N.; Bours, M.; Revoy, F.; Allard, M.R. & Laguerre, G. (1994). *Rhizobium tropici* nodulates field-grown *Phaseolus vulgaris* in France. *Plant and Soil* 161, 147-156.
- Amarger, N.; Macheret, V. & Laguerre, G. (1997). *Rhizobium gallicum* sp. nov. and *Rhizobium giardinii* sp. nov., from *Phaseolus vulgaris* nodules. *International Journal of Systematic Bacteriology* 47, 996-1006.
- Anyango, B.; Wilson, K.J.; Beynon, J.L. & Giller, K.E. (1995). Diversity of rhizobia nodulating *Phaseolus vulgaris* L. in two Kenyan soils with contrasting pHs. *Applied and Environmental Microbiology* 61, 4016-4021.
- Barkay, T.; Fouts, D.L. & Olson, B.H. (1985). Preparation of a DNA gene probe for detection of mercury resistance genes in gram-negative bacterial communities. *Applied and Environmental Microbiology* 49, 686-692.
- Barry, T.; Collieran, G.; Glennon, M.; Dunican, L.K. & Gannon, F. (1991). The 16S/23S ribosomal spacer region as a target for DNA probes to identify eubacteria. *PCR Methods and Applications* 1, 51-56.
- Beijerinck, M.W. (1888). Die bacterien der Papilionaceeknollchen. *Botanische Zeitung* 46, 726-735, 741-750, 757-771, 781-790, 797-804.

- Boddey, R.M.; Chalk, P.M.; Victoria, R.L. & Matsui, E. (1984). Nitrogen fixation by nodulated soybean under tropical field conditions estimated by the ^{15}N isotope dilution technique. *Soil Biology and Biochemistry* 16, 583-589.
- Bromfield, E.S.P.; Wheatcroft, R. & Barran, L.R. (1994). Medium for direct isolation of *Rhizobium meliloti* from soils. *Soil Biology and Biochemistry* 26, 423-428.
- Cadahia, E.; Leyva, A. & Ruiz-Argueso, T. (1986). Indigenous plasmids and cultural characteristics of rhizobia nodulating chickpeas (*Cicer arietinum* L.). *Archives of Microbiology* 146, 239-244.
- Chen, W.X.; Li, G.S.; Qi, Y.L.; Wang, E.T.; Yuan, H.L. & Li, J.L. (1991). *Rhizobium huakuii* sp. nov. isolated from the root nodules of *Astragalus sinicus*. *International Journal of Systematic Bacteriology* 41, 275-280.
- Chen, W.X.; Tan, Z.Y.; Gao, J.L.; Li, Y.; Wang, E.T. (1997). *Rhizobium hainanense* sp. nov., isolated from tropical legumes. *International Journal of Systematic Bacteriology* 47, 870-873.
- Chen, W.; Wang, E.; Wang, S.; Li, Y.; Chen, X. & Li, Y. (1995). Characteristics of *Rhizobium tianshanense* sp. nov., a moderately and slowly growing root nodule bacterium isolated from an arid saline environment in Xinjiang, People's Republic of China. *International Journal of Systematic Bacteriology* 45, 153-159.
- Coutinho, H.L.C. (1993). *Studies of bradyrhizobia from the Brazilian cerrados*. Ph.D. thesis, University of Bristol.
- Coutinho, H.L.C.; Oliveira, V.M.; Hollanda, L.M.; Moreira, F.M.S. & Franco, A.A. (1995). Diversity of rhizobia isolated from nodules of legume species occurring in the Atlantic and Amazonian rainforests p. 162. In *7th International Symposium of Microbial Ecology, Abstracts*, Santos-SP: Brazil.
- Dangeard, P.A. (1926). Recherches sur les tubercles radicaux des Legumineuses. *Botaniste* 13, 1-275.
- de Lajudie, P.; Willems, A.; Pot, B.; Dewettinck, D.; Maestrojuan, G.; Neyra, M.; Collins, M.D.; Dreyfus, B.; Kersters, K. & Gillis, M. (1994). Polyphasic taxonomy of rhizobia: emendation of the genus *Sinorhizobium* and description of *Sinorhizobium meliloti* comb. nov., *Sinorhizobium saheli* sp. nov., and *Sinorhizobium teranga*, sp. nov. *International Journal of Systematic Bacteriology* 44, 715-733.
- Dobert, R.C.; Breil, B.T. & Triplett, E.W. (1994). DNA sequence of the common nodulation genes of *Bradyrhizobium elkanii* and their phylogenetic relationship to those of other nodulating bacteria. *Molecular Plant Microbe Interactions* 7, 564-572.
- Dreyfus, B.; Garcia, J.L. & Gillis, M. (1988). Characterization of *Azorhizobium caulinodans* gen. nov., sp. nov., a stem-nodulating nitrogen-fixing bacterium isolated from *Sesbania rostrata*. *International Journal of Systematic Bacteriology* 38, 89-98.
- Dupuy, N.; Willems, A.; Pot, B.; Dewettinck, D.; Vandenbruaene, I.; Maestrojuan, G.; Dreyfus, B.; Kersters, K.; Collins, M. D. & Gillis, M. (1994). Phenotypic and genotypic characterization of bradyrhizobia nodulating the leguminous tree *Acacia albida*. *International Journal of Systematic Bacteriology* 44:461-473.
- Eardly, B.D.; Young, J.P.W. & Selander, R.K. (1992). Phylogenetic position of *Rhizobium* sp. strain Or 191, a symbiont of both *Medicago sativa* and *Phaseolus vulgaris*, based on partial sequences of the 16S rRNA and *nifH* genes. *Applied and Environmental Microbiology* 58, 1809-1815.
- Evguenieva-Hackenberg, E. & Selenska-Pobell, S. (1995). Variability of the 5'-end of the large subunit rDNA and presence of a new short class of RNA in *Rhizobiaceae*. *Letters in Applied Microbiology* 21, 402-405.
- Faria, S.M.; Lewis, G.P.; Sprent, J.I. & Sutherland, J.M. (1989). Occurrence of nodulation in the Leguminosae. *New Phytologist* 111, 607-619.
- Fischer, S.G. & Lerman, L.S. (1983). DNA fragments differing by single basepair substitutions are separated in denaturing gradient gels: correspondence with melting theory. *Proceedings of the National Academy of Sciences of the USA* 80, 1579-1583.
- Frank, B. (1889). *Berichte der Deutschen Botanischen Gesellschaft* 7, 332-346.
- Fred, E.B.; Baldwin, I.L. & McCoy, E. (1932). *Root nodule bacteria and leguminous plants*. Madison:

University of Wisconsin Press.

- Fredrickson, J.K.; Bezdicsek, D.F.; Brickman, F.J. & Li, S.W. (1988). Enumeration of Tn5 mutant bacteria in soil by using a most-probable-number DNA hybridization technique and antibiotic resistance. *Applied and Environmental Microbiology* 54, 446-453.
- Gamo, T.; Itoh, A.; Sawazaki, A.; Manguiat, I.J. & Mendoza, D.M. (1991). Rhizobia collected from leguminous plants in the Philippines. *Bulletin of the National Institute of Agrobiological Resources* 6, 111-129.
- Graham, P.H. (1976). Identification and classification of root nodule bacteria. In *Symbiotic Nitrogen Fixation in Plants*. Edited by P.S. Nutman. pp. 99-112. Cambridge, Cambridge University Press.
- Graham, P.H.; Sadowsky, M.J.; Keyser, H.H.; Barnett, Y.M.; Bradley, R.S.; Cooper, J.E.; De Ley, D.J.; Jarvis, B.D.W.; Roslycky, E.B.; Strijdom, B.W. & Young, J.P.W. (1991). Proposed minimal standards for the description of new genera and species of root- and stem-nodulating bacteria. *International Journal of Systematic Bacteriology* 41, 582-587.
- Gross, D.C.; Vidaver, A.K. & Klucas, R.U. (1979). Plasmids, biological properties and efficacy of nitrogen fixation in *Rhizobium japonicum* strains indigenous to alkaline soils. *Journal of General Microbiology* 114, 257-266.
- Gürtler, V. & Stanisich, V.A. (1996). New approaches to typing and identification of bacteria using the 16S-23S rDNA spacer region. *Microbiology* 142, 3-16.
- Hahn, M. & Hennecke, H. (1987). Conservation of a symbiotic DNA region in soybean root nodule bacteria. *Applied and Environmental Microbiology* 53, 2253-2255.
- Harrison, S.P.; Jones, D.G.; Schunmann, P.H.D.; Forster, J.W. & Young, J.P.W. (1988). Variation in *Rhizobium leguminosarum* biovar *trifolii* Sym plasmids and the association with effectiveness of nitrogen fixation. *Journal of General Microbiology* 134, 2721-2730.
- Hartmann, A.; Gomez, M.; Giraud, J.J. & Revellin, C. (1996). Repeated sequence RS α is diagnostic for *Bradyrhizobium japonicum* and *Bradyrhizobium elkanii*. *Biology and Fertility of Soils* 23, 15-19.
- Haukka, K. & Lindström, K. (1994). Pulse-field gel electrophoresis for genotypic comparison of *Rhizobium* bacteria that nodulate leguminous trees. *FEMS Microbiology Letters* 119, 215-220.
- Hellriegel, H. & Wilfarth, H. (1888). Untersuchungen über die Stickstoffernährung der Gramineen und Leguminosen. *Beitraege zur Vereinigung Deutschen Zuckerindustrie*. pp. 234.
- Hernandez, B.S. & Focht, D.D. (1984). Invalidity of the concept of slow growth and alkali production in cowpea rhizobia. *Applied and Environmental Microbiology* 48, 206-210.
- Hollanda, L.M.; Coutinho H.L.C. & Manfio G.P. (1996). Caracterização de rizóbios através de perfis de crescimento em carboidratos. In *Anais do XIII Congresso Latino Americano de Ciência do Solo, CD-ROM*.
- Honeycutt, R.J.; Sobral, B.W.S. & McClelland, M. (1995). tRNA intergenic spacers reveal polymorphisms diagnostic for *Xanthomonas albilineans*. *Microbiology* 141, 3229-3239.
- Ishizuka, J. (1992). Trends in biological nitrogen fixation research and application. *Plant and Soil* 141, 197-209.
- Jarvis, B.D.W.; Pankhurst, C.E. & Patel, J.J. (1982). *Rhizobium loti*, a new species of legume root nodule bacteria. *International Journal of Systematic Bacteriology* 32, 378-380.
- Jarvis, B.D.W.; van Berkum, P.; Chen, W.X.; Nour, S.M.; Fernandez, M.P.; Cleyet-Marel, J.C. & Gillis, M. (1997). Transfer of *Rhizobium loti*, *Rhizobium huakuii*, *Rhizobium ciceri*, *Rhizobium mediterraneum* and *Rhizobium tianshanense* to *Mesorhizobium* gen. nov. *International Journal of Systematic Bacteriology* 47, 895-898.
- Jensen, M.A.; Webster, J.A. & Straus, N. (1993). Rapid identification of bacteria on the basis of polymerase chain reaction-amplified ribosomal DNA spacer polymorphisms. *Applied and Environmental Microbiology* 59, 945-952.
- Jordan, D.C. (1982). Transfer of *Rhizobium japonicum* Buchanan 1980 to *Bradyrhizobium* gen. nov., a genus of

- slow growing root-nodule bacteria from leguminous plants. *International Journal of Systematic Bacteriology* 32, 136-139.
- Jordan, D.C. (1984). *Rhizobiaceae*. In *Bergey's Manual of Systematic Bacteriology*, vol. 1, pp. 234-256. Edited by N.R. Kreig & J.G. Holt. Baltimore: Williams and Wilkins.
- Jordan, D.C. & Allen, O.N. (1974). *Rhizobiaceae*. In *Bergey's Manual of Determinative Bacteriology*, 8^o pp. 261-264, edited by R.E. Buchanan & N.E. Gibbons. Baltimore: Williams & Wilkins.
- Kay, H.E.; Coutinho, H.L.C.; Fattori, M.; Manfio, G.P.; Goodacre, R.; Nuti, M.P.; Basaglia, M. & Beringer, J.E. (1994). The identification of *Bradyrhizobium japonicum* strains isolated from Italian soils. *Microbiology* 140, 2333-2339.
- Kennedy, L.D. & Greenwood, R.M. (1982). 6-phosphogluconate and glucose-6-phosphate dehydrogenase activities, growth rate and acid production as taxonomic criteria for *Rhizobium*. *New Zealand Journal of Science* 25, 361-366.
- Kimura, M. (1983). *The neutral theory of molecular evolution*. New York, Cambridge University Press.
- Kramicker, B.J. & Brill, W.J. (1986). Identification of *Bradyrhizobium japonicum* nodule isolates from Wisconsin soybean farms. *Applied and Environmental Microbiology* 51, 487-492.
- Kuykendall, L.D.; Saxena, B.; Devine, T.E. & Udell, S.E. (1992). Genetic diversity in *Bradyrhizobium japonicum* Jordan 1982 and a proposal for *Bradyrhizobium elkanii* sp. nov. *Canadian Journal of Microbiology* 38, 501-505.
- Laguerre, G.; Bardin, M. & Amarger, N. (1993a). Isolation from soil of symbiotic and nonsymbiotic *Rhizobium leguminosarum* by DNA hybridization. *Canadian Journal of Microbiology* 39, 1142-1149.
- Laguerre, G.; Geniaux, E.; Mazurier, S.; Rodriguez-Casartelli, R. & Amarger, N. (1993b). Conformity and diversity among field isolates of *Rhizobium leguminosarum* bv. *viciae*, bv. *trifolii* and bv. *phaseoli* revealed by DNA hybridization using chromosome and plasmid probes. *Canadian Journal of Microbiology* 39, 412-419.
- Laguerre, G.; Mavingui, P.; Allard, M.R.; Charnay, M.P.; Louvrier, P.; Mazurier, S.I.; Rigottier-Gois, L. & Amarger, N. (1996). Typing of rhizobia by PCR DNA fingerprinting and PCR-restriction fragment length polymorphism analysis of chromosomal and symbiotic gene regions: Application to *Rhizobium leguminosarum* and its different biovars. *Applied and Environmental Microbiology* 62, 2029-2036.
- Lane, D.L.; Pace, B.; Olsen, G.J.; Stahl, D.A.; Sogin, M.L. & Pace, N.R. (1985). Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. *Proceedings of the National Academy of Sciences, USA* 82, 6955-6959.
- Liesack, W. & Stackebrandt, E. (1992). Occurrence of novel groups of the domain *Bacteria* as revealed by analysis of genetic material from an Australian terrestrial environment. *Journal of Bacteriology* 174, 5072-5078.
- Lim, G. ? Ng, H.L. (1977). Root nodules of some tropical legumes in Singapore. *Plant and Soil* 46, 317-327.
- Lindström, K. (1989). *Rhizobium galegae*, a new species of legume root nodule bacteria. *International Journal of Systematic Bacteriology* 39, 365-367.
- Lindström, K.; Paulin, L.; Roos, C. & Suominen, L. (1995). Nodulation genes of *Rhizobium galegae*. In *Nitrogen Fixation: Fundamentals and Applications*, pp. 365-370. Edited by I.A. Tikhonovich, N.A. Provorov, V.I. Romanov & W.E. Newton. Dordrecht, The Netherlands, Kluwer Academic Publishers.
- Lindström, K.; van Berkum, P.; Gillis, M.; Martinez, E.; Novikova, N. & Jarvis, B. (1995). Report of the roundtable on *Rhizobium* taxonomy. In *Nitrogen Fixation: Fundamentals and Applications*, pp. # 807-81. Edited by I.A. Tikhonovich, N.A. Provorov, V.I. Romanov & W.E. Newton. Dordrecht, The Netherlands: Kluwer Academic Publishers.
- Loureiro, M.F.; de Faria, S.M.; James, E.K.; Pott, A. & Franco, A.A. (1994). Nitrogen-fixing stem nodules of the legume *Discolobium pulchellum* Benth. *New Phytologist* 128, 283-295.
- Loureiro, M.F.; James, E.K.; Sprent, J.I. & Franco, A.A. (1995). Stem and root nodules on the tropical

- legume *Aeschynomene fluminensis*. *New Phytologist* 130, 531-544.
- Louvrier, P.; Laguerre, G. & Amarger, N. (1995). Semiselective medium for isolation of *Rhizobium leguminosarum* from soils. *Soil Biology and Biochemistry* 27, 919-924.
- Maidak, B.L.; Olsen, G.J.; Larsen, N.; Overbeek, R.; McCaughey, M.J. & Woese, C.R. (1997). The RDP (Ribosomal Database Project). *Nucleic Acids Research* 25, 109-111.
- Manjula, M. & Rakesh, T. (1990). Cluster analysis of genes for nitrogen fixation from several diazotrophs. *Journal of Genetics* 69, 67-78.
- Martinez-Dretz, G. & Arias, A. (1972). Enzymatic basis for differentiation of *Rhizobium* into fast and slow-growing groups. *Journal of Bacteriology* 109, 467-470.
- Martinez-Romero, E.; Segovia, L.; Mercante, F.M.; Franco, A.A.; Graham, P. & Pardo, M.A. (1991). *Rhizobium tropici*, a novel species nodulating *Phaseolus vulgaris* L. beans and *Leucaena* sp. trees. *International Journal of Systematic Bacteriology* 41, 417-426.
- Masterson, R.V.; Prakash, R.K. & Atherly, A.G. (1985). Conservation of symbiotic nitrogen fixation gene sequences in *Rhizobium japonicum* and *Bradyrhizobium japonicum*. *Journal of Bacteriology* 163, 21-26.
- McClelland, M.; Petersen, C. & Welsh, J. (1992). Length polymorphisms in tRNA intergenic spacers detected by using the polymerase chain reaction can distinguish streptococcal strains and species. *Journal of Clinical Microbiology* 30, 1499-1504.
- Minamisawa, K.; Seki, T.; Onodera, S.; Kubota, M. & Asami, T. (1992). Genetic relatedness of *Bradyrhizobium japonicum* field isolates as revealed by repeated sequences and various other characteristics. *Applied and Environmental Microbiology* 58, 2832-2839.
- Moreira, F.M.S. (1991). *Caracterização de Estirpes de Rizóbio Isoladas de Espécies Florestais Pertencentes a Diversos Grupos de Divergência de Leguminosae Introduzidas ou Nativas da Amazônia e Mata Atlântica*. Tese de Doutorado em Ciências do Solo. Seropédica, Universidade Federal Rural do Rio de Janeiro.
- Moreira, F.M.S.; Silva, M.F. & Faria, S.M. (1992). Occurrence of nodulation in legume species in the Amazon region of Brazil. *New Phytologist* 121, 563-570.
- Moreira, F.M.S.; Gillis, M.; Pot, B.; Kersters, K. & Franco, A.A. (1993). Characterization of rhizobia isolated from different divergence groups of tropical Leguminosae by comparative polyacrylamide gel electrophoresis of their total proteins. *Systematic and Applied Microbiology* 16, 135-146.
- Moreira, F.M.S.; Martinez-Romero, E.; Segovia, L. & Franco, A.A. (1995). Genetic diversity of rhizobia and bradyrhizobia from native tropical species characterized by multilocus enzyme electrophoresis, p. 88. In *7th International Symposium of Microbial Ecology, Abstracts*, Santos-SP, Brazil.
- Mullis, K.B. & Faloona, F.A. (1987). Specific synthesis of DNA *in vitro* via a polymerase-catalyzed chain reaction. *Methods of Enzymology* 155, 335-351.
- Muyzer, G.; De Waal, E.C. & Uitterlinden, A.G. (1993). Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Applied and Environmental Microbiology* 59, 695-700.
- Neves, M.C.P. & Rumjanek, N.G. (1997). Diversity and adaptability of soybean and cowpea rhizobia in tropical soils. *Soil Biology and Biochemistry* 29, 889-895.
- Nour, S.M.; Fernandez, M.P.; Normand, P. & Cleyet-Marel, J.C. (1994a). *Rhizobium ciceri* sp. nov., consisting of strains that nodulate chickpeas (*Cicer arietinum* L.). *International Journal of Systematic Bacteriology* 44, 511-522.
- Nour, S.M.; Cleyet-Marel, J.C.; Beck, D.; Effosse, A. & Fernandes, M.P. (1994b). Genotypic and phenotypic diversity of *Rhizobium* isolated from chickpea (*Cicer arietinum* L.). *Canadian Journal of Microbiology* 40, 345-354.
- Nour, S.M.; Cleyet-Marel, J.C.; Normand, P. & Fernandes, M.P. (1995). Genomic heterogeneity of strains

- nodulating chickpea (*Cicer arietinum* L.) and description of *Rhizobium mediterraneum* sp. nov. *International Journal of Systematic Bacteriology* 45, 640 - 648.
- Novikova, N.I.; Pavlova, E.A.; Vorobjev, N.I. & Limenshchenko, E.V. (1994). Numerical taxonomy of *Rhizobium* strains from legumes of the temperate zone. *International Journal of Systematic Bacteriology* 44, 734-742.
- Nutman, P.S. (1987). Centenary lecture. *Philosophical Transactions of the Royal Society of London* 317, 69-106.
- Oliveira, V.M.; Rosato, Y.B.; Coutinho, H.L.C. & Manfio, G.P. (1997). Design of a 16S rRNA-directed oligonucleotide probe for *Bradyrhizobium* tropical strains. In *11th International Congress on Nitrogen Fixation, Proceedings*, p.581. Edited by C. Elmerich, A. Kondorosi & W.E. Newton. Dordrecht, The Netherlands: Kluwer Academic Publishers.
- Olsen, G.J.; Matsuda, H.; Hagstrom, R. & Overbeek, R. (1994). fastDNAm1: A tool for construction of phylogenetic trees of DNA sequences using maximum likelihood. *Computer Applications in Biosciences* 10, 41-48.
- Olsen, P.E.; Rice, W.A.; Bordeleau, L.M. & Biederbeck, V.O. (1994). Analysis and regulation of legume inoculants in Canada: the need for an increase in standards. *Plant and Soil* 161, 127-134.
- Oyaizu, H.; Narubashi, N. & Gamou, T. (1992). Molecular methods of analysing bacterial diversity: the case of rhizobia. *Biodiversity and Conservation* 1, 237-249.
- Pace, N.R.; Stahl, D.A.; Lane, D.J. & Olsen, G.J. (1986). The analysis of natural microbial populations by ribosomal RNA sequences. *Advances in Microbial Ecology* 9, 1-55.
- Paffetti, D.; Scotti, C.; Gnocchi, S.; Fancelli, S. & Bazzicalupo, M. (1996). Genetic diversity of an Italian *Rhizobium meliloti* population from different *Medicago sativa* varieties. *Applied and Environmental Microbiology* 62, 2279-2285.
- Pickup, R.W. (1991). Development of molecular methods for the detection of specific bacteria in the environment. *Journal of General Microbiology* 137, 1009-1019.
- Richardson, A.E.; Viccars, L.A.; Watson, J.M. & Gibson, A.H. (1995). Differentiation of *Rhizobium* strains using the polymerase chain reaction with random and directed primers. *Soil Biology and Biochemistry* 27, 515-524.
- Rinaudo, G.; Orenge, S.; Fernandez, M.P.; Meugnier, H. & Bardin, R. (1991). DNA homologies among members of the genus *Azorhizobium* and other stem- and root- nodulating bacteria isolated from the tropical legume *Sesbania rostrata*. *International Journal of Systematic Bacteriology* 41, 114-120.
- Rome, S.; Brunel, B.; Normand, P.; Fernandez, M. & Cleyet-Marel, J.C. (1996a). Evidence that two genomic species of *Rhizobium* are associated with *Medicago truncatula*. *Archives of Microbiology* 165, 285-288.
- Rome, S.; Fernandez, M.P.; Brunel, B.; Normand, P. & Cleyet-Marel, J.C. (1996b). *Sinorhizobium medicae* sp. nov., isolated from annual *Medicago* spp. *International Journal of Systematic Bacteriology* 46, 972-980.
- Rosado, A.S.; Seldin, L.; Wolters, A.C. & van Elsas, J.D. (1996). Quantitative 16S rDNA-targeted polymerase chain reaction and oligonucleotide hybridization for the detection of *Paenibacillus azotofixans* in soil and the wheat rhizosphere. *FEMS Microbiology and Ecology* 19, 153-164.
- Rumjanek, N.G.; Dobert, R.C.; van Berkum, P. & Triplett, E.W. (1993). Common soybean inoculant strains in Brazil are members of *Bradyrhizobium elkanii*. *Applied and Environmental Microbiology* 59, 4371-4373.
- Scholla, M.H. & Elkan, G.H. (1984). *Rhizobium fredii* sp. nov., a fast-growing species effectively nodulates soybeans. *International Journal of Systematic Bacteriology* 34, 484-486.
- Seal, S.E.; Jackson, L.A. & Daniels, M.J. (1992). Use of tRNA consensus primers to indicate subgroups of *Pseudomonas solanacearum* by polymerase chain reaction amplification. *Applied and Environmental Microbiology* 58, 3759-3761.

- Segovia, L.; Young, J.P.W. & Martinez-Romero, E. (1993). Reclassification of American *Rhizobium leguminosarum* biovar *phaseoli* type I strains as *Rhizobium etli* sp. nov. *International Journal of Systematic Bacteriology* 43, 374-377.
- Selenska-Pobell, S.; Evguenieva-Hackenberg, E.; Radeva, G. & Squartini, A. (1996). Characterization of *Rhizobium 'hedysari'* by RFLP analysis of PCR amplified rDNA and by genomic PCR fingerprinting. *Journal of Applied Bacteriology* 80, 517-528.
- Siqueira, J.O. & Franco, A.A. (1988). *Biotecnologia do Solo: Fundamentos e Perspectivas*. pp. 236. Lavras, FAEPE/ABEAS/MEC/ESAL.
- Smalla, K.; Cresswell, N.; Mendonça-Hagler, L.C.; Wolters, A.C. & van Elsas, J.D. (1993). Rapid DNA extraction protocol from soil for polymerase chain reaction-mediated amplification. *Journal of Applied Bacteriology* 74, 78-85.
- Springer, N.; Ludwig, W. & Hardarson, G. (1993). A 23S rRNA targeted specific hybridization probe for *Bradyrhizobium japonicum*. *Systematic and Applied Microbiology* 16, 468-470.
- Stackebrandt, E.; Liesack, W. & Goebel, B.M. (1993). Bacterial diversity in a soil sample from a subtropical Australian environment as determined by 16S rDNA analysis. *FASEB Journal* 7, 232-236.
- Stanley, J.; Brown, G.G. & Verma, P.S. (1985). Slow-growing *Rhizobium japonicum* comprises two highly divergent symbiotic types. *Journal of Bacteriology* 163, 148-154.
- Stephen, J.R.; McCaig, A.E.; Smith, Z.; Prosser, J.I. & Embley, T.M. (1996). Molecular diversity of soil and marine 16S rRNA gene sequences related to β -subgroup ammonia-oxidizing bacteria. *Applied and Environmental Microbiology* 62, 4147-4154.
- Tan, I.K.P. & Broughton, W.J. (1981). Rhizobia in tropical legumes. XIII. Biochemical basis of acid and alkali reactions. *Soil Biology and Biochemistry* 13, 389-393.
- Tas, E.; Kaijalainen, S.; Saano, A. & Lindström, K. (1994). Isolation of a *Rhizobium galegae* strain-specific DNA probe. *Microbiological Releases* 2, 231-237.
- Tas, E.; Leinonen, P.; Saano, A.; Räsänen, L.A.; Kaijalainen, S.; Piippola, S.; Hakola, S. & Lindström, K. (1996). Assessment of competitiveness of rhizobia infecting *Galega orientalis* on the basis of plant yield, nodulation, and strain identification by antibiotic resistance and PCR. *Applied and Environmental Microbiology* 62, 529-535.
- UNEP (United Nations Environment Programme). (1992). *Convention on Biological Diversity*. Nairobi, UNEP.
- Vallaëys, T.; Topp, E.; Muyzer, G.; Macheret, V.; Laguerre, G.; Rigaud, A. & Soulas, G. (1997). Evaluation of denaturing gradient gel electrophoresis in the detection of 16S rDNA sequence variation in rhizobia and methanotrophs. *FEMS Microbiology and Ecology* 24, 279-285.
- van Berkum, P.; Beyene, D.; Eardly, B.D. & van Berkum, P. (1996). Phylogenetic relationships among *Rhizobium* species nodulating the common bean (*Phaseolus vulgaris* L.). *International Journal of Systematic Bacteriology* 46, 240-244.
- van Rossum, D.; Schuurmans, F.P.; Gillis, M.; Muotcha, A.; van Verseveld, H.W.; Stouthamer, A.H. & Boogerd, F.C. (1995). Genetic and phenetic analyses of *Bradyrhizobium* strains nodulating peanut (*Arachis hypogaea* L.) roots. *Applied and Environmental Microbiology* 61, 1599-1609.
- Vincent, J.M. (1970). *A Manual for the Practical Study of Root-Nodule Bacteria*. Oxford and Edinburgh: Blackwell Scientific Publications.
- Woese, C.R. (1987). Bacterial evolution. *Microbiological Reviews* 51, 221-271.
- Wong, F.Y.K.; Stackebrandt, E.; Ladha, J.K.; Fleischman, D.E.; Date, R.A. & Fuerst, J.A. (1994). Phylogenetic analysis of *Bradyrhizobium japonicum* and photosynthetic stem-nodulating bacteria from *Aeschynomene* species grown in separated geographical regions. *Applied and Environmental Microbiology* 60, 940-946.

- Xu, L.M.; Ge, C.; Cui, Z.; Li, J. & Fan, H. (1995). *Bradyrhizobium liaonigensis* sp. nov. isolated from the root nodules of soybeans. *International Journal of Systematic Bacteriology* **45**, 706-711.
- Yokoyama, T.; Ando, S.; Murakami, T. & Imai, H. (1996). Genetic variability of the common *nod* gene in soybean bradyrhizobia isolated in Thailand and Japan. *Canadian Journal of Microbiology* **42**, 1209-1218.
- Young, J.P.W. (1993). Molecular phylogeny of rhizobia and their relatives. In *New Horizons in Nitrogen Fixation* p. 587-592. Edited by R. Palacios, J. Mora & W.E. Newton. Proceedings of the 9th International Congress on Nitrogen Fixation, Cancun, Mexico, December 6-12, 1992. Dordrecht, The Netherlands: Kluwer Academic Publishers.
- Young, J.P.W. (1996). Phylogeny and taxonomy of rhizobia. *Plant and Soil* **186**, 45-52.
- Young, J.P.W. & Johnston, A.W.B. (1989). The evolution of specificity in the legume-*Rhizobium* symbiosis. *Trends in Ecology and Evolution* **4**, 341-349.
- Zhang, X.; Harper, R.; Karsisto, M. & Lindström, K. (1991). Diversity of *Rhizobium* bacteria isolated from the root nodules of leguminous trees. *International Journal of Systematic Bacteriology* **41**, 104-113.

2.2.Métodos moleculares no estudo da diversidade microbiana do solo

Obs.: O material apresentado a seguir foi publicado como uma revisão: **Evaluating the Microbial Diversity of Soil Samples: Methological Innovations.** 1999. *Anais da Academia Brasileira de Ciências* **71**:491-503.

Evaluating the Microbial Diversity of Soil Samples: Methodological Innovations

HEITOR L. C. COUTINHO¹, VALÉRIA M. DE OLIVEIRA²,
GILSON P. MANFIO² and ALEXANDRE S. ROSADO³

¹Embrapa Solos - 22460-000 Rio de Janeiro, RJ

²Fundação Tropical de Pesquisas e Tecnologia André Tosello - 13087-010 Campinas, SP

³Instituto de Microbiologia Prof. Paulo de Góes, UFRJ,
CCS, Bloco I, Ilha do Fundão - 21944-970 Rio de Janeiro, RJ

Manuscript received February 10, 1999

ABSTRACT

This manuscript is a review of the innovative methodologies that enable more precise evaluations of soil microbial diversity. Highlighting the molecular approach, which does not require the isolation of microorganisms and allows the inclusion of non-culturable genotypes in the analyses, the described methodologies revolutionised the environmental microbiology and opened gateways for an accurate understanding of the ecology and diversity of microorganisms. The application of techniques based on soil total DNA extraction, PCR amplification of genes or gene fragments, and sequence analysis revealed that the microbial universe is far more complex than ever imagined. Examples of applications of the molecular approach to study the diversity of soil diazotrophic bacteria are given.

Key words: microbial diversity, DGGE, molecular methods, soil DNA, 16S rRNA.

INTRODUCTION

The study of microbial diversity is hampered by several problems, such as: 1) the microscopic dimensions of the subject; 2) incomplete taxonomic descriptions for many known species; and 3) lack of selective isolation and growth media for the majority of soil microorganisms. In addition, classical characterization methods based on microbial morphology and physiology are untrustworthy due to the great ability of microorganisms to adapt to a wide range of environmental conditions. The same genotype may reveal different cell shapes or behaviour when grown in the laboratory. There is an urgent need for faster and more efficient meth-

odologies for the evaluation of microbial diversity. Table I lists current methods applied to study microbial diversity in the environment.

Until recently, the detection and identification of microorganisms in environmental samples was accomplished by their growth in artificial selective or non-selective media, or their direct observation using a microscope (Herbert, 1990). However, growth media are, to a greater or lesser extent, selective to particular groups of microorganisms. Even if a selective media for the target organism is available, some unculturable strains might be excluded from the analyses. Cell counts by microscopy can aid a quantitative assessment of the microbial populations, being less informative about their diversity (Pickup, 1991).

One of the methods applied to investigate the metabolic diversity of microbial communities is

Correspondence to: Heitor L. C. Coutinho
Embrapa Solos — Rua Jardim Botânico, 1024
22460-000 Rio de Janeiro, RJ.

TABLE I
Methods to study the different levels of microbial diversity in the environment.

Method	Observations/References
I) Function based methods	
Colony counts	Herbert (1990)
Biolog	Heuer & Smalla (1997a)
Microbial biomass	Morgun & Winstanley (1997)
II) Community structure based methods	
PFLA ("Phospholipid fatty acid")	Morgun & Winstanley (1997)
"In situ" hybridization	Aman <i>et al.</i> (1990)
PCR/DGGE or TGGE	Muyzer <i>et al.</i> (1993) Rosado <i>et al.</i> (1998)
PCR/ARDRA	Smit <i>et al.</i> (1997)

based on the commercial kit named BIOLOG. The microtitre plates of BIOLOG are inoculated with bacterial cell suspensions extracted from soil samples. The wells of the BIOLOG kit are coated with different sources of carbon, nutrients and labelled with tetrazolium. The plates are then incubated and periodically monitored for substrate oxidation and tetrazolium reduction. This methodology was applied to study microbial communities from both soil and rhizosphere (Wünsche *et al.*, 1995), and to investigate the effects of the introduction of genetically modified organisms on soil microbial communities (Heuer & Smalla, 1997a).

During the last decade, the molecular approach to study microbial diversity has been progressively adopted and improved by microbiologists eager to avoid the *biases* associated with classical methods based on growth in artificial media. One of these methods is based on the analysis of total phospholipid fatty acids (PFLA). This technique enables the structural analysis of microbial communities in the environment as well as the detection of shifts in microbial populations (Morgun & Winstanley, 1997). However, this type of analysis is not indicated to detailed structural evaluations of the soil microbial community.

Nucleic acids based techniques are an alternative to these methods. Torsvik *et al.* (1990) estimated the genetic diversity of a bacterial community by analysing the reassociation kinetics

of DNA extracted from soils of a deciduous forest. Their results were compared with those obtained by conventional analysis of populations of bacteria isolated from the same soil samples. The authors found 4,000 different genomes in the soil samples when applying the molecular approach, an estimate 200-fold greater than that obtained by conventional analyses. This indicates that only a minute fraction of the soil microbiota is accounted for by the analysis of phenotypic variants of isolated bacteria.

MOLECULAR PROBES

The need to monitor the release and fate of genetically modified microorganisms in the environment (Droge *et al.*, 1998; Kellenberger, 1994) fostered the development of a plethora of methods designed for the detection of all organisms. These methods are able to cope both with microorganisms in the non-cultured state, as well as cloned genes, which are amenable to transfer to other microbial populations (Giddings, 1998).

New strategies were developed and adapted to cope with the limitations of traditional culture-based approaches for the study of bacterial populations. Notable developments were achieved by nucleic acid based techniques. The use of molecular probes proved to be a versatile approach, allowing both the study of isolated and cultivated microorganisms as well as of specific organisms or

groups of organisms by use of nucleic acids extracted directly from environmental samples (Pickup, 1991).

A probe is a labeled fragment of DNA or RNA with a nucleotide sequence complementary to that of the target gene sequence of interest. Under controlled conditions (particularly ionic concentration and temperature), probe and single-stranded target gene sequence hybridize forming heteroduplexes. The detection of target gene sequences depends on the nature of the labeling system, which can be radioactively or non-radioactively labeled deoxynucleotides present in the probe sequence. The latter include a choice of labels, from enzymes (*e.g.*, alkaline phosphatase or horse radish peroxidase), to fluorescent molecules (*e.g.*, fluorescein), and biotin or digoxigenin (Stahl & Amann, 1991).

Nucleic acid probes can be constructed to target particular genes, and thus be used to detect organisms with the corresponding genotypes or biochemical pathways. Examples are probes directed to bacterial genes coding for antibiotic or heavy metal resistance, as well as for enzymes involved in biosynthetic or catabolic pathways, such as nitrogenase (Rosado *et al.*, 1998), and those involved in the metabolism of xenobiotic compounds. Probes can also be targeted to mobile genetic sequences, which can be transferred between chromosomal and plasmid DNA, such as transposons, or between organisms from different taxa, such as conjugative plasmids (Kellenberger, 1994; Hill & Top, 1998).

The use of molecular probes presents the following advantages (Stahl & Amann, 1991; Schleifer & Ludwig, 1994):

- can be designed to be highly specific and used under stringent conditions to detect a gene or nucleic acid sequence from a particular organism or group of organisms;
- can be used to detect and identify organisms without the need for cultivation and isolation in pure culture;
- probes targeted to regions of the rRNA molecule with different levels of variability may be designed and used to detect specific organisms or broader taxonomic groups;

— in general, the bacterial genome is highly stable and is not affected by growth conditions, contrary to antigenic molecules and phenotypic properties.

The application of nucleic acid probes to the study of natural microbial populations had a quantum leap after the development of the polymerase chain reaction technique (PCR; (Mullis & Faloona, 1987)). The PCR can significantly amplify rare nucleotide sequences against a background of diverse and more abundant sequences, thus enhancing the possibility of detecting rare organisms/sequences in heterogeneous mixtures. Sequences from particular microorganisms can be amplified using PCR primer pairs targeted at species-specific complementary sequences located in the 16S or 23S rDNA (Bej *et al.*, 1990; Roller *et al.*, 1992), or at genes coding for enzymes (Bej *et al.*, 1991), or even at some other genome fragments (Pooler & Hartung, 1995).

Over the last few years, a range of protocols based on the PCR have been reported for the detection of microorganisms in soil samples (Smalla *et al.*, 1993; Briglia *et al.*, 1996). The combined PCR/probing approach significantly enhances the sensitivity of detection protocols. Combined PCR/probe hybridization have raised approximately 1,000 times the sensitivity of detection of genetically engineered *Pseudomonas cepacia* strains in environmental samples (Steffan & Atlas, 1988).

USE OF RIBOSOMAL RNA AS PHYLOGENETIC MARKERS

Ribosomal ribonucleic acids (rRNA) are considered to be the most adequate biopolymers for comparative studies in microbial molecular energy. Their coding genes, the rDNA, are universally distributed, being the molecule with the highest degree of conservation in the living world. Variability can accumulate to a greater or lesser extent in different regions of the molecule (Lane *et al.*, 1985). A major advantage of using rRNA sequence information is their availability in databases (RDP, GenBank, EMBL), in most cases accessible free-of-charge, along with services such as align-

ment and comparison of newly obtained sequences with the data set (Maidak *et al.*, 1997).

Automation of nucleic acid sequencing had a big impact on the use of 16S rRNA sequences as molecular markers, resulting in a large number of microbial rRNA sequences stored in the databases. These sequences can be used to build phylogenetic trees, depicting how the studied organisms relate to one another. Sequence alignments give precious information to aid taxa-specific oligonucleotide design. Specific oligonucleotides can be designed to different taxonomic levels, from universal (for example, encompassing all Eubacteria taxa) to family-, species-, or strain-specific probes or PCR primers. Microbial ecology research has benefited from a great variety of ribosomal probes specific to different genera, species or strains (Pace *et al.*, 1986).

Probes specific to the 16S rRNA of the nitrogen fixing actinomycetes *Frankia* were used to study the ecology of this microorganism in soil samples (Hahn *et al.*, 1990). Ecological and taxonomic studies of this genus have been hampered by their difficulty to grow in artificial media in the laboratory. The use of molecular probes enabled the identification of *Frankia* strains in *Alnus glutinosa* nodules.

A molecular ecology study based on the amplification and sequencing of 16S rRNA molecules amplified from DNA of Australian soils, followed by phylogenetic analysis and hybridization with taxon-specific probes revealed the occurrence of unique bacterial groups, which should be classified as new lines of descent under the domain *Bacteria* (Liesack & Stackebrandt, 1992). A similar approach was applied by Stackebrandt *et al.* (1993) to investigate the genetic diversity of streptomycetes in acid soils sampled from a subtropical Australian forest. Again, the results revealed a significant degree of microbial diversity. The majority of the 113 16S rRNA sequences analysed were new types of actinomycetes, unrelated to the known, culturable strains of these organisms.

The use of ribosomal probes were improved by Amann *et al.* (1990), who coupled fluorescent labels to the oligonucleotides. The fluorescent probes were used in *in situ* hybridization experi-

ments, consisting of the immobilization and permeabilization of cells directly on the soil samples. The labelled probes were detected by fluorescent microscopy, revealing the target organisms. One could use, concomitantly, probes containing different fluorescent labels, allowing the discrimination of different species in soil samples.

Ribosomal probes can also be used to quantify microbial populations in soil samples. A quantitative PCR method was developed by Rosado *et al.* (1996) to detect *Paenibacillus azotofixans* strains in soils and wheat rhizosphere. The 16S rRNA gene regions were PCR amplified with specific primers, and hybridized to a homologous probe to an internal region of the amplified fragment. The method was shown to be adequate to evaluate the impact of water stress on the population of *P. azotofixans* in the soil. The primers and probes are highly specific and the method precludes the isolation or growth of the bacterial strains in the laboratory, as well as the use of other specific markers.

A similar approach, based on the hybridization of total soil rDNA against a taxon-specific probe, enabled quantitative analysis of microbial taxa. The quantification is achieved by expressing the specific signal as a fraction of the hybridization signal obtained with a universal probe (Zheng *et al.*, 1996).

Microbial molecular diversity studies can focus on unique functional groups. The analysis of ribosomal sequences obtained from soil samples can reveal new species, modify concepts, and even reassemble the taxonomy of functional groups. Sequencing of ammonium oxidizing bacteria 16S rDNA fragments amplified from soil and sediment samples revealed that the vast majority of the sequences obtained were unique, meaning that the known culturable genera belonging to this functional group (*Nitrosomonas*, *Nitrospira* and *Nitrosococcus*) are not dominant in any of the analysed samples (Stephen *et al.*, 1996). These findings redefined this functional group, previously considered of low diversity, and highlights the importance of the molecular approach to studies of evaluation and monitoring of microorganisms in the environment.

THE EXTRACTION OF NUCLEIC ACID FROM SOILS AND SEDIMENTS

The study of microbial populations in the environment by using DNA or RNA sequences requires the extraction of the deoxyribonucleic acids from the samples. Various methods of extracting DNA or RNA from environmental samples are available in the literature, each adapted to the different categories of environmental sample (water, soil and sediments, among others). DNA extraction from water samples generally requires the concentration of the microbial biomass by means of filtration or centrifugation of large volumes of material (Somerville *et al.*, 1989). In the case of soils and sediments, both cellular extraction and direct cell lysis can be adopted (Fig. 1).

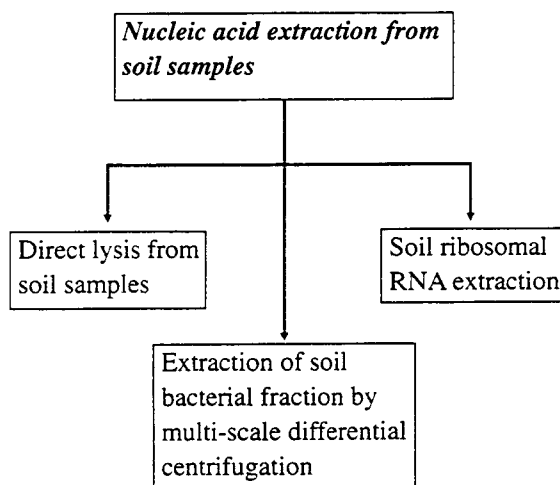


Fig. 1 — Major protocols for nucleic acid extraction from environmental samples.

The direct lysis approach is applied with increasing frequency to extract DNA from soils and sediments. It is based on the physical disruption of the cells in the soil or sediment matrix and subsequent purification of the released DNA (Ogram *et al.*, 1988). Despite being efficient, this process could result in partial lysis or widespread deterioration of the DNA. Therefore, careful standardization of the methodology for the different types of samples is necessary.

Soil and sediment samples also contain a large quantity of humic material, which is extracted

along with the DNA. This occurs due to the polyanionic properties of humic acids, which are linked to free nucleic acids. The greater the organic carbon and clay contents of a soil, the more difficult it is to extract DNA of good quality (Wellington *et al.*, 1994). The humic acids are inhibitors of the constraining endonucleases and DNA polymerases, and may also interfere in the specific nature of the hybridization reactions. The addition of PVPP (polyvinyl polypyrrolidone) is recommended to remove, by adsorption, the phenolic compounds such as humic acids. Other compounds may be added to minimize oxidation of the phenols, such as the reducing agent sodium ascorbate (Holben *et al.*, 1988).

The total quantity of DNA (from procaryotes and eucaryotes) in soils and sediments ranges, on average, from 10 to 200 $\mu\text{g g}^{-1}$ of soil. However, the extraction yield depends on the nature of the sample (the soil type) and on the efficiency of the *in situ* cell lysis, which in turn depends on the composition of the bacterial populations and the lysis method utilized; Gram-positive bacteria, for instance, are more resistant to mechanical lysis. The extraction method used should minimize the period of time that the DNA is in contact with the soil matrix after the lysis has occurred, thus avoiding the adsorption of mineral particles, humic acids and the potential action of free nucleases.

Pillai *et al.* (1991) developed a simple method for purifying soil-extracted DNA before submitting it to PCR amplification. It consists of the separation of DNA from the bulk of the colloidal soil particles by centrifugation in a sucrose gradient before amplification by PCR. The authors demonstrated the potential of this method to extract bacterial DNA from a variety of soils.

A quick method for the direct DNA extraction from soils involves a lysozyme treatment followed by mechanical lysis by bead-beating coupled with detergent (SDS) at low temperatures. The nucleic acids are extracted using phenol and a cold process, followed by DNA precipitation with caesium chlorate and potassium acetate, and separation with HCl spermine or glass-milk (glass micro-beads). The DNA obtained using this methodology is suitable for PCR amplification (Smalla *et al.*, 1993).

An improvement of this method was achieved by van Elsas *et al.* (1997), who introduced a commercial product ("Wizard cleanup system", Promega) during the last stage of DNA purification, and in this way reduced total protocol time. Another simple and quick method for purifying DNA involves the addition of PVP (polyvinylpyrrolidone) to the agarose gel during the electrophoretic separation of the extracted DNA (Young *et al.*, 1993). The addition of PVP eliminates the joint migration of humic acids with the nucleic acids, by delaying the electrophoretic mobility of the phenol compounds.

The extraction of ribosomal RNA from soil samples can be useful, since it enables information to be obtained on the metabolically active members of the microbial population in the environment. Recently, a protocol to obtain DNA or RNA simultaneously was developed by Duarte *et al.* (1998). The protocol enables the extraction of large quantities of both nucleic acids from different soil samples and the subsequent amplification of them using (RT)-PCR ("Reverse Transcriptase-Polymerase Chain Reaction").

rDNA SEQUENCE ANALYSIS

Several approaches can be applied to evaluate microbial diversity by use of rDNA sequences amplified from environmental samples. In general, the choice of approach must take into account the aim of the study, the availability of suitable probes and primers for the subject taxa, laboratory infrastructure, expertise and time.

The automation of sequencing techniques, combined with PCR, led to a rapid increase in the number of rDNA sequences obtained from cultured microorganisms and environmental samples, which are stored in the databases and available for phylogenetic analysis. Ideally, a comprehensive evaluation of microbial diversity should involve the quantification and sequencing of all representative rDNA molecules present in the sample, which is certainly not a practical task to be performed. If one considers the need for replicating samples in order to attain reliable results, then it becomes a daunting task. Thus, the alternative is to analyze only a fraction of the rDNA molecules obtained.

The methods that follow are all based upon PCR-amplified rDNA sequences, which aim at the estimation of microbial diversity and phylogenetic analysis through the evaluation of a moiety of the rDNA sequences present in the samples. A basic protocol for the study of microbial diversity in environmental samples would involve the following steps (Fig. 2):

- extraction and purification of DNA from the sample;
- partial or full amplification of rDNA sequences (16S and/or 23S rDNA, or intergenic regions) using primers with the desired specificity;
- cloning of the PCR products in plasmids/phage and construction of a genomic library representative of the amplified rDNAs;
- if necessary, primary analysis of the cloned sequences (*e.g.*, using *T-tracking* sequencing) to assess the representativeness of the genomic library (rarefaction curves; number of distinct sequences in the sample *versus* number of analyzed clones);
- partial or full sequencing of the rDNA clones;
- alternatively, methods of sequence analysis based on DNA fingerprinting (restriction profiles, ARDRA) or DGGE may be used;
- analysis of data (phylogenetic affiliation, taxonomic diversity indexes).

SEQUENCING AND SEQUENCE ANALYSIS

The diversity of microorganisms may be estimated directly by comparative analysis of the ribosomal sequences obtained from the environmental sample. Partial or full sequence analysis of 16S rDNA or bacteria and archaea provides a comprehensive framework for such studies. An additional advantage is the fact that the 16S rDNA molecule may provide information at both supra- and infra-specific levels as different portions of the molecule present different degrees of variability, and thus may have different levels of taxonomic resolution.

Usually, diversity studies based on 16S rDNA sequence analysis depend on the availability of a good sequencing infrastructure. High throughput and automated sequencing are the options of choice for conducting such studies, though many microbiology laboratories around the globe are not

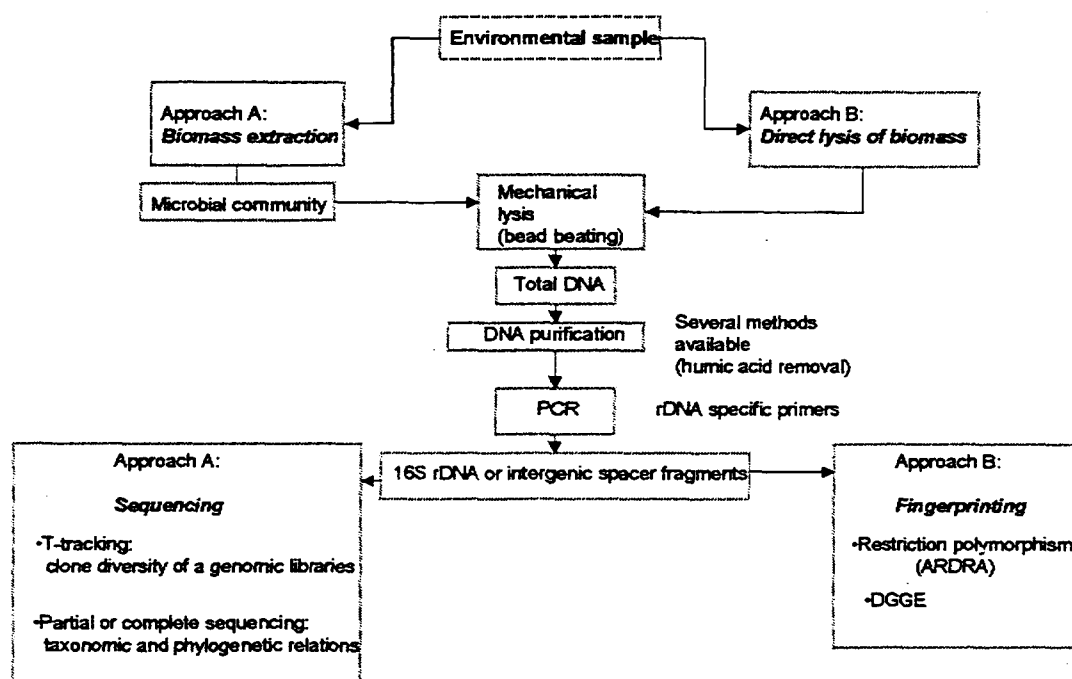


Fig. 2 — Experimental approaches for microbial diversity evaluations through rDNA analysis.

yet familiar with such resources and the necessary expertise.

RAPID METHODS FOR THE COMPARISON OF DNA FRAGMENTS (RFLP AND T-TRACKING)

Redundancy in large genomic libraries can be reduced by performing rapid characterizations of the DNA fragments prior to the more laborious and expensive analyses (e.g., full sequence determination). One commonly used approach is based on the restriction enzyme profiles obtained after digesting the DNA fragments with restriction enzymes, that are selected based on their ability to reveal restriction length polymorphisms (RFLPs) in the DNA fragments analysed. Usually, enzymes that have short recognition sites (4- or 5-cutters), such as *Alu* I, *Dde* I, *Hinf* I, *Msp* I, *Rsa* I, *Sau*3 A and *Taq* I, are selected for RFLP analyses.

Another approach, denominated *T-tracking*, is based on partial sequence analysis of DNA fragments by using only one single terminator dideoxynucleotide (ddTTP) in the sequencing reactions, which are submitted to electrophoresis in

sequencing gels. The results are profiles comprised of bands corresponding to the T positions of the DNA fragment and larger gaps corresponding to the remaining DNA bases. The profiles obtained can be directly compared to those obtained for other DNA fragments. The diversity of patterns represented in the sample of fragments analysed can be correlated to the diversity of the population (Stephen *et al.*, 1996). *T-tracking* is usually applied to construct rarefaction curves to estimate how many clones need to be analyzed in order to have a representative sampling of a given population of PCR-amplified/cloned DNA fragments.

ARDRA

Amplified Ribosomal DNA Restriction Analysis (ARDRA) is a technique based on the restriction enzyme digestion of amplified rDNA fragments followed by the electrophoretic separation of the resulting fragments in agarose gel. This methodology was originally applied to genome characterization as well as to comparative studies of isolated strains (Vanechoutte *et al.*, 1992). The technique is based on the degree of conservation of

the rRNA operon restriction sites, therefore reflecting phylogenetic patterns. ARDRA is suitable for fast evaluations of microbial community genotypic changes with time, and for comparing samples from sites under differing environmental conditions by analysis of amplified rDNA from mixed populations (Massol-Deya *et al.*, 1995). The authors recommend careful selection of the rDNA fragment to be amplified and analysed by ARDRA. If the microbial group analysed has a strong phylogenetic affinity, the amplified fragment should include the 16S-23S rDNA spacer region. This intergenic region has greater variability both in its nucleotide base composition as well as in its sequence size, when compared to the 16S or the 23S rDNAs. If the microbial group under study has greater phylogenetic heterogeneity, the targeted fragment should be the 16S rDNA. This gene generates simple banding patterns, depending on the restriction endonucleases used (usually with restriction sites containing four to five nucleotide bases). This technique was applied recently to evaluate the effects of copper contamination on soil microbial diversity (Smit *et al.*, 1997).

DGGE

Another technique recently developed is the DGGE method (*denaturing gradient gel electrophoresis*), which has been successfully applied to detect variations in the sequences of various microbial genes. This methodology enables the separation of DNA fragments of same length, differing in their nucleotide sequences. This separation is achieved based on the differential mobility of DNA molecules subjected to electrophoresis in polyacrylamide gels under a gradient provided by a denaturing agent. Partially denatured DNA molecules have a lower mobility when compared to fully paired double stranded molecules. Therefore, the electrophoretic migration of DNA fragments with different sequences ceases at different positions in a DGGE gel, depending on base composition, generating fingerprints of the mixture of genes analyzed (Fischer & Lerman, 1983). This technique can be applied to the study of microbial diversity based on the differences between the rDNA se-

quences of an environmental sample. A mixture of amplified rDNA 16S from the environment can be separated using DGGE, generating a fingerprint which can be monitored time and space-wise. The greater the complexity of the fingerprint, the more diverse is the rDNA mixture and consequently, the diversity of the microbial group studied. The loss or addition of one or more bands represents the rate loss or gain (represented by the rDNA 16S sequence) in the molecular sampling carried out. The DGGE method is also suitable for sampling effort studies and for the definition of relatively simple diversity indices in molecular studies of the microbial diversity. Despite the relative simplicity of the method, DGGE requires strict standardization and has the same limitations which are inherent in PCR amplification of rDNA 16S (Wintzingerode *et al.*, 1997; Heuer & Smalla, 1997b).

The first application of DGGE in molecular microbial ecology studies was carried out by Muyzer *et al.* (1993). The objective of the authors was to analyze the genetic diversity of microbial populations. They observed the presence of up to 10 different rDNA fragments in microbial communities obtained from different origins. The specific amplification of DNA fragments associated with microbial strains or defined taxonomic groups, allied with DGGE, is a strategy which enables direct detection of the presence and relative abundance of different microbial species in environmental samples for the first time (Muyzer *et al.*, 1993). The DNA contained in the DGGE gel can be transferred to nylon membranes and hybridized with specific probes for certain microbial groups or the PCR bands can be eluted from the gel, re-amplified and sequenced to enable phylogenetic positioning of the members of the community (see diagram in Fig. 3).

The DGGE technique has been frequently used to study the structure of natural microbial communities. For this purpose, the ribosomal gene 16S has been most frequently used in the published protocols (Muyzer *et al.*, 1993). However, other preserved genes can also be used in DGGE systems (Rosado *et al.*, 1997). Waver & Muyzer (1995) used DGGE to study the genetic diversity of *Desulfovibrio* species by analyzing fragments of

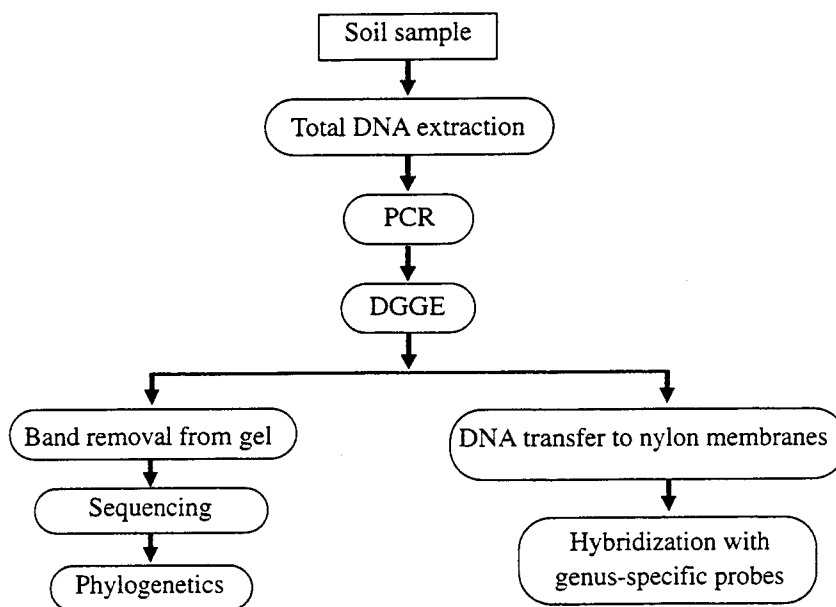


Fig. 3 — The use of DGGE to study microbial diversity in environmental samples.

the functional NiFe gene (hydrogenase) amplified using PCR.

A DGGE system was recently developed based on *nifH* genes from *P. azotofixans* (Rosado *et al.*, 1998). The *nifH* gene was selected because it has preserved and variable regions, useful for the development of primers for PCR. It was first necessary to obtain information on the *nifH* gene from *Paenibacillus* sp., since there was no information available in the data bank. For this purpose, a methodology using degenerate primers was used (Zehr & McReynolds, 1989) to amplify part of the *nifH* gene from *Paenibacillus* nitrogen-fixing species. This methodology proved to be simple and quick. The PCR products obtained were cloned and sequenced. The alignment and analysis of the *Paenibacillus nifH* sequences using parsimony and matrix methods (neighbor joining), enabled the construction of a phylogenetic tree based on the similarities found. The phylogenetic tree suggests that the *Paenibacillus* species are closely grouped amongst themselves and that the *P. azotofixans* strains have sequence deviations but form a very interrelated group. What is even more surprising is the presence of copies of the *nif* gene in *P. azotofixans* strains that are grouped with sequences of an alternative nitrogenase system (*anf*), which sug-

gests the existence of an alternative nitrogen fixing system in *P. azotofixans*. In the future, it will be interesting to study the different genic expressions of the two nitrogen fixing systems in *P. azotofixans*.

DGGE results using the product of PCR *nifH* from *P. azotofixans* indicated that this gene is present with several copies in the bacterial genome and that there is a great diversity amongst the different copies and also between different strains. The use of the DGGE *nifH* system with DNA extracted from environmental samples demonstrates that the DGGE system is a powerful tool for the study of the genetic diversity in natural *Paenibacillus* populations (Rosado *et al.*, 1998).

A DGGE system was also applied to evaluate the diversity of *Rhizobium* species in soils subjected to different types of agricultural handling (direct planting, conventional planting, and the use of a soil insecticide) (Oliveira *et al.*, 1998). The same group described the design of specific primers for sites in the ribosomal spacing region (rDNA 16S-rDNA 23S) of the *Rhizobium leguminosarum* and *R. tropici* species and its use to amplify fragments of the ribosomal genic region of *Rhizobium* species from soil extracted DNA from the "Cerrado" region in the northern part of the State of São Paulo (Oliveira *et al.*, 1999).

METHODOLOGICAL LIMITATIONS

Possibly the greatest current methodological limitation is the definition of sampling parameters, in order to confirm statistical relevance of the results. This is fundamental for monitoring ecosystems subject to natural transformations or not. The fundamental point to be evaluated is the question of the number of sequences to be analyzed in order to define the representative nature of the sampling. This value can change from sample to sample, depending on the richness of the amplified sequences and relative representativeness of each group (abundance). These problems are even more complex, being influenced by the methodological variations involved in the process as a whole, such as: the efficiency of DNA extraction, which can be more efficient in the lysing of a microbial group to the detriment of another; the degree of specificity of the primers or genetic probes, defined as a function of the sequences representing only known and already sequenced microorganisms (a minimal fraction of the environmental diversity); the amplification procedure using PCR, which can exhibit preferential amplification of some sequences and the formation of technical artifacts (*e.g.* chimeric molecules). To this, the problems with respect to sequential variations in the rRNA 16S molecules due to the heterogeneous nature of the *rrn* operon can be added. The possible limitations of using PCR and DGGE in environmental samples were recently reviewed by Wintzingerode *et al.* (1997) and Heuer & Smalla (1997b), respectively.

GENERAL CONCLUSIONS

Molecular microbial ecology is a multidisciplinary theme covering the sciences of microbiology, ecology, molecular biology, statistics and bioinformatics, among others. Its tools aid the investigation of microbial diversity and the advantages and risks involved in environmental applications of microorganisms. The development of agricultural and environmental biotechnology led to a greater demand for microbial strains that exert important functions, such as: (i) supplying nutrients to plants; (ii) promote plant growth; (iii) control or inhibit phytopathogenic activity; (iv) im-

prove soil structure; and (v) degrade polluting compounds. There are many bacterial species to exert these functions, however their widespread application is limited by problems of survival and low efficiency in the environment. The best approach would be to use strains better adapted to conditions of ecological stress (Van Veen *et al.*, 1997). These strains could also be altered genetically so as to enhance its performance in the environment. This means that there is an enormous biotechnological potential represented by the vast quantity of unknown and unexplored microorganisms present in the soil. Therefore, the development of methodologies to enable the study of the diversity of these microorganisms in the environment is essential.

A polyphasic approach to the study of microbial diversity, integrating conventional and molecular techniques, is necessary. In addition, the development of new and improved methods and approaches would benefit greatly by a greater interaction among microbiologists, ecologists, statisticians, and professionals of the different disciplines involved in microbial diversity studies. The emphasis of those studies should be on the evaluation of the current paradigms and theoretical models and on the importance of microbial diversity to the biological processes.

REFERENCES

- AMANN, R. L.; KUMHOLZ, L. & STAHL, D. A., (1990), Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. *J. Bacteriol.*, **172**: 762-770.
- BEJ, A. K.; MCCARTY, S. C. & ATLAS, R. M., (1990), Detection of coliform bacteria in water by polymerase chain reaction and genes probes. *Appl. Environ. Microbiol.*, **56**: 307-314.
- BEJ, A. K.; DICESARE, J. L.; HAFF, L. & ATLAS, R. M., (1991), Detection of *Escherichia coli* and *Shigella* spp. in water by using the polymerase chain reaction and gene probes for *uid*. *Appl. Environ. Microbiol.*, **57**: 1013-1017.

- BRIGLIA, M.; EGGEN, R. I. L.; DE VOS, W. M. & VAN ELSAS, J. D., (1996), Rapid and sensitive method for the detection of *Mycobacterium chlorophenicum* PCP-1 in soil based on 16S rRNA gene-targeted polymerase chain reaction. *Appl. Environ. Microbiol.*, **62**: 1478-1480.
- DROGE, M.; PUHLER, A. & SELBITSCHKA, W., (1998), Horizontal gene transfer as a biosafety issue: A natural phenomenon of public concern. *J. Biotechnol.*, **64**: 75-90.
- DUARTE, G. F.; ROSADO, A. S.; SELDIN, L.; WOLTERS, A. C. K. & VAN ELSAS, J. D., (1998), Extraction of ribosomal RNA and genomic DNA from soil for studying the diversity of the indigenous bacterial community. *J. Microbiol. Methods*, **32**: 21-29.
- FISCHER, S. G. & LERMAN, L. S., (1983), DNA fragments differing by single basepair substitutions are separated in denaturing gradient gels: correspondence with melting theory. *Proc. Natl. Acad. Sci. U.S.A.*, **80**: 1579-1583.
- GIDDINGS, G., (1998), Tansley Review No. 99 – The release of genetically engineered micro-organisms and viruses into the environment. *New Phytol.*, **140**: 173-184.
- HAHN, D.; STARRENBURG, M. J. C. & AKKERMANS, A. D. L., (1990), Oligonucleotide probes that hybridize with rRNA as a tool to study *Frankia* strains in root nodules. *Appl. Environ. Microbiol.*, **56**: 1342-1346.
- HERBERT, R. A., (1990), Methods for enumerating microorganisms and determining biomass in natural environments. *Methods Microbiol.*, **19**: 1-40.
- HEUER, H. & SMALLA, K., (1997a), Evaluation of community-level catabolic profiling using BIOLOG GN microplates to study microbial community changes in potato rhizosphere. *J. Microbiol. Methods*, **30**: 49-61.
- HEUER, H. & SMALLA, K., (1997b), Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis for studying soil microbial communities. In: VAN ELSAS, J. D.; WELLINGTON, E. M. H.; TREVORS, J. eds. *Modern Soil Microbiology*, New York, Marcel Dekker, Inc., p. 353-373.
- HILL, K. E. & TOP, E. M., (1998), Gene transfer in soil systems using microcosms. *FEMS Microbiol. Ecol.*, **25**: 319-329.
- HOLBEN, W. E.; JANSSON, J. K.; CHELM, B. K. & TIEDJE, J. M., (1988), DNA probe method for the detection of specific microorganisms in the soil bacterial community. *Appl. Environ. Microbiol.*, **54**: 703-711.
- KELLENBERGER, E., (1994), Genetic ecology – A new interdisciplinary science, fundamental for evolution, biodiversity and biosafety evaluations. *Experientia*, **50**: 429-437.
- LANE, D. L.; PACE, B.; OLSEN, G. J.; STAHL, D. A.; SOGIN, M. L. & PACE, N. R., (1985), Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. *Proc. Natl. Acad. Sci. U.S.A.*, **82**: 6955-6959.
- LIESACK, W. & STACKEBRANDT, E., (1992), Occurrence of novel groups of the domain *Bacteria* as revealed by analysis of genetic material from an Australian terrestrial environment. *J. Bacteriol.*, **174**: 5072-5078.
- MAIDAK, B. L.; OLSEN, G. J.; LARSEN, N.; OVERBEEK, R.; MCCAGHEY, M. J. & WOESE, C. R., (1997), The RDP (Ribosomal Database Project). *Nucleic Acid Res.*, **25**: 109-111.
- MASSOL-DEYA, A. A.; ODELMAN, D. A.; HICKEY, R. F. & TIEDJE, J. M., (1995), Bacterial community fingerprinting of amplified 16S and 16-23S ribosomal DNA gene sequences and restriction endonuclease analysis (ARDRA). In: AKKERMANS, A. D. L.; VAN ELSAS, J. D.; DE BRUIJN, F. J. eds. *Molecular Microbial Ecology Manual*. Kluwer Academic Press, **3.3.2**: 1-8.
- MORGUN, A. & WINSTANLEY, C., (1997), Microbial biomarkers. In: VAN ELSAS, J. D.; TREVORS, J. T. & WELLINGTON, E. M. H. eds. *Modern Soil Microbiology*. New York, Marcel Dekker, p. 331-352.
- MULLIS, K. B. & FALOONA, F. A., (1987), Specific synthesis of DNA *in vitro* via a polymerase-catalyzed chain reaction. *Methods Enzymol.*, **155**: 335-351.
- MUYZER, G.; DE WAAL, E. C. & UITTERLINDEN, A. G., (1993), Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl. Environ. Microbiol.*, **59**: 695-700.
- OGRAM, A.; SAYLER, G. S. & BARKAY, T., (1988), DNA extraction and purification from sediments. *J. Microbiol. Methods*, **7**: 57-66.

- OLIVEIRA, V. M.; COUTINHO, H. L. C.; VAN ELSAS, J. D. & MANFIO, G. P., (1998), Avaliação da diversidade de rizóbios em amostras ambientais através do uso de *primers* específicos. In: LIMA, J. M.; FAQUIN, V.; SIQUEIRA, J. O. & MOREIRA, F. M. S. eds. *Interrelação Fertilidade, Biologia do Solo e Nutrição de Plantas: Consolidando um Paradigma*. V Simpósio Brasileiro de Microbiologia do Solo, Resumos, Caxambu, p. 808.
- OLIVEIRA, V. M.; COUTINHO, H. L. C.; SOBRAL, B. W. S.; GUIMARÃES, C. T.; VAN ELSAS, J. D. & MANFIO, G. P., (1999), Discrimination of *Rhizobium tropici* and *R. leguminosarum* strains by PCR-specific amplification of 16S-23S rDNA spacer region fragments and denaturing gradient gel electrophoresis (DGGE). *Let. Appl. Microbiol.*, **28**: 137-141.
- PACE, N. R.; STAHL, D. A.; LANE, D. J. & OLSEN, G. J., (1986), The analysis of natural microbial populations by ribosomal RNA sequences. *Adv. Microb. Ecol.*, **9**: 1-55.
- PICKUP, R. W., (1991), Development of molecular methods for the detection of specific bacteria in the environment. *J. General Microbiol.*, **137**: 1009-1019.
- PILLAI, S. D.; JOSEPHSON, K. L.; BAILEY, R. L.; GERBA, C. P. & PEPPER, I. L., (1991), Rapid method for processing soil samples for polymerase chain reaction amplification of specific gene sequences. *Appl. Environ. Microbiol.*, **57**: 2283-2286.
- POOLER, M. R. & HARTUNG, J. S., (1995), Specific PCR detection and identification of *Xylella fastidiosa* strains causing citrus variegated chlorosis. *Current Microbiol.*, **31**: 377-381.
- ROLLER, C.; LUDWIG, W. & SCHLEIFER, K. H., (1992), Gram-positive bacteria with a high DNA G+C content are characterized by a common insertion within their 23S rRNA genes. *J. General Microbiol.*, **138**: 1167-1175.
- ROSADO, A. S.; SELDIN, L.; WOLTERS, A. C. & VAN ELSAS, J. D., (1996), Quantitative 16S rDNA-targeted polymerase chain reaction and oligonucleotide hybridization for the detection of *Paenibacillus azotofixans* in soil and the wheat rhizosphere. *FEMS Microbiol. Ecol.*, **19**: 153-164.
- ROSADO, A. S.; DUARTE, G. F.; SELDIN, L. & VAN ELSAS, J. D., (1997), Molecular microbial ecology: a minireview. *Rev. Microbiol.*, **28**: 135-147.
- ROSADO, A. S.; DUARTE, G. F.; SELDIN, L. & VAN ELSAS, J. D., (1998), Genetic diversity of *nifH* sequences in *Paenibacillus azotofixans* strains and soil samples analysed by Denaturing Gradient Gel Electrophoresis of PCR-amplified gene fragments. *Appl. Environ. Microbiol.*, **64**: 2770-2779.
- SCHLEIFER, K.-H. & LUDWIG, W., (1994), Molecular Taxonomy: Classification and Identification. In: PRIEST, F. G.; RAMOS-CORMENZANA, A.; TYNDALL, B. J. eds. *Bacterial Diversity and Systematics*. Federation of European Microbiological Societies Symposium Series, **75**: 1-15.
- SMALLA, K.; CRESSWELL, N.; MENDONÇA-HAGLER, L. C.; WOLTERS, A. C. & VAN ELSAS, J. D., (1993), Rapid DNA extraction protocol from soil for polymerase chain reaction-mediated amplification. *J. Appl. Bacteriol.*, **74**: 78-85.
- SMIT, E.; LEEFLANG, P. & WERNARS, K., (1997), Detection of shifts in microbial communities structure and diversity in soil caused by copper contamination using amplified ribosomal DNA restriction analysis. *FEMS Microbiol. Ecol.*, **23**: 249-261.
- SOMERVILLE, C. C.; KNIGHT, I. T.; STRAUBE, W. L. & COLWELL, R. R., (1989), Simple, rapid method for direct isolation of nucleic-acids from aquatic environments. *Appl. Environ. Microbiol.*, **55**: 548-554.
- STACKEBRANDT, E.; LIESACK, W. & GOEBEL, B. M., (1993), Bacterial diversity in a soil sample from a subtropical Australian environment as determined by 16S rDNA analysis. *FASEB J.*, **7**: 232-236.
- STAHL, D. A. & AMANN, R., (1991), Development and applications of nucleic acid probes. In: STACKEBRANDT, E. & GOODFELLOW, M. eds. *Nucleic acid techniques in bacterial systematics*, John Wiley & Sons, Chichester, 205-248.
- STEFFAN, R. J. & ATLAS, R. M., (1988), DNA amplification to enhance the detection of genetically engineered bacteria in environmental samples. *Appl. Environ. Microbiol.*, **54**: 2185-2191.
- STEPHEN, J. R.; MCCAIG, A. E.; SMITH, Z.; PROSSER, J. I. & EMBLEY, T. M., (1996), Molecular diversity of soil and marine 16S rRNA gene sequences related to β -subgroup ammonia-oxidizing bacteria. *Appl. Environ. Microbiol.*, **62** (11): 4147-4154.
- TORSVIK, V.; GOKSOYR, J. & DAAE, F. L., (1990), High diversity in DNA of soil bacteria. *Appl. Environ. Microbiol.*, **56**: 782-787.

- VANEECHOUTTE, M.; ROSSAU, R.; DE VOS, P.; GILLIS, M.; JANSSENS, D.; PAEPE, N.; DE ROUCK, A.; FIERIS, T.; CLAEYS, G. & KERSTERS, K., (1992), Rapid identification of bacteria of the *Comamonadaceae* with amplified ribosomal DNA-restriction analysis (ARDRA). *FEMS Microbiol. Lett.*, **93**: 227-234.
- VAN ELSAS, J. D.; MANTYNEN, V. & WOLTERS, A. C., (1997), Soil DNA extraction and assessment of the fate of *Mycobacterium chlorophenolicum* strain PCP-1 in different soils by 16S ribosomal RNA gene sequence based most-probable-number PCR and immunofluorescence. *Biol. Fertil. Soils*, **24**: 188-195.
- VAN VEEN, J. A.; VAN OVERBEEK, L. S. & VAN ELSAS, J. D., (1997), Fate and activity of microorganisms following release into soil. *Microbiol. Mol. Biol. Rev.*, **61**: 121-135.
- WAVER, C. & MUYZER, G., (1995), Genetic diversity of *Desulfovibrio* spp. in environmental samples analyzed by denaturing gradient gel electrophoresis of [NiFe] hydrogenase gene fragments. *Appl. Environ. Microbiol.*, **61**: 2203-2210.
- WELLINGTON, E. M. H.; HUDDLESTON, A. S. & MARSH, P., (1994), The use of molecular markers for the detection and typing of bacteria in soil. In: PRIEST, F. G.; RAMOS-CORMENZANA, A.; TYNDALL, B. J. eds. *Bacterial Diversity and Systematics*. Federation of European Microbiological Societies Symposium Series, **75**: 137-152.
- WINTZINGERODE, F. V.; GÖBEL, U. B. & STACKEBRANDT, E., (1997), Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. *FEMS Microbiol. Rev.*, **21**: 213-229.
- WÜNSCHE, L.; BRÜGGEMANN, L. & BABEL, W., (1995), Determination of substrate utilization patterns of soil microbial communities: an approach to assess population changes after hydrocarbon pollution. *FEMS Microbiol. Ecol.*, **17**: 295-306.
- YOUNG, C. C.; BURGHOFF, R. L.; KEIM, L. G.; MINAK-BERNERO, V.; LUTE, J. R. & HINTON, S. M., (1993), Polyvinylpyrrolidone-agarose gel electrophoresis purification of polymerase chain reaction-amplifiable DNA from soils. *Appl. Environ. Microbiol.*, **59**: 1972-1974.
- ZEHR, J. P. & McREYNOLDS, L. A., (1989), Use of degenerate oligonucleotide primers for amplification of the *nifH* gene from the marine cyanobacterium *Trichodesmium thiebautii*. *Appl. Environ. Microbiol.*, **55**: 2522-2526.
- ZHENG, D. E.; ALM, W.; STAHL, D. A. & RASKIN, L., (1996), Characterization of universal small-subunit rRNA hybridization probes for quantitative molecular microbial ecology studies. *Appl. Environ. Microbiol.*, **62** (12): 4504-4513.

2.3. Diversidade de rizóbios em solos tropicais

A família Leguminosae é bem representada nos trópicos, principalmente a subfamília Caesalpinioidea, dominada por espécies arbóreas. Contudo, estudos sobre a diversidade de leguminosas capazes de nodular e seus rizóbios simbiossantes são escassos.

Trinick (1980) foi um dos pioneiros nos estudos sobre nodulação de leguminosas tropicais, investigando características morfológicas, metabólicas, de crescimento, serológicas e infectivas de bactérias isoladas das leguminosas *Leucaena*, *Mimosa*, *Acacia*, *Sesbania* e *Lablab*. Verificou-se que as bactérias estudadas apresentavam características fenotípicas que as relacionavam às espécies de rizóbios de crescimento rápido associados a leguminosas de clima temperado, assim como características de preferência e compatibilidade com hospedeiros que as relacionavam aos rizóbios de crescimento lento e promíscuos de solos tropicais.

Ahmad *et al.* (1981) empregaram métodos imunológicos para examinar 53 linhagens de rizóbios isolados de leguminosas em três regiões diferentes da África Ocidental e demonstraram a grande diversidade de serotipos nas populações naturais de rizóbios destas regiões. Uma alta diversidade também foi observada por Zhang *et al.* (1991) entre os rizóbios isolados de leguminosas arbóreas, *Acacia senegal* e *Prosopis chilensis*, no Sudão através do uso de taxonomia numérica.

Moreira *et al.* (1993) realizaram um estudo amplo, envolvendo 171 linhagens de rizóbios isoladas de leguminosas tropicais da Floresta Amazônica e Mata Atlântica no Brasil, através de eletroforese de proteínas totais. Vinte e três grupos eletroforéticos foram obtidos, com um coeficiente de correlação média de 0,86, sendo que a maioria dos isolados de crescimento lento formou um grupo que continha a linhagem tipo de *Bradyrhizobium japonicum*. Os perfis de proteínas dos rizóbios de crescimento rápido evidenciaram uma diversidade genética maior que a encontrada para os rizóbios de crescimento lento.

Pinero *et al.* (1988) empregaram a metodologia de eletroforese de isoenzimas (*multiloci enzyme electrophoresis*) para caracterizar 51 linhagens de *Rhizobium leguminosarum* bv. *phaseoli* isoladas de várias regiões tropicais. Os resultados das análises de mobilidade eletroforética de 15 enzimas metabólicas indicaram uma alta heterogeneidade dos genes estruturais das linhagens deste biovar e um nível de diversidade genética e divergência genotípica não registrado antes para qualquer espécie de bactéria.

Um estudo polifásico de rizóbios de crescimento lento típicos isolados de solo sob a leguminosa arbórea *Acacia albida* no Senegal envolveu análises de eletroforese de proteínas, ribotipagem utilizando rRNA da linhagem tipo de *B. japonicum* e

sequenciamento de genes de rRNA 16S (Dupuy *et al.*, 1994). A maioria dos isolados estudados distribuiu-se em oito grupos eletroforéticos contendo linhagens representantes de *B. japonicum*, *B. elkanii*, e *Bradyrhizobium* sp. Os resultados das ribotipagens confirmaram que a maioria dos grupos eletroforéticos de proteínas pertencia ao complexo de rRNA de *Bradyrhizobium-Rhodopseudomonas*. Já o sequenciamento de genes de rRNA indicou considerável heterogeneidade genotípica entre as linhagens que nodulam *Acacia albida*.

O alto grau de diversidade verificado pelos autores citados acima é um indicativo de que novos gêneros e espécies de rizóbios poderão vir a ser descritos à medida que mais linhagens sejam isoladas de leguminosas de regiões tropicais e estudadas por taxonomistas microbianos.

2.4. Efeitos do manejo agrícola sobre a microbiota do solo

Todas as formas de vida dependem dos processos bacterianos para sua sobrevivência. As bactérias são responsáveis por diversas funções metabólicas que afetam o solo e as plantas. A ciclagem de nutrientes, formação e decomposição de matéria orgânica, formação da estrutura do solo, e promoção do crescimento das plantas, estão entre os benefícios desempenhados pelas bactérias. Portanto, a diversidade e funcionamento da comunidade bacteriana do solo é um bioindicador da qualidade de agroecossistemas.

Diversos estudos vêm sendo conduzidos numa tentativa de avaliar e quantificar os efeitos de práticas agrícolas como plantio direto, plantio convencional, rotação de culturas, dentre outras, sobre a comunidade microbiana de solos agrícolas. Medir a biomassa e abundância de fungos e bactérias é uma das estratégias para se comparar os efeitos entre o plantio direto e o plantio convencional. Frey *et al.* (1999) não encontraram uma correlação consistente entre a abundância e biomassa bacteriana e o tipo de manejo empregado, e a maior proporção de biomassa total composta por fungos observada para o manejo do tipo plantio direto foi correlacionada a diferenças de umidade do solo. Os resultados encontrados por Lupwayi *et al.* (1999) demonstraram que a biomassa microbiana do solo era geralmente significativamente maior sob plantio direto do que sob plantio convencional, sugerindo o primeiro tipo de manejo e a rotação de culturas baseada em leguminosas como sistemas agrícolas mais sustentáveis.

Buyer e Kaufman (1997) investigaram a diversidade microbiana na rizosfera de milho sob plantio convencional e sistemas de manejo mínimo utilizando diferentes métodos, entre eles plaqueamento e contagem total e métodos bioquímicos (análises de FAMES - *fatty acid methyl esters*). A diversidade encontrada não foi significativamente

diferente entre os sistemas de manejo avaliados, sugerindo que práticas de agricultura convencional podem manter altos índices de diversidade microbiana na rizosfera.

Outros métodos bioquímicos usados para analisar a diversidade e estrutura de comunidades microbianas em solos sob diferentes manejos incluem a extração de PLFA (*phospholipid fatty acids*) (Yeates *et al.*, 1997) e o sistema BIOLOG™ para detecção de padrões específicos de utilização de substrato (Lupwayi *et al.*, 1998). Os resultados mostraram-se variados. No primeiro estudo, utilizando-se PLFA, não se observou alteração da diversidade bacteriana, mas sim da de fungos, em função do tipo de manejo, convencional e orgânico. No segundo caso, uma diversidade microbiana significativamente maior foi detectada no sistema de rotação de culturas utilizando leguminosas, comparado com o sistema de cultura contínua.

Mais recentemente, as inovações das técnicas moleculares vêm também sendo empregadas na análise da diversidade microbiana em ambientes naturais e perturbados. Torsvik *et al.* (1998) analisaram a comunidade bacteriana total, incluindo os organismos cultivados e os não-cultivados, usando extração de DNA das amostras de solo e sedimento e uma combinação de diferentes métodos moleculares, como análise de reassociação de DNA, DGGE (*denaturing gradient gel electrophoresis*) de genes rRNA amplificados por PCR, hibridização com sondas grupo-específicas e sequenciamento. Os autores demonstraram que o manejo agrícola pode levar a profundas alterações na estrutura da comunidade e a uma redução da diversidade bacteriana.

Estudos dos efeitos de práticas agrícolas específicas sobre populações de rizóbios no ambiente não foram encontrados na literatura. Por outro lado, a interação entre os pesticidas comumente usados na agricultura (herbicidas, fungicidas e inseticidas) e rizóbios tem sido investigada por muitos autores, com ênfase na nodulação e fixação de nitrogênio em leguminosas de interesse comercial, principalmente a soja.

A maior parte dos estudos, principalmente aqueles realizados na década de setenta, considerava a aplicação de pesticidas como fator essencial para garantir altas produtividades. Neste sentido, fungicidas eram testados em diferentes doses para avaliar quais seriam adequadas para o revestimento de sementes a serem inoculadas com rizóbios. Curley e Burton (1975) registraram uma redução significativa da nodulação, principalmente nas raízes pivotantes, e diminuição da sobrevivência de rizóbios inoculados em plantas de soja com a aplicação de doses recomendadas de Captan e pentacloronitrobenzeno (PCNB). Chambers e Montes (1982) obtiveram resultados similares com a aplicação do fungicida Thiram, mas não observaram redução da nodulação quando da utilização de PCNB e Benomil. Estes autores verificaram que os efeitos deletérios dos fungicidas foram maiores quando a linhagem CB1809 de *Bradyrhizobium japonicum* foi utilizada para inocular a

soja, comparativamente com a linhagem USDA110, demonstrando a variabilidade na resposta em função das diferentes linhagens de uma mesma espécie. Mallik e Tesfai (1985) observaram decréscimo significativo no número e massa de nódulos em plantas de soja causado pelo fungicida Carboxin, sendo que a maior parte dos nódulos apresentava tamanho reduzido e encontrava-se concentrada nas raízes laterais, indicando infecção por outros rizóbios presentes no solo.

Além dos fungicidas, os herbicidas também foram bastante investigados quanto aos seus efeitos sobre a simbiose rizóbio-leguminosa. Havia uma preocupação quanto a possíveis efeitos dos herbicidas no metabolismo das leguminosas, os quais pudessem causar alguma inibição da formação de nódulos e translocação de carboidratos para os bacteróides. Em um estudo com amendoim, três herbicidas, Flucloralin, Pendimetalin e Oxadiazon, apresentaram efeitos negativos na proliferação de bactérias totais de uma amostra de solo (Kole & Dey, 1989). Pendimetalin e Metribuzin também causaram redução no número de nódulos de plantas de grão-de-bico, enquanto que Flucloralin produziu resultados variáveis (Pahwa & Prakash, 1992). Em feijão, Schnelle e Hensley (1990) observaram redução significativa da fixação de nitrogênio causada pelo herbicida pós-emergente Bentazone, quando aplicado em concentração três vezes superior à recomendada. Em estudos com feijão fava (*Vicia faba*) o herbicida Metabenzthiazuron (MBT) causou uma diminuição significativa na nodulação (Islam & Afendi, 1980).

Os efeitos de inseticidas aplicados ao solo, ou nas sementes, sobre a nodulação e fixação de nitrogênio não foram extensivamente estudados, comparativamente a fungicidas e herbicidas. Já foram registrados efeitos adversos ou a inocuidade de inseticidas sobre a nodulação, fixação de nitrogênio e produtividade de leguminosas (Pareek & Gaur, 1970; Selim *et al.*, 1970; Smith *et al.*, 1978).

Estudos de avaliação dos impactos de pesticidas sobre a diversidade de rizóbios do solo não foram encontrados na literatura. Alguns pesticidas mais recalcitrantes podem se acumular no solo e persistir por um período de tempo longo. Estes seriam inibidores potenciais de processos vitais para a manutenção da capacidade produtiva deste solo (Mallik & Tesfai, 1985).

3. OBJETIVOS GERAIS

- Estabelecer um sistema de detecção de rizóbios, e/ou de grupos destes, e avaliação da sua diversidade em amostras ambientais baseado no uso de sondas moleculares e *primers* específicos.
- Avaliar a influência da agricultura intensiva sobre a diversidade de rizóbios em amostras de solo através da aplicação do sistema de detecção desenvolvido.
- Comparar estratégias diferentes de abordagem molecular para o estudo da diversidade de rizóbios em amostras ambientais.

4. OBJETIVOS ESPECÍFICOS

- Desenhar *primers* específicos para espécies de rizóbios de maior ocorrência tropical baseado no alinhamento e comparação de sequências de genes ribossomais.
- Demonstrar o poder de resolução da técnica de DGGE na discriminação de rizóbios em nível infra-específico através da separação dos fragmentos de DNAr de linhagens-referência amplificados por PCR.
- Avaliar a diversidade relativa de linhagens de rizóbios em amostras de solo através de extração direta de DNA da comunidade bacteriana, aliada à amplificação de sequências do DNAr com *primers* específicos e separação em DGGE.
- Avaliar o potencial da técnica de DGGE como estratégia rápida de análise de diversidade de rizóbios em amostras ambientais.
- Estabelecer um sistema molecular direto para avaliar a diversidade de rizóbios em amostras ambientais baseado na extração de DNA da comunidade bacteriana do solo,

construção de banco de sequências ribossomais, uso de sondas específicas e análise de perfis de bandas gerados por PCR-RFLP.

- Estimar a influência de diferentes práticas agrícolas, envolvendo plantio direto e convencional e o uso ou não de inseticida de solo, sobre a diversidade de populações de rizóbios no solo.
- Avaliar o impacto de diferentes práticas agrícolas, plantio direto e plantio convencional, sobre a diversidade de rizóbios utilizando-se o método clássico de isolamento (planta-isca e plaqueamento em meio de cultivo) e o índice de diversidade de Shannon-Weaver para analisar os perfis de bandas gerados pela técnica de RAPD.

Obs.: O material apresentado a seguir compreende três artigos publicados e/ou submetidos para publicação em revistas internacionais com corpo editorial, exceto para a seção intitulada “**Capítulo Avulso**”.

Discrimination of *Rhizobium tropici* and *R. leguminosarum* strains by PCR-specific amplification of 16S–23S rDNA spacer region fragments and denaturing gradient gel electrophoresis (DGGE)

V.M. de Oliveira¹, H.L.C. Coutinho², B.W.S. Sobral³, C.T. Guimarães^{3*}, J.D. van Elsas⁴ and G.P. Manfio¹

¹Fundação André Tosello, Campinas, SP, ²Embrapa Solos, Rio de Janeiro, RJ, Brazil, ³National Center for Genome Resources, Santa Fe, NM, USA, and ⁴Research Institute for Plant Protection, Wageningen, The Netherlands

1898/98: received 5 October 1998 and accepted 21 October 1998

V.M. DE OLIVEIRA, H.L.C. COUTINHO, B.W.S. SOBRAL, C.T. GUIMARÃES, J.D. VAN ELSAS AND G.P. MANFIO. 1999. With the aim of detecting *Rhizobium* species directly in the environment, specific PCR primers for *Rh. tropici* and *Rh. leguminosarum* were designed on the basis of sequence analysis of 16S–23S rDNA spacer regions of several *Rh. tropici*, *Rh. leguminosarum* and *Agrobacterium rhizogenes* strains. Primer specificity was checked by comparison with available rDNA spacer sequences in databases, and by PCR using DNA from target and reference strains. Sequence polymorphisms of rDNA spacer fragments among strains of the same species were detected by denaturing gradient gel electrophoresis (DGGE). The specific PCR primers designed in this study could be applied to evaluate the diversity of *Rh. tropici* and *Rh. leguminosarum* by analysing the polymorphisms of 16S–23S spacer rDNA amplified from either whole-cell or soil-extracted DNA.

INTRODUCTION

Rhizobium is a genus of soil bacteria belonging to the family Rhizobiaceae, whose members are able to establish symbiotic relationships with a range of legume plants of agricultural and environmental importance, a process that results in biological nitrogen fixation (BNF) (Hirsh 1992).

The inoculation of cultivated leguminous plants with selected rhizobial strains is recommended to maximize the contribution of BNF to the nitrogen status of the host plant. However, inoculation with commercially available strains may not improve crop yields in the presence of high numbers of indigenous rhizobia in the soil, which may be more competitive for nodulation or nitrogen fixation, and are often better adapted to the prevailing soil and climate conditions (Anyango *et al.* 1995). Therefore, the evaluation and moni-

toring of the indigenous rhizobial population diversity in soils is of great importance in estimating the need for inoculum, and for the screening of novel, highly effective inoculant strains.

The diversity of *Rhizobium* populations has usually been determined by phenotypic and/or genotypic characterization of strains isolated from legume root nodules (Martínez-Romero 1994). This approach may be prone to bias as the isolates obtained represent the bacterial genotypes which were successful in the competition for nodulation of the particular host plant. Recent reports have demonstrated individual plant selection effects (Handley *et al.* 1998).

Methods for extraction of soil DNA (Ogram *et al.* 1988; Steffan *et al.* 1988; Smalla *et al.* 1993) coupled with the use of molecular probes and/or PCR primers for the specific detection of *Rhizobium* (Laguerre *et al.* 1993a, b; Streit *et al.*, 1993; Pooyan *et al.* 1994; Tas *et al.* 1994) may offer an alternative approach to studying the composition of, and monitoring, natural rhizobial populations.

Sequences of 16S rDNA are known to be highly conserved among bacteria (Woese 1987) and, in spite of the fact that

Correspondence to: V.M. de Oliveira, Fundação André Tosello, Rua Latino Coelho, 1301, CEP: 13087-010, Campinas, SP, Brazil (e-mail: maia@bdt.org.br).

*Present address: Bioagro, Universidade Federal de Viçosa, 36571-000, Viçosa, MG, Brazil.

strain-specific regions are sometimes identified, analyses of the genetic variation within this gene are not always sufficient to differentiate between strains within a species. In contrast, sequence analysis of the spacer regions between the 16S and the 23S rDNA can be used to differentiate organisms at the intraspecific level, given the relatively higher sequence divergence of this region compared with rRNA genes (Honeycutt *et al.* 1995; Nour *et al.* 1995; Gürtler and Stanisch 1996).

Denaturing gradient gel electrophoresis (DGGE) allows the separation of DNA fragments with identical length but different nucleotide sequences. This technique, combined with PCR applied to 16S rRNA segments, has recently been introduced in the field of molecular microbial ecology and is used not only to 'profile' complex microbial communities (Muyzer *et al.* 1993; Heuer *et al.* 1997), but also to infer the phylogenetic affiliation of the community members (Muyzer *et al.* 1995). It is also used to test the purity of bacterial strains, to monitor the isolation of bacteria from environmental samples (Brinkhoff and Muyzer 1997), and to study the dynamics of specific populations according to environmental variations (Ferris *et al.* 1996; Teske *et al.* 1996).

In this study, the sequence of the spacer region between 16S and 23S rDNA was analysed to design PCR-specific primers able to differentiate *Rh. tropici* and *Rh. leguminosarum* strains from those of *Agrobacterium rhizogenes*, a phylogenetically closely related organism. This strategy was chosen because of the lack of variability of the 16S rRNA sequences between *Rh. leguminosarum*/*Rh. tropici* and *A. rhizogenes*, as observed on the phylogenetic trees and sequence alignments of the Ribosomal Database Project (Olsen *et al.* 1992). In addition, preliminary restriction enzyme polymorphism analyses of the 16S–23S intergenic spacers among *Rhizobium* and *Agrobacterium* species revealed the occurrence of polymorphic sites (data not shown).

MATERIALS AND METHODS

Strains, growth conditions and DNA extraction

Rhizobium leguminosarum bv. *trifolii* CCT 4179 (←IAM 12613) and CCT 4488 (=LMG 6119), *Rh. leguminosarum* bv. *viceae* CCT 5087^T (←IAM 12609^T), *Rh. leguminosarum* bv. *phaseoli* CCT 4168^T (←IAM 12612^T), *Rh. tropici* CCT 4160^T (=LMG 9503^T) and CCT 4164 (=LMG 9517), *Rh. meliloti* CCT 4167^T (=LMG 6133^T) and CCT 4169 (=LMG 6130), *Rh. loti* CCT 4063^T (=LMG 6125^T) and CCT 4159 (=LMG 4284), *Agrobacterium tumefaciens* CCT 5075 (←IBSBF 304; =IAM 1524) and CCT 5265 (←LMG 196), *A. rhizogenes* CCT 4832^T (←IAM 13570^T) and CCT 4842 (←IBSBF 642; =IAM 13571), and *A. vitis* CCT 6069 (←IBSBF 915) and CCT 6072^T (←IBSBF 919^T; =ATCC 49767^T) were obtained from Coleção de Culturas Tropical

(CCT), Fundação 'André Tosello', Campinas, SP, Brazil; ATCC (American Type Culture Collection); IAM (Institute of Applied Microbiology, Tokyo, Japan); IBSBF (Instituto Biológico, Seção de Bacteriologia Fitopatológica, Campinas, Brazil); and LMG (Laboratorium voor Microbiologie Universiteit, Gent, Belgium). *Rhizobium* strains were grown in YM broth (0.5 g KH₂PO₄, 0.2 g MgSO₄·7H₂O, 0.1 g NaCl, 0.5 g yeast extract, 10.0 g mannitol and 0.5% bromothymol blue litre⁻¹ distilled water), and *Agrobacterium* strains in Nutrient Broth (Difco), for 48 h at 28 °C. Genomic DNA extractions from pure cultures were performed according to Pitcher *et al.* (1989).

PCR amplification

The 16S–23S intergenic spacers were amplified using primers pHr (Massol-Deya *et al.* 1995), located at position 1518–1541 relative to the *Escherichia coli* 16S sequence, and p23 Suni322anti (Honeycutt *et al.* 1995), at position 322 relative to the *E. coli* 23S sequence, generating fragments of approximately 1.5 kb. PCR was performed using 50 ng bacterial DNA in 50 µl reactions containing 2.0 U *Taq* polymerase (CENBIOT, RS, Brazil), 1 × *Taq* buffer, 2.0 mmol l⁻¹ MgCl₂, 0.2 mmol l⁻¹ dNTP mix and 0.4 µmol l⁻¹ of each primer. The PCR amplifications were carried out by using an initial denaturation step at 95 °C for 2 min, followed by 30 cycles of 1 min at 94 °C, 1 min at 60 °C and 3 min at 72 °C, and a final extension at 72 °C for 5 min, in a Perkin Elmer 9600 thermal cycler (Norwalk, USA).

Cloning and sequencing

PCR-amplified 16S–23S rDNA intergenic spacers from *Rh. leguminosarum*, *Rh. tropici* and *A. rhizogenes* strains were cloned into pGEM-T vector (Promega) and partially sequenced in an automated system (ABI 373; Perkin Elmer, Foster City, CA, USA).

Primer design and specificity tests

Sequence data were aligned and analysed using the GDE software environment (Genetic Data Environment, V.2.2; gopher://megasun.bch.umontreal.ca: 70/11/GDE). Primer specificity was checked by comparison with available GenBank/EMBL/ DDBJ database sequences (Genbank database release 107.0 and updates up to July 98). The specificity of the primers was then confirmed in PCR reactions with reference strains under the conditions described above, except that the temperature profile consisted of an initial denaturation step at 95 °C for 2 min, followed by 30 cycles of 1 min at 94 °C, 30 s at 60 °C and 45 s at 72 °C. The amplification products were visualized in 1.4% agarose gels, stained with ethidium bromide.

DGGE analysis

The DGGE technique was carried out using an INGENY apparatus (INGENY phorU-2, Vlissingen, The Netherlands) at 100 V and 60 °C for 16 h in 0.5 × TAE buffer (20 mmol l⁻¹ Tris-acetate, 10 mmol l⁻¹ sodium acetate, 0.5 mmol l⁻¹ Na₂EDTA; pH 7.4). The denaturing gradient (urea and formamide) in 6% acrylamide gels (acrylamide/N,N'-methylenebisacrylamide, 37/1) ranged from 45 to 65% (100% denaturant corresponding to 7 mol l⁻¹ urea and 40% (v/v) deionized formamide). The bands were visualized under u.v. light after staining with SYBR Green (Molecular Probes) diluted 1:4000 in 0.5 × TAE buffer for 1 h in the dark.

RESULTS AND DISCUSSION

The 16S–23S rDNA sequences of strains CCT 4179, CCT 5087^T, CCT 4168^T, CCT 4160^T, CCT 4164, CCT 4832^T and CCT 4842 were deposited in the Genome Sequence DataBase (National Center for Genome Resources, <http://www.ncgr.org>) and GenBank under the accession numbers AF091791 and AF091790, AF091793 and AF091792, AF091789 and AF091788, AF091801 and AF091800, AF091799 and AF091798, AF091795 and AF091794, and AF091797 and AF091796, corresponding to the initial 700 bases and the final 600 bases of the 16S–23S rDNA, respectively.

Three specific primers for *Rh. leguminosarum* and/or *Rh. tropici* were designed based on the alignment of the rDNA spacer sequences: rhizo2f, *Rh. tropici* and *Rh. leguminosarum* forward primer (5'-gAT ggC ACC AgT CAg gTg AC-3'), rhizo3r, *Rh. tropici* and *Rh. leguminosarum* reverse primer (5'-ggA AgA CTT gAA YTT CCg A-3') and trop1f, *Rh. tropici* forward primer (5'-Cgg ACR TgS CCC gAT AT-3'; base degeneracy: R = AG, S = CG, Y = CT).

Specificity checks against sequences in nucleotide databases showed that the rhizo2f forward primer was homologous, with only two mismatches, to a non-coding sequence in the beginning of the 16S–23S spacer region of two strains of *A. vitis* (accession numbers U45329 and U28505). The rhizo3r reverse primer showed partial homology to the tRNA-Ile gene sequence within the 16S–23S spacer region of *Bradyrhizobium japonicum* (one mismatch; Z35330) and *A. vitis* (two mismatches; U45329 and U28505), and presented three or more mismatches to sequences from several other bacteria. No homologous sequences in the database were found for the trop1f forward primer.

In PCR reactions, the primer pair rhizo2f/rhizo3r was shown to be highly specific for reference strains of *Rh. leguminosarum* and *Rh. tropici*, whereas the primer pair trop1f/rhizo3r was shown to be specific for reference strains of *Rh. tropici* (Figs 1 and 2, respectively). Neither primer pair amplified rDNA spacer fragments of the closely related species *A. rhizogenes*. The PCR products, varying in size between

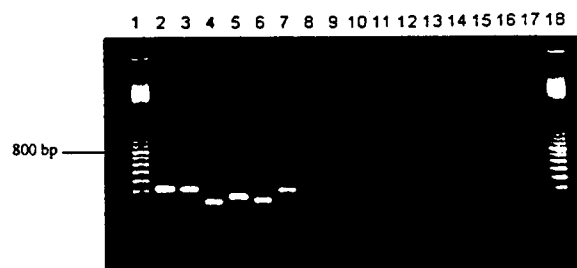


Fig. 1 PCR fragments amplified from pure cultures of reference strains using primer pair rhizo2f/rhizo3r. Lanes 1 and 18: 100 bp marker (Pharmacia); lanes 2 and 3: *Rhizobium leguminosarum* bv. *trifolii* CCT 4179 and CCT 4488; lane 4: *Rh. leguminosarum* bv. *phaseoli* CCT 4168^T; lane 5: *Rh. leguminosarum* bv. *viceae* CCT 5087^T; lanes 6 and 7: *Rh. tropici* CCT 4160^T and CCT 4164; lanes 8 and 9: *Agrobacterium rhizogenes* CCT 4832^T and CCT 4842; lanes 10 and 11: *A. tumefaciens* CCT 5075 and CCT 5265; lanes 12 and 13: *A. vitis* CCT 6069 and CCT 6072^T; lanes 14 and 15: *Rh. meliloti* CCT 4167^T and CCT 4169; lanes 16 and 17: *Rh. loti* CCT 4063^T and CCT 4159

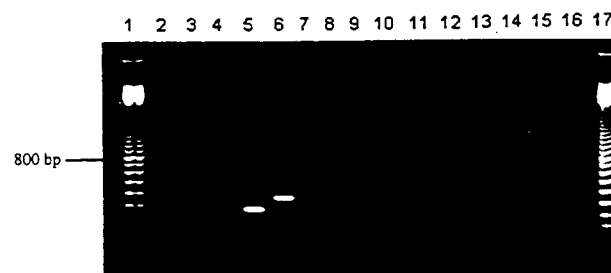


Fig. 2 PCR fragments amplified from pure cultures of reference strains using primer pair trop1f/rhizo3r. Lanes 1 and 17: 100 bp marker (Pharmacia); lane 2: *Rhizobium leguminosarum* bv. *trifolii* CCT 4179; lane 3: *Rh. leguminosarum* bv. *phaseoli* CCT 4168^T; lane 4: *Rh. leguminosarum* bv. *viceae* CCT 5087^T; lane 5 and 6: *Rh. tropici* CCT 4160^T and CCT 4164; lanes 7 and 8: *Agrobacterium rhizogenes* CCT 4832^T and CCT 4842; lanes 9 and 10: *A. tumefaciens* CCT 5075 and CCT 5265; lanes 11 and 12: *A. vitis* CCT 6069 and CCT 6072^T; lanes 13 and 14: *Rh. meliloti* CCT 4167^T and CCT 4169; lanes 15 and 16: *Rh. loti* CCT 4063^T and CCT 4159

200 and 400 bp, showed intraspecific length polymorphism for *Rh. leguminosarum* strains CCT 4179, CCT 5087^T and CCT 4168^T (Fig. 1, lanes 2, 4 and 5) and *Rh. tropici* strains CCT 4160^T and CCT 4164 (Fig. 1, lanes 6 and 7; Fig. 2, lanes 5 and 6). However, *Rh. leguminosarum* bv. *trifolii* CCT 4179 and CCT 4488 (Fig. 1, lanes 2 and 3), which belong to the same biovar, did not present length polymorphism.

Denaturing gradient gel electrophoresis (DGGE) was applied to analyse the sequence polymorphism of the 16S–23S rDNA spacer fragments, and to evaluate the ability of this technique to differentiate *Rhizobium* spp. at the strain

level. The pattern obtained (Fig. 3) confirmed the high resolution power of DGGE gels in separating DNA fragments of similar sizes but different sequence composition. The 16S–23S spacer fragments amplified from *Rh. leguminosarum* biovar *trifolii*, strains CCT 4179 and CCT 4488, which yielded same-sized PCR fragments, were clearly differentiated by DGGE (Fig. 3, lanes 2 and 3). Also, the PCR products of strains CCT 4168^T and CCT 5087^T, biovar *phaseoli* and *viceae*, respectively, which were barely differentiated in agarose gels, showed distinct melting behaviours in DGGE, resulting in a large migration distance between them (Fig. 3, lanes 4 and 5).

The DGGE technique has been largely used in studies of the diversity and dynamics of complex natural microbial communities (Muyzer *et al.* 1995; Ferris *et al.* 1996; Brinkhoff and Muyzer 1997) for the analysis of 16S rRNA gene fragments, which are separated on the basis of differences in



Fig. 3 DGGE pattern of the specific PCR fragments generated with the primer pair rhizo2f and rhizo3r/GC-clamp from DNA of reference strain pure cultures. Lanes 1 and 8: DGGE marker (PCR fragments amplified using the primer pair U968/GC-clamp and L1401 from DNA of *Enterobacter cloacae* BE1; *Listeria innocua* ALM 105; *Rhizobium leguminosarum* bv. *trifolii* R62; *Arthrobacter* sp. and *Pseudomonas cepacia* P2, from top); lanes 2 and 3: *Rh. leguminosarum* bv. *trifolii* CCT 4179 and CCT 4488, respectively; lane 4: *Rh. leguminosarum* bv. *phaseoli* CCT 4168^T; lane 5: *Rh. leguminosarum* bv. *viceae* CCT 5087^T; lanes 6 and 7: *Rh. tropici* CCT 4160^T and CCT 4164, respectively

sequence composition. However, given the limited fragment size that can be resolved by DGGE (Myers *et al.* 1985), the variability of 16S rDNA sequences may not be sufficient to discriminate phylogenetically closely related organisms at lower rank taxa.

In previous studies, it was demonstrated that DGGE analysis of 16S rDNA fragments (200 bp fragments amplified using bacterial universal primers) was not sufficient to discriminate among several *Rhizobium* species (Vallaeys *et al.* 1997). In contrast, 16S–23S rDNA fragments were shown to be highly variable in size and sequence (Laguerre *et al.* 1996), and thus could be of potential value for discriminating between phylogenetically related *Rhizobium* strains.

Data obtained from the current study corroborate the resolution power of 16S–23S rDNA sequences and DGGE in distinguishing phylogenetically closely related organisms, such as in the case of *Rh. leguminosarum*/*Rh. tropici* and *A. rhizogenes*, in contrast to the analysis of 16S rDNA alone. The results demonstrate that the rhizobial-specific primers designed in the present study, and DGGE, can also be used to differentiate rhizobia at the intraspecific level. The use of methods for extracting DNA directly from soil microbial communities, for specific 16S–23S rDNA spacer PCR amplification, and for DGGE, may provide a rapid, accurate and sensitive approach to characterizing and monitoring the diversity of *Rh. tropici* and *Rh. leguminosarum* in the environment.

ACKNOWLEDGEMENTS

V.M.O. was supported by an SPE grant from Conselho Nacional de Desenvolvimento Científico e Tecnológico (RHAE/CNPq, Brazil) and a doctoral fellowship from Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP, Brazil). The authors are grateful to Ana Paula Macêdo Zibordi for helping with photographs.

REFERENCES

- Anyango, B., Wilson, K.J., Beynon, J.L. and Giller, K.E. (1995) Diversity of rhizobia nodulating *Phaseolus vulgaris* L. in two Kenyan soils with contrasting pHs. *Applied and Environmental Microbiology* 61, 4016–4021.
- Brinkhoff, T. and Muyzer, G. (1997) Increased species diversity and extended habitat range of sulfur-oxidizing *Thiomicrospira* spp. *Applied and Environmental Microbiology* 63, 3789–3796.
- Ferris, M.J., Muyzer, G. and Ward, D.M. (1996) Denaturing gradient gel electrophoresis profiles of 16S rRNA-defined populations inhabiting a hot spring microbial mat community. *Applied and Environmental Microbiology* 62, 340–346.
- Gürtler, V. and Stanisich, V.A. (1996) New approaches to typing and identification of bacteria using the 16S–23S rDNA spacer region. *Microbiology* 142, 3–16.
- Handley, B.A., Hedges, A.J. and Beringer, J.E. (1998) The import-

- ance of host plants for detecting the population diversity of *Rhizobium leguminosarum* biovar *viciae* in soil. *Soil Biology and Biochemistry* 30, 241–249.
- Heuer, H., Krsek, M., Baker, P., Smalla, K. and Wellington, E.M.H. (1997) Analysis of actinomycete communities by specific amplification of genes encoding 16S rRNA and gel-electrophoretic separation in denaturing gradients. *Applied and Environmental Microbiology* 63, 3233–3241.
- Hirsh, A.M. (1992) Developmental biology of legume nodulation. *New Phytologist* 122, 211–237.
- Honeycutt, R.J., Sobral, B.W.S. and McClelland, M. (1995) rRNA intergenic spacers reveal polymorphisms diagnostic for *Xanthomonas albilineans*. *Microbiology* 141, 3229–3239.
- Laguerre, G., Geniaux, E., Mazurier, S.I., Rodriguez-Casartelli, R. and Amarger, N. (1993a) Conformity and diversity among field isolates of *Rhizobium leguminosarum* bv. *viciae*, bv. *trifolii*, and bv. *phaseoli* revealed by DNA hybridization using chromosome and plasmid probes. *Canadian Journal of Microbiology* 39, 412–419.
- Laguerre, G., Bardin, M. and Amarger, N. (1993b) Isolation from soil of symbiotic and nonsymbiotic *Rhizobium leguminosarum* by DNA hybridization. *Canadian Journal of Microbiology* 39, 1142–1149.
- Laguerre, G., Mavingui, P., Allard, M.-R. *et al.* (1996) Typing of rhizobia by PCR DNA fingerprinting and PCR-Restriction Fragment Length Polymorphism analysis of chromosomal and symbiotic gene regions: application to *Rhizobium leguminosarum* and its different biovars. *Applied and Environmental Microbiology* 62, 2029–2036.
- Martinez-Romero, E. (1994) Recent developments in *Rhizobium* taxonomy. *Plant and Soil* 161, 11–20.
- Massol-Deya, A.A., Odelson, D.A., Hickey, R.F. and Tiedje, J.M. (1995) Bacterial community fingerprinting of amplified 16S and 16S–23S ribosomal DNA gene sequences and restriction endonuclease analysis (ARDRA). In *Molecular Microbial Ecology Manual* ed. Akkermans, A.D.L., van Elsas, J.D. and de Bruijn, F.J. Dordrecht: Kluwer Academic Publishers.
- Muyzer, G., De Waal, E.C. and Uitterlinden, A.G. (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Applied and Environmental Microbiology* 59, 695–700.
- Muyzer, G., Teske, A., Wirsén, C.O. and Jannasch, H.W. (1995) Phylogenetic relationships of *Thiomicrospira* species and their identification in deep-sea hydrothermal vent samples by denaturing gradient gel electrophoresis of 16S rDNA fragments. *Archives of Microbiology* 164, 165–172.
- Myers, R.M., Fischer, S.G., Lerman, L.S. and Maniatis, T. (1985) Nearly all single base substitutions in DNA fragments joined to a GC-clamp can be detected by denaturing gradient gel electrophoresis. *Nucleic Acids Research* 13, 3131–3145.
- Nour, S.M., Cleyet-Marel, J.-C., Normand, P. and Fernandez, M.P. (1995) Genomic heterogeneity of strains nodulating chickpeas (*Cicer arietinum* L.) and description of *Rhizobium mediterraneum* sp. nov. *International Journal of Systematic Bacteriology* 45, 640–648.
- Ogram, A., Sayler, G.S. and Barkay, T. (1988) DNA extraction and purification from sediments. *Journal of Microbiological Methods* 7, 57–66.
- Olsen, G.J., Overbeek, R., Larsen, N. *et al.* (1992) The ribosomal database project. *Nucleic Acids Research* 20 (suppl.), 2100–2200.
- Pitcher, D.G., Saunders, N.A. and Owen, R.J. (1989) Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. *Letters in Applied Microbiology* 8, 151–156.
- Pooyan, S., George, M.L.C. and Borthakur, D. (1994) Characterization of a *Rhizobium etli* chromosomal gene required for nodule development on *Phaseolus vulgaris* L. *World Journal of Microbiology and Biotechnology* 10, 583–589.
- Smalla, K., Cresswell, N., Mendonça-Hagler, L.C., Wolters, A.C. and van Elsas, J.D. (1993) Rapid DNA extraction protocol from soil for polymerase chain reaction-mediated amplification. *Journal of Applied Bacteriology* 74, 78–85.
- Steffan, R.J., Goksøyr, J., Bej, A.K. and Atlas, R.M. (1988) Recovery of DNA from soils and sediments. *Applied and Environmental Microbiology* 54, 2908–2915.
- Streit, W., Bjourson, A.J., Cooper, J.E. and Werner, D. (1993) Application of subtraction hybridization for the development of a *Rhizobium leguminosarum* biovar *phaseoli* and *Rhizobium tropici* group-specific DNA probe. *FEMS Microbiology and Ecology* 13, 59–68.
- Tas, É., Kaijalainen, S., Saano, A. and Lindström, K. (1994) Isolation of a *Rhizobium galegae* strain-specific DNA probe. *Microbial Releases* 2, 231–237.
- Teske, A., Sigalevich, P., Cohen, Y. and Muyzer, G. (1996) Molecular identification of bacteria from a coculture by denaturing gradient gel electrophoresis of 16S ribosomal DNA fragments as a tool for isolation in pure cultures. *Applied and Environmental Microbiology* 62, 4210–4215.
- Vallaëys, T., Topp, E., Muyzer, G. *et al.* (1997) Evaluation of denaturing gradient gel electrophoresis in the detection of 16S rDNA sequence variation in rhizobia and methanotrophs. *FEMS Microbiology and Ecology* 24, 279–285.
- Woese, C.R. (1987) Bacterial evolution. *Microbiological Reviews* 51, 221–271.

Genetic diversity of *Rhizobium tropici* and *R. leguminosarum* in soil samples under different agricultural practices analyzed by DGGE of 16S-23S rDNA spacer sequences

V.M. de Oliveira¹; G.P. Manfio¹; H.L.C. Coutinho²; A.C. Keijzer-Wolters³ and J.D. van Elsas³

¹Coleção de Culturas Tropical, Fundação André Tosello, Rua Latino Coelho, 1301, CEP 13087-010, Campinas, SP, Brazil; ²Embrapa Solos, Rua Jardim Botânico, 1024, CEP 22460-000, Rio de Janeiro, RJ, Brazil; ³Research Institute for Plant Protection (IPO-DLO), P.O. Box 9060; 6700 GW, Wageningen, The Netherlands.

Running title: Diversity of soil rhizobia by DGGE analysis of rDNA

Corresponding author: Valéria Maia de Oliveira, Fundação André Tosello, Rua Latino Coelho, 1301, CEP: 13087-010, Campinas, SP, Brazil. Phone: +55 (19) 242-7022, Fax: +55 (19) 242-7827, e-mail: maia@bdt.org.br

ABSTRACT

A direct and rapid molecular approach was developed to evaluate the diversity of rhizobia in soil cultivated with common bean (*Phaseolus vulgaris*) under different agricultural practices, namely conventional and no-tillage of soils, with and without application of the insecticide Furadan (Carbofuran). A nested PCR approach was employed using primer sets flanking conserved regions of bacterial 16S-23S rDNA spacer sequences (pHr and p23Suni322anti) and specific primers targeting *Rhizobium leguminosarum* and/or *R. tropici* (27) to amplify soil community rDNA. Rhizobia-specific rDNA fragments were separated using denaturing gradient gel electrophoresis (DGGE). The resulting banding patterns enabled the direct analysis of relative richness (number of bands) and abundance (intensity of bands) of *R. leguminosarum* and *R. tropici* strains in the soils under different agricultural treatments. Data derived from using the *R. tropici*/*R. leguminosarum* primer set allowed us to conclude that the different agricultural practices influenced the occurrence of particular strains in the soil, without significantly affecting the overall diversity. Control samples from pasture soil exhibited a lower number of dominating rhizobial types compared to samples from cultivated soils, suggesting a positive selection of rhizobial strains in the latter. DGGE patterns obtained using the *R. tropici* specific primer set indicated that these organisms were favored under no-tillage practices. Sequencing of DGGE fragments revealed the presence of potentially novel organisms in some soil samples, which were distantly related to the *R. tropici* and *R. leguminosarum* reference strains used in the comparisons.

INTRODUCTION

Rhizobia are widespread soil bacteria capable of establishing symbiotic relationships with plants from the family Leguminosae. The association between plant and bacterium usually results in the development of nodules in the roots or stems of the legumes, where the rhizobia reduce atmospheric nitrogen (N_2) to ammonium (NH_4^+), in a process called biological nitrogen fixation (BNF) (17). BNF is performed by a variety of free-living and symbiotic microorganisms, but rhizobia comprise the most intensively studied group to date, and contribute significantly to BNF in agriculture (21).

BNF may be of significant value to the economics of commercially important legume crops, considering the reduction in fertilizer application. In addition, sustainable agricultural systems may use leguminous plants in consortia with annual crops, supplying fodder, fuelwood or shade. They may also serve as wind barriers or live fences, and, in crop rotation systems, legumes help the fertilization of soils when used as green manure (4). In the first case, the benefits rendered by BNF usually depend on the efficiency of the rhizobial strains used as inoculants, whereas in sustainable agriculture systems the success of BNF is mostly dependent on naturally occurring rhizobial populations. Due to the highly specific nature of the *Rhizobium*-host plant interaction, loss of rhizobial diversity in the soil may adversely affect nitrogen fixation for a range of legume plants.

The response of bacterial communities to environmental perturbation, such as soil management practices, can be assessed by analyzing the structure of these communities, as well as the activity and function of individual organisms. Traditional microbiological techniques based on isolation and cultivation are usually not adequate to address these questions, since most bacteria in the environment are not recovered by conventional, cultivation-based, methods (1, 19, 31).

To overcome the limitations of culture-dependent approaches in assessing bacterial diversity, molecular techniques have become increasingly popular (2, 13, 29, 39). Analysis of ribosomal RNA (rRNA) and/or rDNA has been widely used to determine the species composition of bacterial communities. Current methods encompass a range of different approaches based mainly on direct extraction of genomic DNA or rRNA from mixed microbial populations (6, 26, 37, 41), followed by screening of clone libraries using molecular probes (2) and/or sequence analysis of PCR-amplified 16S rDNA or reverse-transcribed 16S rRNA (9, 10, 28, 38, 42). However, cloning and sequencing, as well as

hybridization with a range of probes, are laborious and time-consuming for routine analyses (2, 19).

The denaturing gradient gel electrophoresis (DGGE) separation of 16S rDNA fragments offers a rapid and comprehensive means for the simultaneous study of different bacterial populations (23). In the DGGE technique, fragments of PCR-amplified rRNA genes or other functional genes, similar in length, but different in sequence composition, are separated into discrete bands during electrophoresis in polyacrylamide gels containing gradients of denaturants (urea and formamide) (12). A GC-rich sequence (GC clamp) is attached to one of the primers used in the PCR to avoid complete strand dissociation during electrophoresis. DGGE was able to detect almost 100% of all single base substitutions in a 500 bp DNA fragment (36), and may thus provide very informative fingerprints of complex microbial populations based on the analysis of relatively conserved genes (23, 25). In addition, bands can be excised from the gel and sequenced for identification of individual components and phylogenetic analysis (24).

In the current study, we evaluated the potential of DGGE to rapidly assess the relative diversity of *R. leguminosarum* and *R. tropici* in soil samples. The use of microbial community DNA extracted from soil, followed by PCR amplification of 16S-23S rDNA spacer regions using rhizobial specific primer sets (27) and DGGE, allowed us to estimate the influence of different agricultural practices on the diversity of rhizobial populations in the soil.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used and sources are listed in Table 1. *Rhizobium* strains were grown in YM broth (0.5 g KH_2PO_4 , 0.2 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 g NaCl, 0.5 g yeast extract, 10 g mannitol and 0.025 g bromothymol blue; per 1 L distilled water) for 48 h at 28°C under constant shaking. Long-term storage was at -80°C in 50% (vol/vol) glycerol and by lyophilization.

TABLE 1. Bacterial strains used in this study, source, accession codes in other collections, and their host plants.

Strain code ^a	Source/accession number in other collections ^b	Host plant
<i>Rhizobium leguminosarum</i>		
CCT 6305	←LMG 4255	<i>Trifolium pratense</i>
CCT 6306	←LMG 4256	<i>Trifolium repens</i>
CCT 6308	←LMG 4259	<i>Vicia sativa</i>
CCT 6309	←LMG 4260	<i>Vigna unguiculata</i>
CCT 6310	←LMG 4267 (t1) ^c	<i>Trifolium procumbens</i>
CCT 6311	←LMG 4267 (t2) ^c	<i>Trifolium procumbens</i>
CCT 6314	←LMG 4292	<i>Phaseolus vulgaris</i>
CCT 6315	←LMG 4293	<i>Phaseolus vulgaris</i>
CCT 6317	←LMG 6120	<i>Trifolium subterraneum</i>
CCT 6318	←LMG 6121	<i>Trifolium repens</i>
CCT 6319	←LMG 6122	<i>Trifolium repens</i>
CCT 6320	←LMG 6293	<i>Lens</i> sp.
CCT 6321	←LMG 6294 (t1)	<i>Lathyrus</i> sp.
CCT 6322	←LMG 6294 (t2)	<i>Lathyrus</i> sp.
CCT 6323	←LMG 6295	<i>Lathyrus</i> sp.
CCT 6324	←LMG 6296	<i>Lathyrus</i> sp.
CCT 6336	←LMG 4257	Unknown
<i>R. leguminosarum</i> bv. <i>trifolii</i>		
CCT 4179	←BR 7606 (=ATCC 14480)	<i>Trifolium pratense</i>
CCT 4488	←BR 7605 (=LMG 6119)	<i>Trifolium repens</i>
<i>R. leguminosarum</i> bv. <i>viceae</i>		
CCT 5087 ^T	←IAM 12609 ^T (=ATCC 10004 ^T)	Unknown
<i>R. leguminosarum</i> bv. <i>phaseoli</i>		
CCT 4168 ^T	←BR 10052 ^T (=ATCC 14482 ^T)	<i>Phaseolus</i> sp.
CCT 4180	←BR 10051 (=LMG 4285)	<i>Phaseolus vulgaris</i>
<i>Rhizobium tropici</i>		
CCT 4160 ^T	←BR 322 ^T (=LMG 9503 ^T)	<i>Phaseolus vulgaris</i>
CCT 4164	←BR 10016 (=LMG 9517)	<i>Phaseolus vulgaris</i>
<i>Soil isolates</i>		
F62	←H.L.C. Coutinho (RJ, Brazil)	<i>Phaseolus vulgaris</i>

CCT 6222	←G47 (5)	<i>Cajanus cajan</i>
CCT 6224	←G49 (5)	<i>Cajanus cajan</i>
CCT 6239	←G72 (5)	<i>Cajanus cajan</i>

^aCCT, Tropical Culture Collection, Fundação André Tosello (Campinas, SP, Brazil).

^bBR, Embrapa Agrobiologia Culture Collection (Seropédica, RJ, Brazil); ATCC, American Type Culture Collection (Manassas, USA); IAM, Institute of Applied Microbiology (Tokyo, Japan); LMG, Laboratorium voor Microbiologie (Univ. Gent, Belgium).

^c(t1) and (t2) are two stable colony types showing minor differences in SDS-PAGE protein profiles.

Field experiment and soil samples. The soil samples were collected from a field experiment conducted by Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA) in Guaíra, located in the North of the State of São Paulo, Brazil. The soil was typical of Brazilian Cerrado (acid oxisol), with an average pH 5.0 (CaCl₂) and low organic matter content (2.8%) in the top layer (0-20 cm). The experimental area comprised four plots, selected at random, where different agricultural treatments were set up: no-tillage treatment (NT), where the remains from the previous soybean crop were treated with 2.0 L/ha Glyphosate Nortox and 1.5 L/ha of 2,4-Dichlorophenoxyacetate-amine 20 days prior to sowing the common bean crop, and the top soil was not ploughed nor disc-harrowed; no-tillage with application of the soil insecticide Furadan® (active ingredient: Carbofuran; 2,3-dihydro-2,2-dimethyl-7-benzofuranylmethylcarbamate; C₁₂H₁₅NO₃; FMC Corporation, Philadelphia, USA) during sowing (NTF); conventional tillage (CT), where the remains from the previous crop were incorporated by ploughing and disc-harrowing the top soil; and conventional tillage plus Furadan (CTF). Common bean (*Phaseolus vulgaris*) was sowed in June 1996, over rows supplemented with 300 kg/ha of combined fertilizer (N-P-K: 2-20-20).

Each plot was divided in four subplots (9 x 18 m) for sampling purposes. Ten individual samples from each subplot were collected at random from the top 20 cm layer of soil, along the rows of beans, 30 days after sowing. The individual samples from each subplot were then pooled and transported to the laboratory in polystyrene boxes containing ice, and stored at -20°C for subsequent DNA extraction. Additional composite samples were collected to be used as control samples: one from the experimental area before sowing of beans, named Time Zero, and the other from a non-cultivated pasture area adjacent to the experimental plots, named Pasture.

Extraction of DNA from pure cultures and soil. Total genomic DNA was isolated from pure cultures of reference strains according to Pitcher *et al.* (32). Soil samples from each of the four subplots comprising a treatment were pooled prior to DNA extraction. Direct

extraction of soil DNA was based on a modification of a previously described protocol (37), using 2 g of soil and two final purification steps with the Wizard DNA Clean-Up System (Promega, Madison, WI, USA) instead of glassmilk (41). Integrity and concentration of purified soil DNA were assessed by electrophoresis on 0.8% agarose gels.

PCR amplification of 16S-23S rDNA spacer fragments. A nested-PCR approach (15) was used with soil DNA samples in order to amplify *R. leguminosarum* and/or *R. tropici* rDNA spacer sequences. Two sets of oligonucleotide primers were used in the nested-PCR approach (Table 2). In the first PCR reaction, 16S-23S rDNA spacer sequences of the whole bacterial community were amplified by using a set of primers targeting conserved rDNA regions [primers pHr (22) and p23Suni322anti (18)]. In a second PCR, the primer sets rhizo2f/rhizo3r and trop1f/rhizo3r (27) were employed in separate reactions, using aliquots of the first PCR reaction as template, in order to amplify *R. leguminosarum*/*R. tropici* and *R. tropici* specific rDNA spacer fragments, respectively.

The first PCR was performed in 50 μ L reactions containing: 5 μ L of a 1:10 dilution of the soil DNA sample (10-20 ng/ μ L), 5 μ L 10X PCR buffer (supplied with the enzyme), 0.2 mM dNTP mix, 3.75 mM MgCl₂, 0.4 μ M each primer, 1% (vol/vol) formamide, 0.20 μ g T4 gene 32 protein (Amersham Pharmacia Biotech, Roosendaal, The Netherlands) and 5 U *Taq* DNA polymerase Stoffel fragment (Perkin-Elmer, Nieuwerkerk a/d IJssel, The Netherlands). T4 gene 32 protein was added to improve the efficiency of target amplification in soil DNA (41). The reaction mixtures were overlaid with 2 drops of mineral oil (Sigma, Zwijndrecht, The Netherlands) and PCR amplifications were carried out using an initial denaturation step at 95°C for 2 min, followed by 30 cycles of 1 min at 94°C, 1 min at 60°C and 3 min at 72°C; and a final extension at 72°C for 5 min, in Perkin Elmer DNA Thermal Cycler (Nieuwerkerk a/d IJssel, The Netherlands).

In the second PCR, 1 μ L of the first PCR product was used as target in a 50 μ L reaction volume containing: 5 μ L 10X PCR buffer, 0.2 mM dNTP mix, 3.75 mM MgCl₂, 0.2 μ M of each primer and 5 U *Taq* DNA polymerase (Stoffel fragment). In order to enhance the specificity, touchdown PCR (24) was performed. An initial denaturation step at 95°C for 2 min and a final extension step at 72°C for 10 min were performed for all samples. For the primer set rhizo2f/rhizo3r, after denaturation at 94°C for 1 min, the annealing temperature was initially set at 62°C for 30 s, and then decreased to 60°C by 1°C every 3 cycles, followed by 26 additional cycles at 58°C; primer extension was performed at 72°C for 45 sec. For the primer set trop1f/rhizo3r, after denaturation at 94°C for 1 min, the annealing temperature was initially set at 55°C for 1 min and then decreased to 51°C by 2°C every 3 cycles; then 26 additional cycles were carried out at 50°C; primer extension was performed at 72°C for 1 min.

TABLE 2. Sequence, target and reference of the primers and probes used in this study.

Primer/probe	Sequence ^a (5'→3')	Target group	Reference
pHr	TGC GGC TGG ATC ACC TCC TT	Bacteria	22
p23S	GGT TCT TTT CAC CTT TCC CTC	Bacteria	18
uni322anti			
U968	AAC GCG AAG AAC CTT AC	Bacteria	16
L1401	CGG TGT GTA CAA GGC CCG GGA ACG	Bacteria	16
rhizo2f	GAT GGC ACC AGT CAG GTG AC	<i>R. leguminosarum</i> and <i>R. tropici</i>	27
trop1f	CGG ACR TGS CCC GAT AT	<i>Rhizobium tropici</i>	27
rhizo3r	GGA AGA CTT GAA YTT CCG A	<i>R. leguminosarum</i> and <i>R. tropici</i>	27
GC clamp ^b	CGCCCGGGGCGCGCCCGGGGCGGGGCGGGGG CACGGGGGG	-	23
Probe A	ATG CTT AAA GCA TTC CGT GG	Clone A	This work
Probe 2	CAA ACC GGC CCA GAT CGC TCT	Clone 2	This work
Probe 34B	AAC GGT CCA GCA TCA CCT GA	Clones 3,4 and B	This work

^aY = T or C; R = A or G; S = C or G.

^bThe GC clamp was attached to the 5' end of the primer rhizo3r.

Specific amplification of 16S-23S rDNA spacer fragments from *R. leguminosarum* and *R. tropici* reference strains were carried out by using 50 ng of genomic DNA and the same conditions described for the second PCR.

Amplification products were checked by electrophoresis in 1.4% agarose gels in 0.5X TBE buffer (35). PCR products were stored at -20°C for subsequent DGGE analyses.

DGGE analysis. DGGE was carried out using the PhorU2 System (Ingeny, Goes, The Netherlands). Gels were run at 100 V and 60°C for 16 h in 0.5X TAE buffer (20 mM Tris-acetate, pH 7.4, 10 mM sodium acetate, 0.5 mM Na₂ EDTA). PCR products (20 µL) were applied directly onto 6% (wt/vol) polyacrylamide gels (acrylamide/N,N'-methylenebisacrylamide, 37.5:1, w/w) containing a linear denaturing gradient of urea and formamide ranging from 45% to 65%. The 100% denaturant corresponded to 7 M urea and 40% (vol/vol) deionized formamide (deionized with AG501-X8 mixed-bed resin, Bio-Rad, Veenendaal, The Netherlands). A DGGE marker, comprised by a mixture of amplification products of 16S rDNA genes from *Enterobacter cloacae* BE1; *Listeria innocua* ALM105;

R. leguminosarum bv. *trifolii* R62; *Arthrobacter* sp. Ar1 and *Burkholderia cepacia* P2 (listed in order of separation of fragments in DGGE gels, from the top to the bottom), generated by using the primer set U968-GC clamp and L1401 (Table 2), was used as a reference in the gels (27). After electrophoresis, the gels were stained in the dark for 1 h with SYBR Gold Nucleic Acid Gel Stain (Molecular Probes Europe, Leiden, The Netherlands), diluted 1:10,000 in 0.5X TAE buffer. Photographs were taken under UV light using a Docugel V system apparatus (Biozym, Landgraaf, The Netherlands).

DGGE patterns generated from soil samples under different agricultural treatments were analyzed by using the GelCompar software package v.4.1 (Applied Maths, Kortrijk, Belgium). Cluster analysis was performed using the Pearson (product-moment) correlation coefficient (30) and dendrograms constructed by using the UPGMA clustering algorithm.

Sequencing of DGGE bands. Small blocks of acrylamide containing DNA fragments of interest were excised from DGGE gels with a razor blade, placed in sterile microfuge tubes and crushed against the tube wall. The tubes were filled with 1 to 2 volumes of elution buffer (0.5 M ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA, pH 8.0, 0.1% SDS) and incubated for 16 h at 37°C in a rotating oven. The samples were then centrifuged at 12,000 g for 1 min (at 4°C), and the recovered supernatants were spin-filtered through glass wool (6,000 g for 5 min). DNA was precipitated with 2 volumes of cold ethanol, kept on ice for 30 min, and pelleted by centrifugation (12,000 g for 10 min, 4°C). DNA was dissolved in 200 µL TE buffer, pH 7.6, and re-precipitated by adding 25 µL 3 M sodium acetate, pH 5.2, and 2 volumes of ethanol. The pellets were washed with 70% ethanol, dried and resuspended in 15 µL TE buffer for subsequent PCR and cloning.

Purified DNA from selected bands was serially diluted (1:10, 1:100, 1:1,000 and 1:10,000) and subjected to PCR. The DNA dilution that yielded one single band equivalent to the band of interest in the DGGE gel was then used as the template in subsequent PCR reactions using primers without the GC clamp. PCR products (2 µL) were cloned into the pCR®2.1 vector (TA cloning kit; Invitrogen, Leek, The Netherlands) and sequenced using Thermo sequenase fluorescently-labelled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Nederland BV, 's Hertogenbosch, The Netherlands). Sequences were determined using an automatic sequence analyzer (ALF DNA sequencer; Pharmacia). Both strands of the amplification products were sequenced for each clone.

Phylogenetic analysis of 16S-23S rDNA spacer fragments. Sequences of 16S-23S rDNA spacer fragments were aligned with reference sequences of *Rhizobium leguminosarum*, *R. tropici* and *Agrobacterium rhizogenes* (27), and with additional sequences from outgroup organisms recovered from GenBank (accession numbers listed in Figure 13). Sequences were analyzed using the GDE software package (Genetic Data Environment, V. 2.2;

[gopher://megasun.bch.umontreal.ca:70/11/GDE](http://megasun.bch.umontreal.ca:70/11/GDE)). Distance matrices were calculated using DNADIST as implemented in PHYLIP V. 3.5 (8), and the Jukes-Cantor model (20). A phylogenetic tree was constructed using the Neighbor-Joining method (34), included in the PHYLIP package.

Blotting and hybridization analysis of the DGGE profiles. Probes A, 2 and 34B (Table 2) were designed based on the sequences obtained from the DGGE bands. The synthesized oligonucleotides were 5'-labelled with digoxigenin (Eurogentec S.A., Seraing, Belgium).

The DGGE gel was equilibrated for 30 min with 0.5X TBE buffer, and DNA was electro-transferred onto a nylon membrane (Boehringer Mannheim, Almere, The Netherlands) for 2 h at a constant amperage of 300 mA. The membrane was then denatured for 15 min in 0.4M NaOH, 0.6 M NaCl solution and washed twice for 10 min in 2.5X SSC (1X SSC is 0.15 M NaCl plus 0.015 M sodium citrate). After cross-linking (20 min at 120°C), the nylon membrane was hybridized for 18 h with either one of the specific probes, A, 2 or 34B, at 40°C, 50°C and 46°C, respectively. Probe concentration was 10 pmol/mL in the hybridization solution. After hybridization, two non-stringent washes were carried out (room temperature, 20 min, 2X SSC plus 0.1% SDS), followed by two stringent washes at the hybridization temperature (20 min, 0.5X SSC plus 0.1% SDS). Detection was performed by chemiluminescence (DIG Luminescent Detection Kit, Boehringer Mannheim, Almere, The Netherlands). Subsequent hybridizations with the remaining probes were carried out after removal of the bound probe (one wash with distilled water, followed by two incubations with 0.2 N NaOH, 0.1% SDS at 37°C for 20 min and a final wash with 2X SSC).

RESULTS

Relative diversity of rhizobia among different agricultural treatments based upon the analysis of DGGE profiles

Analysis of soil DGGE profiles generated with the primer set rhizo2f/rhizo3r-GC in the nested-PCR set up, which targeted both *Rhizobium leguminosarum* and *R. tropici*, revealed great similarity between the different agricultural treatments (Fig. 1 A). Band profiles corresponding to treatments CT, CTF, NT and NTF showed overall similarity levels ranging from $89.4 \pm 3.5\%$ to $92.7 \pm 1.3\%$ (Fig. 1 B). The profiles corresponding to Time Zero and Pasture samples showed greater differences from the remaining treatments ($83.0 \pm 4.4\%$ and $64.3 \pm 8.1\%$, respectively, Fig. 1 B). No remarkable differences in the overall strain “richness” (number of bands) between treatments were detected (Fig. 1 A),

except for the samples from pasture soils (Fig. 1 A, lanes 10 and 11), which exhibited a lower diversity of dominating rhizobial types and lacked reproducibility (see details below). Relatively greater variability was observed in relation to strain abundance (intensity of bands), as exemplified by differences of intensity of individual bands (indicated by black arrows) in the duplicate profiles of treatments NT and NTF (Fig. 1 A, lanes 14, 15, 18 and 19), and treatments CT and CTF (Fig. 1 A, lanes 16, 17, 20 and 21).

Greater differences with respect to the diversity of rhizobia among the agricultural treatments could be detected when soil DNA samples were analyzed by using the primer set *trop1f/rhizo3r*-GC, specific for *R. tropici* (Fig. 2 A). Band profiles corresponding to treatments CT and CTF showed a high level of similarity ($95.9 \pm 0.8\%$, Fig. 2 B), whereas profiles from NT and NTF showed slightly greater differences ($89.7 \pm 0.4\%$, Fig. 2 B). A very low level of similarity was detected between CT and NT treatments (Fig. 2 B). One of the replicates of NT was recovered as a distinct profile, different from all the remaining treatments (Fig. 2 A, lane 8). No-tillage treatments supported a higher number of distinct dominating rhizobial types (Fig. 2 A, lanes 8, 9, 12 and 13), compared to the conventional tillage treatments (Fig. 2 A, lanes 10, 11, 14 and 15). Profiles corresponding to Pasture and Time Zero samples were very divergent from the remaining, presenting a reduced number of bands or no bands at all (Fig. 2 A, lanes 4 to 7). The use of the insecticide Furadan did not seem to have a strong effect on the diversity of *R. tropici* strains in the environment, similarly to the results obtained with the *R. leguminosarum/R. tropici* primer set.

Reproducibility of the DGGE patterns

The reproducibility of the DGGE patterns was evaluated by running duplicate independent nested-PCR amplifications using the primer set *rhizo2f/rhizo3r*-GC (*Rhizobium leguminosarum* and *R. tropici*) and soil DNA derived from two distinct extractions of soil samples from each of the treatments (Fig. 1 A and 2 A). The overall reproducibility of DGGE profiles was high for duplicate samples from all treatments (Fig. 1 A, lanes 14 to 21), ranging between 95.4% and 95.9% (Fig. 1 B), except for Pasture and Time Zero control samples (79.9 and 85.8%, respectively, Fig. 1 B). The former exhibited greater differences between DGGE patterns derived from duplicate PCR's in comparison with the other treatments (Fig. 1 A, lanes 10 and 11). It could also be noticed that some individual bands (indicated by the white arrows in Fig. 1 A), although reproducible, showed greater intensity in one PCR amplification in comparison to its duplicate. Reproducibility results were confirmed in additional nested-PCR and DGGE experiments conducted independently (results not shown).

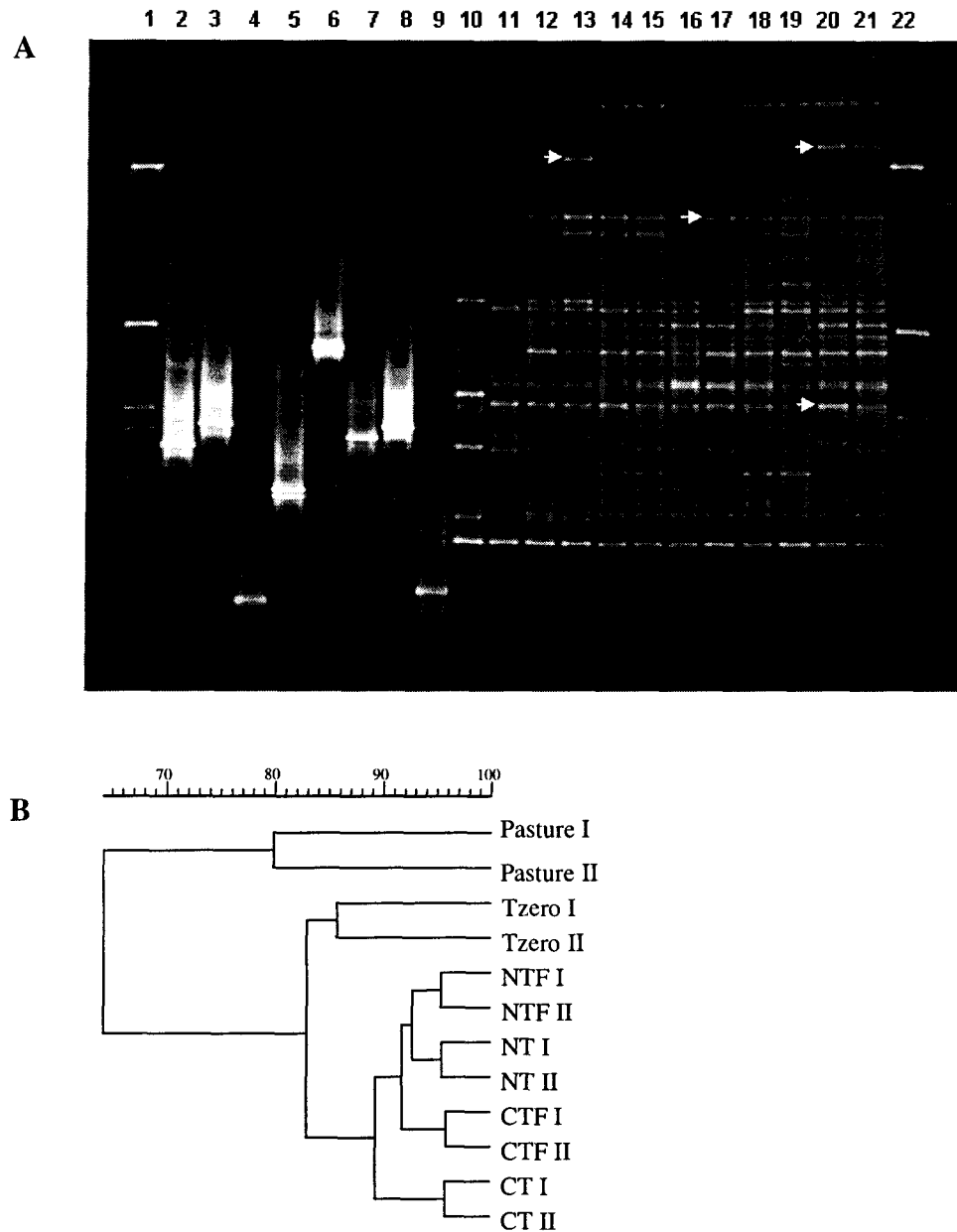


FIG. 1. (A) DGGE analysis of PCR fragments generated from different agricultural treatments and from reference strains, using the primer set rhizo2f/rhizo3r-GC clamp, targeting *R. leguminosarum*/*R. tropici*. Lanes: 1 and 22, DGGE marker; 2 to 9, PCR products from pure cultures of *Rhizobium leguminosarum* bv. *trifolii* CCT 4179 and CCT 4488; *R. leguminosarum* bv. *phaseoli* CCT 4168^T and CCT 4180; *R. leguminosarum* bv. *viceae* CCT 5087^T; *R. tropici* CCT 4160^T and CCT 4164; and of the soil isolate F62, respectively; 10 to 21, amplification products from duplicate soil DNA extractions from Pasture, Time Zero, NT, CT, NTF and CTF plots, in that order. (B) Cluster analysis of DGGE profiles derived from the agricultural treatments with primer set rhizo2f/rhizo3r-GC clamp, generated by using the *Pearson* correlation coefficient and UPGMA method.

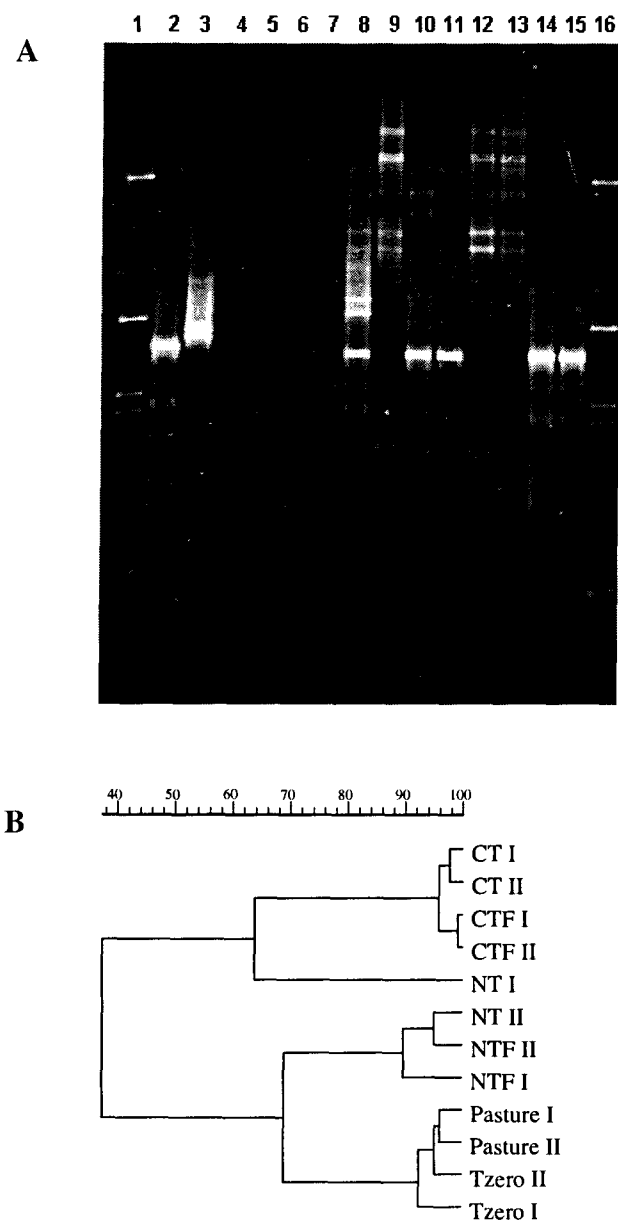


FIG. 2. (A) DGGE analysis of PCR fragments generated from different agricultural treatments and from reference strains, using the primer set trop1f/rhizo3r-GC clamp, targeting *R. tropici*. Lanes: 1 and 16, DGGE marker; 2 and 3, PCR products from pure cultures of *R. tropici* CCT 4160^T and CCT 4164, respectively; 4 to 15, amplification products from duplicate soil DNA extractions from Pasture, Time Zero, NT, CT, NTF and CTF plots, in that order. (B) Cluster analysis of DGGE profiles derived from the agricultural treatments with primer set trop1f/rhizo3r-GC clamp, generated by using the *Pearson* correlation coefficient and UPGMA method.

The lack of reproducibility between DGGE profiles generated from duplicate samples of Pasture soil DNA was observed in independent replicate experiments, and thus investigated in more detail. Two sets of experiments were performed, one using serial dilutions of the same Pasture DNA sample, as outlined in Fig. 3, and the other using DNA samples derived from two different extractions. In the first experiment, serial dilutions of the original DNA sample were used for nested PCR with the specific primer set rhizo2f/rhizo3r-GC. Two aliquots of the same specific PCR products loaded into different gel lanes yielded identical DGGE profiles (Fig. 4, lanes 1 and 2). This was also true when two aliquots of PCR products from the same amplification reaction, using universal primers, were subjected to independent specific PCR's (Fig. 4, lanes 2 and 3), or when two aliquots of the same DNA dilution (1:10) were subsequently subjected to independent nested PCR's (Fig. 4, lanes 3 and 4). On the other hand, when aliquots taken from distinct dilutions of the same Pasture soil DNA sample (1:20, 1:50 and 1:100) were subjected to independent nested PCR's, the resulting band profiles were clearly different (Fig. 4, lanes 5 to 7).

In the second experiment, nested PCR's were performed using two-fold serial dilutions of the DNA samples from separate extractions from pasture soil and the specific primer set rhizo2f/rhizo3r-GC, followed by DGGE (Fig. 5). The results indicated the expected variation between the amplification patterns derived from the two different DNA extracts at equivalent template dilutions. In addition, differences were observed between DGGE patterns derived from amplifications done using serially diluted concentrations of the same DNA sample. In the latter case, bands which appeared with strong intensity in one amplification profile disappeared in the profile from the higher dilution (Fig. 5, lane 3, arrow), whereas other bands which were not visible in one dilution were present with strong intensity in the next one (Fig. 5, lane 5, arrow).

Spatial variability and diversity of rhizobia within plots

The spatial variability of *R. leguminosarum* and *R. tropici* diversity in the environmental samples was evaluated by analyzing DNA extracted from four composite soil samples, originating from the four NT treatment subplots. The composite samples from the subplots were prepared as previously described. Soil DNA was amplified by nested PCR using the primer pair rhizo2f/rhizo3r-GC and subjected to DGGE. Additionally, DNA extracted from three other composite samples, named NT-I, NT-II and NT-III, made by pooling equal amounts of the four soil samples from the NT plot, were also analyzed.

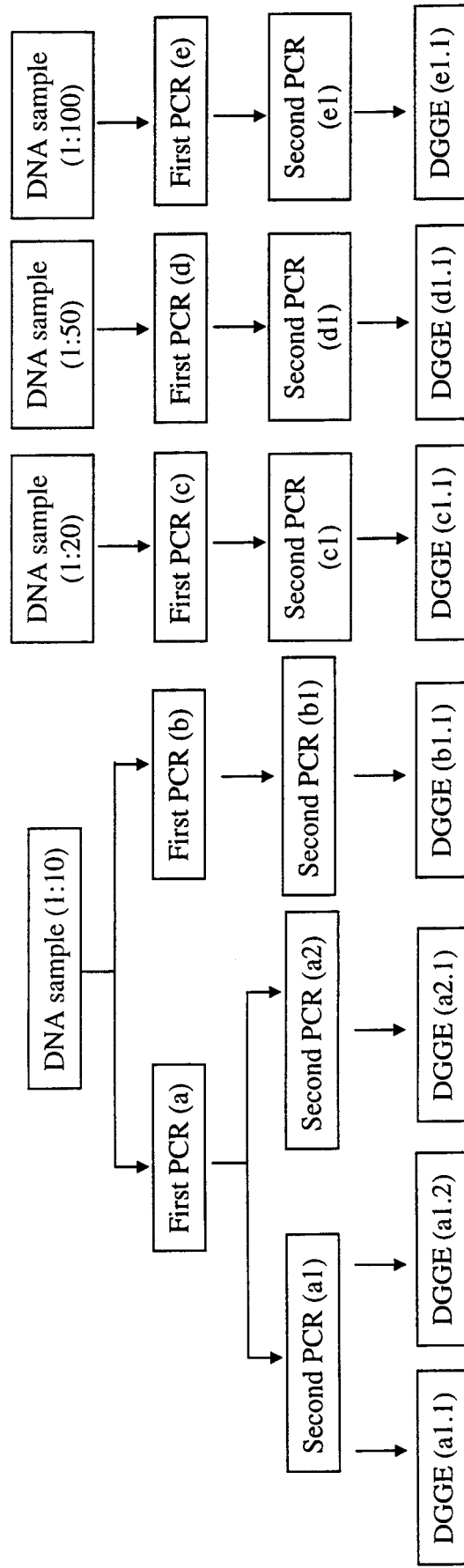


FIG. 3. Schematic representation of the reproducibility experiment carried out using dilutions of a Pasture soil DNA sample, nested-PCR's and DGGE.

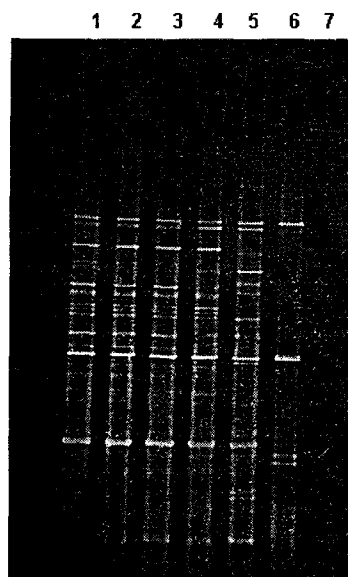


FIG. 4. DGGE analysis of PCR products from the reproducibility experiment using Pasture soil DNA and primer set rhizo2f/thizo3r-GC clamp. Lanes: 1 and 2, duplicate samples (a1.1 and a1.2; see Fig. 3) from specific PCR reaction a1; 3, band pattern (a2.1) from specific PCR reaction a2, using the same first-PCR template (a) as previous samples; 4, band pattern (b1.1) from a separate nested-PCR (b); 5 to 7, band patterns obtained from separate nested PCR's using 1:20, 1:50 and 1:100 dilutions of the same soil DNA sample (c1.1, d1.1 and e1.1, respectively).

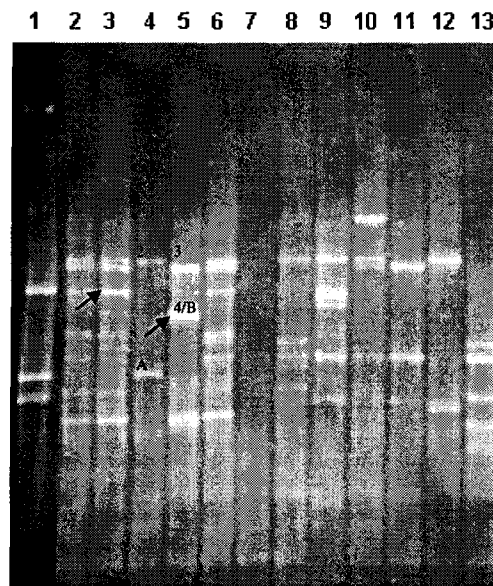


FIG. 5. DGGE analysis of PCR products from the reproducibility experiment using $\frac{1}{2}$ series dilution of DNA sample duplicates from Pasture soil and primer rhizo2f/rhizo3r-GC clamp. Lanes: 1, DGGE marker; 2 to 7, band patterns obtained from separate nested-PCR's using 1:2, 1:4, 1:8, 1:16, 1:32 and 1:64 dilutions, respectively, of soil DNA sample **A**; 8 to 13, band patterns obtained from separate nested-PCR's of dilutions 1:2, 1:4, 1:8, 1:16, 1:32 and 1:64, respectively, of the soil DNA sample **B**.

The overall diversity of rhizobia was not considerably different between the four subplots (Fig. 6 A, lanes 10 to 13), with little variation observed in strain richness (number of bands) among the samples. However, greater differences were observed in relation to strain abundance, with the presence of bands of higher intensity in the profiles from the different subplots (Fig. 6 A, lanes 10, 12 and 13, black arrows). This was also observed in the profiles derived from pooled samples (Fig. 6 A, lanes 14 and 15, black arrows). Cluster analysis of DGGE profiles revealed high levels of similarity between the four subplots, ranging from $88.1 \pm 1.5\%$ to $89.6 \pm 0.2\%$ (Fig. 6 B). PCR and DGGE experiments were repeated twice using the original extracted DNA samples and yielded similar results (data not shown).

The DGGE profiles derived from the analysis of DNA samples from the four NT subplots, generated using the primer set trop1f/rhizo3r-GC (Fig. 7 A), revealed greater differences in the abundance and richness of *R. tropici* among the subplots. In this case, the overall number of bands of *R. tropici* was lower compared to profiles obtained by using the primer set rhizo2f/rhizo3r-GC (Fig. 6 A), which targeted both *Rhizobium leguminosarum* and *R. tropici*. Some bands were more intense in particular subplots, indicating higher numbers of individual *R. tropici* strains (Fig. 7 A, lanes 5 and 7, black arrows), whereas the absence or presence of bands in the different subplots indicated variability in the composition of the *R. tropici* populations (Fig. 7 A, lanes 4 to 7, white arrows). Variation in strain abundance (Fig. 7 A, lanes 8 to 10, black arrows), as well as strain richness (Fig. 7 A, lane 10, white arrows), was also observed in the analysis of the pooled samples NT-I, NT-II and NT-III, comparable to data obtained in the previous experiments. Cluster analysis (Fig. 7 B) revealed a wider range of similarity levels between profiles derived from the different subplots ($66.9 \pm 11\%$ to $90.7 \pm 1.3\%$).

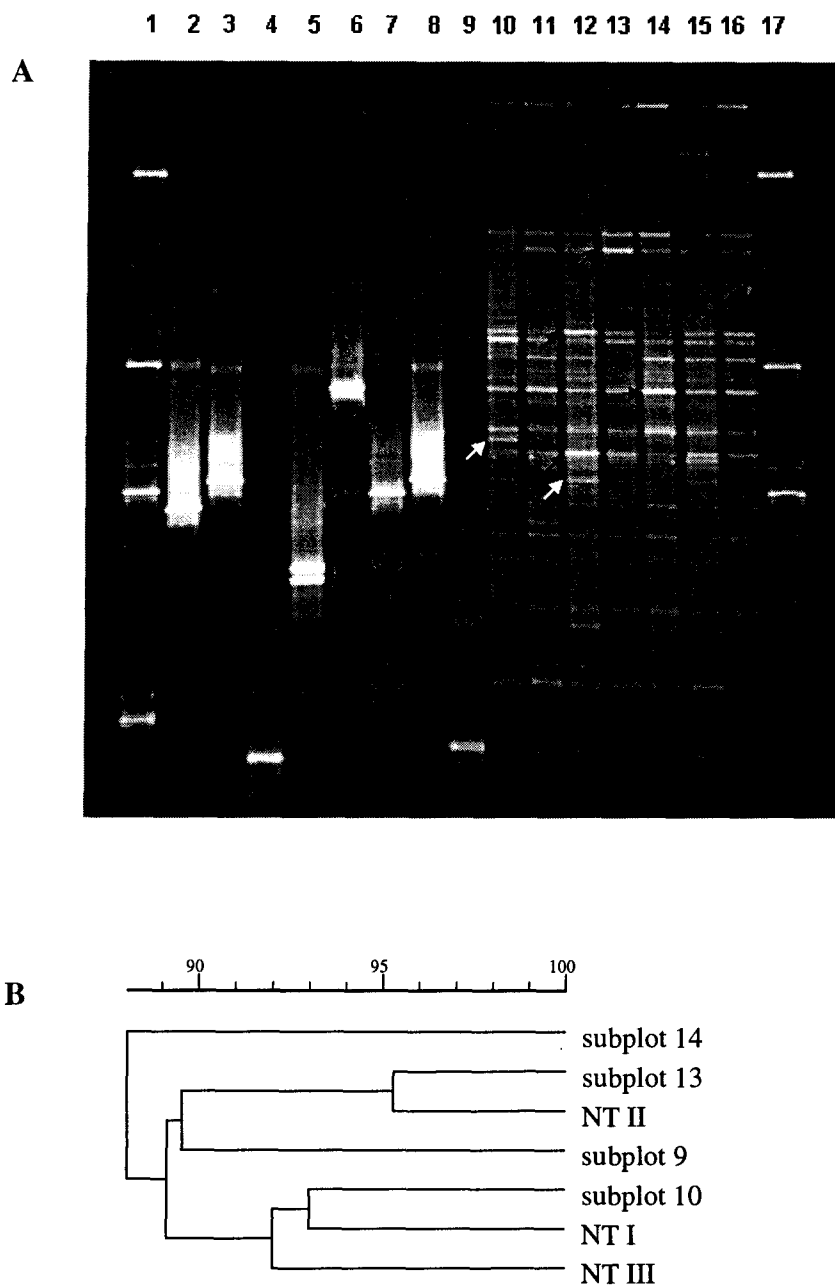


FIG. 6. (A) DGGE analysis of PCR fragments generated from No-tillage subplots and reference strains using the primer set rhizo2f/rhizo3r-GC clamp. Lanes: 1 and 17, DGGE marker; 2 to 9, PCR products from pure cultures of *Rhizobium leguminosarum* bv. *trifolii* CCT 4179 and CCT 4488; *R. leguminosarum* bv. *phaseoli* CCT 4168^T and CCT 4180; *R. leguminosarum* bv. *viceae* CCT 5087^T; *R. tropici* CCT 4160^T and CCT 4164; and the soil isolate F62, respectively; 10 to 16, amplification products from the 4 subplots of NT treatment and from pooled soil samples NT-I, NT-II and NT-III, in that order. **(B)** Cluster analysis of DGGE profiles derived from No-tillage subplots with the primer set rhizo2f/rhizo3r-GC clamp, generated by using the *Pearson* correlation coefficient and UPGMA method.

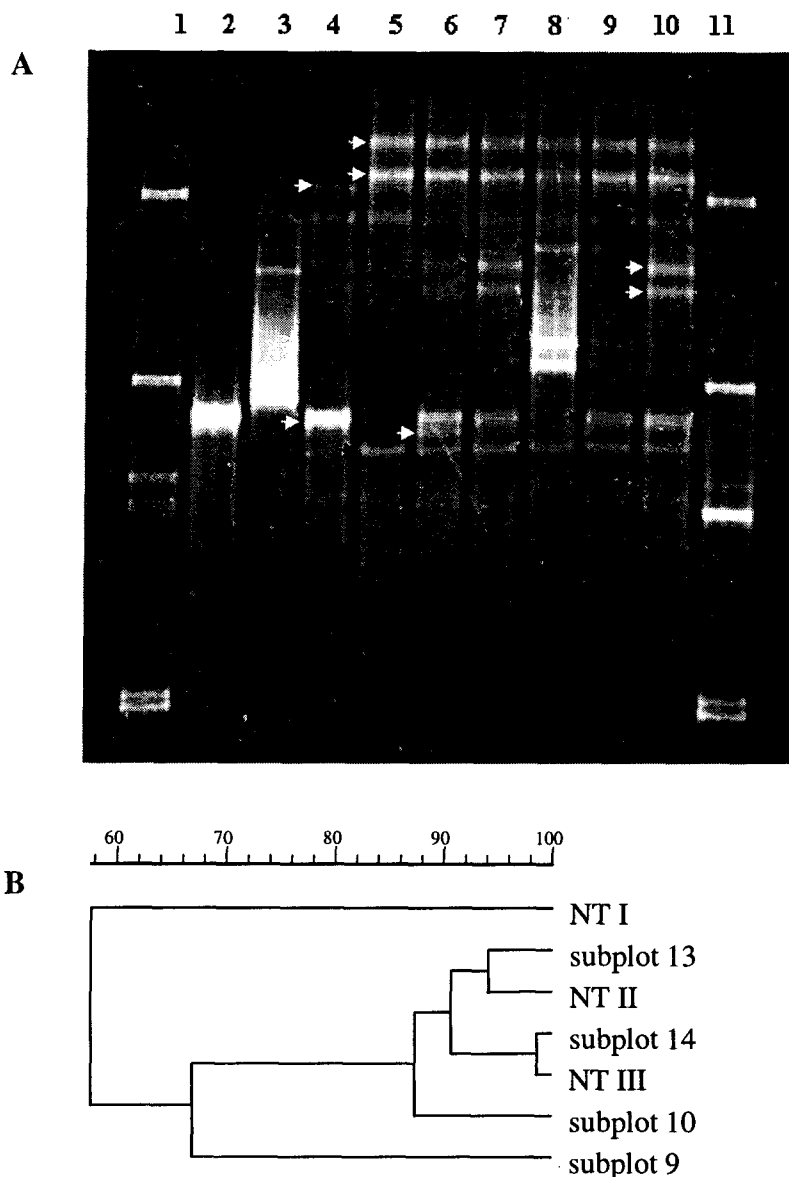


FIG. 7. (A) DGGE analysis of PCR fragments generated from No-tillage subplots and reference strains using the primer set trop1f/rhizo3r-GC clamp. Lanes: 1 and 11, DGGE marker; 2 and 3: PCR products from pure cultures of *R. tropici* CCT 4160^T and CCT 4164; respectively; 4 to 10, amplification products from the 4 subplots of NT treatment and from pooled soil samples NT-I, NT-II and NT-III, in that order. **(B)** Cluster analysis of DGGE profiles derived from No-tillage subplots with the primer set trop1f/rhizo3r-GC clamp, generated by using the *Pearson* correlation coefficient and UPGMA method.

Identification and phylogenetic analysis of individual bands extracted from DGGE gels

The electrophoretic behavior of some of the amplified fragments on DGGE gels was comparable to that of some reference strains included in the study (Table 1). Two fragments in the soil profiles (Fig. 8, arrows) showed identical migration behavior to that from bands derived from *Rhizobium leguminosarum* CCT 6323 and CCT 6308 (Fig. 8, lanes 15 and 20). These, named band 1 and band 1', and the corresponding bands from the *R. leguminosarum* strains, were extracted from the gels and analyzed further by DGGE to confirm their identity, prior to cloning and sequencing. Additionally, individual bands from the Pasture soil sample (Fig. 5, bands 2, 3, 4, A and B), which were not reproducibly amplified in replicate analyses, were also recovered, cloned and sequenced (forward and reverse sequencing of 240 bp fragments) in order to investigate their identities.

Sequence analyses of bands 1 and 1' revealed a low level of sequence similarity with the comigrating bands from *R. leguminosarum* CCT 6323 and CCT 6308 (75.4 % and 72.1%, respectively) suggesting that, although they are similar in the DGGE, they may correspond to different strains. In contrast, bands 3, 4 and B from pasture soils showed a high overall sequence similarity among themselves, ranging from 98.75% to 99.58%, whereas band 2 and band A showed lower sequence similarity (66.66% to 87.08%) with the former and among themselves. Bands 4 and B (99.58% sequence similarity) showed practically the same migration and overlapped in the original DGGE gel (Fig. 5).

Sequence data derived from cloned bands recovered from DGGE gels were deposited in GenBank (accession numbers listed in Fig. 9). Sequences were aligned with those from reference organisms and a phylogenetic tree was constructed (Fig. 9). Band 1 and 1' were recovered in a cluster comprised by *Rhizobium leguminosarum* and *Agrobacterium rhizogenes* reference strains, including the type strains of both species. Clone A was recovered in a cluster with *Rhizobium tropici* CCT 4160^T. Clones 3, 4 and B formed a tight cluster with clone 2, which came out separately from all reference strains used in the analysis.

Hybridization analyses of the DGGE profiles

Sequence analysis of cloned bands from the DGGE patterns of the pasture soil DNA highlighted variable regions which were used in the design of clone-specific oligonucleotide probes (Table 2). These were used in hybridization experiments in order to verify the presence of these target rhizobial strains in the soil samples from the different treatments (Fig. 10).

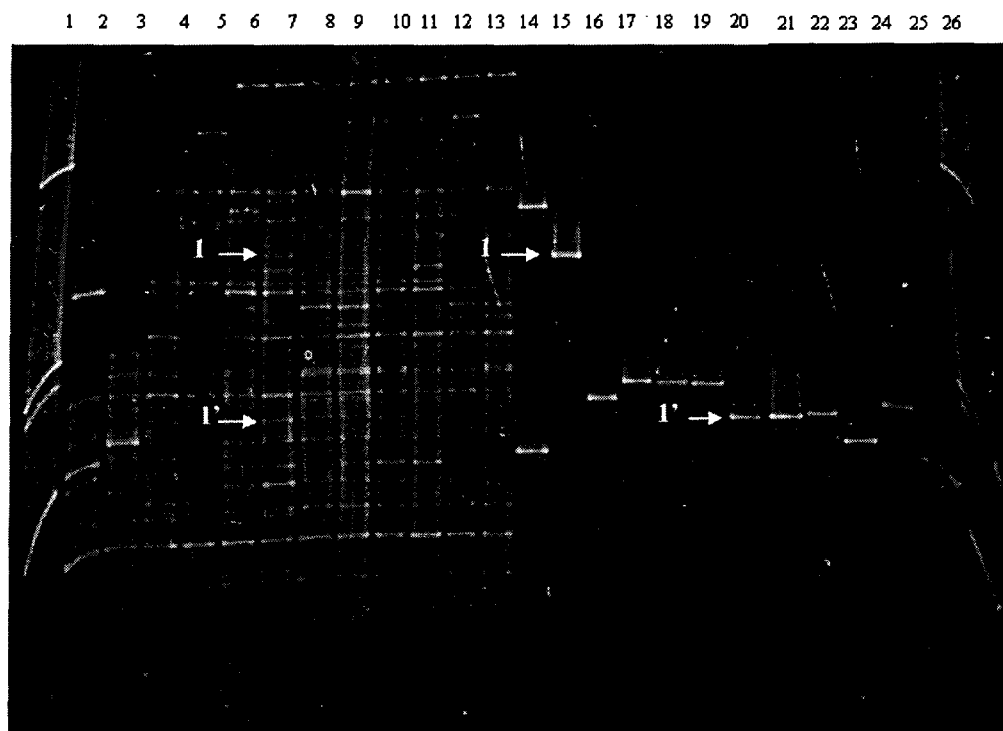


FIG. 8. DGGE analysis of amplification products from reference *R. leguminosarum* strains and different agricultural treatments, using the primer set rhizo2f/rhizo3r-GC clamp, targeting *R. leguminosarum*/*R. tropici*. Lanes: 1 and 26, DGGE marker; 2 to 13, amplification products from duplicate DNA samples from Pasture, Time Zero, NT, CT, NTF and CTF treatments, in that order; 14 to 25, PCR products from pure cultures of strains CCT 6315, CCT 6323, G72, CCT 6305, CCT 6306, CCT 6320, CCT 6308, CCT 6321, G49, CCT 6314, CCT 6310, and CCT 6317, respectively.

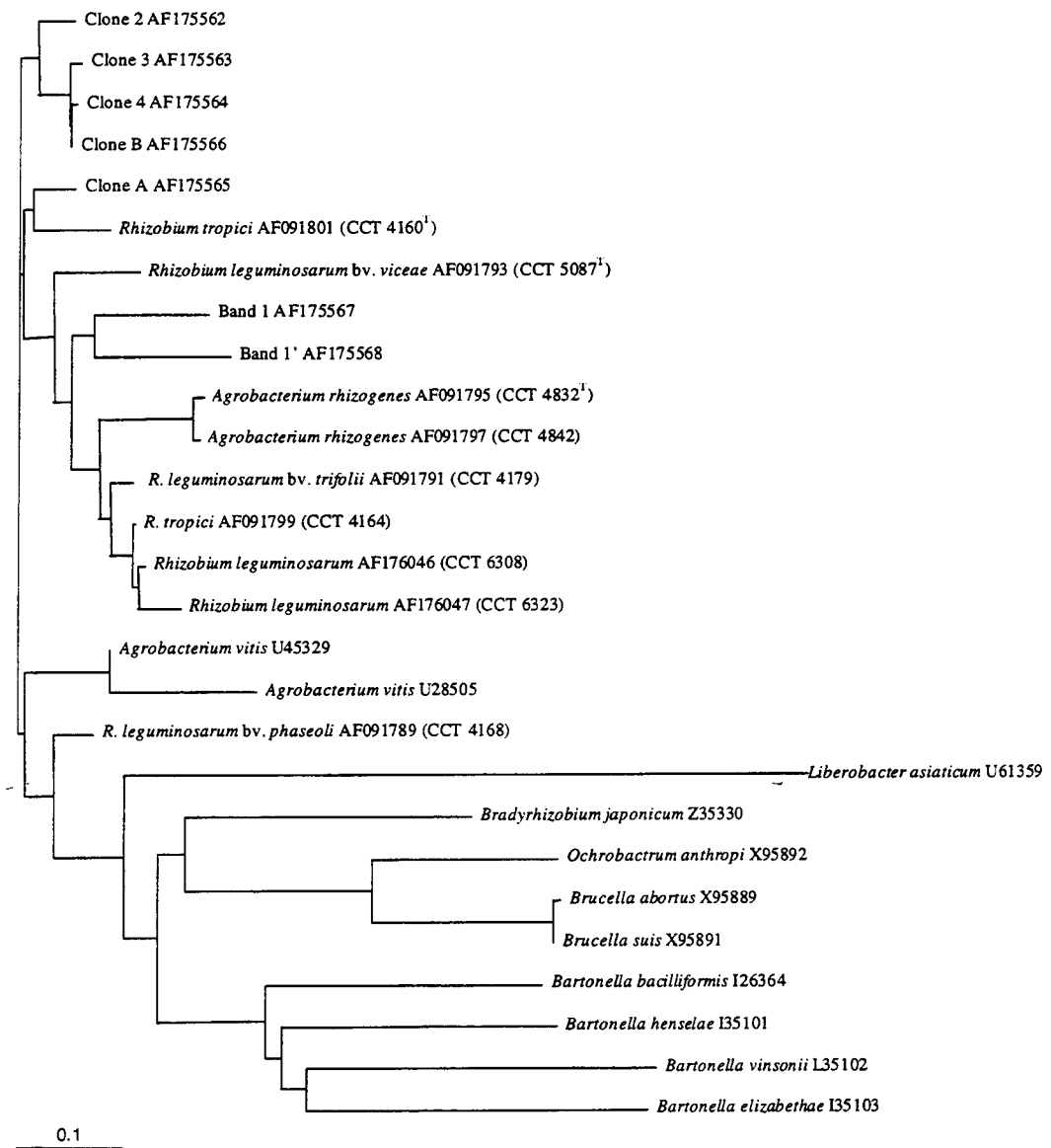


FIG. 9. Phylogenetic analysis of 16S-23S rDNA spacer sequences from cloned DGGE fragments and reference strains, based on Jukes-Cantor/*Neighbor-joining* method. GenBank accession numbers listed after species names.

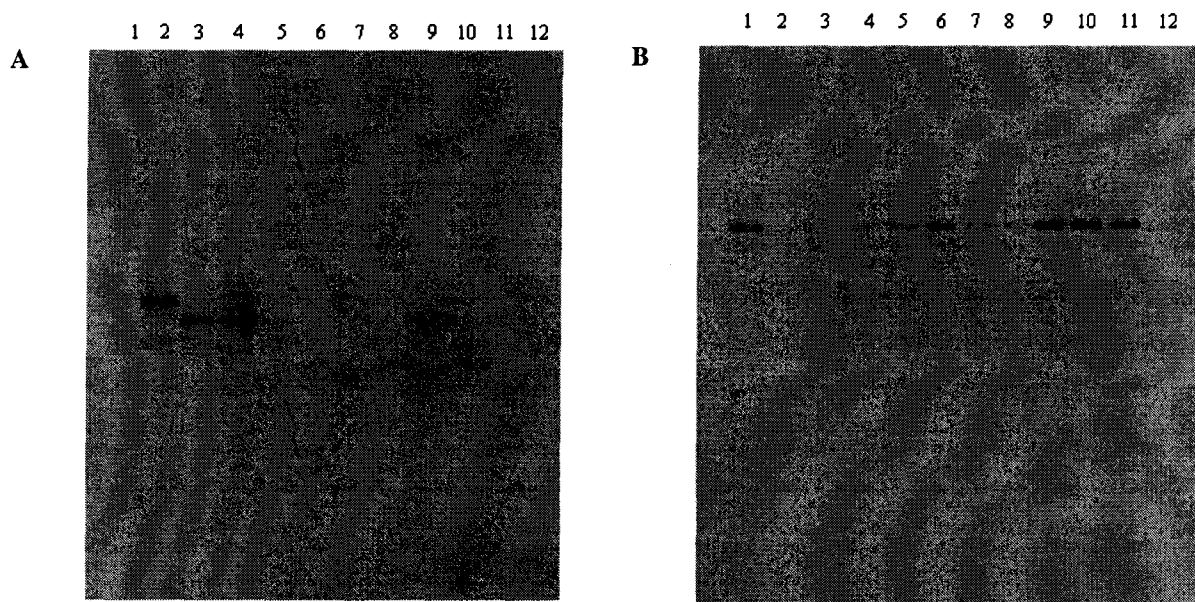


FIG. 10. Hybridization of soil DGGE patterns generated by using the primer set *rhizo2f/rhizo3r*-GC clamp with: (A) probe A, and (B) probe 2. Lanes: 1 to 12, duplicates of Pasture, Time Zero, NT, CT, NTF and CTF treatments, in that order.

Hybridization with probe A, which presumably identified a *R. tropici* related bacterial type, highlighted a single band in the duplicate Time Zero samples (Fig. 10 A, lanes 3 and 4) at a position coincident with that of clone A. Fainter bands in the same position were visualized in both duplicates of the NTF treatment and in one of the duplicates of CTF (Fig. 10 A, lanes 9-11, respectively). A band in a slightly higher position was also detected by probe A in one of the duplicates of the pasture soil (Fig. 10 A, lane 2). Thus, *R. tropici*-related organisms were fairly consistently recognizable in the control (time zero) samples, as well as in some of the treatments.

Hybridization experiments with probe 2, which identified a cluster of novel sequences divergent from the *R. tropici* and *R. leguminosarum* branches, revealed the presence of single DGGE bands that reacted with the probe in all treatments, except for one of the duplicates of pasture soil and one of the CTF samples, which showed no hybridization signal (Fig. 10 B). The intensity of the hybridization signals varied between the different treatments. A similar result was obtained in the hybridization experiment with probe 34B, which reacted with bands overlapping or coinciding with those detected by probe 2 (data not shown). No hybridization signals with bands at migration positions corresponding to clones 4 and B were visualized.

DISCUSSION

The high levels of similarity found among the DGGE profiles derived from treatments CT, CTF, NT and NTF, using the primer set rhizo2f/rhizo3r-GC, which targets both *R. leguminosarum* and *R. tropici* strains, suggest that the different agricultural practices implemented in the experimental areas had low impact on the overall diversity of these organisms in soil. However, this primer set revealed differences in strain abundance between treatments, suggesting that treatments CT and NT favored the selection of particular rhizobial strains.

The diversity of *R. leguminosarum* and *R. tropici* types was higher in plots planted with beans, compared to soil under pasture, probably due to the fact that both species nodulate and fix nitrogen in beans and are under positive selection by the host. The high diversity observed in the soil sample Time Zero may be explained by the fact that the plots had been cultivated with common bean in the year previous to the experiments, and rhizobia associated with this crop may have persisted in the soil. Selection of specific bacterial strains by host plants was also suggested to explain the occurrence of a

Paenibacillus azotofixans strain, originally obtained from a sugarcane rhizosphere, in soils previously cultivated with sugarcane (33).

Relatively greater variability was observed when the primer set trop1f/rhizo3r-GC, specific for *R. tropici*, was used. DGGE profiles generated with these primers revealed the absence, or presence below PCR-detectable levels, of *R. tropici* in the Pasture and Time Zero soil samples. Reduction of diversity on Conventional Tillage samples was also observed, suggesting a deleterious impact of this practice compared to NT. Results also demonstrated a strong selection of *R. tropici* in soil under bean crop, reflected by the higher number of bands in the corresponding treatments and by the occurrence of several bands with high intensity in these profiles. The high diversity profiles obtained in the treatments associated with bean crop may also reflect the introduction of novel strains of *R. tropici* via the legume seeds.

The analysis of DGGE data generated from using both primer sets suggests that species richness of *R. leguminosarum* is higher than that of *R. tropici* in the treatment soils. Additionally, no correlation was observed between richness or abundance of *R. leguminosarum* and/or *R. tropici* and the use of the insecticide Furadan, although data from literature suggest that microbial populations may be affected by the use of pesticides. DGGE analysis of soil microbial communities under long-term application of phenylurea herbicides (diuron, linuron and chlorotoluron) demonstrated that community structure of herbicide-treated and nontreated soils were significantly different, with decreased diversity in soils treated with urea herbicides (7).

The strain *R. leguminosarum* bv. *phaseoli* CCT 4180 showed two different fragments in the DGGE gel (Fig. 1 A, lane 5) as products of the specific PCR with the primer pair rhizo2f/rhizo3r-GC, suggesting the occurrence of two rRNA operons with sequence differences in the spacer region. This has already been observed for *Paenibacillus azotofixans* in analyses of *nifH* amplicons (33) and for *Saccharothrix waywayandensis* in TGGE separation of rRNA fragments (16). Therefore, one must be aware that although estimates of diversity based on the analysis of individual bands in DGGE gels may be useful for overall comparison of community structure, they should not be considered as precise representations of the organism diversity in these communities, since numbers of bands cannot be strictly related to numbers of different strains.

The lack of reproducibility between the DGGE profiles generated from duplicates of Pasture soil DNA samples led us to carry out PCR/DGGE-reproducibility experiments in an attempt to elucidate this problem. Results obtained indicated that neither the DGGE electrophoretic run, nor the specific PCR amplification using the *R. leguminosarum*/*R. tropici*-specific primer set influenced the reproducibility of the band patterns in our

experimental conditions. On the other hand, it was clear that the initial relative amount of template DNA significantly affected the resulting profiles from the first PCR amplification reaction using bacterial universal primers. This phenomenon may be associated with stochastic events during the serial dilution of DNA templates (in our case, rDNA operons from the different organisms in the soil) and initial steps of the PCR amplification; i.e., individual DNA templates which are present at very low concentration in the soil DNA sample may be lost during the dilution process. Conversely, the successful amplification of templates in low copy numbers may be influenced by their relative abundance and by the overall richness of templates in the PCR reaction. The correlation between low abundance of bacterial species in environmental samples and non-representativeness of these in DGGE analyses has been observed in other studies (3, 16), where the authors suggested that target molecules in low numbers could be outcompeted by those present in higher numbers in the PCR reaction.

Additional results obtained in this study corroborated our suspicion that the first PCR amplification may be the key step that controls the generation of reproducible profiles from complex soil DNA samples, and that probability may play a significant role in the amplification of low copy number DNA templates during PCR. In PCR reactions of serially diluted DNA, one could expect fainter bands, or even loss of bands, at increasingly higher DNA dilutions. Accordingly, a progressive attenuation in the intensity of the bands on DGGE gels was observed when increasingly higher dilutions of PCR products amplified by using the universal primers (the first PCR) were used in a second (specific) PCR with the primer pair rhizo2f/rhizo3r-GC (data not shown).

The results derived from the experiments on spatial variability using the primer pairs trop1f/rhizo3r-GC, targeting *R. tropici*, and rhizo2f/rhizo3r-GC, targeting both *Rhizobium leguminosarum* and *R. tropici*, suggested that the number of rhizobial individuals (strain abundance) in soil samples from the same treatment may vary. Even when samples were pooled (*cf* the pooled samples NT-I, NT-II and NT-III), the intensity of a few bands related to individual strains differed between the profiles. Data generated from repetitions of these experiments suggested that the distribution of rhizobial populations in soil is not homogeneous and one must be aware that biases may be introduced during sampling.

Sequence analysis of comigrating bands isolated from the DGGE gels, namely bands 1 and 1', and the fragments from reference strains *R. leguminosarum* CCT 6323 and CCT 6308, respectively (Fig. 8), showed that equivalent migration distance of bands on the gel should not be unequivocally taken as an indicator of sequence identity, since migration on the denaturing gradient is related to the melting behavior of the DNA fragment (G+C content and nucleotide sequence), as already demonstrated in previous studies (11, 24, 40).

Due to the lack of reproducibility verified between the replicates of DGGE profiles from the Pasture soil DNA samples (Fig. 5), some individual bands were excised from the gel, cloned and sequenced in order to investigate their identities. Although the fragments analyzed were relatively short, with 218, 237 and 239 bp, the high sequence similarity found among bands 3, 4 and B suggested that they may correspond to the same or to very related rhizobial strains.

The phylogenetic tree constructed using data from the sequenced bands (Fig. 9) demonstrated that band 1 and band 1' are related to *R. leguminosarum*/*A. rhizogenes*. In a previous study (27), the primer set rhizo2f/rhizo3r, used for PCR amplification of soil DNA in this work, did not amplify 16S-23S rDNA fragments from *A. rhizogenes* reference strains, thus suggesting that band 1 and band 1' may correspond to rDNA spacer sequences from *R. leguminosarum* strains. Clones 2, 3, 4 and B, recovered as a separate cluster in the phylogenetic analysis (Fig. 9), may represent putatively novel rhizobial strains, detected in the soil by using the *R. leguminosarum*/*R. tropici* primer set (27). However, given the relatively small number of rhizobial rDNA spacer sequences available for comparison and the highly variable nature of the 16S-23S rDNA spacer region (14), these conclusions must be considered with caution and need further evaluation.

The results obtained using probe A suggested that rhizobial strains corresponding to clone A, recovered in DGGE analyses from Pasture samples (Fig. 5) and shown to be related to *R. tropici* in the phylogenetic analysis (Fig. 9), are also present in samples from Time Zero, and, probably in lower numbers, in the NTF and CTF treatments (Fig. 10 A).

Data obtained by using probe 2 demonstrated the occurrence of a relatively ubiquitous rhizobial strain belonging to a "novel" sequence cluster in soil samples from the different treatments (Fig. 10 B). It may be speculated that differences in signal intensity could be associated to population densities of these organisms in the soils under distinct agricultural practices. Results suggest that these organisms are favored by the No-tillage with Furadan treatment (Fig. 10 B, lanes 9 and 10) compared to the remaining treatments.

The results from the hybridization analysis demonstrated the high sensitivity of this approach when coupled to the DGGE technique to uncover organisms present in low numbers in environmental samples. DGGE profiles obtained using the *R. tropici* primer set revealed only a few low-intensity bands in the Time Zero samples, and the bands detected by hybridization with probe A were not visible in the original DGGE gel (Fig. 2 A).

The strategy developed in the current study to evaluate the diversity of rhizobia in soil samples, based on DGGE analysis of soil community DNA and hybridization with specific probes, proved to be of great value in unraveling the diversity of *R. leguminosarum* and *R. tropici* strains in soils subjected to different agricultural treatments. This approach

was also successful in the detection of putatively novel organisms by phylogenetic analysis of amplified band fragments, which may prove to be useful in the study of non-cultured organisms in low-density populations in environmental samples.

Acknowledgements

V.M.O was supported by an SPE grant from Conselho Nacional de Desenvolvimento Científico e Tecnológico (RHAE/CNPq, Brazil) and a doctoral fellowship from Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP, Brazil). The authors are grateful to L. Lankwarden for technical assistance.

References

1. Akkermans, A.D.L., M.S. Mirza, H.J.M. Harmsen, H.J. Blok, P.R. Herron, A. Sessitsch, and W.M. Akkermans. 1994. Molecular ecology of microbes: A review of promises, pitfalls and true progress. *FEMS Microbiol. Rev.* **15**:185-194.
2. Amann, R. I., W. Ludwig and K. Schleifer. 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiol. Rev.* **59**:143-169.
3. Brinkhoff, T. and G. Muyzer. 1997. Increased species diversity and extended habitat range of sulfur-oxidizing *Thiomicrospira* spp. *Appl. Environ. Microbiol.* **63**:3789-3796.
4. Coutinho, H.L.C., V. M. Oliveira, and F.M.S. Moreira. 1999a. Systematics of legume nodule nitrogen fixing bacteria (Rhizobia): agronomic and ecological applications. *In*: F. Priest and M. Goodfellow (ed.), *Applied Microbial Systematics*. (In press).
5. Coutinho, H.L.C., V.M. Oliveira, A. Lovato, A.H.N. Maia, and G.P. Manfio. 1999b. The application of the Shannon-Weaver diversity index associated with molecular techniques to evaluate the diversity of rhizobia in Brazilian agricultural soils. *Appl. Soil Ecol.* (In press).
6. Duarte, G.F., A.S. Rosado, L. Seldin, A.C. Keijzer-Wolters, and J.D. van Elsas. 1998. Extraction of ribosomal RNA and genomic DNA from soil for studying the diversity of the indigenous bacterial community. *J. Microbiol. Methods* **32**:21-29.
7. El Fantroussi, S., L. Verschuere, W. Verstraete, and E.M. Top. 1999. Effect of phenylurea herbicides on soil microbial communities estimated by analysis of 16S

- rRNA gene fingerprints and community-level physiological profiles. Appl. Environ. Microbiol. **65**:982-988.
8. **Felsenstein, J.** 1989. Phylip—phylogeny inference package. Cladistics **5**:164-166.
 9. **Felske, A., H. Rheims, A. Wolterink, E. Stackebrandt, and A.D.L. Akkermans.** 1997. Ribosome analysis reveals prominent activity of an uncultured member of the class Actinobacteria in grassland soils. Microbiol. **143**:2983-2989.
 10. **Felske, A., A. Wolterink, R. Van Lis, and A.D.L. Akkermans.** 1998. Phylogeny of the main bacterial 16S rRNA sequences Drentse A grassland soils (The Netherlands). Appl. Environ. Microbiol. **64**:871-879.
 11. **Ferris, M.J., G. Muyzer, and D.M. Ward.** 1996. Denaturing gradient gel electrophoresis profiles of 16S rRNA-defined populations inhabiting a hot spring microbial mat community. Appl. Environ. Microbiol. **62**:340-346.
 12. **Fischer, S.G., and L.S. Lerman.** 1983. DNA fragments differing by single basepair substitutions are separated in denaturing gradient gels: correspondence with melting theory. Proc. Natl. Acad. Sci. U. S. A. **80**:1579-1583.
 13. **Giovannoni, S.J., T.B. Britschgi, C.L. Moyer, and K.G. Field.** 1990. Genetic diversity in Sargasso Sea bacterioplankton. Nature **345**:60-63.
 14. **Gürtler, V., and V.A. Stanisich.** 1996. New approaches to typing and identification of bacteria using the 16S-23S rDNA spacer region. Microbiology **142**:3-16.
 15. **Haqqi, T.M., G. Sarkar, C.S. David, and S.S. Sommer.** 1988. Specific amplification with PCR of a refractory segment of genomic DNA. Nucleic Acids Res. **16**:11844.
 16. **Heuer, H., M. Krsek, P. Baker, K. Smalla, and E.M.H. Wellington.** 1997. Analysis of actinomycete communities by specific amplification of genes encoding 16S rRNA and gel-electrophoretic separation in denaturing gradients. Appl. Environ. Microbiol. **63**:3233-3241.
 17. **Hirsh, A. M.** 1992. Developmental biology of legume nodulation. New Phytologist **122**:211-237.
 18. **Honeycutt, R.J., B.W.S. Sobral, and M. McClelland.** 1995. tRNA intergenic spacers reveal polymorphisms diagnostic for *Xanthomonas albilineans*. Microbiol. **141**:3229-3239.
 19. **Hugenholtz, P., B.M. Goebel, and N.R. Pace.** 1998. Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. J. Bacteriol. **180**:4765-4774.
 20. **Jukes, T.H., and R.R. Cantor.** 1969. Evolution of protein molecules p.21-132. In: H.N. Munro (ed.), Mammalian protein metabolism. Academic Press, New York.

21. **Kahindi, J.H.P., P. Woormer, T. George, F.M.S. Moreira, N.K. Karanja, and K.E. Giller.** 1997. Agricultural intensification, soil biodiversity and ecosystem function in the tropics: the role of nitrogen-fixing bacteria. *Appl. Soil Ecol.* **6**:55-76.
22. **Massol-Deya, A.A., D.A. Odelson, R.F. Hickey, and J.M. Tiedje.** 1995. Bacterial community fingerprinting of amplified 16S and 16-23S ribosomal DNA gene sequences and restriction endonuclease analysis (ARDRA), section 3.3.2, p. 1-8. *In*: A. D. L. Akkermans, J. D. van Elsas, F. J. de Bruijn (ed.), *Molecular microbial ecology manual*. Kluwer Academic Press, Dordrecht, The Netherlands.
23. **Muyzer, G., E.C. De Waal, and A.G. Uitterlinden.** 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl. Environ. Microbiol.* **59**:695-700.
24. **Muyzer, G., S. Hottentrager, A. Teske, and C. Wawer.** 1995a. Denaturing gradient gel electrophoresis of PCR-amplified 16S rDNA—a new molecular approach to analyse the genetic diversity of mixed microbial communities, section 3.4.4, p. 1-23. *In* A.D.L. Akkermans, J.D. van Elsas, and F.J. de Bruijn (ed.), *Molecular microbial ecology manual*. Kluwer Academic Publishers, Dordrecht, The Netherlands.
25. **Muyzer, G., A. Teske, C.O. Wirsén, and H.W. Jannasch.** 1995b. Phylogenetic relationships of *Thiomicrospira* species and their identification in deep-sea hydrothermal vent samples by denaturing gradient gel electrophoresis of 16S rDNA fragments. *Arch. Microbiol.* **164**:165-172.
26. **Ogram, A., G.S. Sayler, and T. Barkay.** 1988. DNA extraction and purification from sediments. *J. Microbiol. Methods* **7**:57-66.
27. **Oliveira, V.M., H.L.C. Coutinho, B.W.S. Sobral, C.T. Guimarães, J.D. van Elsas, and G.P. Manfio.** 1999. Discrimination of *Rhizobium tropici* and *R. leguminosarum* strains by PCR-specific amplification of 16S-23S rDNA spacer region fragments and denaturing gradient gel electrophoresis (DGGE). *Let. Appl. Microbiol.* **28**:137-141.
28. **Pace, N.R., D.A. Stahl, D.J. Lane, and G.J. Olsen.** 1986. The analysis of natural microbial populations by ribosomal RNA sequences. *Adv. Microb. Ecol.* **9**:1-55.
29. **Pace, N.R.** 1996. New Perspective on the Natural Microbial World: Molecular Microbial Ecology. *ASM News.* **62**:463-470.
30. **Pearson, K.** 1926. On the coefficient of racial likeness. *Biometrika.* **18**:105-117.
31. **Pickup, R.W.** 1991. Development of molecular methods for the detection of specific bacteria in the environment. *J. Gen. Microbiol.* **137**:1009-1019.

32. Pitcher, D.G., N.A. Saunders, and R.J. Owen. 1989. Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. *Lett. Appl. Microbiol.* **8**:151-156.
33. Rosado, A.S., G.F. Duarte, L. Seldin, and J.D. van Elsas. 1998. Genetic diversity of *nifH* gene sequences in *Paenibacillus azotofixans* strains and soil samples analyzed by denaturing gradient gel electrophoresis of PCR-amplified gene fragments. *Appl. Environ. Microbiol.* **64**:2770-2779.
34. Saitou, N., and M. Nei. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4**:406-425.
35. Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA.
36. Sheffield, V.C., D.R. Cox, L.S. Lerman, and R.M. Myers. 1989. Attachment of a 40-base pair G+C-rich sequence (GC-clamp) to genomic DNA fragments by the polymerase chain reaction results in improved detection of single-base changes. *Proc. Natl. Acad. Sci. USA.* **86**:232-236.
37. Smalla, K., N. Cresswell, L.C. Mendonça-Hagler, A.C. Wolters, and J.D. van Elsas. 1993. Rapid DNA extraction protocol from soil for polymerase chain reaction-mediated amplification. *J. Appl. Bacteriol.* **74**:78-85.
38. Stackebrandt, E., W. Liesack, and B.M. Goebel. 1993. Bacterial diversity in a soil sample from a subtropical Australian environment as determined by 16S rDNA analysis. *FASEB J.* **7**: 232-236.
39. Torsvik, V., J. Goksoyr, and F.L. Daae. 1990. High diversity in DNA of soil bacteria. *Appl. Environ. Microbiol.* **56**:782-787.
40. Vallaey, T., E. Top, G. Muyzer, V. Macheret, G. Laguerre, A. Rigaud, and G. Souglas. 1997. Evaluation of denaturing gradient gel electrophoresis in the detection of 16S rDNA sequence variation in rhizobia and methanotrophs. *FEMS Microbiol. Ecol.* **24**:279-285.
41. van Elsas, J.D., V. Mantynen, and A.C. Wolters. 1997. Soil DNA extraction and assesment of the fate of *Mycobacterium chlorophenolicum* strain PCP-1 in different soils by 16S ribosomal RNA gene sequence based most-probable-number PCR and immunofluorescence. *Biol. Fertil. Soils* **24**:188-195.
42. Ward, D.M., R. Weller, and M.M. Bateson. 1990. 16S RNAr sequences reveal numerous uncultured microorganisms in a natural community. *Nature* **345**:63-65.



Evaluation of the diversity of rhizobia in Brazilian agricultural soils cultivated with soybeans

Heitor L.C. Coutinho^{a,*}, Valéria M. Oliveira^b, Andrea Lovato^b,
Aline H.N. Maia^c, Gilson P. Manfio^b

^a Embrapa Solos, Rua Jardim Botânico 1024, Rio de Janeiro-RJ, CEP 22460-000, Brazil

^b Fundação André Tosello, Rua Latino Coelho 1301, Campinas-SP, CEP 13087-010, Brazil

^c Embrapa Meio Ambiente, Rod. SP-340 Km. 127, Jaguariúna-SP, CEP 13820-000, Brazil

Handwritten: # 1 x 6

Received 20 May 1998; received in revised form 1 November 1998; accepted 10 March 1999

Abstract

The diversity of rhizobia in agricultural soils planted with soybean (*Glycine max* L.) and managed under conventional or no-tillage practices was evaluated by using a combination of trap-host capture and DNA fingerprinting approaches. Fifty-eight rhizobia isolates were captured using pigeonpea (*Cajanus cajan* L.) as a trap-host and characterised by using the RAPD DNA fingerprinting technique, yielding 25 different RAPD profiles. The application of the Shannon–Weaver diversity index demonstrated that the diversity of rhizobia was significantly reduced in soil samples from plots cultivated with soybean compared with original uncultivated pasture plots. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Rhizobium; Bradyrhizobium; Biodiversity; Agricultural soils; Tillage; DNA fingerprinting

1. Introduction

Rhizobia are nitrogen-fixing bacteria which usually develop symbiotic associations with leguminous plants. Recent taxonomic revision of this group of organisms has defined five different genera: *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium* and *Sinorhizobium* (Young, 1996), taking into account phenotypic and phylogenetic characteristics of the organisms.

Studies of the diversity of rhizobia have been hampered by methodological difficulties regarding sampling, identification, and available methods for

environmental monitoring (Coutinho, 1996), related to an overall high diversity of this group of micro-organisms in nature (Laguerre et al., 1993; Moreira et al., 1993). Oyaizu et al. (1992) performed an extensive survey of 117 rhizobial strains isolated from 91 legume species, suggesting the existence of at least 16 species among the strains studied. Data from Moreira et al. (1993) demonstrated that the majority of rhizobia from soils of the Brazilian Atlantic and Amazonian rainforests were of the slow-growing, alkali-producing type, leading the authors to classify them as *Bradyrhizobium* sp. Other researchers have shown a high degree of diversity among rhizobia isolated from nodules of both woody and non-woody legumes (Haukka and Lindström, 1994; Novikova et al., 1994; van Rossum et al., 1995). However, there are

*Corresponding author. Tel.: +55-021-274-4999; fax: +55-021-274-5291; e-mail: heitor@cnpes.embrapa.br

not many studies on the effects of agricultural practices on the diversity of rhizobia in natural environments in the tropics.

In this study, we assess the impact of different soil-management practices (conventional and no-tillage) on the diversity of rhizobia in plots planted with soybean (*Glycine max* L.), using the Shannon–Weaver diversity index to analyse RAPD data derived from strains captured with the legume pigeonpea (*Cajanus cajan* L.) as trap-host.

2. Material and methods

2.1. Field experiment and soil samples

The field experiment was set up in Fazenda Barcelona (Guaíra, state of São Paulo, Brazil), in April 1995. Soils were of the acidic oxisol type, typical of the Brazilian Cerrado, and the area had been under pasture (*Brachiaria decumbens*) for several years prior to setting up the experiment. Three replicate plots, $9 \times 18 \text{ m}^2$ each, were prepared by using conventional tillage (CT), characterised by one mouldboard ploughing of the soil followed by two disc-harrowing procedures. The same number of replicate plots were prepared with no-tillage (NT), that is, the seeds and fertilisers were applied directly to the furrows opened over the residues from the previous crop. The plots were sown with beans (*Phaseolus vulgaris* L.) (April–July 1995) and left fallow until soybean (*Glycine max* L.) was sown (December 1995), and managed according to the common agricultural practices in the region. Four composite soil samples, consisting of pooled samples taken from 10 randomly selected points at a depth of 20 cm, were collected prior to the preparation of the pasture plots for planting (PO; April 1995). Thirty days after sowing soybean in the CT and NT plots (January 1996), composite soil samples were taken from each plot, with a total of three composite samples per treatment. The soil samples were refrigerated at 4°C during transport to the laboratory prior to the trap-host experiments.

2.2. Isolation procedures and phenotypic characterisation of strains

Rhizobia isolates were obtained from the soil samples using pigeonpea plants (*C. cajan* L.) as trap-hosts. This legume species was chosen due to its widespread utilisation as green manure in Brazilian agriculture. Capture experiments were performed in a greenhouse using Leonard jars and artificial sterile substrate (vermiculite) in the upper compartment and plant nutrient solution in the lower compartment. Leonard jars setup and plant nutrient solution were performed as described previously (Somasegaran and Hoben, 1985). Three pre-germinated pigeonpea plants were each inoculated with 2 ml of a 10^{-2} soil dilution. Thirty days after planting, three nodules per plant were harvested, surface-sterilised and macerated onto the surface of the growth media, as described previously (Somasegaran and Hoben, 1985). Isolation and growth media consisted of YMA (0.5 g KH_2PO_4 ; 0.2 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 0.1 g NaCl; 0.5 g yeast extract; 10 g mannitol; 0.5% bromthymol blue solution; 15 g agar; per litre). The bacterial culture obtained around the nodule debris, after 3–7 days of incubation at 28°C was streaked again over YMA plates for colony isolation. The isolates obtained were checked for purity after incubation at 28°C, and their growth rates determined. Strains were classified as slow- (5–7 days) or fast- (3–4 days) growers according to their ability to form single colonies of $\approx 1\text{--}2 \text{ mm}$ over the surface of the culture media. Only one pure isolate was selected per nodule. Authentication of strains was performed using nodulation tests in pigeonpea plants, as described by Somasegaran and Hoben (1985). Isolates were preserved by storage of cell suspensions in 20% glycerol at -20°C (strains representative of distinct RAPD profiles were also preserved by lyophilisation) and deposited at the Tropical Culture Collection (CCT, Fundação André Tosello, Campinas–SP, Brazil).

2.3. Characterisation of strains by RAPD

Strains were characterised by using the RAPD technique (Coutinho et al., 1993). Rhizobial cells were lysed in a solution containing 50 ng μl proteinase K, according to Young and Blakesley (1991), and their DNA concentration was estimated by agarose gel

electrophoresis and ethidium bromide staining. The 25 μ l-volume reactions were prepared in duplicate, each containing either 5 or 10 ng of rhizobial DNA, 2 U of *Taq* DNA polymerase (CENBIOT, RS, Brazil), 2 mM $MgCl_2$, 0.2 mM dNTPs and 1.0 μ M of a single 10-mer oligonucleotide primer (UBC#4, 5'-CCT GGG CTG G-3', University of British Columbia, Vancouver, BC). Cycling conditions were: 1 cycle at 94°C (2 min), followed by 30 cycles at 94°C (30 s), 36°C (30 s) and 72°C (1 min), with a final extension cycle at 72°C (3 min). Agarose gel electrophoresis was performed according to Sambrook et al. (1989), using 1.2% gels. RAPD profiles were registered on Polaroid films after ethidium bromide staining of gels.

2.4. Hybridisation with *Bradyrhizobium* sp./*B. elkani* probe

A *Bradyrhizobium* sp./*B. elkani*-specific probe (5'-CCg TCT CTg gAg TCC gCg ACC-3'), designed by Oliveira et al. (1997), was used to check the identity of some of the soil isolates. This probe was shown to hybridise with DNA from *Bradyrhizobium* sp. and *B. elkani*, but not to *Bradyrhizobium japonicum*. Genomic DNA from pure cultures of rhizobia was purified according to Pitcher et al. (1989). 16S rDNA was then amplified by using universal primers p27f and p1401r (Lane, 1991). PCR reactions (50 μ l) contained 50 ng of genomic DNA, 2 U of *Taq* DNA polymerase (CENBIOT, RS, Brazil), 2 mM $MgCl_2$, 0.2 mM dNTPs and 0.4 μ M of each primer. Amplification conditions consisted of one cycle at 95°C (2 min), followed by 30 cycles at 94°C (1 min), 60°C (1 min) and 72°C (3 min), with a final extension cycle at 72°C (5 min), in a Perkin-Elmer 9600 thermal cycler. Amplified 16S rDNA were electrophoresed on 1.2% agarose gels, denatured and vacuum-transferred (VacuGene XL-Pharmacia) to nylon membranes (Boehringer-Mannheim), according to the manufacturer's instructions. The *Bradyrhizobium* sp./*B. elkani* probe was 3' labelled with digoxigenin (DIG-11-dUTP) using the terminal transferase enzyme (GIBCO-BRL), according to the manufacturer's instructions. Labelling reactions (20 μ l) contained 50 U of terminal transferase, 12 μ M oligonucleotide, 0.5 mM dATP and 0.05 mM DIG-11-dUTP (Boehringer-Mannheim) in 1X reaction buffer. The labelling

reaction was carried out overnight at 37°C. Hybridisations were carried at 50°C for 18 h, followed by two washes with 6X SSC for 15 min at room temperature and one stringent wash at 60°C for 3 min. Probe signal was detected by chemiluminescence (DIG Luminescent Detection Kit, Boehringer-Mannheim), using CSPD, and recorded on X-ray film by exposure for 1 min.

2.5. Data analysis and evaluation of diversity

The rhizobia isolates were grouped according to the degree of similarity of their RAPD banding patterns. The Shannon-Weaver index (Shannon and Weaver, 1949) was applied to determine the diversity index (H) for each treatment, and was calculated by the following equation:

$$H = - \sum_{i=1}^k p_i \ln p_i$$

where k is the number of operational taxonomic units (OTU; defined by similar RAPD profiles) and p_i , the relative abundance of isolates of each OTU ($i = 1, 2, \dots, k$). An adaptation of the Pielou's pooled quadrates method (Pielou, 1969), consisting of 3000 random ordinations of the data and calculation of the diversity indices in a cumulative manner, was applied. Confidence limits were determined by calculating the jackknife estimate of the variance of H (Magurran, 1988).

3. Results

3.1. Isolation and phenotypic characterisation of strains

A total of 58 strains were isolated from pigeonpea nodules taken from plants cultivated in substrates inoculated with soil samples from pasture, conventional tillage and no-tillage plots. All putative rhizobial strains isolated were able to form nodules in pigeonpea (*C. cajan* L.), thus characterising them as rhizobia. Strains which formed nodules were further classified as slow- and fast-growers according to their growth rates (Table 1).

Table 1

Classification of rhizobial isolates according to their growth rates, RAPD banding patterns and hybridisation signal with *Bradyrhizobium* sp./*B. elkanii*-specific probe

Soil sample	Isolate	Growth rate	OTU ^{a,b}	Hybridisation signal with <i>Bradyrhizobium</i> sp./ <i>B. elkanii</i> probe ^c
Pasture (PO)				
	CCT 6186	slow	SMC	+
	CCT 6187	slow	I	+
	CCT 6188	slow	I	ND
	CCT 6189	slow	SMC	+
	CCT 6190	slow	I	ND
	CCT 6191	slow	SMC	-
	CCT 6192	slow	SMC	ND
	CCT 6193	slow	SMC	-
	CCT 6194	slow	IV	+
	CCT 6195	slow	IV	ND
	CCT 6196	slow	IV	ND
	CCT 6197	slow	SMC	-
	CCT 6198	slow	IV	ND
	CCT 6199	slow	SMC	+
	CCT 6200	slow	SMC	+
	CCT 6201	slow	SMC	+
	CCT 6202	slow	SMC	ND
	CCT 6203	slow	SMC	+
	CCT 6204	slow	III	+
	CCT 6205	slow	V	+
	CCT 6206	slow	V	ND
	CCT 6207	slow	SMC	+
	CCT 6208	slow	I	ND
	CCT 6209	slow	I	ND
	CCT 6210	slow	I	ND
	CCT 6211	slow	I	ND
	CCT 6212	slow	III	+
	CCT 6213	slow	SMC	+
	CCT 6214	slow	SMC	+
	CCT 6215	slow	SMC	+
	CCT 6216	slow	SMC	+
Conventional tillage (CT)				
	CCT 6217	fast	II	-
	CCT 6218	fast	II	ND
	CCT 6219	slow	I	ND
	CCT 6220	slow	VII	+
	CCT 6221	slow	I	ND
	CCT 6222	fast	VIII	-
	CCT 6223	slow	I	+
	CCT 6224	fast	VIII	ND
	CCT 6225	slow	VII	ND
	CCT 6226	slow	I	ND
	CCT 6227	fast	II	ND
	CCT 6228	slow	VII	ND
	CCT 6229	fast	VI	-
	CCT 6230	slow	I	ND
	CCT 6231	slow	IX	+
	CCT 6232	slow	IX	ND
	CCT 6233	slow	I	ND

No tillage (NT) --->

Table 1 (Continued)

Soil sample	Isolate	Growth rate	OTU ^{a,b}	Hybridisation signal with <i>Bradyrhizobium</i> sp./ <i>B. elkanii</i> probe ^c
	CCT 6234	fast	VI	ND
	CCT 6235	slow	SMC	+
	CCT 6236	fast	VI	ND
	CCT 6237	fast	VI	ND
	CCT 6238	fast	VI	ND
	CCT 6239	fast	SMC	–
	CCT 6240	slow	I	ND
	CCT 6241	fast	VI	ND
	CCT 6242	fast	SMC	–
	CCT 6243	fast	VI	ND

^a Operational taxonomic units: named I to IX (see text for details). (OTU)

^b Single-membered cluster: isolate with unique banding pattern. (SMC)

^c Not determined. (ND)

3.2. DNA fingerprinting of rhizobial isolates

The characterisation of the 58 rhizobia isolates yielded 28 different RAPD banding profiles (Table 1 and Fig. 1), corresponding to nine operational taxonomic units (OTUs), which included more than one

strain, and to nineteen unique fingerprint patterns. Strains derived from pasture soil (P0) comprised a diverse group of isolates, which included 16 organisms with unique RAPD profiles. OTU I isolates were found in all soil samples. OTUs III, IV, and V were found solely in the pasture soil. Likewise, OTUs II, VI,

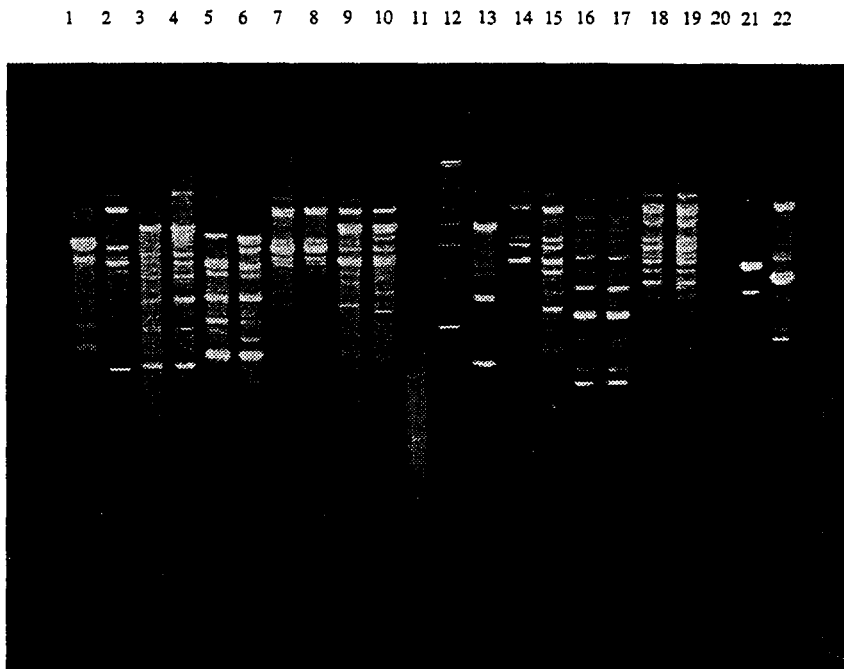


Fig. 1. RAPD fingerprints of some of the rhizobial strains isolated from the soil samples by using the trap-host capture method. Lanes 1–11, isolates CCT 6187, 6223, 6217, 6227, 6204, 6212, 6194, 6198, 6205, 6206, 6236, respectively; lane 12, 1 Kb DNA ladder (Pharmacia); lanes 13–22: CCT 6241, 6220, 6228, 6222, 6224, 6231, 6232, 6191, 6197, and 6201, respectively.

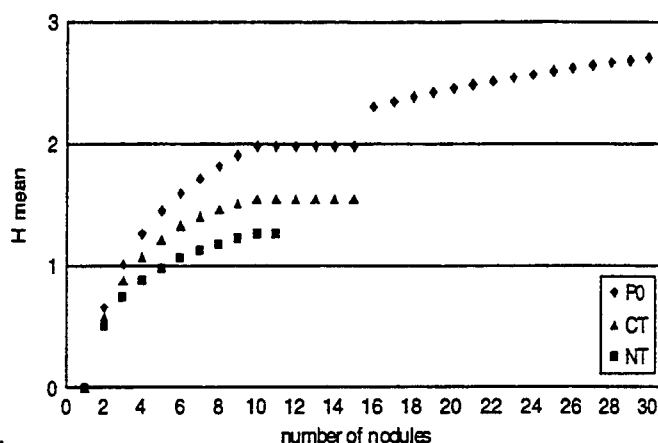


Fig. 2. Shannon-Weaver cumulative diversity curve for the different agricultural treatments: means of H indices as function of sample size. P0, pasture; CT, conventional tillage; and NT, no tillage.

VII, VIII, and IX were detected only in soil samples from the cultivated plots. Fast-growing rhizobia from group VI prevailed among isolates from soil samples of the no tillage (NT) treatment.

3.3. Analysis of diversity

The Shannon-Weaver diversity index (H) was calculated, and the corresponding curves constructed, showing its variation as a function of the number of isolates considered in the analysis (Fig. 2). For treatments CT (conventional tillage) and NT (no-tillage), the diversity-index curves stabilised at nodule numbers >10. Treatment P0 comprised data from 31 strains and its curve followed the overall pattern of the others. However, the diversity-index values increased gradually with isolate numbers >16, suggesting that the H values obtained are underestimates and more isolates are needed. Nevertheless, the rhizobial diversity in the P0 soil was greater than that in the cultivated plots. The Shannon-Weaver index jackknife estimates

demonstrate that the difference between the index for P0 and that for the cultivated plots is statistically significant (Table 2 and Fig. 3). No significant difference was found between the diversity indices of the CT and NT treatments.

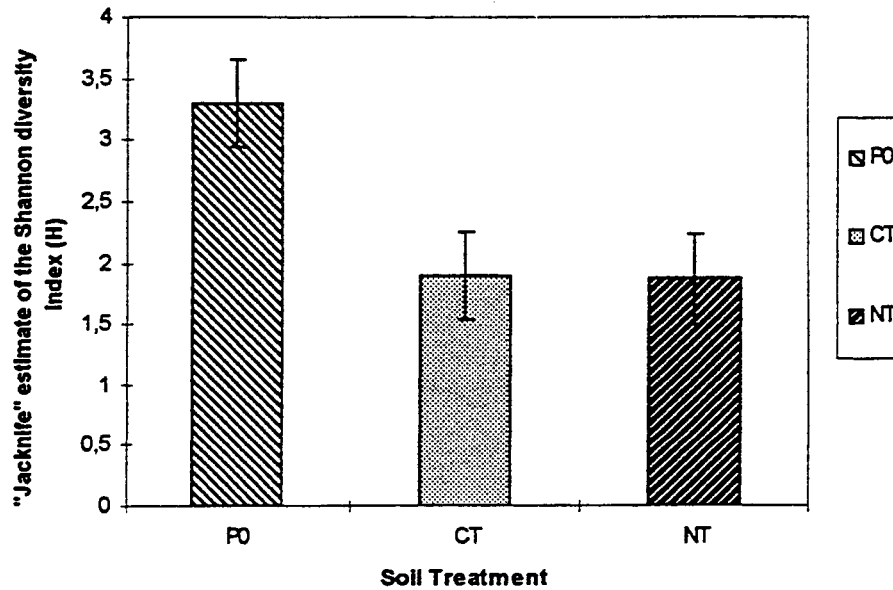
4. Discussion

The use of pigeonpea (*C. cajan* L.) to capture rhizobia proved to be an efficient strategy. Its low host-specificity was demonstrated by the diversity of isolates recovered. Rhizobia of both slow- and fast-growing types were isolated, indicating that species of different genera were recovered (Table 1). Some of the slow-growing isolates could be identified as belonging to the genus *Bradyrhizobium*, and were putatively assigned to *Bradyrhizobium* sp./*B. elkani* by their hybridisation signal with the *Bradyrhizobium* sp./*B. elkani* probe.

Table 2

Jackknife estimate of the Shannon-Weaver diversity indices (H_j) for the different agricultural treatments

Treatment	n	Shannon-Weaver diversity indices (H_j)			
		mean	SD	confidence limit (lower)	confidence limit (upper)
Pasture (P0)	31	3.30	0.22	2.84	3.76
Conventional tillage (CV)	16	1.90	0.16	1.55	2.24
No tillage (NT)	11	1.87	0.12	1.60	2.14



P0, pasture soil; CT, conventional tillage; NT, no tillage.

Fig. 3. Shannon–Weaver index of the diversity of rhizobia in soil under different management practices. Confidence limits (P0, 0.92; CT, 0.70; and NT, 0.54) were determined by using the jackknife estimate of variance of the diversity index (H).

No fast-growing rhizobia were recovered from the P0 soil samples (prior to planting of soybean). On the other hand, the majority of isolates from the NT samples (no tillage) were fast-growers. Clearly, the different soil-management practices produced alterations in the profile of the rhizobial populations in the soil. However, the trends observed were contrary to the expectation that the soybean crop should favour an increase of bradyrhizobia.

The number of operational taxonomic units (OTUs) and the number of individuals belonging to each OTU ('richness' and 'abundance', respectively) were used to calculate the Shannon–Weaver diversity index (Shannon and Weaver, 1949) of each treatment. The P0 soil samples presented a significantly higher rhizobial diversity when compared to that of NT and CT (Table 2; Figs. 2 and 3). In fact, 16 out of 31 isolates recovered from P0 produced unique RAPD fingerprints (Table 1). This may explain why the diversity index curve for this treatment did not stabilise, unlike treatments NT and CT (Fig. 2). As the rhizobial diversity in a soil increases, a larger sample size, i.e., more isolates, should be analysed in order to achieve a more precise estimation of their diversity.

The variance of the diversity-index estimates was calculated using the 'jackknife' procedure, which provided a statistical significance test to the comparative analysis undertaken. The jackknives of the diversity index (H_j) of treatments CT and NT were statistically identical (Table 2) and different from those presented in Fig. 2. This could be explained by the fact that the jackknife has a bias of the order $1/n$ (Quenouille, 1956).

The changes in rhizobial diversity observed in soils converted to soybean cultivation are probably a result of the profound modifications imposed on the soil environment by the application of agrochemicals (fertilisers, herbicides, and pesticides), by physical disturbance (ploughing and tillage), and by the soybean crop itself. One does not know whether alterations in rhizobial diversity will affect the nodulation potential of a soil. In order to address these matters, further investigation should focus on the symbiotic performance of the rhizobial strains isolated in this study.

Although diversity evaluations based on isolated rhizobia may be biased, since only those strains able to nodulate the legume species used for capture are

analysed, this approach proved to be useful for comparative analyses, such as the one presented in this work. This study could be complemented by an analysis of the sequence diversity of rhizobial gene fragments amplified from total bacterial DNA extracted from the soil samples (Stackebrandt et al., 1993). PCR primers or DNA probes, specific to different rhizobial taxa, are expected to be developed in the near future, which will enable the evaluation of the diversity of a broader range of rhizobia in soil, most of them likely to be unknown. However, conventional isolation and characterisation methods will still be important, since they enable subsequent screening for novel legume seed inoculants.

The data obtained and the statistical framework adopted in the current study is, to our knowledge, pioneering in the study of rhizobia diversity. This methodology will be applicable to a variety of comparative studies based on molecular data of microorganisms. It is currently being applied to evaluate how much each individual host plant influences the selection of nodulating strains. This has been shown to greatly influence population-diversity studies of *Rhizobium leguminosarum* biovar *viciae* in British soils (Handley et al., 1998).

5. Conclusions

A methodology for comparative analysis of rhizobial diversity in soils has been proposed. It was used to evaluate how the diversity of pigeonpea (*C. cajan* L.) rhizobia is affected by different types of land use and tillage practices in a tropical soil in the Brazilian cerrado. The results indicate that conversion of the land use from pasture (*Brachiaria decumbens*) to soybean (*Glycine max* L.) led to a significant decrease in the diversity of pigeonpea rhizobia. No statistical difference was found between the Shannon–Weaver diversity indices of rhizobia in soils prepared by no-till and conventional tillage practices. However, DNA fingerprinting showed that the strains recovered from soils under no-tillage were different from those under conventional tillage. The determination of growth rates and hybridisation with a [*Bradyrhizobium* sp./*B. elkanii*]-specific DNA probe demonstrated that the majority of pigeonpea nodule bacteria recovered from the soils prepared by conventional tillage or under

pasture belong to the *Bradyrhizobium* genus. The use of the jackknife procedure enabled statistical analysis of the Shannon–Weaver diversity indices, in spite of the low number of isolates studied. However, higher numbers of isolates and advanced molecular systematics procedures, such as soil DNA extraction and sequence analysis, are required to allow the observation of changes in rhizobial population structures in soils under different types of land use.

Acknowledgements

H.L.C. Coutinho and V.M. Oliveira were supported by a CNPq Research Grant and FAPESP Doctoral Grant, respectively. Presentation of results in the XVI World Congress of Soil Science was sponsored by CNPq and FAPERJ. The authors wish to thank Dr. Pedro Valarini for the support to set up the field experiment and Ms. Carla M. Soares and Mr. Jefferson Mineiro for valuable technical assistance.

References

- Coutinho, H.L.C., 1996. Diversidade microbiana e agricultura sustentável. In: Workshop: Biodiversidade—Perspectivas e Oportunidades Tecnológicas. On-line publication, <http://www.bdt.org.br/bdt/paper/padctbio/cap9/>.
- Coutinho, H.L.C., Handley, B.A., Kay, H.E., Stevenson, L., Beringer, J.E., 1993. The effect of colony age on PCR fingerprinting. *Lett. App. Microbiol.* 17, 282–284.
- Handley, B.A., Hedges, A.J., Beringer, J.E., 1998. The importance of host plants for detecting the population diversity of *Rhizobium leguminosarum* biovar *viciae* in soil. *Soil Biol. Biochem.* 30(2), 241–249.
- Haukka, K., Lindström, K., 1994. Pulse-field gel electrophoresis for genotypic comparison of *Rhizobium* bacteria that nodulate leguminous trees. *FEMS Microbiol. Lett.* 119, 215–220.
- Lane, D.J., 1991. 16S/23S rRNA sequencing. In: Goodfellow, M., Stackebrandt, E. (Eds.), *Nucleic Acid Techniques in Bacterial Systematics*. John Wiley & Sons, Chichester. pp. 115–147.
- Laguette, G., Geniaux, E., Mazurier, S., Rodriguez-Casartelli, R., Amarger, N., 1993. Conformity and diversity among field isolates of *Rhizobium leguminosarum* by *viciae*, by *Trifolii* and by *phaseoli* revealed by DNA hybridisation using chromosome and plasmid probes. *Can. J. Microbiol.* 39(4), 412–419.
- Magurran, A.E., 1988. *Ecological Diversity and its Measurement*. Croom Helm, London.
- Moreira, F.M.S., Gillis, M., Pot, B., Kersters, K., Franco, A.A., 1993. Characterization of rhizobia isolated from different divergence groups of tropical Leguminosae by comparative

- polyacrylamide gel electrophoresis of their total proteins. *Syst. Appl. Microbiol.* 16, 135–146.
- Novikova, N.I., Pavlova, E.A., Vorobjev, N.I., Limenshenko, E.V., 1994. Numerical taxonomy of *Rhizobium* strains from legumes of the temperate zone. *Int. J. Syst. Bacteriol.* 44, 734–741.
- Oliveira, V.M., Rosato, Y.B., Coutinho, H.L.C., Manfio, G.P., 1997. Design of a 16S rRNA-directed oligonucleotide probe for *Bradyrhizobium* tropical strains. In: Elmerich, C., Kondorosi, A., Newton, W.E. (Eds.), *Biological Nitrogen Fixation for the 21st Century. Proceedings of the 11th International Congress on Nitrogen Fixation*, Paris, France. Kluwer Academic Publishers, Dordrecht, pp. 581.
- Oyaizu, H., Naruhashi, N., Gamou, T., 1992. Molecular methods of analysing bacterial diversity: the case of Rhizobia. *Biodiv. Conserv.* 1, 237–249.
- Pielou, E.C., 1969. *An Introduction to Mathematical Ecology*. Wiley, New York.
- Pitcher, D.G., Saunders, N.A., Owen, R.J., 1989. Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. *Lett. Appl. Microbiol.* 8, 151–156.
- Quenouille, M.H., 1956. Notes on bias in estimation. *Biometrika* 43, 353–360.
- Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA.
- Shannon, C.E., Weaver, W., 1949. *The Mathematical Theory of Communication*. University of Illinois Press, Urbana.
- Somasegaran, P., Hoben, H.J., 1985. *Methods in legume-Rhizobium technology*. University of Hawaii NifTAL Project and MIRCEN, USA.
- Stackebrandt, E., Liesack, W., Goebel, B.M., 1993. Bacterial diversity in a soil sample from a subtropical Australian environment as determined by 16S rDNA analysis. *FASEB J.* 7, 232–236.
- van Rossum, D., Schuurmans, F.P., Gillis, M., Muotcha, A., van Verseveld, H.W., Stouthamer, A.H., Boogerd, F.C., 1995. Genetic and phenetic analyses of *Bradyrhizobium* strains nodulating peanut (*Arachis hypogaea* L.) roots. *Appl. Environ. Microbiol.* 61, 1599–1609.
- Young, J.P.W., 1996. Phylogeny and taxonomy of rhizobia. *Plant Soil* 186, 45–52.
- Young, A., Blakesley, R., 1991. Sequencing plasmids from single colonies with the dsDNA cycle sequencing system. *Focus* 13, 13.

Capítulo avulso:

Abordagem Molecular Direta no Estudo da Diversidade de *Bradyrhizobium* sp.

1. Introdução

As bactérias pertencentes ao gênero *Bradyrhizobium* compreendem um grupo muito diverso de microrganismos comumente encontrados em solos tropicais (Moreira *et al.*, 1993). Estas bactérias são microssimbiontes que nodulam e fixam nitrogênio em raízes de leguminosas e que podem ser usados como inoculantes para melhorar a produção de plantações comerciais. Ainda, rizóbios nativos ou inoculados podem desempenhar importante papel em sistemas de agricultura sustentável, os quais utilizam leguminosas em consórcio com culturas anuais, ou em rotação de culturas, como adubo verde.

Até recentemente, estudos de detecção, identificação e diversidade de rizóbios eram baseados no uso de plantas-isca para a sua captura e isolamento em meios de cultivo (Pafetti *et al.*, 1996; Sullivan *et al.*, 1996). Considerando a especificidade de hospedeiro destes simbioses, esta estratégia pode levar a grandes desvios da estimativa da diversidade dos rizóbios no ambiente, como já extensivamente tratado em capítulos anteriores deste trabalho.

Dentre as novas estratégias que vêm sendo desenvolvidas e adaptadas a fim de superar as limitações impostas pelos métodos clássicos de estudo de comunidades microbianas, a aplicação de sondas de ácidos nucleicos oferece uma ferramenta rápida e confiável (Pickup, 1991; Steffan & Atlas, 1991; Ludwig *et al.*, 1998).

Um refinamento dos métodos baseados em ácidos nucleicos surgiu com o uso da sequência de RNA ribossomal (RNAr) como molécula-alvo para as sondas (Ward *et al.*, 1990; Stackebrandt *et al.*, 1993). Devido à sua distribuição universal, alto grau de conservação, ausência de transferência interespecífica e alto número de cópias, os RNAr's são geralmente considerados os biopolímeros mais úteis para análise comparativa de sequências e posterior desenho de sondas de oligonucleotídeo com diferentes graus de

especificidade a serem empregados em análises por hibridização (Pace *et al.*, 1986; Pace, 1996). Desde meados dos anos 80, vários grupos de pesquisa têm desenhado e aplicado sondas de oligonucleotídeo para a identificação de organismos e estudos da diversidade de populações microbianas em diferentes tipos de ambientes (Pace *et al.*, 1986; Amann *et al.*, 1995; Hugenholtz *et al.*, 1998).

Este estudo teve como objetivo estabelecer um sistema direto para avaliar a diversidade do grupo *Bradyrhizobium* sp. em amostras ambientais baseado na extração de DNA da comunidade bacteriana do solo e uso de sondas específicas para o gene RNAr 16S.

2. Material e Métodos:

2.1. Desenho das sondas

A etapa inicial do trabalho consistiu no desenho das sondas e confirmação da sua especificidade através da comparação com sequências de RNA ribossômico (RNAr) 16S existentes na base de dados RDP (Ribosomal Database Project, <http://www.cme.msu.edu/RDP>).

As sequências de RNAr 16S, recuperadas sob a forma alinhada, foram então inseridas no programa GDE (Genetic Data Environment, V. 2.2; gopher://megasun.bch.umontreal.ca:70/11/GDE). O alinhamento das sequências foi verificado visualmente e estas foram então comparadas, procurando-se regiões potencialmente adequadas para a construção de sondas homólogas às sequências de *Bradyrhizobium* sp. disponíveis na base de dados, e capazes de discriminar estas de grupos filogeneticamente relacionados.

2.2. Linhagens de bactérias e condições de crescimento

As linhagens bacterianas empregadas nesta etapa do trabalho estão listadas na Tabela 1 e foram cedidas pela Coleção de Culturas Tropical (CCT - Fundação André Tosello, Campinas - SP).

Tabela 1. Linhagens bacterianas empregadas no estudo.

Bactéria	Código*	Código em outras coleções**	Planta Hospedeira
Linhagens referência			
<i>Bradyrhizobium elkanii</i>	CCT 4068	BR 29/ LMG 9520	<i>Glycine max</i> L.
	CCT 4066	BR 96	<i>Glycine max</i> L.
<i>Bradyrhizobium</i> sp.	CCT 4039	BR 4406/ LMG 9980	<i>Enterolobium ellipticum</i>
	CCT 5266	BR 3621/ LMG 9966	<i>Acacia mangium</i>
	CCT 5483	BR 6011/ LMG 9514	<i>Lonchocarpus costatus</i>
<i>Bradyrhizobium japonicum</i>	CCT 4182	BR 110/ LMG 6136	<i>Glycine max</i> L.
	CCT 4184 ^T	BR 111 ^T / LMG6138 ^T	<i>Glycine max</i> L.
<i>Rhizobium loti</i>	CCT 4159	BR 10057/ LMG 4284	<i>Caragana arborescens</i>
	CCT 4063 ^T	BR 7801 ^T /LMG 6125 ^T	<i>Lotus corniculatus</i>
<i>Rhodopseudomonas palustris</i>	CCT 5355 ^T	ATCC 17001 ^T	-
Isolados			
<i>Bradyrhizobium</i> sp.	CCT 4191	INPA 46B/ LMG 10043	<i>Clitoria racemosa</i>
	CCT 4270	INPA 183B/ LMG 10075	<i>Pentaclethra macroloba</i>
	CCT 4117	INPA 223A/ LMG 10085	“
	CCT 4233	INPA 549A/ LMG 10118	<i>Tachigalia paniculata</i>
	CCT 4219	INPA 609A/ LMG 10123	<i>Derris negrensis</i>
	CCT 4661	FL 276/ LMG 10025	<i>Abrus</i> sp.
	CCT 4118	BR 5609/ LMG 9997	<i>Albizia falcata</i>
	CCT 4119	BR 5611/ LMG 9998	“
	CCT 6186, CCT 6187,	-	***
	CCT 6189, CCT 6191,		
	CCT 6193, CCT 6194,		
	CCT 6197, CCT 6199,		
	CCT 6200, CCT 6201,		
	CCT 6203, CCT 6204,		
	CCT 6205, CCT 6207,		
	CCT 6212, CCT 6213,		
	CCT 6214, CCT 6215,		
	CCT 6216, CCT 6217,		
	CCT 6220, CCT 6222,		
	CCT 6223, CCT 6229,		
	CCT 6231, CCT 6235,		
	CCT 6239, CCT 6242		

*CCT, Coleção de Culturas Tropical, Fundação André Tosello (Campinas, SP, Brazil). **BR e FL, Coleção de Culturas Embrapa Agrobiologia (Seropédica, RJ, Brazil); INPA, Coleção de Culturas do Instituto Nacional de Pesquisas da Amazônia; ATCC, American Type Culture Collection (Manassas, EUA); IAM, Institute of Applied Microbiology (Tokyo, Japão); LMG, Laboratorium voor Microbiologie (Univ. Gent, Bélgica). ***Linhagens isoladas de solo de Cerrado, no estado de São Paulo, empregando-se guandu (*Cajanus cajan* L.) como planta-isca (Artigo 3).

Condições de crescimento: Para a extração de DNA das culturas bacterianas, realizada segundo Pitcher *et al.* (1989), as linhagens de *Rhizobium* e *Bradyrhizobium* foram cultivadas em 3 mL de meio YM (KH₂PO₄, 0,5 g; MgSO₄ . 7H₂O, 0,2 g; NaCl, 0,1 g; extrato de levedura, 0,5 g; manitol, 10,0 g; solução de azul de bromotimol, 0,5%; água destilada qsp. 1000 mL). O cultivo se deu por 48 horas a 28°C sob agitação. A linhagem de *Rhodopseudomonas palustris* utilizada foi cultivada em 3 mL de YB (KH₂PO₄, 1,0 g; MgSO₄, 0,5 g; extrato de levedura, 10,0 g; água qsp. 1000 mL), em condições de anaerobiose e luz a 30°C por 48 horas.

2.3. Amplificação do DNAr 16S

O DNA genômico de linhagens tipo e referência foi submetido à reação de amplificação do gene para RNAr 16S visando posterior hibridização com a sonda #5.

Primers: os *primers* utilizados para amplificar o DNA das linhagens bacterianas são homólogos a regiões conservadas situadas no início do gene para RNAr 16S, *primer* p27f (9-27, posição relativa à sequência de *E. coli*) (Massol-Deya *et al.*, 1995), e no final deste gene, *primer* p1401r (1401-1378, relativo a *E. coli*) (Heuer *et al.*, 1997).

Condições do PCR: Foram utilizados 50 ng de DNA de cada uma das linhagens em reações de 50 µl contendo 2,0 U de *Taq* polimerase (CENBIOT, RS); 1X Tampão da *Taq* (670 mM de Tris, pH 8,8); 2,0 mM de MgCl₂; 0,2 mM de uma mistura de dNTP e 0,4 µM de cada *primer*. O programa utilizado para a amplificação consistiu de 1 ciclo a 95°C por 2 min., 30 ciclos a 94°C/1 min., 60°C/1 min. e 72°C/3 min., e 1 ciclo a 72°C por 5 min., em ciclizador térmico Perkin Elmer modelo 9600.

2.4. Southern Blot

O oligonucleotídeo #5 foi marcado com digoxigenina na extremidade 3' de maneira a formar uma cauda (3'- *Tailing*), empregando-se DIG-dUTP e transferase terminal (GIBCO-BRL), de acordo com especificações do fabricante. As reações de marcação (20 µl) continham 15 µM do oligonucleotídeo; 50 U da enzima transferase terminal; 0,5 mM de

dATP e 0,05 mM de DIG-dUTP (Boehringer-Mannheim) em tampão de reação 1 X. A reação foi realizada a 37°C por 16 hs.

A especificidade da sonda # 5 foi avaliada através de hibridização com DNAr 16S de linhagens referência e isolados de *Bradyrhizobium* sp. (Tabela 1).

O DNAr 16S amplificado foi submetido à eletroforese em gel de agarose 1,2%, desnaturado e transferido à vacuo (*VacuGene XL*-Pharmacia) para membranas de nylon (Boehringer-Mannheim), de acordo com especificações do fabricante. A concentração de sonda utilizada foi de 50 pmol/mL de solução de hibridização. As hibridizações foram realizadas a 50°C por 18 hs, seguidas por 2 lavagens à temperatura ambiente por 15 min. e uma lavagem estridente a 61°C por 3 min., utilizando 6X SSC. A detecção do sinal de hibridização se deu por quimioluminescência (*DIG Luminescent Detection Kit*, Boehringer-Mannheim), usando CSPD, e exposição a filme raio-X por 1 min.

2.5. Amostras de DNA do solo

As amostras de DNA da comunidade bacteriana do solo utilizadas nos experimentos que se seguem foram as mesmas descritas no **Artigo 2**. Cabe ressaltar que as amostras de DNA foram mantidas congeladas a -20°C.

2.6. PCR do operon RNAr de *Bradyrhizobium* sp. a partir de DNA ambiental

Parte do operon RNAr, incluindo o gene para RNAr 16S e a região espaçadora 16S-23S, foi amplificada a partir do DNA da comunidade de microrganismos extraído de duas amostras de solo, **Pastagem** e **Tempo Zero**, visando posterior clonagem, seleção por *Colony blot* e análise de diversidade por PCR-RFLP.

Primers: os *primers* utilizados, homólogos a regiões conservadas do DNAr para a maioria dos organismos do domínio *Bacteria*, foram o p27f e o p23Suni322anti (Honeycutt *et al.*, 1995).

Condições do PCR: foram utilizados 5 µL de uma diluição 1:10 da amostra de DNA do solo em reações de 50 µL contendo 2,0 U de *Taq* polimerase (CENBIOT, RS); tampão da enzima *Taq* 1 X; 2,0 mM de MgCl₂; 0,2 mM de uma mistura de dNTP; e 0,4 µM de cada *primer*. O programa utilizado para a amplificação consistiu de 1 ciclo a 95°C por 2 min., 30 ciclos a 94°C/1 min., 55°C/1 min. e 72°C/3 min., e 1 ciclo a 72°C por 5 min., em ciclizador térmico Perkin Elmer modelo 9600. Os produtos de PCR foram visualizados em gel de agarose 1,2% corado com brometo de etídio.

2.7. Banco das sequências do operon RNAr amplificadas do DNA ambiental

As sequências do operon RNAr amplificadas a partir do DNA extraído da amostra de solo **Tempo Zero** foram clonadas utilizando-se o vetor pGEM-T (Promega), segundo especificações do fabricante. Os produtos de PCR, com cerca de 3000 pb, foram submetidos diretamente a reações de ligação com o vetor, sem purificação prévia.

2.8. Colony blot

Os transformantes obtidos foram selecionados através de hibridização por *Colony blot* com a sonda #5 para o grupo *Bradyrhizobium* sp./*B. elkanii*, visando posterior análise de diversidade por PCR-RFLP.

As colônias bacterianas foram crescidas por cerca de 18 hs a 37°C, até atingirem um diâmetro de 1-2 mm. Após o crescimento, as placas foram esfriadas por 1 h a 4°C. As colônias foram, a seguir, transferidas para membranas de nylon (Boehringer-Mannheim). As membranas foram submetidas à desnaturação por 15 min. sobre papel de filtro saturado com solução desnaturante (NaOH 0,5 N; NaCl 1,5 M; SDS 0,1%), transferidas para uma outra folha de papel de filtro saturada com solução de neutralização (Tris-HCl 1,0 M, pH 7,5; NaCl 1,5 M), onde foram deixadas por 5 min., e, por último, colocadas por 15 min. sobre papel de filtro saturado com solução 2X SSC (20X SSC = NaCl 17,5%; citrato de sódio 8,8%; pH 7,0). O DNA foi fixado à membrana por UV-*crosslinking*. Antes de proceder à hibridização, os restos celulares das colônias foram removidos lavando-se as membranas com 5X SSC/0,5% SDS/1 mM Na₂EDTA por 2 hs a 50°C, com agitação. A hibridização das membranas com a sonda # 5 e a detecção foram processadas como descrito anteriormente no item 2.4.

Com a finalidade de se obter controles positivo e negativo para os experimentos de hibridização por *Colony blot*, o DNAr 16S foi amplificado a partir de cultura pura das linhagens *B. japonicum* CCT 4182 (controle negativo) e *Bradyrhizobium* sp. CCT 4039 (controle positivo), utilizando-se o par de *primers* universal p27f e L1401r. Foram utilizados 50 ng de DNA de cada uma das linhagens em reações de 50 µL contendo 2,0 U de *Taq* polimerase (Pharmacia); 1X Tampão da *Taq* (Pharmacia); 2,0 mM de MgCl₂; 0,2 mM de uma mistura de dNTP e 0,2 µM de cada *primer*. O programa utilizado para a amplificação consistiu de 1 ciclo a 95°C por 2 min., 30 ciclos a 94°C/1 min., 60°C/1 min. e 72°C/3 min., e 1 ciclo a 72°C por 5 min., em ciclizador térmico Perkin Elmer modelo 9600. A clonagem dos produtos de PCR foi realizada diretamente, sem purificação prévia, empregando-se o vetor pGEM-T (Promega). A presença do inserto foi confirmada através de *miniprep* plasmidial, segundo protocolo da Promega, e digestão com enzima de restrição

Pst I (Amersham) a 37°C por 2 h. Em cada uma das placas a serem testadas por *Colony blot*, foram crescidos um controle positivo e um negativo.

2.9. PCR do DNAr 16S a partir de DNA ambiental

O gene para RNAr 16S foi amplificado a partir do DNA da comunidade bacteriana dos solos **Pastagem**, **Tempo Zero** e **Plantio Direto**, a fim de se proceder à hibridização por *Slot blot* com a sonda #5. Os *primers* utilizados, assim como as condições de reação, foram as mesmas descritas no item anterior, à exceção de que a concentração de $MgCl_2$ utilizada foi 3,0 mM e a temperatura de anelamento dos *primers* no programa de amplificação foi de 55°C. Ainda, a solução de DNA do solo foi diluída 1:10 e 5 µL dessa diluição foram utilizados no PCR. O DNAr 16S das linhagens empregadas como controle positivo e negativo nos experimentos de *Slot blot*, *B. elkanii* CCT 4066 e *R. loti* CCT 4159, respectivamente, foi amplificado como descrito no item acima.

2.10. Slot blot

O produto de PCR do DNAr 16S da amostra do solo **Tempo Zero**, assim como das linhagens controle, foi primeiramente quantificado através de espectrofotometria (*GeneQuant*, Pharmacia) e as concentrações confirmadas através de eletroforese em gel de agarose 0,8% corado com brometo de etídio (0,1 µg/mL).

O DNA foi, a seguir, diluído para as concentrações de 50 ng, 10 ng, 5 ng, 2 ng, 1 ng, 500 pg, 200 pg e 100 pg, em solução de SSC 10X (20X SSC = NaCl 17,5%; citrato de sódio 8,8%; pH 7,0) em um volume final de 200 µL. As amostras de diferentes concentrações de DNA foram desnaturadas a 95°C por 10 min., aplicadas nas canaletas do aparato de *Slot blot* (*Hybri-Slot*TM, Gibco-BRL) e transferidas à vácuo, de acordo com as especificações do fabricante, para membrana de nylon (*Hybond-N*⁺, Amersham). A membrana foi posteriormente desnaturada por 20 min. sobre papel de filtro saturado com solução de desnaturação (NaOH 0,5 N; NaCl 1,5 M; SDS 0,1%) e transferida para uma outra folha de papel saturada com solução de neutralização (Tris-HCl 1,0 M, pH 7,5; NaCl 1,5 M). O DNA foi fixado à membrana por UV-crosslinking. A hibridização das membranas com a sonda # 5 e a detecção foram processadas como descrito anteriormente no item 2.4.

2.11. PCR específico usando o oligonucleotídeo #5 como forward primer

- *Teste de condições de amplificação com linhagens referência*

Primers: os *primers* utilizados, foram o oligonucleotídeo #5 como *forward primer* (posição 917 dentro do 16S, relativo à sequência de *Bradyrhizobium* sp., este estudo), e o *primer* universal L1401r.

Condições do PCR: as concentrações dos reagentes utilizados nas reações de PCR foram as mesmas descritas no item 2.8. Entretanto, vários perfis de temperatura, com programação de *touch down*, foram testados como descrito a seguir:

- i) *Touch down* 65-55°C- 1 passo de desnaturação inicial a 94°C/2 min.; 10 ciclos a 94°C/1 min, anelamento/1 min. e 72°C/2 min., onde a temperatura de anelamento abaixou de 2 em 2 graus a cada 2 ciclos, de 65° para 57°C; 30 ciclos a 94°C/1 min, 55°C/1 min. e 72°C/2 min.; e um passo de extensão final a 72°C por 10 min.;
- ii) *Touch down* 70-60°C – ídem ao perfil anterior, à exceção de que a temperatura de anelamento abaixou de 2 em 2 graus de 70 para 60°C;
- iii) *Touch down* 75-65°C; e
- iv) *Touch down* 68-62°C, cujo perfil consistia de 1 passo de desnaturação inicial a 94°C/2 min.; 9 ciclos a 94°C/1 min, anelamento/1 min. e 72°C/1 min., onde a temperatura de anelamento abaixou de 2 em 2 graus a cada 3 ciclos, de 68° para 64°C; 31 ciclos a 94°C/1 min, 62°C/1 min. e 72°C/1 min.; e um passo de extensão final a 72°C por 10 min.

- *Nested-PCR do DNA ambiental* (Figura 1)

Primers: o par de *primers* utilizado no primeiro PCR (de “enriquecimento”), a partir do DNA do solo, foi p27f e L1401r, visando a amplificação do DNAr 16S da comunidade bacteriana total do solo. No PCR específico, o par de *primers* utilizado foi o p5f (sonda #5 usada como *forward primer*) e o L1401r, visando a amplificação específica de fragmentos do DNAr 16S do grupo *Bradyrhizobium* sp./*B. elkanii* (Figura 1).

Condições do PCR: no primeiro PCR, as condições de reação foram as mesmas descritas no item 2.9, inclusive o perfil de temperatura empregado. Para a amplificação específica, 1 µL do produto de PCR de enriquecimento foi usado como DNA-molde na reação. As concentrações dos outros reagentes foram as mesmas descritas no item 2.8. O perfil de temperatura empregado foi aquele que apresentou melhor resultado no teste de condições com linhagens-referência.

Todas as reações de amplificação foram realizadas em ciclizador térmico Perkin Elmer 480 (Nieuwerkerk a/d IJssel, The Netherlands). Os produtos de PCR foram visualizados em gel de agarose 1,2% corado com brometo de etídio.

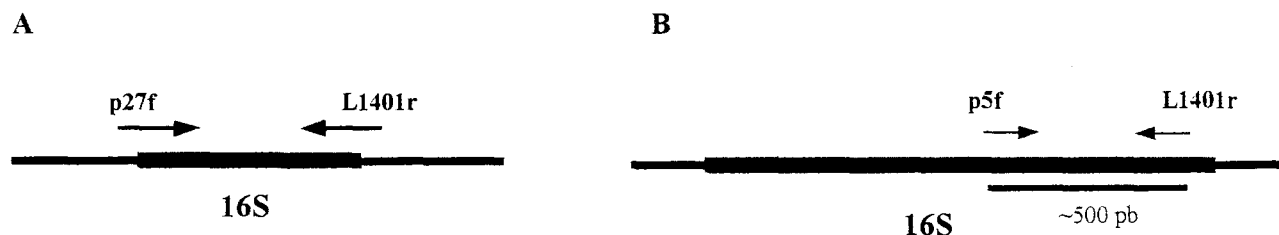


Figura 1 – Esquema ilustrando *nested-PCR* do DNA ambiental. Painel (A): PCR de enriquecimento do DNAr 16S a partir do DNA do solo, usando os *primers* p27f e L1401r. Painel (B): PCR específico para o grupo *Bradyrhizobium* sp./*B. elkanii* a partir do produto do PCR de enriquecimento, usando os *primers* p5f e L1401r.

2.12. PCR específico usando o oligonucleotídeo #5 como reverse primer

Primers: o *primer* p27f foi usado como *forward primer* e o oligonucleotídeo #5 como *reverse primer*.

Condições do PCR: foram utilizados 50 ng de DNA genômico de cada uma das linhagens, extraído e purificado segundo Pitcher *et al.* (1989), em reações de 50 µl contendo 1,0 U de *Taq* polimerase (CENBIOT, RS); 1X Tampão da *Taq* (670 mM de Tris, pH 8,8); 2,0 mM de MgCl₂; 0,2 mM de uma mistura de dNTP e 0,2 µM de cada *primer*. O programa utilizado para a amplificação consistiu de 1 ciclo a 95°C por 2 min., 30 ciclos a 94°C/1 min., 75°C/30 seg. e 72°C/1 min., em ciclizador térmico Perkin Elmer modelo 9600.

2.13. Amplificação da região espaçadora 16S-23S

Primers: os *primers* utilizados, homólogos a regiões conservadas do DNAr para a maioria dos organismos do domínio *Bacteria*, foram o pHr (1518-1541 dentro do 16S, posição relativa à sequência de *E. coli*), e o p23Suni322anti (posição 322 dentro do 23S, relativo a *E. coli*) (Honeycutt *et al.*, 1995).

Condições do PCR: as condições de reação utilizadas, assim como a concentração dos reagentes, foram as mesmas descritas anteriormente para a amplificação do DNAr 16S no item 1.3.

2.14. Restrição enzimática da região espaçadora 16S-23S

Os produtos de PCR correspondentes às sequências da região espaçadora 16S-23S das linhagens pertencentes ao grupo de *Bradyrhizobium* foram submetidos à restrição enzimática por 18hs a 37 °C. As enzimas empregadas foram *Pst*I, *Bcl*II, *Hind*III, *Bam*HI e *Eco*RV (Pharmacia).

3. Resultados e Discussão:

3.1. Desenho das sondas

Dezenove sequências de RNAr 16S, incluindo sequências parciais e integrais de *Bradyrhizobium*, foram recuperadas do RDP (<http://www.cme.msu.edu/RDP>). Como a finalidade foi se obter uma sonda específica para o grupo de *Bradyrhizobium* sp., de grande ocorrência nos trópicos, e capaz de discriminar grupos relacionados, 14 sequências de RNAr 16S de espécies filogeneticamente próximas (com base na filogenia construída a partir de análises de sequências de RNAr 16S do próprio RDP), consideradas como *outgroups*, foram também recuperadas. Por fim, foram recuperadas ainda três sequências do gênero *Photrhizobium*, proposto por Eaglesham *et al.* (1990) e filogeneticamente muito próximo de *Bradyrhizobium* (Young *et al.*, 1996).

Duas sondas de oligonucleotídeo foram desenhadas com base na comparação das sequências de RNAr 16S alinhadas (Tabela 2), e testadas contra todas as sequências de RNAr 16S disponíveis no RDP através da rotina *CHECK-PROBE*, com um número máximo permitido de 4 *mismatches*.

Tabela 2 - Sequências das sondas potenciais para RNAr 16S de *Bradyrhizobium* sp. e possíveis *mismatches* com sequências de espécies relacionadas.

Sequência da sonda (5' → 3')	Nº. da sonda	nt	Espécies-alvo	Espécies com <i>mismatches</i>	Nº. de <i>mismatches</i>
ccg tct ctg gag tcc gcg acc	#5	21	<i>Bradyrhizobium</i> sp./	<i>R. loti</i>	3
			<i>B. elkanii</i>	<i>Mycoplana dimorpha</i>	4
gaa ggg tcg cc	#7	11	<i>B. japonicum</i> /	<i>N. hamburgensis</i> /	3
			<i>Bradyrhizobium</i> sp./	<i>N. winogradskyi</i>	
			<i>Rhodopseudomonas</i> <i>palustris</i>	<i>A. spiritensis</i>	4

3.2. Southern Blot

Apenas a especificidade da sonda #5 (Figura 2) foi avaliada através de hibridização com DNAr 16S de linhagens-referência e isolados de *Bradyrhizobium* sp. (Tabela 1), uma vez que esta abrange rizóbios de ocorrência tropical e potencialmente discrimina *Bradyrhizobium* sp. de *Rhodopseudomonas palustris* (Tabela 2).

	1511	1521	1531	1541	1551	1561	1571
Afp.felis	UUGACA-UG	-----	U	CCAGG	ACCGGU-C	GCA-GAGA-UGU-G	ACCUU-
Aqb.spirit			G	AG	-GAU..U	C.....	G.A.U.UC..
Azr.cauli3			G	AG	-GAC..U	C.G.....	C.G.A.UUUC..
Bdr.japon2							
Bdr.japon5							
Bdr.japoni							
Bdr.jpnl36							
Bdr.sp0689		C		GGUC	G.G.AC.U	C.....	C.G.AGUU....
Bdr.sp9514		C		GGUC	G.G.AC.U	C.....	C.G.AGUU....
Bdr.sp9520		C		GGUC	G.G.AC.U	C.....	C.G.AGUU....
Bdr.sp9966		C		GGUC	G.G.AC.U	C.....	C.G.AGUU....
Bdr.sp9980		C		GGUC	G.G.AC.U	C.....	C.G.AGUU....
Phr.MKAa_2				UUG			C.....-
Phr.sp23				UUG			C.....-
Phr.thps				UUG			C.....-
Rps.palus2							
Rps.palus3					V	V	C.C.....
Rps.palus5				A	-		C.C.....
Rps.palust							C.C.....
Ntb.hambur				U			
Ntb.winogr				U			
Cau.FWC14			C	UG	-A.C.C	A	GNN.-
Lib.spNels	A			GUU	-A.A.A	U	A.U.UU.-
Mlb.extorq			G	AU	-UUACC.U	CG	CG...GnA.-
Mlb.rhode2			G	AU	-UUACC.U	CG	CG...GGA.-
Rhh.orient	C		C	G.U	-C.G.G.U.A	C.G	C.G.U.UU.C.
Rps.marin2	C		C	G.U	-C.G.G.U.A	C.G	C.G.U.U.C.

Figura 2 - Alinhamento e comparação de sequências de RNAr 16S; os nucleotídeos em destaque ilustram a região alvo da sonda #5 para o grupo *Bradyrhizobium* sp./*B. elkanii*. Denominações no lado esquerdo do alinhamento referem-se ao código do RDP para o organismo correspondente a cada sequência alinhada. Abreviações: Afp. = *Azotobacter*; Aqb. spirit = *Aquabacter spiritensis*; Azr. cauli = *Azorhizobium caulinodans*; Bdr. japoni = *Bradyrhizobium japonicum*; Bdr. sp = *Bradyrhizobium* sp.; Phr. = *Photobacterium*; thps = *thompsoniano*; Rps. palust = *Rhodopseudomonas palustris*; marin = *marina*; Ntb. hambur = *Nitrobacter hamburgensis*; winogr = *winogradskyi*; Cau. = *Caulobacter*; Lib. = *Liberobacter*; Mlb. extorq = *Methylobacterium extorquens*; rhode = *rhodesianum*; Rhh. orient = *Rhodohalobium orientum*. “.” significa o mesmo nucleotídeo que aquele especificado na primeira sequência do alinhamento; “-” significa ausência de nucleotídeo naquela posição específica; os números na parte superior do alinhamento indicam a posição dos nucleotídeos dentro do RNAr 16S. Os hospedeiros e locais de origem dos rizóbios correspondentes às sequências de RNAr Bdr. sp0689, Bdr. sp9514, Bdr. sp9520, Bdr. sp9966 e Bdr. sp9980 são *Acacia alba*/norte do Senegal, *Lonchocarpus costatus*/Brasil, desconhecido/Brasil, *Acacia mangium*/Brasil e *Enterolobium ellipticum*/Brasil, respectivamente.

O DNAr 16S amplificado das linhagens-referência de *Bradyrhizobium* usadas como controles positivos mostrou sinal de hibridização com a sonda #5 (Figura 3/A, canaletas 1 a 4). Por outro lado, nenhum sinal de hibridização foi detectado com o DNAr 16S de linhagens de *Bradyrhizobium japonicum*, *Rhodopseudomonas palustris* ou *Rhizobium loti* (Figura 3/A, canaletas 5 a 9), usadas como controles negativos, como esperado. Cabe salientar que a sequência do DNAr 16S de *R. loti*, homóloga à sequência de 21 nt da sonda, apresenta apenas 3 *mismatches* com esta (Tabela 2). Isto demonstra que as condições de estringência utilizadas durante a hibridização com a sonda # 5 foram adequadas para eliminar o sinal de hibridização das linhagens desta espécie utilizadas. Dentre 28 isolados de rizóbios capturados usando-se guandu (Tabela 1), vinte mostraram sinal positivo de hibridização com a sonda #5 (Figura 3/A, canaletas 10 a 12, 15, 17 a 23; 2/B, canaletas 1 a 5, 7, 9 a 11). Dos oito isolados (CCT 6191, CCT 6193, CCT 6197, CCT 6217, CCT 6222, CCT 6229, CCT 6239 e CCT 6242) que não hibridizaram com a sonda (Figura 3/A, canaletas 13, 14 e 16; Figura 3/B, canaletas 6, 8, 12 a 14), apenas 3 isolados (CCT 6191, CCT 6193 e CCT 6197) apresentam características fenotípicas típicas de *Bradyrhizobium* sp., como crescimento lento, colônias brancas e pequenas, e alcalinização em meio de cultivo YM ágar. Os outros 5 isolados apresentam características típicas do gênero *Rhizobium*, ou seja, crescimento rápido, colônias amarelas, bastante gomosas e grandes, e reação de acidificação do meio, o que explica o sinal negativo de hibridização com a sonda #5. Estes resultados estão apresentados sob a forma de tabela no **Artigo 3**. Ainda, a figura 3/B (canaletas 15 a 22) mostra sinal positivo de hibridização para o DNAr 16S de 8 linhagens de *Bradyrhizobium* sp. (CCT 4191, CCT 4270, CCT 4117, CCT 4233, CCT 4219, CCT 4661, CCT 4118 e CCT 4119) isoladas de leguminosas arbóreas da Amazônia e Mata Atlântica (Moreira *et al.*, 1993).

Portanto, de um total de 23 isolados com características fenotípicas que suportam sua classificação preliminar dentro do gênero *Bradyrhizobium*, apenas 3 isolados não hibridizaram com a sonda #5. A análise da sequência de RNAr 16S destes isolados poderia indicar a sua posição taxonômica real.

Os resultados obtidos demonstram a especificidade da sonda #5 para linhagens de *Bradyrhizobium* sp., assim como para *B. elkanii*, e permitem o uso desta sonda na análise da diversidade destes rizóbios em amostras ambientais, como mostrarão experimentos posteriores.



Figura 3 - *Southern blot* com a sonda #5 do DNAr 16S amplificado a partir de culturas puras de linhagens referência e de isolados utilizando o par de *primers* 27f/1401r. Canaletas: (A) 1 e 4, *Bradyrhizobium elkanii* CCT 4068 e CCT 4066; 2 e 3, *Bradyrhizobium* sp. CCT 4039 e CCT 5266; 5 e 6, *Bradyrhizobium japonicum* CCT 4182 e CCT 4184^T; 7, *Rhodopseudomonas palustris* CCT 5355^T; 8 e 9, *Rhizobium loti* CCT 4159 e CCT 4063^T; 10 a 23, isolados CCT 6186, CCT 6187, CCT 6189, CCT 6191, CCT 6193, CCT 6194, CCT 6197, CCT 6199, CCT 6200, CCT 6201, CCT 6203, CCT 6204, CCT 6205 e CCT 6207, respectivamente. (B) 1 a 22, isolados CCT 6212, CCT 6213, CCT 6214, CCT 6215, CCT 6216, CCT 6217, CCT 6220, CCT 6222, CCT 6223, CCT 6231, CCT 6235, CCT 6229, CCT 6239, CCT 6242, CCT 4191, CCT 4270, CCT 4117, CCT 4233, CCT 4219, CCT 4661, CCT 4118 e CCT 4119, respectivamente.

3.3. PCR do operon RNAr de *Bradyrhizobium* sp. a partir de DNA ambiental

A figura 4 ilustra a amplificação de parte do operon RNAr, abrangendo o RNAr 16S e a região espaçadora 16S-23S, da comunidade bacteriana das amostras de solo **Pastagem** e **Tempo zero**. As reações de PCR foram realizadas a partir de cada duplicata da extração de DNA das amostras de solo, sendo que os fragmentos gerados apresentaram cerca de 3 kb. Entretanto, a amplificação não foi muito eficiente, como refletem as bandas de intensidade fraca (Figura 4, canaletas 2, 3 e 5). Isto provavelmente se deu devido à existência de compostos inibidores da polimerase *Taq* nas preparações de DNA do solo, e poderia ser superado com o emprego da proteína p32 do fago T4. A amostra A de DNA do solo **Tempo zero** gerou uma banda de amplificação mais intensa (canaleta 4). Apenas este produto de amplificação, cuja concentração estimada em gel de agarose foi de cerca de 20

ng/μL, foi utilizado nos experimentos preliminares que se seguem de clonagem e análise dos transformantes por *Colony blot*.

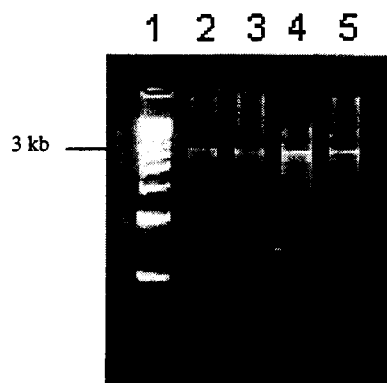


Figura 4 - Amplificação de parte do operon RNAr a partir do DNA da comunidade bacteriana do solo. Canaletas: 1, marcador de peso molecular 100 pb; 2 a 5, PCR das duplicatas da extração de DNA das amostras de solo **Pastagem** e **Tempo zero**, nesta ordem.

3.4. Banco das sequências do operon RNAr amplificadas do DNA ambiental

Um total de 2150 colônias, obtidas da clonagem das sequências de DNAr amplificadas a partir do DNA do solo **Tempo zero**, foi transferido para placas de Petri contendo LB, ampicilina, IPTG e X-gal, a fim de se confirmar a interrupção do gene que codifica para a enzima β-galactosidase e se proceder ao teste de hibridização com a sonda #5 por *Colony blot*.

3.5. Colony blots

Um total de 2.000 colônias de bactérias transformantes selecionadas nos experimentos de clonagem das sequências do operon RNAr foi analisado através de hibridização com a sonda #5 por *Colony blot*. Apenas **uma** colônia transformante apresentou sinal positivo de hibridização com a sonda, detectada através de quimioluminescência (Figura 5), representando uma frequência de 0,05%. Os clones contendo DNAr dos organismos utilizados como controle nas membranas (CCT 4039, controle positivo, e CCT 4182, controle negativo) apresentaram sinais de hibridização conforme o esperado, indicando que a baixa frequência de sinais positivos encontrada era realmente reflexo do baixo número de cópias do DNAr-alvo na população de fragmentos clonados.

Não foi possível, portanto, proceder com a estratégia previamente proposta de avaliação de diversidade do grupo *Bradyrhizobium* sp./*B. elkanii* através da análise de

RFLP do operon RNAr destes organismos. Se considerarmos uma quantidade mínima de 20 clones para realização de uma análise de diversidade, uma amostragem de pelo menos 40.000 clones deveria ser testada com a sonda #5, o que torna inviável a condução do estudo utilizando a estratégia de clonagem em plasmídeo empregada. Neste caso, a clonagem em fagos e posterior seleção de placas de lise poderia ser uma alternativa.

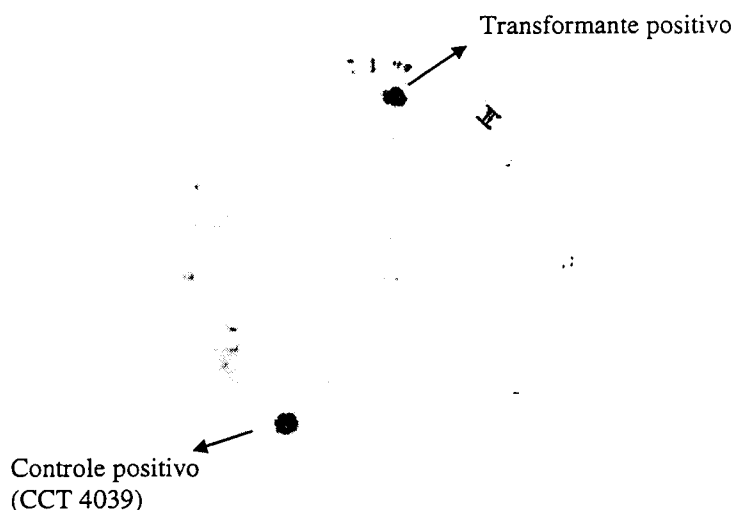


Figura 5 – *Colony blot* com a sonda #5 de 50 transformantes resultantes do banco das seqüências do operon RNAr amplificado a partir do DNA ambiental.

Dados de literatura indicam que a proporção de rizóbios é geralmente menor que 0,01% da população bacteriana total do solo (Heuer & Smalla, 1997). Considerando uma população bacteriana hipotética ao redor de 10^6 em 1 grama de solo, a frequência de rizóbios seria ao redor de 100 células. A frequência de clones positivos detectada neste experimento (0,05%) encontra-se na mesma ordem de grandeza e corrobora os resultados encontrados na literatura.

3.6. Slot blots

O gene RNAr 16S foi amplificado a partir do DNA da comunidade bacteriana das amostras de solo **Pastagem**, **Tempo Zero** e **Plantio Direto**, como um passo inicial nos experimentos de detecção e quantificação do sinal de hibridização com a sonda #5 por *Slot blot*. A Figura 6 ilustra a amplificação do DNAr 16S a partir das amostras ambientais, a qual mostrou-se eficiente, como refletido pelas bandas de intensidade forte, com tamanho em torno de 1,5 kb. Entretanto, houve amplificação inespecífica de uma banda com cerca de 2,5 kb. Esta banda não foi mais visualizada após diluição dos produtos de PCR e

eletroforese em gel de agarose, realizada visando a quantificação do produto e posterior hibridização por *Slot blot* (dados não apresentados). A concentração do DNAr 16S da amostra de solo foi estimada em cerca de 75 ng/ μ L, ao passo que das linhagens referência foi em torno de 100 ng/ μ L.

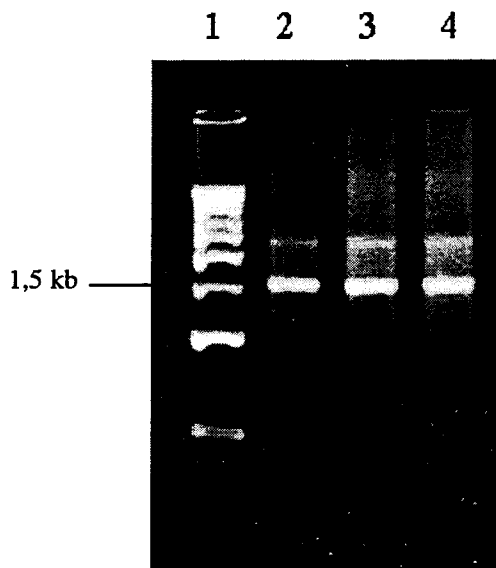


Figura 6 – Amplificação do DNAr 16S a partir do DNA da comunidade bacteriana do solo. Canaletas: 1, marcador de peso molecular Kilobase (Pharmacia); 2 a 4, 5 μ L do produto de PCR dos solos **Pastagem**, **Tempo Zero** e **Plantio Direto**, nesta ordem.

Os resultados obtidos com os experimentos de *Slot blot* revelaram sinal positivo de hibridização do DNAr 16S do solo **Tempo Zero** com a sonda #5 (Figura 7). O sinal de hibridização de 2 ng do DNAr 16S ambiental correspondeu ao sinal de hibridização de 200 pg detectado para a linhagem controle positiva *B. elkanii* CCT 4066, sugerindo que, do total de DNAr 16S amplificado a partir da amostra de solo **Tempo Zero**, aproximadamente 10% é representado por DNAr 16S do grupo *Bradyrhizobium* sp./*B. elkanii* (Figura 7/A). Entretanto, este resultado não pode ser considerado absoluto, uma vez que o DNAr 16S da linhagem controle negativa *Rhizobium loti* CCT 4159 (que apresenta 3 *mismatches* com a sequência da sonda) também apresentou sinal de hibridização com a sonda #5. Neste caso, o sinal de hibridização de 2 ng do DNAr 16S da linhagem controle negativa correspondeu ao sinal de hibridização de 100 pg detectado para a linhagem controle positiva, o que não pode ser muito bem percebido pela figura 7/B, mas é visível no filme original de detecção da hibridização.

Portanto, muito provavelmente, parte do sinal de hibridização detectado na amostra de solo deve ser devida ao anelamento inespecífico da sonda com DNA-alvo de outros

grupos componentes da comunidade bacteriana que não *Bradyrhizobium* sp./*B. elkanii*. Este experimento foi repetido duas vezes, e os mesmos resultados foram encontrados.

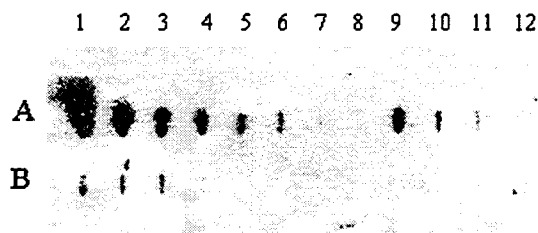


Figura 7 – Slot blot do DNAr 16S com a sonda #5. (A) Canaletas 1 a 8: DNAr 16S amplificado a partir de cultura pura da linhagem controle positiva CCT 4066 de *B. elkanii* nas concentrações de 50 ng, 10 ng, 5 ng, 2 ng, 1 ng, 500 pg, 200 pg e 100 pg, nesta ordem; canaletas 9 a 12: DNAr 16S amplificado a partir de cultura pura da linhagem controle negativa CCT 4159 de *Rhizobium loti* nas concentrações de 50 ng, 10 ng, 5 ng e 2 ng, nesta ordem. (B) DNAr 16S amplificado a partir do DNA da comunidade bacteriana da amostra de solo **Tempo Zero** nas concentrações de 50 ng, 10 ng, 5 ng, 2 ng, 1 ng, 500 pg, 200 pg e 100 pg, nesta ordem.

Apesar do experimento ter sido realizado três vezes, nas condições previamente estabelecidas para uso da sonda #5, e ainda com temperaturas de lavagem mais estridentes, os resultados não são conclusivos, e seria necessário a realização de experimentos adicionais e ajustes nas condições de lavagem para remoção do sinal de hibridização inespecífico.

Embora não conclusivos, os resultados estão de acordo com o que foi encontrado nos experimentos de *Colony blot*, ou seja, a proporção de *Bradyrhizobium* sp./*B. elkanii* com relação às outras bactérias presentes no solo é muito baixa. Neste caso, a amplificação por PCR do DNAr 16S das bactérias presentes no solo tende a aumentar ainda mais o número das moléculas que são mais abundantes, as quais são competitivamente favorecidas como alvos na reação em relação às moléculas-molde menos abundantes.

Resultados similares foram encontrados por Heuer *et al.* (1997) em análises de comunidades de actinomicetos. Através de experimentos de amplificação seletiva a partir de comunidades-modelo, de solo e de rizosfera, os autores verificaram que fragmentos do gene para RNAr 16S amplificados com *primers* específicos para actinomicetos e visualizados como bandas individuais em géis de DGGE, não eram visualizados, ou eram fracamente visualizados, no perfil da comunidade total obtido quando *primers* universais para bactérias eram empregados e o DNA molde dos actinomicetos era diluído 1:10 ou 1:100. Os dados encontrados levaram à conclusão de que os grupos de actinomicetos foram

deslocados competitivamente na reação de PCR devido, provavelmente, ao pequeno tamanho das suas populações.

3.7. PCR específico usando o oligonucleotídeo #5 como forward primer

Frente aos resultados obtidos nos experimentos de *Colony blot* e *Slot blot*, tornou-se evidente a necessidade da utilização de um sistema de detecção para o grupo *Bradyrhizobium* sp./*B. elkanii* baseado em *primers* específicos. O emprego destes permitiria a amplificação de fragmentos do DNAr 16S apenas dos organismos de interesse frente às outras bactérias presentes na amostra ambiental. A posterior análise da diversidade populacional poderia ser realizada através de géis de DGGE, à semelhança do sistema desenvolvido para *R. tropici* e *R. leguminosarum* usando *primers* específicos para a região espaçadora 16S-23S (vide **Artigo 1**). Com essa finalidade, o oligonucleotídeo #5, usado como sonda nos experimentos anteriores, foi testado quanto à sua especificidade como *forward primer* (p5f) em reações de PCR.

- *Teste de condições com linhagens referência*

A Figura 8 ilustra a amplificação do fragmento p5f-L1401r, de cerca de 500 pb, das linhagens tipo e referência sob as condições de temperatura descritas para o programa com *touch down* de 65-55°C. Houve amplificação de apenas uma única banda, em torno de 500 pb, para as linhagens referência positivas (Figura 8, canaletas 2 a 6), conforme esperado.

Entretanto, também houve amplificação para as linhagens controle-negativo *R. loti* CCT 4159 e CCT 4063^T (Figura 8, canaletas 10 e 11), cujas sequências de DNAr 16S apresentam 3 *mismatches* com a sequência do oligonucleotídeo #5. Com a finalidade de se eliminar a amplificação inespecífica, empregou-se um programa de PCR com *touch down* de 70-60°C. Os produtos de amplificação visualizados em gel revelaram ainda a presença de bandas inespecíficas, embora comparativamente bem mais fracas (dados não apresentados). O *touch down* de 75-65°C, por outro lado, eliminou a amplificação inespecífica das linhagens controle-negativo, porém diminuiu consideravelmente a amplificação específica das linhagens controle-positivo, refletida como bandas finas de intensidade fraca (dados não apresentados).

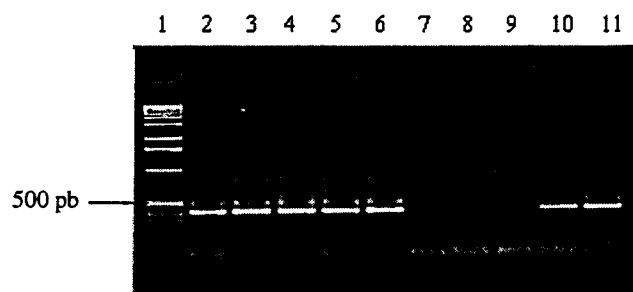


Figura 8 – Amplificação de parte do DNAr 16S a partir do DNA de culturas puras de linhagens tipo e referência utilizando o par de primers p5f/L1401r e *touch down* de 65-55°C. Canaletas: 1, marcador de peso molecular X (Boehringer Mannheim); 2 a 11, 10 µL do produto de PCR das linhagens CCT 4068, 4066, 4039, 5266, 5483, 5355^T, 4182, 4184^T, 4159 e 4063^T, respectivamente.

Os resultados obtidos com o *touch down* de 68-62°C indicaram serem estas as condições mais adequadas para amplificação específica do fragmento de DNAr 16S do grupo *Bradyrhizobium* sp./*B. elkanii*, usando o oligonucleotídeo #5 como *forward primer* (Figura 9).

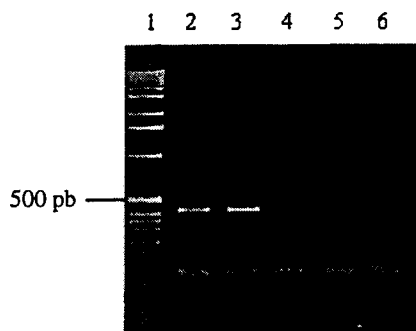


Figura 9 - Amplificação de parte do DNAr 16S a partir do DNA de culturas puras de linhagens tipo e referência utilizando o par de *primers* p5f/L1401r e *touch down* de 68-62°C. Canaletas: 1, marcador de peso molecular X (Boehringer Mannheim); 2 a 5, 10 µL do produto de PCR das linhagens CCT 4068, 4039, 4159 e 4063^T, respectivamente; 6, controle da reação usando água no lugar do DNA-molde.

- *Nested-PCR do DNA ambiental*

A amplificação do DNAr 16S da comunidade bacteriana do solo, ou PCR de enriquecimento, gerou um produto de PCR de cerca de 1,5 kb, como esperado, para todas as amostras (Figura 10).

A figura 11 ilustra a amplificação a partir do produto de PCR do DNAr 16S do solo, utilizando o p5f como *forward primer*, na tentativa de se obter uma banda única de amplificação para o grupo *Bradyrhizobium* sp./*B. elkanii*. Entretanto, o que se observou foi,

além da banda esperada em torno de 500 pb, várias outras bandas correspondentes a fragmentos maiores e resultantes de amplificação inespecífica (Figura 11, canaletas 2 a 13). Estas bandas não foram observadas para as linhagens usadas como controle positivo da amplificação específica (Figura 11, canaletas 14 a 17), e resultam, provavelmente, do anelamento inespecífico do *primer* p5f com outras regiões do DNAr 16S de organismos presentes na comunidade bacteriana do solo e não relacionados às linhagens-referência e tipo incluídas nos testes de especificidade dos *primers*. Estas regiões, situadas entre o nucleotídeo 1 e o 900 do DNAr 16S, apresentariam homologia com a sequência do oligonucleotídeo #5 suficiente para permitir a amplificação de fragmentos maiores que 500 pb e menores que 1,5 kb, sendo que o *primer* L1401r funcionaria como *reverse primer*.

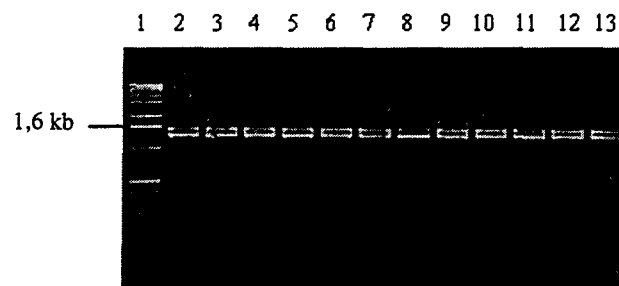


Figura 10 – Amplificação do DNAr 16S a partir do DNA da comunidade bacteriana presente nas amostras de solo utilizando os *primers* p27f e L1401r. Canaletas: 1, marcador de peso molecular X (Boehringer-Mannheim); 2 a 13, PCR das duplicatas da extração de DNA das amostras de solo **Pastagem**, **Tempo Zero**, **Plantio Direto (PD)**, **Plantio Convencional (PC)**, **PD com o inseticida Furadan (PDF)** e **PC com o inseticida Furadan (PCF)**, nesta ordem.

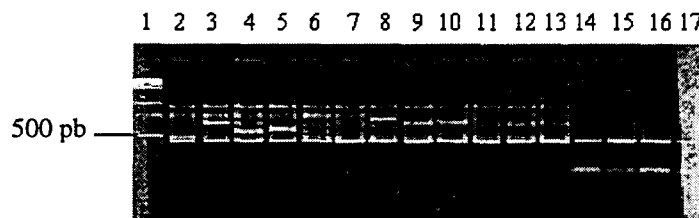


Figura 11 – Amplificação de parte do DNAr 16S a partir do PCR de enriquecimento das amostras de solo utilizando o par de *primers* p5f/L1401r e *Touch down* de 68-62°C. Canaletas: 1, marcador de peso molecular X (Boehringer-Mannheim); 2 a 13, PCR específico a partir do PCR de enriquecimento das duplicatas da extração de DNA das amostras de solo **Pastagem**, **Tempo Zero**, **PD**, **PC**, **PDF** e **PCF**, nesta ordem; 14 a 17, PCR específico a partir de culturas puras das linhagens-referência usadas como controles positivos CCT 4068, 4066, 4039 e 5266, respectivamente.

Frente aos resultados encontrados, a possibilidade de se empregar o p5f juntamente com um *reverse primer* universal, no caso o L1401r, para amplificação específica do DNAr 16S do grupo *Bradyrhizobium* sp./*B. elkanii* a partir de DNA ambiental, e posterior separação dos fragmentos em gel de DGGE, foi desconsiderada.

3.8. PCR específico usando o oligonucleotídeo #5 como *reverse primer*

A amplificação do DNAr 16S de linhagens-referência usando a sonda #5 como *reverse primer* e o *primer* p27f como *forward primer* foi uma segunda tentativa realizada com a finalidade de se verificar a possibilidade de utilização direta destes oligonucleotídeos para amplificação específica de sequências do DNAr 16S do grupo *Bradyrhizobium* sp.

A figura 12 ilustra a amplificação de um fragmento de cerca de 1000 pb a partir do DNA da linhagem *Bradyrhizobium* sp. CCT 5266 (canaleta 2), cuja sequência do RNAr 16S apresenta pareamento perfeito com a sequência do *primer* #5. O tamanho deste produto de PCR é compatível com o esperado, considerando a localização dos sítios de anelamento dos *primers* utilizados. Um produto de tamanho similar mas muito fraco também foi observado para a linhagem *R. loti* CCT 4063^T (Figura 12, canaleta 4), cuja sequência do RNAr 16S homóloga à sequência do oligonucleotídeo #5 apresenta 3 *mismatches* com relação a esta última. Observou-se também uma banda de cerca de 1500 pb resultante da amplificação do DNA da linhagem *B. japonicum* CCT 4182 (Figura 12, canaleta 3). Este produto pode ter sido resultante da amplificação de uma sequência situada entre a região conservada homóloga ao *primer* p27f e uma região homóloga à sequência do *primer* #5 posicionada em uma outra região do operon ribossomal.

Alterações das condições de amplificação utilizando o oligonucleotídeo #5 como *reverse primer* foram testadas com o intuito de aumentar a especificidade da reação, sem, entretanto, gerar resultados satisfatórios (dados não mostrados).

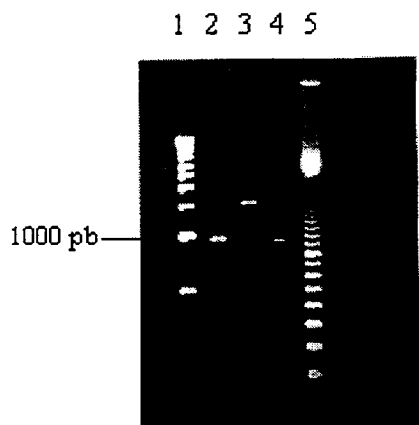


Figura 12 - Amplificação de parte do DNAr 16S a partir do DNA de culturas puras de linhagens tipo e referência utilizando o p27f como *forward primer* e o oligonucleotídeo #5 como *reverse primer*. Canaletas 1 e 5: marcadores de peso molecular 1 kb e 100 bp *ladder* (Pharmacia), respectivamente. Canaletas 2 a 4: linhagens *Bradyrhizobium* sp. CCT 5266, *B. japonicum* CCT 4182 e *R. loti* CCT 4063^T, respectivamente.

Uma alternativa à estratégia utilizada nos experimentos anteriores seria a **busca de outra região na sequência de DNAr 16S que pudesse servir como um segundo *primer* específico para o grupo *Bradyrhizobium* sp./*B. elkanii***, o que facilitaria a amplificação específica de DNA pela polimerase durante o PCR. A recuperação, alinhamento e análise de sequências de RNAr 16S disponíveis em bases de dados (item 3.1) permitiu a seleção de uma região potencial para o desenho de um oligonucleotídeo, sonda #7 (Tabela 2), capaz de discriminar as espécies *Bradyrhizobium* sp., *B. elkanii*, *B. japonicum* e *Rhodopseudomonas palustris* de outros rizóbios, ou *outgroups* filogeneticamente relacionados (Figura 13). Esta sequência poderia ser futuramente testada, como *reverse primer*, em experimentos de PCR juntamente com o oligonucleotídeo #5, numa tentativa de amplificar especificamente fragmentos do DNAr do grupo *Bradyrhizobium* sp./*B. elkanii*.

	2401	2411	2421	2431	2441	2451	2460
Afp.felis	G-CG-CGAGCCC--UA-GCAAA-UCUCA--AAAAGCC-GUC-UCAGUUCG						GAUUGGACUC
Aqb.spirit	..G....C.U...G...U.....C.....A.....						C....
Azr.cauli3	C.U.....GGU..G.....C.....						C....
Bdr.japon2	..G....C..U...C.....						G....
Bdr.japon5	..G....C..U...C.....						G....
Bdr.japoni	..G....C..U...C.....						G....
Bdr.sp0689	..G....C..U...C.....						G....
Bdr.sp9514	..G....C..U...C.....						G....
Bdr.sp9520	..G....C..U...C.....						G....
Bdr.sp9966	..G....C..U...C.....						G....
Bdr.sp9980	..G....C..U...C.....						G....
Rps.palus2	..G....C..U...C.....				A.....		G....
Rps.palus3	..G....C..U...C.....						G....
Rps.palus5	..G....C..U...C.....						G....
Rps.palust	..G....C..U...C.....						G....
Ntb.hambur	..G...A.C.....				A.....		G....
Ntb.winogr	..G..U..C.....				A.....		G....
Cau.FWC14	-.--.-----.-.-.-U....CUU.....U.....						UC....
Lib.spNels	U.....G.G..G...U....C.C....GU...A.....N.....						C....
Mlb.extorq	C.....GUG..G.....C.C....A.....						C....
Mlb.rhode2	C.....GUU..G...GG....C.C.....						C....
Rhh.orient	U..U....GAG..G...U....C.C.....						UU....
Rps.marin2	-.--.-----.-.-.-C.C....A.....						UC....

Figura 13 – Alinhamento e comparação de sequências de RNAr 16S; os nucleotídeos em destaque ilustram a região potencial para o desenho de sonda/primer para o grupo *Bradyrhizobium* sp./*B. elkanii*/*B. japonicum*/*Rhodopseudomonas palustris*. Denominações no lado esquerdo do alinhamento referem-se ao código do RDP para o organismo correspondente a cada sequência alinhada. Abreviações: Afp. = *Afixia*; Aqb. spirit = *Aquabacter spiritensis*; Azr. cauli = *Azorhizobium caulinodans*; Bdr. japoni = *Bradyrhizobium japonicum*; Bdr. sp = *Bradyrhizobium* sp.; Rps. palust = *Rhodopseudomonas palustris*; marin = *marina*; Ntb. hambur = *Neorhizobium elkanii*; winogr = *winogradskyi*; Cau. = *Caulobacter*; Lib. = *Liberobacter*; Mlb. extorq = *Methylobacterium extorquens*; rhode = *rhodesianum*; Rhh. orient = *Rhodohalobium orientum*. “.” significa o mesmo nucleotídeo que aquele especificado na primeira sequência do alinhamento; “-” significa ausência de nucleotídeo naquela posição específica; os números na parte superior do alinhamento indicam a posição dos nucleotídeos dentro do RNAr 16S.

3.9. Análise da região espaçadora 16S-23S

Uma estratégia alternativa ao DNAr 16S, visando a discriminação das espécies de rizóbios de outras espécies filogeneticamente relacionadas, seria a análise da região existente entre o DNAr 16S e o DNAr 23S. Essa região apresenta sequências com variações interespecíficas denominadas ISR (*intergenic spacer regions*), as quais separam genes para RNAt. Diversos autores vêm se valendo destas variações nas ISR, refletidas como variações no tamanho dos produtos de PCR, como meio para diagnosticar espécies ou sub-

espécies de bactérias (Seal *et al.*, 1992, Nour *et al.*, 1995; Gürtler & Stanisich, 1996; Laguerre *et al.*, 1996). Esta foi a estratégia utilizada neste trabalho de tese para análise de diversidade de *R. tropici* e *R. leguminosarum* em amostras ambientais (vide Artigos 1 e 2).

Com a finalidade de investigar a existência de regiões suficientemente variáveis que permitissem discriminar *Bradyrhizobium* sp. e *B. elkanii* de *B. japonicum*, assim como de outros grupos de bactérias filogeneticamente relacionados (*Rhodopseudomonas palustris* e *Nitrobacter hamburgensis*), foi realizada a amplificação da região espaçadora 16S-23S das linhagens *B. elkanii* CCT 4068, *Bradyrhizobium* sp. CCT 4039 e CCT 5266, *B. japonicum* CCT 4182 e CCT 4184^T, e *Rhodopseudomonas palustris* CCT 5355, e subsequente restrição enzimática.

A análise dos perfis de restrição revelou a existência de polimorfismos entre as linhagens utilizadas das espécies de *B. elkanii*, *B. japonicum*, *Bradyrhizobium* sp. e *Rhodopseudomonas palustris* (Figura 14). Foi observado também variação de sequência entre as duas linhagens de *Bradyrhizobium* sp. utilizadas, CCT 4039 e CCT 5266, quando os fragmentos 16S-23S foram digeridos com *Hind*III. As duas linhagens de *B. japonicum* apresentaram o mesmo perfil de bandas com três das enzimas utilizadas (*Pst*I, *Bcl*II, *Hind*III). Não houve digestão com as enzimas *Bam*HI e *Eco*RV (dados não mostrados).

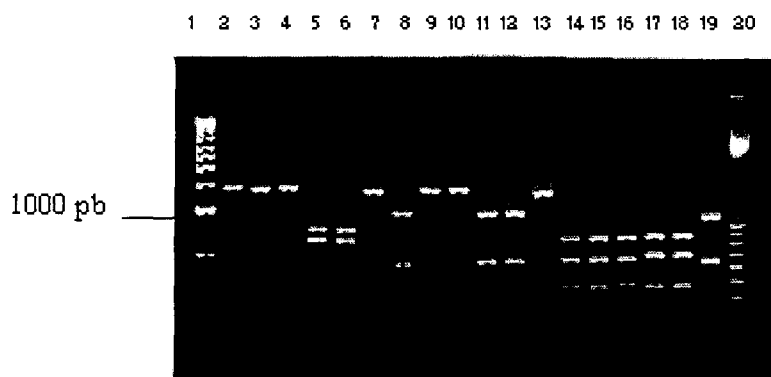


Figura 14 - Canaletas 2 a 7: Digestão com *Pst*I da região espaçadora 16S-23S das linhagens *B. elkanii* CCT 4068, *Bradyrhizobium* sp. CCT 4039 e CCT 5266, *B. japonicum* CCT 4182 e CCT 4184^T, e *Rhodopseudomonas palustris* CCT 5355, respectivamente. Canaletas 8 a 13: Digestão com *Bcl*II da região espaçadora 16S-23S das mesmas linhagens, na mesma ordem. Canaletas 14 a 19: Digestão com *Hind*III da região espaçadora 16S-23S das mesmas linhagens, na mesma ordem. Canaletas 1 e 20: marcadores de peso molecular 1 kb e 100 pb *ladder*, respectivamente

A evidência da existência de polimorfismos nas regiões espaçadoras 16S-23S das diferentes espécies de *Bradyrhizobium* analisadas, assim como da espécie de *Rhodopseudomonas* analisada, dá suporte à idéia de se procurar regiões nessa sequência capazes de discriminar o grupo *Bradyrhizobium* sp./*B. elkanii* das espécies relacionadas, não-diferenciáveis através do RNAr 16S, o que poderá vir a ser feito em estudos futuros de diversidade de rizóbios tropicais.

5. DISCUSSÃO GERAL

A abordagem inicial do presente trabalho consistiu no desenho de sondas/*primers* para grupos de rizóbios com base no alinhamento e comparação de sequências de RNA ribossômico 16S existentes nas bases de dados consultadas e confirmação da sua especificidade através da rotina *CHECK-PROBE* do RDP. Esta rotina compara a sequência de oligonucleotídeo potencial a ser usada como sonda/*primer* com todas as sequências de RNAr 16S disponíveis na base de dados, permitindo a detecção, se for o caso, de sequências que apresentem hibridização cruzada.

Como os gêneros *Rhizobium* e *Bradyrhizobium* são filogeneticamente distantes, apesar de pertencerem à mesma família, seria difícil procurar por uma sonda que abrangesse os dois gêneros e simultaneamente os discriminasse de gêneros próximos, como *Agrobacterium*, no primeiro caso, e *Rhodopseudomonas* e *Nitrobacter*, no segundo. Portanto, as sequências das espécies de *Rhizobium* foram analisadas em separado das sequências de *Bradyrhizobium japonicum* e *Bradyrhizobium* sp.

Entretanto, dentro do gênero *Rhizobium* não foi possível encontrar uma sonda única para RNAr 16S que pudesse agrupar todas as espécies e, ao mesmo tempo, discriminá-las das espécies filogeneticamente relacionadas. Apesar dos organismos da família Rhizobiaceae apresentarem grande diversidade taxonômica (Young, 1996; Ludwig *et al.*, 1998), a sequência dos genes de RNAr 16S dentro desta família é altamente conservada (Vallayes *et al.*, 1997).

A estratégia alternativa empregada envolveu a clonagem e análise da sequência da região espaçadora entre o RNAr 16S e 23S. Esta região apresenta relativamente maior variabilidade de sequência comparado com o RNAr 16S (Honeycutt *et al.*, 1995; Gürtler & Stanisich, 1996) e possibilitou o desenho de *primers* específicos, capazes de diferenciar *Rhizobium leguminosarum* e *R. tropici* de *Agrobacterium rhizogenes*, um organismo filogeneticamente relacionado (**Artigo 1**). As espécies de *Rhizobium leguminosarum* e *R. tropici* se tornaram o alvo de interesse do presente trabalho por serem, dentro do gênero *Rhizobium*, aquelas de maior ocorrência nos trópicos.

Experimentos de amplificação por PCR utilizando linhagens-referência confirmou a especificidade dos *primers* desenhados para *Rhizobium leguminosarum* e/ou *R. tropici*. A utilização da técnica de eletroforese em gel de gradiente desnaturante (DGGE) na separação dos fragmentos amplificados de linhagens-referência corroborou o alto poder de resolução

deste método na separação de sequências de DNA similares em tamanho, porém com diferenças na composição de nucleotídeos (Fisher & Lerman, 1983). Fragmentos amplificados de duas linhagens de *R. leguminosarum* bv. *trifolii*, que apresentaram mesma migração em gel de agarose, puderam ser claramente discriminados em gel de DGGE, demonstrando a possibilidade de utilização desta metodologia para diferenciação de rizóbios em nível intra-específico.

Os resultados obtidos no **Artigo 1** demonstraram que os *primers* específicos para *R. leguminosarum* e/ou *R. tropici* poderiam ser utilizados em reações de PCR, e subsequente análise de DGGE, para estudos de diversidade de rizóbios em amostras ambientais, empregando extração direta de DNA da comunidade bacteriana. Assim, o potencial do DGGE para avaliar a diversidade relativa de *R. leguminosarum* e *R. tropici* em amostras de solo e a influência de diferentes práticas agrícolas sobre a diversidade de populações destes organismos foram avaliados no **Artigo 2**.

Neste estudo, as práticas agrícolas avaliadas foram **Plantio Direto** com (PDF) e sem (PD) o inseticida de solo Furadan®, e **Plantio Convencional** com (PCF) e sem (PC) o Furadan® (vide **Artigo 2**). Dois controles foram utilizados nesta análise: **Tempo Zero**, correspondente à área experimental amostrada antes do plantio do feijão, e **Pastagem**, correspondente a uma área adjacente não sujeita ao manejo agrícola e ao cultivo.

Os altos níveis de similaridade encontrados entre os perfis de DGGE derivados dos tratamentos **PD**, **PDF**, **PC** e **PCF**, usando o par de *primers* rhizo2f/rhizo3r, específico para ambas as espécies *R. leguminosarum* e *R. tropici*, sugerem que as diferentes práticas agrícolas implementadas na área experimental tiveram pouco impacto na diversidade geral destes organismos no solo. Entretanto, este par de *primers* revelou diferenças na abundância de linhagens entre os tratamentos, sugerindo que os tratamentos **PD** e **PC** favoreceram a seleção de algumas linhagens específicas de rizóbios.

A diversidade de *R. leguminosarum* e *R. tropici* foi maior nas parcelas plantadas com feijão, quando comparada com o solo sob Pastagem. Isto se deveu provavelmente ao fato de que ambas espécies nodulam e fixam nitrogênio em feijão e, portanto, estão sob seleção positiva pelo hospedeiro.

Uma variabilidade relativamente maior foi observada quando o par de *primers* específico para *R. tropici* foi utilizado. Os perfis de DGGE gerados revelaram a ausência, ou presença em níveis inferiores àqueles detectados por PCR, de *R. tropici* nas amostras de solo **Pastagem** e **Tempo Zero**, o que demonstra, também neste caso, uma forte seleção de *Rhizobium tropici* nas amostras de solo sob cultivo do feijão. Os perfis com maior diversidade obtidos nos tratamentos associados ao cultivo do feijão podem ainda refletir a introdução de novas linhagens de *R. tropici* através das sementes desta leguminosa. Uma

redução na diversidade nas amostras de solo originadas do **PC** também foi observada, sugerindo um impacto deletério do **PC** comparado ao **PD**.

A análise combinada dos dados de DGGE gerados com ambos os pares de *primers* sugerem que *R. leguminosarum* é a espécie que ocorre em maior abundância nas amostras de solo derivadas dos tratamentos agrícolas analisados. Ainda, não foi observada nenhuma correlação entre riqueza ou abundância de linhagens de *R. leguminosarum* e/ou *R. tropici* e o uso do inseticida Furadan®, embora dados de literatura sugiram que populações microbianas podem ser afetadas pelo uso de pesticidas (El Fantroussi *et al.*, 1999).

O sequenciamento e análise de bandas obtidas em amplificações de DNA de solo e de produtos de PCR de linhagens-referência de *R. leguminosarum* que co-migraram nos géis de DGGE demonstraram que distâncias de migração equivalentes em gel de DGGE não devem ser consideradas inequivocamente como uma indicação de identidade de sequência. Estes resultados corroboram aqueles previamente encontrados por outros autores (Muyzer *et al.*, 1995; Ferris *et al.*, 1996; Vallaey *et al.*, 1997), que sugerem a necessidade do sequenciamento para confirmação de identidade de fragmentos co-migrantes.

Algumas bandas específicas foram excisadas dos perfis de DGGE do solo **Pastagem**, clonadas e sequenciadas a fim de se investigar a identidade dos organismos correspondentes. A árvore filogenética derivada da análise dos dados de sequenciamento sugere que um dos clones, denominado clone A, é relacionado a *R. tropici*, ao passo que os clones, 2, 3, 4 e B podem representar linhagens pertencentes às espécies *R. tropici* ou *R. leguminosarum*, porém filogeneticamente distantes das linhagens-referência utilizadas. Uma outra possibilidade é que estes clones representem novas espécies que puderam ser detectadas utilizando-se o par de *primers* para *R. leguminosarum/R. tropici*.

Os resultados obtidos no **Artigo 2** demonstraram o desenvolvimento de uma metodologia para avaliar a diversidade de rizóbios em amostras de solo que provou ser de grande valor para desvendar a diversidade de linhagens de *R. leguminosarum* e *R. tropici* em solos sujeitos a diferentes tratamentos agrícolas. A estratégia utilizada também mostrou ser útil na detecção putativa de novos organismos, sugerindo seu potencial no estudo de organismos não-cultivados e de linhagens que ocorrem em baixa densidade populacional em amostras ambientais.

O **Artigo 3** avalia o impacto de práticas agrícolas (**Plantio Direto** e **Plantio Convencional**) sobre a diversidade de rizóbios em parcelas plantadas com soja através da caracterização de RAPD de organismos isolados por captura utilizando guandu.

O uso de guandu para capturar rizóbios mostrou ser uma estratégia eficiente, sendo a sua baixa especificidade como planta hospedeira demonstrada pela diversidade de isolados recuperada. Um total de 58 linhagens de rizóbios foi isolado das amostras de solo

analisadas, sendo 14 de crescimento rápido e 44 de crescimento lento, classificadas preliminarmente como *Rhizobium* sp. e *Bradyrhizobium* sp., respectivamente. Vinte e oito perfis de RAPD foram observados, e, dentre estes, 19 mostraram-se como padrões únicos. Os perfis de RAPD foram analisados empregando-se o índice de diversidade de Shannon-Weaver, o que permitiu a comparação entre os diferentes tratamentos.

As diferentes práticas de manejo agrícola produziram, claramente, alterações no perfil das populações de rizóbios no solo. Entretanto, os resultados observados foram contrários à expectativa de que a plantação de soja favoreceria um aumento na diversidade de bradirrizóbios. As amostras derivadas de solo sob **Pastagem** apresentaram diversidade de rizóbios significativamente maior do que aquelas sob o cultivo da soja (**PD** e **PC**). A diversidade encontrada entre os tratamentos **PD** e **PC** não foi significativamente diferente. Rizóbios de crescimento rápido não foram recuperados do solo **Pastagem** (área experimental amostrada antes do plantio da soja), ao passo que a maioria dos isolados das amostras originadas do **PD** foram de crescimento rápido.

A menor diversidade de rizóbios verificada nos solos cultivados provavelmente é resultado das profundas modificações impostas pela aplicação de agroquímicos e perturbações físicas decorrentes do manejo agrícola. A dominância de isolados de *Bradyrhizobium* sp. observada no solo **Pastagem** pode refletir a maior abundância de linhagens deste gênero no ambiente nos trópicos, corroborando resultados obtidos previamente (Moreira *et al.*, 1993).

A avaliação da diversidade baseada no isolamento de rizóbios pode se mostrar tendenciosa, uma vez que apenas aquelas linhagens capazes de nodular a espécie de leguminosa usada como planta-isca serão analisadas. Entretanto, esta estratégia se mostrou adequada para estudos comparativos, como o realizado no **Artigo 3**. Apesar das limitações, métodos de isolamento e caracterização convencionais são importantes, pois permitem a subsequente seleção para novos inoculantes de leguminosas.

Contrariamente aos resultados encontrados no **Artigo 3**, a diversidade de rizóbios observada no **Artigo 2** foi menor no solo **Pastagem**, quando comparada com os solos cultivados. Isto poderia ser explicado pelo fato de que rizóbios pertencentes ao gênero *Rhizobium*, grupo-alvo dos *primers* específicos utilizados no **Artigo 2** em conjunto com a técnica de DGGE e extração direta de DNA do solo, se encontram em menor número nos solos tropicais do que *Bradyrhizobium* spp., como sugerido anteriormente. É, portanto, plausível que uma maior diversidade de *R. leguminosarum* e *R. tropici* seja encontrada nas parcelas plantadas com feijão, como observado, devido à seleção pela planta hospedeira.

Os resultados descritos no **Artigo 3** indicam que não houve diferença significativa na diversidade de rizóbios entre os tratamentos **PD** e **PC**. Analogamente, estes mesmos

resultados foram encontrados no **Artigo 2** quando o par de *primers* para ambas as espécies *R. leguminosarum* e *R. tropici* foi utilizado. Entretanto, um certo impacto deletério do **Plantio Convencional** sobre a diversidade de rizóbios é observado quando o par de *primers* específico para *R. tropici* é utilizado. Isto pode sugerir uma maior suscetibilidade desta espécie aos distúrbios físicos decorrentes da aragem e gradagem, utilizadas no **PC**.

Os experimentos realizados visando o desenvolvimento de uma abordagem molecular direta no estudo da diversidade de *Bradyrhizobium* sp. demonstraram que a estratégia baseada na construção de um banco de sequências ribossomais da comunidade bacteriana do solo, associado ao uso de sondas específicas para seleção de transformantes e posterior análise de diversidade por PCR-RFLP, não é adequada no estudo de organismos que ocorrem em baixa proporção no ambiente, como no caso de rizóbios (Heuer & Smalla, 1997). Portanto, não foi possível comparar esta estratégia com aquela utilizada no **Artigo 3**, a qual permitiu o isolamento e caracterização da diversidade genética de linhagens de *Bradyrhizobium* sp. nas amostras de solo.

O desenvolvimento de conjuntos de *primers* específicos, baseados na sequência altamente variável da região espaçadora 16S-23S, aliado ao emprego de PCR e análises de DGGE, é sugerido para estudos futuros como uma estratégia possivelmente mais sensível para a detecção de organismos pertencentes ao grupo *Bradyrhizobium* sp., semelhantemente ao que foi desenvolvido para *R. leguminosarum* e *R. tropici* (**Artigos 1 e 2**).

Como um primeiro passo neste sentido, a análise de restrição da região espaçadora do DNAr 16S-23S de linhagens-referência de diferentes espécies de *Bradyrhizobium* já foi realizada (vide **Capítulo Avulso**). Os resultados desta análise revelaram a existência de polimorfismos nas regiões espaçadoras 16S-23S das diferentes espécies de *Bradyrhizobium* analisadas, dando suporte à idéia de se procurar sítios nessa sequência capazes de discriminar o grupo *Bradyrhizobium* sp./*B. elkanii* das espécies relacionadas, não-diferenciáveis através do RNAr 16S.

Entretanto, não se pode deixar de ressaltar aqui a utilidade potencial da sonda #5 em estudos futuros de diversidade como uma das etapas no processo de isolamento de linhagens de *Bradyrhizobium* sp./*B. elkanii* de amostras ambientais, para posterior caracterização genotípica, ou em estudos de detecção utilizando tecidos de nódulos de leguminosas para hibridização *in situ*, no monitoramento da persistência e competitividade de linhagens inoculantes liberadas no ambiente, ou mesmo no controle de qualidade de inoculantes.

6. CONCLUSÕES

- A estratégia empregada na análise de diversidade dos organismos pertencentes às espécies *Rhizobium leguminosarum* e *R. tropici* em amostras de solo, baseada na amplificação de fragmentos da região espaçadora do DNAr 16S-23S com *primers* específicos e resolução em géis de DGGE, foi bem sucedida, permitindo a comparação da diversidade destes organismos em solos agrícolas sob diferentes regimes de manejo.
- Os dados derivados dos experimentos empregando-se o par de *primers* específico para *R. leguminosarum* e *R. tropici* permitiram concluir que as diferentes práticas agrícolas analisadas (**Pastagem**, **Tempo Zero**, **Plantio Direto** e **Plantio Convencional**, com e sem aplicação de inseticida) influenciaram a ocorrência de linhagens particulares de rizóbios no solo, porém sem efeitos significativos na diversidade geral destes organismos.
- Analisando-se separadamente os dados de polimorfismo em géis de DGGE com *primers* específicos apenas para *R. tropici*, pôde ser verificado uma maior diversidade destes organismos nas amostras de solo originadas do tratamento **Plantio Direto**.
- A diversidade de *R. tropici* e *R. leguminosarum* foi maior nas parcelas plantadas com feijão, comparada com o solo sob **Pastagem**, provavelmente devido ao fato de que ambas as espécies nodulam e fixam nitrogênio em feijão, sendo assim positivamente selecionadas pelo hospedeiro. A maior diversidade observada no solo **Tempo Zero** sugere que linhagens de rizóbio podem persistir em solos agrícolas por pelo menos um ano.
- Ainda, o sequenciamento de fragmentos isolados dos géis de DGGE permitiu a descoberta de organismos potencialmente novos nas amostras de solo, os quais não se mostraram proximamente relacionados às linhagens-referência de *R. leguminosarum* e *R. tropici* utilizadas nesta análise.
- Não foi observada correlação entre o uso do inseticida de solo Furadan® e a diversidade de *R. leguminosarum* e/ou *R. tropici* nas amostras de solo.
- Os resultados obtidos também ressaltaram uma potencial limitação desta estratégia, exemplificada pela falta de reprodutibilidade entre as duplicatas das amostras do solo **Pastagem**. O PCR pode estar sujeito a eventos estocásticos nos passos iniciais de enriquecimento. Isto é, moléculas-molde com baixo número de cópia podem não ser

selecionadas durante o PCR, não sendo assim representadas na segunda etapa de amplificação, o PCR específico, e gerando dados inconsistentes.

- O uso de guandu como planta-isca mostrou ser uma estratégia eficiente para capturar linhagens de rizóbios no estudo de diversidade em amostras de solo sob o cultivo da soja e sujeitas a diferentes tratamentos agrícolas, dada a diversidade de isolados recuperada.
- As diferentes práticas de manejo do solo produziram alterações visíveis no perfil das populações de rizóbios nas amostras ambientais. Os resultados observados sugerem que as amostras derivadas de solo sob pastagem, não-cultivado com leguminosas, apresentaram diversidade de rizóbios significativamente maior do que aquelas sob o cultivo da soja (**PD** e **PC**).
- A menor diversidade de rizóbios verificada nos solos cultivados, comparativamente ao solo **Pastagem**, provavelmente é resultado das profundas modificações impostas pela aplicação de agroquímicos e perturbações físicas decorrentes do manejo agrícola. Por outro lado, pode estar havendo favorecimento de algumas linhagens mais eficientes na nodulação do cultivar de soja plantado, em detrimento de outras, resultando na redução da diversidade natural de bradirrizóbios nos solos cultivados.
- A diversidade encontrada entre os tratamentos **PD** e **PC** não foi significativamente diferente. Portanto, um impacto da prática agrícola convencional sobre a diversidade de rizóbios em relação ao plantio direto é descartado neste estudo.
- Apenas rizóbios de crescimento lento foram recuperados do solo **Pastagem**. A dominância de *Bradyrhizobium* spp. observada no solo **Pastagem** pode refletir a maior abundância de linhagens deste gênero em solos tropicais.
- A estratégia empregada na análise de diversidade dos organismos do grupo *Bradyrhizobium* sp./*B. elkanii* em amostras de solo, baseada na amplificação do DNAr 16S da comunidade bacteriana e seleção de sequências de DNAr com sinal de hibridização positivo para a sonda #5, não foi adequada. O baixo número de clones positivos obtido (1/2.000) reflete o baixo número de *Bradyrhizobium* spp. na amostra ambiental. Uma estratégia baseada na utilização de *primers* específicos para *Bradyrhizobium* spp. seria mais adequada, permitindo a amplificação de fragmentos de DNAr apenas deste grupo de organismos a partir do DNA da comunidade bacteriana do solo. A diversidade e variação das populações de *Bradyrhizobium* spp. nas amostras ambientais, sujeitas às diferentes práticas agrícolas, poderiam ser posteriormente avaliadas através da separação dos fragmentos amplificados em géis de DGGE.

Referências Gerais

- AHMAD, M.H.; EAGLESHAM, A.R.J. & HASSOUNA, S. 1981. Examining serological diversity of "cowpea" rhizobia by the ELISA technique. *Arch. Microbiol.* **130**:281-287.
- AMANN, R. I.; BINDER, B. J.; OLSON, R. J.; CHISHOLM, S. W.; DEVEREUX, R. & STAHL, D. A. 1990b. Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. *Appl. Environ. Microbiol.* **56**:1919-1925.
- AMANN, R. I.; KRUMHOLZ, L. & STAHL, D. A. 1990a. Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. *J. Bacteriol.* **172**:762-770.
- AMANN, R. I.; LUDWIG, W. & SCHLEIFER, K. 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiol. Rev.* **59**:143-169.
- ANYANGO, B.; WILSON, K.J.; BEYNON, J.L. AND GILLER, K.E. 1995. Diversity of rhizobia nodulating *Phaseolus vulgaris* L. in two Kenyan soils with contrasting pHs. *Appl. Environ. Microbiol.* **61**:4016-4021.
- BARRY, T.; COLLERAN, G.; GLENNON, M.; DUNICAN, L.K. & GANNON, F. 1991. The 16s/23s ribosomal spacer region as a target for DNA probes to identify eubacteria. *PCR Methods Appl.* **1**:51-56.
- BODDEY, R.M.; CHALK, P.M.; VICTORIA, R.L. & MATSUI, E. 1984. Nitrogen fixation by nodulated soybean under tropical field conditions estimated by the ^{15}N isotope dilution technique. *Soil Biol. Biochem.* **16**:583-589.
- BORRESEN, A.L.; HOVIG, E. & BROGGER, A. 1988. Detection of base mutations in genomic DNA using denaturing gradient gel electrophoresis (DGGE) followed by transfer and hybridization with gene-specific probes. *Mutat. Res.* **202**:77-83.
- BUYER, J.S. & KAUFMAN, D.D. 1997. Microbial diversity in the rhizosphere of corn grown under conventional and low-input systems. *Appl. Soil Ecol.* **5**:21-27.
- CHAMBERS, M.A. & MONTES, F.J. 1982. Effects of some seeds desinfectants and methods of rhizobial inoculation on soybeans (*Glycine max* L. Merrill). *Plant and Soil* **66**:353-360.
- COUTINHO, H.L.C.; OLIVEIRA, V. M. & MOREIRA, F.M.S. 1999. Systematics of legume nodule nitrogen fixing bacteria (Rhizobia): agronomic and ecological applications. In: **Applied Microbial Systematics**. F. Priest and M. Goodfellow (eds.) (No prelo).
- CURLEY, R. L. & BURTON, J. C. 1975. Compatibility of *Rhizobium japonicum* with chemical seed protectants. *Agron. J.* **67**:807-808.
- DE LAJUDIE, P.; LAURENT-FULELE, E.; WILLEMS, A.; TORCK, U.; COOPMAN, R.; COLLINS, M.D.; KERSTERS, K.; DREYFUS, B. & GILLIS, M. 1998. *Allorhizobium undicola* gen. nov., sp. nov., nitrogen-fixing bacteria that efficiently nodulate *Neptunia natans* in Senegal. *I. J. Syst. Bacteriol.* **48**:1277-90.
- DE LAJUDIE, P.; WILLEMS, A.; NICK, G.; MOREIRA, F.; MOLOUBA, F.; HOSTE, B.; URBAIN, T.; NEYRA, M.; COLLINS, M.D.; LINDSTRÖM, K.; DREYFUS, B. & GILLIS, M. 1998. Characterization of tropical tree rhizobia and description of *Mesorhizobium plurifarum* sp. nov. *Int. J. Syst. Bacteriol.* **48**:369-382.

- DE-POLLI, H.; SOUTO, S. M. & FRANCO, A. A. 1986. Compatibilidade de agrotóxicos com *Rhizobium* spp. e a simbiose das leguminosas. Ministério da Agricultura. EMBRAPA. U.A.P.N.P.B.S. Seropédica, 71p.
- DÖBEREINER, J. & DUQUE, F. F. 1980. Contribuição da pesquisa em fixação biológica de nitrogênio para o desenvolvimento do Brasil. *Rev. Econ. Rural* **18**:447-460.
- DREYFUS, B. L.; DIEM, H. G. & DOMMERGUES, Y. R. 1988a. Future directions for biological nitrogen fixation research. *Plant and Soil* **108**:191-199.
- DREYFUS, B.; GARCIA, J. L. & GILLIS, M. 1988b. Characterization of *Azorhizobium caulinodans* gen. Nov., sp. Nov., a stem-nodulating nitrogen-fixing bacterium isolated from *Sesbania rostrata*. *Int. J. Syst. Bacteriol.* **38**:89-98.
- DUPUY, N.; WILLEMS, A.; POT, B.; DEWETTINCK, D.; VANDENBRUAENE, I.; MAESTROJUAN, G.; DREYFUS, B.; KERSTERS, K.; COLLINS, M. D. & GILLIS, M. 1994. Phenotypic and genotypic characterization of bradyrhizobia nodulating the leguminous tree *Acacia albida*. *Int. J. Syst. Bacteriol.* **44**:461-473.
- EAGLESHAM, A. R. J.; ELLIS, J. M.; EVANS, W. R.; FLEISCHMAN, D. E.; HUNGRIA, M. & HARDY, R. W. F. 1990. The first photosynthetic N₂-fixing rhizobium: characteristics. p. 805-811. In: P. M. Gresshoff, L. E. Roth, G. Stacey, and W. L. Newton (ed), **Nitrogen fixation: achievements and objectives**. Chapman and Hall, New York.
- EL FANTROUSSI, S., VERSCHUERE, L.; VERSTRAETE, W., & TOP, E.M.. 1999. Effect of phenylurea herbicides on soil microbial communities estimated by analysis of 16S rRNA gene fingerprints and community-level physiological profiles. *Appl. Environ. Microbiol.* **65**:982-988.
- FELSKE, A.; RHEIMS, H.; WOLTERINK, A.; STACKEBRANDT, E. & AKKERMANS, A.D.L. 1997. Ribosome analysis reveals prominent activity of an uncultured member of the class Actinobacteria in grassland soils. *Microbiol.* **143**:2983-2989.
- FELSKE, A.; WOLTERINK, A.; VAN LIS, R. & AKKERMANS, A.D.L. 1998. Phylogeny of the main bacterial 16S rRNA sequences Drentse A grassland soils (The Netherlands). *Appl. Environ. Microbiol.* **64**:871-879.
- FERGUSON, R. L.; BUCKLEY, E. N. & PALUMBO, A. V. 1984. Response of marine bacterioplankton to differential centrifugation and confinement. *Appl. Environ. Microbiol.* **47**:49-55.
- FERRIS, M.J.; MUYZER, G. & WARD, D.M. 1996. Denaturing gradient gel electrophoresis profiles of 16S rRNA-defined populations inhabiting a hot spring microbial mat community. *Appl. Environ. Microbiol.* **62**:340-346.
- FISHER, D. J. 1976. Effects of some fungicides on *Rhizobium trifolii* and its symbiotic relationship with white clover. *Pestic. Sci.* **7**:10-18.
- FISCHER, S.G. & LERMAN, L.S. 1983. DNA fragments differing by single basepair substitutions are separated in denaturing gradient gels: correspondence with melting theory. *Proc. Natl. Acad. Sci. USA* **80**:1579-1583.
- FORD, S. & OLSON, B. H. 1988. Methods for detecting genetically engineered microorganisms in the environment. *Adv. Microb. Ecol.* **10**:45-73.
- FREY, S.D; ELLIOTT, E.T & PAUSTIAN, K. 1999. Bacterial and fungal abundance and biomass in conventional and no-tillage agroecosystems along two climatic gradients. *Soil Biol. Biochem.* **31**:573-585.

- GENIAUX, E.; LAGUERRE, G. & AMARGER, N. 1993. Comparison of geographically distant populations of *Rhizobium* isolated from root nodules of *Phaseolus vulgaris*. *Mol. Ecol.* **2**:295-302.
- GIOVANNONI, S. J.; BRITSCHGI, T. B.; MOYER, C. L. & FIELD, K. G. 1990. Genetic diversity in Sargasso Sea bacterioplankton. *Nature* **345**:60-63.
- GRAHAM, P. H.; OCAMPO, G.; RUIZ, L. D. & DUQUE, A. 1980. Survival of *Rhizobium phaseoli* in contact with chemical seed protectants. *Agron. J.* **72**:625-627.
- GÜRTLER, V. & STANISICH, V.A. 1996. New approaches to typing and identification of bacteria using the 16S-23S rDNA spacer region. *Microbiology* **142**:3-16.
- HAHN, D.; STARRENBURG, M. J. C. & AKKERMANS, A. D. L. 1990. Oligonucleotide probes that hybridize with rRNA as a tool to study *Frankia* strains in root nodules. *Appl. Environ. Microbiol.* **56**:1342-1346.
- HERBERT, R. A. 1990. Methods for enumerating microorganisms and determining biomass in natural environments. *Methods Microbiol.* **19**:1-40.
- HEUER, H.; KRSEK, M.; BAKER, P.; SMALLA, K. & WELLINGTON, E.M.H. 1997. Analysis of actinomycete communities by specific amplification of genes encoding 16S rRNA and gel-electrophoretic separation in denaturing gradients. *Appl. Environ. Microbiol.* **63**:3233-3241.
- HEUER, H. & SMALLA, K. 1997. Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis for studying soil microbial communities. In: van Elsas, J. D.; Wellington, E. M. H.; Trevors, J. eds., *Modern Soil Microbiology*. New York, Marcel Dekker, Inc., p. 353-373.
- HIRSCH, A. M. 1992. Developmental biology of legume nodulation. *New Phytol.* **122**:211-237.
- HOLBEN, W. E.; JANSSON, J. K.; CHELM, B. K. & TIEDJE, J. M. 1988. DNA probe method for the detection of specific microorganisms in the soil bacterial community. *Appl. Environ. Microbiol.* **54**:703-711.
- HONEYCUTT, R.J.; SOBRAL, B.W.S. & McCLELLAND, M. 1995. tRNA intergenic spacers reveal polymorphisms diagnostic for *Xanthomonas albilineans*. *Microbiology* **141**:3229-3239.
- HUGENHOLTZ, P.; GOEBEL, B.M. AND PACE, N.R. 1998. Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. *J. Bacteriol.* **180**:4765-4774.
- ISLAM, R. & AFENDI, F. 1980. Effect of several herbicides on the nodulation and yield of faba bean. *Fabis Newsletter* **2**:36-37.
- JENSEN, M.A.; WEBSTER, J.A. & STRAUS, N. 1993. Rapid identification of bacteria on the basis of polymerase chain reaction-amplified ribosomal DNA spacer polymorphisms. *Appl. Environ. Microbiol.* **59**:945-952.
- JENSEN, S.; ØVREÅS, L.; DAAE, F.L. & TORSVIK, V. 1998. Diversity in methane enrichments from agricultural soil revealed by DGGE separation of PCR amplified 16S rDNA fragments. *FEMS Microbiol. Ecol.* **26**:17-26.
- JORDAN, D. C. 1984. *Rhizobiaceae* Conn 1938, 321^{AL}, p.234-256. In: N. R. Krieg and J. G. Holt (ed.), *Bergey's manual of systematic bacteriology*, vol. 1. The Williams & Wilkins Co., Baltimore.
- KOLE, S.C. & DEY, B.K. 1989. Effect of aromatic amine herbicides on bacterial population and nitrogen fixing power of the rhizosphere soils of groundnut. *Environ. Ecol.* **7**:850-853.

- KUYKENDALL, L.D.; SAXENA, B.; DEVINE, T.E. & UDELL, S.E. 1992. Genetic diversity in *Bradyrhizobium japonicum* (Jordan, 1982) and a proposal for *Bradyrhizobium elkanii* sp. nov. *Can. J. Microbiol.* **38**:501-505.
- LAGUERRE, G.; MAVINGUI, P.; ALLARD, M.-R.; CHARNAY, M.-P.; LOUVRIER, P.; MAZURIER, S.-I.; RIGOTTIER-GOIS, L. & AMARGER, N. 1996. Typing of rhizobia by PCR DNA fingerprinting and PCR-Restriction Fragment Length Polymorphism analysis of chromosomal and symbiotic gene regions: application to *Rhizobium leguminosarum* and its different biovars. *Appl. Environ. Microbiol.* **62**:2029-2036.
- LIESACK, W. & STACKEBRANDT, E. 1992. Occurrence of novel groups of the domain *Bacteria* as revealed by analysis of genetic material from an Australian terrestrial environment. *J. Bacteriol.* **174**:5072-5078.
- LUDWIG, W.; AMMAN, R.; MARTINEZ-ROMERO, E.; SHÖNHUBER, W.; BAUER, S.; NEEF, A. & SCHLEIFER, K.-H. 1998. rRNA based identification and detection systems for rhizobia and other bacteria. *Plant and Soil* **204**:1-19.
- LUPWAYI, N.Z.; RICE, W.A. & CLAYTON, G.W. 1998. Soil microbial diversity and community structure under wheat as influenced by tillage and crop rotation. *Soil Biol. Biochem.* **30**:1733-1741.
- LUPWAYI, N.Z.; RICE, W.A. & CLAYTON, G.W. 1999. Soil microbial biomass and carbon dioxide flux under wheat as influenced by tillage and crop rotation. *Can. J. Soil Sci.* **79**:273-280.
- MALLIK, M.A.B. & TESFAI, K. 1985. Pesticidal effect on soybean-rhizobia symbiosis. *Plant and Soil* **85**:33-41.
- MARTINEZ-ROMERO, E. & CABALLERO, J.M. 1996. *Rhizobium* phylogenies and bacterial genetic diversity. *Crit. Rev. Plant Sci.* **15**:113-140.
- MASSOL-DEYA, A.A.; ODELSON, D.A.; HICKEY, R.F. & TIEDJE, J.M. 1995. Bacterial community fingerprinting of amplified 16S and 16S-23S ribosomal DNA gene sequences and restriction endonuclease analysis (ARDRA). In *Molecular microbial ecology manual*. ed. Akkermans, A.D.L.; van Elsas, J.D. and de Bruijn, F.J. **3.3.2**, 1-8. Dordrecht: Kluwer Academic Publishers.
- McCLELLAND, M.; PETERSEN, C. & WELSH, J. 1992. Length polymorphisms in tRNA intergenic spacers detected by using the polymerase chain reaction can distinguish streptococcal strains and species. *J. Clin. Microbiol.* **30**:1499-1504.
- MOREIRA, F. M. S.; GILLIS, M.; POT, B.; KERSTERS, K. & FRANCO, A. A. 1993. Characterization of rhizobia isolated from different divergence groups of tropical Leguminosae by comparative polyacrylamide gel electrophoresis of their total proteins. *Syst. Appl. Microbiol.* **16**:135-146.
- MULLIS, K.B. & FALOONA, F.A. 1987. Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. *Methods Enzymol.* **155**:335-351.
- MUYZER, G.; WAAL, E. C.; UITTERLINDEN, A.G. 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl. Environ. Microbiol.* **59**:695-700.
- MUYZER, G.; HOTTENTRAGER, S.; TESKE, A. and WAWER, C. 1995. Denaturing gradient gel electrophoresis of PCR-amplified 16S rDNA—a new molecular approach to analyse the genetic diversity of mixed microbial communities, section 3.4.4, p. 1-23. In A.D.L. Akkermans, J.D. van Elsas, and F.J. de Bruijn (ed.), *Molecular microbial ecology manual*. Kluwer Academic Publishers, Dordrecht, The Netherlands.

- NEVES, M.C.P. & RUMJANEK, N.G. 1997. Diversity and adaptability of soybean and cowpea rhizobia in tropical soils. *Soil Biol. Biochem.* **29**: 889-895.
- NOUR, S.M.; CLEYET-MAREL, J.-C.; NORMAND, P. & FERNANDEZ, M.P. 1995. Genomic heterogeneity of strains nodulating chickpeas (*Cicer arietinum* L.) and description of *Rhizobium mediterraneum* sp. nov. *Int. J. Syst. Bacteriol.* **45**:640-648.
- NÜSSLEIN, K. & TIEDJE, J.M. 1998. Characterization of the dominant and rare members of a young Hawaiian soil bacterial community with small-subunit ribosomal DNA amplified from DNA fractionated on the basis of its guanine and cytosine composition. *Appl. Environ. Microbiol.* **64**:1283-1289.
- NUTMAN P.S. 1987. Centenary lecture. *Philosophical Transactions of the Royal Society of London.* **317**:69-106.
- OGRAM, A.; SAYLER, G. S. & BARKAY, T. 1988. DNA extraction and purification from sediments. *J. Microbiol. Methods* **7**:57-66.
- PACE, N. R.; STAHL, D. A.; LANE, D. J. & OLSEN, G. J. 1986. The analysis of natural microbial populations by ribosomal RNA sequences. *Adv. Microb. Ecol.* **9**:1-55.
- PACE, N.R. 1996. New Perspective on the Natural Microbial World: Molecular Microbial Ecology. *ASM News.* **62**:463-470
- PAFFETTI, D.; SCOTTI, C.; GNOCCHI, S.; FANCELLI, S. & BAZZICALUPO, M. 1996. Genetic diversity of an Italian *Rhizobium meliloti* population from different *Medicago sativa* varieties. *Appl. Environ. Microbiol.* **62**:2279-2285.
- PAHWA, S.K. & PRAKASH, J. 1992. Effect of some herbicides on the growth, nodulation and nitrogen fixation in chickpea (*Cicer arietinum* L.). *Ind. J. Plant Physiol.* **35**:207-212.
- PAREEK, R. P. & GAUR, A. C. 1970. Effect of dichlorodiphenyltrichloro ethane (DDT) on symbiosis of *Rhizobium* sp. with *Phaseolus aureus* (green gram). *Plant and Soil* **33**:297-304.
- PICKUP, R. W. 1991. Development of molecular methods for the detection of specific bacteria in the environment. *J. Gen. Microbiol.* **137**:1009-1019.
- PINERO, D.; MARTINEZ, E. & SELANDER, R. K. 1988. Genetic diversity and relationships among isolates of *Rhizobium leguminosarum* biovar *phaseoli*. *Appl. Environ. Microbiol.* **54**:2825-2832.
- PITCHER, D. G.; SAUNDERS, N.A. & OWEN, R.J. 1989. Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. *Lett. Appl. Microbiol.* **8**:151-156.
- ROSADO, A.S.; SELDIN, L.; WOLTERS, A.C. & van ELSAS, J.D. 1996. Quantitative 16S rDNA-targeted polymerase chain reaction and oligonucleotide hybridization for the detection of *Paenibacillus azotofixans* in soil and the wheat rhizosphere. *FEMS Microbiol. Ecol.* **19**:153-164.
- SCHNELLE, M.A. & HENSLEY, D.L. 1990. Effects of pesticides upon nitrogen fixation and nodulation by dry bean. *Pestic. Sci.* **28**:83-88.
- SEAL, S.E.; JACKSON, L.A. & DANIELS, M.J. 1992. Use of tRNA consensus primers to indicate subgroups of *Pseudomonas solanacearum* by polymerase chain reaction amplification. *Appl. Environ. Microbiol.* **58**:3759-3761.
- SELIM, K.G.; MAHMOUD, S.A.Z. & EL-MOKADEN, M.T. 1970. Effect of dieldrin and lindane on the growth and nodulation of *Vicia faba*. *Plant and Soil* **33**:325-329.
- SMALLA, K.; CRESSWELL, N.; MENDONÇA-HAGLER, L.C.; WOLTERS, A.C. & van ELSAS, J.D. 1993. Rapid DNA extraction protocol from soil for polymerase chain reaction-mediated amplification. *J. Appl. Bacteriol.* **74**:78-85.

- SMITH, C.R.; FUNKE, B.R. & SCHULZ, J.T. 1978. Effects of insecticides on acetylene reduction by alfafa, red clover and sweet clover. *Soil Biol. Biochem.* **10**:463-466.
- STACKEBRANDT, E.; LIESACK, W. & GOEBEL, B. M. 1993. Bacterial diversity in a soil sample from a subtropical Australian environment as determined by 16S rDNA analysis. *FASEB J.* **7**:232-236.
- STEFFAN, R. J. & ATLAS, R. M. 1988. DNA amplification to enhance the detection of genetically engineered bacteria in environmental samples. *Appl. Environ. Microbiol.* **54**:2185-2191.
- STEFFAN, R. J.; GOKSØYR, J.; BEJ, A. K. & ATLAS, R. M. 1988. Recovery of DNA from soils and sediments. *Appl. Environ. Microbiol.* **54**:2908-2915.
- STEFFAN, R. J. & ATLAS, R. M. 1991. Polymerase chain reaction: applications in environmental microbiology. *Annu. Rev. Microbiol.* **45**:137-161.
- SULLIVAN, J.T.; EARDLY, B.D.; VAN BERKUM, P. & RONSON, C.W. 1996. Four unnamed species of nonsymbiotic rhizobia isolated from the rhizosphere of *Lotus corniculatus*. *Appl. Environ. Microbiol.* **62**:2818-2825.
- TEREFEWOR, Z.; NICK, G.; SUOMALAINEN, S.; PAULIN, L. & LINDSTRÖM, K. 1998. Phylogeny of *Rhizobium galegae* with respect to other rhizobia and agrobacteria. *Int. J. Syst. Bacteriol.* **48**:349-356.
- TESKE, A.; SIGALEVICH, P.; COHEN, Y. AND MUYZER, G. 1996. Molecular identification of bacteria from a coculture by denaturing gradient gel electrophoresis of 16S ribosomal DNA fragments as a tool for isolation in pure cultures. *Appl. Environ. Microbiol.* **62**:4210-4215.
- TORSVIK, V.; GOKSOYR, J. & DAAE, F. L. 1990. High diversity in DNA of soil bacteria. *Appl. Environ. Microbiol.* **56**:782-787.
- TORSVIK, V.; DAAE, F.L.; SANDAA, R.A. & OVREAS, L. 1998. Novel techniques for analysing microbial diversity in natural and perturbed environments. *J. Biotechnol.* **64**:53-62.
- TRINICK, M. J. 1980. Relationships amongst the fast-growing rhizobia of *Lablab purpureus*, *Leucaena leucocephala*, *Mimosa* spp., *Acacia farnesiana* and *Sesbania grandiflora* and their affinities with other rhizobial groups. *J. Appl. Bacteriol.* **49**:39-53.
- VALLAEYS, T.; TOPP, E.; MUYZER, G.; MACHERET, V.; LAGUERRE, G.; RIGAUD, A. & SOULAS, G. 1997. Evaluation of denaturing gradient gel electrophoresis in the detection of 16S rDNA sequence variation in rhizobia and methanotrophs. *FEMS Microbiol. Ecol.* **24**:279-285.
- VAN BERKUM, P.; BEYENE, D.; BAO, G.; CAMPBELL, T.A.; & EARDLY, B.D. 1998. *Rhizobium mongolense* sp. nov. is one of three rhizobial genotypes identified which nodulate and form nitrogen-fixing symbioses with *Medicago ruthenica*. *Int. J. Syst. Bacteriol.* **48**:13-22.
- XU, L.M.; GE, C.; CUI, Z.; LI, J. & FAN, H. 1995. *Bradyrhizobium liaonigensis* sp. nov. isolated from the root nodules of soybeans. *Int. J. Syst. Bacteriol.* **45**:706-711.
- WANG, E.T.; VAN BERKUM, P.; BEYENE, D.; SUI, X.-H.; DORADO, O.; CHEN, W.X. & MARTINEZ-ROMERO, E. 1998. *Rhizobium huautlense* sp. nov., a symbiont of *Sesbania herbacae* that has a close phylogenetic relationship with *Rhizobium galegae*. *Int. J. Syst. Bacteriol.* **48**:687-699.
- WANG, E.T.; VAN BERKUM, P.; SUI, X.-H.; BEYENE, D.; CHEN, W.X. & MARTINEZ-ROMERO, E. 1999. Diversity of rhizobia associated with *Amorpha fruticosa* isolated from Chinese soils and description of *Mesorhizobium amorphae* sp. nov. *Int. J. Syst. Bacteriol.* **49**:51-65.

- WARD, D. M.; WELLER, R. & BATESON, M. M. 1990. 16S rRNA sequences reveal numerous uncultured microorganisms in a natural community. *Nature* **345**:63-65.
- WAVER, C. & MUYZER, G. 1995. Genetic diversity of *Desulfovibrio* spp. in environmental samples analyzed by denaturing gradient gel electrophoresis of [NiFe] hydrogenase gene fragments. *Appl. Environ. Microbiol.* **61**:2203-2210.
- WIEDMANN-AL-AHMAD, M.; TICHY, H.-V. & SCHOEN, G. 1994. Characterization of *Acinetobacter* type strains and isolates obtained from wastewater treatment plants by PCR fingerprinting. *Appl. Environ. Microbiol.* **60**:4066-4071.
- YEATES, G.W.; BARDGETT, R.D.; COOK, R.; HOBBS, P.J.; BOWLING, P.J. & POTTER, J.F. 1997. Faunal and microbial diversity in three Welsh grassland soils under conventional and organic management regimes. *J. Appl. Ecol.* **34**:453-470.
- YOUNG, J.P.W. 1996. Phylogeny and taxonomy of rhizobia. *Plant Soil* **186**:45-52.
- ZHANG, X.; HARPER, R.; KARSISTO, M. & LINDSTRÖM, K. 1991. Diversity of *Rhizobium* bacteria isolated from the root nodules of leguminous trees. *Int. J. Syst. Bacteriol.* **41**:104-113.