Universidade Federal do Rio Grande do Sul
Programa de Pós Graduação em Genética e Biologia Molecular

Diversidade de *Bradyrhizobium elkanii* e *B. japonicum* que nodulam soja em solos do Rio Grande do Sul

Tese de Doutorado

Adriana Giongo

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Tese submetida ao Programa de Pós Graduação em Genética e Biologia Molecular da Universidade Federal do Rio Grande do Sul, como requisito parcial para a obtenção do Título de Doutor em Ciências.

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La culpa, por lo que cuentan, es del nitrógeno... Las bacterias y plantas han patentado su propio sistema para fijar el nitrógeno en las células merced a trucos muy engeniosos y sin molestar a nadie. Pero los animales para ganar tiempo y no darle más vueltas al asunto, han resuelto el problema comiéndose las plantas y asimilando de este modo el nitrógeno ya manufacturado... De los seres humanos para qué hablarte. Comemos plantas, animales herbívoros y también carnívoros: todo vale. Si algún ser en el mundo ha hecho divisa del “todo vale”, somos nosotros... el hombre es el depredador total, la fiera más completa de las conocidas. La culpa de esta feroz condición, si es que nos empeñamos en hablar de culpas, la tiene, ya digo, el nitrógeno: ¿no se podia haber fijado en las células él solito, sin tantos melindres ni complicaciones?

(Fernando Savater)

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que disse que eu podia

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Sumário

Lista de Abreviaturas

AFLP	*amplified fragment length polymorphism*
	(polimorfismo do comprimento do fragmento amplificado)
ATP	*adenosine triphosphate*
	(trifosfato de adenosina)
BOX	*elementos de seqüências repetitivas*
DNA	*desoxyribonucleic acid*
	(ácido desoxirribonucléico)
rDNA	*ribossomal desoxyribonucleic acid*
	(ácido desoxirribonucléico ribossomal)
ERIC	*enterobacterial repetitive intergenic consensus*
	(seqüências intergênicas de consenso em enterobactérias)
FBN	*fixação biológica do nitrogênio*
N_2	*nitrogênio atmosférico*
NH_4	*amônia*
PGPB	*plant growth promoting bacteria*
	(bactéria promotora de crescimento vegetal)
PCR	*polymerase chain reaction*
	(reação de polimerização em cadeia)
SEMIA	*Sessão de Microbiologia Agrícola*
T3SS	*type III secretion system*
	(sistema de secreção do tipo III)

Resumo

Rizóbios são bactérias aeróbias, Gram-negativas, que fixam nitrogênio atmosférico quando associadas a leguminosas. O gênero *Bradyrhizobium* é de grande importância na agricultura, pois essas bactérias fixam nitrogênio em simbiose com soja [*Glycine max* (L.) Merrill]. A caracterização dos rizóbios é fundamental para estudos relacionados à diversidade e à distribuição ecológica desses microrganismos. Três estudos foram conduzidos nesse trabalho: i) uma estratégia baseada em amplificação por PCR para diferenciar *Bradyrhizobium japonicum* de *B. elkanii*, utilizando-se seqüências 16S rDNA; ii) a caracterização da variabilidade genética de uma população de bradirrizóbios isolada de um campo experimental, trinta anos após a inoculação de estirpes padrão; iii) a avaliação da variabilidade genética de bradirrizóbios isolados de cinco regiões produtoras de soja no Estado do Rio Grande do Sul (RS) e dos possíveis fatores ambientais que poderiam ser os responsáveis por tal diversidade. Técnicas de biologia molecular foram utilizadas na identificação, ocorrência, distribuição e estudo populacional dos rizóbios, especialmente a amplificação do gene 16S rRNA por PCR, rep-PCR e AFLP. Essas duas últimas técnicas quando combinadas permitiram uma análise mais precisa da variabilidade genética das populações estudadas. O índice de diversidade de Shannon foi utilizado para comparar o grau de diversidade observado nas diferentes populações. Também foi observada uma correlação direta entre o grau de diversidade e o pH do solo. Os resultados obtidos permitiram concluir que as populações de bradirrizóbios que nodulam as lavouras de soja do RS são altamente variáveis, podem persistir nos solos, mesmo na ausência da planta hospedeira, e sofrem influência de fatores bióticos e abióticos.

Abstract

Rhizobia are Gram-negative aerobic bacteria that fix atmospheric nitrogen in symbiosis with leguminous plants. Bacteria belonging to the *Bradyrhizobium* genus are very important because they are able to nodulate and fix nitrogen in symbiosis with soybean [*Glycine max* (L.) Merrill]. Characterization of rhizobia is fundamental in studies concerning the diversity and ecological distribution of these microorganisms. Three studies have been conducted in this work: i) a strategy based on amplification by PCR to differentiate *Bradyrhizobium japonicum* and *B. elkanii* using 16S rDNA sequences; ii) the genetic variability assessment of a bradyrhizobial population isolated from an experimental field thirty years after the inoculation with reference strains; iii) the genetic variability characterization of bradyrhizobia isolated from five soybean fields in different regions in Rio Grande do Sul (RS) State and the environmental factors that can influence such diversity. Molecular biology techniques were used for the identification, occurrence, distribution, and studies of rhizobia population, specially the 16S rRNA gene amplification by PCR, rep-PCR and AFLP. When combined, these last two techniques provided an accurate analysis of the genetic diversity of the analyzed populations. Shannon diversity index was used to compare the diversity among different populations. A direct correlation was observed between diversity degree and soil pH. The results obtained have shown that bradyrhizobia nodulating soybean in RS are highly variable and were able to persist in soil even when the host legume was lacking. Besides they have shown to be influenced by abiotic and biotic parameters.

1 Introdução

Rizóbios são bactérias estritamente aeróbias, quimiorganotróficas, em forma de bastonetes não formadores de esporos, Gram-negativas, com tamanho que varia de 0,5-0,9 μm x 1,2-3,0 μm. Sua mobilidade é dada por um flagelo polar único ou dois a seis flagelos peritríqueos (Somasegaram e Hoben, 1994). Normalmente encontrados no solo, são capazes de fixar nitrogênio atmosférico quando em associação simbiótica com plantas da Família Fabaceae [aproximadamente 650 gêneros, 18.000 espécies (Sprent, 1995)]. Além das leguminosas, *Parasponia andersonii*, pertencente à Família Ulmaceae, é a única planta não-leguminosa conhecida capaz de realizar simbiose com rizóbios (Trinick *et al*., 1988; Rhijn e Vanderleyden, 1995).

Os rizóbios foram inicialmente agrupados em um único gênero, *Rhizobium* Frank 1889 (Kuykendall *et al*., 2005). Jordan (1982) sugeriu uma separação taxonômica baseada em curvas de crescimento, para diferenciar os rizóbios de multiplicação rápida (*Rhizobium*) daqueles de multiplicação lenta (*Bradyrhizobium*). Esse último gênero era composto por apenas uma espécie, *B. japonicum*, isolada a partir de nódulos de raízes de soja. Embora o termo rizóbio tenha sido utilizado inicialmente para designar bactérias pertencentes ao gênero *Rhizobium*, mais recentemente ele vem sendo utilizado para todas bactérias capazes de formar nódulos e fixar nitrogênio em associação com leguminosas e/ou que pertençam a um gênero correlato (Willems, 2007).

Atualmente, os rizóbios estão distribuídos nos gêneros *Rhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Sinorhizobium* (*Ensifer*), *Azorhizobium* (Lafay e Burdon, 1998; Michiels *et al*., 1998; Vásquez-Arroyo *et al*., 1998; Perret *et al*., 2000; Andrade *et al*., 2002; Willems *et al*., 2003) e *Allorhizobium* (Sessitsch *et al*., 2002). A partir da década de

1980, o uso de ferramentas de genética molecular (hibridização DNA-DNA e seqüenciamento do rDNA), juntamente com técnicas moleculares mais modernas, como a reação em cadeia da polimerase (PCR, *Polymerase Chain Reaction*) e seqüenciamentos genômicos, levaram à modificação e reorganização taxonômica dos gêneros existentes e à descrição de novos gêneros (Willems, 2007). Além dos gêneros citados acima, *Methylobacterium* (Sy *et al*., 2001), *Burkholderia* (Moulin *et al*., 2001), *Ralstonia* (Chen *et al*., 2001), *Devosia* (Rivas *et al*., 2003), *Phyllobacterium* (Valverde *et al*., 2005), *Ochrobactrum* (Trujillo *et al*., 2005) e *Cupriavidus* (Barrett e Parker, 2006) possuem espécies capazes de fixar nitrogênio atmosférico em nódulos formados em raízes e em caules de plantas hospedeiras, podendo ser, também, denominados de rizóbios, como mencionado anteriormente (Willems 2007).

Entre os rizóbios melhor estudados, as espécies pertencentes ao gênero *Bradyrhizobium* possuem um grande interesse agronômico, pois tais bactérias são capazes de fixar nitrogênio atmosférico em simbiose com muitas leguminosas, especialmente com a soja [*Glycine max* (L.) Merrill]. *Bradyrhizobium japonicum* (Jordan, 1982), *B. elkanii* (Kuykendall *et al*., 1992) e *B. liaoningense* (Xu *et al*., 1995) são os rizóbios de multiplicação lenta associados a essa leguminosa. No entanto, no Brasil, apenas linhagens das espécies *B. japonicum* e *B. elkanii* são utilizadas comercialmente como inoculantes para a soja (Freire, 1977; Peres e Vidor, 1980; Alberton *et al*., 2006).

1.1 Fixação Biológica do Nitrogênio e a Formação dos Nódulos

A fixação biológica do nitrogênio (FBN), processo que consiste na redução do nitrogênio atmosférico (N_2) em amônia (NH_4), é realizada por microrganismos chamados diazotróficos. Embora a habilidade de reduzir o nitrogênio atmosférico à amônia esteja presente em vários gêneros procariontes (Burns e Hardi, 1975), as condições fisiológicas necessárias para a FBN variam de espécie para espécie. Os rizóbios são as únicas bactérias diazotróficas capazes de fixar nitrogênio em uma relação de simbiose com plantas leguminosas. Além destes, existem ainda as bactérias fixadoras de nitrogênio de vida livre, as

que se associam com raízes de plantas e alguns tipos de algas azuis. Vários dos eventos que resultam na fixação de nitrogênio são muito complexos, envolvendo mudanças bioquímicas, fisiológicas e morfológicas, tanto na bactéria quanto na planta hospedeira ou associada (Beringer *et al*., 1979).

Embora os microrganismos diazotróficos vivam em ambientes distintos e apresentem diferenças significativas quanto à morfologia, fisiologia e genética, todos compartilham de um mesmo mecanismo bioquímico para a fixação do nitrogênio atmosférico em amônia, o complexo enzimático denominado de Nitrogenase. Este complexo é formado por duas proteínas essenciais para a redução do substrato. O componente maior, ou MoFe-proteína, é um tetrâmero de FeMoS consistindo de dois pares de subunidades não idênticas, enquanto que o componente menor da Nitrogenase é a Fe-proteína, um dímero de subunidades idênticas. No processo de fixação do nitrogênio, a Fe-proteína é primeiramente reduzida, sendo esta forma capaz de transferir elétrons para a MoFe-proteína, com consumo de ATP. Finalmente, a MoFe-proteína, que contém o sítio ativo para o substrato, efetua a redução do nitrogênio até amônia. A amônia, assim produzida, é incorporada ao metabolismo destes microrganismos sob a forma de glutamato e glutamina, que irão doar este nitrogênio reduzido a todas as rotas biossintéticas de compostos que contenham nitrogênio.

A bactéria de vida livre *Klebsiella pneumoniae* foi o primeiro microrganismo diazotrófico a ter os genes envolvidos na FBN identificados e caracterizados, bem como foi nessa bactéria que o processo de FBN foi primeiramente estudado (Arnold *et al*., 1988). Nela, os genes de fixação do nitrogênio encontram-se agrupados em uma região cromossômica abrangendo 24.206 pares de base (Arnold *et al*., 1988), onde estão dispostos 20 genes, denominados genes *nif* (do ingles, *nitrogen fixation*), organizados em 8 operons: *nifJ*, *nifHDKTY*, *nifENX*, *nifUSVWZ*, *nifM*, *nifF*, *nifLA* e *nifBQ*.

A organização dos genes de fixação de nitrogênio de *K. pneumoniae* em agrupamentos de operons *nif* consiste em um modelo estrutural que também é extrapolado, até certo ponto, para os demais organismos diazotróficos, como os rizóbios, *Azotobacter* e *Azospirillum*. No entanto, a associação de bactérias fixadoras de nitrogênio com plantas mais estudada em nível molecular é a relação das leguminosas e os rizóbios (Vanderleyden e Pieternel, 1995).

Os rizóbios diferenciam-se de outras bactérias diazotróficas devido à sua capacidade de nodulação. Durante a associação, a bactéria induz a planta a desenvolver um novo órgão, o nódulo. A formação dessa estrutura depende de um reconhecimento específico entre a bactéria e a planta hospedeira, ocasionando a invasão dos rizóbios no tecido vegetal. Embora o microssimbionte possa viver independente no solo, somente quando em simbiose com leguminosas é que a FBN é possível (Sandowsky *et al*., 1995). Esse processo ocorre após as bactérias terem se diferenciado em bacteróides. A reação química de conversão do nitrogênio à amônia não apenas tem uma grande demanda energética (16 moléculas de ATP para cada molécula de N_2 reduzida), como também é muito sensível à presença de oxigênio no nódulo e de compostos nitrogenados no solo (Sandowsky *et al*., 1995).

No solo ocorre uma troca de sinalizações moleculares entre a planta hospedeira e a bactéria, na qual compostos flavonóides liberados pela planta nos exudatos das raízes irão induzir nos rizóbios a expressão de operons contendo genes *nod* de forma altamente seletiva (López-García *et al*., 2002; Ausmees *et al*., 2004). Os genes *nod* codificam enzimas de biossíntese e liberação de lipo-quito-oligossacarídeos, conhecidos como fatores Nod, que são secretados e essenciais para o processo de infecção (Gresshoff, 2003; Ausmees *et al*., 2004). Após a infecção, sinais bacterianos adicionais, como polissacarídeos de superfície ou proteínas secretadas pelas bactérias, também são requeridos para uma eficiente nodulação de plantas hospedeiras específicas (Ausmees *et al*., 2004).

Os genes de nodulação de rizóbios estão organizados em diversos operons, que podem estar localizados no cromossomo ou em grandes plasmídeos, dependendo da espécie em questão (Chen *et al*., 2005). Os genes são positivamente regulados pela proteína NodD (produto do gene *nodD*). Essa proteína se liga a uma seqüência chamada Box-nod, localizada na região promotora dos genes *nod*, *nol* e *noe*, ativando a transcrição dos operons, mediante a presença de uma molécula flavonóide indutora (Viprey *et al*., 1998; Chen *et al*., 2005).

Dentre os genes envolvidos com a nodulação já descritos para as espécies de rizóbios estudadas existem muitos envolvidos na faixa de amplitude de hospedeiros para a simbiose. Esse é o caso dos genes *nolXWBTUV* de *Sinorhizobium fredii* USDA257, os quais, quando mutados, alteraram a especificidade de hospedeiros da bactéria. Análises moleculares e o seqüenciamento da região do lócus *nolXWBTUV* (que pode ser referido como lócus de

especificidade de cultivar) de *Rhizobium* sp. NGR234 (Viprey *et al*., 1998) e *Bradyrhizobium japonicum* USDA110 (Gottfert *et al*., 2001) revelaram a presença de um sistema de secreção do tipo III (T3SS, *Type III Secretion System*). Inicialmente descrito em bactérias patógenas de animais e vegetais (Baker *et al*., 1997; Hueck, 1998), ele é composto por aproximadamente 20 genes, cujos produtos geram um sistema de patogenicidade que capacita as bactérias Gram-negativas a secretar e injetar proteínas patogênicas diretamente no citosol dos hospedeiros eucarióticos (Hueck, 1998). Os genes e proteínas do sistema T3SS foram amplamente estudados na estirpe *Rhizobium* sp. NGR234. Entretanto, em outros rizóbios, o sistema ainda não foi plenamente elucidado. Na maior parte dessas bactérias, apenas algumas seqüências foram isoladas, identificadas e/ou deduzidas para o sistema (Gottfert *et al*., 2001; Krishnan 2002; Krause *et al*., 2002). No gênero *Bradyrhizobium* somente a espécie *B. japonicum*, amplamente utilizada na FBN em soja, teve seus genes isolados. Estas seqüências foram identificadas como pertencentes ao sistema T3SS, porém, a completa caracterização desse sistema nesse organismo ainda não foi realizada (Göttfert *et al*., 2001; Krause *et al*., 2002). Apesar do sistema T3SS já ter sido descrito na estirpe *B. japonicum* USDA110, existem poucos estudos mostrando a presença desse sistema em *B. elkanii* (Viprey *et al*., 1998; Marie *et al*., 2001).

1.2 Rizóbios e a fixação biológica de nitrogênio em soja

O nitrogênio é um dos nutrientes mais limitantes para as culturas em geral, e, na cultura de soja, isso não é diferente. Estima-se ser necessário 80 kg de N para se produzir 1.000 kg de grãos de soja (Zilli *et al*., 2005). A FBN, através da simbiose rizóbio/leguminosa, é uma forma barata e eficiente de suprir as necessidades de cultivo. Para a soja, por exemplo, a inoculação com estirpes de bradirrizóbio supre as necessidades da planta, dispensando o uso de fertilizantes nitrogenados.

O Brasil é o segundo maior produtor mundial de soja, participando com quase 25% desta produção em 2004. Em 2006, a produção de grãos chegou a 117 milhões de

toneladas (IBGE, 2007a), com 20,5 milhões de hectares plantados em 2007 (IBGE, 2007b). Desde 1998, a cultura da soja tem se expandido significativamente, ampliando a produção no país em 38,7% (Atlas Socioeconômico do Rio Grande do Sul, 2007). O Rio Grande do Sul é o terceiro maior produtor de soja do Brasil, com 16,8% do total produzido, relativo à produção média de 2001 a 2003. Já a safra 2006 produziu 7,6 milhões de toneladas. Em 2007, a produção de soja no Rio Grande do Sul promoveu um acréscimo de 2,3% na produção nacional. A produtividade observada nessa safra (2.586 kg. ha^{-1}) é a segunda maior obtida no Estado, superada apenas pela de 2003 (2.667 kg. ha^{-1}) (IBGE, 2007b).

Teoricamente, as fontes de N disponíveis para a cultura da soja são os fertilizantes nitrogenados e a FBN (Hungria *et al*., 2001). Entretanto, no Rio Grande do Sul, como no resto do país, a adubação nitrogenada não é recomendada para soja (Comissão de Química e Fertilidade do Solo, 2004), pois a inoculação com bactérias fixadoras de nitrogênio fornece totalmente a necessidade de utilização de adubos nitrogenados nas lavouras. Além da economia gerada pela inoculação com estirpes de rizóbios em soja, que se estima ser, no Brasil, de pelo menos R$ 2,87 bilhões anualmente (Campo e Hungria, 2004; Menna *et al*., 2006), a inoculação não origina poluição ambiental, conseqüência comum da utilização de fertilizantes nitrogenados.

1.3 Diversidade genética dos rizóbios

Uma vez que a soja é uma cultura exótica, os solos brasileiros originalmente não possuíam rizóbios eficientes para essa leguminosa (Ferreira e Hungria, 2002; Hungria *et al*., 2006; Loureiro *et al*., 2006). Uma possibilidade bem aceita é que os atuais bradirrizóbios possivelmente tenham vindo com as primeiras sementes de soja dos Estados Unidos. Com a inoculação massiva nas últimas décadas, essa população aumentou de forma significativa (Ferreira e Hungria, 2002). Em 1975, o governo brasileiro instituiu normas à produção e comercialização de inoculantes, definindo que os inoculantes deveriam conter apenas estirpes recomendadas por instituições públicas de pesquisa. A Coleção de Cultura de *Rhizobium*

SEMIA (Seção de Microbiologia Agrícola) da Fundação Estadual de Pesquisa Agropecuária (FEPAGRO) é responsável, desde então, pela manutenção e distribuição das estirpes utilizadas comercialmente no país. A partir de 1992, quatro estirpes são autorizadas para uso em inoculantes comerciais no Brasil: *Bradyrhizobium elkanii* SEMIA 587 e SEMIA 5019 e *Bradyrhizobium japonicum* SEMIA 5079 e SEMIA 5080 (FEPAGRO, 1999).

O sucesso da inoculação com as estirpes recomendadas depende, em parte, da sua capacidade de persistir no solo e, assim, competir com a microflora nativa já estabelecida. A competição entre estirpes no solo é complexa e é causada por diferentes fatores, como as características de cada estirpe, o hospedeiro com o qual se relaciona, a interação planta-microrganismo e o impacto do ambiente sobre eles. Alguns experimentos mostram que as estirpes de rizóbios podem se naturalizar, sofrendo alterações nas características morfológicas, fisiológicas, genéticas e simbióticas, o que as tornam distintas das estirpes introduzidas inicialmente (Boddey e Hungria, 1997; Ferreira e Hungria, 2002). Além disso, a diversidade e o tamanho da população rizobiana nativa no solo podem variar com a presença de leguminosas hospedeiras (Parker, 1999; Andrade *et al*., 2002). A presença de uma determinada espécie de leguminosa em determinado solo pode resultar no desenvolvimento de uma população simbiótica específica (Rodriguez-Navarro *et al*., 2000; Carelli *et al*., 2000). Em estudos realizados sobre diversidade de *R. leguminosarum* bv. *viciae*, observou-se que a planta *Vicia faba* (feijão de fava) poderia ser responsável pela seleção de um certo grupo de rizóbios no solo (van Berkum *et al*., 1995; Labes *et al*., 1996). Além disso, na ausência de uma planta hospedeira os estresses ambientais, como os efeitos tóxicos relacionados à acidez do solo, diminuem significativamente a população de rizóbios no solo (Kahindi *et al*., 1997; Santos *et al*., 1999; Hungria e Vargas, 2000; Andrade *et al*., 2002). Essas observações reforçam a interação entre estresses ambientais e a presença do legume hospedeiro na sobrevivência e manutenção das populações rizobianas no solo.

Um dos temas mais interessantes nos estudos de genomas microbianos é a transferência de genes entre rizóbios (Sullivan *et al*., 1995). Por essa transferência, genes necessários às interações simbióticas, que estão localizados em plasmídeos (como é o caso de *Rhizobium*) ou no cromossomo sob a forma de ilhas genômicas (como ocorre em *Bradyrhizobium*), podem ser transferidos para estirpes, gêneros relacionados e até mesmo para

gêneros diferentes (Finan, 2002), podendo, inclusive, converter uma bactéria saprofítica em simbiótica (Sullivan e Ronson, 1998). O plasmídeo simbiótico Sym, de *Sinorhizobium* e *Rhizobium*, contém os genes responsáveis pela infecção, fixação biológica de nitrogênio e especificidade hospedeira na associação rizóbio/leguminosa (Rogel *et al*., 2001). Análises de genética populacional nestes microrganismos indicaram a ocorrência de transferência plasmidial intensa entre estirpes (Souza e Eguiarte, 1997). Embora os genes relacionados à nodulação estejam integrados no cromossomo dos bradirrizóbios, foi relatada a transmissão vertical de genes relacionados à simbiose (Moulin *et al*., 2004) e à fixação de nitrogênio (Qian *et al*., 2003) de *Bradyrhizobium* a bactérias relacionadas. Possivelmente, essa transferência foi favorecida pela organização dos genes sob a forma de ilhas genômicas, que facilita a transmissão completa dos genes simbióticos (Göttfert *et al*., 2001; Kaneko *et al*., 2000). Essa constatação afeta diretamente o entendimento sobre a diversidade e ecologia dos rizóbios. Tais frações do genoma podem ser transferidas de uma bactéria à outra, através de um processo rápido no ambiente solo/rizosfera (Finan, 2002).

Muitos trabalhos relacionaram a alta diversidade de rizóbios à transferência lateral de genes (Galli-Terasawa *et al*., 2003; Loureiro *et al*., 2006). Além disso, plasmídeos transmissíveis são considerados fatores importantes na divergência e adaptabilidade das populações bacterianas no solo, contribuindo para a plasticidade genética dos indivíduos (Wernegreen *et al*., 1997). Algumas características do solo também podem influenciar as populações de rizóbios quanto à transferência gênica. Wernegreen *et al*. (1997) demonstraram que as quantidades de matéria orgânica e de argila, juntamente com o pH do solo, podem influir nas taxas de transferência plasmidial entre isolados de *Sinorhizobium fredii*, o que afetaria o nível de diversidade genética dessa população. Por outro lado, a perda de regiões plasmidiais relacionadas às características simbióticas pode ocorrer em rizóbios sujeitos a longas exposições a estresses ambientais (Moawad *et al*., 1998).

1.4 Técnicas para o estudo da diversidade de rizóbios

O acesso à diversidade genética das bactérias fixadoras de nitrogênio é fundamental para os estudos relacionados à variabilidade e à distribuição ecológica desses microrganismos. O uso de técnicas moleculares tem estimulado o desenvolvimento de métodos simples e rápidos para a caracterização de populações microbianas, inclusive para estudos em nível de gênero, espécie e até mesmo estirpe (Schneider e de Bruijn, 1996). A técnica de amplificação de seqüências específicas de DNA, pela reação em cadeia da polimerase (PCR), vem sendo bastante utilizada em estudos filogenéticos (Eardly *et al*., 1992; Ueda *et al*., 1995) e para detecção, identificação e caracterização de estirpes de rizóbio (Harrison *et al*., 1992; Laguerre *et al*., 1994; Watson *et al*., 1995). Esse método pode ser executado utilizando-se oligonucleotídeos iniciadores arbitrários ou complementares a determinada seqüência do genoma bacteriano.

Uma variação da técnica de PCR está baseada na utilização de oligonucleotídeos iniciadores, correspondentes a seqüências repetitivas de consenso, dispersas no genoma das eubactérias [rep-PCR (de Bruijn, 1992)]. A amplificação de seqüências repetitivas intergênicas, conhecidas como ERIC (*Enterobacterial Repetitive Intergenic Consensus*) e elementos BOX (seqüências repetitivas espalhadas no genoma), geram padrões altamente característicos, quando separadas em gel de agarose (Selenska-Pobell,1995). Com a metodologia de rep-PCR é possível se obter uma boa discriminação das amostras em nível de estirpe (Olive e Bean, 1999), embora ela não seja capaz de agrupá-las quanto à espécie ou gênero (Ferreira e Hungria, 2002; Galli-Terasawa *et al*., 2003; Grange e Hungria, 2004; Kaschuk *et al*., 2006a, 2006b). Como os oligonucleotídeos iniciadores não são específicos para gênero, espécie ou estirpe, a técnica permite que os oligonucleotídeos iniciadores generalizados sejam utilizados para comparar diferentes bactérias dentro de uma mesma população. A técnica de rep-PCR é uma ferramenta universal para os estudos de variações genômicas em organismos procariontes e reflete a variabilidade do genoma por inteiro (Igual *et al*., 2001).

As seqüências ERIC e BOX são altamente conservadas entre os rizóbios e foram utilizadas para distinguir e classificar diferentes estirpes em estudos de diversidade da

população de *Bradyrhizobium* em solos na Polônia, onde não havia bradirrizóbios nativos capazes de nodular soja (Madrzak *et al*., 1995); para avaliar o impacto de vários parâmetros ambientais em uma população de *Rhizobium leguminosarum* bv. *viciae* (Labes *et al*., 1996); e em diversos outros estudos populacionais de rizóbios (de Bruijn, 1992; Selenska-Pobell, 1995; Selenska-Pobell *et al*., 1996; Laguerre *et al*., 1997; Vinuesa *et al*., 1998; Sikora *et al*., 2002). Especialmente no Brasil, muitos trabalhos utilizaram a técnica de rep-PCR e obtiveram resultados satisfatórios quanto à análise de diversidade e variabilidade genética das populações de rizóbios (Chen *et al*., 2000; Ferreira e Hungria, 2002; Galli-Terasawa *et al*., 2003; Grange e Hungria, 2004; Alberton *et al*., 2006; Hungria *et al*., 2006; Kaschuk *et al*., 2006 a, b; Loureiro *et al*., 2006).

Outra técnica utilizada para diferenciação de estirpes de rizóbios é o estudo do polimorfismo do comprimento do fragmento amplificado (AFLP, *Amplified Fragment Length Polymorphism*). Essa técnica baseia-se na amplificação seletiva, por PCR, de fragmentos de DNA genômico total, gerados pela digestão com enzimas de restrição (Vos *et al*., 1995; Lin *et al*., 1996; Olive e Bean, 1999). A AFLP é uma técnica relativamente rápida e simples, cuja reprodutibilidade tem sido testada em vários laboratórios com bastante sucesso. Outro ponto importante dessa metodologia é que não é necessário o conhecimento prévio do genoma a ser analisado e o mesmo protocolo pode ser aplicado para uma ampla variedade de organismos (Lucchini, 2003).

Inicialmente utilizada para diferenciação genotípica de plantas, a AFLP tem sido cada vez mais aplicada para distinguir linhagens de microrganismos (Huys *et al*., 2000; Ventura *et al*., 2002; Hahm *et al*., 2003), inclusive para a diferenciação de bactérias fixadoras de nitrogênio (Gao *et al*., 2001; Wolde-meskel *et al*., 2004), como *Rhizobium galegae* (Terefework *et al*., 2001; Andronov *et al*., 2003) e *Bradyrhizobium* (Olive e Bean, 1999; Savelkoul *et al*., 1999; Doignon-Bourcier *et al*., 2000; Willems *et al*., 2000; Willems *et al*., 2001; Willems *et al*., 2003; Kalita e Malek, 2004).

Terefework *et al*. (2001) demonstraram que a AFLP é uma técnica excelente para diferenciar estirpes ou espécies intimamente relacionadas, mas não se aplica como uma ferramenta filogenética. Alguns autores têm utilizado a técnica de rep-PCR concomitantemente com a AFLP (Ventura e Zink, 2002; Guimarães *et al*., 2003). Com isso

torna-se possível aliar a simplicidade e rapidez da rep-PCR na distinção de linhagens bacterianas e nos estudos de diversidade, com a capacidade discriminatória da AFLP. Segundo Gao *et al*. (2004), uma vez que a AFLP pode ser aplicada em qualquer DNA, independente da origem ou complexidade, ela amplia a faixa de genomas que podem ser analisados (Vos *et al*., 1995; Restrepo *et al*., 2000).

2 Justificativa

Procuram-se, cada vez mais, estirpes de rizóbios que sejam eficientes, competitivas e de melhor qualidade, que possam ser usadas comercialmente nas plantações de leguminosas. Paralelamente a isso, faz-se necessário o aprofundamento dos estudos sobre a ecologia e a genética dos microrganismos já utilizados para essa finalidade. Por fim, em um país como o Brasil, onde as leguminosas têm papel fundamental na economia, o estudo de bactérias simbióticas fixadoras de nitrogênio e seus aspectos genético-ecológicos, torna-se decisivo para o incremento da produção de grãos e forrageiras.

3 Objetivos

O objetivo geral deste trabalho foi o isolamento e a caracterização microbiológica e genética de estirpes de *Bradyrhizobium japonicum* e *B. elkanii* em lavouras de soja do Rio Grande do Sul (RS), Brasil.

Os objetivos específicos foram:

a) desenvolver uma metodologia rápida e eficiente para a diferenciação das duas espécies de bradirrizóbio, *B. japonicum* de *B. elkanii*, utilizadas como inoculantes comerciais em lavouras de soja do Brasil;

b) isolar, identificar e avaliar o grau de variabilidade genética de uma população de bradirrizóbios que nodulam soja, de um campo experimental, inoculado há 30 anos com sete estirpes padrão;

c) isolar, identificar e avaliar a diversidade genética de bradirrizóbios capazes de nodular soja, isolados de cinco regiões produtoras do RS, correlacionando a diversidade com parâmetros ambientais do solo de cada região, como pH, matéria orgânica e argila.

Capítulo I

A strategy based on amplification of 16S rDNA sequences to differentiate *Bradyrhizobium japonicum* and *B. elkanii*

Submetido ao periódico *Pesquisa Agropecuária Brasileira*

A strategy based on amplification of 16S rDNA sequences to differentiate *Bradyrhizobium japonicum* and *B. elkanii*

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Abstract - As soybean is an exotic crop, Brazilian soils does not contain naturally *Bradyrhizobium* nodulating soybean. Since 1975, the government demands that inoculants commercialized in the country must contain only strains recommended by Brazilian public research institutions. The inoculants are prepared only with strains of *B. japonicum* and *B. elkanii* species. As these two *Bradyrhizobium* species have been largely used together in Brazilian soybean fields, sometimes it is practically impossible to classify strains isolated from soybean nodules. This problem is particularly important when the objective of the screening is to identify more competitive strains and with higher nitrogen fixation ability to be used in the formulation of the commercial inoculants. In this study, we report the development of a PCR-based assay on the 16S rRNA gene, which was able to discriminate those two related *Bradyrhizobium* species from 11 reference strains and 37 strains isolated from soybean fields. The methodology described in this work can be used in any laboratory that possesses simple molecular biology equipment with relatively low cost.

Index terms*: Bradyrhizobium,* PCR, 16S rRNA gene, rhizobia diversity, commercial inoculant

Título em Português: Uma estratégia baseada na amplificação de seqüências do rDNA 16S para diferenciar *Bradyrhizobium japonicum* e *B. elkanii.*

Resumo - Uma vez que a soja é uma planta exótica, os solos brasileiros não contêm espécies nativas de *Bradyrhizobium* capazes de nodular soja. Desde 1975, o governo exige que os inoculantes comercializados no país devem conter apenas estirpes recomendadas por instituições brasileiras de pesquisa públicas. Esses inoculantes contêm somente estirpes das espécies *B. japonicum* e *B. elkanii.* Já que essas duas species de *Bradyrhizobium* têm sido amplamente utilizadas em conjunto em lavouras de soja brasileiras, algumas vezes é praticamente impossível classificar as estirpes isoladas de nódulos de soja. Esse problema é particularmente importante quando o objetivo da busca é a identificação de estirpes mais competitivas e com maior capacidade de fixação de nitrogênio, para serem utilizadas na formulação dos inoculantes comerciais. Nesse estudo, relatamos o desenvolvimento de uma metodologia baseada em PCR do gene do rDNA 16S, a qual foi capaz de discriminar essas duas espécies de *Bradyrhizobium* relacionadas em 11 estirpes padrão e 37 estirpes isoladas de lavouras cultivadas com soja. A metodologia descrita nesse trabalho pode ser aplicada em qualquer laboratório que possua equipamento simples de biologia molecular com um custo relativamente baixo.

Termos para indexação: *Bradyrhizobium*, PCR, gene do rRNA 16S, diversidade de rizóbios, inoculantes comerciais.

Introduction

Accurate and reproducible techniques of species identification are essential to explore the phenotypic and genotypic variation in the family Rhizobiaceae. Conventional phenotypic methods based on serotyping have been widely applied to differentiate species within this family (Weber et al., 1989, Leung et al., 1994, Sprent, 1997) and DNA-DNA homology based on quantitative DNA-DNA hybridization was considered the standard method for species designation (Graham et al., 1991). AFLP (*amplified fragment length polymorphism*) is another technique that corroborates with DNA-DNA hybridization and can be useful for genus determination in bradyrhizobia (Willems et al., 2003). However, these methods are expensive and time consuming not been appropriated for routine identification (Laguerre et al., 1994, Willems et al., 2003).

In the last years, the amplification of rRNA gene sequences by the polymerase chain reaction (PCR) especially the 16S rRNA, has become the basis to identify the species of nodule bacteria. This methodology has been used to provide adequate information for studying the occurrence, distribution and relationship of different rhizobia species (Ludwig et al., 1994, Amann et al., 1995, Willems et al., 2003). Nevertheless, the species of the genus *Bradyrhizobium* present low genetic variability in their rRNA gene sequences (Chueire et al., 2003) which difficult their discrimination. Even the utilization of RFLP (*restriction fragment length polymorphism*) technique in the internally transcribed spacer (ITS) between 16S-23S rRNA genes amplified by PCR is not enough to differentiate species closely related (Laguerre et al., 1994, Chueire et al., 2000, Chueire et al., 2003, Willems et al., 2003).

B. japonicum (Jordan 1982), *B. elkanii* (Kuykendall et al. 1992) and *B. liaoningense* (Xu et al., 1995) are the only three rhizobia species capable of nodulate

soybean. As soybean is an exotic crop, Brazilian soils did not contain naturally *Bradyrhizobium* species nodulating this legume. Since 1975, the government demands that inoculants commercialized in the country must contain only strains recommended by Brazilian public research institutions, which maintenance of the strains and their distribution to the inoculant industry has been a responsibility of the Fundação Estadual de Pesquisa Agropecuária (FEPAGRO, 1999). The brazilian inoculants contained only strains of *B. japonicum* and *B. elkanii* species.

The massive inoculation of Brazilian soils with few bradyrhizobia strains has resulted in an established population in most soils cropped with soybean (Ferreira & Hungria, 2002). However, these populations can naturalize and show morphological, biochemical, physiological, genetic and symbiotic variability related to adaptation processes and genetic transfer (Paffetti et al., 1996, Santos et al., 1999, Ferreira et al., 2000, Hungria & Vargas, 2000, Ferreira & Hungria, 2002, Galli-Terasawa et al., 2003). These new bacterial populations differ significantly from the original introduced strains (Boddey & Hungria, 1997; Ferreira & Hungria, 2002) including a high percentage of nodules occupied by strains with unknown serological reaction after some years of soybean cropping (Freire et al., 1983, Vargas et al., 1993, Vargas & Hungria, 1997, Ferreira et al., 2000).

Bradyrhizobium japonicum and *B. elkanii* have been largely used together in Brazilian soybean fields, sometimes being practically impossible to classify strains isolated from soybean nodules. This problem is particularly important when the objective of the screening is to identify more competitive strains, with greater nitrogen fixation ability to be used in the formulation of the commercial inoculants.

In this study, we report the development of a PCR-based assay on the 16S rRNA gene, which was able to discriminate those two related *Bradyrhizobium* species from 11 reference strains and 37 strains isolated from fields cultivated with soybean. DNA from these same bacteria were also utilized in RFLP analysis of 16S-23S rDNA ITS and *nifH* gene regions in order to confirm their taxonomic classification, although these two RFLP approaches were not able to distinguish strains of *B. japonicum* from strains of *B. elkanii*.

Material and Methods

The bacterial strains used in this study are listed in Table 1. *B. betae* (Rivas et al., 2004), *B. canariense* (Vinuesa et al., 2005), *B. elkanii and B. japonicum* strains were cultivated in yeast extract-mannitol (YEM) broth (Somasegaran and Hoben, 1994) for seven days at 28^{o}C. Other bacteria utilized in this work were cultivated in LB medium (Sambrook and Russel, 2001) for 24 h at 37ºC. Fifty μl of the liquid cultures were heated in a boiling water bath and 5 μl of the supernatant obtained after a brief spin were used as a template for PCR reactions.

Partial nucleotide sequences of the 16S RNA gene from 15 strains of *B. japonicum* (GenBank accession numbers: AB195991, AB195985, AB070570, AB070571, X66024, AF234888, DQ133343, AY996780, DQ133442, AB070569, AF236087, AF234884, AY904786, AY904732 and AY904774) and 11 strains from *B. elkanii* (GenBank accession numbers: AB195989, AY649438, AB195990, AY904780, AF417553, AY904789, AY904778, AY568513, AF234890, AF293380 and AF237422), which were isolated from diverse habitats all over the world were compared to design three potentially specific primers. The forward primer sequence (Brady: AMTKCCTTTGAKWYTKAAGATCTTG) was the same for all DNAs analyzed. The

reverse primers were specific for each *Bradyrhizobium* species [Bjap for *B. japonicum* (GTCACATCTCTGCGACCGGTC) and Belk for *B. elkanii* (AACTCCGTCTCTGGAGTCCGCGA)]. The amplified fragment was about 401 bp for each species. Amplification reactions (25 μL) contained 5 μl of the culture's suspension as a DNA source, 1 U *Taq* DNA polymerase with the correspondent 1 X Buffer (Invitrogen), 5 mM of dNTP and 10 pmol of each opposing primers (Brady combined with Belk or Bjap). To determinate the most suitable annealing temperature the PCR were performed under a temperature gradient varying from 45 to 64ºC, where the temperature of 55ºC presented the best amplifications results (data not shown). All final PCR amplifications were performed in a Thermo Hybaid thermal cycler using an initial denaturation step at 94º for 1 min, 30 cycles of 1 min of denaturation at 94ºC, 30 s of annealing at 55ºC, 1 min of extension at 72ºC, followed by a final extension for 5 min at 72ºC. The amplification products were electrophoresed in 1% agarose gel containing ethidium bromide in 1 X Tris-EDTA buffer (TBE) and the gels were photographed under UV light.

For the 16S-23S rDNA ITS spacer RFLP fingerprinting primers pHr and p23S uni322anti described previously (Oliveira et al., 2006) were used. Fragments of *nifH* gene were amplified using the primers PolF and PolR defined by Poly et al. (2001) as the most successful to amplify a 360 base pair *nifH* fragment from DNA isolated from a wide range of diazotrophic microorganisms. PCR were conducted as described next. Ten μl of the purified amplification products were digested with *Mbo*I and *Hae*III endonucleases (Promega Life Sciences) for ITS region and *Hae*III and *Sal*I endonucleases (Promega Life Sciences) for *nifH* gene fragment. Digestions were performed overnight to ensure that complete fragmentation was achieved. The restricted fragments were separated on 8% acrylamide-bisacrylamide gel stained with silver nitrate (Sambrook and Russel, 2001).

Results and Discussion

Specific detection and identification of organisms by PCR requires a high degree of specificity of the oligonucleotide primers for the DNA sequences of their target organism. The comparison between sequences of the 16S rDNA region from 26 bradyrhizobia strains allowed us to identify one specie-specific region. This region was used to design two pairs of group-specific primers (Brady + Belk and Brady + Bjap) that produced a predicted PCR product either using intact cells directly from YEM liquid culture or from single colonies grown on YEM agar (data not shown).

Willems et al. (2003) working with 16S-23S ITS sequence from 32 isolates of *Bradyrhizobium* had shown that this region was not suitable for the design of specific primers adequate for differentiation among *Bradyrhizobium* species because it is very variable within the same species. These authors suggested that the ITS sequence similarity can be used as a guideline to decide which bradyrhizobia groups should be included in DNA-DNA hybridizations when identifying these bacteria. They also proposed that sequence data for additional genes should be necessary to provide further insights into the inter- and intrageneric relationships between several bradyrhizobia groups.

Our assay have proven to be efficient to discriminate a group of 37 isolates obtained from fields cultivated with soybean, 24 belonging to the *B. japonicum* and 13 belonging to the *B. elkanii* species. The results obtained by PCR for isolates were confirmed by the application of the same methodology with DNA obtained from cultures of well-characterized *Bradyrhizobium* species, which are traditionally recommended as commercial inoculants in Brazilian fields (Fig. 1). In order to confirm their taxonomic classification all the strains investigated were also subjected to the RFLP analysis of the 16S-23S rDNA ITS spacer, to corroborate the results obtained by Willems et al. (2003),

and of the *nifH* gene, which phylogeny has been reported to be largely consistent with the 16S rDNA tree (Young, 1992, 1993, Zehr et al., 2003) (data not shown). The group-specific primers developed in this work were also utilized with DNA obtained from other bacterial strains available in the laboratory, *Rhizobium leguminosarum* bv trifolii, *Agrobacterium tumefaciens* AGL1, *Escherichia coli* XL1-Blue, *Azospirillum brasilense* Sp7 ATCC 2914 and *Paenibacillus macerans* ATCC8244, as well with DNA obtained from two other *Bradyrhizobium* species, *B. betae* and *B. canariense*. Either no PCR products were obtained or some DNAs amplified fragments with sizes bigger than 400 bp (Fig. 1 and data not shown).

The technique here described has several clear advantages, such as it is fast, since the PCRs can start from DNA obtained directly from pure cultures or single colonies, and presents a relatively low cost, since its requires only two PCR to identify each isolate. These features can be very useful when the objective of the work is to characterize genotipically bradyrhizobia species for agricultural practices.

Conclusions

1) A new and efficient methodology to discriminate *B. japonicum* from *B. elkanii* strains is described.

2) This methodology is specific for strains of *B. japonicum* and *B. elkanii* species.

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References

AMANN, L.; LUDWIG, W.; SCHLEIFER K. Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. **Microbiology Review**, v. 59, p. 143-169, 1995.

BODDEY, L. H.; HUNGRIA, M. Phenotypic grouping of Brazilian *Bradyrhizobium* strains which nodulate soybean. **Biology and Fertility of Soils**, v. 25, p. 407-415, 1997.

CHUEIRE, L. M. O.; NISHI, C. Y. M.; LOUREIRO, M. F.; HUNGRIA, M. Identificação de estirpes de *Bradyrhizobium* e *Rhizobium* utilizadas em inoculantes comerciais para as culturas da soja e do feijoeiro pela técnica de PCR com "primers" aleatórios ou específicos. **Agricultura Tropical**, v. 4, p. 80-95, 2000.

CHUEIRE, L. M. O.; BANGEL, E. V.; MOSTASSO, F. L.; CAMPO, R. J.; PEDROSA, F. O.; HUNGRIA, M. Classificação taxonômica das estirpes de rizóbio recomendadas para a cultura da soja e do feijoeiro baseada no seqüenciamento do gene 16S rRNA. **Revista Brasileira de Ciência do Solo**, v. 27, p. 833-840, 2003.

FEPAGRO (Fundação Estadual de Pesquisa Agropecuária). **Culture Collection Catalogue**, 8th ed, FEPAGRO, Porto Alegre, Brazil, 1999.

FERREIRA, M. C.; ANDRADE, D. S.; CHUEIRE, L. M. O.; TAKEMURA, S. M.; HUNGRIA, M. Tillage method and crop rotation effects on the population sizes and diversity of bradyrhizobia nodulating soybean. **Soil Biological and Biochemistry**, v. 32, p. 627–637, 2000.

FERREIRA, M. C.; HUNGRIA, M. Recovery of soybean inoculant strains from uncropped soils in Brazil. **Field Crops Research**, v. 79, p. 139-152, 2002.

FREIRE, J. R. J.; KOLLING, J.; VIDOR, C.; PEREIRA, J. S.; KOLLING, I. G.; MENDES, N. G. Sobrevivência e competição por sítios de nodulação de estirpes de *Rhizobium japonicum* na cultura da soja. **Revista Brasileira de Ciência do Solo**, v. 7, p. 47–53, 1983.

GALLI-TERASAWA, L. V.; GLIENKE-BLANCO, C.; HUNGRIA, M. Diversity of a soybean rhizobial population adapted to a Cerrados soil. **World Journal of Microbiology and Biotechnology**, v. 19, p. 933–939, 2003.

GRAHAM, P. H.; SADOWSKY, M. J.; KEYSER, H. H.; BARNET, Y. M.; BRADLEY, R. S.; COOPER, J. E.; DELEY, D. J.; JARVIS, B. D. W.; ROSLYCKY, E. B.; STRIJDOM, B. W.; YOUNG, J. P. W. Proposal minimal standards for the description of new genera and species of root- and stem-nodulating bacteria. **International Journal of Systematic Bacteriology**, v. 41, p. 582-587, 1991.

HUNGRIA, M.; VARGAS, M. A. T. Environmental factors affecting N_2 fixation in grain legumes in the tropics, with an emphasis on Brazil. **Field Crops Research**, v. 65, p. 151-164, 2000.

JORDAN, D. C. 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**, v. 32, p. 136-139, 1982.

KUYKENDALL, L. D.; SAXENA, B.; DEVINE, T.E.; UDELL, S.E. Genetic diversity in *Bradyrhizobium japonicum* Jordan (1982) and a proposal for *Bradyrhizobium elkanii* sp. nov. **Canadian Journal of Microbiology**, v. 38, p. 501-505, 1992.

LAGUERRE, G.; ALLARD, M. R.; REVOY, F.; AMARGER, N. Rapid identification of rhizobia by restriction fragments length polymorphism analysis of PCR-amplified 16S rRNA genes. **Applied and Environmental Microbiology,** v. 60, p. 58-63, 1994.

LEUNG, K.; YAP, K.; DASHTI, N.; BOTTOMLEY, P. J. Serological and ecological characteristics of a nodule-dominant serotype from an indigenous soil population of *Rhizobium leguminosarum* bv. *trifolii*. **Applied and Environmental Microbiology,** v. 60, p. 408-415, 1994.

LAZO, G. R.; STEIN, P. A.; LUDWIG, R. A DNA transformation-competent *Arabidopsis* genomic library in *Agrobacterium*. **BioTechnology,** v. 9, p. 963-967, 1991.

LUDWIG, W.; DORNS, S.; SPRINGER, N.; KIRCHHOF, G.; SCHLEIFER, K. PCR-based preparation of 23S rRNA-targeted group-specific polynucleotide probes. **Applied and Environmental Microbiology**, v. 60, p. 3236-3244, 1994.

OLIVEIRA, V. M.; MANFIO, G. P.; COUTINHO, H. L. C.; KEIJZER-WOLTERS, A. C.; van ELSAS, J. D. A ribosomal RNA gene intergenic spacer based PCR and DGGE fingerprinting method for the analysis of specific rhizobial communities in soil. **Journal of Microbiological Methods**, v 64, p. 366-379, 2006.

PAFFETTI, D.; SCOTTI, C.; GNOCCHI, S.; FANCELLI, S.; BAZZICALUPO, M. Genetic diversity of an Italian *Rhizobium meliloti* population from different *Medicago sativa* varieties. **Applied and Environmental Microbiology**, v. 62, p. 2279-2285, 1996.

POLY, F.; MONTOZIER, L. J.; BALLY, R. Improvement in the RFLP procedure for studying the diversity of *nifH* genes in communities of nitrogen fixers in soil. **Research in Microbiology**, v. 152, p. 95-103, 2001.

RIVAS, R.; WILLEMS, A.; PALOMO, J. L.; GARCIA-BENAVIDES, P.; MATEOS, P. F.; MARTINEZ-MOLINA, E.; GILLIS, M.; VELAZQUEZ, E. *Bradyrhizobium betae* sp. nov., isolated from roots of *Beta vulgaris* affected by tumour-like deformations.

International Journal of Systematic and Evolutionary Microbiology, v. 54, p. 1271–1275, 2004.

SAMBROOK, J.; RUSSEL, D. W. **Molecular cloning: a laboratory manual**. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 2001.

SANTOS, M. A.; VARGAS, M. A. T.; HUNGRIA, M. Characterization of soybean bradyrhizobia strains adapted to the Brazilian cerrados region. **Microbial Ecology**, v. 30, p. 261–272, 1999.

SOMASEGARAN, P.; HOBEN, J. H. **Handbook for Rhizobia: methods in legume-*Rhizobium* technology**. Springer-Verlag, New York, 1994.

SPRENT, J. I. Phenotypic characteristics and composition of rhizobia associated with woody legumes growing in diverse Kenyan conditions. **Plant and Soil**, v. 188, p. 65-75, 1997.

TARRAND, J. J.; KRIEG, N. R.; DÖBEREINER, J. A taxonomic study of the *Spirillum lipoferum* group, with descriptions of a new genus, *Azospirillum* gen. nov. and two species, *Azospirillum lipoferum* (Beijerinck) comb. nov. and *Azospirillum brasilense* sp. nov. **Canadian Journal of Microbiology**, v. 24, p. 967-980, 1978.

VARGAS, M. A. T.; MENDES, I. C.; SUHET, A. R.; PERES, J. R. R. Serological distribution of *Bradyrhizobium japonicum* from Brazilian cerrados areas under soybean cultivation. **Research in Microbiology**, v. 24, p. 239–243, 1993.

VARGAS, M. A. T.; HUNGRIA, M. **Biologia dos Solos de Cerrados.** EMBRAPA-CPAC, Planaltina, p. 297-360, 1997.

VINUESA, P.; LEÓN-BARRIOS, M.; SILVA, C.; WILLEMS, A.; JARABO-LORENZO, A.; PÉREZ-GALDONA, R.; WERNER, D.; MARTÍNEZ-ROMERO, E. *Bradyrhizobium canariense* sp. nov., an acid-tolerant endosymbiont that nodulates endemic genistoid

legumes (Papilionoideae: Genisteae) from the Canary Islands, along with *Bradyrhizobium japonicum* bv. *genistearum*, *Bradyrhizobium* genospecies alpha and *Bradyrhizobium* genospecies beta. **International Journal of Systematic and Evolutionary Microbiology**, v. 55, p. 569-575, 2005.

WEBER, D. F.; KEYSER, H. H.; URATSU, S. L. Serological distribution of *Bradyrhizobium japonicum* from U.S. soybean production areas. **Agronomical Journal,** v. 81, p. 786-789, 1989.

WILLEMS, A.; MUNIVE, A.; de LAJUDIE, P.; GILLIS, M. In most *Bradyrhizobium* groups sequence comparison of 16S-23S rDNA internal transcribed spacer regions corroborates DNA-DNA hybridizations. **Systematic and Applied Microbiology**, v. 26, p. 203-210, 2003.

YOUNG, J. P. W. Phylogenetic classification of nitrogen-fixing organisms. In: STACY, G.; BURRIS, R. H.; EVANS, H. J. (Ed). **Biological nitrogen fixation**. Chapman and Hall, New York, 1992. p. 43-86.

YOUNG, J. P. W. Molecular phylogeny of rhizobia and their relatives. In: PALACIOS, R.; MORA, J.; NEWTON, W. E. (Ed). **New horizons in nitrogen fixation**. Kluwer Academic Publishers, London, 1993. p. 587-592.

XU, L. M.; GE, C.; CUI, Z.; LI, J.; FAN, H. *Bradyrhizobium liaoningense* sp. nov., isolated from the root nodules of soybean. **International Journal of Systematic Bacteriology**, v. 45, p. 706-711, 1995.

ZEHR, J. P.; JENKINS, B. D.; SHORT, S. M.; STEWARD, G. F. Nitrogenase gene diversity and microbial community structure: a cross-system comparison. **Environmental Microbiology**, v. 5, p. 539-554, 2003.

Table 1. Bacterial strains utilized in this work.

Strains	Origin/Source	
Reference Strains		
Bradyrhizobium betae		
PL7HG1 (= LMG 21987T = CECT 5829T).		Rivas et al., 2004.
Bradyrhizobium canariense		
BTA-1 (= ATCC BAA-1002 = LMG 22265 = CFNE 1008).		Vinuesa et al., 2005.
Bradyrhizobium elkanii		
SEMIA 587	Brazilian reference strain	Fepagro/Brazil
SEMIA 5019 (= 29w; = BR 29)	Brazilian reference strain	Fepagro/Brazil
USDA 31 (= SEMIA 5086)	Isolated from USA soybean fields	Fepagro/Brazil
USDA 76 (= SEMIA 5087)	Isolated from USA soybean fields	Fepagro/Brazil
USDA 94 (= SEMIA 5088)	Isolated from USA soybean fields	Fepagro/Brazil
Bradyrhizobium japonicum		
SEMIA 586 (= CB 1809; = USDA 136b; =TAL 379)	Isolated from USA soybean fields	Fepagro/Brazil
SEMIA 5079 (= CPAC 15)	Brazilian reference strain	Fepagro/Brazil
SEMIA 5080 (= CPAC 7)	Brazilian reference strain	Fepagro/Brazil
USDA 6 (= ATCC 10324; = SEMIA 5052)	Isolated from USA soybean fields	Fepagro/Brazil
USDA 110 (= TAL 102)	Group Ia[(1)] isolated from USA soybean fields	Fepagro/Brazil
USDA 123 (= SEMIA 5074)	Isolated from USA soybean fields	Fepagro/Brazil
Isolates identified as *B. japonicum*		
A30, D29, D32, D41, D45, D50, D54, D56, F54, I9, J1, J5, J8, J36, K2, K5, K7, K8, K16, K17, K39, K40, K47, L54	Isolated from Brazilian soybean fields	This work
Isolates identified as *B. elkanii*		
D23, D24, D61, D65, D66, D67, G21, I4, I5, I8, I57, J7, K9	Isolated from Brazilian soybean fields	This work
Other bacterial strains		
Rhizobium leguminosarum bv trifolii	Isolated from Brazilian's fields	L.K.Vargas, Fepagro/Brazil
Agrobacterium tumefaciens	AGL1	Lazo et al., 1991.
Escherichia coli	XL1-Blue	Stratagene, La Jolla, U. S. A
Azospirillum brasilense Sp7	ATCC 29145	Tarrand et al., 1978.
Paenibacillus macerans	ATCC 8244	Fiocruz, RJ, Brazil

(1) Kuykendall et al., 1988.

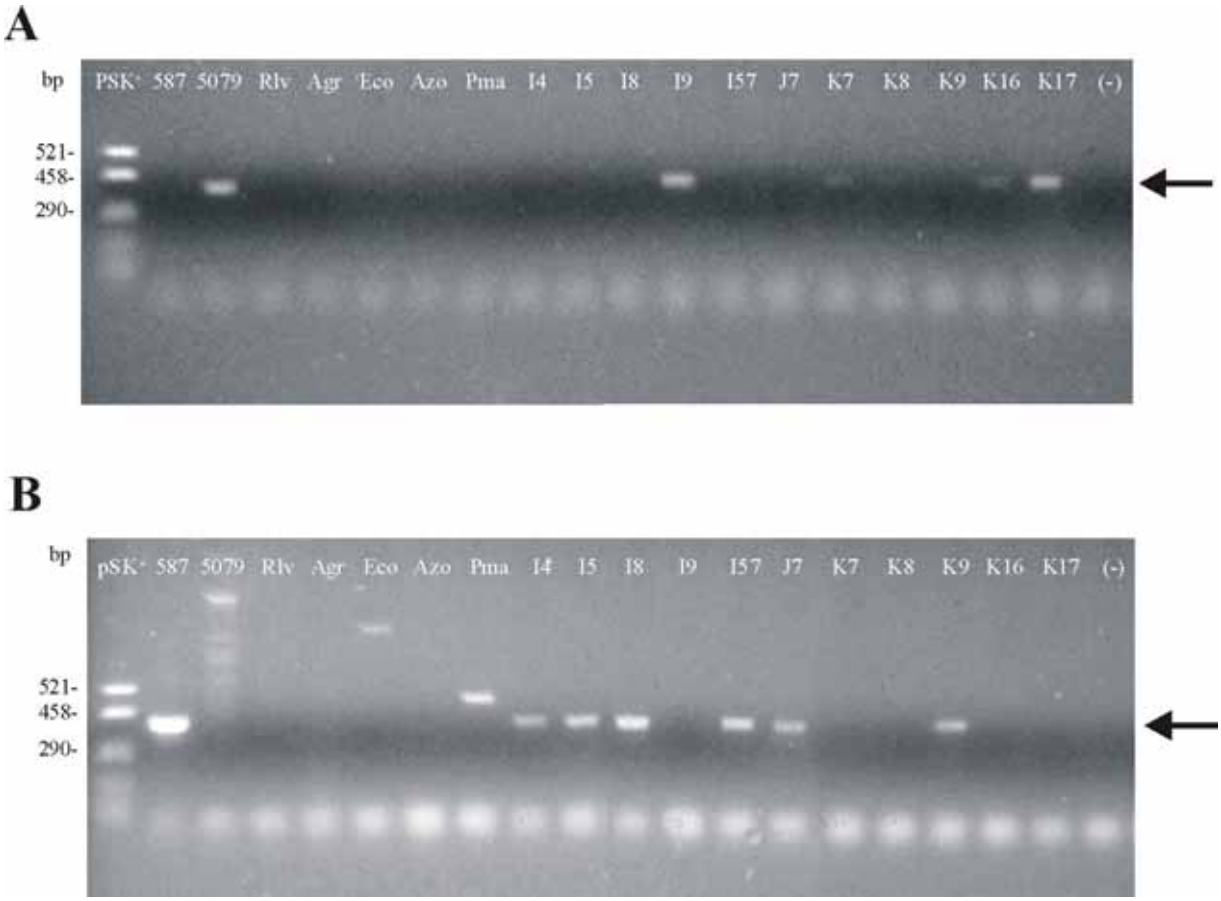

Figure 1. Amplification of the 16S rRNA gene region using the group-specific primers from a representative sample of the bacterial strains utilized in this work. In (A) the primer combination was Brady + Bjap and in (B) was Brady + Belk. The bacterial strains are: 587 = *B. elkanii* SEMIA 587; 5079 = *B. japonicum* SEMIA 5079; Rlv = *Rhizobium leguminosarum* bv trifolii; Agr = *Agrobacterium tumefaciens* AGL1; Eco = *Escherichia coli* XL1-Blue; Azo = *Azospirillum brasilense* Sp7 ATCC 2914; Pma = *Paenibacillus macerans* ATCC 8244; I9, I57, K7, K8, K16 and K17 = strains identified as *B. japonicum*; I4, I5, I8, J7 and K9 = strains identified as *B. elkanii*. The molecular weight marker (PSK^+) in (A) and (B) is $pBSSK^+$ plasmid DNA cleaved with *HaeIII*. Negative control (-) was reaction without DNA. Arrows indicate the expected amplifications products with sizes of approximately 400 bp.

Capítulo II

Rescue and genetic assessment of *Bradyrhizobium japonicum* and *B. elkanii* strains thirty years after the first inoculation in an experimental in the South Brazilian fields

Submetido ao periódico *Plant and Soil*

Rescue and genetic assessment of *Bradyrhizobium japonicum* and *B. elkanii* strains thirty years after the first inoculation in an experimental experimental in the South Brazilian fields

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Abstract

In order to study the survival and competitiveness of bradyrhizobia strains for soybean nodule site a field experiment in Eldorado do Sul, Brazil, beginning in 1974, was conducted for six consecutive years. At the fifth year of analysis, besides the different ability of the strains to compete for nodule sites, it was observed a high percentage of nodules without serological reaction with any of the antisera used to identify the original strains. This indicated that the nodules were formed by naturalized strain or strains of higher competitiveness. As the long-term phenotype and genetic stability of bacteria used as inoculant is an important parameter in their ecology as well as for agricultural purposes, the aim of this study was genotypically characterize several-rescued bradyrhizobia of that experimental field. Thirty bradyrhizobia isolates were obtained using the IAS-5 soybean variety as trap host and the soil deriving from the same field used in the former experiment. Genetic diversity of the isolates was evaluated by rep-PCR and AFLP techniques. The Shannon index (*H*) was used to assess the diversity among the isolates on the basis of 70% similarity. A very high genetic diversity among the isolates was observed either by AFLP (H = 4.87) or rep-PCR (H = 4.18). The results indicate that the *Bradyrhizobium* population, which was able to persist in the Eldorado soil for more than 30 years after inoculation, is genetically very diverse and different from the original strains. All isolates were infective and trapped in IAS-5 soybean variety maintaining their nodulation and nitrogen fixation properties. Given that many rhizobia in a soil can loose the infective capacity and that the host genotype can affect the spectrum of rhizobial genotype selected from a soil, the genetic diversity of the complete bradyrhizobia population in Eldorado soil could be even higher than the identified in this work.

Keywords: *Bradyrhizobium*, genetic diversity, AFLP, rescued bradyrhizobia, soybean.

Introduction

Bacteria belonging to the genus *Bradyrhizobium* are of enormous agricultural value since they are able to fix atmospheric nitrogen in symbiosis with several leguminous plants, especially soybean [*Glycine max* (L.) Merrill]. *B. japonicum* or *B. elkanii* strains are the only two species capable of nodulate soybean in Brazil (Freire, 1977; Peres and Vidor, 1980; Alberton et al., 2006). Since soybean is an exotic crop, Brazilian soils did not contain naturally such *Bradyrhizobium* species nodulating this legume. Probably these bacteria came with seeds and inoculants from USA and its population in Brazilian soils increased due to the massive commercial inoculation during the last decades (Ferreira and Hungria, 2002).

Besides some intrinsic host or symbiont characteristics and environmental stresses like high temperature, soil acidity and aluminum toxicity (Hungria et al., 1993; Hungria and Vargas, 2000), bradyrhizobia introduced with seeds or inoculation usually survive in the soil for many years (Revellin et al., 1996). However, some experiments have shown that bradyrhizobia can naturalize and present morphology, physiology, genetic and symbiotic differences from the original introduced strains (Boddey and Hungria, 1997; Ferreira and Hungria, 2002) including a high percentage of nodules occupied by strains with unknown serological reaction after some years of soybean cropping (Freire et al., 1983; Vargas et al., 1993; Vargas and Hungria, 1997; Ferreira et al., 2000). Moreover, the diversity and the size of indigenous population in soil can vary with the presence of the host legume (Parker, 1999; Andrade et al., 2002). Coutinho et al (1999) demonstrated that the diversity of rhizobia was reduced in plots cultivated with soybean compared with original uncultivated pasture. They also observed that the soybean crop did not increase the number of bradyrhizobia strains.

In order to study the survival and competitiveness of bradyrhizobia strains for soybean nodule site, Freire et al. (1983) conducted a field experiment for six consecutive years beginning at agriculture year of 1973/1974. The experimental area, not cropped with soybean before, located at Eldorado do Sul, Rio Grande do Sul, Brazil, was inoculated with seven *Bradyrhizobium* reference strains and has not been reinoculated or cropped with soybean since then. At the fifth year of analysis, besides the different ability of the strains to compete for nodule sites, it was observed a high percentage of nodules without serological reaction with any of the antisera used to identify the original strains. According to the authors (Freire et al., 1983) this result indicated that the nodules were formed by naturalized strain or strains of higher competitiveness. As the long-term phenotype and genetic stability of bacteria used as inoculant is an important parameter in their ecology as well as for agricultural purposes, the aim of this study was genotypically characterize several-rescued bradyrhizobia of that experimental field. The purpose was to investigate whether these bacteria were still able to persist for 30 years in the soil maintaining their nodulation and nitrogen fixation properties.

Material and methods

Bacterial isolates, reference strains and trap host

Soil samples were collected at the Estação Experimental Agronômica da Universidade Federal do Rio Grande do Sul (EEA - UFRGS), located at Eldorado do Sul, Rio Grande do Sul, Brazil [30º05'27"S, 51º 40'18"W]. Based on Soil Survey Staff (1998) the soil of the experimental area is a Paleudult with pH of 5.5, 34% of clay and 4.2% of organic matter. In 1974 this area was inoculated with seven strains of *Bradyrhizobium* (SEMIA 509, 527, 531, 532, 566, 586 and 587) for a field experiment, which was carried

out for six consecutive years. Each strain was inoculated as an individual inoculant only at the first year.

In this work we tried to use the same seven reference strains for comparison, however, strains SEMIA 531 and SEMIA 532 were replaced respectivelly by strains SEMIA 5038 and SEMIA 5039. We also included in our analysis the currently recommended strains SEMIA 5019, SEMIA 5079, SEMIA 5080 and the type reference strain USDA 110. All reference strains were obtained from the Biological Nitrogen Fixation Center/Fundação Estadual de Pesquisa Agropecuária (FEPAGRO), Brazil, and are described in Table 1.

The IAS-5 soybean cultivar was used as trap-host. The bradyrhizobia isolates were obtained from 50 fresh root nodules collected from plants that had grown in pots filled with the soil derived from the field cited above. Bacteria were isolated on yeast mannitol agar (YEM, Somasegaran and Hoben, 1994) supplemented with 0.025 g. l^{-1} of Congo red dye using standard procedures (Somasegaran and Hoben, 1994). The isolates were purified by repeated streaking (Vincent, 1970). To confirm their purity, all the isolates and reference strains were streaked on YEM agar supplemented with 0.1 g l^{-1} of bromothymol blue (Somasegaran and Hoben, 1994). The bacterial isolates were reinoculated in pouches with IAS-5 soybean cultivar according to standard procedures. Those that had confirmed their ability to nodulate soybean were selected for this study. Pure cultures were stored at -10°C in 25% glycerol-YEM broth (Somasegaran and Hoben, 1994).

DNA isolation

Bacterial cells were grown in YEM broth for seven days at 28ºC at 128 rpm. Cells were rinsed with TES buffer (50 mM Tris pH 8, 0.5 mM EDTA, 50 mM NaCl), and

ressuspended in EDTA saline (150 mM NaCl, 10 mM EDTA pH 8). Cell lyses took place in 20% sodium dodecyl sulfate (SDS) warmed at 55ºC. DNA was extracted using chloroform/isoamylic alcohol (24:1) and precipitated with ethanol.

rep-PCR

Rep-PCR reactions were carried out using enterobacterial repetitive intergenic consensus primers ERIC1-R (ATGTAAGCTCCTGGGGATTCAC) and ERIC-2 (AAGTAAGTGACTGGGGTGAGCG) (de Bruijn, 1992) and enterobacterial repetitive sequences (BOX A1) primer (CTACGGCAAGGCGACGCTGACG, Versalovic et al., 1994). The reactions were performed in a 25-µl volume, containing 50 ng of DNA template, 1 U *Taq* DNA polymerase, 1 X *Taq* DNA polymerase buffer, 15 mM $MgCl_2$, 200 mM dNTPs (Life Technologies) and 10 pmoles of each primer. A total of 32 cycles took place, as follows: one initial denaturation cycle at 95ºC for 7 min, 30 cycles of denaturation at 94ºC for 1 min, annealing at 52ºC for 1 min, and extension at 65ºC for 8 min, and one final extension cycle at 65ºC for 16 min. Reactions were performed in a PCR Express Temperature Cycling System (Thermo Hybaid) and fragments were visualized after electrophoresis at 100 V for 3 h on a 1% agarose gel stained with ethidium bromide. Molecular size marker was 1 Kb Plus DNA ladder (Gibco BRL).

AFLP

All AFLP procedures were carried out as described by Dougnon-Bourcier et al (2000). A 100 µg aliquot of genomic DNA of each isolate was overnight digested with 1.25 U of both *Apa*I and *Taq*I restriction enzymes (Invitrogen) at 37^{o}C and 65^{o}C, respectively, in a final volume of 25 µl. The restriction reaction was then ligated to the

*Apa*I and *Taq*I adapters (*Apa*I adapters: TCGTAGACTGCGTACAGGCC and CATCTGACGCATGT; *Taq*I adapters: GACGATGAGTCCTGAC and TACTCAGGACTGGC, Wong et al., 1999; Singh et al., 2001). The ligation mixture consisted of 25 µl of digested DNA, 10 pmol of each adapter and 1 U of T4 DNA ligase (Invitrogen) in correspondent buffer. Ligated DNA was 1:10 diluted in ultra pure water and heated at 80^{o}C for 10 min to inactivate T4 DNA ligase. Five µl of ligated DNA was then used as template for PCR DNA amplification. The reactions were performed in a 25 µl volume, containing 1 U *Taq* DNA polymerase (Invitrogen), 1 X *Taq* DNA polymerase reaction buffer, 15 mM $MgCl_2$, 200 mM dNTPs (Invitrogen) and 10 pmoles of each primer (*ApaI* GACTGCGTACAGGCCCE, where E means ATT/CCC/CTA/CTG/GAC/TTG and *TaqI* CGATGAGTCCTGACCGAE, where E means CCC/CTG/GAC/GTA). PCR amplification was performed using the *touchdown* protocol: 94°C for 3 min, followed by 10 cycles of 94°C for 30 s, 65°C to 56°C for 1 min and 72°C for 2 min, followed by 23 cycles of 94°C for 30 s, 56°C for 30 s, and a final extension of 72 °C for 1 min. Reactions were performed in a PCR Express Temperature Cycling System (Thermo Hybaid). The fragments were visualized after electrophoresis for 3 hours at 80 V in an 8% acrylamide-bisacrylamide gel stained with silver nitrate (Sambrook and Russel, 2001). Molecular size marker (λ DNA digested with *Eco*RI/*Hind*III, Invitrogen) was run in all gels.

Differentiation of *Bradyrhizobium japonicum* and *B. elkanii* by PCR amplification of 16S rRNA gene sequences

The bradyrhizobia isolates were differentiated as strains of *B. japonicum* and *B. elkanii* using a protocol described by A. Giongo (personal communication). The differentiation was based in a selective PCR amplification of 16S rRNA gene sequences

using the forward primer sequence (Brady AMTKCCTTTGAKWYTKAAGATCTTG), and the reverse primers, specific for each *Bradyrhizobium* species [Bjap for *B. japonicum* (GTCACATCTCTGCGACCGGTC) and Belk for *B. elkanii* (AACTCCGTCTCTGGAGTCCGCGA)].

DNA fingerprint analysis

Fingerprint patterns were visualized under UV illumination. The size of the fragments obtained ranged from 650 to 5,000 bp. They were converted into a two-dimensional binary matrix (1, presence of a band; 0, absence of a band) and analyzed using Jaccard (*J*) coefficient, which do not consider the negative similarities. The matrix was analyzed by NTSYS-PC package. UPGMA (Unweighted Pair Group Method with Arithmetic mean) algorithm was used to perform hierarchical cluster analysis and construct a dendrogram. Index of diversity (*H*, Shannon and Weaver, 1949) was estimated based on the number of isolates belonging to each group of profiles in rep-PCR and AFLP dendrograms, considering a 70% of similarity in the cluster analysis (Alberton et al., 2006). The *H* index was calculated using the formula $H = C/N(N\log N - \Sigma n\log ni)$ (Atlas and Bartha, 1998), where *ni* corresponds to the number of isolates with the same rep-PCR/AFLP fingerprints, N corresponds to the total number of isolates and C is a constant that equals 23. The *H* values obtained were submitted to Student test (*t*, Magurran, 1987).

Results and Discussion

The assessment of long-term genetic stability of bacteria used as a soil inoculant is an important parameter in microbial ecology as well as for agricultural purposes. In 1974, a field experiment was carried out on soils of Estação Experimental Agronômica of the

Universidade Federal do Rio Grande do Sul (UFRGS), located at Eldorado do Sul, RS, Brazil (Freire et al., 1983). The objective of that experiment was to study the survival and competitiveness for nodule sites of seven *Bradyrhizobium* strains of soybean for six consecutive years. It was reported that the strains showed different ability to compete for nodule sites. Moreover, at the fifth year of nodule analysis it was observed a high percentage of nodules without serological reaction with any of the antisera used to identify the seven original strains. This result indicated that the nodules were formed by naturalized strain or strains of higher competitiveness, even higher than SEMIA 587, which had showed the highest competitiveness and ability to colonize the soil in the previous years (Freire et al., 1983).

Using the IAS-5 soybean cultivar as trap host and the soil deriving from the same field used in the former experiment, 30 bradyrhizobia isolates were obtained. These bacteria were genotypic and symbiotically characterized according to standard procedures used to isolate *Bradyrhizobium* species (Somasegaran and Hoben, 1994). All the isolates failed to absorb Congo red and their colonies were well visualized after 5-7 days of incubation at 28ºC in plates containing YEMA medium. All isolates also alkalinized YEM medium supplemented with bromothymol blue, which is a typical characteristic of *Bradyrhizobium* genus (Batista et al., 2006). Although Hungria et al. (2001) and Galli-Terasawa et al. (2003) reported that fast growing rhizobia represent 17 to 29% of soybean rhizobia population in Brazil, this kind of bacteria did not appear among the isolates.

Genetic diversity of the bradyrhizobia isolates was evaluated by electrophoretic profiles of rep-PCR and AFLP amplification products. It was possible to distinguish well-defined groups of bradyrhizobia using ERIC1-R, ERIC-2 and BOX primers (data not shown). These groups were confirmed by AFLP analysis, although a higher diversity

among isolates was observed within AFLP-groups. Figure 1 shows the AFLP-relationships among all the strains analyzed. According to the dendrogram, two main groups (I and II) were obtained with a similarity index of 28%. Group I was subdivided in two other groups, IA and IB. The group IB was again subdivided in IB1 and IB2. Group IA was composed by 15 isolates, from which five where identified as *B. japonicum* and ten as *B. elkanii.* Reference strains were clustered within groups IB1 and IB2. Group IB1 was composed by four reference strains: *B. elkanii* SEMIA 587 and SEMIA 5019, *B. japonicum* SEMIA 566, and SEMIA 5038 which belong to a group of still not defined bacteria. Group IB2 was composed by seven reference strains: SEMIA 5079, SEMIA 5080, SEMIA 586 and USDA 110, all of them belonging to *B. japonicum* specie, and SEMIA 509, SEMIA 527 and SEMIA 5039, which also belong to a group of undefined bacteria. Group II was composed by another set of isolates, as occurred with group IA, being eight identified as strains of *B. japonicum* and seven as strains of *B. elkanii.*

When used in combination rep-PCR and AFLP can provide accurate information of strains relationship as well as to show the extent of genetic diversity among field population. The Shannon index (*H*) was used in order to assess the diversity among our isolates. The rep-PCR and AFLP fingerprints were compared on the basis of a 70% similarity. The results revealed a very high genetic diversity among the isolates either by AFLP ($H = 4.87$) or rep-PCR ($H = 4.18$) techniques. These results do not differ statistically (test *t*). Similar results were obtained by Vargas et al. (2007) that found a *H* of 4.3 studying the genetic diversity of *Acacia mearnsii* (black wattle) nodulating rhizobia in Rio Grande do Sul, Brazil. Andrade et al. (2002) also found a similar diversity index ($H = 3.93$) analyzing common bean rhizobia in Brazilian acid soils altered by liming. Lõhmus et al. (2006) obtained Shannon indexes of 4.63 and 4.56 among cultivable bacterial communities

extracted from soil–root interface and rhizosphere bulk soil, respectively. A high level of diversity within a sample of 100 soybean rhizobia isolated from an uncropped area in Cerrados, Brazil, was also reported by Galli-Terasawa et al. (2003). Although this area had received inoculants 15 years before, nowadays it lacks rhizobia able to nodulate soybean. Chen et al. (2000) also confirmed the high level of genetic diversity among the Paraguayan soybean rhizobia. They showed that DNA profiles of the slow growers differed from the reference strains used in inoculants. Soybean rhizobia from field sites in Croatia were also genotypically analyzed using RFLP, rep-PCR and RAPD and most of the isolates significantly diverged from inoculant strains (Sikora and Redzepovic, 2003). The authors suggested that even with inoculation, indigenous rhizobia remained predominant in these areas, as could be the case of the bradyrhizobia population analyzed in the present work.

On the other hand, some authors have reported lower diversity indexes when studying rhizobial communities in different situations (Marilley et al., 1998; Palmer and Young, 2000; Zilli et al., 2004). Gibson et al. (1991) found stability with no exchange of RFLP pattern among strains of *B. japonicum* that have survived for 9 years in the soil. Brunel et al. (1988) reported that a population of *Bradyrhizobium* strains did not show any significant genomic alteration after 8-13 years of release in a soybean field. Soybean was not grown in that field after the year of bacterial inoculation. Besides DNA hybridization using *nifD* gene as a probe the authors confirmed the stability of the bradyrhizobia population regarding morphology, serology, sugar utilization, antibiotic resistance, and enzymatic activity properties. They concluded that once introduced into a suitable soil without indigenous *B. japonicum* populations, *B. japonicum* inoculants would integrate into the indigenous soil communities without significant modifications. Ferreira and Hungria (2002) also have found genetic similarity among most of the isolates obtained

from a Cerrado field (an uncropped area) and the strains used in commercial inoculants. In this case, the similarity found was probably due to a high level of dispersion of bacteria from neighbor cropped areas. Similar result was obtained by PCR-RAPD for 48 isolates from two French regions, Dijon and Toulouse, where none obvious genomic rearrangement was observed (Obaton et al., 2002). The authors attributed this result to a low or absent saprophytic competition and/or to a low probability of contact between colonies.

Variability in rhizobial population has been attributed to several factors, including mutation and recombination in isolated strains and lateral gene transfer to local strains (Sullivan and Ronson, 1998); furthermore, these processes can be affected by the interaction with the host plant, by agricultural practices (Martinez-Romero and Caballero-Mellado, 1996; Provorov and Vorob'ev, 2000; Galli-Terasawa et al., 2003; Silva et al., 2003) or environmental stress condition, common in tropical regions (Santos et al., 1999; Hungria and Vargas, 2000). Some authors have assumed that nodule populations are more sensitive to environmental disturbances and may be a more valuable biological indicator of ambient variation that free living populations (Depret et al., 2004). It was also suggested that the acquisition of the symbiosis island by another rhizobia can converts saprophyte bacteria into a symbiont one (Sullivan and Ronson, 1998). Therefore, as well as encoding genes required for nodule formation and symbiotic nitrogen fixation, the island is likely to contain other genes that contribute specifically to the success of the plant–microbe interaction.

Another possibility is that insertion sequences elements in rhizobia can promote genetic diversification through genomic rearrangements and recombination (Laberge et al., 1995; Freiberg et al., 1997; Provorov and Vorob'ev, 2000). One example was provided by

B. japonicum that presented a high number of transposases, repetitive RSα elements, and insertion sequences (Göttfert et al., 2001; Kaneko et al., 2002). The occurrence of spontaneous mutation or recombination in bradyrhizobia is a preoccupying point in culture collections once this variability represents a potential change in maintenance and indication of strains to taxonomy and inoculant production (E. Bangel, personal communication).

The present study indicates that the *Bradyrhizobium* population that was able to persist in the Eldorado soil for more than 30 years after inoculation is genetically very diverse and different from the parental strains. It should be recognized that only a small fraction of strains from the total soil population was sampled here. All isolates analyzed were infective in IAS-5 soybean cultivar and were trapped using the same cultivar maintaining their nodulation and nitrogen fixation properties. Given that many rhizobia in a soil can loose the infective capacity and that the host genotype can affect the spectrum of rhizobial genotype selected from a soil (Laguerre et al., 2003), it is likely that genetic diversity of the complete bradyrhizobia population in Eldorado soil could be even higher than the identified in this work.

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References

Alberton O, Kaschuk G and Hungria M 2006 Sampling effects on the assessment of genetic diversity of rhizobia associated with soybean and common bean. Soil Biol. Biochem. 38, 1298–1307.

Andrade D S, Murphy P J and Giller K E 2002 The diversity of phaseolus-nodulating rhizobial populations is altered by liming of acid soils planted with *Phaseolus vulgaris* L. in Brazil. Appl. Environ. Microbiol. 68, 4025–4034.

Atlas R M and Bartha R 1998 Microbial Ecology. Benjamin Cummings Science Publishing, Menlo Park, 694 pp.

Batista J S S, Hungria M, Barcellos F G, Ferreira M C and Mendes I C 2006 Variability in *Bradyrhizobium japonicum* and *B. elkanii* seven years after introduction of both the exotic microsymbiont and the soybean host in a Cerrados soil. Microbiol. Ecol. 53, 270-284.

Boddey L H and Hungria M 1997 Phenotypic grouping of brazilian *Bradyrhizobium* strains which nodulate soybean. Biol. Fertil. Soils 25, 407–415.

de Bruijn F J 1992 Use of repetitive extragenic palindromic and enterobacterial repetitive intergenic consensus sequences and the polymerase chain reaction to fingerprint the genomes of *Rhizobium meliloti* isolates and other soil bacteria. Appl. Environ. Microbiol. 58, 2180-2187.

Brunel B, Cleyet-Marel J C, Normand P and Bardin R 1988 Stability of *Bradyrhizobium japonicum* inoculants after introduction into soil. Appl. Environ. Microbiol. 54, 2636–2642.

Chen L S, Figueredo A, Pedrosa F O and Hungria M 2000 Genetic characterization of soybean rhizobia in Paraguay. Appl. Environ. Microbiol. 66, 5099–5103.

Coutinho H L C, Oliveira V M, Lovato A, Maia A H N and Manfio G P 1999 Evaluation of the diversity of rhizobia in Brazilian agricultural soils cultivated with soybeans. Appl. Soil Ecol. 13, 159-167.

Depret G, Houot S, Allard M R, Breuil M C, Nouaim R and Laguerre G 2004 Long-term effects of crop management on *Rhizobium leguminosarum* biovar *viciae* populations. Microbiol. Ecol. 51, 87-97.

Doignon-Bourcier F, Willems A, Coopman R, Laguerre G, Gillis M and de Lajudie P 2000 Genotypic characterization of *Bradyrhizobium* strains nodulating small Senegalese legumes by 16s-23s rRNA intergenic gene spacers and amplified fragment length polymorphism fingerprint analyses. Appl. Environ. Microbiol. 66, 3987–3997.

Ferreira M C, Andrade D S, Chueire L M O, Takemura S M and Hungria M 2000 Tillage method and crop rotation effects on the population sizes and diversity of bradyrhizobia nodulating soybean. Soil Biol. Biochem. 32, 627–637.

Ferreira M C and Hungria M 2002 Recovery of soybean inoculant strains from uncropped soils in Brazil. Field Crops Res. 79, 139–152.

Freiberg C, Fellay R, Bairoch A, Broughton W, Rosenthal A and Perret X 1997 Molecular basis of symbiosis between *Rhizobium* and legumes. Nature 387, 394–401.

Freire J R J 1977 Inoculation of soybean. In Exploiting the legume-*Rhizobium* symbiosis in tropical agriculture Hawaii. Ed. J M Vincent, A S Whitney and J Bose. pp. 335-379. University of Hawaii, Honolulu.

Freire J R J, Kolling J, Vidor C, Pereira J S, Kolling I G and Mendes N G 1983 Sobrevivência e competição por sítios de nodulação de estirpes de *Rhizobium japonicum* na cultura da soja. Rev. Bras. Ci. Solo 7, 47–53.

Galli-Terasawa L V, Glienke-Blanco C and Hungria M 2003 Diversity of a soybean rhizobial population adapted to a Cerrados soil. World J. Microbiol. Biotechnol. 19, 933–939.

Gibson A H, Demezas D H, Gault R R, Bhuvaneswari T V and Brockwell J 1991 Genetic stability of rhizobia in the field. In The Rhizosphere and Plant Growth. Ed D L Keister, P B Cregan. pp.141-148. Kluwer Academic Publishers, Dordrecht.

Göttfert M, Röthlisberger S, Kündig C, Beck C, Marty R and Hennecke H 2001 Potential symbiosis-specific genes uncovered by sequencing a 410-kilobase DNA region of the *Bradyrhizobium japonicum* chromosome. J. Bacteriol. 183, 1405-1412.

Hungria M, Franco A A and Sprent J I 1993 New sources of high-temperature tolerant rhizobia for *Phaseolus vulgaris* L. Plant Soil 149, 103-109.

Hungria M and Vargas M A T 2000 Environmental factors affecting N_2 fixation in grain legumes in the tropics, with an emphasis on Brazil. Field Crops Res. 65, 151-164.

Hungria M, Chueire L M O, Coca R G and Megias M 2001 Preliminary characterization of fast growing rhizobial strains isolated from soybean nodules in Brazil. Soil Biol. Biochem. 33, 1349-1361.

Kaneko T, Nakamura Y, Sato S, Minamisawa K, Uchiumi T, Sasamoto S, Watanabe A, Idesawa K, Iriguchi M, Kawashima K, Kohara M, Matsumoto M, Shimpo S, Tsuruoka H, Wada T, Yamada M and Tabata S 2002 Complete genomic sequence of nitrogen-fixing symbiotic bacterium *Bradyrhizobium japonicum* USDA110. DNA Res. 9, 189-197.

Laberge S, Middleton A T and Wheatcroft R 1995 Characterization, nucleotide sequence, and conserved genomic locations of insertion sequence ISRm5 in *Rhizobium meliloti*. J. Bacteriol. 177, 3133–3142.

Laguerre G, Louvrier P, Allard M R and Amarger N 2003 Compatibility of rhizobial genotypes within natural populations of *Rhizobium leguminosarum* biovar *viciae* for nodulation of host legumes. Appl. Environ. Microbiol. 69, 2276–2283.

Lõhmus K, Truu M, Truu J, Ostonen I, Kaar E, Vares A, Uri V, Alama S and Kanal A 2006 Functional diversity of culturable bacterial communities in the rhizosphere in relation to fine-root and soil parameters in alder stands on forest, abandoned agricultural and oil-shale mining areas. Plant Soil 283, 1–10.

Magurran A E 1987 Ecological diversity and its measurement. Princeton, New Jersey, 179 pp.

Marilley L, Vogt G, Blanc M and Aragno M 1998 Bacterial diversity in the bulk soil and rhizosphere fractions of *Lolium perenne* and *Trifolium repens* as revealed by PCR restriction analysis of 16S rDNA. Plant Soil 198, 219–224.

Martinez-Romero E and Caballero-Mellado J 1996 *Rhizobium* phylogenies and bacterial diversity. Crit. Rev. Plant Sci. 15, 113–140.

Obaton M, Bouniols A, Piva G and Valdez V 2002 Are *Bradyrhizobium japonicum* stable during a long stay in soil? Plant Soil 245, 315–326.

Palmer K M and Young J P W 2000 Higher diversity of *Rhizobium leguminosarum* biovar *viciae* populations in arable soils than in grass soils. Appl. Environ. Microbiol. 66, 2445–2450.

Parker M A 1999 Relationships of bradyrhizobia from the legumes *Apios americana* and *Desmodium glutinosum*. Appl. Environ. Microbiol. 65, 4914–4920.

Peres J R R and Vidor C 1980 Seleção de estirpes de *Rhizobium japonicum* e competitividade por sítios de infecção nodular em estirpes de soja. Agron. Sulriograndense 16, 205-219.

Provorov N A and Vorobev N I 2000 Evolutionary genetics of nodule bacteria: molecular and population aspects. Rus. J. Gen. 36, 1323–1335.

Revellin C, Pinochet X, Beauclair P and Catroux G 1996 Influence of soil properties and soybean cropping history on the *Bradyrhizobium japonicum* population in some French Soils. Eur. J. Soil Sci. 47, 505–510.

Sambrook J and Russell D W 2001 Molecular Cloning: A Laboratory Manual. Cold Spring Harbor, New York.

Santos M A, Vargas M A T and Hungria M 1999 Characterization of soybean bradyrhizobia strains adapted to the Brazilian cerrados region. Microb. Ecol. 30, 261–272.

Shannon C E and Weaver W 1949 The mathematical theory of communication. University of Illinois Press, Urbana.

Sikora S and Redzepovic S 2003 Genotypic characterization of indigenous soybean rhizobia by PCR-RFLP of 16S rDNA, rep-PCR and RAPD analysis. Food Technol. Biotechnol. 41, 61-67.

Silva C, Vinuesa P, Eguiarte L E, Martinez-Romero E and Souza V 2003 *Rhizobium etli* and *Rhizobium gallicum* nodulate common bean (*Phaseolus vulgaris*) in a traditionally managed milpa plot in Mexico: population genetics and biogeographic implications. Appl. Environ. Microbiol. 69, 884–893.

Singh D V, Matte M H, Matte G R, Jiang S, Sabeena, F, Shukla B N, Sanyal S C, Huq A and Colwell R R 2001 Molecular analysis of *Vibrio cholerae* O1, O139, non-O1, and non-O139 strains: clonal relationships between clinical and environmental isolates. Appl. Environ. Microbiol. 67, 910–921.

Soil Survey Staff (1998) Keys to soil taxonomy USDA-NRCS. US Gov Print Office, Washington.

Somasegaran P and Hoben J H 1994 Handbook for Rhizobia: methods in legume-*Rhizobium* technology. Springer-Verlag, New York.

Sullivan J T and Ronson C W 1998 Evolution of rhizobia by acquisition of a 500-kb symbiosis island that integrates into a phe-tRNA gene. Proc. Nat. Acad. Sci. 95, 5145–5149.

Vargas M A T, Mendes I C, Suhet A R and Peres J R R 1993 Serological distribution of *Bradyrhizobium japonicum* from Brazilian cerrados areas under soybean cultivation. Res. Microbiol. 24, 239–243.

Vargas M A T and Hungria M 1997 Biologia dos Solos de Cerrados. EMBRAPA-CPAC, Planaltina, pp. 297-360.

Vargas L K, Lisboa B B, Scholles D, Silveira J R P, Jung G C, Granada C E, Neves A G, Braga M M and Negreiros T 2007 Genetic diversity and symbiotic efficiency of black wattle nodulating rhizobia from soils of Rio Grande do Sul. Rev. Bras. Ci Solo 31, 647-654.

Versalovic J, Schneider M, Bruijn F J D and Lupski J R 1994 Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. Methods Mol. Cell. Biol. 5, 25-40.

Vincent J M 1970 A manual for the practical study of root nodule bacteria. Blackwell Scientific, Oxford.

Wong H L, Yeoh H H and Lim S H 1999 Customization of AFLP analysis for cassava varietals identification. Phytochem. 49, 808-813.

Zilli J E, Valisheski R R, Freire Filho F R, Neves M C P and Rumjanek N G 2004 Assessment of cowpea *Rhizobium* diversity in Cerrado areas of northeastern Brazil. Braz. J. Microbiol. 35, 281-287.

Table 1. *Bradyrhizobium japonicum* and *B elkanii* reference strains utilized in this work.

Strains	Relevant characteristics	Source
Bradyrhizobium elkanii		
SEMIA 587	Brazilian (RS) reference strain (Recommended commercially from 1968 to 1975 and since 1979)	Fepagro/Brazil
SEMIA 5019 (=29w; =BR 29)	Brazilian (RJ) reference strain (Recommended commercially since 1979)	Fepagro/Brazil
Bradyrhizobium japonicum		
USDA 110 (=TAL 102)	Group Ia Isolated from USA soybean fields	Fepagro/Brazil
SEMIA 586 (=CB 1809;=USDA 136b; =TAL 379)	Isolated from USA soybean fields	Fepagro/Brazil
SEMIA 5079 (=CPAC 15)	Brazilian reference strain (Natural variant of SEMIA 566) (Recommended commercially since 1992)	Fepagro/Brazil
SEMIA 5080 (=CPAC 7)	Brazilian reference strain (Natural variant of CB 1809) (Recommended commercially since 1992)	Fepagro/Brazil
SEMIA 566	Brazilian (RS) soybean fields (Recommended commercially from 1966 to 1978)	Fepagro/Brazil
Undefined *Bradyrhizobium*		
SEMIA 509		Fepagro/Brazil
SEMIA 527		Fepagro/Brazil
SEMIA 531		Fepagro/Brazil
SEMIA 532		Fepagro/Brazil

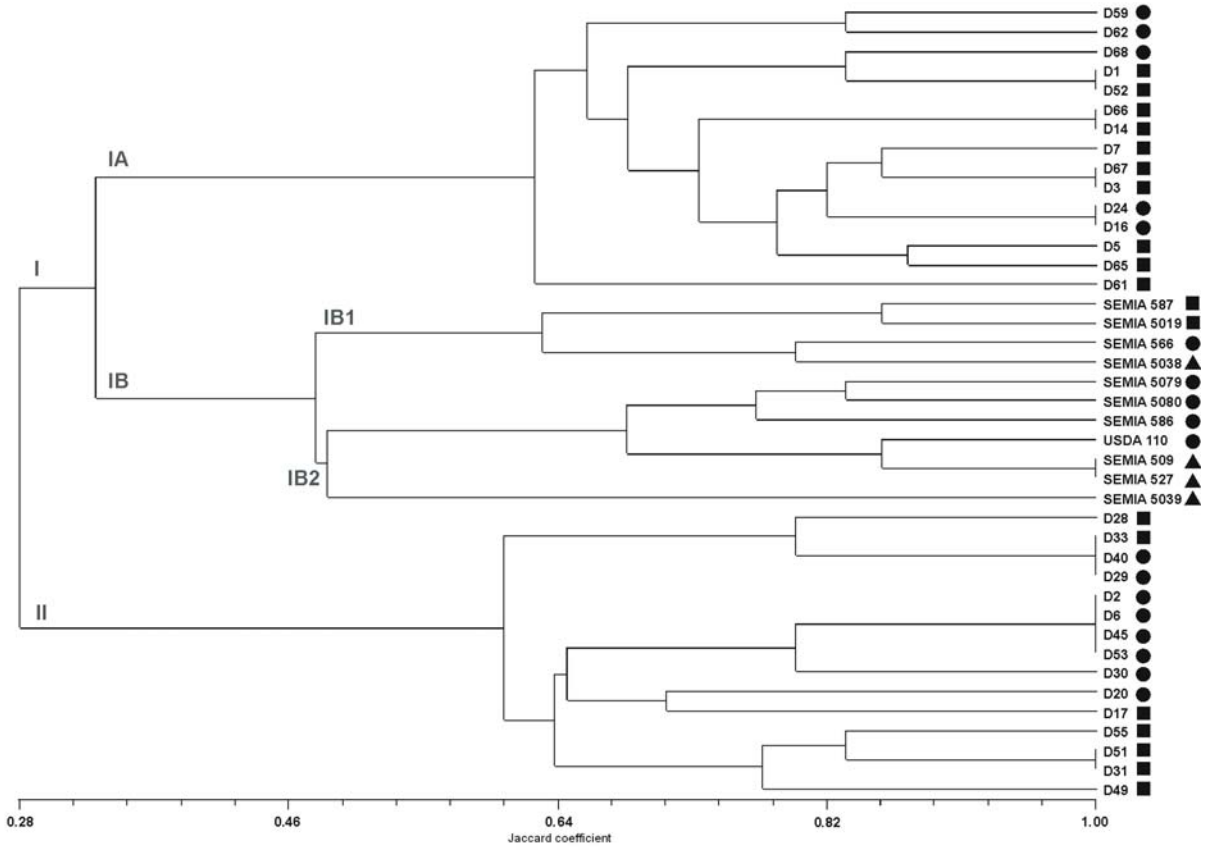

Figure 1. UPGMA-dendrogram derived from AFLP fingerprints of reference strains and soybean bradyrhizobia isolates. Strains are described in Material and Methods and Table 1. Scale shows percentage similarity. Symbols represent: ● *B japonicum* isolates and reference strains; ■ *B elkanii* isolates and reference strains; ▲ *Bradyrhizobium* undefined strains.

Capítulo III

Diversity of bradyrhizobia strains nodulating soybean [*Glycine max* (L.) Merrill] isolated from South Brazilian fields

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Diversity of bradyrhizobia strains nodulating soybean [*Glycine max* (L.) Merrill] isolated from South Brazilian fields

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Abstract

Aims: To characterize and to compare the bradyrhizobia nodulating soybean populations from five distinct regions of Rio Grande do Sul (RS) State under frequent inoculation in order to determine the genetic diversity of these populations and verify the major soil environmental factors that can affect their diversity.

Methods and Results: Bacterial populations were analyzed by rep-PCR and AFLP methodologies. A high level of genetic diversity within populations was observed with diversity indexes varying from 3.95 to 6.17. Using the principal coordinate analysis as statistical approach it was found that pH, clay and organic matter contents were the major soil factors affecting diversity of the analyzed populations. Soil pH was the characteristic more related to bradyrhizobial diversity, whereas clay and organic matter contents had less influence in bacterial diversity.

Conclusions: The results obtained showed that bradyrhizobia nodulating soybean in RS are highly variable and have shown to be influenced by abiotic parameters.

Significance and Impact of this Study: The high level of genetic diversity presented by the analyzed bradyrhizobia populations emphasizes the influence of local environmental conditions on the bacterial populations. This influence should be considered in the moment of inoculation in order to increase soybean yields.

Keywords: *Bradyrhizobium,* diversity, genotypic characterization, rep-PCR, AFLP, principal coordinate analysis.

Introduction

Soybean (*Glycine max* L. Merrill), a summer annual herb native from China, is widely cultivated in the South and North Americas. It can establish effective nitrogen fixing symbiosis with several species of slow-growing rhizobia, like *Bradyrhizobium japonicum* (Jordan, 1982), *B. elkanii* (Kuykendall et al., 1992), and *B. liaoningense* (Xu et al., 1995). In Brazil, however, only *B. japonicum* and *B. elkanii* have been used as commercial inoculants to increase soybean yields. As Brazilian soils lack indigenous soybean bradyrhizobia (Freire, 1977, Peres and Vidor, 1980, Ferreira and Hungria, 2002, Alberton et al., 2006), its entire naturalized bradyrhizobia population nodulating soybean possibly came with seeds and inoculants from United States.

The massive inoculation of Brazilian soils with few bradyrhizobia strains recommended for soybean has resulted in an established population in most soils cropped with this legume (Ferreira and Hungria, 2002). These populations show morphological, biochemical, physiological, genetic and symbiotic variability related to adaptation processes and genetic transfer (Paffetti et al., 1996, Santos et al., 1999, Ferreira et al., 2000, Hungria and Vargas, 2000, Ferreira and Hungria, 2002, Galli-Terasawa et al., 2003). Some studies on bradyrhizobia species isolated from soybean (Abaidoo et al., 2000, Chen et. al., 2000), *Lupinus* spp. (Barrera et al., 1997), *Acacia albida* (Dupuy and Dreyfus, 1992), *Aeschynomene* spp. (Wong et al., 1994), and other legumes (Parker and Lunk, 2000, Willems et al., 2003, Wolde-meskel et al., 2004) cultivated in Brazilian soils indicated that the genus *Bradyrhizobium* represents a huge heterogeneous population, which is more diverse than would be if derived solely from the *B. japonicum* and *B. elkanii* species (Jordan, 1984, Chueire et al., 2003).

Due to the ecological and economic importance, the bradyrhizobia species and their diversity have been extensively investigated in the last years (Liu et al., 2005). The diversity and the size of indigenous population in soil can vary with the presence of the host legume (Parker, 1999, Andrade et al., 2002) and the history of the land use pattern at the sampling site (Sharma et al., 2005). Several environmental conditions, like extremes of pH, are also limiting factors to the growth and activity of rhizobia in soil (Brockwell et al., 1991, Kahindi et al., 1997, Zahran, 1999). These features can shape soil and plant-associated habitats, modifying the composition and activities of their microbial communities (Paffetti et al., 1996, Bever et al., 1997, Wieland et al., 2001).

Since rhizobia are taxonomically very diverse (Wolde-meskel et al., 2004), efficient strain classification methods are required to identify genotypes displaying, for example, superior nitrogen-fixation capacity (Sikora et al., 2002). Molecular techniques have helped to develop easy and quick methods to microbial characterization including studies discriminating genera, species and even strains (Schneider and de Bruijn, 1996, Botha et al., 2004). The polymerase chain reaction (PCR), a DNA-based typing technique, has greatly increased the power of studying, comparing and sorting bacteria (Sikora et al., 2002, Girvan et al., 2003, Muresu et al., 2005). PCR has added a wide array of possibilities such as rep-PCR [*repetitive extragenic palindromic* (de Bruijn, 1992)] and AFLP [*amplified fragment length polymorphism* (de Vos et al., 1995)], which target random regions of the genome that can contribute to the genetic polymorphism and strain-level typing (Huys et al., 1996, Janssen and Dijkshoorn, 1996). Primers corresponding to consensus repetitive sequences dispersed in the eubacteria genome, known as ERIC (*enterobacterial repetitive intergenic consensus*) and BOX (*enterobacterial repetitive sequences*) can also create highly characteristic patterns when separated in agarose gels

(Selenska-Pobell et al., 1995), providing good discrimination on strain level (Olive and Bean, 1999, Gomez-de-Leon et al., 2000, Saldanã et al., 2003, Wang et al., 2006). ERIC sequences are highly conserved among rhizobia genomes and they were used to distinguish and classify different rhizobia strains in population studies (de Bruijn, 1992, Madrzak et al., 1995, Selenska-Pobell, 1995, Selenska-Pobell et al., 1996, Laguerre et al., 1997, Vinuesa et al., 1998, Chen et al., 2000, Mostasso et al., 2002) and to evaluate the environmental impact in defined populations (Labes et al., 1996).

Quantifying the effects of the factors that best explain the variation of diversity communities and populations is a central goal in ecology (Tuomisto et al., 2003). Considering that only four bradyrhizobia reference strains have been widely used in most soybean fields in Brazil, especially in the State of Rio Grande do Sul (RS), the objectives of this work were (1) to characterize and to compare the bradyrhizobia nodulating soybean populations from five distinct regions of RS under frequent inoculation; (2) to determine the genetic diversity of the bradyrhizobia populations using rep-PCR and AFLP methodologies; (3) to assess the major soil environmental factors that can affect the local diversity of the bradyrhizobia populations.

Material and Methods

Collection sites and soil samples

Bradyrhizobia nodules were collected from five different regions of RS, Brazil: (1) Ibirubá [28°32'52"S, 53°10'16"W], (2) Cachoeira do Sul [30°02'21"S, 52°53'38"W], (3) Santa Rosa [27°52'15"S, 54°34'50"W], (4) Vacaria [28°30'44"S, 50°56'02"W] and (5) Dom Pedrito [30°58'58"S, 54°40'23"W]. These areas have been used as soybean crops for at least 10 years and have been inoculated with soybean inoculants every two years,

following standard soil management practices. Ten sub samples of soil (0-15 cm layer) of each field were taken and bulked to obtain a representative soil sample. Sampled soils were analyzed and results are shown in Table 1.

Bacterial isolates and reference strains

About 100 fresh root nodules from plants growing in fields during the summer (January, 2004) were collected from each sampled site, and kept on silica gel. Rhizobia were isolated on yeast-extract mannitol agar [YEMA (Somasegaran and Hoben, 1994)] using standard procedures and purified by repeated streaking (Vincent, 1970). To confirm their purity, all the isolates and the reference strains were streaked on YEMA supplemented with 0.025 g. l^{-1} of Congo red dye, and YEMA supplemented with 0.1 g. l^{-1} of bromothymol blue (Somasegaran and Hoben, 1994). Pure cultures were stored at -20°C in 25% glycerol–YEM broth (Somasegaran and Hoben, 1994).

Four reference strains representing two bradyrhizobia species were used: *Bradyrhizobium elkanii* SEMIA 587 and SEMIA 5019 and *B. japonicum* SEMIA 5079 and SEMIA 5080. These strains were obtained from the Biological Nitrogen Fixation Center/Fundação Estadual de Pesquisa Agropecuária (Fepagro), RS, Brazil. Isolates were named as follow: Ibirubá (A, B, C), Cachoeira do Sul (H, I), Santa Rosa (J, K), Vacaria (F) and Dom Pedrito (L).

DNA isolation

Bacterial cells were grown in YEM broth for seven days at 28ºC at 128 rpm. Cells were rinsed with TES buffer (50 mM Tris pH 8, 0.5 mM EDTA, 50 mM NaCl), and ressuspended in EDTA saline (150 mM NaCl, 10 mM EDTA pH 8). Cell lyses took place

in 20% sodium dodecyl sulfate (SDS) warmed at 55ºC. DNA was extracted using chloroform/isoamylic alcohol (24:1) and precipitated with ethanol.

Genotyping characterization

Genotypic characterization by rep-PCR

Rep-PCR reactions were carried out using enterobacterial repetitive intergenic consensus primers ERIC1-R (ATGTAAGCTCCTGGGGATTCAC) and ERIC-2 (AAGTAAGTGACTGGGGTGAGCG) (de Bruijn, 1992) and enterobacterial repetitive sequences (BOX A1) primer [CTACGGCAAGGCGACGCTGACG (Versalovic et al., 1994)]. The reactions were performed in a 25-µl volume, containing 50 ng of DNA template, 1 U *Taq* DNA polymerase, 1 X *Taq* DNA polymerase buffer, 15 mM $MgCl_2$, 200 mM dNTPs (Life Technologies) and 10 pmoles of each primer. A total of 32 cycles took place, as follows: one initial denaturation cycle at 95ºC for 7 min, 30 cycles of denaturation at 94ºC for 1 min, annealing at 52ºC for 1 min, and extension at 65ºC for 8 min, and one final extension cycle at 65ºC for 16 min. Reactions were performed in a PCR Express Temperature Cycling System (Thermo Hybaid) and fragments were visualized after electrophoresis at 100 V for 3 h on a 1% agarose gel stained with ethidium bromide. Molecular size marker was 1 Kb Plus DNA ladder (Gibco BRL).

AFLP

AFLP procedures were carried out as described by Dougnon-Bourcier et al. (2000). A 100 µg aliquot of genomic DNA of each isolate was overnight digested with 1.25 U of both *Apa*I and *Taq*I restriction enzymes (Invitrogen) at 37°C and 65°C, respectively, in a final volume of 25 µl. The restriction reaction was then ligated to the *Apa*I and *Taq*I

adapters [*Apa*I adapters: TCGTAGACTGCGTACAGGCC and CATCTGACGCATGT; *Taq*I adapters: GACGATGAGTCCTGAC and TACTCAGGACTGGC (Wong et al., 1999, Singh et al., 2001)]. The ligation mixture consisted of 25 µl of digested DNA, 10 pmol of each adapter and 1 U of T4 DNA ligase (Invitrogen) in correspondent buffer. Ligated DNA was 1:10 diluted in ultra pure water and heated at 80^{o}C for 10 min to inactivate T4 DNA ligase. Five µl of ligated DNA was then used as template for PCR DNA amplification. The reactions were performed in a 25 µl volume, containing 1 U *Taq* DNA polymerase (Invitrogen), 1 X *Taq* DNA polymerase reaction buffer, 15 mM $MgCl_2$, 200 mM dNTPs (Invitrogen) and 10 pmoles of each primer (*Apa*I GACTGCGTACAGGCCCE, where E means ATT/CCC/CTA/CTG/GAC/TTG and *Taq*I CGATGAGTCCTGACCGAE, where E means CCC/CTG/GAC/GTA). PCR amplification was performed using the *touchdown* protocol: 94°C for 3 min, followed by 10 cycles of 94°C for 30 s, 65°C to 56°C for 1 min and 72°C for 2 min, followed by 23 cycles of 94°C for 30 s, 56°C for 30 s, and a final extension of 72 °C for 1 min. Reactions were performed in a PCR Express Temperature Cycling System (Thermo Hybaid). The fragments were visualized after electrophoresis for 3 h at 80 V in an 8% acrylamide-bisacrylamide gel stained with silver nitrate (Sambrook and Russel 2001). Molecular size marker (λ DNA digested with *Eco*RI / *Hind*III, Invitrogen) was run in all gels.

DNA fingerprint analysis

Fingerprint patterns were visualized under UV illumination. The size of the fragments obtained ranged between 550 and 5,000 bp. They were converted into a two-dimensional binary matrix (1, presence of a band; 0, absence of a band) and analyzed using Jaccard (*J*) coefficient, which do not consider the negative similarities. The matrix was

analyzed by NTSYS-PC package. UPGMA (Unweighted Pair Group Method with Arithmetic mean) algorithm was used to perform hierarchical cluster analysis and construct a dendrogram. Index of diversity (*H*, Shannon and Weaver, 1949) was estimated based on the number of isolates belonging to each group of profiles in rep-PCR and AFLP, considering a 70% of similarity in the cluster analysis (Kaschuk et al., 2006a; Alberton et al., 2006). Principal coordinate analysis (PCA) was used to determine statistical correlation between soil properties and population diversity (Rico et al., 2004). Pairwise squared Euclidean distances based on different soil properties were calculated for the five analyzed soils in order to derive a double-centered distance matrix for factoring (Rohlf, 1990).

Results and Discussion

A collection of 417 isolates was obtained from soybean nodules harvested from five different soybean fields of Rio Grande do Sul State. The number of isolates analyzed in each sampled site is shown in Table 2. All bacterial isolates presented slow growth rate and alkaline reaction in mannitol as carbon source, characteristics of the genus *Bradyrhizobium* (Kuykendall et al., 1992). The isolation of fast-growing rhizobia able to nodulate soybean in South America fields has been reported by Hungria et al. (2001, 2006) and Galli-Terasawa et al. (2003). These bacteria were identified as indigenous *Rhizobium tropici* and *Agrobacterium* spp. in Brazil and Paraguay (Chen et al., 2000, Hungria et al., 2001, Hungria et al., 2006). However, this kind of bacteria did not appear in our isolates.

Each bacterial population was analyzed by rep-PCR (ERIC and BOX) methodology and a high level of genetic diversity within populations was observed. It was possible to distinguish well-defined groups of rhizobia, indicating the occurrence of intragenic diversity (data not shown). Aiming to obtain a more detailed cluster analysis the data of

ERIC and BOX were combined. Hungria et al. (2000) and Sikora et al. (2002) also combined two rep-PCR analyses in order to maximize the detection of genotypic differences, which provided a more consistent result (Rademaker et al., 2000). When analyzed by UPGMA, the combined rep-PCR data clustered the tested strains in 12 to 22 groups at the similarity of 70%, depending on the population analyzed (Table 2).

In order to quantify the diversity among the isolates of each bacterial population the Shannon-Weaver index (*H*) was used, maintaining the basis of a 70% similarity. The results revealed a high genetic diversity among these different bradyrhizobia populations: Cachoeira do Sul (*H* = 6.17), Santa Rosa (*H* = 6.04), Ibirubá (*H* = 5.79), Dom Pedrito (*H* = 4.41) and Vacaria (*H* = 3.95). Similar result was obtained by Vargas et al. (2007) that found an *H* of 4.3 studying the genetic diversity of black wattle nodulating rhizobia in RS. Andrade et al. (2002) also found a diversity index of 3.93 analyzing common bean rhizobia in Brazilian acid soils altered by liming. Lõhmus et al. (2006) obtained Shannon indexes of 4.63 and 4.56 among cultivable bacterial communities extracted from soil–root interface and rhizosphere bulk soil, respectively. Moreover, a *Bradyrhizobium* population persisting in a soil for more than 30 years after inoculation, without the host plant, was as diverse as the fields populations analyzed in this work, with an *H* of 4.18 (A. Giongo, personal communication).

A subset of 20 bacteria of each population previously analyzed by rep-PCR was used for AFLP analysis, totalizing 100 strains. A dendrogram obtained using the UPGMA analysis clustered those 100 strains into 19 AFLP groups at the similarity level of 70% (Fig. 1). The isolates were joined in five principal groups that represent the isolates from each sampled site. Group I comprised preferentially isolates from Vacaria (F), group II from Ibirubá (A, B, C), group III from Cachoeira do Sul (H, I), group IV from Santa Rosa

(J, K), and group V from Dom Pedrito (L). Similarly to other authors (Wang et al., 2003, Wang et al., 2006) some differences were observed in grouping results obtained from different analysis. AFLP joined isolates that presented different band patterns in rep-PCR profile (data not shown). However, AFLP genotyping has proved to be a highly sensitive method for subtyping and discriminating bradyrhizobial strains (Dougnon-Bourcier et al., 2000) as the patterns obtained are reproducible and are not dependent on technical factors, such as $MgCl_2$ concentration or the *Taq* DNA polymerase used (Terefework et al., 2001).

Aiming to determine whether there are specific soil parameters that could influence rhizobial diversity, soils of the sampled sites were taken to analysis. Zhang et al. (2006) had showed that different environmental parameters could affect the diversity of soil bacteria, including nitrogen-fixing bacteria. However, low information is available about such parameters (Ramette and Tiedje, 2007). Using the principal coordinate analysis (PCA) as statistical approach to correlate the bacterial diversity (*H*) to the soil parameters, it was possible to sort out that pH, clay and organic matter contents were the major soil factors affecting diversity of the five different bradyrhizobia populations (Fig. 2). Palmer and Young (2000) observed that these parameters have shown some influence in bacterial diversity and survival and they can be expected to change according to the land management. Thus they possible have some impact on bacterial dynamics in soil.

The first two dimensions of PCA (PCA1 and PCA2) explained 72.47% of the total variation. Component 1 accounted for 42.61% of the variance while component 2 for 29.86%. The diversity index was higher in Ibirubá, Santa Rosa and Cachoeira and lower in Vacaria and Dom Pedrito (Table 2). Soil pH was the characteristic more related to bradyrhizobial diversity, whereas clay and organic matter contents, although were closely related to each other, had less influence in bacterial diversity (Fig. 2).

It was demonstrated that pH can limit the presence of microorganisms in soils and can be a barrier to diversity (Brockwell et al., 1991). Fierer and Jackson (2006) considered that pH was the best predictor of soil bacterial diversity and richness, with the lower levels of bacteria diversity and richness observed in acid soils. The soils of the sampled sites presented differences in pH values ranging from 5.4 to 6.7 (Table 1). Although rhizobia optimum pH is between 6 and 7 (Jordan, 1984) the tolerance can vary among the strains within the species (Brockwell et al., 1995). Some rhizobia have been reported to grow in pH ranging between 3.5 and 12 (Hung et al., 2005). Many authors have found an apparent correlation between genetic variation and environmental stress including extremes of pH (Harrison et al., 1989). Population density of indigenous *Rhizobium leguminosaram* biovar *trifolii* in pastures is increased when the soil is liming (Richardson and Simpson, 1988). Studies of Anyango et al. (1995) showed that rhizobial diversity is reduced under stressed conditions such as those associated with soil acidity. Laranjo et al. (2002) studied chickpea rhizobia populations in soils with different pH (ranging between 5.1 and 8.2). They observed an apparent positive correlation between genetic diversity and soil pH being the genetic diversity higher in alkaline soils. Lafay and Burdon (1998), Bala et al. (2003) and Abaidoo et al. (2007) have found correlation between rhizobia population density and soil pH. The results obtained in this work reinforce the correlation between soil alkalinity and genetic diversity since the sampled sites with higher diversity indexes also presented the highest soil pH. Otherwise, Vacaria and Dom Pedrito, which presented the lower diversity indexes, also presented the lowest soil pH (Tables 1 and 2).

The chemical and physical differences between soils may be at least partially responsible for the genetic differences among the strains (Paffetti et al., 1996). Soil particle size not only affects the bacterial biomass, but also determines the structure of these

communities (Gonzalez-Acosta et al., 2006). Some authors also suggested that parameters like clay contents and soil texture influence on survival and proliferation of bacteria in the soil and rhizosphere (Fages, 1992, Bashan et al., 1995). Sessitsch et al. (2002) observed that soils composed predominantly of clay and fine silt particles showed a greater diversity of bacteria than soils with large particles. On the other hand, Bottomley (1991) suggested that specific abiotic parameters like pH, clay and organic matter contents are much less important than the interactive effects among individuals within populations. In this study, clay and organic matter soil contents did not seem to have much influence in bradyrhizobial diversity.

There are scarce studies of rhizobial diversity in Southern Brazilian fields (Kaschuk et al., 2006a, 2006b, Giongo et al., 2007, Vargas et al., 2007). The Brazilian population of bradyrhizobia nodulating soybean is a result of adaptation of few strains introduced as inoculants to Brazilian soil conditions (Alberton et al., 2006). Nevertheless, the present study emphasizes how diverse a local population of *Bradyrhizobium* could be corroborating the high level of diversity in morphology, physiology, genetic, and symbiotic properties demonstrated by Loureiro et al. (2006). Similar results concerning rhizobial populations were early obtained by Laguerre et al. (1997), Lafay and Burdon (1998), and Parker (1999). It should be mentioned that a small fraction of strains from the total soil population was sampled and that diversity of the bradyrhizobia in RS soils is no doubt greater than that observed in this work.

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References

Abaidoo, R.C., Keyser, H.H., Singleton, P.W., Borthakur, D., 2000. *Bradyrhizobium* spp. (TGx) isolates nodulating the new soybean cultivars in Africa are diverse and distinct from bradyrhizobia that nodulate North American soybeans. Int. J. Syst. Evol. Microbiol. 50, 225–234.

Abaidoo, R.C., Keyser, H.H., Singleton, P.W., Dashiell, K.E., Sanginga, N., 2007. Population size, distribution, and symbiotic characteristics of indigenous *Bradyrhizobium* spp. that nodulate TGx soybean genotypes in Africa. Appl. Soil Ecol. 35, 57-67.

Alberton, O., Kaschuk, G., Hungria, M., 2006. Sampling effects on the assessment of genetic diversity of rhizobia associated with soybean and common bean. Soil Biol. Biochem. 38, 1298–1307.

Andrade, D.S., Murphy, P.J., Giller, K.E., 2002. Effects of liming and legume/cereal cropping on populations of indigenous rhizobia in an acid Brazilian Oxisol. Soil Biol. Biochem. 34, 477-485.

Anyango, B., Wilson, J.K., Beynon, J.L., Giller, K.E., 1995. Diversity of rhizobia nodulation *Phaseolus vulgaris* L. in two kenyan soils with contrasting pHs. Appl. Environ. Microbiol. 61, 4016-4021.

Bala, A., Murphy, P., Giller, K.E., 2003. Distribution and diversity of rhizobia nodulating agroforestry legumes in soils from three continents in the tropics. Mol. Ecol. 12, 917–930.

Barrera, L.L, Trujillo, M.E., Goodfellow, M., Garcia, F.J., Hernandez-Lucas, I., Davila, G., van Berkum, P., Martinez-Romero, E., 1997. Biodiversity of bradyrhizobia nodulating *Lupinus* spp. Int. J. Syst. Bacteriol. 47, 1086–1091.

Bashan, Y., Puente, M.E., Rodriguez-Mendoza, M.N., Toledo, G., Holguin, G., Ferrera-Cerrato, R., Pedrin, S., 1995. Survival of *Azospirillum brasilense* in the bulk soil and rhizosphere of 23 soil types. Appl. Environ. Microbiol. 61, 1938–1945.

Bever, J.D., Westoven, K.M., Antonovics, J., 1997. Incorporation the soil community into plant population dynamics: the utility of the feedback approach. J. Ecol. 85, 561-573.

Botha, W.J., Jaftha, J.B., Bloem, J.F., Habig, J.H., Law, I.J., 2004. Effect of soil bradyrhizobia on the success of soybean inoculant strain CB 1809. Microbiol. Res. 159, 219–231.

Bottomley, P., 1991. Ecology of *Rhizobium* and *Bradyrhizobium* In: Staceym, G., Burris, R.H., Evans, H.J. (Eds.), Biological Nitrogen Fixation. Chapman and Hall, New York, pp.292-347.

Brockwell, J., Pilka, A., Holliday, R.A., 1991. Soil pH is a major determinant of the numbers of naturally occurring *Rhizobium meliloti* in non-cultivated soils of New South Wales. Aust. J. Exp. Agric. 31, 211–219.

Brockwell, J., Bottomley, P.J., Thies, J.E., 1995. Manipulation of rhizobia microflora for improving legume productivity and soil fertility: a critical assessment. Plant Soil 174, 143-180.

Chen, L.S., Figueredo, A., Pedrosa, F.O., Hungria, M., 2000. Genetic characterization of soybean rhizobia in Paraguay. Appl. Environ. Microbiol. 66, 5099–5103.

Chueire, L.M.O., Bangel, E.V., Mostasso, F.L., Campo, R.J., Pedrosa, F.O., Hungria, M., 2003. Classificação taxonômica das estirpes de rizóbio recomendadas para a cultura da soja e do feijoeiro baseada no seqüenciamento do gene 16S rRNA. Rev. Bras. Ci. Solo 27, 833-840.

de Bruijn, F.J., 1992. Use of repetitive extragenic palindromic and enterobacterial repetitive intergenic consensus sequences and the polimerase chain reaction to fingerprint the genomes of *Rhizobium meliloti* isolates and other soil bacteria. Appl. Environ. Microbiol. 58, 2180-2187.

Doignon-Bourcier, F., Willems, A., Coopman, R., Laguerre, G., Gillis, M., de Lajudie, P., 2000. Genotypic characterization of *Bradyrhizobium* strains nodulating small Senegalese

legumes by 16s-23s rRNA intergenic gene spacers and amplified fragment length polymorphism fingerprint analyses. Appl. Environ. Microbiol. 66, 3987–3997.

Dupuy, N.C., Dreyfus, B.L., 1992. *Bradyrhizobium* populations occur in deep soil under the leguminous tree *Acacia albida.* Appl. Environ. Microbiol. 58, 2415-2419.

Fages, J., 1992. An industrial review of *Azospirillum* inoculant: formulation and application technology. Symbiosis 13, 15–26.

Ferreira, M.C., Andrade, D.S., Chueire, L.M.O., Takemura, S.M., Hungria, M., 2000. Tillage method and crop rotation effects on the population sizes and diversity of bradyrhizobia nodulating soybean. Soil Biol. Biochem. 32, 627–637.

Ferreira, M.C., Hungria, M., 2002. Recovery of soybean inoculant strains from uncropped soils in Brazil. Field Crops Res. 79, 139–152.

Fierer, N., Jackson, R.B., 2006. The diversity and biogeography of soil bacterial communities. PNAS 103, 626–631.

Freire, J.R.J., 1977. Inoculation of soybean. In: Vincent, J.M., Whitney, A.S., Bose, J. (Eds.) Exploiting the Legume-*Rhizobium* Symbiosis in Tropical Agriculture. University of Hawaii, Honolulu, pp. 335-379.

Galli-Terasawa, L.V., Glienke-Blanco, C., Hungria, M., 2003. Diversity of a soybean rhizobial population adapted to a Cerrados soil. World J. Microbiol. Biotechnol. 19, 933–939.

Giongo, A., Passaglia, L.M.P., Freire, J.R.J., Sá, E.L.S., 2007. Genetic diversity and symbiotic efficiency of population of rhizobia of *Phaseolus vulgaris* L. in Brazil. Biol. Fertil. Soils 43, 593–598.

Girvan, M.S., Bullimore, J., Pretty, J.N., Osborn. A.M., Ball, A.S., 2003. Soil type is the primary determinant of the composition of the total and active bacterial communities in arable soils. Appl. Environ. Microbiol. 69, 1800–1809.

Gomez-de-Leon, P., Santos, J.I., Caballero, J., Gomez, D., Espinosa, L.E., Moreno, I., Pinero, D., Cravioto, A., 2000. Genomic variability of *Haemophilus influenzae* isolated from Mexican children determined by using enterobacterial repetitive intergenic consensus sequences and PCR. J. Clin. Microbiol. 38, 2504–2511.

Gonzalez-Acosta, B., Bashan, Y., Hernandez-Saavedra, N.Y., Ascencio, F., de la Cruz-Aguero, G., 2006. Seasonal seawater temperature as the major determinant for populations of culturable bacteria in the sediments of an intact mangrove in an arid region. FEMS Microbiol. Ecol. 55, 311–321.

Harrison, S.P., Jones, D.G., Young, J.P.W., 1989. *Rhizobium* population genetics: genetic variation within and between populations from diverse locations. J. Gen. Microbiol. 135, 1061–1069.

Huys, G., Coopman, R., Janssen, P., Kersters, K., 1996. High-resolution genotypic analysis of the genus *Aeromonas* by AFLP fingerprinting. Int. J. Syst. Bacteriol. 46, 572-580.

Hung, M.H., Bhagwath, A.A., Shen, F.T., Devasya, R.P., Young, C.C., 2005. Indigenous rhizobia associated with native shrubby legumes in Taiwan. Pedobiologia 49, 577-584.

Hungria, M., Vargas, M.A.T., 2000. Environmental factors affecting N_2 fixation in grain legumes in the tropics, with an emphasis on Brazil. Field Crops Res. 65, 151-164.

Hungria, M., Chueire, L.M.O., Coca, R.G., Megias, M., 2001. Preliminary characterization of fast growing rhizobial strains isolated from soybean nodules in Brazil. Soil Biol. Biochem. 33, 1349-1361.

Hungria, M., Chueire, L.M.O., Megıas, M., Lamrabet, Y., Probanza, A., Guttierrez-Manero, F.J., Campo, R.J., 2006. Genetic diversity of indigenous tropical fast-growing rhizobia isolated from soybean nodules. Plant Soil 288, 343–356.

Janssen, P., Dijkshoorn, L., 1996. High resolution DNA fingerprinting of *Acinetobacter* outbreak strains. FEMS Microbiol. Lett. 142, 191-194.

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. Int. J. Syst. Bacteriol. 32, 136-139.

Jordan, D.C., 1984. Rhizobiaceae Conn 1938. In: Krieg, N.R., Holt, J.G. (Eds.) Bergey's manual of systematic bacteriology: Williams and Wilkins, Baltimore/London, pp. 235-244.

Kahindi, J.H.P., Woomer, P., George, T., de Souza Moreira, F.M., Karanja, N.K., Giller, K.E., 1997. Agricultural intensification, soil biodiversity and ecosystem function in the tropics: the role of nitrogen-fixing bacteria. Appl. Soil Ecol. 6, 55-76.

Kaschuk, G., Hungria, M., Andrade, D.S., Campo, R.J., 2006a. Genetic diversity of rhizobia associated with common bean (*Phaseolus vulgaris* L.) grown under no-tillage and conventional systems in Southern Brazil. Appl. Soil. Ecol. 32, 210-220.

Kaschuk, G., Hungria, M., Santos, J.C.P., Berton-Junior, J.F., 2006b. Differences in common bean rhizobial populations associated with soil tillage management in southern Brazil. Soil Tillage Res. 87, 205–217.

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.

Labes, G., Ulrich, A., Lentzsch, P., 1996. Influence of bovine slurry deposition on the structuture of nodulating *Rhizobium leguminosarum* bv *viciae* soil populations in a natural habitat. Appl. Environ. Microbiol. 62, 1717-1722.

Lafay, B., Burdon, J.J., 1998. Molecular diversity of rhizobia occurring on native shrubby legumes in southeastern Australia. Appl. Environ. Microbiol. 64, 3989-3997.

Laguerre, G., van Berkum, P., Amarger, N., Prevost, D., 1997. Genetic diversity of rhizobial symbionts isolated from legume species within the genera *Astragalus, Oxytropis* and *Onobrychis*. Appl. Environ. Microbiol. 63, 4748-4758.

Laranjo, M., Branco, C., Soares, R., Alho, L., Carvalho, M.D.E., Oliveira, S., 2002. Comparison of chickpea rhizobia isolates from diverse Portuguese natural populations based on symbiotic effectiveness and DNA fingerprint. J. Appl. Microbiol. 92, 1043-1050.

Liu, J., Wang, E.T., Chen, W.X., 2005. Diverse rhizobia associated with woody legume *Wisteria sinensis*, *Cercis racemosa* and *Amorpha fruticosa* grown in the temperate zone of China. Int. J. Syst. Evol. Bacteriol. 55, 1762-1770.

Lõhmus, K., Truu, M., Truu, J., Ostonen, I., Kaar, E., Vares, A., Uri, V., Alama, S., Kanal, A., 2006. Functional diversity of culturable bacterial communities in the rhizosphere in relation to fine-root and soil parameters in alder stands on forest, abandoned agricultural, and oil shale mining areas. Plant Soil 283, 1-10.

Loureiro, M.F., Kaschuk, G., Alberton, O., Hungria, M., 2006. Soybean [*Glycine max* (L.) Merrill] rhizobial diversity in Brazilian oxisols under various soil, cropping, and inoculation managements. Biol. Fertil. Soils doi: 10.1007/s00374-006-0146-x

Madrzak, C.J., Golinska, B., Kroliczak, J., Pudelko, K., Lazewska, D., Lampka, D., Sadowsky, M.J., 1995. Diversity among field populations of *Bradyrhizobium japonicum* in Poland. Appl. Environ. Microbiol. 61, 1194-1200.

Mostasso, L., Mostasso, F.L., Dias, B.G., Vargas, M.A.T., Hungria, M., 2002. Selection of bean (*Phaseolus vulgaris* L.) rhizobial strain for the Brazilian Cerrados. Fields Crop Res. 73, 121-132.

Muresu, R., Sulas, L., Polone, E., Squartini, A., 2005. PCR primers based on different portions of insertion elements can assist genetic relatedness studies, strain fingerprinting and species identification in rhizobia. FEMS Microbiol. Ecol. 54, 445–453.

Olive, D.M., Bean, P., 1999. Minireview: Principles and applications of methods for DNA-based typing of microbial organisms. J. Clin. Microbiol. 37, 1661-1669.

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.

Palmer, K.M., Young, J.P.W., 2000. Higher diversity of *Rhizobium leguminosarum* biovar *viciae* populations in arable soils than in grass soils. Appl. Environ. Microbiol. 66, 2445–2450.

Parker, M.A., 1999. Relationship of *Bradyrhizobium* from the legumes *Apios americana* and *Desmodium glutinosum*. Appl. Environ. Microbiol. 65, 4914-4920.

Parker, M.A., Lunk, A., 2000. Relationships of bradyrhizobia from *Platypodium* and *Machaerium* (Papilionoideae tribe Dalbergieae) on Barro Colorado Island, Panama. Int. J. Syst. Evol. Microbiol. 50, 1179–1186.

Peres, J.R.R. and Vidor, C. (1980) Seleção de estirpes de *Rhizobium japonicum* e competitividade por sítios de infecção nodular em estirpes de soja. Agronomia Sulriograndense 16, 205-219.

Rademaker, L.W., Hoste, B., Louws, F.J., Kersters, K., Swings, J., Vauterin, L., Vauterin, P., de Bruijn, F.J., 2000. Comparison of AFLP and rep-PCR genomic fingerprinting with DNA–DNA homology studies: *Xanthomonas* as a model system. Int. J. Syst. Evol. Microbiol. 50, 665–677.

Ramette, A., Tiedje, J.M., 2007. Multiscale responses of microbial life to spatial distance and environmental heterogeneity in a patchy ecosystem. PNAS 104, 2761–2766.

Richardson, A.E., Simpson, R.J., 1988. Enumeration and distribution of *Rhizobium trifolii* under a subterranean clover based pasture growing in acid soil. Soil Biol. Biochem. 20, 431-438.

Rico, A., Ortiz-Barredo, A., Ritter, E., Murillo, J., 2004. Genetic characterization of *Erwinia amylovora* strains by amplified fragment length polymorphism. J. Appl. Microbiol. 96, 302–310.

Rohlf, F.J, 1990. NTSYS-pc numerical taxonomy and multivariate system. Version 2.01. Exeter Software. Setauket, New York.

Saldaña, G., Martinez Alcantara, V., Vinardell, J.M., Bellogín, R., Ruíz-Sainz, J., Balatti, P.A., 2003. Genetic diversity of fast-growing rhizobia that nodulates soybean (*Glycine max* L. Merr.). Arch. Microbiol. 180, 45-52.

Sambrook, J., Russel, D.W., 2001. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, New York.

Santos, M.A., Vargas, M.A.T., Hungria, M., 1999. Characterization of soybean bradyrhizobia strains adapted to the Brazilian Cerrados Region. FEMS Microbiol. Ecol. 30, 261–272.

Schneider, M., de Bruijn, F.J., 1996. Rep-PCR mediated genomic fingerprinting of rhizobia and computer-assisted phylogenetic patterns analysis. World J. Microbiol. Biotechnol. 12, 163-174.

Selenska-Pobell, S., 1995. Direct and simultaneous extraction of DNA and RNA from soil. In: Ackermans, A.D.L., van Elsas, J.D., de Bruijn, F.D. (Eds.) Molecular Microbial Ecology Manual, Kluwer Academic, Dordrech, pp.1-17.

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 fingerprint. J. Appl. Bacteriol. 80, 517-528.

Sessitsch, A., Howieson, J.G., Perret, X., Antoun, H., Martinez-Romero, E., 2002. Advances in *Rhizobium* research. Crit. Rev. Plant Sci. 21, 323-378.

Shannon, C.E., Weaver, W., 1949. The mathematical theory of communication. University of Illinois Press, Urbana.

Sharma, R.S., Mohmmed, A., Mishra V., Babu, C.R., 2005. Diversity in a promiscuous group of rhizobia from three *Sesbania* spp. colonizing ecologically distinct habitats of the semi-arid Delhi region. Res. Microbiol. 156, 57–67.

Sikora, S., Dedzepovic, S., Bradic, M., 2002. Genomic fingerprinting of *Bradyrhizobium japonicum* isolates by RAPD and rep-PCR. Microbiol. Res. 157, 213-219.

Singh, D.V., Matte, M.H., Matte, G.R., Jiang, S., Sabeena, F., Shukla, B.N., Sanyal, S.C., Huq, A., Colwell, R.R., 2001. Molecular analysis of *Vibrio cholerae* O1, O139, non-O1, and non-O139 strains: clonal relationships between clinical and environmental isolates. Appl. Environ. Microbiol. 67, 910–921.

Somasegaran, P., Hoben, J.H., 1994. Handbook for Rhizobia: Methods in legume-*Rhizobium* technology. Springer-Verlag, New York.

Terefework, Z., Kaijalainen, S., Lindstrom, K., 2001. AFLP fingerprinting as a tool to study the genetic diversity of *Rhizobium galegae* isolated from *Galega orientalis* and *Galega officinalis*. J. Biotechnol. 91, 169–180.

Tuomisto, H., Ruokolainen, K., Yli-Halla, M., 2003. Dispersal, environment and floristic variation of Western Amazonian Forests. Science 299, 241–244.

Vargas, L.K., Lisboa, B.B., Scholles, D., Silveira, J.R.P., Jung, G.C., Granada, C.E., Neves, A.G., Braga, M.M., Negreiros, T.A., 2007. Genetic diversity and symbiotic efficiency of black wattle nodulating rhizobia from soils from Rio Grande do Sul. Rev. Bras. Ci. Solo 31, 647-654.

Versalovic, J., Schneider, M., de Bruijn, F.J., Lupski, J.R., 1994. Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. Meth. Mol. Cell Biol. 5, 25-40.

Vincent, J.M., 1970. A manual for the practical study of root nodule bacteria. Blackwell Scientific, Oxford.

Vinuesa, P., Rademaker, J.L.W., de Bruijn, F.J., Werner, D., 1998. Genotypic characterization of *Bradyrhizobium* strains nodulation endemic woody legumes of the Canary Islands by PCR-RFPL of genes encoding 16S rRNA and 16S-23S rDNA intergenic spacers, REP-PCR genomic fingerprinting and partial 16S rDNA sequencing. Appl. Environ. Microbiol. 64, 2096-2104.

de Vos, P., Hogers, R., Bleeker, M., 1995. AFLP: a new technique for DNA fingerprinting. Nucleic Acids Res. 23, 4407-4414.

Wang, E.T., Kan, F.L., Tan, Z.Y., Toledo, I., Chen, W.X., Martınez-Romero, M., 2003. Diverse *Mesorhizobium plurifarium* populations native to Mexican soils. Arch. Microbiol. 180, 444–454.

Wang, F.Q., Wang, E.T., Zhang, Y.F., Chen, W.X., 2006. Characterization of rhizobia isolated from *Albizia* spp. in comparison with microsymbionts of *Acacia* spp. and *Leucaena* leucocephala grown in China. Syst. Appl. Microbiol. 29, 502–517.

Wieland, G., Neumann, R., Backhaus, H., 2001. Variation of microbial communities in soil, rhizosphere and rhizoplane in response to crop species, soil type and crop development. Appl. Environ. Microbiol. 67, 5849-5854.

Willems, A., Munive, A., de Lajudie, P., Gillis, M., 2003. In most *Bradyrhizobium* groups sequence comparison of 16S-23S rDNA internal transcribed spacer regions corroborates DNA-DNA hybridizations system. Appl. Microbiol. 26, 203–210.

Wolde-meskel, E., Terefework, Z., Lindstrom, K., Frostegard, A., 2004. Rhizobia nodulating African *Acacia* spp. and *Sesbania sesban* trees in southern Ethiopian soils are metabolically and genomically diverse. Soil Biol. Biochem. 36, 2013–2025.

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. Appl. Environ. Microbiol. 60, 940-946.

Wong, H.L., Yeoh, H.H., Lim, S.H., 1999. Customization of AFLP analysis for cassava varietal identification. Phytochemistry 49, 808-813.

Xu, L.M., Ge, C., Cui, Z., Li, J., Fan, H., 1995. *Bradyrhizobium liaoningense* sp. nov., isolated from the root nodules of soybean. Int. J. Syst. Bacteriol. 45, 706-711.

Zahran, H.H., 1999. *Rhizobium*-legume symbiosis and nitrogen fixation under severe conditions and in an arid climate. Microbiol. Mol. Biol. Rev. 63, 968-989.

Zhang, Y., Li, D., Wang, H., Xiao, Q., Liu, X., 2006. Molecular diversity of nitrogen-fixing bacteria from the Tibetan plateau, China. FEMS Microbiol. Lett. 260, 134–142.

Table 1. Chemical properties of the soils from each sampled site.

Sampled site	pH	Clay	O.M.	SMP-	P	K	Fe	Al+ H	Al exc	Ca exc	Mg exc
	H_20	%		pH	mg/dc^3		g/dc_3	$cmol_c/dc^3$			
Ibirubá	6.7	45	4.1	6.8	6.4	227	6.2	1.6	6.3	3.8	6.1
Cachoeira do Sul	6.2	13	2.4	6.8	8	67	1.3	1.7	0	4.1	1
Santa Rosa	6.1	54	4.4	6.2	28	252	1.4	3.5	0	4.5	2.5
Vacaria	5.8	56	6.6	6.2	2.1	261	2.3	3.5	0	6.2	5.1
Dom Pedrito	5.4	24	1.2	5.2	6.8	121	1.8	4.1	0	1.2	4.7

Abbreviations: O.M. = organic matter; SMP = potential soil acidity buffer; exc = exchangeable.

Table 2. Number of isolates, rep-PCR data and Shannon diversity index of each sampled site.

Sampled site	**Number of isolates**	**Number of rep-PCR clusters at 70% similarity**	**Diversity index (*H*)**
Ibirubá	103	19	5.79
Cachoeira do Sul	90	22	6.17
Santa Rosa	95	20	6.04
Vacaria	92	17	3.95
Dom Pedrito	57	12	4.41

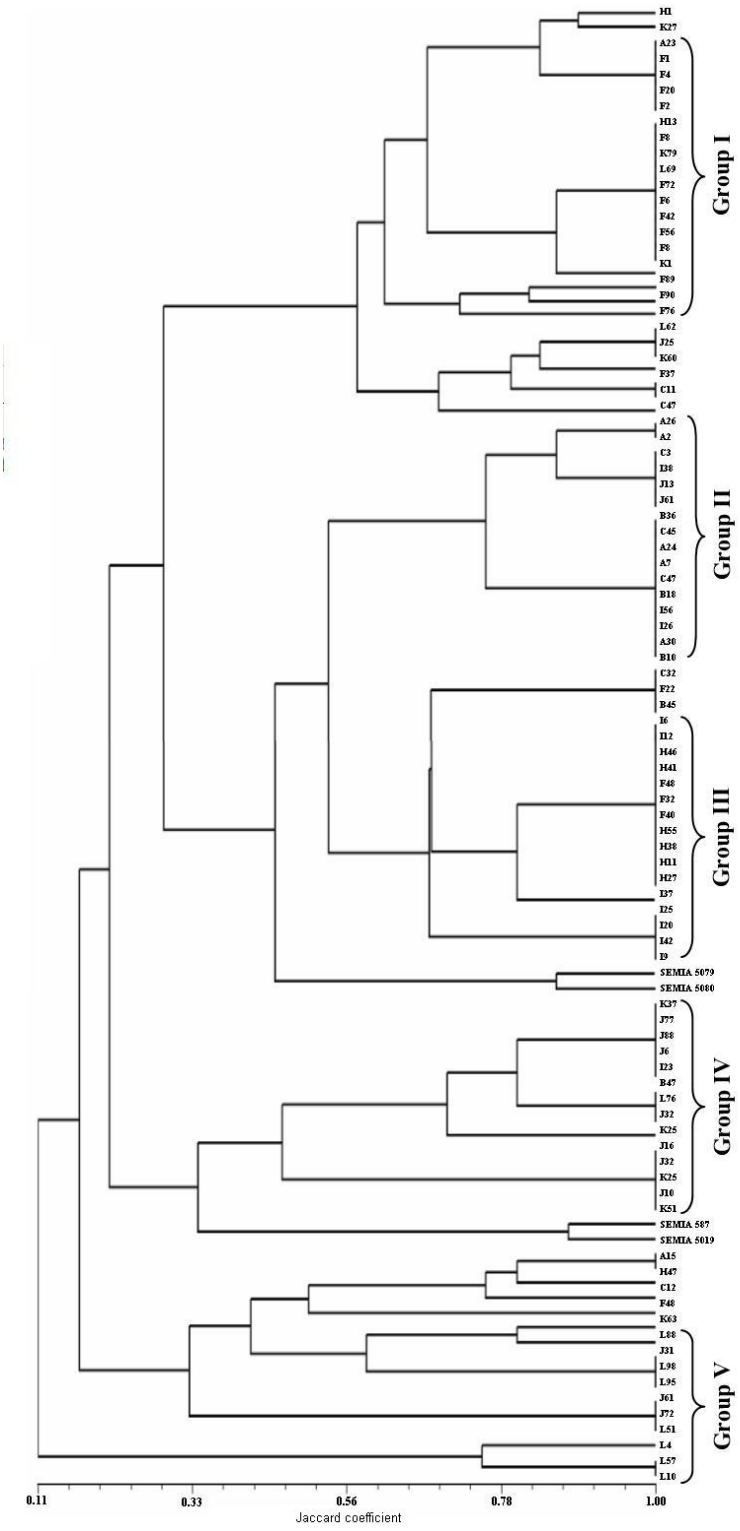

Figure 1. Dendrogram of *Bradyrhizobium* strains based on UPGMA cluster analysis with the NTSYS-PC program using the AFLP data obtained from 100 isolates. Groups I to V and their respective letters are described in Material and Methods and through the text.

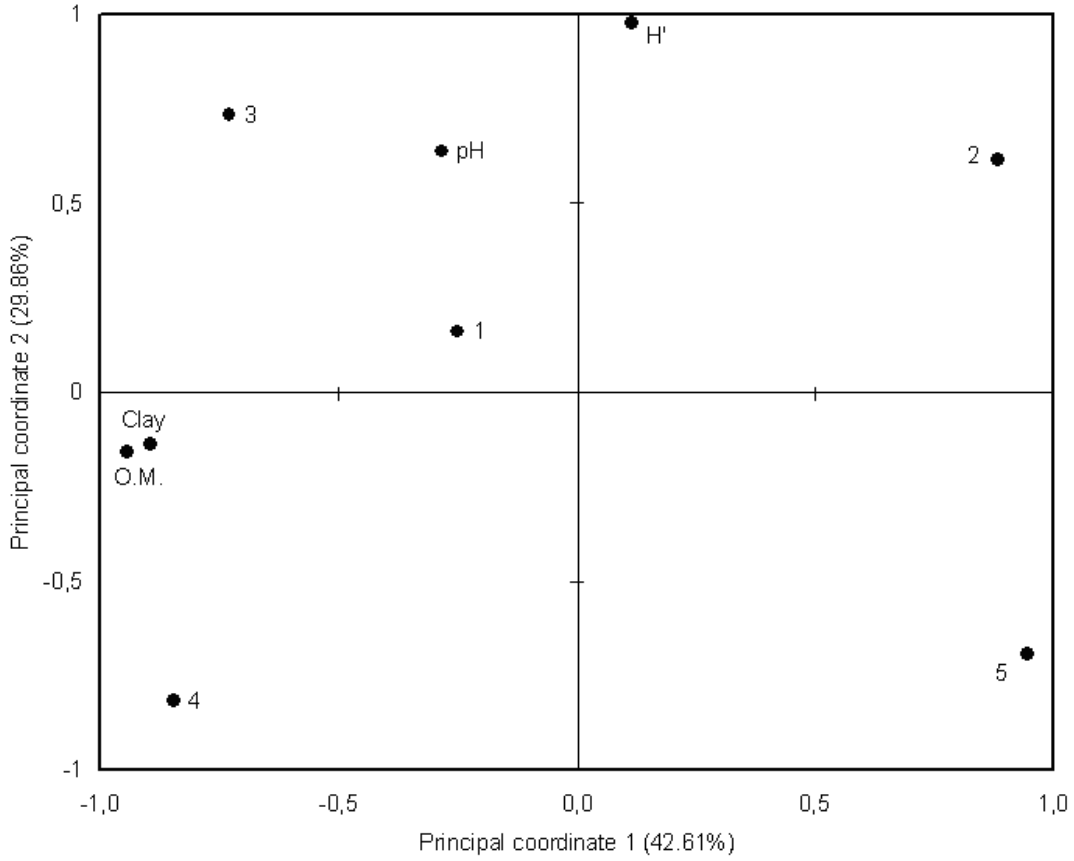

Figure 2. Principal coordinate analysis of sampled sites (1 = Ibirubá; 2 = Cachoeira do Sul; 3 = Santa Rosa; 4 = Vacaria; and 5 = Dom Pedrito) related with three different soil properties [pH, clay, organic matter (OM)] and diversity index (H').

4 Considerações finais

O nitrogênio é um elemento essencial a todos os seres vivos. Embora seja o quarto elemento mais abundante na atmosfera, a sua forma molecular (N_2) é metabolicamente indisponível para plantas e animais. Alguns microrganismos desenvolveram um mecanismo chamado de Fixação Biológica do Nitrogênio (FBN), no qual o nitrogênio atmosférico (N_2) é convertido em amônia, através de um complexo sistema enzimático. Esses microrganismos são chamados de diazotróficos e podem ser organismos de vida livre ou simbióticos. O processo mais bem conhecido de FBN consiste na simbiose de microrganismos diazotróficos, denominados coletivamente de rizóbios, com as raízes de plantas leguminosas. Primeiramente descrita por Beijerinck em 1888, a simbiose rizóbio-leguminosa tem sido extensivamente estudada até os dias de hoje, dado à importância agronômica e ambiental. Por um lado, os legumes representam uma das maiores fontes protéicas para os seres humanos e animais, por outro, a utilização de bactérias fixadoras de nitrogênio supre totalmente a demanda das plantas por esse elemento, baixando de forma significativa os custos das plantações.

O Brasil é o segundo maior produtor mundial de soja e o Estado do Rio Grande do Sul é o terceiro maior produtor de soja do Brasil. Essa alta produtividade é resultado da combinação de condições ambientais e climáticas favoráveis com a substituição da adubação química nitrogenada (de alto custo econômico e ambiental) pela utilização de inoculantes contendo bactérias do gênero *Bradyrhizobium*.

Por ser uma leguminosa exótica, não há relatos da existência de bradirrizóbios nativos, em solos brasileiros, eficientes na fixação biológica do nitrogênio na soja. A introdução de espécies bacterianas exóticas nos solos do país foi iniciada, possivelmente, com sementes contendo essas bactérias, trazidas dos Estados Unidos.

Existem, atualmente, seis espécies pertencentes ao gênero *Bradyrhizobium*, três das quais capazes de fixar nitrogênio em associação com a soja: *B. elkanii*, *B. japonicum* e *B.*

liaoningense (Willems, 2007). No Brasil foram criadas, em 1975, normas referentes à escolha, produção e comercialização de bactérias utilizadas como inoculantes comerciais para as culturas. Desde 1992, são utilizadas quatro estirpes, recomendadas pela RELARE (Rede de Laboratórios para Recomendação, Padronização e Difusão de Tecnologia de Inoculantes Microbianos de Interesse Agrícola), para o cultivo de soja no país: *B. elkanii* SEMIA 587 e SEMIA 5019 e *B. japonicum* SEMIA 5079 e SEMIA 5080.

Considerando que somente duas espécies de bradirrizóbios são utilizadas como inoculantes no Brasil, muitas vezes se faz necessária a distinção entre elas em estudos referentes à diversidade, capacidade simbiótica, análises moleculares de sistemas bioquímicos e estudos taxonômicos. Os métodos convencionais de classificação fenotípica, baseados em sorologia, foram, por muito tempo, utilizados para diferenciar as espécies pertencentes à família Rhizobiaceae (Weber et al., 1989, Leung et al., 1994, Sprent, 1997). A hibridização quantitativa DNA-DNA foi considerada a metodologia padrão para a designação de espécies pertencentes a essa família (Graham et al., 1991). Com o desenvolvimento de técnicas moleculares mais modernas, como a PCR e suas técnicas derivadas (rep-PCR e AFLP), a diferenciação entre espécies, e mesmo entre estirpes de uma mesma espécie, ficou mais precisa. Entretanto, esses métodos apresentam um custo relativamente elevado e demandam um tempo razoável para a obtenção de resultados, o que não é desejável em procedimentos rotineiros para a identificação de um grande número de isolados.

Uma vez que um dos principais objetivos desse trabalho era a avaliação da diversidade de estirpes de *Bradyrhizobium* isoladas de nódulos de soja obtidos de diferentes regiões do Estado, fez-se necessário o desenvolvimento de uma metodologia que permitisse uma rápida e precisa classificação dessas bactérias. Levando-se em consideração que o gene do rDNA de 16S é o mais utilizado para a classificação de espécies bacterianas, procuramos, através do alinhamento de diversas seqüências disponíveis no banco de dados, regiões dentro desse gene que distinguissem *B. elkanii* de *B. japonicum*. Assim, foi possível o desenho de dois pares de oligonucleotídeos iniciadores que produziram um produto de PCR esperado espécie-específico: Brady + Belk, para identificação de estirpes de *B. elkanii* e Brady + Bjap, para identificação de estirpes de *B. japonicum*. Desse modo, conseguiu-se discriminar as duas espécies de bradirrizóbios fixadores de nitrogênio em soja, através de uma metodologia rápida e de baixo custo. Essa

abordagem permitiu identificar a espécie correspondente a cada estirpe, fosse ela oriunda de coleções de cultura ou isolada de campos de soja. Os resultados obtidos nessa etapa do trabalho foram apresentados no artigo que constitui o Capítulo I.

A população de rizóbios inoculados no solo e que consegue se estabelecer no local é variável e sofre a ação de fatores abióticos, como fertilidade e propriedades físicas do solo (por exemplo, pH e argila); fatores bióticos, como a distribuição da planta hospedeira, relações sinérgicas e antagônicas com os outros representantes da biota do solo, inclusive com outros rizóbios; e efeitos climáticos, como precipitação e temperatura.

Em 1974, na Estação Experimental Agronômica da Universidade Federal do Rio Grande do Sul, em Eldorado do Sul, foi iniciado um estudo a campo sobre a sobrevivência e competitividade de sete estirpes padrão de bradirrizóbio que nodulam soja. No quinto ano de análise, apesar das estirpes permanecerem competitivas por sítios de nodulação em soja, foi observada uma alta percentagem de nódulos com ausência de reação sorológica com qualquer uma das estirpes inoculadas inicialmente (Freire et al., 1983). A área utilizada para esse estudo foi mantida sem o cultivo de leguminosas e sem posteriores inoculações com rizóbios até os dias atuais, o que corresponde há mais de 30 anos. A situação apresentada nessa área experimental despertou um grande interesse para a realização de um estudo que verificasse a variabilidade genética e a capacidade de nodulação da população de bradirizóbios capaz de sobreviver três décadas em uma área campo nativo, sem a presença da planta hospedeira. Estudos semelhantes foram realizados por outros autores (Brunel et al., 1988; Gibson et al., 1991; Galli-Terasawa et al., 2003). Entretanto, em nenhum desses trabalhos o intervalo de tempo entre a inoculação e a análise foi tão extenso quanto o relatado no artigo que constitui o Capítulo II dessa tese. Utilizando-se uma planta isca (variedade de soja IAS-5), foram obtidos 30 isolados de bradirrizóbios, que foram analisados por rep-PCR e AFLP, ferramentas muito utilizadas para a elucidação da diversidade genética em uma população (Gao et al., 2004). A população de bradirrizóbios analisada mostrou-se geneticamente muito diferente das estirpes padrão inicialmente inoculadas. Entretanto, não foram obtidas evidências de que a população atual tenha perdido a capacidade simbiótica em relação à soja. Deve-se ressaltar aqui que apenas uma pequena fração da população de bradirrizóbios foi analisada. Essa fração foi composta pelos indivíduos que não tiveram suas características simbióticas afetadas por mutações e/ou recombinação.

O Estado do Rio Grande do Sul (RS) tem um papel importante na produção nacional de soja. Como já mencionado, ele é o terceiro estado brasileiro na produção desse grão, cultivando-o em quase todas as suas regiões. Desde 1979 tem ocorrido a inoculação massiva, em todo o país e especialmente no RS, de apenas quatro estirpes de bradirrizóbio recomendadas para a cultura da soja. No entanto, têm se observado que as estirpes de bradirrizóbio são capazes de se "naturalizar", adaptando-se ao ambiente e apresentando características morfológicas, fisiológicas, genéticas e simbióticas diferentes das observadas nas estirpes originais (Ferreira e Hungria, 2002). Em vista disso, populações de bradirrizóbios de cinco regiões produtoras de soja (Ibirubá, Cachoeira do Sul, Santa Rosa, Vacaria e Dom Pedrito) foram analisadas. O objetivo principal desse trabalho foi a avaliação da variabilidade da população rizobiana local e o quanto o ambiente poderia estar influenciando nessa variabilidade, contribuindo, talvez, para uma melhor adaptação das bactérias em cada região. Em todas as populações foi evidenciada uma alta variabilidade genética, confirmando resultados obtidos por outros autores. Além disso, uma correlação importante foi demonstrada entre a variabilidade e fatores ambientais, principalmente em relação ao pH do solo. As regiões com maior índice de variabilidade (Ibirubá, Cachoeira do Sul e Dom Pedrito) também foram as que apresentaram valores mais elevados de pH.

Existem poucos estudos relatando a diversidade e/ou variabilidade genética das populações de rizóbios no sul do Brasil. Basicamente, três deles são relacionados a rizóbios de feijão (Kaschuk et al. 2006a, 2006b; Giongo et al., 2007) e um com rizóbios que nodulam uma leguminosa arbórea do gênero *Acacia* (Kayser et al., 2007). Os resultados obtidos nos trabalhos que compõem o Capítulo III dessa tese vêm se somar a estes poucos relatos, reforçando a importância de estudos populacionais, visando a busca por estirpes cada vez mais eficientes na nodulação e FBN, adaptadas às condições de cada região.

No desenvolvimento de um trabalho de tese de doutorado, muitas vezes surgem abordagens paralelas, que, embora não diretamente relacionadas ao tema central da tese, mostram-se de grande interesse. Os estudos que são apresentados como Anexos 1 e 2 foram desenvolvidos concomitantemente com os trabalhos apresentados nos Capítulos I, II e III.

Assim como a variabilidade genética das populações rizobianas, a FBN em leguminosas nativas do sul do Brasil também têm sido pouco estudada, mesmo com o crescente interesse por novas espécies que possam vir a ter alguma utilização em sistemas de manejo conservacionistas. A região sudoeste do Rio Grande do Sul apresenta 0,26% de sua área sujeita ao processo de arenização, estando esse associado à estrutura geológica e ao uso intensivo destas áreas com cultivo agrícola e pecuária. Na esperança de reverter esse quadro, várias práticas conservacionistas têm sido propostas. Entre estas, o uso de leguminosas nativas como plantas de cobertura parece ser uma boa estratégia para a recuperação de áreas degradadas, diminuindo os efeitos do impacto da gota da chuva e da erosão eólica. *Lupinus albescens* (popularmente conhecida como tremoço) é uma leguminosa nativa do Rio Grande do Sul. Essa planta apresenta várias características interessantes: alta rusticidade, adaptabilidade a solos arenosos, capacidade de redução do processo de erosão eólica, produção de matéria seca, cobertura do solo e FBN (Stroischen, 2007), que a capacitam para a utilização como planta de cobertura. Entretanto, como ocorre com as demais leguminosas nativas da região sul, muito pouco é conhecido sobre as bactérias fixadoras de nitrogênio e as promotoras de crescimento vegetal (PGPB, *Plant Growth Promoting Bacteria*) relacionadas a esta planta. Uma vez conhecida a relação entre o tremoço e os microorganismos rizosféricos, essa informação pode ser de grande importância no manejo da planta em condições de estresse ambiental. O primeiro passo para o entendimento das relações entre o tremoço nativo *Lupinus albescens* H. et Arn e as bactérias a ele associadas foi o isolamento de duas bactérias do rizoplano, que apresentavam características promotoras de crescimento vegetal. Por viverem em um ambiente com constante estresse ambiental, essas bactérias demonstraram ter características de PGPB importantes, como tolerância a pH extremos, alta salinidade e temperatura, produção de ácido indol acético e sideróforo. Os resultados obtidos nesse trabalho estão apresentados no Anexo 1. A continuidade desse trabalho contará com novas coletas de nódulos e raízes de tremoço plantado nas regiões arenosas do estado, visando ao isolamento de outras bactérias PGPB e rizóbios. Além da caracterização das bactérias isoladas, estudos de diversidade populacional de rizóbios que fixam nitrogênio em *Lupinus albescens* ajudarão a esboçar a estrutura ecológica das bactérias associadas a essa leguminosa.

Os resultados apresentados no Anexo 2 dessa tese foram obtidos a partir da oportunidade de realização de um estágio de curta duração no laboratório do Dr. Michael Gottfert, no Institut fur Genetik, Technische Universitat Dresden, em Dresden, Alemanha. A equipe liderada pelo Dr. Gottfert há anos se dedica, entre outros assuntos, a investigar os sistemas que controlam o diálogo bioquímico entre rizóbios e as plantas hospedeiras. De particular interesse é o estudo do sistema de secreção do tipo III (T3SS, *Type III Secretion System*), o qual é capaz de mediar interações com a planta hospedeira nos primeiros estágios de infecção, translocando proteínas importantes relacionadas ao processo infeccioso, tais como os fatores Nod. No laboratório do Dr. Gottfert foi possível a realização de experimentos que resultaram no isolamento de alguns genes envolvidos no sistema T3SS em *Bradyrhizobium elkanii*, demonstrando a presença do tal sistema nessa bactéria. Igualmente importante foi a obtenção de evidências de diferenças na organização genômica de *B. elkanni* e *B. japonicum*, em relação ao referido sistema. Os dados obtidos, embora não conclusivos, abriram uma nova linha de pesquisa no laboratório de Genética Molecular Vegetal, a qual é o tema de uma outra tese de doutorado.

Após um período de quatro anos de dedicação ao tema relacionado ao estudo da diversidade de bradirizóbios em solos rio-grandenses, resultados extremamente importantes foram obtidos nesse trabalho. Hoje se sabe um pouco mais sobre essas bactérias tão fundamentais ao cultivo da soja no estado. Aprendeu-se o quanto elas são diferentes, mesmo sendo originárias de poucas estirpes; o quanto elas podem se modificar de um ambiente para outro; sua capacidade de persistir nos solos, mesmo na ausência da planta hospedeira; e como os fatores ambientais influenciam na sua diversidade. Uma metodologia que permitiu uma identificação rápida e eficiente das duas espécies amplamente utilizadas nas lavouras gaúchas foi desenvolvida e adaptou-se às condições do laboratório de Genética Molecular Vegetal, metodologias clássicas para o estudo da diversidade de microrganismos, que ainda não haviam sido utilizadas pelo grupo. Também se teve a oportunidade de aplicar técnicas de biologia molecular, rotineiras e mais sofisticadas, buscando-se sempre responder, da melhor forma possível, todas as questões propostas. Além disso, como não poderia ser diferente, uma tese de doutorado terminada não é um trabalho com ponto final. Ela é o início de uma nova frente de pesquisa, o ponto de partida para novas investigações, como é o caso dos trabalhos com o sistema T3SS e com as bactérias que interagem com o tremoço. Obviamente, muitas perguntas ainda não

foram completamente respondidas e várias outras surgiram. Isso significa que o trabalho continua, sempre com o objetivo de se aprender, cada vez mais, os processos que regulam e comandam uma associação tão antiga e eficiente como essa dos rizóbios e suas leguminosas hospedeiras.

5 Referências bibliográficas

Alberton O, Kaschuk G e Hungria M (2006) Sampling effects on the assessment of genetic diversity of rhizobia associated with soybean and common bean. Soil Biol Biochem 38:1298–1307.

Andrade DS, Murphy PJ e Giller KE (2002) Effects of liming and legume/cereal cropping on populations of indigenous rhizobia in an acid Brazilian Oxisol. Soil Biol Biochem 34:477-485.

Andronov EE, Terefework Z, Roumiantseva ML, Dzyubenko NI, Onichtchouk OP, Kurchak ON, Dresler-Nurmi A, Young PW, Simarov BV e Lindström K (2003) Symbiotic and genetic diversity of *Rhizobium galegae* isolates collected from the *Galega orientalis* gene center in the Caucasus. Appl Environ Microbiol 69:1067-1074.

Arnold W, Rump A, Klipp W, Priefer UB e Pühler A (1988) Nucleotide sequence of a 24,206-base-pair DNA fragment carrying the entire nitrogen fixation gene cluster of *Klebsiella pneumoniae*. J Mol Biol 203:715–738.

Atlas Socioeconômico do Rio Grande do Sul (SCP – Atlas), http://www.scp.rs.gov.br/ATLAS/ (ultima atualização 23 de maio de 2007).

Ausmees N, Kobaiashi H, Deakin WJ, Marie C, Krishnan HB, Broughton WJ e Perret X (2004) Characterization of NopP, a type III secreted effector of *Rhizobium* sp. Strain NGR234. J Bacteriol 186:4774-4780.

Baker B, Zambryski P, Staskawicz B e Dinesh-Kumar SP (1997) Signaling in Plant-Microbe Interactions. Science 286:726-733.

Barrett CF e Parker MA (2006) Coexistence of *Burkholderia*, *Cupriavidus*, and *Rhizobium* sp nodule bacteria on two *Mimosa* spp. in Costa Rica. Appl Environ Microbiol 72:1198–1206.

Beringer JE, Brewin N, Johnston AWB, Schulman HM e Hopwood DA (1979) The *Rhizobium* legume symbiosis. Proc R Soc London Ser B 204:219-233.

van Berkum P, Beyene D, Vera FT e Keyser HH (1995) Variability among *Rhizobium* strains originating from nodules of *Vicia faba*. Appl Environ Microbiol 61:2649-2653.

Boddey LH e Hungria M (1997) Phenotypic grouping of brazilian *Bradyrhizobium* strains which nodulate soybean. Biol Fertil Soils 25:407–415.

de Bruijn FJ (1992) Use of repetitive extragenic palindromic and enterobacterial repetitive intergenic consensus sequences and the polimerase chain reaction to fingerprint the genomes of *Rhizobium meliloti* isolates and other soil bacteria. Appl Environ Microbiol 58:2180-2187.

Brunel B, Cleyet-Marel JC, Normand P e Bardin R (1988) Stability of *Bradyrhizobium japonicum* inoculants after introduction into soil. Appl Environ Microbiol 54:2636–2642.

Burns RC e Hardy RWF (1975) Nitrogen Fixation in Bacteria and Higher Plants. Springer, New York.

Campo RJ e Hungria M (2004) Economical and environmental benefits of inoculation and biological nitrogen fixation with the soybean: situation in South America. In: Proceedings VII World Soybean Research Conference, IV International Soybean Processing and Utilization Conference, III Congresso Brasileiro de Soja (Brazilian Soybean Congress), Foz do Iguassu, PR, Brazil.

Carelli M, Gnocchi S, Fancelli S, Mengoni A, Paffetti D, Scotti C e Bazzicalupo M (2000) Genetic diversity and dynamics of *Sinorhizobium meliloti* populations nodulating different alfalfa cultivars in Italian soil. Appl Environ Microbiol 66:4785-4789.

Chen LS, Figueredo A, Pedrosa FO e Hungria M (2000) Genetic characterization of soybean rhizobia in Paraguay. Appl Environ Microbiol 66:5099–5103.

Chen WM, Laevens S, Lee TM, Coenye T, Vos PD, Mergeay M e Vandamme P (2001) *Ralstonia taiwanensis* sp. nov., isolated from root nodules of *Mimosa* species and sputum of cystic fibrosis patients. Int J Syst Evol Microbiol 51:1729–1735.

Chen X, Feng J, Hou B, Li F, Li Q e Hong G (2005) Modulating DNA bending affects NodD-mediated transcriptional control in *Rhizobium leguminosarum*. Nucleic Acids Res 33:2540-2548.

Comissão de Química e Fertilidade do Solo - RS/SC (2004) Manual de adubação e calagem para os estados do Rio Grande do Sul e Santa Catarina. 10th edition. SBCS-NRS/EMBRAPA-CNPT, Porto Alegre, 400pp.

Doignon-Bourcier F, Willems A, Coopman R, Laguerre G, Gillis M e de Lajudie P (2000) Genotypic characterization of *Bradyrhizobium* strains nodulating small Senegalese legumes by 16s-23s rRNA intergenic gene spacers and amplified fragment length polymorphism fingerprint analyses. Appl Environ Microbiol 66:3987–3997.

Eardly BD, Young JPW e Selander RK (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 *nif*H genes. Appl Environ Microbiol 58:1809-1815.

FEPAGRO - Fundação Estadual de Pesquisa Agropecuária (1999) Culture Collection Catalogue. 8ed. FEPAGRO, Porto Alegre.

Ferreira MC e Hungria M (2002) Recovery of soybean inoculant strains from uncropped soils in Brazil. Field Crops Res. 79:139–152.

Finan TM (2002) Evolving insights: symbiosis islands and horizontal gene transfer. J Bacteriol 184:2855-2856.

Freire JRJ (1977) Inoculation of soybean. In: Vincent JM, Whitneye AS e Bose J (eds) Exploiting the legume-*Rhizobium* symbiosis in tropical agriculture Hawaii. University of Hawaii, Honolulu, pp 335-379.

Freire JRJ, Kolling J, Vidor C, Pereira JS, Kolling IG e Mendes NG (1983) Sobrevivência e competição por sítios de nodulação de estirpes de *Rhizobium japonicum* na cultura da soja. Rev Bras Ci Solo 7:47–53.

Gao JL, Terefework Z, Chen W e Lindstrom K (2001) Genetic diversity of rhizobia isolated from *Astragalus adsurgens* growing in different geographical regions of China. J Biotech 91:155–168.

Gao JL, Turner SL, Kan FL, Wang ET, Tan ZY, Qiu YH, Gu J, Terefework Z, Young JPW, Lindstrom K e Chen WX (2004) *Mesorhizobium septentrionale* sp. nov. and *Mesorhizobium temperatum* sp. nov., isolated from *Astragalus adsurgens* growing in the northern regions of China. Int J Syst Evol Microbiol 54:2003–2012.

Galli-Terasawa LV, Glienke-Blanco C e Hungria M (2003) Diversity of a soybean rhizobial population adapted to a Cerrados soil. World J Microbiol Biotechnol 19:933–939.

Gibson AH, Demezas DH, Gault RR, Bhuvaneswari TV e Brockwell J (1991) Genetic stability of rhizobia in the field. In: Keister DL e Cregan PB (eds) The Rhizosphere and Plant Growth. Kluwer Academic Publishers, Dordrecht, pp.141-148.

Giongo A, Passaglia LMP, Freire JRJ e Sá ELS (2007) Genetic diversity and symbiotic efficiency of population of rhizobia of *Phaseolus vulgaris* L. in Brazil. Biol Fertil Soils 43:593–598.

Göttfert M, Rothlisberger S, Kundig C, Beck C, Marty R e Hennecke H (2001) Potential symbiosis-specific genes uncovered by sequencing a 410-kilobase DNA region of the *Bradyrhizobium japonicum* chromosome. J Bacteriol 183:1405-1412.

Graham PH, Sadowsky MJ, Keyser HH, Barnet YM, Bradley RS, Cooper JE, Deley DJ, Jarvis BDW, Roslycky EB, Strijdom BW e Young JPW (1991) Proposal minimal standards for the description of new genera and species of root- and stem-nodulating bacteria. Int J Syst Bacteriol 41:582-587.

Grange L e Hungria M (2004) Genetic diversity of indigenous common bean (*Phaseolus vulgaris*) rhizobia in two Brazilian ecosystems. Soil Biol Biochem 36:1389-1398.

Gresshoff PM (2003) Post-genomic insights into nodulation. Gen Biol 4: 201.

Guimarães PM, Smith JJ, Palmano S e Saddler GS (2003) Characterisation of *Curtobacterium flaccumfaciens* pathovars by AFLP, rep-PCR and pulsed-field gel electrophoresis. Eur J Plant Pathology 109:817–825.

Hahm BK, Maldonado Y, Schreiber E, Bhuni AK e Nakatsu CH (2003) Subtyping of foodborne and environmental isolates of *Escherichia coli* by multiplex-PCR, rep-PCR, PFGE, ribotyping and AFLP. J Microbiol Meth 53:387-399.

Harrison SP, Mytton LR, Skøt L, Dye M e Cresswell A (1992) Characterization of *Rhizobium* isolates by amplification of DNA polymorphisms using random primers. Can J Microbiol 38:1009-1015.

Hueck CJ (1998) Type III protein secretion systems in bacterial pathogens of animals and plants. Microbiol Mol Biol Rev 62:379-433.

Hungria M e Vargas MAT (2000) Environmental factors affecting N_2 fixation in grain legumes in the tropics, with an emphasis on Brazil. Field Crops Res 65:151-164.

Hungria M, Campo RJ e Mendes IC (2001) Fixação biológica do nitrogênio na cultura da soja. Embrapa Soja, Londrina. 48pp.

Hungria M, Chueire LMO, Megıas M, Lamrabet Y, Probanza A, Guttierrez-Manero FJ e Campo RJ (2006) Genetic diversity of indigenous tropical fast-growing rhizobia isolated from soybean nodules. Plant Soil 288: 343–356.

Huys G, Rigouts L, Chemla K, Portaels F e Swings J (2000) Evaluation of amplified fragment length polymorphism analysis for inter- and intraspecific differentiation of *Mycobacterium bovis*, *M. tuberculosis*, and *M. ulcerans.* J Clin Microbiol 38:3675–3680.

IBGE (2007a) Levantamento Sistemático da Produção Agrícola. Comunicação Social, 09 de abril de 2007. http://www.ibge.gov.br/home/presidencia/noticias/noticia_impressao.php?id_noticia=859

IBGE (2007b) Levantamento Sistemático da Produção Agrícola. Comunicação Social, 12 de junho de 2007. http://www.ibge.gov.br/home/presidencia/noticias/noticia_impressao.php?id_noticia=905

Igual JM, Valverde A, Cervantes E e Velásquez E (2001) Phosphate-solubilizing bacteria as inoculants for agriculture: use of updated molecular techniques in their study. Agronomie 21:561–568.

Jordan DC (1982) Transfer of *Rhizobium japonicum* Buchanan 1980 to *Bradyrhizobium* gen. nov., a genus of slow-growing, root nodule bacteria from leguminous plants. Int J Syst Bacteriol 32:136-139.

Kahindi JHP, Woomer P, George T, de Souza Moreira FM, Karanja NK e Giller KE (1997) Agricultural intensification, soil biodiversity and ecosystem function in the tropics: the role of nitrogen-fixing bacteria. Appl Soil Ecol 6:55-76.

Kalita M e Malek W (2004) Phenotypic and genomic characteristics of rhizobia isolated from *Genista tinctoria* root nodules. Syst Appl Microbiol 27:707–715.

Kaneko T, Nakamura Y, Sato S, Asamizu E, Kato T, Sasamoto S, Watanabe A, Idesawa K, Ishikawa A e Kawashima K (2000) Complete genome structure of the nitrogen-fixing symbiotic bacterium *Mesorhizobium loti*. DNA Res 7:331-338

Kaschuk G, Hungria M, Andrade DS e Campo RJ (2006a) Genetic diversity of rhizobia associated with common bean (*Phaseolus vulgaris* L.) grown under no-tillage and conventional systems in Southern Brazil. Appl Soil Ecol 32:210-220.

Kaschuk G, Hungria M, Santos JCP e Berton-Junior JF (2006b) Differences in common bean rhizobial populations associated with soil tillage management in southern Brazil. Soil Tillage Res 87:205–217.

Krause A, Doerfel A e Göttfert M (2002) mutational and transcriptional analysis of the type III secretion system of *Bradyrhizobium japonicum*. Mol Plant Microbe Int 15:1228-1235.

Krishnan HB (2002) NolX of *Sinorhizobium fredii* USDA257, a type III-secreted protein involved in host range determination, is localized in the infection threads of cowpea (*Vigna unguiculata* [L.] Walp) and soybean (*Glycine max* [L.] Merr.) nodules. J Bacteriol 184: 831-839.

Kuykendall LD, Saxena B, Devine TE e Udell SE (1992) Genetic diversity in *Bradyrhizobium japonicum* Jordan (1982) and a proposal for *Bradyrhizobium elkanii* sp. nov. Can J Microbiol 38:501-505.

Kuykendall LD, Young JM, Martinez-Romero E, Kerr A e Sawada H (2005) *Rhizobium*. Bergey's Manual. Volume Two. The Proteobacteria Part C The Alpha-, Beta-, Delta-, and Epsilonproteobacteria. New York: Springer, pp 325-340.

Labes G, Ulrich A e Lentzsch P (1996) Influence of bovine slurry deposition on the structure of nodulating *Rhizobium leguminosarum* bv. *viciae* soil populations in a natural habitat. Appl Environ Microbiol 62:1717–1722.

Lafay B e Burdon JJ (1998) Molecular diversity of rhizobia occurring on native shrubby legumes in southeastern Australia. Appl Environ Microbiol 64:3989–3997.

Laguerre G, Allard MR, Revoy F e Amarger N (1994) Rapid identification of rhizobia by restriction fragment length polymorphism analysis of PCR-amplified 16S rRNA. Appl Environ Microbiol 60:56-63.

Laguerre G, van Berkum P, Amarger N e Prevost D (1997) Genetic diversity of rhizobial symbionts isolated from legume species within the general *Astragalus*, *Oxytropis* and *Onobrychis*. Appl Environ Microbiol 63:4748-4758.

Leung K, Yap K, Dashti N e Bottomley PJ (1994) Serological and ecological characteristics of a nodule-dominant serotype from an indigenous soil population of *Rhizobium leguminosarum* bv. *trifolii*. Appl Environ Microbiol 60:408-415.

Lin JJ, Kuo J e Ma J (1996) A PCR-based DNA fingerprinting technique: AFLP for molecular typing of bacteria Nucleic Acids Res 24:3649–3650.

López-García SL, Vasqués TEE, Favelukes G e Lodeiro AR (2002) Improved soybean root association of N-starved *Bradyrhizobium japonicum*. J Bacteriol 183:7241-7252.

Loureiro MF, Kaschuk G, Alberton O e Hungria M (2006) Soybean [*Glycine max* (L.) Merrill] rhizobial diversity in Brazilian oxisols of the State of Mato Grosso under various soil, cropping and inoculation managements. Biol Fert Soils 1-9.

Lucchini V (2003) AFLP: a useful tool for biodiversity conservation and management. CR Biologies 326:S43–S48.

Madrzak CJ, Golinska B, Kroliczak J, Pudelko K, Lazewska D, Lampka D e Sadowsky MJ (1995) Diversity among field populations of *Bradyrhizobium japonicum* in Poland. Appl Environ Microbiol 61:1194-1200.

Marie C, Broughton WJ e Deakin WJ (2001) *Rhizobium* type III secretion systems: legume charmers or alarmers? Curr Opin Plant Biol 4:336-342.

Menna P, Hungria M, Barcellos FG, Bangel EV, Hess PN e Martınez-Romero E (2006) Molecular phylogeny based on the 16S rRNA gene of elite rhizobial strains used in Brazilian commercial inoculants. Syst Appl Microbiol 29:315–332.

Michiels J, Dombrecht B, Vermeiren N, Xi CW, Luyten E e Vanderleyden J (1998) *Phaseolus vulgaris* is a non-selective host for nodulation. Microb Ecol 26:193-205.

Moawad H, El-Din B e Abdel-Aziz RA (1998) Improvement of biological nitrogen fixation in Egyptian winter legumes through better management of *Rhizobium*. Plant Soil 204:95-106.

Moulin L, Munive A, Dreyfus B e Boivin-Masson C (2001) Nodulation of legumes by members of the β-subclass of Proteobacteria. Nature 411:948–950.

Moulin L, Béna G, Boivin-Masson C e Stepkowski T (2004) Phylogenetic analyses of symbiotic nodulation genes support vertical gene co-transfer within the *Bradyrhizobium* genus. Mol Phylogenet Evol 30:720–732.

Mylona P, Pawlowski K e Bisseling T (1995) Symbiotic nitrogen. fixation. Plant Cell 7: 869–885.

Olive DM e Bean P (1999) Minireview: Principles and applications of methods for DNA-based typing of microbial organisms. J Clin Microbiol 37:1661-1669.

Parker MA (1999) Relationship of *Bradyrhizobium* from the legumes *Apios americana* and *Desmodium glutinosum*. Appl Environ Microbiol 65: 4914-4920.

Peres JRR e Vidor C (1980) Seleção de estirpes de *Rhizobium japonicum* e competitividade por sítios de infecção nodular em estirpes de soja. Agron Sulriograndense 16: 205-219.

Perret X, Staehelin C e Broughton WJ (2000) Molecular basis of symbiotic promiscuity. Microbiol Mol Biol Rev 64:180-201.

Qian J, Kwon SW e Parker MA (2003) rRNA and *nifD* phylogeny of *Bradyrhizobium* from sites across the Pacific Basin. FEMS Microbiol Lett 219:159–165.

Restrepo S, Vélez CM e Verdier V (2000) Measuring the genetic diversity of *Xanthomonas axonopodis* pv. *manihotis* within different fields in Colombia. Phytopatology 90:683-690.

van Rhijn P e Vanderleyden J (1995) The *Rhizobium*-plant symbiosis. Microbiol Rev 59:124–142.

Rivas R, Willems A, Subba-Rao N, Mateos P F, Dazzo F B, Martınez-Molina E, Gillis M e Velazquez E (2003) Description of *Devosia neptunia* sp. nov. that nodulates and fixes nitrogen in symbiosis with *Neptunia natans*, an aquatic legume from India. Syst Appl Microbiol 26:47–53.

Rodriguez-Navarro DN, Buendia AM, Camacho M, Lucas MM e Santamaria C (2000) Characterization of *Rhizobium* spp. bean isolates from South West Spain. Soil Biol Biochem 32:1601-1613.

Rogel MA, Hernández-Lucas I, Kuykendall LD, Balkwill DL e Martinez-Romero E (2001) Nitrogen-fixing nodules with *Ensifer adhaerens* harboring *Rhizobium tropici* symbiotic plasmids. Appl Environ Microbiol 67:3264-3268.

Sandowsky J, Kosslar M, Madrzak J, Golinsk B. e Cregan B (1995) Restriction of nodulation by *Bradyrhizobium japonicum* is mediated by factors present in the roots of *Glycine max*. Appl Environm Microbiol 61: 832-836.

Santos MA, Vargas MAT e Hungria M (1999) Characterization of soybean bradyrhizobia strains adapted to the Brazilian Cerrados Region. FEMS Microbiol Ecol 30:261–272.

Savelkoul PH, Aarts HJ, de Haas J, Dijkshoorn L, Duim B, Otsen M, Rademaker JL, Schouls L e Lenstra JA (1999) Amplified-fragment length polymorphism analysis: the state of an art. J Clin Microbiol 37:3083-3091.

Schneider M e de Bruijn FJ (1996) rep-PCR mediated genomic fingerprinting of rhizobia and computer-assisted phylogenetic patterns analysis. World J Microbiol Biotechnol 12: 163-174.

Selenska-Pobell (1995) Direct and simultaneous extraction of DNA and RNA from soil. In: Ackermans ADL, van Elsas JD e de Bruijn FD (eds) Molecular Microbial Ecology Manual. Kluwer Academic, Dordrech, pp.1-17.

Selenska-Pobell S, Evguenieva-Hackenberg E, Radeva G e Squartini A (1996) Characterization of *Rhizobium* '*hedysari*' by RFLP analysis of PCR amplified rDNA and by genomic PCR fingerprint. J Appl Bacteriol 80:517-528.

Sessitsch A, Howieson JG, Perret X, Antoun H e Martínez-Romero E (2002) Advances in *Rhizobium* Research. Crit Rev Plant Sci 21:323-378.

Sikora S, Dedzepovic S e Bradic M (2002) Genomic fingerprinting of *Bradyrhizobium japonicum* isolates by RAPD and rep-PCR. Microbiol Res 157:213-219.

Somasegaran P e Hoben JH (1994) Handbook for Rhizobia: methods in legume-*Rhizobium* technology. Springer-Verlag, New York.

Souza V e Eguiarte LE (1997) Bacteria gone native vs. Bacteria gone awry? Plasmidic transfer and bacterial evolution. Proc Nat Acad Sci USA 94:5501-5503.

Sprent JI (1995) Legume trees and shrubs in the tropics: N_2 fixation in prespective. Soil Biol Biochem 27:401–407.

Stroschein MRD (2007) Caracterização fenotípica, genética e eficiência simbiótica de bactéria fixadora de nitrogênio capaz de nodular um tremoço nativo (*Lupinus albescens* H. Et Arn) do Rio Grande do Sul. Dissertação de Mestrado, Universidade Federal de Santa Maria, Santa Maria.

Sullivan JT, Patrick HN, Lowther WL, Scott DB e Ronson CW (1995) Nodulating strains of *Rhizobium loti* arise through chormosomal symbiotic gene transfer in the environment. Proc Nat Acad Sci USA 92:8985– 8989.

Sullivan JT e Ronson CW (1998) Evolution of rhizobia by acquisition of a 500-kb symbiosis island that integrates into a phe-tRNA gene. Proc Nat Acad Sci 95:5145–5149.

Sy A, Giraud E, Jourand P, Garcia N, Willems A, de Lajudie P, Prin Y, Neyra M, Gillis M, Boivin-Masson C e Dreyfus B (2001) Methylotrophic *Methylobacterium* bacteria nodulate and fix nitrogen in symbiosis with legumes. J Bacteriol 183:214–220.

Terefework Z, Kaijalainen S e Lindstrom K (2001) AFLP fingerprinting as a tool to study the genetic diversity of *Rhizobium galegae* isolated from *Galega orientalis* and *Galega officinalis*. J Biotechnol 91:169–180.

Trinick MJ (1988) Biology of the *Parasponia-Bradyrhizobium* symbiosis. Plant Soil 110:177–185.

Trujillo ME, Willems A, Abril A, Planchuelo AM, Rivas R, Ludena D, Mateos PF, Martınez-Molina E e Velazquez E (2005) Nodulation of *Lupinus albus* by strains of *Ochrobactrum lupini* sp. nov. Appl Environ Microbiol 71:1318–1327.

Ueda T, Suga Y, Yahiro N e Matsuguchi T (1995) Phylogeny of sym plasmids of rhizobia by PCR-based sequencing of a *nod*C segment. J Bacteriol 177:468-472.

Valverde A, Velazquez E, Fernandez-Santos F, Vizcaıno N, Rivas R, Gillis M, Mateos P F, Martınez-Molina E, Igual J M e Willems A (2005) *Phyllobacterium trifolii* sp. nov. nodulating *Trifolium* and *Lupinus* in Spanish soils. Int J Syst Evol Microbiol 55:1985–1989.

Vanderleyden J e Pieternel R (1995) The *Rhizobium*-Plant symbiosis. Microbiol Rev 59:124-142.

Vargas LK, Lisboa BB, Scholles D, Silveira JRP, Jung GC, Granada CE, Neves AG, Braga MM e Negreiros TA (2007) Genetic diversity and symbiotic efficiency of black wattle nodulating rhizobia from soils from Rio Grande do Sul. Rev Bras Ci Solo 31:647-654.

Vásquez-Arroyo J, Sessitsch A, Martínez E e Peña-Cabriales JJ (1998) Nitrogen fixation and nodule occupancy by native strains of *Rhizobium* on different cultivars of common bean (*Phaseolus vulgaris* L.) Plant Soil 204:147-154.

Ventura M e Zink R (2002) Specific identification and molecular typing analysis of *Lactobacillus johnsonii* by using PCR-based methods and pulsed-field gel electrophoresis. FEMS Microbiol Lett 217:141-154.

Vinuesa P, Rademaker JLW, de Bruijn FJ e Werner D (1998) Genotypic characterization of *Bradyrhizobium* strains nodulation endemic woody legumes of the Canary Islands by PCR-RFPL of genes encoding 16S rRNA and 16S-23S rDNA intergenic spacers, REP-PCR genomic fingerprinting and partial 16S rDNA sequencing. Appl Environ Microbiol 64:2096-2104.

Viprey V, Greco AD, Golinowski W, Broughton WJ e Perret X (1998) Symbiotic implications of type III protein secretion machinery in *Rhizobium*. Mol Microbiol 28:1381-1389.

Vos P, Hogers R, Bleeker M, Reijans M, van de Lee T, Hornes M, Frijters A, Pot J, Peleman J, Kluper M e Zabeau M (1995) AFLP: A new technique for DNA fingerprinting. Nucleic Acids Res 23:4407-4414.

Watson RJ, Haitas-Crockett C, Martin T e Heys R (1995) Detection of *Rhizobium meliloti* cells in field soil and nodules by polymerase chain reaction. Can J Microbiol 41:815-825.

Weber DF, Keyser HH e Uratsu SL (1989) Serological distribution of *Bradyrhizobium japonicum* from U.S. soybean production areas. Agron J 81:786-789.

Wernegreen JJ, Harding EE e Riley MA (1997) *Rhizobium* gone native: Unexpected plasmid stability of indigenous *Rhizobium leguminosarum*. Proc Nat Acad Sci USA 94:5483-5488.

Willems A, Doignon-Bourcier F, Coopman R, Hoste B, de Lajudie P e Gillis M (2000). AFLP fingerprint analysis of *Bradyrhizobium* strains isolated from *Faidherbia albida* and *Aeschynomene* species. Syst Appl Microbiol 23:137-147.

Willems A, Coopman C e Gillis M (2001) Comparison of sequence analysis of 16S–23S rDNA spacer regions, AFLP analysis and DNA–DNA hybridizations in *Bradyrhizobium*. Int J Syst Evol Microbiol 51:623–632.

Willems A, Fernandez-Lopez M, Munoz-Adelantado E, Goris J, Vos P, Martınez-Romero E, Toro N e Gillis M (2003) Description of new *Ensifer* strains from nodules and proposal to transfer *Ensifer adhaerens* Casida 1982 to *Sinorhizobium* as *Sinorhizobium adhaerens* comb. nov. Request for an Opinion. Int J Syst Evol Microbiol 53:1207–1217.

Willems A (2007) The taxonomy of rhizobia: an overview. In: Velazquez E e Rodrıguez-Barrueco C (eds) First International Meeting on Microbial Phosphate Solubilization. Springer, pp. 3–14.

Wolde-meskel E, Terefework Z, Lindstrom K e Frostegard A (2004) Rhizobia nodulating African *Acacia* spp. and *Sesbania sesban* trees in southern Ethiopian soils are metabolically and genomically diverse. Soil Biol Biochem 36:2013–2025.

Xu LM, Ge C, Cui Z, Li J e Fan H (1995) *Bradyrhizobium liaoningense* sp. nov., isolated from the root nodules of soybean. Int J Syst Bacteriol 45:706-711.

Zilli JE, Campo RJC, Ribeiro KG, Gianluppi V, Smiderler OJ e Hungria M (2005) Utilização de inoculantes de *Bradyrhizobium* no cultivo de soja nos cerrados de Roraima. In: Fixação Biológica de Nitrogênio (FBN) na Cultura da Soja no Brasil. Boa Vista: Embrapa Roraima, pp.9.

Anexo I

Isolation and characterization of plant growth promoting bacteria from the rhizoplane of *Lupinus albescens* H. et Arn

Submetido ao periódico *Applied Soil Ecology*

Isolation and characterization of plant growth promoting bacteria from the rhizoplane of *Lupinus albescens* H. et Arn

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Abstract

Plant growth promoting bacteria (PGPB) can stimulate plant development directly by producing growth hormones and improving nutrient uptake or indirectly by changing microbial balance in the rhizosphere in favor of beneficial microorganisms. Two bacterial strains isolated from the rhizoplane of *Lupinus albescens* were tested *in vitro* according to several parameters: indole-3-acetic acid (IAA) and siderophore production, phosphate solubilization, ability to growth in different carbon sources, and salt, pH and temperature tolerances. The results showed that the two isolates are Gram-negative, rod-shaped motile and facultative anaerobic bacteria. They were very fast growers, with colonies reaching diameters of 3-4 mm within 24 h of incubation at 28°C. Besides they were also able to grow at temperature as high as 40°C, in the presence of high concentrations of NaCl (2-3% w/v), and at pH ranging from 4 to 10. Strain RP1p was able to utilize ten out of 14 carbon sources evaluated while RP2p utilized nine carbon sources. The isolates produced a large amount of IAA, ranging from 53 to 85 µg. ml^{-1} and they were also able to produced siderophore. None of the isolates was able to solubilize phosphate. Since the benefits from the biological nitrogen fixation could be synergistic with PGPR effects, further studies on the performance of these isolates on the growth of plants will uncover the real mechanism and potential of these PGPR that exhibit multiple PGP traits.

Keywords

Lupinus albescens

plant growth promoting bacteria

siderophore

indole-3-acetic acid

sandy soils

Introduction

It is widely accepted that rhizosphere and rhizoplane microorganisms can influence plant development. The term plant growth promoting rhizobacteria (PGPR) was coined for the bacterial bio-control agents of rhizosphere (Kloepper et al., 1980). Some years later, the term plant growth promoting bacteria (PGPB) was proposed to encompass rhizobacteria, which enhance plant growth by other means (Bashan and Holguin, 1998). Many bacteria including species of *Pseudomonas*, *Azospirillum*, *Azotobacter*, *Klebsiella*, *Enterobacter*, *Alcaligens*, *Arthobacter*, *Burkholderia*, *Bacillus* and *Serratia* have been reported to display PGP activities (Kloepper et al., 1989; Glick, 1995; Glick et al., 1999; Han et al., 2005; Tilak et al., 2005). Although the exact mechanisms by which PGPB promote plant growth are not totally understood (Glick, 1995; Ahmad et al., 2006), these bacteria can improve plant development by nitrogen fixation, phytohormone and siderophore production, solubilization of phosphorus and disease control (Kloepper et al., 1992; Lippmann et al., 1995; Bashan and Holguin, 1998; Barea et al., 2005). Moreover, a place surrounding plant roots is a dynamic environment being a site of nutrient turnover in soil. Bacteria which live surrounding plant roots should exhibit a wide range of metabolic activities and should be able to utilize a wide range of low molecular mass organic compounds, and some more complex compounds, as carbon and energy sources (Misko and Germida, 2002). These microorganisms play major roles in nutrient transformations and element cycling, thus affecting the availability of these nutrients for plant uptake (Dunfield and Germida, 2001).

Leguminosae family comprises about 650 genera and more than 18,000 species, from which only 8% are cultivated plants (Sprent, 1995). Little information is available on wild legumes and their root associated bacteria. These interactions have special importance

when they result in tolerance to extreme environmental conditions such as severe drought, elevated temperature and salinity (Rao and Sharma, 1995; Raza et al., 2001; Moschetti et al., 2005). The high tolerance to extreme abiotic situations is very important in some regions of Rio Grande do Sul State (RS), Brazil, where about 5.2% of the area is occupied by sandy and acid soils, which are characteristics of a subtropical arid climate (Schumaker, 2000). The genus *Lupinus* is composed by about 200 species of leguminous plants (Barrera et al., 1997), most of them originally of the Americas. *Lupinus albescens* H. et Arn, an indigenous specie (Planchuelo & Dunn, 1984), has been used in the recovery of sandy soils of RS due to its high rusticity, strong adaptability to sandy regions, ability to reduce the eolic erosion process, dry matter upgrade, soil improvement and covering, and biological nitrogen fixation when associated to rhizobia (Stroschein, 2007).

Up to now, none study has described or selected PGPB or other bacteria associated to *L. albescens* plants. Moreover, a better knowledge of these bacteria and its implications on plant physiology could change traditional crop management practices regarding plant nutrition and defense mechanisms (Richardson, 2001). In order to obtain the maximum enhancing of plant-bacteria association, effective bacteria must be selected through vegetation trials taking in consideration specific ecological conditions, like crop management, soil, temperature etc. (Egamberdiyeva et al., 2002). The primary aim of the present investigation was to isolate and characterize rhizoplane bacteria associated with native *Lupinus albescens* H. et Arn. cultivated in sandy soils of RS. These bacteria may help their host plant support the adverse ecological conditions through their PGP features.

Material and Methods

Collection site and soil samples

Small root pieces from *Lupinus albescens* H. et Arn. were collected from a field experimental area located at Alegrete, Rio Grande do Sul, Brazil, situated at 29°39'58"S and 55°23'43"W (Figure 1). The region climate is classified by Köppen as Cfa, humid subtropical without dry season, with a raining fall about 1,400 mm per year (Souto, 1984). That area has never been inoculated with commercial bacteria inoculants.

Bacteria isolation and reference strains

Bacteria from the rhizoplane of *L. albescens* were isolated in yeast-mannitol agar (YEMA) medium following standard procedures (Somasegaran and Hoben, 1994). They were purified by repeated streaking (Vincent, 1970) and pure cultures were stored at -10°C in 30% glycerol–yeast mannitol broth. Two rhizobial reference strains (*Bradyrhizobium* sp. SEMIA 928 and SEMIA 938), obtained from the Biological Nitrogen Fixation Center, Fepagro, Brazil, and three PGPB (*Enterobacter* sp., *Pseudomonas* sp. and *Klebsiella* sp.) available in the laboratory were used as control. To confirm their purity, all the isolates and control strains were streaked on YEMA medium plus 25 $mg.ml^{-1}$ Congo red dye, and glucose-peptone agar (GPA) medium plus 100 $mg.\ ml^{-1}$ bromothymol blue (Somasegaran and Hoben 1994) as a pH indicator.

Phenotypic characterization

Growth rates

Bacterial colony size was determined on YEMA after 48 hours at 28^{o}C. The strains were grown in YEMA plates and were categorized as very fast (colonies visible in less

than one day), fast (colonies visible in 2-3 days) and slow (colonies visible in 4-7 days) growers.

Carbohydrate utilization

Carbon sources evaluated included monosaccharides (L-arabinose, D-fructose, D-galactose, D-glucose, D-mannose, rhamnose, and D-xylose), disaccharides (lactose, maltose, and sucrose) and sugar alcohol (glycerol, inositol, mannitol, and sorbitol). Basal medium used for carbon assimilation was a minimum salt medium (MSM, Brown and Dillwort, 1975). Carbon compounds were sterilized by filtration (pore size 0.45 μm, Millipore) and added aseptically to the autoclaved MSM at a final concentration of 1 $g.l^{-1}$ (Amarger et al., 1997). The medium contained 2 ml of 0.5% phenol red per liter as pH indicator. About 50 μl of culture of each bacterium grown in YEM broth for 48 hours at 28^{o}C were added in one individual culture tube containing MSM and the designed carbon source and let to grow at 128 rpm at 28^{o}C. Those isolates that were able to grow in determined carbon source after 72 h modified the pH of the medium turning it from red to yellow. This behavior was recorded as growth (+) or no growth (-) in relation to the controls. All experiments were done in triplicate.

Salt, pH and temperature tolerance

Salt tolerance was evaluated using 0.5, 1, 2, 3 or 4% (w/v) NaCl in YEM broth (Odee et al., 1997). pH tolerance was evaluated using YEM broth with pH ranged between 4 and 10 (Somasegaran and Hoben, 1994). About 50 μl of culture of each isolated grown in YEM broth for 48 hours at 28^{o}C were added in individual tubes containing the different pH or NaCl concentration and let to grow at 128 rpm. It was recorded as growth (+) or no

growth (-) in relation to the controls after 72 h. Temperature tolerance of isolates was tested at 28ºC (control), 37ºC and 40°C on YEMA for two days. All tests were carried out in triplicate.

Siderophore production

Isolates were tested about their capacity for siderophore production. Two different media were used, King B (King et al., 1954) and TSA (Cattelan et al., 1999) supplemented with a complex cromoazurol S [CAS/iron(III)/hexadeciltrimetil ammonium bromide], as described by Schwyn and Neilands (1987). One single drop of bacterium culture grown in King B broth for 48 hours at 28^{o}C was deposited in those TSA plates and let for incubation for seven days at 28°C. Bacterium that was able to produce siderophore grew and formed a yellow halo in the blue-green medium. It was recorded as siderophore production (+) or no siderophore production (-) in relation to the controls.

Phosphate solubilization

The method described by Sylvester-Bradley et al. (1982) was used to identify isolates able to solubilize phosphates. Bacteria were grown in glucose-yeast broth (GYB, Sylvester-Bradley et al., 1982), containing 10 g of glucose, 2 g of yeast extract and 15 g of agar per liter. Two other solutions were prepared separately, one containing 5 g of K_2HPO_4 in 50 ml of distilled water and the other containing 10 g of $CaCl_2$ in 100 ml of distilled water. These solutions were added to one liter of GY medium just before pouring into Petri dishes, forming insoluble calcium phosphate that made the medium opaque. Bacterial isolates previously grown in YEM broth were dropped (10 µl per culture) into the GY

plates and incubated for seven days at 28°C. Those isolates that formed visible clearing halos around their colonies were considered phosphate solubilizers.

Indole-3-acetic acid production

The production of indole-3-acetic acid (IAA) by the isolates was evaluated according to Asghar et al. (2002). Briefly, bacterial strains were grown in YEM broth supplemented with 0.05 mg. ml^{-1} of tryptophan. After 48 h, bacterial cultures were centrifuged at 10,000 rpm for 5 min and 60 µl of their supernatants were placed into micro plates to react with 40 µl of Salkowski reagent (2 ml 0.5 M $FeCl_3$ + 98 ml 35% $HClO_4$) for 30 min. The mixture was left in the dark for 30 min at room temperature. The visualization of a red color in the mixture was considered a positive result (Ehmann, 1977). Samples were further analyzed in spectrophotometer at 535 nm in order to quantify the IAA production (Asghar et al., 2002).

Anaerobiosis

All the isolates were tested about their capacity of grow in an anaerobiosis chamber. The isolates were streaked in two media, YEMA and Tiamine-biotine agar (TBA, Seldin et al., 1983) and incubated in anaerobiosis jars for seven days at 28°C.

Genotyping characterization

Genotypic characterization by rep-PCR and RFLP of the *nifH* gene

Bacterial total DNA was extracted as described by Giongo et al (2007). rep-PCR reactions were performed using enterobacterial repetitive intergenic consensus primers ERIC1-R (ATGTAAGCTCCTGGGGATTCAC) and ERIC-2

(AAGTAAGTGACTGGGGTGAGCG) (de Bruijn, 1992) and enterobacterial repetitive sequences (BOX A1) primer (CTACGGCAAGGCGACGCTGACG, Versalovic et al., 1994). Reactions were performed in a PCR Express Temperature Cycling System (Thermo Hybaid) and the fragments were visualized after electrophoresis on 1% UltraPure agarose gels (Invitrogen Life Technologies). A molecular size marker (1 Kb Plus DNA ladder, Gibco BRL) was run in all gels.

One hundred ng of DNA were used as template in RFLP procedures. Selected primers PolF and PolR (TGCGAYCCSAARGCBGACTC and ATSGCCATCATYTCRCCGGA, respectively, Poly et al., 2001) were used to amplify a 360 bp region of *nifH* gene. PCR amplifications were carried out as described by Soares et al. (2006). Ten μl of each PCR product was directly used for restriction enzyme cleavage. The reaction enzyme mixture contained 1 X restriction enzyme buffer and 1.25 U of restriction endonuclease. *Taq*I and *Hae*III were selected for their specificity for the amplified region of *nifH*. The PCR products were digested overnight. Digested DNA samples were analyzed by electrophoresis in a 30% polyacrylamide gel (Sambrook and Russel, 2001). The electrophoresis conditions were 5 h at 150 V in 1 X Tris-borate-EDTA buffer (TBE) at room temperature, followed by 30 min of silver nitrate staining (Sambrook and Russel, 2001). All fingerprint patterns were converted into a two-dimensional binary matrix (1, presence of a band; 0, absence of a band) and analyzed using Jaccard (*J*) coefficient, which do not consider the negative similarities. The matrix was analyzed by NTSYS-PC package. UPGMA (Unweighted Pair Group Method with Arithmetic mean) algorithm was used to perform hierarchical cluster analysis and construct a dendrogram.

Partial sequencing of the 16S rRNA gene

PCR fragments obtained by the amplification of a 434 bp DNA fragment corresponding to 16S rDNA gene region of each isolate were sequenced. This region was amplified using primers U968 (AACGCGAAGAACCTTAC) and L1401 (CGGTGTGTACAAGACCC) (Felske et al. 1997). PCR reactions were performed in a 25 µl volume, containing 50 ng of DNA template, 1 U of *Taq* DNA polymerase (Life Technologies), 1 X *Taq* DNA polymerase reaction buffer, 1.5 mM $MgCl_2$, 200 mM dNTPs (Life Technologies) and 10 pmoles of each primer. Amplification was carried out for 32 cycles as follows: one initial denaturation cycle at 95ºC for 5 min, 30 denaturation cycles for 45 s at 94°C, annealing for 45 s at 52°C, and extension for 45 s at 72°C, and one final extension cycle for 5 min at 72ºC. Reactions were performed in a PCR Express Temperature Cycling System (Thermo Hybaid) and the fragments were visualized after electrophoresis at 80 V for 1 h on a 1.5% agarose gel stained with ethidium bromide. A molecular size marker (1 Kb plus DNA ladder, Gibco BRL) was run in all gels. Sequences of partial 16S rDNA genes were determined in both the forward and reverse directions with sequencing primers M13F and M13R in the ACTGene Laboratory (Centro de Biotecnologia, UFRGS, RS, Brazil) using the automatic sequencer ABI-PRISM 3100 Genetic Analyzer (Applied Biosystems). Sequences were submitted to the GenBank database, using the program BLASTN (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/BLAST/). The nucleotide sequences of the two partial 16S rDNA segments determined in this study have been deposited in GenBank database under accession numbers EF585401 (for Rp2p isolate) and EF585402 (for Rp1p isolate).

Results and Discussion

Aiming to identify some of the PGPB strains that habit the rhizoplane of *L. albescens* small root pieces of plants cultivated in a sandy area of an experimental field in Alegrete, Rio Grande do Sul, Brazil, were collected. Five bacterial strains were selected among all bacteria isolated. Genotypic characterization of these strains was assessed by electrophoretic profiles of amplification products using rep-PCR and RFLP methodologies. The RFLP analysis was based in a fragment of the *nif*H gene, which is one of the most important genes in the biological nitrogen fixation system and encodes the nitrogenase reductase protein (Passaglia et al., 1991). Although the existence of *nif*H gene does not always mean nitrogenase activity, as this enzyme is regulated at both pre- and postranslational levels (Dean and Jacobson, 1992) its presence in the genome is a good evidence that the bacterium is a nitrogen-fixer (Soares et al., 2006). The genotypic results obtained with the isolates (RP1p, RP2p, RP2g, RP3p and RP3g) and the reference strains (*Bradyrhizobium* sp. SEMIA 928 and SEMIA 938, *Klebsiella* sp., *Enterobacter* sp., *Pseudomonas* sp.) were used to construct a dendrogram (Figure 2). The dendrogram obtained indicated that in fact there were only two different isolates, RP1p and RP2p, and that both contained a *nif*H gene. These bacteria presented about 30% of similarity with the reference strains SEMIA 928 and 938, which belong to the *Bradyrhizobium* genus, and differ about 30% of each other (Figure 2). For this reason, all the following experiments were carried out with these two strains together with the reference strains.

According to the sequence analysis of the 16S rDNA fragments RP1p strain showed genetic similarity with *Enterobacter* sp. (92% homology) and RP2p strain was identified as *Serratia* sp. (94% homology) (data not shown). These results indicated that strains RP1p and RP2p belong to the Enterobacteriaceae family that contains several

nitrogen-fixing organisms. Although rhizosphere bacteria are largely represented by species of *Pseudomonas* and *Bacillus* genera, some authors have found bacteria belonging to the Enterobacteriaceae family including *Serratia*, *Pantoea* and *Enterobacter* strains in the rhizoplane of leguminous plants (Alström, 2001; Bent, 2006).

The capacity to utilize different carbon sources can permit a greater insight into the ecology and metabolism of microbial species in rhizosphere (Barboza et al., 2000). Carbon substrate utilization by strains RP1p and RP2p are listed in Table 1. Strain RP1p was able to utilize ten out of 14 carbon sources evaluated while RP2p utilized nine carbon sources. Both strains were unable to utilize neither L-arabinose nor lactose as sole carbon source. Although glucose is the preferred carbon source for diazotrophic strains of Enterobacteriaceae, bacteria from this family vary a lot in respect to carbon sources utilization (Cannon et al., 1974). *Serratia marcercens* (Greenup and Blazevic, 1971) *Enterobacter agglomerans* and *Klebsiella pneumoniae* (Haahtela et al., 1983) were not able to grow when lactose was the sole carbon source. Strains belonging to *Pseudomonadas*, one of the most famous PGPBs genera, are especially well suited to the rhizosphere because they are able to use a wide variety of carbon sources as nutrients (Misko and Germida, 2002). PGPBs that exhibit a wide range of metabolic activities and are able to utilize a wide range of carbon and energy sources can be well suited in the rhizosphere (Dunfield and Germida, 2001; Misko and Germida, 2002). This parameter can be useful not only to observe if isolates can utilize determinate carbon source but whether the strains were metabolically similar. Understanding the metabolic profile of the strains could help to disclose how bacteria can compete and survive in the root environment.

Based in the experiments performed for phenotypic characterization, the two isolates correspond to Gram-negative, rod-shaped motile and facultative anaerobic

bacteria. Under optical microscopy cells occur often in pairs when grown on YEMA (data not shown). They were very fast growers forming convex, white, single, circular and mucoid colonies, reaching diameters of 3-4 mm within 24 h of incubation at 28°C on YEMA.

The sensitivity of the isolates to extreme pH, high salt concentration and high temperatures was also analyzed (Table 1). While 28°C is the optimum temperature for the growth of many strains, the two isolates were able to grow at temperature as high as 40°C. Although *in vitro* temperature selection is not considered a promising approach for fields appliance (Hungria and Vargas, 2000) this high temperature tolerance can be useful when we intent to select competitive PGPR in oscillatory temperature fields. Both isolates were also able to grow in the presence of high NaCl (2-3% w/v) concentrations. This result indicates that these bacteria could be potential candidates for applications in soils with elevate saline influence.

Extremes of pH can be one of the major factor-limiting the presence of microorganisms in soils (Brockwell et al., 1991). Some microorganisms have been reported to grow in pH ranging from 3.5 to 12 (Hung et al., 2005). RP1p and RP2p strains were able to grow in pH ranging from 4 to 10. This result indicates that these isolates could also be strong candidates for improving highly alkaline or acidic soil, as is the Alegrete soil, which pH ranges from 4.5 to 5.0 (Eltz and Rovedder, 2005). The two bacterial isolates were also capable to produce a huge amount of mucous and exopolysaccharides. These compounds probably protect them against desiccation and help them to grow on extreme temperatures, salinity and pH conditions (Hung et al., 2005).

Various soil microorganisms live on plant rhizosphere and rhizoplane due to rich nutrient availability (Glick, 2003; Han et al., 2005). Although bacteria like *Rhizobium,*

Azotobacter and *Azospirillum* enhance plant growth as a result of their ability to fix nitrogen, bacteria with mechanisms such as production of phytohormones in the rhizosphere and other PGP activities can contribute to improve the ability of the host plant live in extreme environments (Glick, 1995; Patten and Glick, 1996; Bashan and Holguin, 1997; Asghar et al., 2002). Some studies showed that isolates from the rhizosphere are more efficient auxin producers than isolates from soils not associated with plant roots (Sarwar and Kremer, 1995). RP1p and RP2p strains produced 85 and 53 μg of IAA. ml^{-1} *in vitro*, respectively, in the presence of very low amounts of tryptophan. It has been reported that PGPR vary greatly in their intrinsic ability to produce IAA. The IAA production can vary among different species and strains and is also influenced by culture condition, growth stage and availability of the substrate (Mirza et al., 2001). Barazani and Friedman (1999) reported PGPR that were able to secrete until 13.5 μg of IAA. ml^{-1}. However, Ahmad et al. (2006) showed that *in vitro* IAA production of some *Azotobacter* sp. and *Pseudomonas* sp. strains, in the presence of 5 mg. ml^{-1} of tryptophan, varied from 32.8 to 53.2 μg of IAA. ml^{-1}. Strains of *Enterobacter* sp. isolated from the rhizosphere of sugarcane produced *in vitro* about 2.21 μg of IAA. ml^{-1} when tryptophan was added in the medium (Mirza et al., 2001). The comparison with the above results allowed us to conclude that the two PGPB isolated in the present study are very efficient IAA producers. Further studies will be needed to explore the exact contribution of IAA production in the promotion of *Lupinus albescens* growth.

Siderophore production is another PGP feature that may influence the plant growth by binding to the available form of iron (Fe^{3+}) in the rhizosphere. Through this process, iron is made unavailable to the phytopathogens. At the same time, the siderophore protects the plant health (Siddiqui, 2005). As observed with IAA, both isolates produced a large

amount of siderophore on medium provided with CAS. Press et al. (2001) reported that the *Serratia marcescens* catechol siderophore biosynthesis gene is associated with resistance of cucumber against anthracnose. Among all PGPB features tested in this work, phosphate solubilization was the only one not present in the two strains isolated. However this characteristic was absent in the reference strains as well (Table 1).

Usually a search for PGPR involves screening of a large number of isolates and the identification of some desired phenotypic traits. Once isolates are purified, the main goal is to keep the maximum genetic diversity in the minimum number of isolates for further biological assays (Barriuso et al., 2005; Solano et al., 2006). In the present study we decided to work with only two isolates aiming to obtain an initial screening of bacteria that can exert positive effects on plants by various mechanisms that stimulate metabolic activities in the roots and supply the plant with biologically fixed-nitrogen. Since the benefits from the biological nitrogen fixation could be synergistic with PGPR effects (Cocking, 2003; Ahmad et al., 2006), further studies on the performance of RP1p and RP2p isolates on the growth of plants will uncover the real mechanism and potential of these PGPR that exhibit multiple PGP traits.

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References

Ahmad, F., Ahmad, I., Khan, M.S., 2006. Screening of free-living rhizospheric bacteria for their multiple plant growth promoting activities. doi:10.1016/j.micres.2006.04.001

Alström, S., 2001. Characteristics of bacteria from oilseed rape in relation to their biocontrol activity against *Verticillium dahliae*. J. Phytopath. 149, 57-64.

Amarger, N., Macheret, V., Laguerre, G., 1997. *Rhizobium gallicum* sp. nov. and *Rhizobium giardinii* sp. nov. from *Phaseolus vulgaris* nodules. Int. J. Syst. Bacteriol. 47, 996–1006.

Asghar, H.N., Zahir, Z.A., Arshad, M., Khaliq, A., 2002. Relationship between *in vitro* production of auxins by rhizobacteria and their growth-promoting activities in *Brassica juncea* L. Biol. Fertil. Soils 35, 231–237.

Barazani, O.Z., Friedman, J., 1999. Is IAA the major root growth factor secreted from plant-growth-mediating bacteria? J. Chem. Ecol. 25, 2397-2406.

Barboza, F., Correa, N.S., Rosas, S.B., 2000. Metabolic and physiological characteristics of salt-tolerant strains of *Bradyrhizobium* spp. Biol. Fertil. Soils 32, 368–373.

Barea, J.M., Pozo, M.J., Azcon, R., Azcon-Aguilar, C., 2005. Microbial co-operation in the rhizosphere. J. Experim. Bot. 56, 1761–1778.

Barrera, L.L., Trujillo, M.E., Goodfellow, M., Garcia, F.J., Hernandez-Lucas, I., Davila, G., Berkum, P., Martinez-Romero, E., 1997. Biodiversity of bradyrhizobia nodulating *Lupinus* spp. Intern. J. System. Bacteriol. 47, 1086–1091.

Barriuso, J., Pereyra, M.T., Lucas-Garcıa, J.A., Megıas, M., Gutierrez–Manero, F.J., Ramos, B., 2005. Screening for putative PGPR to improve establishment of the symbiosis *Lactarius deliciosus* - *Pinus* sp. Microbiol. Ecol. 50, 82–89.

Bashan, Y., Holguin, G., 1997. *Azospirillum*-plant relationships: environmental and physiological advances (1990–1996). Can. J. Microbiol. 43, 103–121.

Bashan, Y., Holguin, G., 1998. Proposal for the division of plant growth-promoting rhizobacteria into two classifications: biocontrol-PGPB (plant growth-promoting bacteria) and PGPB. Soil Biol. Biochem. 30, 1225–1228.

Bent, E., 2006. Induced systemic resistance mediated by plant growth-promoting rhizobacteria (PGPR) and fungi (PGPF). In: Tuzun, S., Bent, E. (Eds.), Multigenic and induced systemic resistance in plants. Springer, New York, pp. 225-258.

Brockwell, J., Pilka, A., Holliday, R.A., 1991. Soil pH is a major determinant of the numbers of naturally occurring *Rhizobium meliloti* in non-cultivated soils of New South Wales. Aust. J. Exp. Agric. 31, 211–219.

Brown, C.M., Dilworth, M.J., 1975. Ammonia assimilation by *Rhizobium* cultures and bacteroids. J. Gen. Microbiol. 86, 39-48.

de Bruijn, F.J., 1992. Use of repetitive extragenic palindromic and enterobacterial repetitive intergenic consensus sequences and the polymerase chain reaction to fingerprint the genomes of *Rhizobium meliloti* isolates and other soil bacteria. Appl. Environ. Microbiol. 58, 2180-2187.

Cannon, F.C., Dixon, R.A., Postgate, J.R., Primrose, S.B., 1974. Chromosomal integration of *Klebsiella* nitrogen fixation genes in *Escherichia coli*. J. Gen. Microbiol. 80, 227–239.

Cattelan, A.J., Hartel, P.G., Fuhrmann, J. J., 1999. Screening for plant growth-promoting rhizobacteria to promote early soybean growth. Soil Sci. Soc. Amer. J. 63, 1670-1680.

Cocking, E.C., 2003. Endophytic colonization of plant roots by nitrogen-fixing bacteria. Plant Soil 252, 169–175.

Dean, D.R., Jacobson, M.R., 1992. Biochemical genetics of nitrogenase. In: Stacy, G., Burris, R. H., Evans, H. J. (Eds.), Biological Nitrogen Fixation. Chapman and Hall, New York, pp. 763-834.

Dunfield, K.E., Germida, J.J., 2001. Diversity of bacterial communities in the rhizosphere and root interior of field-grown genetically modified *Brassica napus*. Microbiol. Ecol. 38, 1-9.

Egamberdiyeva, D., Juraeva, D., Gafurova, L., Höflich, G., 2002. Promotion of plant growth of maize by plant growth promoting bacteria in different temperatures and soils. In: van Santen, E. (Ed.), Making conservation tillage conventional: building a future on 25 years of research. Proc. of 25th Annual Southern Conservation Tillage Conference for Sustainable Agriculture. Auburn, pp. 239-244.

Ehmann, A., 1977. The van urk-Salkowski reagent — a sensitive and specific chromogenic reagent for silica gel thin-layer chromatographic detection and identification of indole derivatives. J. Chromatogr. 132, 267–276.

Eltz, F.L.F., Rovedder, A.P.M., 2005. Revegetação e temperatura do solo em áreas degradadas no sudoeste do Rio Grande do Sul. Revista Brasileira de Agrociência 11, 193-200.

Felske, A., Rheims, H., Wolterink, A., 1997. Ribosome analysis reveals prominent activity of an uncultured member of the class Actinobacteria in grasslands soils. Microbiology 143, 2983-2989.

Greenup, P., Blazevic, D.J., 1971. Antibiotic susceptibilities of *Serratia marcescens* and *Enterobacter liquefaciens*. Appl. Microbiol. 22, 309-314.

Giongo, A., Passaglia, L.M.P., Freire, J.R.J., Sá, E.L.S., 2007. Genetic diversity and symbiotic efficiency of population of rhizobia of *Phaseolus vulgaris* L. in Brazil. Biol Fertil Soils, in press.

Glick, B.R., 1995. The enhancement of plant growth by free living bacteria. Can. J. Microbiol. 41, 109–114.

Glick, B.R., Patten, C.L., Holguin, G., Penrose, D.M., 1999. Biochemical and genetic mechanisms used by plant growth-promoting bacteria. London, Imperial College Press, 267 pp.

Glick, B.R., 2003. Phytoremediation: synergistic use of plants and bacteria to clean up the environment. Biotechnol. Adv. 21, 383–393.

Haahtela, K., Kari, K., Sundman, V., 1983. Nitrogenase activity (acetylene reduction) of root-associated, cold-climate *Azospirillum*, *Enterobacter*, *Klebsiella*, and *Pseudomonas* species during growth on various carbon sources and at various partial pressures of oxygen. Appl. Environ. Microbiol. 45, 563-570.

Han, J., Sun, L., Dong, X., Cai, Z., Sun, X., Yang, H., Wang, Y., Song, W., 2005. Characterization of a novel plant growth-promoting bacteria strain *Delftia tsuruhatensis* HR4 both as a diazotroph and a potential biocontrol agent against various plant pathogens. Syst. Appl. Microbiol. 28, 66–76.

Hung, M.H., Bhagwath, A.A., Shen, F.T., Devasya, R.P., Young, C.C., 2005. Indigenous rhizobia associated with native shrubby legumes in Taiwan. Pedobiol. 49, 577-584.

Hungria, M., Vargas, M.A.T., 2000. Environmental factors affecting N_2 fixation in grain legumes in the tropics, with an emphasis on Brazil. Field Crops Res. 65, 151-164.

King, E.O., Ward, M.K., Raney, D.E., 1954. Two simple media for the demonstration of pyocyanin and fluorescin. J. Lab. Clin. Med. 44, 301-307.

Kloepper, J.W., Leong, J., Teintze, M., Scrhorth, M.N., 1980. Enhanced plant growth by siderophores produced by plant growth promoting rhizobacteria. Nature 286, 885–886.

Kloepper, J.W., Lifshitz, R., Zablotowicz, R.M., 1989. Free-living bacterial inocula for enhancing crop productivity. Trends Biotechnol. 7, 39–43.

Kloepper, J.K., Schippers, B., Bakker, P.A.H.M., 1992. Proposed elimination of the term endorhizosphere. Phytopathol. 82, 726–727.

Lippmann, B., Leinhos, V., Bergmann, H., 1995. Influence of auxin producing rhizobacteria on root morphology and nutrient accumulation of crops. I. Changes in root morphology and nutrient accumulation in maize (*Zea mays* L.) caused by inoculation with indole-3-acetic acid (IAA) producing *Pseudomonas* and *Acinetobacter* strains or IAA applied exogenously. Angew Bot. 69, 31–36.

Mirza, M.S., Ahmad, W., Latif, F., Haurat, J., Bally, R., Normand P., Malik, K.A., 2001. Isolation, partial characterization, and the effect of plant growth-promoting bacteria (PGPB) on micro-propagated sugarcane in vitro. Plant Soil 237, 47–54.

Misko, A.L., Germida, J.J., 2002. Taxonomic and functional diversity of pseudomonads isolated from the roots of field-grown canola. Microbiol. Ecol. 42, 399-407.

Moschetti, G., Peluso, A.L., Protopapa, A., Anastasio, M., Pepe, O., Defez, R., 2005. Use of nodulation pattern, stress tolerance, *nod*C gene amplification, RAPD-PCR and RFLP–16S rDNA analysis to discriminate genotypes of *Rhizobium leguminosarum* biovar *viciae*. Syst. Appl. Microbiol. 28, 619–631.

Odee, D.W., Sutherland, J.M., Makatiani, E.T., McInroy S.G., Sprent, J.I., 1997. Phenotypic characteristics and composition of rhizobia associated with woody legumes growing in diverse Kenyan conditions. Plant Soil 188, 65–75.

Passaglia, L.M.P., Nunes, C.P., Zaha, A., Schrank, I.S., 1991. The *nif*HDK operon in the free-living nitrogen-fixing bacteria *Azospirillum brasilense* sequentially comprises genes H,D,K, a 353-bp ORF and gene Y. Braz. J. Med. Biol. Res. 24, 649-675.

Patten, C.L., Glick, B.R., 1996. Bacterial biosynthesis of indole-3- acetic acid. Can. J. Microbiol. 42, 207–220.

Planchuelo, A.M., Dunn, D., 1984. The simple leaved lupines and their relatives in Argentina. Ann. Missouri Bot. Gard. 71, 90-103.

Poly, F., Monrozier, L.J., Bally, R., 2001. Improvement in RFLP procedure to study the community of nitrogen fixers in soil through the diversity of *nif*H gene. Res. Microbiol. 152, 95-103.

Press, C.M., Loper, J.E., Kloepper, J.W., 2001. Role of iron in rhizobacteria-mediated induced systemic resistance of cucumber. Phytopath. 91, 593-598.

Rao, D.C.N., Sharma, P.C., 1995. Effectiveness of rhizobial strains for chickpea under salinity stress and recovery of nodulation on desalinization. Indian J. Exper. Biol. 33, 500-504.

Raza, S., Jornsgard, B., Abou-Taleb, H., Christiansen, J.L., 2001. Tolerance of *Bradyrhizobium* sp. (*Lupinus*) strains to salinity, pH, $CaCO_3$ and antibiotics. Lett. Appl. Microbiol. 32, 379-383.

Richardson, A.E., 2001. Prospects for using soil microorganism to improve the acquisition of phosphorus by plants. Aust. J. Plant Physiol. 28, 897–906.

Sambrook, J., Russel, D.W., 2001. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, New York.

Sarwar, M., Kremer, R.J., 1995. Determination of bacterially derived auxin using microplate method. Lett. Appl. Microbiol. 20, 282-285.

Schumacher, V.M., 2000. “Deserto de Alegrete” - Florestas, uma alternativa de controle? Sociedade Brasileira de Recuperação de Áreas Degradadas. Universidade Federal de Viçosa, Viçosa, pp. 21-23.

Schwyn, B., Neilands, J.B., 1987. Universal chemical assay for the detection and determination of siderophores. Anal. Biochem. 160, 47-56.

Seldin, L., van Elsas, J.D., Penido, E.G.C., 1983. *Bacillus* nitrogens fixers from Brazilian soils. Plant Soil 70, 243-255.

Siddiqui, Z.A., 2005. PGPR: Prospective biocontrol agents of plant pathogens. In: PGPR: Biocontrol and Biofertilization. Springer, Netherlands, pp. 111-142.

Soares, R.A., Roesch, L.F., Zanatta, G., Camargo, F.A.O., Passaglia, L.M.P., 2006. Occurrence and distribution of nitrogen fixing bacterial community associated with oat (*Avena sativa*) assessed by molecular and microbiological techniques. Appl. Soil Ecol. 33, 221-234.

Solano, R.B., la Iglesia, M.T.P., Probanza, A., Garcia, J.A.L., 2006. Screening for PGPR to improve growth of *Cistus ladanifer* seedlings for reforestation of degraded mediterranean ecosystems. Plant Soil 287, 59-68.

Somasegaran, P., Hoben, J.H., 1994. Handbook for Rhizobia: methods in legume-*Rhizobium* technology. Springer-Verlag, New York, 450 pp.

Souto, J.J., 1984. Deserto, uma ameaça? Secretaria da Agricultura, Porto Alegre, 169 pp.

Sprent, J.I., 1995. Legume trees and shrubs in the tropics: N_2 fixation in prespective. Soil Biol. Biochem. 27, 401–407.

Stroschein, M.R.D., 2007. Caracterização fenotípica, genética e eficiência simbiótica de bactéria fixadora de nitrogênio capaz de nodular um tremoço nativo (*Lupinus Albescens* H.

Et Arn) do Rio Grande do Sul. Master of Science Dissertation, Universidade Federal de Santa Maria, Santa Maria.

Sylvester-Bradley, R., Asakawa, N., la Torraca, S., Magalhães, F.M.M., Oliveira, L., Pereira, R.M., 1982. Levantamento quantitativo de microrganismos solubilizadores de fosfatos na rizosfera de gramíneas e leguminosas forrageiras na Amazônia. Acta Amazônica 12, 15-22.

Tilak, K.V.B.R., Ranganayaki, N., Pal, K.K., De, R., Saxena, A.K., Nautiyal, C.S., Mittal, S., Tripathi, A.K., Johri, B.N., 2005. Diversity of plant growth and soil health supporting bacteria. Curr. Sci. 89, 1-10.

Versalovic, J., Schneider, M., Bruijn, F.J.D., Lupski, J.R., 1994. Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. Meth. Mol. Cel. Biol. 5, 25-40.

Vincent, J.M., 1970. A manual for the practical study of root nodule bacteria. Blackwell, Oxford.

Figure 1. Geographical localization of sandy region of Rio Grande do Sul, Brazil. The name of sampling site of this study, Alegrete, is highlight on the map.

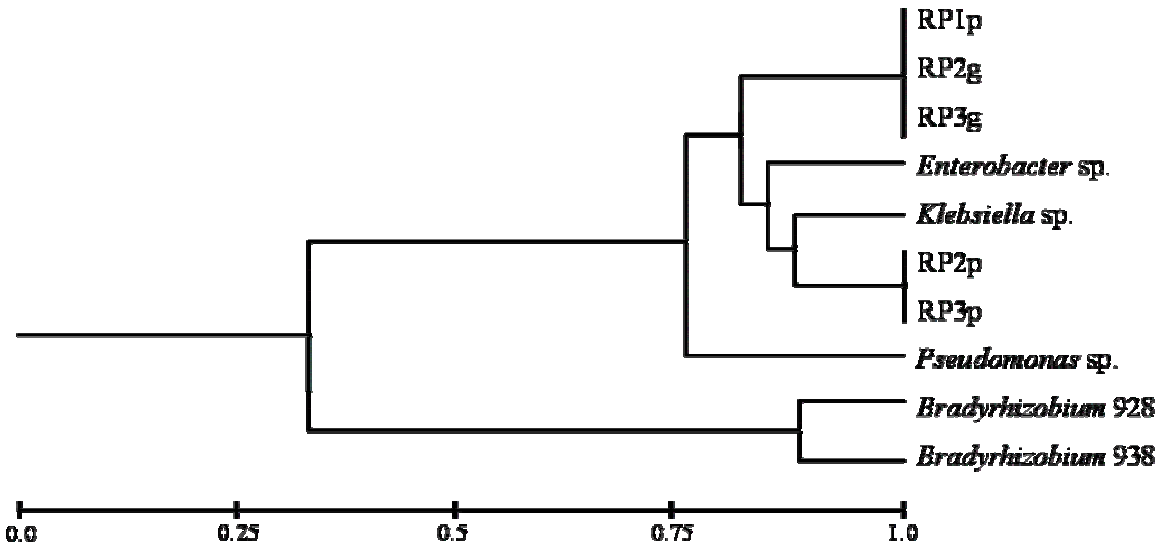

Figure 2. Dendrogram built with the UPGMA method and the Jaccard coefficient based on rep-PCR and RFLP products obtained of *L. albescens* isolates and references strains.

Table 1. Morphological, physiological and biochemical characteristics of the isolates from *L. albescens* rhizosphere and two reference strains.

Characteristics		Isolates from *L. albescens*		Rhizobial reference strains	
		RP1p	RP2p	SEMIA928	SEMIA938
Gram staining		-	-	-	-
Congo red utilization		-	-	-	-
Monosaccharides	L-arabinose	-	-	nd	nd
	D-fructose	+	+	+	+
	D-galactose	+	+	+	+
	D-glucose	+	+	-	-
	D-mannose	+	+	nd	nd
	Rhamnose	+	-	nd	nd
	D-xylose	+	-	nd	nd
Disaccharides	Lactose	-	-	-	-
	Maltose	+	-	+	+
	Sucrose	+	+	-	-
Sugar alchool	Glycerol	+	+	-	-
	Inositol	-	+	+	+
	Mannitol	+	+	+	+
	Sorbitol	-	+	+	+
0.5% NaCl		+	+	+	+
1% NaCl		+	+	+	+
2% NaCl		+	+	-	-
3% NaCl		+	+	-	-
4% NaCl		-	-	-	-
Siderophore production		+	+	-	-
IAA production		+	+	-	-
Phosphate solubilization		-	-	-	-
Anaerobiosis		+	+	-	-
Growth at 40ºC		+	+	-	-
Growth at pH 4		+	+	-	-
Growth at pH10		+	+	-	-
Growth rate		very fast	very fast	slow	slow

nd = not determined

Anexo II

Identification and analysis of genes and proteins related to type III secretion system in *Bradyrhizobium japonicum* and *B. elkanii* Brazilian strains

A ser submetido ao periódico *Applied Microbiology and Biotechnology*

Identification and analysis of genes and proteins related to type III secretion system in *Bradyrhizobium japonicum* and *B. elkanii* Brazilian strains

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Keywords: type three secretion system, *rhc* genes, T3SS, *Bradyrhizobium elkanii*, nodulation

Abstract

Soybean (*Glycine max* (L.) Merrill) is one of the most important and oldest cultivated crops. It nodulates and fixes nitrogen in symbiosis with Gram-negative soil bacteria *Bradyrhizobium japonicum, B. elkanii* and *B. liaoningense*. Many bacteria including rhizobial strains have developed a secretion system, termed type III secretion system (T3SS), which mediates the interaction with the host, including the entrance of these bacteria into the eukaryotic cells. Although the T3SS of *B. japonicum* strain USDA110 has been described, few studies have shown the presence of T3SS in *B. elkanii*. Aiming for the identification of the T3SS genes of Brazilian *B. elkanii* and *B. japonicum* reference strains and isolates, Southern blot hybridization was performed using bradyrhizobia total DNA and two probes, pBJ100 and pBJ106. Sequences of the genes *rhcC2* and *rhcJ* of *B. japonicum, Mesorhizobium loti, Rhizobium sp.* NGR234 and *Sinorhizobium (Ensifer) meliloti* were aligned and the primers RhcC2for and RhcC2rev, and RhcJfor and RhcJrev were designed. PCR assays for detection of the r*hcC2* and *rhcJ* genes were performed using *Bradyrhizobium* chromosomal DNA. PCR fragments were cloned and sequenced. All the *B. japonicum* and *B. elkanii* DNA amplified expected size bands. The species differ about the patterns when hybridized with the two different probes. These results indicated that T3SS does not have the same gene organization in these two bacterial species. Protein assays were also performed using 2D-electrophoresis technique and genistein was used as a T3SS protein inductor. *B. japonicum* was well induced by genistein while *B. elkanii* did not respond to it. These were the first experiments conducted in order to elucidate the T3SS in *B. elkanii*.

Introduction

Many Gram-negative bacteria are able to establish pathogenic and symbiotic association with animals and plants through the development of secretion systems, which helps them to interact with their hosts. One of these systems, termed type three secretion system (T3SS), deliver a variety of bacterial effectors directly into the host cytoplasm, allowing the bacteria interact with the host, including the entrance of them into the eukaryotic cells (Hueck 1998; Galan and Collmer 1998; Lahaye and Bonas 2001; Dale et al. 2002; Gophna et al. 2003). T3SS are highly conserved among different bacteria and are evolutionarily and functionally related to the flagellar export apparatus (Nguyen et al. 2000). Secreted proteins (effectors) are loaded into a central channel of the T3SS and exported from its distal end. The amino acid sequences of these effectors are often poorly conserved but a strong similarity is observed in many proteins comprising the secretion apparatus (Marie et al. 2001; Gophna et al. 2003).

The T3SS export mechanism was first identified in pathogenic bacteria, like *Yersinia* spp. (Straley et al. 1993), *Salmonella* (Galan 1996), *Shigella* (Menard et al. 1996), *Escherichia coli, Pseudomonas syringae* and *Ralstonia solanacearum* (Jarvis et al. 1995; Gophna et al. 2003), and more recently in *Aeromonas* sp. (Chacón et al. 2004). Moreover a complete rhizobial T3SS was identified after sequencing the symbiotic plasmid of *Rhizobium* sp. strain NGR234 (pNGR234*a*) (Freiberg et al. 1997). Additional T3SS were found by sequencing of the genome of *Mesorhizobium loti* strain MAF303099 (Kaneko et al. 2000) and the “symbiotic island” (a 410 kilobase region) of *Bradyrhizobium japonicum* USDA110 (Göttfert et al. 2001). Genomic hybridizations have shown that the T3SS genes are also present in *B. elkanii* USDA76 (Viprey et al. 1998) and in two strains of *Sinorhizobium (Ensifer) fredii*, USDA257 and HH103 (Meinhardt et al. 1993).

Interestingly, genes for the type III secretion system were lack in *Sinorhizobium (Ensifer) meliloti* 2011 and NZP4010 strains, indicating that a different secretion mechanism may be used (Viprey et al. 1998; Kamoun and Hogenhout 2001). Thus, although T3SS are widespread, they are apparently not ubiquitous among phytosymbiotic prokaryotes (Marie et al. 2001).

In Gram-negative phytopatogenic bacteria, the T3SS is required for the colonization of the host tissue, through the secretion and probable translocation of effector proteins into the plant cell (Lahaye and Bonas 2001). However, the presence of the system was also verified in comensal bacteria (such as *Photorhabdus luminscens, Sodalis glossinidius* e *Sitophilus zeamais*), as well as in plant symbiotic rhizobia and some others non-pathogen prokaryotes (Tampakaki et al. 2004). It was suggested that the system function in symbiotic organisms would be to simulate the one that occurs in pathogens, the secretion of effector proteins directly into the cytosol of the host eukaryotic cell (Krishnan 2002).

Among the rhizobia, the genus *Bradyrhizobium* is of enormous agricultural value since these bacteria are able to fix atmospheric nitrogen in symbiosis with soybean [*Glycine max* (L.) Merrill]. Two species, *B. japonicum* and *B. elkanii*, have been used together as inoculant in brazilian soybean fields (Ferreira and Hungria 2002). Although the T3SS of *B. japonicum* strain USDA110 has been well described (Göttfert et al. 2001; Krause et al. 2002), few studies have shown and analyzed the presence of T3SS in strains of *B. elkanii* (Viprey et al. 1998; Marie et al. 2001).

Here, we reported the presence and analysis of genes and proteins related to type III secretion system in *B. japonicum* and *B. elkanii* Brazilian references strains and strains isolated from fields cultivated with soybean in the Rio Grande do Sul State.

Material and Methods

Bacterial strains, grown conditions and plasmids

Bacterial strains used in this study are listed in Table 1. *Escherichia coli* strains were grown in Luria-Bertani (LB) medium (Sambrook and Russel 2001) supplied with ampicilin (final concentration of 50 μg ml^{-1}). *Bradyrhizobium japonicum* and *B. elkanii* strains were cultivated at 28ºC in yeast extract-mannitol (YEM) medium (Somasegaran and Hoben 1994).

DNA isolation

Bacterial cells were grown in YEM broth for seven days at 28ºC at 128 rpm. Cells were rinsed with TES buffer (50 mM Tris pH 8, 05 mM EDTA, 50 mM NaCl), and ressuspended in EDTA saline (150 mM NaCl, 10 mM EDTA pH 8). Cell lyses took place in 20% sodium dodecyl sulfate (SDS) warmed at 55ºC DNA was extracted using chloroform/isoamylic alcohol (24:1) and precipitated with ethanol.

Hybridization analysis

DNA extracted from five *B. japonicum* and three *B. elkanii* strains isolated from soils cultivated with soybean in Rio Grande do Sul, and four Brazilian reference strains (Table 1) were used in the hybridization analysis. The fragments carried by the plasmids pBJ100 and pBJ106 were used as hybridization probes (Table 1). Southern blot hybridizations were performed using the DIG High Prime System (Roche Molecular Biochemicals) in accordance with the manufacturer's instructions.

Primers alignment

The RhcC2 and RhcJ amino acid sequences of *B. japonicum* USDA110, *Mesorhizobium loti* MAFF303099, *Rhizobium sp.* NGR234 and *Sinorhizobium (Ensifer) fredii* USDA234 were aligned using ClustalW software. The consensus sequences were used to design two degenerate set of primers: RhcC2for and RhcC2rev and RhcJfor and RhcJrev (Table 2). Primers were synthesized by MWG Biotech (Munchenstein, Switzerland).

Gene detection by PCR

PCR assays for detection of the *rhcC2* and *rhcJ* genes were performed using all chromosomal DNAs extracted from the strains described above as template. The PCR reactions were performed in a 25 µL volume, containing 50 ng of DNA template, 1 U *Taq* DNA polymerase (Life Technologies), 1 X *Taq* DNA polymerase buffer, 1.5 mM $MgCl_2$, 200 mM dNTPs (Life Technologies) and 100 pmoles of each primer. PCR amplification of *rhcC2* was performed using the *touchdown* protocol: 94°C for 7 min, followed by 25 cycles of 94°C for 30 s, 55°C to 40°C for 30 s more 25 cycles of 40°C, 72°C for 1min, followed by 72 °C for 5 min. PCR products were electrophoresed through agarose gels (0.8% w/v), stained with ethidium bromide and visualized using UV radiation. PCR amplification of *rhcJ* was performed as follow: 96°C for 7 min, 35 cycles of 96°C for 30 s, 55°C for 30 s and 72°C for 70 s, followed by 72°C for 5 min.

Cloning and sequencing PCR fragments

Before cloning, PCR fragments were analyzed by electrophoresis in a 0.9% agarose gel in TAE. The TOPO TA Cloning Version R (Invitrogen) was used for cloning as recommended. Nucleotide sequences of the cloned fragments were determined on

Beckman Coulter DNA capillary sequencer. Nucleotide sequence homology searches against major sequence databases were done with programs BLAST 2.0.

Isolation of extra cellular proteins

Six hundred milliliters AG medium were inoculated 1:200 from *B. japonicum* USDA 110 and *B. elkanii* Strain A26 precultures. For induction 1 M genistein (dissolved in methanol) was added. Bacteria were incubated for 48 h at 28ºC at 200 rpm. Uninduced cultures were treated with the corresponding amount of methanol. Bacterial cells and exopolysaccharides were separated from the supernatant by two centrifugation steps (first step: 1 h, 4000 *g*, 4°C; second step: 30 min, 8000 *g*, 4°C). Phenol extraction and precipitation of proteins were carried out similar as described (Watt et al., 2005). Twenty µL 10% (w/v) SDS, 1 ml 1 M DTT, 40 ml 0.5 M Tris–HCl pH 6.8 and 120 ml saturated aqueous phenol were added to 500 ml culture supernatant. After incubation at room temperature for 30 min the mixture was centrifuged (30 min, 5000 *g*). The phenol phase was collected in a flask. To precipitate the proteins, 400 ml methanol, 6 ml 8 M ammonium acetate and 400 µl 1 M DTT were added to the phenol phase. The mixture was incubated over night at -20ºC. Proteins were pelleted by centrifugation (1 h, 10,000 *g*, 4ºC). The protein pellet was washed twice with 70% ethanol and was ressuspended in 400 µl rehydration buffer (8 M urea; 2%, w/v CHAPS; 0.01%, w/v bromophenol blue). Proteins were used for gel electrophoresis or stored at -20ºC.

Two-dimensional gel electrophoresis

For isoelectric focussing, 355 µl of protein solution (ca. 200 µg protein) plus 1.8 µl IPG-buffer pH 3–10 (Amersham Biosciences) were mixed with 3.6 µl 28% (w/v) DTT and

incubated for 30 min at 37ºC. Three-hundred forty µl of protein solution were used for rehydration loading onto an Immobiline DryStrip (pH 3–10, 18 cm, Amersham Biosciences). The strips were rehydrated and the samples were focused for 1 h at 0 V, 12 h at 30 V, 2 h at 60 V, 1 h at 500 V, 1 h at 1,000 V and 7 h at 8,000 V at 20ºC (maximum 50 µA per strip). The strips were equilibrated for 30 min in 4 ml equilibration buffer I (containing 50 mM Tris–HCl pH 8.8, 6 M urea, 30%, v/v glycerol, 2%, w/v SDS, 0.01%, w/v bromophenol blue and 1%, w/v DTT) and for 30 min in 4 ml equilibration buffer II (same as buffer I, instead of DTT 4.5%, w/v iodoacetamide). The strips were embedded with sealing solution (containing 0.25%, w/v agarose in SDS-electrophoresis running buffer and 0.01%, w/v bromophenol blue) on the top of 1 mm thick, 15% polyacrylamide gels. Gels were run at 8–10 mA/gel and 20ºC over night. Proteins were stained with Coomassie Brilliant Blue R250.

Results and Discussion

To verify the presence of T3SS genes in the genomes of the *Bradyrhizobium* Brazilian strains a Southern blot experiment was performed. All DNAs analyzed showed hybridization patterns with the two probes used (data not shown). However, the hybridization patterns displayed by *B. japonicum* strains differed notably when compared to those displayed by *B. elkanii* strains for both probes (Figure 1). Besides to confirm the presence of T3SS related genes into the genome of *B. elkanii* strains, the hybridization data also indicated that there is a different organization of these genes between the genomes of the two *Bradyrhizobium* species. Göttfert et al. (2001) had demonstrated that within a 47-kb region of the *B. japonicum* chromosome, ten ORFs showed similarity to genes encoding T3SS in rhizobia and pathogenic bacteria and, apart from a few rearrangements and the

presence of several IS-related elements within the *B. japonicum* cluster, the gene order within these *tts* clusters (as was designed this region) of different rhizobia is relatively well conserved. Perhaps the differences observed within the *B. elkanii tts* region could indicate that these genes are scattered on its chromosome since the sum of the *B. elkanii* fragment sizes that hybridized with both probes was approximately twice the sum of the sizes obtained for *B. japonicum* fragments.

All DNA analyzed also amplified fragments corresponding to portions of *rhcC2* and *rhcJ* genes (Figure 2). The amplified fragments of strains *B. japonicum* A19 and *B. elkanii* A26 were cloned into TOPO vector and their nucleotide and the predicted amino acid sequences were determined. The predicted *B. japonicum* A19 *rhcC2* and *rhcJ* amino acid sequences are 99 and 100% homologous to the corresponding predicted sequences of *B. japonicum* USDA110 (GI 47118316), 68 and 81% to those of *Mesorhizobium loti* MAFF303099 (GI 47118328), 70 and 79% to those of *Rhizobium sp.* NGR234 (GI 2182716) and 63 and 79% to those of *Sinorhizobium(Ensifer) fredii* USDA257 (GI 19749308). The predicted *B. elkanii* A26 *rhcC2* and *rhcJ* amino acid sequences are 90 and 96% homologous to the corresponding predicted sequences of *B. japonicum* USDA110, 70 and 79% to those of *Mesorhizobium meliloti* MAFF303099, 71 and 77% to those of *Rhizobium sp.* NGR234 and 66 and 77% to those of *Sinorhizobium (Ensifer) fredii* USDA234 (Figure 3). In rhizobia, the genes encoding type III secretion machines are clustered within regions of 35-47 kilobases (Marie et al. 2001) Three groups of genes, *rhcC1-rhcU*, *y4yQ-y4yS* and *y4xI-y4xK*, appear to be present in all of the strains for which complete sequence information is available. At the nucleotide level, the degree of homology with the *Rhizobium sp*. NGR234 sequences ranges from 98% identical for *S. fredii*, through approximately 75% for *M. loti* MAF303099, to 68% for *B. japonicum*

USDA110 (Marie et al. 2001). A high level of homology was also found between members of the *avrBs3* gene family, which are involved with the ability of some phytopathogernic bacteria to betray the plant surveillance system. AvrB3-homologous proteins share 90-97% amino acid sequence identity and are secreted via the Hrp type III pathway (Lahaye and Bonas 2001).

It has been reported that several rhizobial strains secrete a number of proteins in a T3SS-dependent manner. For NGR234 and USDA257 T3SS systems, the secretion occurs after flavonoid induction, and apigenin (a strong inducer of NGR234 *nod* genes [Kobayashi et al. 2004]) or genistein (used predominantly with USD257) appears to activate T3SS-related genes with the same efficiency (Ausmees et al., 2004). Both of these inducers have also been reported to be potent activators of expression of nodulation genes in NGR234 and *S. fredii* (Fellay et al. 1995; Krishnan et al. 1992). However, when genistein was used to induce the expression of extracellular proteins by *B. elkanii* strain A26 the profile of proteins secreted was identical to the profile obtained when methanol was used as inducer (data not shown). Nevertheless, according to the Figure 4, the mixtures of proteins secreted by the two strains analyzed, *B. elkanii* A26 and *B. japonicum* BJDΔ283, were markedly different. This result suggest that despite the many genes that are found in both species, several loci that are active in only one of the two strains might contribute to the extracellular protein signature. Further experiments using different kinds of inducers, as well as the extension of T3SS-gene characterization will help understand the detailed mechanisms of the T3SS system and the flavonoid-dependent gene regulation in *B. elkanii*.

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References

Ausmees N, Kobayashi H, Deakin WJ, Marie C, Krishnan HB, Broughton WJ, Perret X (2004) Characterization of NopP, a type III secreted effector of *Rhizobium* sp. strain NGR234. J Bacteriol 186: 4774–4780

Chacon MR, Soler L, Groisman EA, Guarro J, Figueras MJ (2004) Type III Secretion System genes in clinical *Aeromonas* isolates. J Clin Microbiol 42: 1285–1287

Dale C, Plague GR, Wang B, Ochman H, Moran NA (2002) Type III secretion systems and the evolution of mutualistic endosymbiosis. PNAS 99: 12397–12402

Fellay R, Rochepeau P, Relic B, Broughton WJ (1995) Signals to and emanating from *Rhizobium* largely control symbiotic specificity. In: Singh US, Singh RP, Kohmoto K (eds) Pathogenesis & host specificity in plant diseases: histopathological, biochemical, genetic and molecular bases, vol. 1. Prokaryotes. Pergamon/Elsevier Science Ltd, Oxford, United Kingdom, pp 199–220

Ferreira MC, Hungria M (2002) Recovery of soybean inoculant strains from uncropped soils in Brazil. Field Crops Res 79: 139–152

Freiberg C, Fellay R, Bairoch A, Broughton WJ, Rosenthal A, Perret X (1997) Molecular basis of symbiosis between *Rhizobium* and legumes. Nature 387: 394-401

Galan JE (1996) Molecular genetic bases for *Salmonella* entry into host cells. Mol Microbiol 20: 263-271

Galan JE, Collmer A (1998) Type III secretion machines: bacterial devices for protein delivery into host cells. Science 284: 1322-1328

Gophna U, Ron EZ, Graur D (2003) Bacterial type III secretion systems are ancient and evolved by multiple horizontal-transfer events. Gene 312: 151–163

Göttfert M, Rothlisberger S, Kundig C, Beck C, Marty R, Hennecke H (2001) Potential symbiosis-specific genes uncovered by sequencing a 410 kilobase DNA region of the *Bradyrhizobium japonicum* chromosome. J Bacteriol 183: 1405-1412

Hueck CJ (1998) Type III protein secretion systems in bacterial pathogens of animals and plants. Microbiol Mol Biol Rev 62: 379–433

Jarvis KG, Giron JA, Jerse AE, McDaniel TK, Donnenberg MS, Kaper JB (1995) Enteropathogenic *Escherichia coli* contains a putative type III secretion system necessary for the export of proteins involved in attaching and effacing lesion formation. Proc Natl Acad Sci USA 92: 7996-8000

Kamoun S, Hogenhout SA (2001) Agricultural microbes genome 2: first glimpses into the genomes of plant-associated microbes. Plant Cell 13: 451-458

Kaneko T, Nakamura Y, Sato S, Asamizu E, Kato T, Sasamoto S, Watanabe A, Idesawa K, Ishikawa A, Kawashima K (2000) Complete genome structure of the nitrogen-fixing symbiotic bacterium *Mesorhizobium loti*. DNA Res 7: 331-338

Kobayashi H, Naciri-Graven Y, Broughton WJ, Perret X **(**2004) Flavonoids induce temporal shifts in gene-expression of *nod*-box controlled loci in *Rhizobium* sp. NGR234. Mol Microbiol 51: 335–347

Krause A, Doerfel A, Gottfert M (2002) Mutational and transcriptional analysis of the Type III Secretion System of *Bradyrhizobium japonicum*. Mol Plant Microbe Int 15: 1228-1235

Krishnan HB, Lewin A, Fellay R, Broughton WJ, Pueppke SG (1992) Differential expression of *nodS* accounts for the varied abilities of *Rhizobium fredii* USDA257 and *Rhizobium* sp. strain NGR234 to nodulate *Leucaena* spp. Mol Microbiol 6: 3321–3330

Krishnan HB (2002) NolX of *Sinorhizobium fredii* USDA257, a Type III-Secreted Protein involved in host range determination, is localized in the infection threads of cowpea (*Vigna unguiculata* [L.] Walp) and soybean (*Glycine max* [L.] Merr.) nodules. J Bacteriol 184: 831-839

Lahaye T, Bonas U (2001) Molecular secrets of bacterial type III effector proteins. Trends Plant Sci 6: 479-485

Marie C, Broughton WJ, Deakin WJ (2001) *Rhizobium* type III secretion systems: legume charmers or alarmers? Curr Opin Plant Biol 4: 336–342

Meinhardt LW, Krishnan HB, Balatti PA, Pueppke SG (1993) Molecular cloning and characterization of a sym plasmid locus that regulates cultivar-specific nodulation of soybean by *Rhizobium fredii* USDA257. Mol Microbiol 9: 17-29

Menard R, Dehio C, Sansonetti PJ (1996) Bacterial entry into epithelial cells: the paradigm of *Shigella*. Trends Microbiol 4: 220-225

Nguyen L, Paulsen IT, Tchieu J, Hueck CJ, Saier Jr MH (2000) Phylogenetic analyses of the constituents of type III protein secretion systems. J Mol Microbiol Biotech 2: 125–144

Regensburger B, Hennecke H (1983) RNA polymerase from *Rhizobium japonicum*. Arch Microbiol 135: 103–109

Sambrook J, Russel DW (2001) Molecular Cloning: a laboratory manual. Cold Spring Harbor, New York.

Somasegaran P, Hoben JH (1994) Handbook for Rhizobia: Methods in legume-*Rhizobium* technology. Springer-Verlag, New York

Straley SC, Skrzypek E, Plano GV, Bliska JB (1993) Yops of *Yersinia* spp. pathogenic for humans. Infect Imm 61: 3105-3110

Süss C, Hempel J, Zehner S, Krause A, Patschkowski T, Göttfert M (2006) Identification of genistein-inducible and type III-secreted proteins of *Bradyrhizobium japonicum* J Biotech 126: 69-77

Tampakaki AP, Fadouloglou VE, Gazi AD, Panopoulos NJ, Kokkinidis M (2004) Conserved features of type III secretion. Cell Microbiol 6: 805-816

Viprey V, del Greco A, Golinowski W, Broughton WJ, Perret X (1998) Symbiotic implications of type III protein secretion machinery in *Rhizobium.* Mol Microbiol 28: 1381-1389

Watt SA, Wilke A, Patschkowski T, Niehaus K (2005) Comprehensive analysis of the extracellular proteins from *Xanthomonas campestris* pv. *campestris* B100. Proteomics 5: 153-167

Table 1. Bacterial strains and plasmids used in this work

Strains and plasmids	Relevant characteristics	Source
Bradyrhizobium elkanii		
SEMIA 587	Brazilian reference strain	Fepagro/Brazil
SEMIA 5019	Brazilian reference strain	Fepagro/Brazil
A5	Isolated from soybean fields in RS, Brazil	
A26	Isolated from soybean fields in RS, Brazil	
B5	Isolated from soybean fields in RS, Brazil	
Bradyrhizobium japonicum		
110spc4	Referred to as wild type; Sm^r	Regensburger and Hennecke 1983
BJDΔ283	110spc4 mutant [Δ(bll6865, bll6866)]; Sp^r, Km^r	Süss et al. 2006
SEMIA 5079	Brazilian reference strain	Fepagro/Brazil
SEMIA 5080	Brazilian reference strain	Fepagro/Brazil
A19	Isolated from soybean fields in RS, Brazil	
A42	Isolated from soybean fields in RS, Brazil	
B27	Isolated from soybean fields in RS, Brazil	
B34	Isolated from soybean fields in RS, Brazil	
C26	Isolated from soybean fields in RS, Brazil	
Escherichia coli		
TOPO10		Invitrogen
Plasmids		
pBJ100	pBlueScript II SK+ derivative carrying a 6,099 bp fragment containing the *B. japonicum Bj5* to *rhc*V region	personal comm. Göttfert
pBJ106	pBlueScript II SK+ derivative carrying a 2,961 bp fragment containing the *B. japonicum nolB* to *rhcC* region	personal comm. Göttfert

Table 2. Primers used for detection of type III secretion genes in *Bradyrhizobium* strains

Primers	**Expected fragment size**
RhcC2for TA(CT)CAGGC(GC)CC(GC)(AT)(GC)(GC)(AT)(GC)(GC)(AT)(GC)(GC)AC(GC)A TCTTCGT(GC)TTCGGCAAGAA RhcC2rev GAA(GC)AG(GC)GCGCC(GC)AGGAT(GC)GG(GC)AC(AG)TCGCC(GC)AGCCA	948 bp
RhcJfor TA(CT)AC(GC)AAGATCCAGGA(AG)CG(GC)GA(AG)GC(GC)AACGA(AG)ATG RhcJrev ACCTT(AG)TC(AG)TA(GC)(GC)(AT)(GC)AGGCC(CT)TCGAT(GC)(GC)(AT)GT T(GC)GC(GC)AC(GC)AGCATCTT	474 bp

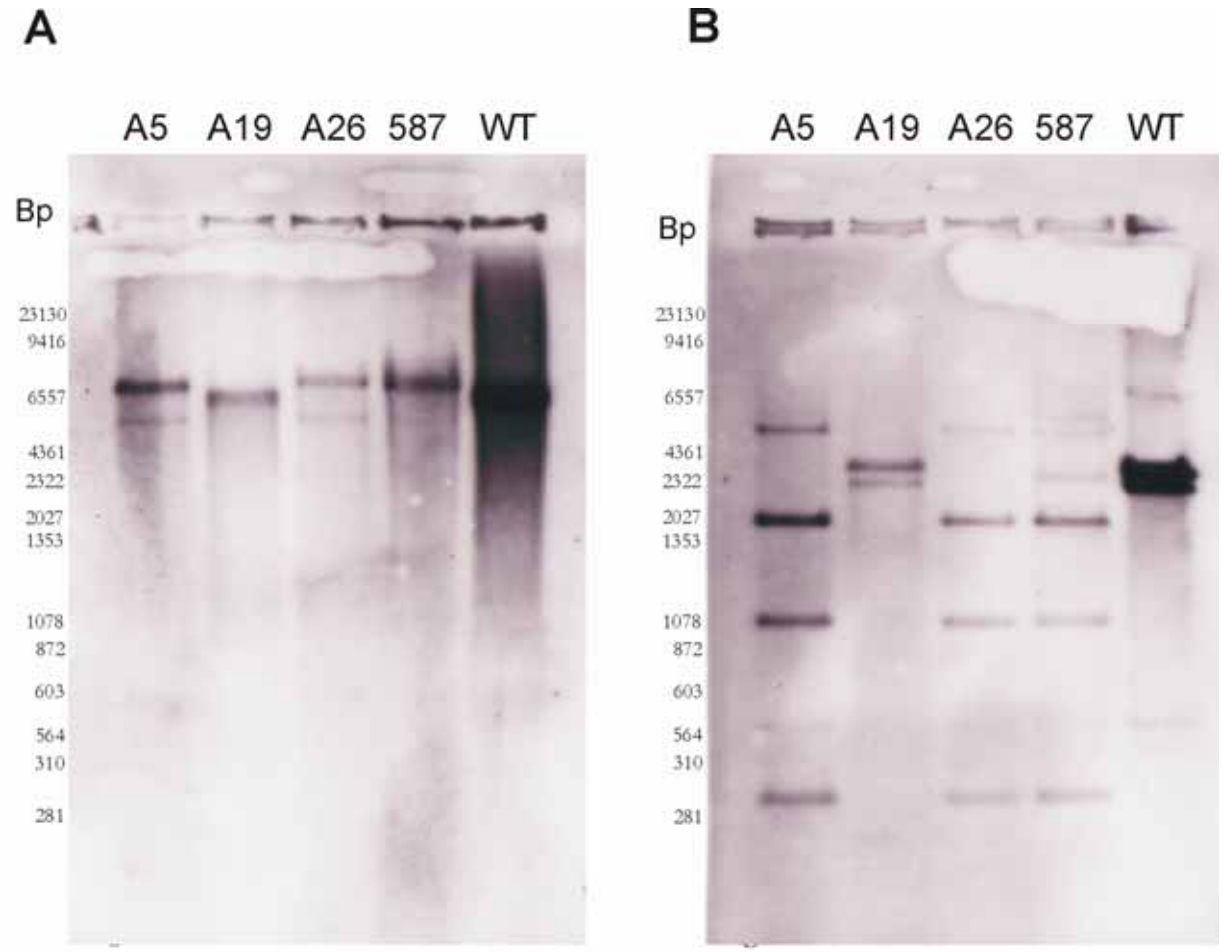

Figure 1. Hybridizations patterns showed by different *Bradyrhizobium* strains with the two probes: pBJ100 (A) and pBJ106 (B). Bp = Molecular marker (λ DNA digested with *Eco*RI/*Hind*III, Invitrogen); A5 and A26 = *B. elkanii* isolates; A19 = *B. japonicum* isolate; 587 = *B. elkanii* SEMAI 587 reference strain; WT = *B. japonicum* USDA110, referred as wild type.

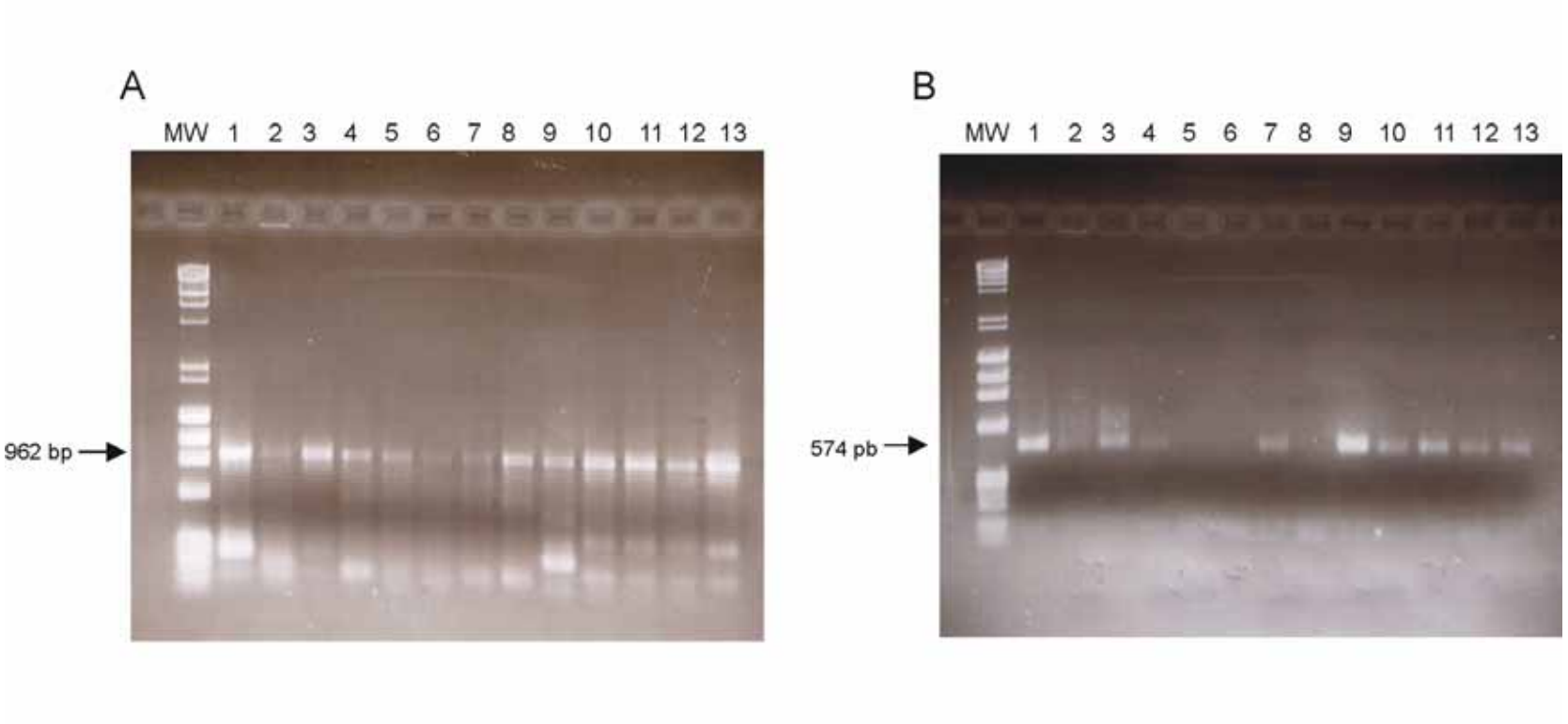

Figure 2. Agarose gels showing the PCR amplified fragments corresponding to portions of *rhcC2* (A) and *rhcJ* genes (B). Arrows indicated the size of the amplified fragments. MW = Molecular marker (λ DNA digested with *Eco*RI/*Hind*III, Invitrogen); 1 – 8) *Bradyrhizobium* isolates: A5 (1), A19 (2), A26 (3), A42 (4), B5 (5), B27 (6), B34 (7) and C26 (8); 9) *B. elkanii* SEMIA 587; 10) *B. elkanii* SEMIA 5019; 11) *B. japonicum* SEMIA 5079; 12) *B. japonicum* SEMIA 5080; 13) *B. japonicum* USDA 110.

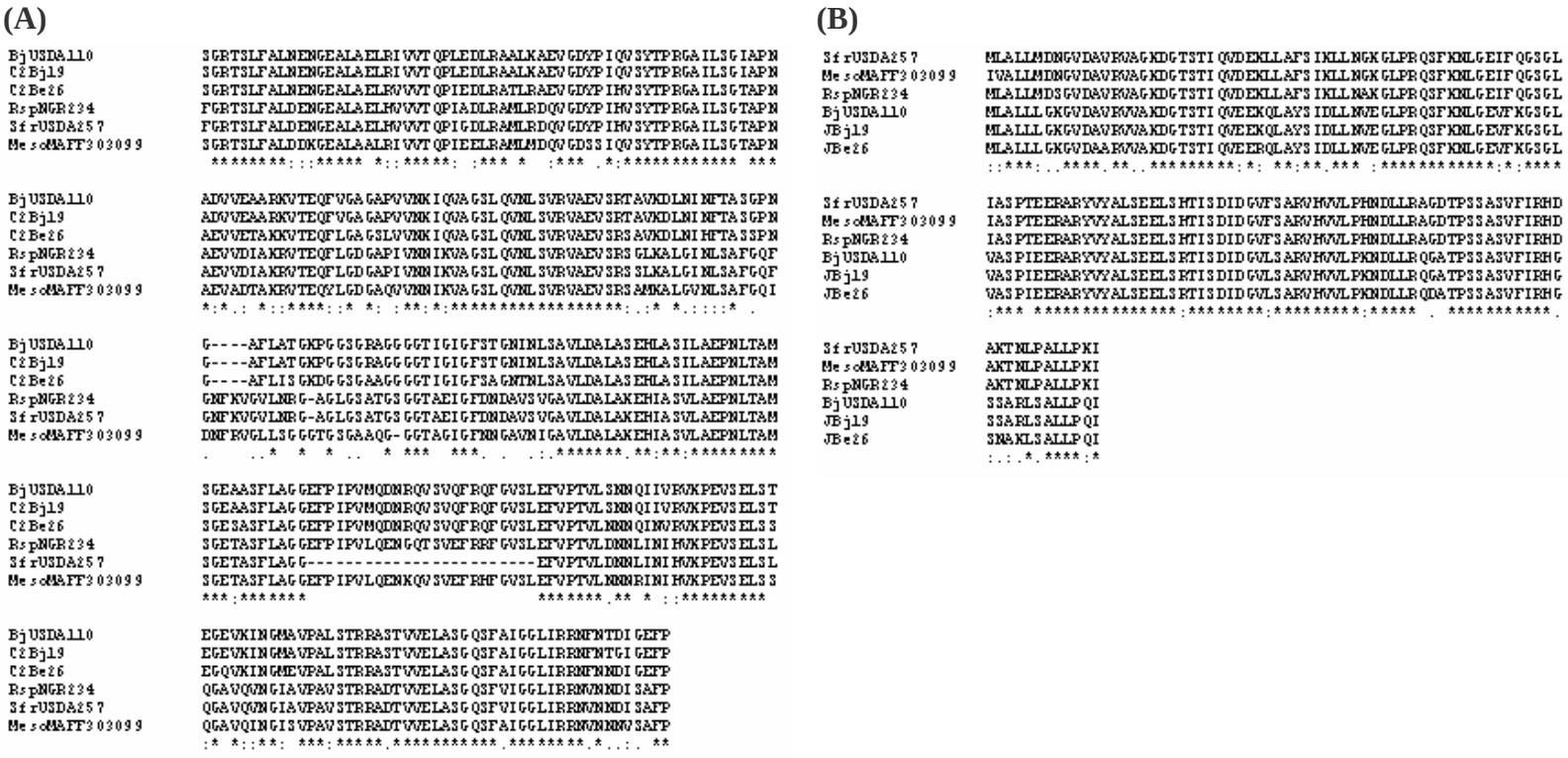

Figure 3. CLUSTAL X multiple sequence alignment between amino acid sequences derived from PCR amplified fragments of *rhcC2* (A) and *rhcJ* (B) genes from *B. japonicum* A19 isolate [C2Bj19 (A) and JBj19 (B)] and *B. elkanii* A26 isolate [C2Be26 (A) and JBe26 (B)] with the correspondent sequences of *B. japonicum* USDA110 (BjUSDA110; GI 47118316) *Mesorhizobium meliloti* MAFF303099 (MesoMAFF303099; GI 47118328), *Rhizobium sp.* NGR234 (RspNGR234; GI 2182716) and *Sinorhizobium (Ensifer) fredii* USDA257 (SfrUSDA257; GI 19749308).

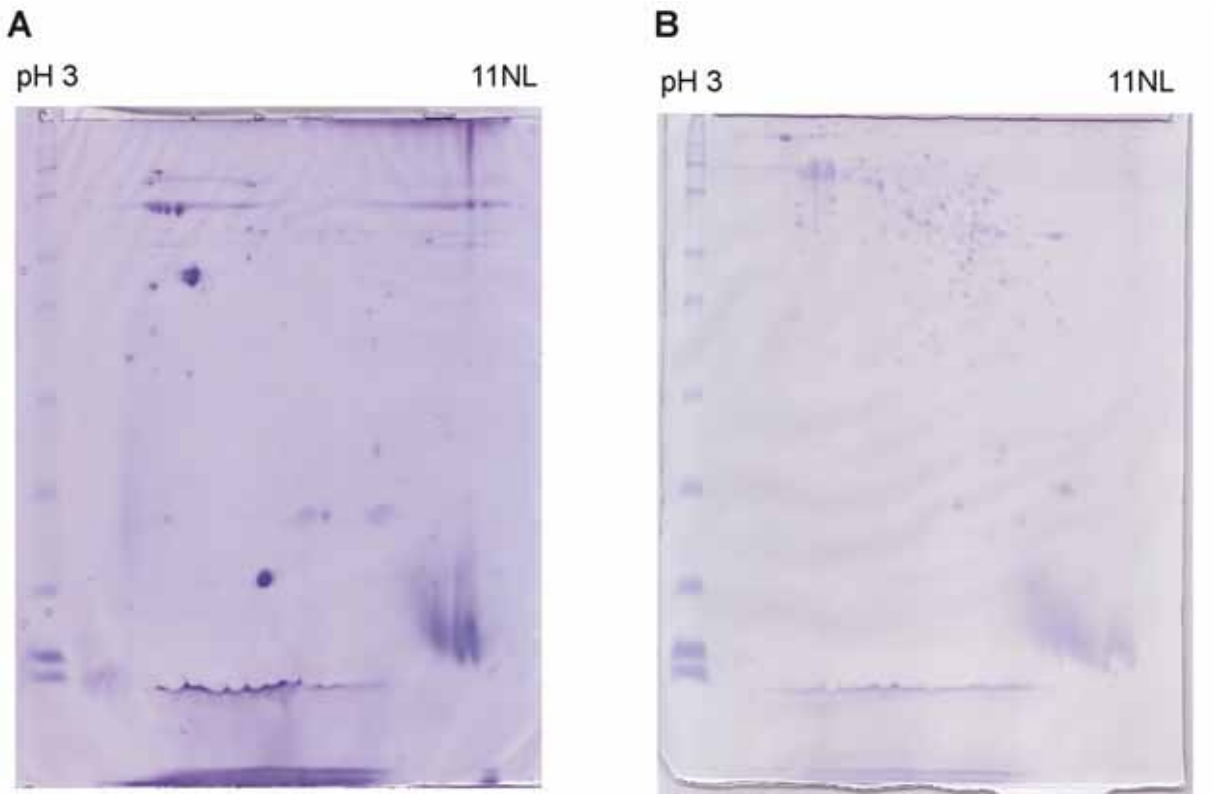

Figure 4. 2D gel eletrophoresis profiles showed by *B. elkanii* A26 (A) and *B. japonicum* BJDΔ283 (B) strains after induction with methanol.