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

Isolamento e caracterização de linhagens de *Bacillus* e *Paenibacillus* promotores de crescimento vegetal em lavouras de arroz e trigo do Rio Grande do Sul

Tese de Doutorado

Anelise Beneduzi da Silveira

Porto Alegre
2008

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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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Porto Alegre, março de 2008.

Este trabalho foi realizado nas instalações do Laboratório de Genética Molecular Vegetal do Departamento de Genética desta Universidade. O projeto foi subvencionado pelo Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) e Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS).


L'essentiel
est invisible
pour
les yeux.
Le Petit Prince

A Heraldo José de Amorim (Zé),

pelo amor de tantos anos, carinho, dedicação, amizade e compreensão

Aos meus pais,
Venilda e José,

pelo amor incondicional, constante carinho e dedicação, por minha formação e por estarem sempre ao meu lado.

DEDICO

Agradecimentos

- À Dra. Luciane Passaglia pela orientação, paciência, apoio, estímulo, amizade e, principalmente, pelo privilégio de fazer parte de sua equipe de pesquisa.

- Aos professores do Laboratório de Genética Vegetal, principalmente a minha co-orientadora Dra. Maria Helena Bodanese-Zanettini pela ajuda sempre bem-vinda.

- Aos colegas de curso, de laboratório e amigos Adriana Ambrosini, Adriana Giongo, Clarisse Palma, Gecele Mattos, Milena Homrich, Milena Cardoso, Natália Cobalchini, Pedro Costa, Rafael Oliveira, René Soares, Roberto Farina e Samanta Campos, pelo coleguismo, sugestões e amizade.

- À técnica e também amiga Sílvia Richter pela valiosa ajuda de sempre e pela amizade.

- À Dra. Gertrudes Corção (Depto Microbiologia/UFRGS) pela ajuda e disponibilidade do seu laboratório.

- À Dra. Lucy Seldin (UFRJ) pelo fornecimento de linhagens-padrão.

- Ao Dr. Leon Rabinovitch (Coleção de Culturas do Gênero Bacilos e Gêneros Correlatos – FIOCRUZ) pelo fornecimento de linhagens-padrão.

- Ao Elmo e a Ellen pela eficiência e por estarem sempre prontos a nos auxiliar com a parte burocrática.

- A todos que contribuíram de alguma maneira para a realização deste trabalho.

- Ao CNPq pelo apoio financeiro.

MUITO OBRIGADA!

Sumário

Lista de Abreviaturas

ACC desaminase	1-aminociclopropano-1-carboxilato desaminase
AIA	ácido indol-acético
ATP	*adenosine triphosphate* (trifosfato de adenosina)
C	carbono
Ca	cálcio
DNA	*desoxyribonucleic acid* (ácido desoxirribonucléico)
ePGPR	*extracellular plant growth promoting rhizobacteria* (rizobactéria promotora do crescimento vegetal extracelular)
FBN	fixação biológica de nitrogênio
Fe	ferro
Fe^{3+}	íon férrico
Fe-Mo	ferro-molibdênio
G+C	guanina + citosina
HPLC	*high performance liquid chromatography* (cromatografia líquida de alto desempenho)
IAM	*indole-3-acetamide* (indol-acetamida)
iPGPR	*intracellular plant growth promoting rhizobacteria* (rizobactéria promotora do crescimento vegetal intracelular)
IPyA	*indole-3-pyruvate* (ácido indol-pirúvico)
K	potássio
M	mol
Mg	magnésio
Mg^{2+}	íon magnésio
Mn	manganês
N	nitrogênio
N_2	nitrogênio atmosférico
P	fósforo
PCR	*polymerase chain reaction* (reação de polimerização em cadeia)
PGP	*plant growth promoting*
PGPR	*plant growth promoting rhizobacteria* (rizobactéria promotora de crescimento vegetal)
pH	potencial hidrogeniônico
RFLP	*restriction fragment length polymorphism* (polimorfismo dos fragmentos de restrição)
RNA	*ribonucleic acid* (ácido ribonucléico)
rRNA	*ribossomal ribonucleic acid* (ácido ribonucléico ribossomal)
YIB	*yield increasing bacteria* (bactéria que aumenta a produção)
Zn	zinco

Resumo

Bacilos são bactérias aeróbias ou anaeróbias facultativas, Gram positivas ou Gram variáveis, produtoras de endósporos, que lhes conferem resistência ao estresse ambiental. Os gêneros *Bacillus* e *Paenibacillus* são os que possuem as espécies reconhecidamente fixadoras de nitrogênio. Outra característica dos bacilos é o grande potencial em produzir substâncias capazes de promover o crescimento vegetal, como hormônios, sideróforos, antibióticos e a capacidade de solubilização de fosfatos. Enquanto múltiplas espécies de bacilos podem ser detectadas nos solos e na rizosfera de várias plantas, muito pouco tem sido feito para estimar a sua diversidade e para indicar quais são as espécies mais comumente isoladas. Dois estudos semelhantes foram conduzidos nesse trabalho, com os seguintes objetivos: i) isolar as espécies de bacilos fixadores de nitrogênio predominantes em diferentes regiões orizícolas e tritícolas do Estado do Rio Grande do Sul, ii) estimar a sua diversidade; e iii) avaliar suas atividades como bactérias promotoras de crescimento de plantas, para utilização como futuros inoculantes. Das espécies que foram identificadas, através do seqüenciamento parcial do gene do RNA ribossomal 16S, as mais comuns foram *P. borealis* e *P. graminis*. Para a grande maioria das bactérias isoladas não foi possível a identificação em nível de espécie. Há uma alta probabilidade de que tais bactérias constituam espécies ainda não descritas, mas filogeneticamente muito próximas a *P. borealis* e *P. graminis*. Dentre os isolados da rizosfera de arroz e trigo, dois foram descritos como novas espécies neste trabalho: *Bacillus oryzae* e *Paenibacillus riograndensis*. Uma característica marcante entre os isolados da rizosfera foi a capacidade destes de produzir grandes quantidades de ácido indol-acético. Poucos isolados, tanto do solo quanto da rizosfera, produziram sideróforos e solubilizaram fosfato. As linhagens selecionadas para experimentos *in vivo* em casa de vegetação provaram ser muito eficientes na promoção do crescimento de suas plantas hospedeiras. Tais linhagens poderão ser usadas na formulação de novos inoculantes, melhorando o sistema de cultivo em que eles venham a ser aplicados. A identificação e o isolamento de bacilos de solos temperados e subtropicais, que combinem a habilidade de fixar nitrogênio com a produção de substâncias capazes de promover o crescimento vegetal, poderá aumentar significativamente a produtividade das lavouras graníferas no Brasil e no Estado do Rio Grande do Sul, em especial.

Abstract

Bacilli are aerobic or facultatively anaerobic, Gram positive or variable, endospore-forming bacteria that exhibit resistance to environmental stress. *Bacillus* and *Paenibacillus* genera include the best knowing nitrogen-fixing species. Another bacilli characteristic is their great potential in producing substances that promote plant growth by the production of hormones, siderophores and phosphate solubilization. While multiple species of bacilli can be detected in the soils and rhizosphere, scarce research has been carried out to estimate their diversity and indicate the most commonly isolated species. In this work, two similar studies have been conducted with the objectives of: i) isolate the predominant nitrogen-fixing bacilli species from different rice and wheat crops of Rio Grande do Sul State, ii) estimate their diversity, and iii) evaluate their plant growth promoting (PGP) activities in order to use them further as inoculant strains. Among the species that have been identified through partial 16S rRNA gene sequence analysis, *P. borealis* and *P. graminis* were the most common species in both locations, soil and rhizosphere. It was not possible to identify the vast majority of isolates at the level of species. There is a high probability that these bacteria constitute species not yet described, but phylogenetically very closely related to *P. borealis* and *P. graminis*. Two isolates from rice and wheat rhizospheres were described as new species: *Bacillus oryzae* and *Paenibacillus riograndensis*. A remarkable characteristic among isolates of the rhizosphere was their ability to produce high amounts of indole-acetic acid. Few isolates, both from bulk soil and rhizosphere, produced siderophores and/or solubilized phosphates. The strains selected for *in vivo* experiments in a greenhouse proved to be very effective in promoting the growth of their host plants. Such strains can be used in the formulation of new inoculants, improving the cropping system in which they will be applied. The identification and the isolation of PGP bacilli from temperate and subtropical soils, which combine the ability to fix nitrogen with the production of substances capable of promoting plant growth, could also significantly increase the productivity of grain crops in Brazil and Rio Grande do Sul State in special.

1 Introdução

Bactérias em forma de bastonetes, aeróbias ou anaeróbias facultativas e formadoras de esporos são geralmente onipresentes em sistemas agrícolas. Os traços fisiológicos comuns importantes para a sobrevivência de tais bactérias incluem a produção de uma parede celular com estrutura em multicamada, a formação de endósporos resistentes ao estresse e a secreção de vários compostos como antibióticos, moléculas sinalizadoras e enzimas extracelulares (Gardener, 2004). Entretanto, uma variação significativa existe em outros traços-chave, incluindo a utilização de determinados nutrientes, a motilidade e a presença de características fisioquímicas de multiplicação (Gardener, 2004). Variações quantitativas e qualitativas nestas características possibilitam a essas bactérias ocupar diferentes nichos nos agrossistemas, com grande abundância nos solos, o que facilita a colonização de plantas e animais. De fato, linhagens de *Bacillus* e *Paenibacillus* podem ser facilmente isoladas do solo e da rizosfera de várias plantas (Seldin *et al.* 1998). No entanto, a localização dos nichos preferenciais de muitas espécies não tem sido extensivamente estudada. Além disso, a significância ecológica da diversidade genotípica e fenotípica de espécies reconhecidas de *Bacillus* e gêneros relacionados ainda permanece um grande mistério (Gardener, 2004) e muito pouco tem sido feito para indicar quais são as espécies mais comumente isoladas.

Análises de DNA extraído diretamente do solo, principalmente através do seqüenciamento de regiões do gene do RNA ribossomal 16S (rRNA 16S), têm confirmado a presença de espécies bacterianas facilmente cultiváveis, mas também revelado uma grande diversidade de linhagens não-cultiváveis de espécies pertencentes aos gêneros *Bacillus* e *Paenibacillus,* (Borneman *et al.,* 1996; Felske *et al.,* 1999; Garbeva *et al.,* 2003; Smalla *et al.,* 2001). Entretanto, existem evidências contraditórias na quantidade relativa de representantes cultiváveis e não-cultiváveis desses gêneros em diferentes solos. Alguns estudos indicam que a grande maioria de seqüências de rRNA 16S de bacilos isoladas diretamente do solo seja altamente similar a seqüências de espécies conhecidas e cultiváveis (Garbeva *et al.,* 2003; Smalla *et al.,* 2001). Outros autores reportam que as seqüências predominantes encontradas em diferentes solos não são as mesmas daquelas apresentadas por bacilos isolados e facilmente cultiváveis (Borneman *et al.,* 1996; Felske *et al.,* 1999).

A especificidade de nichos e atividades ecológicas importantes em *Bacillus* e *Paenibacillus* spp. parecem se estender para além das fronteiras filogenéticas. Muitas espécies podem sobreviver como saprófitas no solo, que é considerado a fonte primária destas bactérias. Contudo, é provável que muitas células viáveis estejam na forma de esporos inativos em um determinado período de tempo (Nicholson, 2002). Adicionalmente, muitas espécies podem ser recuperadas como epífitas e endofíticas de plantas e animais, bem como de gêneros alimentícios e compostos derivados deles (Priest, 1993; Stahly *et al.*, 1992; Slepecky e Hemphill, 1992). A rica variedade de substratos orgânicos e micronichos presentes nestes ambientes suporta um complexo conjunto de espécies microbianas. Dessa maneira, não é surpresa que múltiplas espécies de *Bacillus* e *Paenibacillus* habitem os mesmos (Gardener, 2004).

Muitas espécies de *Bacillus* e *Paenibacillus* podem contribuir para a saúde das plantas de várias maneiras. Inúmeros isolados destes gêneros têm sido utilizados como agentes de controle biológico de fitopatógenos (Lacey *et al.*, 2001; Siddiqui e Mahmood, 1999). Para aplicar com sucesso tais agentes, é necessário um grande entendimento de sua ecologia. Adicionalmente, a segurança e eficácia dos inoculantes serão determinadas, em grande parte, pelo sucesso ecológico das linhagens aplicadas nos ambientes dentro dos quais elas são introduzidas. Um maior conhecimento em relação à diversidade, distribuição e atividades de bactérias pertencentes aos gêneros *Bacillus* e *Paenibacillus* é de extrema importância para a identificação de novas linhagens, para a formulação de inoculantes, e determinação de quais as lavouras em que eles poderão ser aplicados (Gardener, 2004).

Membros do gênero *Paenibacillus* são organismos em forma de bastonetes, aeróbios ou anaeróbios facultativos que produzem esporos elípticos. Fenotipicamente, as espécies deste grupo reagem fracamente com a coloração de Gram e até mesmo colônias jovens podem aparecer como Gram negativas, apesar de terem a estrutura de Gram positivas. O DNA é composto de G+C (mol %) entre 40 e 54 (Shida *et al.*, 1996). São organismos móveis por meio de flagelos peritríquios e não produzem pigmentos (Ash *et al.*, 1993). Também podem excretar uma variedade de enzimas extracelulares de hidrólise de polissacarídeos complexos como alginato, condroitina, quitina, curdlano e outros (Shida *et al.*, 1997). Alguns membros são conhecidos por produzirem polissacarídeos (Yoon *et al.*, 2002) e compostos antifúngicos e antimicrobianos, tais como polimixina, octopitina, bacifelacina (Chung *et al.*, 2000).

Espécies de *Paenibacillus* têm sido isoladas de uma grande variedade de fontes, incluindo solo, água, rizosfera de plantas, raízes de árvores, materiais vegetais, alimentos, forragem, fezes e larvas de insetos (Daane *et al.*, 2002). Isolados bacterianos pertencentes a esse gênero também têm sido encontrados no lago Vostok na Antártida (Christner *et al.*, 2001), na cobertura de gelo de montanhas na China (Christner *et al.*, 2000), em pinturas biodeterioradas (Heyrman e Swings, 2001), em águas marinhas (Siefert *et al.*, 2000), em biofilmes redutores de mercúrio (Wagner-Döbler *et al.*, 2000) e em sedimentos estuarinos contaminados com petróleo (Daane *et al.*, 2002). Esse gênero também foi abundantemente encontrado em empresas que produzem papéis para a indústria alimentícia (Raaska *et al.*, 2002).

Várias espécies descritas como bacilos fixadores de nitrogênio pertencem ao gênero *Paenibacillus*, tais como: *P. polymyxa* (Grau e Wilson, 1962), *P. macerans*, *P. durus* (*P. azotofixans*), *P. peoriae* (Montefusco *et al.*, 1993), *P. borealis* (Elo *et al.*, 2001), *P. graminis* e *P. odorifer* (Berge *et al.*, 2002), *P. brasilensis* (von der Weid *et al.*, 2002), *P. massiliensis* (Roux e Raoult, 2004), *P. wynnii* (Rodríguez-Diaz *et al.*, 2005) *P. sabinae* (Ma *et al.*, 2007a), *P. zanthoxyli* (Ma *et al.*, 2007b) *P. donghaensis* (Choi et al., 2008) e *P. forsythiae* (Ma e Chen, 2008).

Em relação às bactérias pertencentes ao gênero *Bacillus*, Xie e colaboradores (1998) detectaram atividade da enzima nitrogenase nas espécies *B. megaterium*, *B. cereus*, *B. pumilus*, *B. circulans*, *B. licheniformis*, *B. subtilis*, *B. brevis* e *B. firmus*. Em um outro trabalho (Xie *et al.*, 2003) esses mesmos autores relataram o isolamento de 14 linhagens de *Bacillus* capazes de reduzir o acetileno, em lavouras de arroz de oito localidades no rio Yangtze, China. Li e colaboradores, em 1992, também já haviam identificado, através da redução de acetileno, uma espécie de *Bacillus* que fixava nitrogênio em associação com ectomicorrizas. Ahmad e colaboradores (*in press*) encontraram isolados de *Bacillus* fixadores de nitrogênio em diferentes solos rizosféricos em Aligarh, Índia. *Bacillus fusiformis* (linhagens PM-5 e PM-24) também foi identificado como fixador de nitrogênio, através da redução de acetileno, exibindo uma alta atividade de nitrogenase, em diferentes lavouras na província de Chungbuk, Coréia do Sul. Também foram encontrados *Bacillus* diazotróficos na rizosfera de pinheiro e carvalho (Rózycki *et al.*, 1999). Entretanto, somente em 2005, Ding e colaboradores demonstraram, pela primeira vez, que a fixação biológica de nitrogênio ocorria em *Bacillus marisflavi* e *Paenibacillus massiliensis*. Esses

autores também encontraram fragmentos do gene *nifH* em *Bacillus megaterium* e *Bacillus cereus*. Dessa forma, ficou demonstrado que o gene *nifH* está presente no genoma de ambos os gêneros, *Bacillus* e *Paenibacillus*.

Atualmente, o interesse em microrganismos do solo tem aumentado significativamente, uma vez que eles desempenham um importante papel na manutenção da fertilidade do solo. Uma das principais alternativas para o desenvolvimento de uma agricultura sustentável é a utilização de bactérias fixadoras de nitrogênio, hábeis em assimilá-lo da atmosfera (Seldin *et al.*, 1998).

1.1 Fixação Biológica de Nitrogênio

O nitrogênio é um dos principais constituintes das biomoléculas e, em sua forma molecular (N_2), compõe quase 80% da atmosfera. Apesar de abundante, o nitrogênio do ar é quimicamente inerte e poucos são os organismos capazes de utilizá-lo. Determinadas bactérias utilizam como fonte de nitrogênio para o seu metabolismo o enorme reservatório gasoso da atmosfera e estas possuem a capacidade de catalisar a redução do nitrogênio molecular à amônia e incorporar esse nitrogênio em suas moléculas orgânicas (aminoácidos e proteínas), processo denominado de Fixação Biológica do Nitrogênio (FBN, Newton, 2000).

A capacidade de fixar nitrogênio é determinada pelo complexo enzimático da nitrogenase e as bactérias que a possuem são chamadas de diazotróficas. Até o homem conseguir realizar a fixação do nitrogênio industrialmente, as bactérias diazotróficas eram responsáveis pela fixação de cerca de 90% do nitrogênio do planeta, enriquecendo os oceanos e os solos com formas de nitrogênio assimiláveis pelas algas e plantas e, desta forma, sustentando toda a biosfera. Com o aumento explosivo da população humana no último século (passando de 1,6 bilhão, em 1990, para 6,1 bilhões de pessoas, em 2000; Newton, 2000) o homem passou a interferir significativamente nesta equação, colocando fertilizantes nitrogenados na lavoura para suprir a crescente demanda de alimentos. Nos dias de hoje, os fertilizantes nitrogenados representam cerca de 25% do nitrogênio fixado no planeta (Newton, 2000).

O nitrogênio, por ser essencial, é freqüentemente limitante na produção agrícola. São muitos os processos envolvidos na ciclagem desse importante elemento:

desnitrificação, volatilização da amônia, queimadas (processos que retornam o nitrogênio à forma gasosa) e lixiviação de nitratos para as camadas profundas do solo, conduzindo à perda do nitrogênio nos ecossistemas. Para repor o nitrogênio perdido são usados fertilizantes nitrogenados ou a FBN (Neves e Rumjanek, 1998). No entanto, o uso extensivo de fertilizantes nitrogenados na agricultura representa um problema econômico, social, ambiental e de saúde pública. Grande parte dos fertilizantes nitrogenados adicionados ao solo é consumida por bactérias (que voltam a oxidar o nitrogênio, liberando óxido nitroso e óxido nítrico na atmosfera) ou lixiviada, ou seja, não se adere ao solo e acaba sendo levada pela água até o lençol freático. Contaminando o lençol freático, o excesso de nitrogênio pode chegar a rios e lagoas e causar a eutroficação destes ambientes (Lewis *et al.*, 1984).

A produção de soja no Brasil é um exemplo da eficiência da FBN, pois, desde que foram realizados programas para o incremento da fixação de N_2, que resultaram no desenvolvimento de inoculantes com linhagens de *Rhizobium*, poucas aplicações de fertilizantes nitrogenados foram realizadas (Alves *et al.*, 2003).

Os microrganismos fixadores de nitrogênio são muito diversos, com representantes cultiváveis em todos os grupos filogenéticos de procariotos (Gram positivos, Gram negativos, Archaea e Cianobactérias). Os genes de fixação de nitrogênio não são distribuídos entre todos os representantes relacionados filogeneticamente, isto é, os genes de fixação de nitrogênio são encontrados em diversos grupos filogenéticos, mas gêneros fixadores e não fixadores podem estar em grupos fortemente relacionados (Affourtit *et al.*, 2001).

Apesar da variedade de organismos capazes de fixar nitrogênio, o complexo da nitrogenase é muito similar na maioria destes. A nitrogenase é formada por duas proteínas sensíveis ao oxigênio. O componente I ou dinitrogenase é uma proteína ferro-molibdênio, formada por duas subunidades. O componente II ou dinitrogenase redutase é uma proteína ferro-enxofre, que transfere os elétrons para a dinitrogenase. Estas proteínas, juntamente com ATP, Mg^{+2}, e uma fonte de elétrons são essenciais para a atividade de fixação de nitrogênio (Moat e Foster, 1995).

Um ambiente anaeróbio ou microaeróbico é exigido para a atividade da nitrogenase, devido à sensibilidade desta ao oxigênio. Assim, uma hidrogenase acoplada a

uma rota bioquímica que consome oxigênio, ajuda a manter a anaerobiose do sistema. A fixação de nitrogênio é energeticamente muito dispendiosa e é inibida pela presença de nitrogênio fixado, principalmente amônia (Zehr *et al.*, 2000).

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 (*nif*, do ingles, *nitrogen fixation*), encontram-se agrupados em uma região cromossômica abrangendo 24.206 pares de base (Arnold *et al.*, 1988), onde estão dispostos 20 genes, organizados em 8 operons: *nifJ*, *nifHDKTY*, *nifENX*, *nifUSVWZ*, *nifM*, *nifF*, *nifLA* e *nifBQ*.

Como mencionado anteriormente, o complexo da enzima nitrogenase é composto de duas proteínas conservadas: a proteína ferro-molibdênio, um tetrâmero composto de quatro subunidades codificadas pelos genes *nifD* e *nifK* e a ferro-proteína, um dímero com duas subunidades idênticas, codificadas pelo gene *nifH*. O gene da ferro-proteína, *nifH*, é um dos genes funcionais mais antigos da história da evolução e as relações entre as bactérias baseadas nas divergências de seqüências deste gene tem estado de acordo com a filogenia inferida pelas seqüências do gene do rRNA 16S. Esta característica do gene *nifH* tem possibilitado o estudo da diversidade dos genes de fixação de nitrogênio em bactérias de interesse, bem como a caracterização de tais genes em comunidades microbianas do solo (Rosado *et al.*, 1998b).

Os genes *nifH*, *nifD* e *nifK* são normalmente encontrados juntos em um único operon e estão fisicamente adjacentes a outros genes *nif*, fazendo parte de um grande regulon. Freqüentemente encontrados posteriores ao *nifK*, em um outro operon, estão os genes *nifE*, *nifN* e *nifX*. Os genes *nifE* e *nifN* codificam subunidades de um cofator Fe-Mo, essencial para que o complexo da nitrogenase seja reunido. A função do *nifX* ainda não está clara, mas ele pode ter um papel na biossíntese de cofatores ou na regulação (Kessler *et al.*, 1998). A expressão dos genes envolvidos na FBN exige o produto do gene *nifA*, um ativador transcricional deste regulon (Rudnick *et al.*, 1997).

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).

Seqüências do gene *nifH* têm sido amplificadas e seqüenciadas de um grande número de ambientes, incluindo raízes de arroz, solos, oceanos e invertebrados (Zani *et al.*, 2000). A amplificação do *nifH* pode fornecer informações sobre quais tipos de organismos podem estar envolvidos na fixação de N_2, confirmar a presença dos genes em um determinado organismo que se espera ser fixador ou identificar a presença de organismos que são suspeitos de serem pequenos contribuintes na fixação de N_2 (Zehr *et al.*, 1996). Apesar do gene *nifH* ser evolutivamente conservado em nível de aminoácidos, ele pode não ser tão homólogo em nível de DNA, devido à degeneração do código genético. Foi observado que a partir de regiões de seqüências conservadas de aminoácidos, podem ser desenhados oligonucleotídeos iniciadores para amplificar uma seqüência de DNA alvo com alta especificidade (Zehr e McReynolds, 1989).

A reação em cadeia da polimerase (PCR) é uma das ferramentas mais úteis para a investigação dos tipos e abundância de organismos fixadores de nitrogênio, e os fatores que limitam a sua distribuição e atividade. Se organismos fixadores de nitrogênio são detectados, o tipo de microrganismo pode ser identificado pelo seqüenciamento dos fragmentos do gene *nifH* amplificados por PCR. A amplificação de *nifH* de culturas mistas de bactérias é possível através da utilização de oligonucleotídeos degenerados, desenhados a partir de seqüências de genes *nifH* de diferentes bactérias. Assim, a amplificação de regiões bastante conservadas do gene *nifH* é quase universal, e os resultados indicam que os sítios de anelamento não são seletivos para amostras específicas de *nifH*, mesmo que eles variem no conteúdo de bases G+C. Até mesmo seqüências *nifH* de arqueobactérias são amplificadas usando tais oligonucleotídeos, embora tais seqüências estejam distantemente relacionadas às seqüências de *nifH* de outras espécies bacterianas (Zehr *et al.*, 1996).

1.2 Rizobactérias promotoras do crescimento de plantas (*Plant Growth Promoting Rhizobacteria* ou PGPR)

A rizosfera pode ser definida como o solo influenciado especificamente pelas raízes das plantas e/ou em associação com raízes, pêlos e material produzido pela planta. Este espaço inclui o solo ligado às raízes vegetais e, freqüentemente, se estende uns poucos

milímetros da superfície da raiz. Os exsudatos de plantas na rizosfera, tais como aminoácidos e açúcares, provêm uma rica fonte de energia e nutrientes para as bactérias, resultando em uma maior população bacteriana nesta área do que fora da mesma (Gray e Smith, 2005). De fato, a concentração de bactérias (por grama de solo) que é encontrada em volta das raízes (i.e., na rizosfera) é geralmente muito maior do que a densidade bacteriana, ou concentração, que é encontrada em outras porções do solo (Glick, 1995). No entanto, embora haja um grande número de bactérias na rizosfera, somente 7-15% da superfície total da raiz é ocupada por células microbianas (Gray e Smith, 2005).

A interação entre bactérias e as raízes das plantas pode ser benéfica, nociva, ou neutra para a planta e, às vezes, o efeito de uma bactéria em particular pode variar como conseqüência das condições do solo (Lynch, 1990). Assim, por exemplo, é pouco provável que um organismo em particular, que facilite o crescimento vegetal por fixar nitrogênio, irá beneficiar a planta caso nitrogênio fixado exógeno seja adicionado ao solo (Glick, 1995).

As bactérias que beneficiam as plantas são de dois tipos gerais. Existem as que estabelecem uma relação simbiótica com a planta hospedeira e aquelas que vivem livremente no solo, mas que freqüentemente são encontradas perto ou até mesmo dentro das raízes das plantas. Os simbiontes, especialmente rizóbios, têm sido extensivamente estudados e desenvolvidos como alternativa para aumentar a produção das lavouras. As bactérias benéficas de vida livre no solo são geralmente chamadas de rizobactérias promotoras do crescimento de plantas (*plant growth promoting rhizobacteria* ou PGPR), ou, como definido por um grupo de pesquisadores da China, bactérias que aumentam a produção (*yield increasing bacteria* ou YIB) (Piao *et al.*, 1992). Porém, para Gray e Smith (2005), PGPRs também incluem os rizóbios, devido à bem caracterizada simbiose legume-rizóbio. Independentemente dos mecanismos de promoção do crescimento vegetal, as PGPRs têm que colonizar a rizosfera em volta das raízes, o rizoplano (superfície da raiz) ou a raiz (dentro dos tecidos radiculares). Entre as rizobactérias há um gradiente de proximidade da raiz e intimidade: (i) bactérias vivendo no solo perto das raízes, utilizando metabólitos excretados pela raiz como fontes de C e N, (ii) bactérias colonizando o rizoplano (superfície radicular), (iii) bactérias residindo no tecido radicular, habitando os espaços entre células corticais, (iv) bactérias vivendo dentro das células em estruturas radiculares especializadas, ou nódulos. Nessa última categoria encontram-se dois grupos: os rizóbios associados com legumes e espécies de *Frankia* associadas às plantas lenhosas.

Cianobactérias simbiontes fixadoras de nitrogênio das cicadáceas são, também, consideradas PGPRs (Gray e Smith, 2005). Ainda, uma variedade de bactérias diferentes pode ser considerada PGPR, incluindo espécies de *Azotobacter*, *Azospirillum*, *Pseudomonas*, *Acetobacter*, *Burkholderia* e bacilos (Bashan e Levanony, 1990; Brown, 1974; Elmerich, 1984; Kloepper *et al.*, 1988; 1989; Okon e Labandera-González, 1994; Tang, 1994).

Gray e Smith (2005) também adotaram duas divisões de PGPRs: PGPRs intracelulares – iPGPR, que englobam as bactérias que residem dentro das células vegetais, produzem nódulos e estão localizadas dentro dessas estruturas especializadas; e PGPRs extracelulares – ePGPR, bactérias que vivem fora das células vegetais e não produzem nódulos, mas acentuam o crescimento da planta, através da produção de compostos sinalizadores que estimulam diretamente o crescimento vegetal, aumentam a resistência das plantas ou melhoram a mobilização dos nutrientes do solo. A categoria ePGPR pode ser subdividida em três tipos, baseados no grau de associação com as raízes das plantas: bactérias que vivem perto, mas não em contato com as raízes; bactérias que colonizam a superfície radicular e bactérias que vivem nos espaços entre as células do córtex radicular.

A promoção do crescimento vegetal pode ocorrer direta ou indiretamente (Glick, 1995). Há várias maneiras pelas quais bactérias promotoras do crescimento de plantas podem afetar diretamente o crescimento vegetal: através da fixação de nitrogênio atmosférico, da solubilização de minerais, tais como fósforo, produção de sideróforos (moléculas que solubilizam e seqüestram ferro), ou pela produção de reguladores de crescimento vegetal (hormônios), que acentuam o crescimento vegetal em vários estágios de desenvolvimento. A promoção indireta de crescimento ocorre quando a PGPR diminui ou impede os efeitos deletérios de um ou mais organismos fitopatogênicos. Isto pode acontecer pela produção de substâncias antagonistas, ou pela indução de resistência a patógenos (Glick, 1995). A bactéria pode afetar o crescimento vegetal por um ou mais desses mecanismos, e também utilizar diferentes habilidades para promover o crescimento da planta em vários estágios durante o seu ciclo de vida (Glick *et al.*, 1999).

As pesquisas com ePGPRs inicialmente focaram *Bacillus* e *Anthrobacter* spp. (Brown, 1974). Aplicações destas associações têm sido investigadas em milho, trigo, aveia, cevada, ervilha, canola, soja, batata, tomate, lentilha, radite e pepino (Gray e Smith, 2005). Entre os bacilos mais estudados como ePGPRs estão *Bacillus cereus* (Handelsman *et al.*,

1990; Ryder *et al.*, 1999), *Bacillus circulans* (Berge *et al.*, 1990), *Bacillus firmus, Bacillus licheniformis* (Chen *et al.*, 1996), *Bacillus subtilis* (Turner e Blackman, 1991; Zhang e Smith, 1996) e *Bacillus thurigiensis* (Bai *et al.*, 2002a,b).

1.3 Benefícios diretos das ePGPRs para as plantas

Os extensivos estudos bioquímicos e moleculares sobre os diazotróficos simbióticos, tais como rizóbios, têm servido como "ponto de partida" para o entendimento dos mecanismos de promoção de crescimento estimulados pelas PGPRs. Uma vez que uma das maiores contribuições para o crescimento que os rizóbios fornecem às plantas é o nitrogênio fixado, inicialmente pensou-se que todas as PGPRs diazotróficas exercessem seus benefícios dessa maneira. Entretanto, nem todas as PGPRs são diazotróficas. Muitas que o são fixam nitrogênio somente em quantidades limitadas, que, na maioria das vezes, não é suficiente para o seu próprio consumo, muito menos para os requerimentos de nitrogênio da planta hospedeira (Hong *et al.*, 1991). Algumas PGPRs diazotróficas fornecem às suas plantas hospedeiras uma parte da quantidade do nitrogênio fixado que elas precisam; entretanto, o nitrogênio fixado é somente um componente menor do benefício geral para a planta (Chanway e Holl, 1991).

As maneiras pelas quais as ePGPRs podem influenciar o crescimento vegetal diferem de espécie para espécie, bem como de linhagem para linhagem. As rizobactérias de vida livre geralmente não têm um único mecanismo de promoção do crescimento das plantas (Glick *et al.*, 1999). Em adição à fixação de nitrogênio, várias ePGPRs são também capazes de fornecer à planta ferro (Fe) suficiente em solos com limitação desse composto (Wang *et al.*, 1993), ou outros minerais importantes, como, por exemplo, fosfatos (Singh e Kapoor, 1998).

Algumas ePGPRs podem produzir e secretar moléculas de baixo peso molecular que se ligam ao ferro com uma alta afinidade, denominadas de sideróforos (Gray e Smith, 2005). Embora o ferro seja um dos minerais mais abundantes na Terra, ele está relativamente indisponível no solo para a assimilação direta pelos microrganismos. A razão é que em solos aeróbios, o Fe é encontrado predominantemente na forma Fe^{3+}, principalmente como constituinte de polímeros oxihidroxi com uma solubilidade extremamente baixa, 10^{-18} M em pH neutro. As concentrações mínimas requeridas para o

crescimento normal das plantas são de 10^{-9} a 10^{-4} M e, para muitos microrganismos, de aproximadamente 10^{-5} a 10^{-7} M. Para superar esse problema, os microrganismos do solo secretam os sideróforos. Esses compostos se ligam ao ferro, são transportados de volta à célula microbiana e, então, o ferro fica disponível para o crescimento da bactéria (Dobbelaere *et al.*, 2003). Essa ligação ferro-sideróforo também impede a proliferação de patógenos, devido ao seqüestro do ferro do meio ambiente. Exemplos de ePGPRs com esta atividade incluem *Pseudomonas putida* e *P. aeruginosa*. *P. putida* inibe o crescimento de *Fusarium oxysporum*, um patógeno de tomate (Vandendergh e Gonzalez, 1984), enquanto *P. aeruginosa* inibe o crescimento de *Pythium*, também um patógeno do tomate (Buysens *et al.*, 1994). Ao contrário dos fitopatógenos microbianos, as plantas não são prejudicadas com a depleção de ferro pelas ePGPRs. Algumas plantas podem capturar o complexo ferro-sideróforo bacteriano, transportando-o para dentro de suas células, onde o ferro é liberado do sideróforo e fica disponível para a planta (Crowley *et al.*, 1988). A produção de sideróforos também tem sido estudada em *Azospirillum lipoferum* (Saxena *et al.*, 1986; Shah *et al.*, 1992), *Azospirillum brasilense* (Bachhawat e Ghosh, 1987) e *Azotobacter vinelandii* (Demange *et al.*, 1988; Knosp *et al.*, 1984).

O fósforo existe na natureza em uma variedade de formas orgânicas (derivadas de microrganismos e plantas) e inorgânicas (originada de fertilizantes fosfatados), que são muito pouco solúveis (Paul e Clarck, 1989). Na verdade, o fosfóro é um dos nutrientes menos solúveis no ambiente, com menos do que 5% do fosfato total do solo estando disponível para as plantas (Dobbelaere *et al.*, 2003). Sendo o fósforo um elemento nutricional essencial para o crescimento das plantas, a adição de fertilizantes fosfatados é uma prática comum na agricultura moderna. Entretanto, uma grande porção do fosfato inorgânico solúvel aplicado ao solo como fertilizante é rapidamente imobilizada pelo ferro e pelo alumínio, em solos ácidos, e por cálcio, em solos calcáreos, logo depois da aplicação, tornando-se, assim, indisponível para as plantas (Holford, 1997). Microrganismos presentes no solo são capazes de solubilizar fosfato mineral insolúvel pela produção de vários ácidos orgânicos que acidificam o solo, liberando íons ortofosfato solúveis, que podem ser captados pelas plantas (Jones, 1998). Adicionalmente, tais microrganismos são capazes de solubilizar compostos orgânicos fosfatados pela liberação de enzimas fosfatases (Garcia *et al.*, 1992). A presença de quantidades significativas de atividade de fosfatases tem sido reportada e a maior fonte dessa atividade no solo é

considerada como sendo de origem microbiana. Tal atividade é substancialmente aumentada na rizosfera (Rodríguez e Fraga, 1999).

Freqüentemente, a solubilização de fosfato tem sido citada como um possível mecanismo de promoção de crescimento vegetal por ePGPRs. Um grande número de bactérias solubilizadoras de fosfato tem sido isolado da rizosfera de várias lavouras. Foi estimado que esses microrganismos podem constituir de 20 a 40% da população de microrganismos cultiváveis do solo e que uma proporção significativa deles pode ser isolada do solo da rizosfera (Chabot *et al.*, 1993). Embora haja uma boa evidência para a solubilização de fosfato por estes microrganismos em culturas puras, é difícil demonstrar a solubilização de fosfato nos sistemas planta-microrganismo. A produção por estas linhagens bacterianas de outros metabólitos benéficos para as plantas, tais como fito-hormônios, antibióticos ou sideróforos, entre outros, tem criado confusão sobre o papel específico da solubilização de fosfato no crescimento vegetal e na estimulação da produção vegetal (Kloepper *et al.*, 1989).

Experimentos feitos com diazotróficos solubilizadores de fosfato são poucos, e os resultados obtidos são muito diversos, variando de acordo com a planta ou a espécie bacteriana. *Bacillus megaterium* e *Paenibacillus polymyxa* são capazes de acentuar o crescimento e a produção, mas não a captação de fósforo em canola, indicando que a solubilização de fosfato não é o principal mecanismo responsável pela resposta positiva de crescimento (de Freitas *et al.*, 1997). Entretanto, estudos com *Bacillus firmus* (Datta e Dupta, 1982) e *P. polymyxa* (Gaur e Ostwal, 1972) comprovaram o aumento na captação do fósforo e na produção em lavouras de arroz e trigo, respectivamente, depois da inoculação destas bactérias.

Vários estudos têm examinado a habilidade de diferentes espécies bacterianas em solubilizar compostos fosfatados inorgânicos insolúveis, tais como fosfato tricálcio, fosfato dicálcio, hidroxiapatita e fosfato rochoso. Entre os gêneros bacterianos com esta capacidade estão *Pseudomonas, Bacillus, Rhizobium, Burkholderia, Achromobacter, Agrobacterium, Micrococcus, Aereobacter, Flavobacterium e Erwinia,* sendo que *Rhizobium, Pseudomonas e Bacillus* são os mais poderosos solubilizadores, enquanto o fosfato tricálcio e a hidroxiapatita são os substratos mais degradáveis. Como mencionado anteriormente, existe uma enorme quantidade de bactérias solubilizadoras de fosfato no solo e na rizosfera. Estas incluem linhagens aeróbias e anaeróbias, sendo que a maior

concentração de bactérias solubilizadoras de fosfato é encontrada na rizosfera, em comparação com solo não rizosférico (Rodríguez e Fraga, 1999).

Os reguladores de crescimento vegetal são hormônios vegetais, ou fito-hormônios, ou seja, são reguladores naturais do crescimento das plantas, influenciando os processos fisiológicos, quando em baixas concentrações. Os reguladores de crescimento vegetal podem ser classificados como auxinas (diferenciação celular, crescimento radicular, crescimento de frutos e controle da abscisão), citocininas (regulação do crescimento, diferenciação celular e senescência vegetal), giberilinas (divisão e alongamento celular, interrupção da dormência e aumento do desenvolvimento dos frutos), ácido abscísico (regulação da transpiração, quebra de dormência e desenvolvimento inicial das sementes) e etileno (amadurecimento dos frutos, promoção da abscisão de folhas, frutos e flores e influência na expressão do sexo feminino) (Arshad e Frankenberger-Jr, 1998; Raven *et al.*, 1996). A produção destes reguladores já foi relatada em bactérias dos gêneros *Gluconoacetobacter*, *Azospirillum*, *Herbaspirillum*, *Methylobacterium*, *Erwinia*, *Pantoea* e *Pseudomonas* (Bastián *et al.*, 1998; Cassán *et al.*, 2001; Fuentes-Ramirez *et al.*, 1993; Koenig *et al.*, 2002; Lucangeli e Bottini, 1997; Patten e Glick, 1996; Verma *et al.*, 2001), sendo, portanto, essa habilidade amplamente encontrada em bactérias associadas às plantas.

A produção de fito-hormônios é um mecanismo de interação bactéria-planta, sendo influenciada por vários fatores, como o genótipo da planta e do próprio microrganismo. Este fato foi relatado por Jain e Patriquin (1985), que mostraram que linhagens endofíticas puras de *Azospirillum*, ou filtrados das culturas, induzem a ramificação da raiz de trigo. Esses autores observaram que este fenômeno era influenciado pelo genoma bacteriano, em nível de linhagem, e pelo genoma da planta, em nível de cultivar. Nesse mesmo trabalho foi isolada e caracterizada uma auxina, ácido indol-acético (AIA), produzida pela bactéria, a qual apresentou relação com o efeito de ramificação da raiz (ver adiante).

Apesar de algumas espécies de *Paenibacillus* serem altamente eficientes em fixar nitrogênio, a razão para a estimulação do crescimento da planta não está diretamente correlacionada com esta habilidade e, sim, com a sua capacidade de produção e excreção de fito-hormônios, tais como citocininas (Timmusk *et al.*, 1999) e auxinas, principalmente o AIA (Lebuhn *et al.*, 1997).

As auxinas (do grego *auxein*= aumentar) são hormônios vegetais originárias do aminoácido triptofano, sendo a auxina natural chamada de ácido indol-acético (AIA). O triptofano é precursor do AIA. Apesar de serem conhecidos quatro vias diferentes para a biossíntese do AIA, todos se originam a partir do triptofano (Raven *et al.*, 1996). A habilidade para sintetizar fito-hormônios é amplamente distribuída entre bactérias associadas a plantas – 80% dos isolados bacterianos de rizosferas são capazes de produzir AIA (Zakharova *et al.*, 1999). Os primeiros estudos datam do final da década de 70. Tien e colaboradores (1979) demonstraram que *Azospirillum brasilense*, quando exposto ao triptofano, produz AIA e ácido láctico e a produção de AIA aumenta com a idade da cultura. *Bacillus* spp., isolado da rizosfera de *Phaseolus vulgaris* produz quantidades significativas de AIA (Srinivasan *et al.*, 1996). Os isolados de rizosfera dos gêneros *Enterobacter, Xanthomonas, Pseudomonas, Alcaligenes, Azotobacter, Acetobacter* e *Agrobacterium* produzem uma maior quantidade de AIA do que os isolados de solo não associados à raiz (Asghar *et al.*, 2002). Em bactérias fito-patogênicas, tais como *Agrobacterium tumefaciens* e patovares de *Pseudomonas syringae*, o AIA é produzido do triptofano via o intermediário indol-acetoamida e tem sido relacionado a tumores em plantas. As bactérias benéficas sintetizam AIA predominantemente por um caminho alternativo dependente de triptofano, através do ácido indol-pirúvico.

A promoção do crescimento radicular é um dos marcadores pelo qual o efeito benéfico das bactérias promotoras de crescimento é medido. O estabelecimento rápido de raízes por alongamento das raízes primárias ou por proliferação de raízes laterais e adventícias é vantajoso para plantas jovens. Com muitas raízes, essas plantas aumentam a sua habilidade de se ancorar ao solo e obter água e nutrientes do ambiente, acentuando, assim, as suas chances de sobrevivência. Muitas bactérias promotoras do crescimento sintetizam AIA, e seu efeito na planta mimetiza o do AIA exógeno (Patten e Glick, 2002). O ácido indol-acético aparentemente não funciona como um hormônio em células bacterianas e sua produção pelas bactérias pode ter surgido devido a sua importância na relação bactéria-planta. Este regulador, quando secretado por bactérias, poderia promover o crescimento da raiz diretamente pela estimulação do alongamento da célula vegetal ou divisão celular, pela influência da ACC desaminase (1-aminociclopropano-1-carboxilato, um precursor do etileno) (Patten e Glick, 2002). Entretanto, o papel do AIA bacteriano na

promoção do crescimento vegetal ainda não está totalmente esclarecido (Patten e Glick , 2002).

A resposta das plantas ao AIA liberado por bactérias pode variar de efeitos benéficos a deletérios, dependendo de sua concentração. Quando em baixas concentrações, o AIA pode estimular o crescimento e, quando em altas concentrações, pode inibir o desenvolvimento da raiz. Os níveis de AIA produzidos pelas bactérias dependem da multiplicação bacteriana, da atividade metabólica e da expressão de genes que codificam enzimas da rota biossintética de AIA (Lambrecht *et al.*, 2000). Para a avaliação destes efeitos, diferentes metodologias estão sendo aplicadas, tais como a inoculação de raízes com bactérias mutantes quanto à produção de AIA ou aplicação de inóculos bacterianos em diferentes concentrações. Patten e Glick (2002) utilizaram uma linhagem de *Pseudomonas putida* que excretava altos níveis de AIA e constataram um aumento de 35 a 50% no crescimento primário das raízes de canola. Eles demonstraram diretamente que o AIA bacteriano tem um papel importante no alongamento da raiz, quando a bactéria produtora está associada à planta. Além disso, esses pesquisadores deram um maior suporte à hipótese de que bactérias benéficas produzem AIA via ácido indol-pirúvico (IPyA), em contraste com patógenos, que produzem este composto via ácido indol-acetamida (IAM) (Manulis *et al.*, 1998; Patten e Glick, 1996; Prinsen *et al.*, 1993). Extensivas pesquisas têm sido realizadas com bactérias mutantes deficientes na síntese de AIA e os resultados demonstram a existência de mais de uma rota biossintética, dependente de triptofano, para a produção de AIA em um mesmo microrganismo. Estas vias biossintéticas são classificadas segundo seus compostos intermediários, tais como IAM ou IPyA (Dobbelaere *et al.*, 1999; Patten e Glick, 1996).

A detecção de auxinas pode ser realizada por um método bastante preciso e quantitativo, como HPLC (cromatografia líquida de alto desempenho) ou por um método menos preciso, uma reação colorimétrica específica e sensível, na qual se utiliza o reagente de Salkowski (Crozier *et al.*, 1988). Existem diferentes metodologias que utilizam o reagente de Salkowski, algumas utilizam microplacas e outras, membranas de nitrocelulose (Bric *et al.*, 1991; Sarwar e Kremer, 1992).

No estudo de Lebuhn e colaboradores (1997), linhagens de *P. polymyxa* isoladas de raízes de trigo produziram AIA e metabólitos indólicos relacionados a esse composto em meios sem e com adição de triptofano, sendo que o último acentuou grandemente a

produção das auxinas por parte destas linhagens. Esse resultado indicou a importância do triptofano como um precursor do AIA. Os isolados obtidos da rizosfera produziram AIA em maior quantidade do que os isolados do solo não associado à rizosfera, sugerindo que genótipos bacterianos específicos são selecionados pelas plantas.

1.4 Promoção do crescimento vegetal por bacilos

Há uma extensa lista de estudos que demonstram os efeitos benéficos das PGPRs, em que estas melhoram significativamente o crescimento e a produção de vários tipos de lavouras. Alguns dos exemplos mais interessantes são citados a seguir.

Linhagens de *Bacillus* e *Paenibacillus*, ePGPRs Gram positivas, podem ser inoculadas isoladamente ou em consórcio com estirpes de *Rhizobium* ou *Bradyrhizobium*, iPGPRs Gram negativas, para aumentar o crescimento vegetal (Gray e Smith, 2005). A co-inoculação de *P. polymyxa*, linhagem H5, uma bactéria solubilizadora de fosfato, com *Rhizobium* resultou em um aumento da produtividade de lavouras de grão-de-bico, devido à captação do fósforo e de nitrogênio (Alagawadi e Gaur, 1988). A colonização e nodulação de soja com estirpes de *B. japonicum* (Li e Alexander, 1988) pode ser acentuada na presença de *Bacillus* spp., resultando em um aumento do peso seco da planta e da produção de sementes. *Bacillus* spp. isolado da rizosfera de *Phaseolus vulgaris* produziu quantidades significativas do fito-hormônio ácido-indol acético (AIA) (Srinivasan *et al.*, 1996). O AIA promoveu o crescimento radicular e/ou a nodulação, quando adicionado com *R. etli* em *P. vulgaris*, resultando no aumento do peso e número de nódulos, atividade da nitrogenase e conteúdo de leghemoglobina. Aplicações de *Bacillus* spp. isoladamente, também têm efeitos promotores de crescimento em várias plantas e isto pode variar dependendo do tipo de solo (Ramos *et al.*, 2003). Linhagens de *Bacillus* têm, também, aumentado o crescimento de tomates, quando aplicadas ao meio de cultivo das plantas (Yan *et al.*, 2003). *B. subtilis* promoveu o crescimento de plântulas de amendoim, resultando em um aumento de 3,5 a 37% na produção, bem como melhorias na germinação, na emergência de plântulas, na nutrição da planta e aumento do crescimento radicular (Turner e Blackman, 1991). A inoculação com *B. licheniformis* CECT 5106 e *B. pumilus* CECT 5105 acentuou o crescimento de plântulas de *Pinus*.

B. cereus UW85 acentuou a nodulação de soja, tanto em experimento a campo, como em câmaras de crescimento (Halverson e Handelsman, 1991). Interessantemente, nos experimentos de campo, diferenças na nodulação foram detectadas em 25 a 35 dias após o plantio; entretanto, com o progresso do experimento, aos 49 dias, diferenças na nodulação não foram mais observadas. Estes resultados demonstraram um efeito promotor de crescimento inicial, mas não efetivo em longo prazo. No entanto, efeitos iniciais de crescimento podem ser benéficos no aumento do crescimento de plantas jovens, auxiliando-as a lidar melhor com os estresses ambientais que elas venham a enfrentar mais tarde, ao longo do desenvolvimento (Gray e Smith, 2005).

Além da fixação de nitrogênio, *P. polymyxa* solubiliza o fósforo no solo, produz antibióticos, quitinase e outras enzimas hidrolíticas, acentua a porosidade do solo e produz compostos promotores de crescimento em plantas similares em atividade ao ácido indol acético (AIA) (Timmusk *et al.*, 1999). Também foi demonstrado que essa espécie aumenta a captação de nutrientes pela planta e age como biocontrolador de microrganismos patógenos (Chanway, 1995; Mavingui e Heulin, 1994), razão pela qual ela tem sido usada como controle biológico contra *Fusarium* e *Pythium* (Guenouri-Athmani *et al.*, 2000).

Paenibacillus durus (P. azotofixans) é encontrado em solos e raízes de cana-de-açúcar, trigo e outras gramíneas. A característica interessante dessa espécie está relacionada com a sua capacidade de fixação biológica de nitrogênio, mesmo em presença de altos níveis de nitrato e à produção de substâncias antimicrobianas (Neves e Rumjanek, 1998). *P. azotofixans* apresenta uma capacidade *in vitro* de fixar nitrogênio superior às demais espécies de *Paenibacillus*, e por não ser afetada pela presença de nitrato, a fixação em áreas com fertilizantes é possível (Rosado *et al.*, 1996).

No estudo de Li e colaboradores (2008), um total de 98 linhagens bacterianas endofíticas não simbióticas foram isoladas de nódulos de soja e identificadas como *Pantoea, Serratia, Acinetobacter, Bacillus, Agrobacterium* e *Burkholderia*. A inoculação de bactérias endofíticas não apresentou nenhum efeito significante no crescimento e nodulação da soja, mas muitas linhagens produziram AIA, solubilizaram fosfato e fixaram nitrogênio, sugerindo que elas são promissoras para futuros estudos de promoção de crescimento vegetal.

Na procura por PGPRs eficientes, Ahmad e colaboradores (*in press*), isolaram 72 bactérias identificadas como *Azotobacter, Pseudomonas, Mesorhizobium* e *Bacillus* das

rizosferas de trigo, cana de açúcar, cebola e de nódulos de grão de bico na região de Aligarh, na Índia. Estes isolados foram testados para produção de AIA, amônia, ácido cianídrico, sideróforos, solubilização de fosfato e atividade antifúngica. As linhagens de *Bacillus* e *Pseudomonas* isoladas foram as mais eficientes, sendo candidatas a estudos posteriores de promoção de crescimento vegetal.

No estudo de Park e colaboradores (2005), bactérias fixadoras de vida livre foram isoladas da rizosfera de sete plantas diferentes: gergelim, milho, trigo, soja, lentilha, pimenta e arroz, na província de Chungbuk, Coréia do Sul. As linhagens que exibiram atividade de nitrogenase foram identificadas como *Stenotrophomonas maltophila*, *Bacillus fusiformis* e *Pseudomonas fluorescens*. *Bacillus fusiformis* exibiu a mais alta atividade de nitrogenase e produção de AIA, demonstrando seu potencial como rizobactéria promotora de crescimento vegetal.

Outras PGPRs, isoladas da rizosfera de melão, alfafa, tomate, algodão e trigo, foram analisadas quanto à produção de auxinas, atividade de nitrogenase, atividade antifúngica e, também, quanto à melhora da captação de nutrientes em algodão e ervilha em uma região semi-árida do Uzbequistão (Egamberdiyeva e Höflich, 2004). As linhagens produziram auxinas, foram capazes de fixar nitrogênio e reagiram antagonisticamente com o fungo *Verticillium loteritum*. A inoculação das plantas testadas com as linhagens identificadas como *Pseudomonas alcaligenes* PsA15, *P. denitrificans* PsD6, *Paenibacillus polymyxa* BcP26 e *Mycobacterium phlei* MbP18 aumentou significantemente o crescimento da raiz e da parte aérea e, também, o conteúdo de N, P e K destas plantas.

Várias linhagens de *Bacillus subtilis* e *B. cereus* foram isoladas e selecionadas na China por sua habilidade em promover o crescimento vegetal e controlar doenças fúngicas que atacam a raiz de trigo (Ryder *et al.*, 1999). Entre essas linhagens, *B. cereus* A47 tem sido usada em várias lavouras da China, estimulando a produção de trigo em cerca de 11%. Já *B. subtilis* B908 foi usado para controlar a queima da bainha de plantas de arroz, a qual é causada por *Rhizoctonia solani*. Ambas as linhagens também foram testadas em dois solos diferentes na Austrália, em experimentos de casa de vegetação. Tais bactérias reduziram consideravelmente a severidade de doenças causadas por *R. solani* e *Gaeumannomyces graminis* var. *tritici* e estimularam o crescimento de plântulas de trigo (Ryder *et al.*, 1999).

No estudo de Mena-Violante e Olalde-Portugal (2007), o efeito da inoculação de raízes de tomates com *B. subtilis*, linhagem BS13, na produção e qualidade da fruta foi avaliado. A produção, o peso e o comprimento da fruta foram aumentados com a inoculação de BS13, quando comparados com o controle não inoculado. A textura das frutas também foi acentuada, demonstrando que essa PGPR teve efeitos positivos na qualidade da fruta, particularmente no tamanho e na textura.

Orhan e colaboradores (2006) estudaram os efeitos promotores de crescimento de duas linhagens de *Bacillus*, OSU-142 (fixador de nitrogênio) e M3 (fixador de nitrogênio e solubilizador de fosfato), combinadas ou inoculadas isoladamente em framboesas orgânicas. As plantas foram avaliadas de acordo com a produção, crescimento, composição de nutrientes das folhas e variação da composição de nutrientes. Os resultados mostraram que *Bacillus* M3 estimulou o crescimento vegetal e aumentou significativamente a produção. A inoculação das raízes e da rizosfera de framboesa com M3 e/ou OSU-142 em conjunto com M3, aumentou significativamente a produção (33,9% e 74,9%), quando comparada com o controle. Os conteúdos de N, P e Ca das folhas também apresentaram aumentos significativos. As inoculações igualmente afetaram os conteúdos totais de N, P, K, Ca, Mg, Fe, Mn e Zn do solo. Os resultados demonstraram que *Bacillus* M3, isolado ou em combinação com OSU-142, apresentou um enorme potencial para aumentar a produção, crescimento e nutrição de plantas de framboesa crescidas sob condições orgânicas.

Karlidag e colaboradores (2007) também testaram os efeitos dos bacilos citados anteriormente, M3 e OSU-142, e de *Microbacterium* FS01, isolados ou combinados sobre o crescimento de macieiras. A inoculação dessas linhagens isoladamente ou combinadas aumentou o crescimento vegetal (parte aérea 16,4-29,6%, diâmetro da parte aérea 15,9-18,4%) e a produção (26-88%, tamanho do fruto em 13,9-25,5%). Todos os nutrientes (N, P, K, Mg, Ca, Fe, Mn e Zn) investigados, exceto Mg, foram significativamente afetados. Os resultados desse estudo sugeriram que *Bacillus* M3 e/ou OSU-142 e/ou *Microbacterium* FS0 têm o potencial para aumentar a produção, crescimento e nutrição em macieiras.

A mesma linhagem de *Bacillus*, OSU-142, também foi avaliada nos experimentos de Esitken e colaboradores (2006), isolada ou em consórcio com *Pseudomonas* BA-8, na produção, crescimento e composição de nutrientes em plantações de cereja. A presença de *Pseudomonas* BA-8, *Bacillus* OSU-142 e a combinação BA-8 + OSU-142 estimulou o

crescimento vegetal e resultou em aumento significativo da produção. Aplicações foliares e florais de BA-8, OSU-142 e BA-8 + OSU-142 significativamente aumentaram a produção (16,3, 10,9 e 21,7%), o peso das frutas (4,14, 5,37 e 1,24%) e a parte aérea (11,3, 11,8 e 29,6%), respectivamente, quando comparados com os controles. Adicionalmente, o conteúdo de N, P e K das folhas de cereja com todos os tratamentos aplicados, o conteúdo de Fe e Zn com a aplicação de BA-8 + OSU-142 e o conteúdo de Mn com a aplicação de BA-8 e OSU-142 aumentaram significativamente. Os resultados sugerem que a inoculação de *Pseudomonas* BA-8 e *Bacillus* OSU-142 sozinhos ou combinados têm um grande potencial para aumentar a produção, crescimento e nutrição das cerejeiras.

O crescente interesse em PGPRs está baseado no desejo de estudar um sistema biológico complexo e fascinante e no potencial de utilização desses organismos no aumento da produtividade das lavouras. As linhagens que demonstram possuir uma característica específica desejável são selecionadas, testadas e, então, usadas como um componente da prática agrícola, embora, até agora, apesar dos diversos exemplos citados anteriormente, isso tenha sido feito em uma limitada extensão. Se mais e melhores linhagens forem selecionadas e efetivas, o uso de PGPRs na agricultura irá aumentar. Esforços nos últimos 10-15 anos têm fornecido um melhor entendimento das PGPRs e dos mecanismos que estas bactérias utilizam para promover o crescimento das plantas, renovando também, o interesse comercial nestas (Glick, 1995). Enquanto as PGPRs têm sido identificadas dentro de muitos taxa bacterianos diferentes, muitas PGPRs desenvolvidas comercialmente são espécies de *Bacillus*, que na forma de endósporos conferem estabilidade populacional durante a formulação e estocagem do produto. Entre os bacilos, as linhagens de *B. subtilis* são as PGPRs mais largamente usadas, devido a sua capacidade de produção de antibióticos e redução de doenças, quando aplicadas como tratamentos em sementes (Kokalis-Burelle *et al.*, 2006).

2 Justificativa

A maioria dos estudos da genética da fixação de nitrogênio tem como alvo principal as bactérias Gram negativas, como *Klebsiella*, *Rhizobium*, *Azospirillum* e *Bradyrhizobium.* Os inoculantes a base de bactérias simbiontes, especialmente rizóbios, têm sido extensivamente estudados e utilizados como possíveis alternativas para o aumento da produtividade das lavouras.

As bactérias que apresentarem mais de uma característica para a promoção de crescimento vegetal como, por exemplo, fixar nitrogênio e solubilizar fosfato ou produzir auxina e sideróforos, entre outras, são almejadas e rastreadas para uma possível aplicação no campo, objetivando o aumento da produção agrícola (Verma *et al.*, 2001).

Infelizmente, faltam informações a respeito dos microrganismos fixadores de nitrogênio Gram positivos de vida livre, grupo do qual fazem parte principalmente espécies dos gêneros *Bacillus* e *Paenibacillus*. Poucas tentativas foram feitas para avaliar a diversidade das espécies fixadoras de nitrogênio pertencentes a esses gêneros. Desta forma, os estudos de ecologia microbiana que buscam aumentar e consolidar os conhecimentos a respeito dos processos interativos no ambiente podem auxiliar no entendimento destes, visto que já foi observado que tais bactérias podem conferir às plantas maior resistência a condições de estresse, alterações nas condições fisiológicas, proteção contra organismos patogênicos e herbívoros, além da produção de substâncias promotoras do crescimento vegetal.

A identificação e o isolamento de linhagens bacterianas de bacilos fixadores de nitrogênio em solos subtropicais, que aliem características extremamente importantes como a elevada capacidade de fixar nitrogênio, mesmo na presença de fertilizantes, e a capacidade de excreção de substâncias capazes de promover o crescimento das plantas, contribuirá, de maneira significativa, para um aumento na produtividade das lavouras graníferas do Estado do Rio Grande do Sul.

3 Objetivos

O objetivo geral deste trabalho foi caracterizar, por meio de técnicas moleculares e microbiológicas, a diversidade de bacilos fixadores de nitrogênio que ocorrem no Estado do Rio Grande do Sul, buscando-se o isolamento de linhagens produtoras de substâncias que auxiliem o crescimento vegetal.

Os objetivos específicos foram:

a) Isolamento e caracterização molecular de linhagens de bacilos fixadores de nitrogênio provenientes de amostras de rizosfera e de solo não associado à raiz, coletadas em plantações de arroz e trigo em regiões distintas do Estado do Rio Grande do Sul, por meio da técnica de PCR-RFLP do gene *nifH*;

b) Analisar e comparar os isolados coletados nas diferentes regiões geográficas do Estado, com diferentes tipos de solo, em relação às suas características moleculares e microbiológicas;

c) Avaliar a capacidade de excreção de substâncias promotoras de crescimento vegetal *in vitro* de cada isolado, para a identificação das linhagens mais eficientes;

d) Testar as linhagens mais eficientes em experimentos *in vivo* em casa de vegetação.

Capítulo I

Evaluation of genetic diversity and plant growth promoting activities of nitrogen-fixing bacilli isolated from rice fields in South Brazil

Artigo aceito para publicação na revista *Applied Soil Ecology* em fevereiro de 2008

APSOIL 1154 1–10

ARTICLE IN PRESS

APPLIED SOIL ECOLOGY XXX (2008) XXX–XXX

available at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/apsoil

Evaluation of genetic diversity and plant growth promoting activities of nitrogen-fixing bacilli isolated from rice fields in South Brazil

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ARTICLE INFO

Article history:
Received 29 August 2007
Received in revised form
24 January 2008
Accepted 28 January 2008

Keywords:
Bacillus
Paenibacillus
Plant growth promoting rhizobacteria (PGPR)
Nitrogen fixation
PCR-RFLP

ABSTRACT

Several strains of bacilli, mainly species of the genera *Bacillus* and *Paenibacillus*, displaying important plant growth promoting (PGP) characteristics were isolated from seven distinct rice production zones of the Rio Grande do Sul State, Brazil. Of 296 isolates, 155 were from rhizospheric soil and 141 from bulk soil. In order to evaluate the diversity among the isolates of each bacterial population the Shannon index was used on a 70% similarity basis. Diversity indices varying from 2.27 to 5.51 were obtained. Using principal coordinate analysis (PCA) to correlate bacterial diversity with soil parameters, it was found that soil pH was the characteristic most closely related to bacilli diversity. The bacilli isolated were also analyzed for some PGP activities. Of those 296 isolates, 94 and 148 produced between 0.1 and 30 μg of indole-3-acetic acid (IAA) ml^{-1} *in vitro* after 72 and 144 h of incubation, respectively. Twenty-two isolates were able to solubilize phosphate and 32 isolates produced siderophores. *Paenibacillus* and *Bacillus* genera were the most prominent groups in the rhizosphere and soil populations analyzed. *Paenibacillus borealis* was the most frequent species in both locations. The isolate SVPR30, identified by 16S rRNA gene sequence analysis as a strain of *Bacillus* sp., was chosen for *in vivo* greenhouse experiments and proved to be very efficient in promoting a significant increase in the roots and shoot parts of rice plants. The identification and isolation of PGP bacilli from temperate and subtropical soils, which combine the ability to fix nitrogen with the production of substances capable to promote the plant growth, could significantly increase productivity of grain crops in Brazil.

1. Introduction

Previously confined to inoculation of legume seeds with *Rhizobium*, the management of the rhizosphere bacterial population has nowadays advanced toward the concept of plant growth promoting rhizobacteria (PGPR) (Lalande et al., 1989). These bacteria stimulate host plant growth, and have been the object of studies due to the effects produced in commercially important crops. The exact mechanism by which PGPR stimulate plant growth is not clearly established,

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0929-1393/$ – see front matter © 2008 Published by Elsevier B.V.
doi:10.1016/j.apsoil.2008.01.006

although several hypotheses such as production of plant growth substances, suppression of deleterious organisms, and promotion of the availability and uptake of mineral nutrients are usually believed to be involved (Lalande et al., 1989). Albeit variations in the plant response to PGPR in laboratory and field assays are evident, the full potential of rhizobacteria and other microorganisms to promote plant growth should be more extensively investigated.

The widely studied *Bacillus* genus represents one of the most diverse genera in the Bacilli group (Garbeva et al., 2003). Numerous *Bacillus* and *Paenibacillus* strains express plant growth promoting (PGP) activities and a number of these strains have already been commercially developed as biological fungicides, insecticides, and nematicides or generic plant growth promoters. The use of these strains in agriculture has recently been reviewed (Gardener, 2004).

Rice is the most important staple crop in the developing world, and nitrogen is the most important input required for rice production. In order to make rice cultivation sustainable and less dependent on fertilizer nitrogen, it is extremely important to know how to use PGPRs that can biologically fix nitrogen and produce substances (for example, indole-3-acetic acid and siderophores) that can contribute to rice growth improvement (Verma et al., 2001).

The diversity of bacilli species in Southern Brazilian soils remains largely unknown, especially of those strains that, besides having several PGP properties, can also fix nitrogen. The objectives of this study were to (i) isolate the predominant nitrogen-fixing bacilli species from different rice crops, (ii) estimate their diversity, and (iii) evaluate their PGP activities in order to use them further as inoculant strains.

2. Materials and methods

2.1. Sampling and sample preparation

Samples of rhizosphere and bulk soil were collected in seven distinct currently rice production zones of the Rio Grande do Sul State, Brazil: Guaíba [Gu (30°06′50″S, 51°19′30″W)], São Lourenço do Sul [SLS (31°21′55″S, 51°58′42″W)], Santa Vitória do Palmar [SVP (33°31′08″S, 53°22′04″W)], Hulha Negra [HN (31°24′14″S, 53°52′08″W)], Santana do Livramento [Li (30°53′27″S, 55°31′58″W)], Uruguaiana [U (29°45′18″S, 57°05′16″W)] and Osório [O (29°53′13″S, 50°16′12″W)]. Three c. 1 ha fields were sampled per zone. Ten ~0.5 kg fresh weight samples of flooded soil were taken to a depth of 15 cm and bulked to obtain a representative composite sample. Subsamples (0.5 kg fresh weight) were analyzed for pH, clay, P, K, Fe, exchangeable Al, Ca, Mg, and organic matter (OM) contents, using standard methods and the results are shown in Table 1. All samples were collected between January and February 2005.

Diazotrophic bacilli were isolated according to Seldin et al. (1983). Three plants were selected for each individual field within each production zone. One gram of the soil adhering to the roots, considered the rhizospheric soil, or 1 g of bulk soil (at a depth of 10 cm) was mixed with 9 ml of distilled water and used for the bacterial isolation procedures. The different soil suspensions were pasteurized (10 min, 80 °C) to eliminate non-sporulated bacterial forms, and twofold serial dilutions obtained therefrom were plated onto thiamine-biotin agar (TB N-free medium, Seldin et al., 1983) and anaerobically incubated in anaerobic jars (Permution) for 7 days at 28 °C. Typical anaerobic bacilli colonies were transferred to fresh TB agar plates for another period of anaerobic incubation. Single colonies were then transferred to aerobic glucose medium (GB, Seldin et al., 1983). Pure cultures were stored at −10 °C in 20% glycerol.

Paenibacillus durus P3L5T, *Paenibacillus polymyxa* LMG 24.16, *Paenibacillus peoriae* LMG 14832^T, *P. polymyxa* LMG 13294^T, *P. polymyxa* Loutit, *Paenibacillus odorifer* TOD45^T, *P. polymyxa* ATCC10343, *Paenibacillus graminis* RSA19^T, *Paenibacillus rhizosphaerae* CECAPO6^T, and *Paenibacillus borealis* KK19^T were used in this study as standards for nitrogen-fixing bacilli. They were supplied by Dr Lucy Seldin (UFRJ/Brazil), Oswaldo Cruz Foundation (Brazil), and by Dr Encarna Velázquez (Universidad de Salamanca, Spain).

2.2. DNA isolation

DNA was directly extracted from bacterial cultures by a direct-lysis method that consisted of boiling the samples for 5 min at 100 °C in 200 μl 0.1 M NaCl. DNA was electrophoresed on 0.8% agarose ethidium bromide gel.

2.3. PCR amplification and RFLP analysis of the nifH gene

One hundred nanograms of DNA was used as template in PCR procedures. Selected primers PolF and PolR (TGCGAYCCSAARGCBGACTC and ATSGCCATCATYTCRCCGGA, respectively, Poly et al., 2001) were used to amplify a very well

Table 1 – Abiotic characteristics of the soils of the sampled sites

Sampled site	Clay (%)	pH H_2O	P (mg kg^{-1})	K (mg kg^{-1})	Al exc (mg kg^{-1})	Ca exc (mg kg^{-1})	Mg exc (mg kg^{-1})	Fe (%)	Organic matter (%)
1. Guaíba	13	6.9	18	48	0	840	145.9	0.1	1.4
2. Hulha Negra	18	4.3	6	121	107.9	940	206.6	0.6	0.5
3. Osório	7	5.5	18	67	0	640	182.3	0.1	0.8
4. Santana do Livramento	22	5.1	18	222	9.0	2160	401.1	0.2	2.6
5. Santa Vitória do Palmar	13	4.8	14	75	36.0	640	182.3	0.2	2.4
6. São Lourenço do Sul	15	5.1	18	115	27.0	540	218.8	0.3	3.8
7. Uruguaiana	20	4.7	2.6	127	36.0	2180	510.5	1.0	2.9

exc: exchangeable.

Please cite this article in press as: Beneduzi, A. et al., Evaluation of genetic diversity and plant growth promoting activities of nitrogen-fixing bacilli isolated from rice fields in South Brazil, Appl. Soil Ecol. (2008), doi:10.1016/j.apsoil.2008.01.006

conserved 360-bp region of the *nifH* gene. PCR amplifications were carried out as described by Soares et al. (2006). The specificity of amplified DNA bands was checked by hybridization of the PCR products with a probe for the *nifH* gene of *Paenibacillus polymyxa* ATCC 10343 previously amplified and sequenced, using the ECL Direct Nucleic Acid Labeling and Detection System (GE Healthcare). All obtained PCR products were hybridized with the *nifH* gene probe (data not shown). Five thousand nanograms of each PCR product was directly used for restriction enzyme cleavage. The enzyme reaction mixture contained 1× restriction enzyme buffer and 1 U restriction endonuclease. TaqI and HaeIII (Promega) were selected for their specificity for the amplified region of *nifH* (Poly et al., 2001). Digestions were performed overnight to ensure complete fragmentation. Digested DNAs were analyzed in a 10% polyacrylamide gel by electrophoresis for 8 h at 90 V in 1× Tris–borate–EDTA buffer, followed by 30 min of silver nitrate staining (Sambrook and Russel, 2001). This procedure was repeated at least twice for each sample to verify the consistency of the patterns. The whole restriction profile information obtained was used to discriminate each isolate. The statistical analysis and the construction of dendrograms were executed using the NTSYS-PC package, with 1 assigned for the presence and 0 for the absence of band, using the Jaccard coefficient. The UPGMA (Unweighted Pair Group Mathematical Average) algorithm was used to perform hierarchical cluster analysis (Dias, 1998).

2.4. Diversity index

The diversity index (*H'*, Shannon and Weaver, 1949) was estimated based on the number of isolates belonging to each group of profiles in *nifH*-PCR-RFLP. The isolates differed in few bands. Clusters were defined at a 70% similarity cut-off (Borges et al., 2003; Kaschuk et al., 2006). Principal coordinate analysis (PCA) was used to determine the 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 seven soils analyzed in order to obtain a double-centered distance matrix for factoring (Rohlf, 1990).

2.5. In vitro auxin production

Each isolate was grown in GB medium. Optical density was used to control inoculum size (10^5 to 10^6 cfu ml^{-1}). Inocula were transferred (100 μl) to King B medium, which according to Glickmann and Dessaux (1995) is the medium used to quantify auxin production. Sterilized King B medium containing the inoculum was incubated at 30 °C for 72 and 144 h with occasional shaking. The tubes were centrifuged at 15,500 × *g* for 2 min and auxin production was measured as indole-3-acetic acid (IAA) equivalents, as follows: 1 ml of culture supernatant was placed into test tubes containing 1 ml of Salkowski reagent (12 g l^{-1} $FeCl_3$ + 7.9 M H_2SO_4). The tubes containing the mixture were allowed to stand for 30 min, for color development. Intensity of color was spectrophotometrically measured at 550 nm using a standard curve for calibration (Sarwar and Kremer, 1992).

2.6. Siderophore production

Bacterial samples were evaluated for their capacity of siderophore production in Petri dishes containing King B medium supplemented with a complex chrome azurol S [CAS/iron(III)/hexadeciltrimethyl ammonium bromide] as described by Schwyn and Neilands (1987). One drop of culture grown in GB medium for 48 h at 28 °C was inoculated in plates and incubated for at least 7 days. Bacteria that were able to produce siderophores grew and formed a yellow halo in the blue-green media. They were recorded for siderophore production (+) or no siderophore production (−) in relation to the control plates.

2.7. 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 medium (GY, Sylvester-Bradley et al., 1982) containing 10 g glucose, 2 g yeast extract and 15 g agar per liter. Two other solutions were prepared separately, one containing 5 g K_2HPO_4 in 50 ml distilled water, and the other containing 10 g $CaCl_2$ in 100 ml distilled water. These solutions were added to one liter of glucose yeast (GY) medium just before pouring onto Petri dishes, forming insoluble calcium phosphate that made the medium opaque. Bacterial isolates previously grown in GY medium were dropped (10 μl per culture) into the GY plates and incubated for 7 days at 28 °C. Those isolates that formed visible clearing halos around their colonies were considered phosphate solubilizers.

2.8. Partial sequencing of the 16S rRNA gene

The amplification of 16S rRNA gene portions from different bacterial samples was performed in a PCR Express (Thermo Hybaid) thermal cycler in 25 μl reaction volume containing 0.1 mM of each primer, 1.5 mM $MgCl_2$ (Invitrogen), 10 mM of each dNTP (Amersham Biosciences), and 1 U *Taq* DNA polymerase (Invitrogen). The primers used were BacF (GGGAAACCGGGGCTAATACCGGAT, Garbeva et al., 2003) and R1378 (CGGTGTGTACAAGGCCCGGGAACG, Heuer et al., 1997). The thermal cycling was performed as follows: initial denaturation at 94 °C for 5 min, followed by 30 cycles of 94 °C for 1 min, 63 °C for 1 min, 72 °C for 1.5 min, and a final extension at 72 °C for 8 min. The PCR products (expected sizes about 1300 bp) were analyzed by running 5–10 μl aliquots of the reaction mixtures in 1% agarose ethidium bromide gels. Sequences of partial 16S rRNA genes were determined in both the forward and reverse directions with BacF and R1378 primers in the ACTGene Laboratory (Centro de Biotecnologia, UFRGS, RS, Brazil) using the automatic sequencer ABI-PRISM 3100 Genetic Analyzer (Applied Biosystems). Sequences analyses were performed by alignment of the partial 16S rRNA gene sequences to those from the GenBank database, using the program BLAST (NCBI BLAST® homepage). The nucleotide sequences of the 35 partial 16S rRNA gene segments determined in this study have been deposited in GenBank database under accession numbers EU122022 to EU122057.

Please cite this article in press as: Beneduzi, A. et al., Evaluation of genetic diversity and plant growth promoting activities of nitrogen-fixing bacilli isolated from rice fields in South Brazil, Appl. Soil Ecol. (2008), doi:10.1016/j.apsoil.2008.01.006

2.9. *In vitro biological nitrogen fixation assay*

The SVPR30 isolate and control strains were grown in GB medium. Optical density was used to control inoculum size (10^5 to 10^6 cfu ml^{-1}). Inocula were transferred (100 μl) to thiamine-biotin (TB) N-free medium and the amount of *in vitro* biological nitrogen fixation was measured by sulfur digestion and distillation with NaOH 10 mol l^{-1}, as described by Bremner and Keeney (1966). Inoculated TB N-free media were incubated at 30 °C for 7 days in anaerobic jars. The samples were digested with 2 ml concentrated H_2SO_4 ($d = 1.84$), 1 ml H_2O_2 30% and 0.7 g digestion mixture (100 g Na_2SO_4 + 10 g $CuSO_4{\cdot}5H_2O$ + 1 g selenium) at 350–375 °C for 3 h. The products of digestion were distilled with 10 ml NaOH 10 mol l^{-1} and ammonia was measured by titration with H_2SO_4 0.0025 mol l^{-1}.

2.10. *In vivo plant growth promotion experiment with a native PGPR isolate*

A plant growth experiment was carried out with *Oriza sativa* plants, variety BR-IRGA 409, according to Mariano and Silveira (2005) and Döbereiner et al. (1995). Pure bacterial cultures were grown in King B medium at 28 °C and diluted to a final concentration of 10^9 cfu ml^{-1} in sterile distilled water. Rice seeds were surface-sterilized in 70% ethanol for 2 min and 1.2% sodium hypochlorite for 10 min, and rinsed 10 times in sterile tap water. Pots (15 cm × 20 cm) were sterilized with 0.7% sodium hypochlorite solution, filled with sterile vermiculite and seeded. Plants were inoculated with 1 ml aliquots of the bacterial cultures by directly irrigating the substrate. The following treatments were investigated: (1) negative control: plants were irrigated only with distilled water; (2) positive control: plants were irrigated with a 50 ml mixture (v/v) of two mineral fertilizer solutions [Solution 1 was prepared with 4.2 g l^{-1} $MgSO_4$, 1.4 g l^{-1} K_2HPO, and 5.8 g l^{-1} KNO_3; Solution 2 was prepared with 8.5 g l^{-1} Ca $(NO_3)_2$]; (3) type strain: plants were inoculated with *P. polymyxa* ATCC 10343 strain; (4) isolated strain: plants were inoculated with the SVPR30 strain. Treatments (3) and (4) were irrigated only with distilled water. Three seeds were placed at the same depth (2.5 cm below the soil surface) in all pots. Fifteen and thirty days after sprouting, three plants of each treatment were dried at 65 °C to constant weight. Next, dry weight, root and plant sizes were determined. Data obtained from the different treatments were statistically analyzed using the Tukey test at $P = 0.05$. The experiment was conducted three times using a completely randomized design in a greenhouse (12-h photoperiod) with nine plants per treatment. The results obtained in these experiments were very similar; therefore, data from only one experiment are presented.

3. Results

3.1. *Isolation and diversity of PGP bacilli*

A total of 296 bacilli were selectively isolated based on their resistance to high temperature, growth on nitrogen-free medium under anaerobic conditions, and the presence of the *nifH* gene. Of those 296 isolates, 155 were from rhizospheric soil and 141 from bulk soil, representing, respectively, 52.4% and 47.6% of the bacilli isolated.

Strains of nitrogen-fixing bacilli belonging to different species were discriminated by the RFLP-PCR of the *nifH* gene approach using the two selected restriction enzymes, HaeIII and TaqI. The RFLP patterns obtained allowed the construction of a dendrogram for each site sampled. The analysis of each dendrogram revealed that certain genotypes were typical of the rhizospheric soil or of the bulk soil (data not shown).

High genetic diversity was found within the different populations of bacilli: São Lourenço do Sul ($H' = 5.51$), Santa Vitório do Palmar ($H' = 5.23$), Guaíba ($H' = 4.56$), Santana do Livramento ($H' = 3.39$), Uruguaiana ($H' = 2.82$), and Hulha Negra ($H' = 2.27$). It was not possible to calculate the diversity index for the Osório site, since all isolates presented identical band patterns in *nifH*-PCR-RFLP analysis, indicating that the bacilli population in this locality was very uniform and composed of strains belonging to the same genus or even to the same species.

Principal coordinate analysis was used to investigate relationships between bacterial diversity (H') and abiotic soil parameters. The first two dimensions of PCA (PCA1 and PCA2) explained 77.63% of the total variation, with component 1 accounting for 51.56% and component 2 for 26.07% of the variance (Fig. 1). This analysis showed that pH, clay, and organic matter contents were the major soil factors affecting diversity of the six different populations of bacilli (Fig. 1).

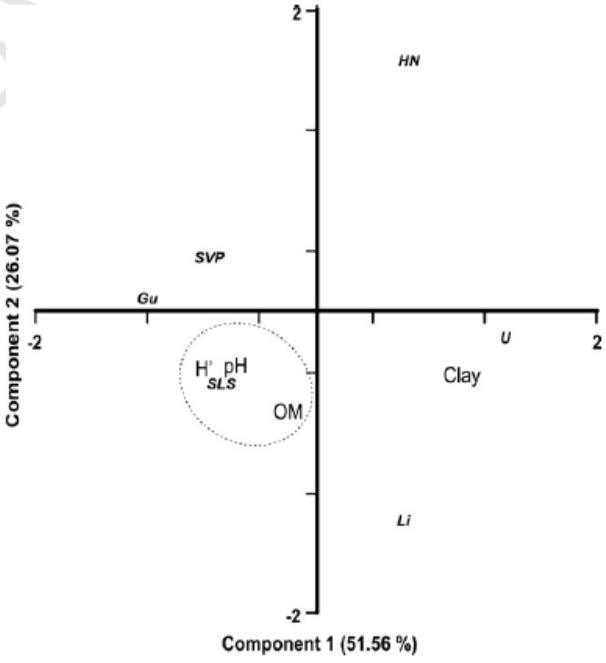

Fig. 1 – Principal coordinate analysis of the diversity indices (H') of the *nifH*-PCR-RFLP profiles of the sampled sites (Gu: Guaíba, HN: Hulha Negra, Li: Santana do Livramento, SVP: Santa Vitória do Palmar, SLS: São Lourenço do Sul, and U: Uruguaiana) in relation to three different soil properties [pH, clay, and organic matter (OM)]. Component 1 and component 2 accounted for 51.56% and for 26.07% of the total variation, respectively.

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Table 2 – IAA and siderophore production, and phosphate solubilization abilities of bacilli isolates

Sampled site		Number of isolates analyzed	Siderophore production	Phosphate solubilization	Production [IAA] (μg ml^{-1})					
					0.1–10, 72 h	11–30, 72 h	>30, 72 h	0.1–10, 144 h	11–30, 144 h	>30, 144 h
1. Guaíba	Rhizosphere	20	0	0	4	1	0	16	2	0
	Soil	20	0	0	0	0	0	13	0	0
2. Hulha Negra	Rhizosphere	22	10	0	17	0	0	17	5	0
	Soil	20	0	0	7	0	0	7	0	0
3. Osório	Rhizosphere	19	1	2	1	0	0	5	1	0
	Soil	21	0	0	0	1	0	2	1	0
4. Santana do Livramento	Rhizosphere	20	4	11	7	4	0	6	5	1
	Soil	21	7	0	6	0	0	6	0	0
5. Santa Vitória do Palmar	Rhizosphere	22	3	1	6	0	1	13	0	1
	Soil	20	4	1	7	3	0	5	3	0
6. São Lourenço do Sul	Rhizosphere	23	1	5	0	0	0	1	0	0
	Soil	20	1	1	5	0	0	5	0	0
7. Uruguaiana	Rhizosphere	28	1	0	10	3	0	14	5	0
	Soil	20	0	1	8	4	0	4	12	0
Total		296	32	22	78	16	01	114	34	02

3.2. *PGP traits of bacilli isolates*

Of the 296 isolates, 94 and 148 produced between 0.1 and 30 μg IAA ml^{-1} *in vitro* after 72 and 144 h of incubation, respectively. Two isolates produced more than 30 μg IAA ml^{-1} 1 after 144 h of incubation. In particular, the isolate SVPR30, from Santa Vitória do Palmar, was the most efficient IAA producer, with 68.27 μg IAA ml^{-1} after 144 h of incubation (Table 2). The control strains *P. polymyxa* LMD 24.16 produced 50 μg IAA ml^{-1} after 72 h of incubation, while *P. polymyxa* ATCC 10343 produced 180 μg IAA ml^{-1} after 144 h of incubation.

Only 22 out of the 296 isolates were able to solubilize phosphate. The rhizospheric soils from Santana do Livramento and São Lourenço do Sul had the highest number of phosphate solubilizer strains in relation to the other sites (Table 2). Of the 296 isolates, 32 were able to produce siderophores. The Hulha Negra site presented the highest

Table 3 – Number of isolates that displayed more than one plant growth promoting trait at the same time per samples site

Sampled site		IAA and siderophore production	Siderophore production and phosphate solubilization	Phosphate solubilization and IAA production	IAA, siderophore production and phosphate solubilization
1. Guaíba	Rhizosphere	0	0	0	0
	Soil	0	0	0	0
2. Hulha Negra	Rhizosphere	0	0	10	0
	Soil	0	0	0	0
3. Osório	Rhizosphere	2	0	1	0
	Soil	0	0	0	0
4. Santana do Livramento	Rhizosphere	7	0	4	0
	Soil	0	0	2	0
5. Santa Vitória do Palmar	Rhizosphere	0	0	3	1
	Soil	1	0	4	0
6. São Lourenço do Sul	Rhizosphere	5	1	1	1
	Soil	1	0	1	0
7. Uruguaiana	Rhizosphere	0	0	1	0
	Soil	1	0	0	0
Total		17	1	27	2

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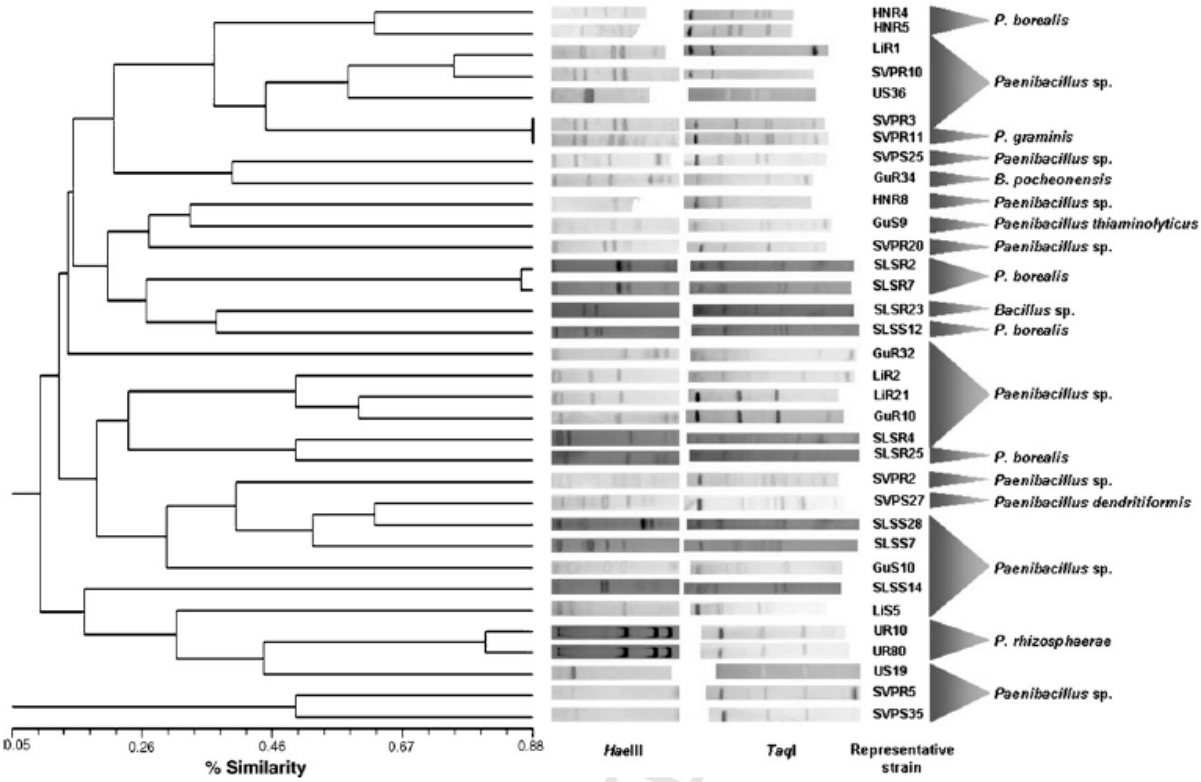

Fig. 2 – Dendrogram based on UPGMA cluster analysis with the NTSYS-PC program using the *nifH*-PCR-RFLP data obtained from 34 representative isolates. The profiles obtained with restriction endonucleases HaeIII and TaqI are shown. Each isolate is identified by the sampled site (Gu: Guaíba, HN: Hulha Negra, Li: Santana do Livramento, SVP: Santa Vitória do Palmar, SLS: São Lourenço do Sul, and U: Uruguaiana) and the place of sampling (S: soil; R: rhizosphere). All these isolates had their 16S rRNA gene partially sequenced and were grouped according to its genus or species designation.

number of rhizospheric soil isolates that produce siderophores (Table 2).

Of the 296 isolates, 17 were able to produce indole-3-acetic acid and siderophores, 27 were able to produce indole-3-acetic acid and solubilize phosphates, only one was able to produce siderophores and solubilize phosphates and two isolates were able to produce indole-3-acetic acid, siderophores and solubilize phosphates at the same time. These bacteria were isolated mainly from the rhizospheric soil of Hulha Negra, Santana do Livramento and São Lourenço do Sul sites, as is demonstrated in Table 3.

3.3. *Identification of PGP bacilli*

From each *nifH*-PCR-RFLP pattern obtained a representative bacterium was chosen for the partial 16S rRNA gene sequence analysis (Fig. 2). This allowed the classification of all isolates in at least a specific genus. The results obtained indicated that *Paenibacillus* and *Bacillus* genera were the most prominent groups in the rhizosphere and soil populations. *P. borealis* was the most frequent species in both locations (Table 4). The two highly related species *Paenibacillus thiaminolyticus* and *P. dendritiformis* were found only in the soil not associated with the roots (Table 4).

Many partial 16S rRNA gene sequences obtained were not similar to any sequence available in the databases. These sequences represent 65.2% and 83.7% of the isolates from rhizosphere and soil, respectively, indicating that these

Table 4 – Distribution of the bacterial population from the rhizosphere and soil of all rice zones sampled

Bacteria	Percentage of the bacterial population from	
	Rhizosphere	Soil
Paenibacillus sp.	31.6	48.2
Bacillus sp.[a]	32.9	35.5
Paenibacillus borealis	14.8	9.2
Paenibacillus rhizosphaerae	7.8	ND
Bacillus pocheonensis	5.8	ND
Paenibacillus pabuli	3.2	ND
Paenibacillus graminis	3.2	ND
Bacillus sp.[b]	0.7	ND
Paenibacillus thiaminolyticus	ND	5.0
Paenibacillus dendritiformis	ND	2.1

ND = Not detected.
[a] *Bacillus* strains with different *nifH*-PCR-RFLP profiles.
[b] *Bacillus* strains with different *nifH*-PCR-RFLP profiles.

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Table 5 – Effects of different experimental conditions on root and shoot length and dry matter of inoculated rice plants within 15 and 30 days after sprouting

Treatments	Root (mm)	Shoot (mm)	Dry matter (mg)
15 days after sprouting			
Negative control	93 b	135 b	30 a
Positive control	190 a	180 ab	38 a
Inoculated with *Paenibacillus polymyxa* ATCC 10343	193 a	203 a	47 a
Inoculated with SVP_R30	235 a	220 a	47 a
30 days after sprouting			
Negative control	151 d	168 d	59 a
Positive control	216 c	230 c	64 a
Inoculated with *Paenibacillus polymyxa* ATCC 10343	250 b	260 b	72 a
Inoculated with SVP_R30	298 a	336 a	82 a

Data presented are mean results from three replicates; each replicate consisted of three plants per jar. Values in the same column followed by the same letter did not differ significantly at $P = 0.05$ (Tukey test).

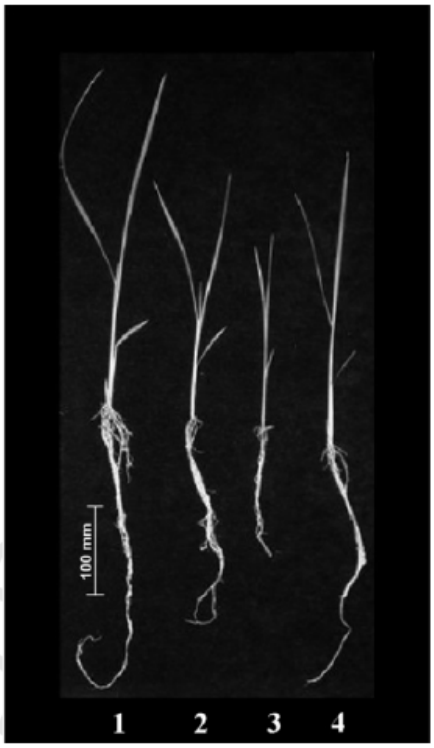

Fig. 3 – The effect of inoculation of a native PGPR isolate on rice growth promotion, 30 days after sprouting. (1) Plant was inoculated with SVPR30 strain; (2) positive control (plant was irrigated with mineral fertilizer solution); (3) negative control (plant was irrigated with distilled water); and (4) plant was inoculated with *Paenibacillus polymyxa* ATCC 10343 strain.

bacteria represent species not yet identified. Nevertheless, some *Paenibacillus* sp. strains identified in this work were phylogenetically very closely related to *P. borealis* and *P. graminis* species (data not shown).

3.4. *Growth promoting effect of bacterial treatment in rice*

In order to test the effective contribution to plant growth of one PGP strain isolated, the isolate SVPR30, identified by 16S rRNA gene sequence as a strain of *Bacillus* sp., was chosen for *in vivo* experiments. This strain was characterized as a high IAA and siderophore producer, being also able to solubilize phosphate and fix a considerably high amount of nitrogen. The amount of *in vitro* biological nitrogen presented by this strain was 6.8 μg N ml^{-1}.

The inoculation of rice with *Bacillus* sp. SVPR30 strain showed a significant increase in the roots and shoot parts of rice plants when compared with the controls within 15 and 30 days after sprouting (Table 5 and Fig. 3).

4. Discussion

4.1. *Isolation and diversity of PGP bacilli*

RFLP-PCR of the *nifH* gene showed that certain genotypes were typical of the rhizosphere or of the bulk soil. Similar results were obtained by Bardgett et al. (1999), which suggested that plant species could affect the soil microbial community more than the physical or chemical soil properties. Elo et al. (2000) also indicated that the bacteria associated with the roots were selected by the plant from the bacterial pool in the soil. On the other hand, Gelsomino et al. (1999) resorted to PCR-DGGE genetic fingerprinting to demonstrate that soil type could be an important determinant of bacterial community structure. In another study, strains of *Paenibacillus graminis* isolated from sorghum and maize sown in a Cerrado soil in Brazil were grouped together. This result could indicate that the diversity of these strains was more affected by soil type than by the plant from which they had been isolated (Vollú et al., 2006).

Zhang et al. (2006) showed that different environmental parameters like the content of soil organic carbon, total nitrogen and altitude could affect the diversity of soil bacteria, including nitrogen-fixing bacteria. However, little information is available about the effects of such parameters (Ramette and Tiedje, 2007). Palmer and Young (2000) also observed that pH, clay and organic matter content may exert some influence on bacterial diversity and survival, and that they can be expected to change according to land management techniques. It was demonstrated that pH could limit the presence of micro-organisms in soils, putting up a barrier to diversity (Brockwell et al., 1991). The soils of the sites sampled in the present study had pH values that ranged from 4.3 to 6.9 (Table 1). Although for bacilli the optimum pH is 7 (Ash et al., 1993), pH tolerance can vary among strains within species. Some species of *Paenibacillus* have been reported to grow in pH ranging between 5 and 9 (Rivas et al., 2005). Fierer and Jackson (2006) considered pH as the best predictor of soil bacterial diversity and richness, with the lower levels of bacterial diversity and richness observed in acid soils. The results

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obtained in this work reinforce the correlation between soil alkalinity and genetic diversity, since the sites with higher diversity indices also had highest soil pH and vice versa (Table 1). Literature data also suggest that parameters like clay contents and soil texture influence survival and proliferation of bacteria in the soil and rhizosphere (Bashan et al., 1995). Sessitsch et al. (2002) observed that soils composed predominantly of clay and fine silt particles had greater diversity of bacteria as compared to soils with large particles.

4.2. PGP traits of bacilli isolates

Our results in regard to IAA production agree with those of Sarwar and Kremer (1992) who also reported that isolates from the rhizosphere were more efficient auxin producers than isolates from bulk soil. Moreover, it has been reported that IAA production by plant growth promoting rhizobacteria (PGPR) can vary among different species and strains, and that it is also influenced by culture condition, growth stage and substrate availability (Mirza et al., 2001). Barazani and Friedman (1999) reported PGPRs that were able to secrete up to 13.5 μg IAA ml^{-1}. Strains of *Enterobacter* sp. isolated from the rhizosphere of sugarcane produced about 2.21 μg IAA ml^{-1} *in vitro*, when tryptophan was added to the medium (Mirza et al., 2001). Taking into account that IAA production by the isolates was measured in a medium lacking extra addition of tryptophan, it is possible to conclude that several of the bacilli isolated in the present study are very efficient IAA producers.

The ability of bacteria to solubilize mineral phosphates and produce siderophores has been of interest to agricultural microbiologists, as it can enhance the availability of phosphorus and iron for microbial and/or plant growth. Therefore, the ability of rhizobacteria to solubilize precipitated phosphates and enhance phosphate availability to rice represents a possible mechanism of plant growth promotion under field conditions (Verma et al., 2001). Only 22 out of the 296 isolates were able to solubilize phosphate. It has been known for long that a considerably higher concentration of phosphate-solubilizing bacteria is commonly found in the rhizosphere, in comparison with non-rhizospheric soil (Raghu and MacRae, 1966). Although several phosphate-solubilizing bacilli occur in soil (Skrary and Cameron, 1998), their numbers are not usually high enough to compete with other bacteria commonly established in the rhizosphere (Lifshitz et al., 1987). The results obtained in this work support this conclusion, since few phosphate solubilizers in the soil not associated to the roots were found (3 out of 22).

Siderophore production is another PGP feature that may influence plant growth by binding to the available iron form (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). Of the 296 isolates, 32 were able to produce siderophores. It has often been assumed that competition for Fe in the rhizosphere is controlled by the Fe affinity of the siderophores, whereby the ligands produced by the biocontrol agent have higher formation constants than those of the pathogen. Other important factors include the concentrations of the various siderophores involved, the kinetics of exchange, and the availability of the Fe complexes to microorganisms that are present (Jurkevitch et al., 1992). It will be important to further investigate the kind of siderophores produced by these isolates and their affinities for different Fe complexes.

4.3. Identification of PGP bacilli

While multiple species of bacilli can be detected in the soils and rhizosphere (Garbeva et al., 2003; Ding et al., 2005; Nishijima et al., 2005), less research has been carried out to indicate the most commonly isolated species. Nitrogen fixation ability in species of the genera *Bacillus* was demonstrated for the first time by Ding et al. (2005). However, Xie et al. (2003) isolated many species of nitrogen-fixing *Bacillus* (*B. megaterium*, *B. cereus*, *B. subtilis*, *B. licheniformis* and *B. azotoformans*) from the rhizospheric zone of rice fields in central China. A substantial number of *Bacillus* species from both environments, rhizosphere and soil, was detected in this work with all isolates belonging to the *Bacillus* or *Paenibacillus* genera. According to Rösch et al. (2002), bacteria belonging to these groups are widely distributed in the soil.

Although several studies reported that the nitrogen-fixing *P. durus* (*P. azotofixans*) and *P. polymyxa* were the most common *Paenibacillus* species isolated from Cerrado soils of Brazil (Seldin et al., 1983, 1998; Rosado et al., 1998), those bacterial species were not detected in our isolates. Screening Japanese soils for the predominant nitrogen-fixing bacilli species, Nishijima et al. (2005) could not find any strain able to fix nitrogen. This result suggested that species like *P. polymyxa* and *P. durus* (*P. azotofixans*) were not present in these soils. A similar result was obtained by Ding et al. (2005), who also could not find these two species when analyzing plant rhizospheres from Beijing. In our samples, it is possible that the differences in temperature and physical properties of Southern Brazilian soils could be responsible for the absence of those *Paenibacillus* species.

Based on the 16S rDNA data, many species of *Paenibacillus* sp. and *Bacillus* sp. were not similar to any sequence available in the databases. Elo et al. (2000) analyzed 16S rDNA sequences and also found unknown species of seven nitrogen-fixing *Paenibacillus* and two Gram-negative bacteria isolated from humus layer of Norwegian spruce stands. Similar results were obtained by Felske et al. (2003). In an effort to isolate novel *Bacillus*-related lineages from soil, these authors obtained 401 bacterial strains from Dutch soil. They found that 60% of those bacteria belonged to unknown species. In another study, Nishijima et al. (2005), in an attempt to reveal the predominant cultivable bacilli present in Japanese arable soils, also reported the isolation of 16% unknown *Paenibacillus* species.

4.4. Growth promoting effect of bacterial treatment in rice

In order to achieve the best results in growth promotion experiments with the strain SVPR30, the same variety of rice from which it was isolated was used. Bhattarai and Hess (1993) had already demonstrated that strains isolated from the same host plant were more efficient in providing benefits to the plants. This strain will be tested in a field experiment in order to confirm its PGP abilities indicated in the glasshouse.

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5. Conclusion

Although it is well known that many species of *Bacillus* and *Paenibacillus* can contribute to plant growth and health in many ways, there are only a few studies concerning the Gram-positive spore forming bacteria. In this work, several bacilli strains displaying important PGP characteristics were isolated, and one (SVPR30) proved to be very efficient in promoting the growth of rice plants. This strain could be useful in the formulation of new inoculants, improving the cropping systems into which it can be most profitably applied. The identification and the isolation of PGP bacilli from temperate and subtropical soils, which combine the ability to fix nitrogen with the production of substances capable of promoting plant growth, could also significantly increase the productivity of grain crops in Brazil.

Q1 Uncited reference

Gillis et al. (1995).

Acknowledgments

The authors are grateful to Dr. Fernanda Bered for its statistical support. This work was financed by a grant and fellowships from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq/Brazil).

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Please cite this article in press as: Beneduzi, A. et al., Evaluation of genetic diversity and plant growth promoting activities of nitrogen-fixing bacilli isolated from rice fields in South Brazil, Appl. Soil Ecol. (2008), doi:10.1016/j.apsoil.2008.01.006

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Please cite this article in press as: Beneduzi, A. et al., Evaluation of genetic diversity and plant growth promoting activities of nitrogen-fixing bacilli isolated from rice fields in South Brazil, Appl. Soil Ecol. (2008), doi:10.1016/j.apsoil.2008.01.006

Capítulo II

Bacillus oryzae sp. nov., a new plant growth promoting rhizobacteria isolated from rhizosphere of rice (_Oryza sativa_) in South Brazil

Submetido ao período *International Journal of Systematic and Evolutionary Microbiology*

Bacillus oryzae sp. nov., a new plant growth promoting rhizobacteria isolated from rhizosphere of rice (*Oryza sativa*) in South Brazil

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ABSTRACT

A bacterial strain, designated SVPR30^T, was isolated from the rhizosphere of *Oryza sativa* in a crop screening devised to isolate strains with plant growth promoting characteristics. SVPR30^T was able to fix nitrogen, produce indole-3-acetic acid and siderophores, and solubilize phosphate. A phylogenetic analysis based on the 16S rRNA gene sequence placed the isolate within the genus *Bacillus*, being most closely related to *B. simplex* species. The isolate comprised Gram positive, motile, facultatively anaerobic bacterium, with spores in terminal position in cells. Starch, casein and aesculin are utilized and dihydroxyacetone and catalase are produced. The DNA G + C content was 43.97 mol %. The main fatty acids are iso and anteiso $C_{15:0}$. DNA relatedness data and the results of phylogenetic and phenotypic analyses showed that strain SVPR30^T should be considered as the type strain of a novel species of the genus *Bacillus*, for which the name *Bacillus oryzae* sp. nov. is proposed. The type strain is SVPR30^T (CCGB 1314 = CECT 7329).

The widely studied *Bacillus* genus represents one of the most diverse genera in the bacilli class (Garbeva *et al*., 2003). Numerous *Bacillus* strains express plant growth promoting (PGP) activities and a number of these strains have already been commercially developed as biological fungicides, insecticides, nematicides or generic plant growth promoters. The use of

these strains in agriculture has recently been reviewed (Gardener, 2004) and there is a need for a better understanding of the factors affecting the ecology and the establishment of PGP rhizobacteria associated with various crops (Lalande *et al*., 1989).

In the present report, we describe the morphological, phylogenetic and physiological characteristics of a novel PGP bacterium, SVPR30^T, isolated from the rhizosphere of *Oryza sativa* cultivated in Rio Grande do Sul State, Brazil.

Aliquots of serially diluted pasteurized (10 min, 80°C) rhizosphere suspensions of rice (*Oryza sativa*) were inoculated onto thiamine-biotin agar (TB N-free medium, Seldin *et al.*, 1983) and incubated in anaerobic jars (Permution) for 7 days at 28°C. Typical anaerobic bacilli colonies were transferred to fresh TB agar plates for another period of anaerobic incubation. Single colonies were then transferred to aerobic GB broth (Seldin *et al*., 1983). A bacterial strain, designated SVPR30^T, was isolated and a pure culture was maintained in a glycerol suspension (20%) at -20ºC.

The morphology of cells was examined by phase-contrast microscopy. Flagellum and spore types were examined with transmission electron microscope (Fig.1) using cells from 48-h cultures in GB broth. Cells were fixed according to Borges *et al*. (2004) and the grids were examined using a model XL-30 transmission electron microscope (Philips). Gram behaviour was ascertained by staining (Doetsch, 1981). Motile was verified by the SIM test (Mac Faddin, 2000). Cells of strain SVPR30^T were Gram positive, rod shaped, sporulated and motile.

The evaluation of desirable characteristics for a PGPR bacterium was performed for nitrogen fixation, indole-3-acetic acid (IAA) and siderophore production and phosphate solubilization. Each strain was grown in TB broth N-free media for biological nitrogen fixation, King B for auxin quantification (Glickmann & Dessaux, 1995), and glucose yeast broth (GYB, Sylvester-Bradley *et al*., 1982) for phosphate solubilization. *Paenibacillus polymyxa* ATCC10343 and *Paenibacillus durus* P3L5^T were used as controls.

For IAA production, the method described by Glickmann and Dessaux (1995) was used. Intensity of color was spectrophotometrically measured at 550 nm after 30 min. A standard curve was used for calibration (Sarwar *et al*., 1992). The isolate SVPR30^T produced 68.27 µg IAA ml^{-1} after 144 h of incubation in a medium without tryptophan.

As mentioned above, the SVPR30^T strain was obtained by screening on nitrogen-free medium. To confirm its nitrogen-fixing ability, an assay for nitrogenase activity and a PCR amplification of the *nifH* gene were carried out. Two degenerate primers (PolF 5' TGC GAY CCS AAR GCB GAC TC 3' and PolR 5' ATS GCC ATC ATY TCR CCG GA 3') were used to amplify a 360-bp fragment of the *nifH* gene, as described by Poly *et al*. (2001). The specificity of the amplified fragment obtained when DNA from SVPR30^T strain was used as template was checked by hybridization, using the *nifH* gene of *Azospirillum brasilense* previously amplified and sequenced as probe. A positive hybridization signal was obtained through the utilization of the ECL Direct Nucleic Acid Labeling and Detection System (GE Healthcare). The amount of *in vitro* biological nitrogen fixation was measured by sulfur digestion and distillation with NaOH 10 mol l^{-1}, as described by Bremner & Keeney (1966). The products of digestion were distillated with 10 ml NaOH 10 mol l^{-1} and the ammonia was measured by titration with H_2SO_4 0.0025 mol l^{-1}. The amount of *in vitro* biological nitrogen presented by this strain was 12.8 µg N ml^{-1}.

The sample was evaluated for its siderophore production capacity in Petri dishes containing King B medium (Glickmann & Dessaux, 1995) supplemented with a complex cromoazurol S [CAS/iron(III)/hexadeciltrimethyl ammonium bromide], as described by Schwyn & Neilands (1987). SVPR30^T strain grew producing a yellow halo in the blue-green media, which indicated its ability to produce siderophores. The method described by Sylvester-Bradley *et al*. (1982) was used to test the ability of SVPR30^T strain to solubilize phosphates. Bacterial strains previously grown in GB broth were dropped (10 µl per culture) into the GY plates and incubated for seven days at 28°C. SVPR30^T isolate formed a visible clearing halo around its colonies; therefore it was considered a phosphate solubilizer.

For 16S rRNA gene sequencing, DNA was directly extracted from bacterial cultures by a direct-lysis method that consisted in boiling the samples for 5 min at 100°C in 200 µl 0.1 M NaCl. Amplification and sequencing of the 16S rRNA gene were performed as described by Heuer *et al*. (1997) and Garbeva *et al*. (2003). An almost-complete (1,171 bp) 16S rRNA sequence was obtained and compared with those deposited in public databases. Sequences were aligned using the CLUSTAL X software (Thompson *et al*., 1997). Evolutionary distances were calculated using the method of Kimura (1980). Phylogenetic trees were inferred using the neighbour-joining method (Saitou & Nei, 1987). Bootstrap analysis was

based on 1,000 resamplings. The MEGA 2.1.0 package (Kumar *et al*., 2001) was used for all analyses.

A comparison of the 16S rRNA gene sequence of strain SVPR30^T and sequences held in GenBank indicated that the organism is phylogenetically related to members of the genus *Bacillus*. Fig. 2 shows the phylogenetic tree obtained with the neighbour-joining method. The closest related recognized species are *Bacillus simplex* WN579^T (98 % similarity) and *Bacillus muralis* LGM20238^T (98 %).

DNA-DNA hybridization was carried out using the initial renaturation-rate method (De Ley *et al*., 1970). The DNA relatedness values for strain SVPR30^T versus *Bacillus simplex* WN579^T and *Bacillus muralis* LMG20238^T were 18 and 18.1 %, respectively. The results show that the isolate SVPR30^T was not closely related to these *Bacillus* species, since the recommended threshold value of DNA-DNA similarity for species definition is 70 % (Wayne *et al*., 1987).

For base composition analysis, DNA was prepared according to the method of Chun & Goodfellow (1995), and the G + C content was determined using the thermal denaturation method (Mandel & Marmur, 1968). The DNA G + C content of strain SVPR30^T was 43.97 mol %, which is between the values obtained for *Bacillus muralis* (41.2 mol %, Heyrman *et al*., 2005) and *Bacillus cibi* (45 mol %, Yoon *et al*., 2005).

The predominant fatty acids were analyzed by GLC as described by MIS Operating Manual (2001) and the results are shown in Table 1. The predominant fatty acids in SVPR30^T strain were iso and anteiso-$C_{15:0}$. According to these results, the fatty acid composition of strain SVPR30^T is similar to those reported for *Bacillus* species (Heyrman *et al*., 2005).

Details of phenotypic characteristics that differentiate strain SBR5^T and phylogenetically related species are given in Table 2. Other characteristics determined are given under the species description below. Phenotypic characterization was performed according to the standard methods described by Claus & Berkeley (1986) and Mac Faddin (2000). The isolate was a Gram-positive, spore-forming, rod-shaped facultatively anaerobic bacterium. These results suggest that this isolate belonged to the genus *Bacillus*. However, the strain did not produce acid from any carbohydrates tested. This characteristic is quite rare among members

of the genus *Bacillus* (Priest *et al*., 1988). SVPR30^T differs from all in growth at 50°C, production of urease and nitrate reduction.

On the basis of the phylogenetic, chemotaxonomic and phenotypic data, we propose that isolate SVPR30^T (= LFB-FIOCRUZ 1314 = CECT 7329) represents a novel species of the genus *Bacillus*, for which the name *Bacillus oryzae* sp. nov. is proposed.

Description of *Bacillus oryzae* sp. nov.

Bacillus oryzae (o.ry'zae. L. gen. n. *oryzae* of rice, from where the type strain was isolated). Cells are rod-shaped, measuring 4.02 x 0.8 µm, Gram-positive, motile by means of peritrichous flagella, facultatively anaerobic and nitrogen-fixing. Spores are in terminal position in cells. Colonies on GB medium are circular, convex, white, and translucent. Usually they are 1-2 mm in diameter within 24 h at 28°C. Optimal growth temperature is 28°C, optimal growth pH is 7. Could grow in the presence of 5% NaCl and at 50°C. Catalase positive. This species is phylogenetically most closely related to *B. simplex*. DNA G + C content is 43.97 mol %. The main fatty acid is anteiso-$C_{15:0}$. Gas is not produced from D-glucose. Acid is not produced from D-glucose, sucrose, D-mannose, lactose, D- xylose, mannitol, L- arabinose, galactose, glycerol, D- fructose, trehalose, meso-inositol, dulcitol, maltose and D- raffinose. Citrate does not serve as carbon source for growth. Starch, casein and aesculin are hydrolyzed and acetoin is produced. Gelatinase, phenylalanine deaminase, indol, hydrogen sulfide and acetoin (in Voges-Prokauer medium) are not produced. Urease is produced. Nitrate is not reduced to nitrite. Strain SVPR30^T, besides its nitrogen-fixing ability, also displays another PGPR characteristics: is able to produce siderophores and indole-3-acetic acid, and to solubilize phosphates. This strain could be useful in the formulation of news inoculants.

The type strain, SVPR30^T (LFB-FIOCRUZ 1314 = CECT 7329), was isolated from the rhizosphere of rice (*Oryza sativa)* in Santa Vitória do Palmar, Rio Grande do Sul State, South Brazil. GenBank accession number EU273353.

Acknowledgements

This work was supported by a grant and fellowships from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Brazil).

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Table 1. Cellular fatty acid composition of strain SVPR30^T and phylogenetically related species of the genus *Bacillus*. Species: 1, *B. oryzae*; 2, *B. simplex*; 3, *B. muralis*. Data for fatty acid composition of strains of *Bacillus* were taken from Heyrman *et al*. (2005).

Fatty acid	**1**	**2**	**3**
Straight-chain:			
$C_{14:0}$	1.53	2.95	4.35
$C_{16:0}$	4.09	6.49	2.27
Iso-branched:			
$C_{14:0}$	6.54	3.92	8.67
$C_{15:0}$	10.94	9.65	22.51
$C_{16:0}$	5.71	2.81	1.61
Anteiso-branched:			
$C_{15:0}$	64.15	61.01	42.69
$C_{17:0}$	3.78	2.70	>1.0
$C_{16:1}$ $\omega 7c$ alcohol	1.85	1.84	4.11
$C_{16:1}$ $\omega 11c$	1.41	6.22	10.33

Table 2. Phenotypic characteristics that differentiate *Bacillus oryzae* sp. nov. from its closest relatives in the genus *Bacillus*.

Species:1. *B. oryzae* sp. nov.; 2. *B. simplex*, data from Heyrman *et al*. (2005), Yoon et al. (2005), Yoon & Oh (2005) and Yumoto *et al*. (2004); 3. *B. muralis*, Heyrman *et al*. (2005). +, Positive reaction; -, negative reaction; v, variable reaction; w, weakly positive. All species are rod-shaped, motile and are positive for starch hydrolysis and catalase. All species are negative for dulcitol, gas production from glucose, indole, hydrogen sulphide production and citrate utilization.

Characteristic	**1**	**2**	**3**
Spore shape *	E	E	E
Spore position †	T	C or S	C
Sporangia swollen	-	-	w
Anaerobic growth	+	v	w
Casein hydrolysis	+	v	v
Gelatin hydrolysis	-	v	-
Aesculin hydrolysis	+	v/w	+
Nitrate reduction	-	+	+
Acid from:			
L-Arabinose	-	v/w	w
Galactose	-	-	w
D-glucose	-	+	+
Glycerol	-	+	+
Fructose	-	+	+
meso-inositol	-	v/w	-
Lactose	-	-	+/w
Maltose	-	-	+
D-Mannitol	-	v	+
Mannose	-	-	+
Raffinose	-	v/w	w
Sucrose	-	+	v
Trehalose	-	+	+
D-Xylose	-	w	-
Growth in the presence of 5% NaCl	+	+	w
Growth at 50°C	+	-	-
Urease	+	-	-

* E, ellipsoidal or oval.

† C, Central or paracentral; S, subterminal; T, terminal.

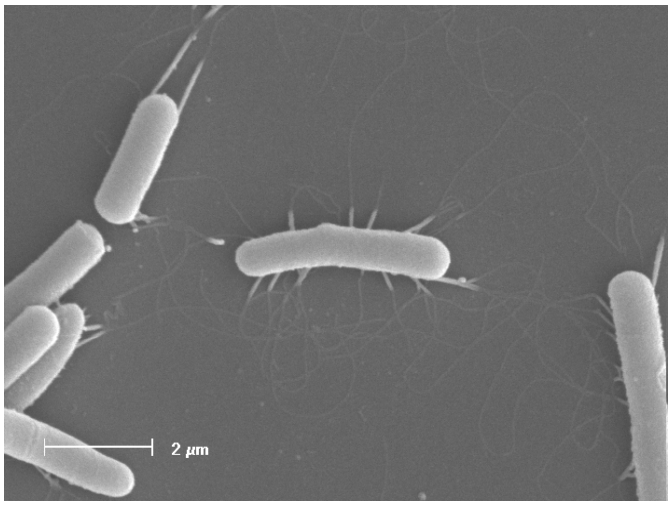

Fig. 1. Scanning electron microscopy of vegetative cells of strain SVPR30^T showing peritrichous flagella

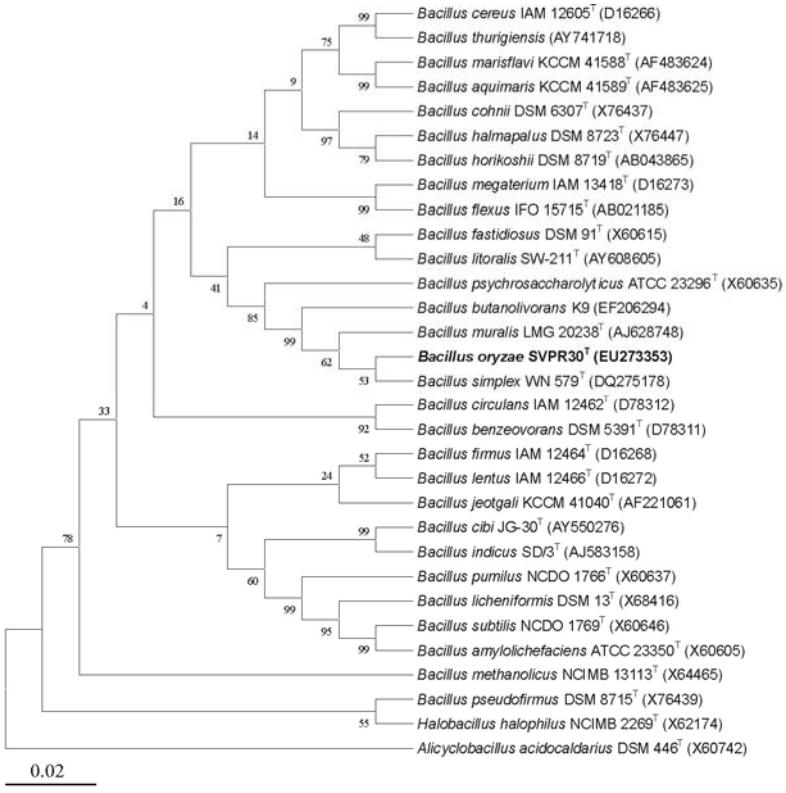

Fig. 2. Neighbour-joining of 16S rDNAs showing the phylogenetic position of strain SVPR30^T compared with species of the genus *Bacillus*. Sequence of *Lactobacillus delbrueckii* subsp. *lactis* was used to root the dendrogram. Bootstrap analyses were made with 1000 cycles. Bar, 2 substitutions per 100 nt.

Capítulo III

Genetic and phenotypic diversity of nitrogen-fixing bacilli isolated from wheat fields in South Brazil

Artigo aceito para publicação na revista *Research in Microbiology*

Genetic and phenotypic diversity of plant growth promoting bacilli isolated from wheat fields in South Brazil

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Abstract

In this work, a total of 311 putative nitrogen-fixing bacilli were isolated from seven distinct wheat production zones of the Rio Grande do Sul State, Brazil. Strains belonging to several species were grouped into 40 different *nifH*-RFLP-PCR profiles. The genus *Paenibacillus* was the most prominent group either in rhizosphere (77.8%) and soil (79%). *P. borealis* was the most abundant identified species followed by *P. graminis*. The remainder of the isolated bacteria belonged to genus *Bacillus* sp. Indolic compounds production [indole 3-acetic acid (IAA), indolepyruvic acid (IPyA), and indoleacetamide (IAM)] was detected in 33.6% and 26% of the isolates from rhizosphere and soil, respectively. Out of the 311 isolates, nine were able to solubilize phosphate and 48 were able to produce siderophores. The isolates SBR5, CSR16 and EsR7 identified by 16S rRNA gene sequence as strains of *Paenibacillus* sp., were chosen for the *in vivo* experiments in a greenhouse and proved to be very efficient in promoting a significant increase in the shoot and dry matter of wheat plants. Those strains could be useful in the formulation of new inoculants, improving the cropping systems into which they can be most profitably applied.

Keywords: Paenibacillus, *Bacillus*, plan growth promoting rhizobacteria, wheat, indolic compounds production, siderophores.

1. Introduction

In general, beneficial free-living soil bacteria are usually referred to as plant growth promoting rhizobacteria or PGPR. PGPR can affect plant growth directly or indirectly. The indirect promotion of plant growth occurs when PGPR lessen or prevent the deleterious effects of one or more phytopathogenic organisms. The direct promotion of plant growth for the most part of PGPR entails either providing the plant with a compound that is synthesized by the bacterium or facilitating the uptake of certain nutrients, like nitrogen (N) or phosphorus (P), from the environment [13].

The restricted availability of major nutrients, like N and P, limits plant growth and yield. The widespread application of single element fertilizers in the cultivation of major crops has led to accelerated exhaustion of other major and minor nutrients leading to nutrient imbalances and poor soil fertility [18]. Bio-fertilizers including microorganisms may add nitrogen to the soil by symbiotic or asymbiotic N_2-fixation. On a worldwide basis, it is estimated that about 175 million tons of nitrogen per year is added to soil through biological nitrogen fixation. Meanwhile super-phosphate fertilizer is expensive and in short supply, but bio-fertilizers can bridge the gap [23]. There are several microorganisms, which can also solubilize the cheaper sources of phosphorus, such as rock phosphate. Bacteria like *Pseudomonas* and *Bacillus* are widely used in organic production system and they are also important phosphorus solubilizing microorganisms, resulting in improved growth and yield of crops [7].

Spore-forming bacteria, typically *Bacillus* species, are one of the major types of soil bacteria. Common physiological traits important to their survival include production of a multilayered cell wall structure, formation of stress-resistant endospores, and secretion of peptide antibiotics, peptide signal molecules, and extra cellular enzymes [11]. Quantitative and qualitative variations in these traits allow for these bacteria to inhabit diverse niches in

agro ecosystems. Their microscopic size and omnipresence in soils facilitate their colonization of plants and animals, but the degree of niche localization of most species has not been thoroughly studied [11].

While multiple species of *Paenibacillus* and *Bacillus* can be detected in the soils and rhizosphere, less work has been done to indicate which might be the most commonly isolated nitrogen-fixing species, in spite of the important ecological role of many bacilli in soil. The objectives of this study were to (i) isolate the predominant nitrogen-fixing bacilli species from different wheat crops, (ii) estimate their diversity, and (iii) evaluate their plant growth promoting activities in order to use them further as inoculant strains.

2. Materials and methods

2.1. Sampling and sample preparation

Samples of rhizosphere and bulk soils were collected from the same wheat variety (v. BRS Louro) in seven distinct wheat production zones of the Rio Grande do Sul State, Brazil: Cachoeira do Sul [CS, (30°02'20"S, 52°53'38"W)], Cruz Alta [CA, (28°38'20"S, 53°36'21"W)], Espumoso [Es, (28°43'30"S, 52°51'00"W)], Passo Fundo [PF, (28°14'46"S, 52°24'25"W)], São Borja [SB, (28°39'39"S, 56°00'14"W)], São Luiz Gonzaga [SLG, (28°24'28"S, 54°57'39"W)] and Vacaria [Va, (28°30'43"S, 50°56'02"W)]. Ten sub-samples of soil (0-15 cm layer) of each field were taken and bulked to obtain a representative soil sample. All samples were collected between July and August 2004.

Putative diazotrophic bacilli were isolated according to Seldin et al. [28]. One g of the soil adhered to the roots, considered the rhizosphere soil, or one g of bulk soil (at a depth of 10 cm) was mixed with nine ml of distilled water and used for the bacterial isolation procedures. The different soil suspensions were pasteurized (10 min, 80°C) to eliminate non-sporulated bacterial forms, and twofold serial dilutions obtained therefrom were plated onto

thiamine-biotin agar (TB N-free medium) and anaerobically incubated in anaerobic jars (Permution) for seven days at 28°C [28]. Typical anaerobic bacilli colonies were transferred to fresh TB agar plates for another period of anaerobic incubation. Single colonies were then transferred to aerobic glucose medium (GB) [28]. Pure cultures were stored at -10°C in 20% glycerol.

2.2. DNA isolation

DNA was directly extracted from bacterial cultures by a direct-lysis method that consisted in boiling the samples for 5 min at 100°C in 200 μl 0.1 M NaCl. DNA quality and integrity was checked by electrophoresis on 0.8% agarose ethidium bromide gel.

2.3. PCR amplification and RFLP analysis of the nifH *gene*

One hundred ng of DNA was used as template in PCR procedures. Selected primers PolF and PolR [24] were used to amplify a 360-bp region of the *nifH* gene. PCR amplifications were carried out as described by Soares et al. [31]. The specificity of amplified DNA bands was checked by hybridization of the PCR products with a probe for the *nifH* gene of *Paenibacillus polymyxa* ATCC 10343 previously amplified and sequenced, using the ECL Direct Nucleic Acid Labeling and Detection System (GE Healthcare). All PCR products obtained hybridized with the *nifH* gene probe (data not shown). Five hundred ng of each PCR product was directly used for restriction enzyme cleavage. *Taq*I and *Hae*III (Promega) were selected for their specificity for the amplified region of *nifH* [24]. Digestions were performed overnight to ensure complete fragmentation. Digested DNAs were analyzed in a 10% polyacrylamide gel stained with silver [25]. The electrophoresis conditions were 8 h at 90 V in 1X Tris-borate-EDTA buffer, followed by 30 min of silver nitrate staining. This procedure was repeated at least twice for each sample to verify the consistency of the patterns. The whole restriction profile information obtained was used for discriminate each isolate. The statistical analysis and the construction of dendrograms were executed using the NTSYS-PC

package, with 1 assigned for the presence and 0 for the absence of band, using the Jaccard coefficient. The UPGMA (Unweighted Pair Group Mathematical Average) algorithm was used to perform hierarchical cluster analysis [6].

2.4. In vitro *indolic compound production*

Each isolate was grown in GB medium. Optical density was used to control inoculum size (10^5 - 10^6 cfu ml^{-1}). Inocula were transferred (100 µl) to King B medium, which according to Glickmann and Dessaux [14] is the medium used to quantify indolic compounds production. This method make use of the Salkowski reagent (12 g l^{-1} $FeCl_3$ + 7.9 M H_2SO_4), and was used to quantify indole 3 acetic acid (IAA), indolepyruvic acid (IPyA), and indoleacetamide (IAM) production, which will be referred in the text collectively as indolic compounds.

2.5. Siderophore production

Bacterial samples were evaluated for their capacity of siderophore production in Petri dishes containing King B medium supplemented with a complex chrome azurol S [CAS/iron(III)/hexadeciltrimethyl ammonium bromide] as described by Schwyn and Neilands [27]. Bacteria that were able to produce siderophore grew and formed a yellow halo in the blue-green media.

2.6. Phosphate solubilization

The method described by Sylvester-Bradley et al. [32] was used to identify isolates able to solubilize phosphates. The medium contained insoluble calcium phosphate that made the medium opaque. The isolates that formed visible clearing halos around their colonies were considered phosphate solubilizers.

2.7. Partial sequencing of the 16S rRNA gene

The amplification of 16S rRNA gene portions from different bacterial samples was performed in 25 µl reaction volume containing 0.1 mM of each primer, 1.5 mM $MgCl_2$

(Invitrogen), 10 mM each dNTP (GE Healthcare), and 1 U *Taq* DNA polymerase (Invitrogen). The primers used were BacF – GGGAAACCGGGGCTAATACCGGAT [10] and R1378 – CGGTGTGTACAAGGCCCGGGAACG [16]. The thermal cycling was performed according Garbeva et al. [10]. The PCR products (expected sizes about 1,300 bp) were analyzed by running 5 to 10 μl aliquots of the reaction mixtures in 1% agarose ethidium bromide gels. Sequences of partial 16S rRNA genes were fully determined in both the forward and reverse directions with BacF and R1378 primers in the ACTGene Laboratory (Centro de Biotecnologia, UFRGS, RS, Brazil) using the automatic sequencer ABI-PRISM 3100 Genetic Analyzer (Applied Biosystems). Analysis of sequences was determined by alignment of the partial 16S rRNA gene sequences to those from the GenBank database, using the program BLAST (NCBI BLAST^{R} homepage). The nucleotide sequences of the 40 partial 16S rRNA gene segments determined in this study have been deposited in GenBank database under accession numbers EU410571 to EU410610.

2.8. In vitro *biological nitrogen fixation assay*

SBR5, CSR16 and EsR7 isolates were grown in GB medium. Optical density was used to control inoculum size (10^5 - 10^6 cfu ml^{-1}). Inocula were transferred (100 μl) to TB N-free medium and the amount of *in vitro* biological nitrogen fixation was measured by sulfur digestion and distillation with NaOH 10 mol l^{-1}, as described by Bremner and Keeney [4].

2.9. In vivo *experiment of plant growth promotion by native PGPR isolates*

Plant growth experiment was carried out with *Triticum aestivum* v. BRS Louro plants according to Mariano and Silveira [21]. Pure bacterial cultures were grown in King B medium (in order to produce indolic compounds) at 28°C and diluted to a final concentration of 10^9 cfu ml^{-1} in sterile distilled water. Wheat seeds were surface-sterilized in 70% ethanol for 2 min and 1.2% sodium hypochlorite for 10 min, and rinsed 10 times in sterile tap water. Pots (15 x 20 cm) were sterilized with 0.7% sodium hypochlorite solution, filled with sterile

vermiculite and seeded. Sterilized wheat seeds were inoculated with one ml aliquots of the bacterial cultures by directly irrigating the substrate. The following treatments were investigated: (1) Control: plants were irrigated with a mixture (v/v) of two mineral fertilizer solutions [Solution 1 was prepared with 4.2 $g.l^{-1}$ $MgSO_4$, 1.4 $g.l^{-1}$ K_2HPO, and 5.8 $g.l^{-1}$ KNO_3; Solution 2 was prepared with 8.5 $g.l^{-1}$ $Ca(NO_3)_2$; (2) sample tested: seeds were inoculated with SBR5, CSR16 and EsR7 strains, separately, and afterwards plants were irrigated only with distilled water. Three seeds were placed at the same depth (2.5 cm below the soil surface) in all pots. Thirty and 45 days after sprouting, three plants of each treatment were dried at 65ºC until a constant weight was reached. Next, dry weight, root and shoot sizes were determined. Data obtained from the different treatments were statistically analyzed using the Tukey test at $P = 0.05$. The experiment was conducted three times using a completely randomized design in a greenhouse (12-h photoperiod) with 9 plants per treatment. The results obtained in these experiments were very similar; therefore, data from only one experiment are presented.

3. Results

3.1. Isolation and identification of isolated bacilli

In this study, a total of 311 putative nitrogen-fixing bacilli, 140 from rhizosphere and 171 from soil, representing, respectively, 45% and 55% of the isolates, were selectively isolated based on their resistance to 80°C for 10 min, their growth on nitrogen-free medium, anaerobic incubation and the amplification of the *nifH* gene.

Strains of putative nitrogen-fixing bacilli belonging to different species were discriminated by the RFLP-PCR of the *nifH* gene approach. The 311 isolates were grouped into 40 different profiles (Fig. 1). One isolate of each cluster was chosen for the 16S rRNA partial gene sequence analysis, in order to identify the 40 isolates that represent each RFLP

profile in at least a specific genus. Although the amplified fragments corresponding to 16S rRNA gene sequences did not contain the full 16S rRNA gene, the determined nucleotide sequences of the 1,300 pb of each isolate analyzed allowed the discrimination between several bacilli species. Table 1 shows the predominant genera identified and their frequencies in rhizosphere and soil. As can be observed, the genus *Paenibacillus* was the most prominent group either in rhizosphere (77.8%) and soil (79%). *P. borealis* was the most abundant identified species followed by *P. graminis*. The remainder of the isolated bacteria belonged to genus *Bacillus* sp. with two different *nifH*-PCR-RFLP profiles. A high percentage of isolates belonging to genus *Paenibacillus*, 63.5% in rhizosphere and 67.3% in soil, presented 16S rDNA sequences that were not similar to any sequence available in the databases, indicating that these bacteria represent species not yet identified.

3.2. Plant growth promoting traits of bacilli isolates

Screening results of plant growth promoting (PGP) traits are depicted in Table 2. Indolic compounds production was detected in 33.6% and 26% of the isolates from rhizosphere and soil, respectively, with amounts varying between 0.1 - 269.4 µg of indolic compounds ml^{-1}. The control strains *P. polymyxa* LMD 24.16 produced 50 µg of indolic compounds ml^{-1} after 72 h of incubation, and *P. polymyxa* ATCC 10343 produced 180 µg of indolic compounds ml^{-1} after 144 h of incubation. The indolic compounds production of the isolates increased significantly after 144 hours of incubation. The sampled sites of Cachoeira do Sul, Cruz Alta and São Borja presented the highest number of indolic compounds producers, while the sampled sites of Passo Fundo and São Luiz Gonzaga showed a very low number of indolic compounds producers. Three isolates, SBR5 from São Borja, CSR16 from Cachoeira do Sul and EsR7 from Espumoso, had produced a very high amount of indolic compounds after 144 h of incubation: 269.4, 112.9 and 112.0 µg of indolic compounds ml^{-1}, respectively.

Only nine out of the 311 isolates were able to solubilize phosphate. The sampled sites of São Borja and São Luiz Gonzaga presented the highest number of phosphate solubilizers strains in relation to the other sites (Table 2). By the other hand, of the 311 isolates, 48 were able to produce siderophores. Cruz Alta and São Borja sites presented the highest number of isolates that produce siderophores (Table 2).

3.3. Growth promoting effect of bacterial treatment in wheat

In order to test the effective contribution to plant growth of PGP strains isolated, the isolates SBR5, CSR16 and EsR7 identified by 16S rRNA gene sequence as strains of *Paenibacillus* sp., were chosen for the *in vivo* experiments in a greenhouse. These strains were characterized as a high indolic compounds and siderophore producers, being also able to solubilize phosphate and fix a considerably amount of nitrogen. The amount of *in vitro* biological nitrogen presented by these strains was of 2.0 µg N ml^{-1} for CSR16, 4.0 µg N ml^{-1} for EsR7 and 8.5 µg N ml^{-1} for SBR5.

Under greenhouse conditions, the inoculation of wheat with SBR5, CSR16 and EsR7 strains showed a significant increase in the shoot and dry matter of wheat plants when compared with the control within 30 and 45 days after sprouting (Table 3). The roots were not significantly affected by the inoculations.

4. Discussion

The occurrence and distribution of microbial communities in the soil and rhizosphere are reported be influenced by many factors like root morphology, the stage of plant growth, root exudates, and the physical and chemical properties of the soil. Previous isolations of nitrogen-fixing bacteria have revealed a broad diversity of diazotrophs that inhabit the crop rhizosphere [35] and many different nitrogen-fixing bacteria have been isolated from roots of several plants [12].

In this study the rhizosphere and soil bacterial populations of wheat, an agriculturally important crop widely cultivated in South Brazil, were screening for the presence of putative nitrogen-fixing and plant growth promoting bacilli. Making use of the RFLP-PCR of the *nifH* gene methodology as preliminary approach, the diversity of the putative nitrogen-fixing bacilli communities isolated in this work was investigated. Forty different RFLP profiles were obtained, indicating that exist a high level of genetic diversity within all analyzed populations. A subset of representative bacteria of each population previously analyzed by the *nifH*-RFLP-PCR was chosen for the 16S rRNA partial gene sequence analysis. This allowed the classification of all isolates in at least a specific genus. The results obtained indicated that *Paenibacillus* and *Bacillus* genera were the most prominent groups in the rhizosphere and soil populations. *P. borealis,* a typical nitrogen-fixing bacterium, was the most known frequent species in both locations (Table 1).

Many partial 16S rRNA gene sequences obtained, corresponding to species of *Paenibacillus* and *Bacillus* genera, were not similar to any sequence available in the databases. These sequences represent 85.7% and 88.3% of the isolates from rhizosphere and soil, respectively, indicating that these bacteria represent species not yet identified. Nevertheless, some *Paenibacillus* sp. strains identified in this work were phylogenetically very closely related to *P. borealis* and *P. graminis* species (data not shown). Elo et al. [8] analyzing 16S rDNA sequences also found unknown species of seven nitrogen-fixing *Paenibacillus* and two Gram-negative bacteria isolated from humus layer of Norwegian spruce stands. In Garbeva et al. [10] most of the unidentified Gram-positive bacteria with low-G+C% content showed close phylogenetic relationships to sequences of *Bacillus* sp. Similar results were obtained by Felske et al. [9] in an effort to isolate novel *Bacillus*-related lineages from Dutch soil. These authors obtained 401 bacterial strains, from which 60% belonged to unknown species.

Screening results for the search for efficient indolic compounds producers showed that isolates from rizosphere produced higher amounts of indolic compounds in comparison to the isolates from soil, especially after 144 h of incubation. Sarwar and Kremer [26] also reported that isolates from the rhizosphere were more efficient auxin producers than isolates from bulk soil. Barazani and Friedman [1] reported PGPRs that were able to secrete up to 13.5 μg of indolic compounds ml^{-1}. Strains of *Enterobacter* sp. isolated from the rhizosphere of sugarcane produced about 2.21 μg of indolic compounds ml^{-1} *in vitro*, when tryptophan was added to the medium [22]. Taking into account that the indolic compounds production by the isolates was measured in a medium lacking extra addition of tryptophan, it is possible to conclude that several of the bacilli isolated in the present study are very efficient indolic compounds producers.

The ability of rhizobacteria to solubilize precipitated phosphates and enhance phosphate availability to wheat represents a possible mechanism of plant growth promotion under field conditions [34]. Only 9 out of the 311 isolates in this work were able to solubilize phosphate. Hameeda et al. [15] in order to improve the growth of maize in India also found only five of the 207 isolates with phosphate-solubilizing ability. These bacteria were isolated from farm waste, rice straw, *Gliricidia* vermicompost, body surface and excreta of macro fauna. Although several phosphate-solubilizing bacteria occur in soil [30], their numbers are not usually high enough to compete with other bacteria commonly established in the rhizosphere [20]. Also, the pH of soils of the sampled regions ranged from 6.7 to 5.9, classified as almost neutral (data not shown). Chen et al. [5] observed that there is an inverse relationship between the pH and soluble phosphate concentration. This observation could indicate that organic acid production by the phosphate-solubilizing strains plays a significant role in the acidification of the medium facilitating the P solubilization. The results obtained in our work support this conclusion, since few phosphate solubilizers were found.

Siderophore production is another PGP feature that may influence plant growth by binding to the available iron form (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 [29]. Of the 311 isolates, 48 were able to produce siderophore (Table 2). It has often been assumed that competition for Fe in the rhizosphere is controlled by the Fe affinity of the siderophores, whereby the ligands produced by the biocontrol agent have higher formation constants than those of the pathogen. Other important factors include the concentrations of the various siderophores involved, the kinetics of exchange, and the availability of the Fe complexes to microorganisms that are present [17]. It will be important to further investigate the kind of siderophores produced by these isolates and their affinities for different Fe complexes.

The results presented in this study indicate the presence of a diverse population of plant promoting growth bacilli in the soil and in the rhizosphere of wheat roots. In order to test the effective contribution to plant growth, the isolates CSR16, EsR7 and SBR5 identified by 16S rRNA gene sequence as strains of *Paenibacillus* sp., were chosen for the *in vivo* experiments in a greenhouse due to their PGPR traits. Aiming to achieve the best *in vivo* results with the strains, the same variety of wheat from which they were isolated was used. Bhattarai and Hess [2] had already demonstrated that strains isolated from the same host plant were more efficient in providing benefits to the plants. The inoculation of wheat with three strains of *Paenibacillus* sp. showed a significant increase in the shoot and dry matter of plants when compared with the control (Table 3). The tested strains had shown a high indolic compounds production that could have affected the elongation of the roots. At relatively high concentrations, natural auxins, such as IAA, stimulate shoot elongation and root induction while reducing root elongation [33]. Indeed, lateral root growth of wheat was reduced at high

inoculation density of *P. polymyxa* CF43 (10^{10} cfu l^{-1}) in the spermosphere model. This might be due to indolic compounds overproduction by this strain at the rhizoplane [19].

Although it is well known that many species of *Bacillus* and *Paenibacillus* can contribute to plant growth and health in many ways, there are only a few studies concerning the Gram-positive spore forming bacteria. While PGPR have been identified within many different bacterial taxa, most commercially developed PGPR are species of *Bacillus* which form endospores that confer population stability during formulation and storage of products [3]. In this work, several bacilli strains displaying important PGP characteristics were isolated. Our results indicated that selected PGPR are able to promote plant growth. Those strains could be useful in the formulation of new inoculants, improving the cropping systems into which they can be most profitably applied. The identification and the isolation of PGP bacilli from temperate and subtropical soils, which combines the ability to fix nitrogen with the production of substances capable to promote the plant growth, will significantly increase productivity of grain crops in Brazil.

Acknowledgments

The authors are grateful to Dr. Fernanda Bered for its statistical support. This work was financed by a grant and fellowships from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq/Brazil).

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Table 1. Distribution of the bacterial population from the rhizosphere and soil of all wheat zones sampled.

Bacteria	Percentage of the bacterial population from	
	Rhizosphere	Soil
Paenibacillus sp.	63.5%	67.3%
Bacillus sp.[a]	8.6%	12.8%
Paenibacillus borealis	10.7%	11.7%
Paenibacillus graminis	3.6%	ND
Bacillus sp.[b]	13.6%	8.2%

ND = Not detected

[a, b] *Bacillus* strains with different *nifH*-PCR-RFLP profiles

Table 2. IAA and siderophore production, and phosphate solubilization abilities of bacilli isolates.

Sampled site		Number of isolates analyzed	Siderophore production	Phosphate solubilization	Production [IAA] µg mL^{-1}					
					72 hours			144 hours		
					0.1-10	11-100	>100	0.1-10	11-100	>100
1. Cachoeira do Sul	Rhizosphere	20	5	1	8	3	0	0	2	1
	Soil	21	1	0	10	1	0	2	2	0
2. Cruz Alta	Rhizosphere	20	10	0	10	2	0	11	2	0
	Soil	22	3	0	7	2	0	4	4	0
3. Espumoso	Rhizosphere	20	3	1	4	3	0	3	4	1
	Soil	22	4	0	4	2	0	7	4	0
4. Passo Fundo	Rhizosphere	20	0	0	1	0	0	3	0	0
	Soil	20	0	0	0	0	0	0	0	0
5. São Borja	Rhizosphere	20	5	1	8	2	1	8	1	1
	Soil	40	9	2	11	0	0	11	0	0
6. São Luiz Gonzaga	Rhizosphere	20	3	2	1	5	0	0	7	0
	Soil	20	2	2	2	4	0	3	3	0
7. Vacaria	Rhizosphere	20	1	0	0	0	0	0	0	0
	Soil	26	2	0	0	0	0	0	0	0
Total		**311**	**48**	**9**	**66**	**24**	**1**	**52**	**29**	**3**

Table 3. Effects of different experimental conditions on root, overground part length and dry matter of inoculated wheat plants within 30 and 45 days after sprouting.

30 days after sprouting

Treatments	Root (mm)	Overground parts (mm)	Dry matter (μg)
Control	11.3	28.8	27.0
Inoculated with SBR5	14.0	35.2*	31.0*
Inoculated with CSR16	13.5	32.6*	31.0*
Inoculated with EsR7	12.9	33.4*	32.0*

45 days after sprouting

Treatments	Root (mm)	Overground parts (mm)	Dry matter (μg)
Control	12.0	35.6	26.0
Inoculated with SBR5	14.0	39.2*	34.0*
Inoculated with CSR16	13.9	37.5*	32.0*
Inoculated with EsR7	14.3	38.1*	31.0*

Data presented are mean results from three replicates: each replica consisted of three plants per jar. *Means are significantly different from control at $P = 0.05$ (Tukey test).

Figure

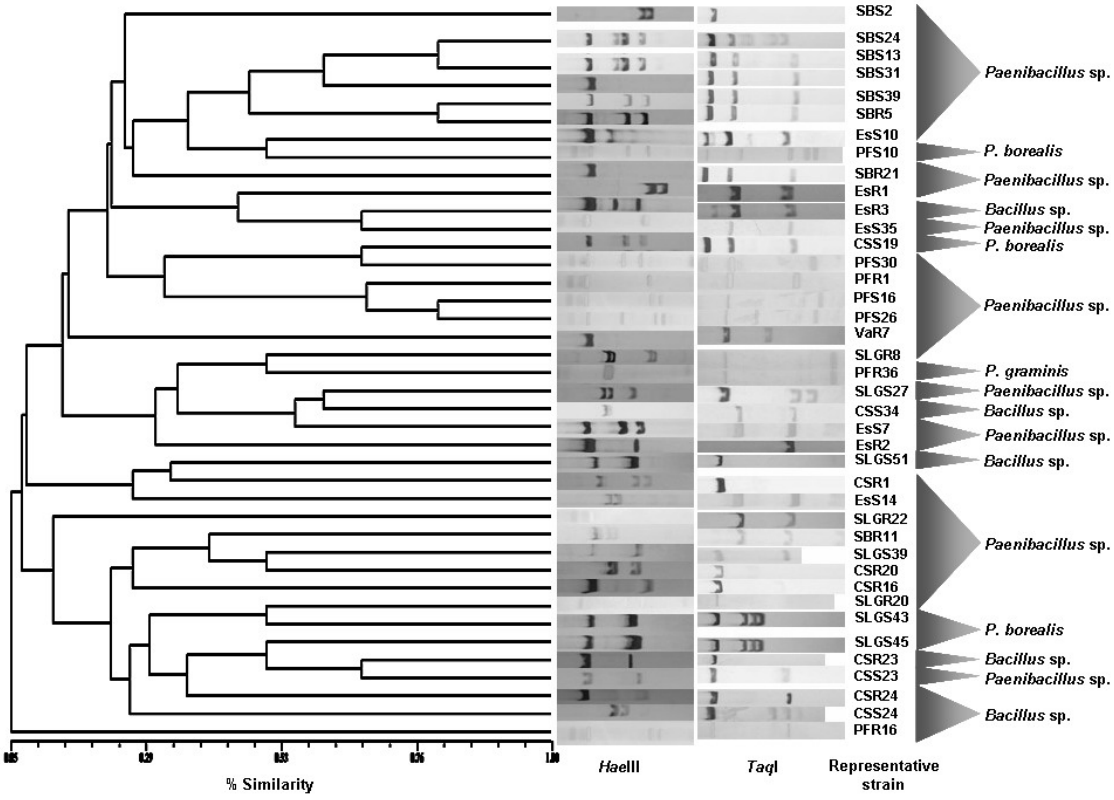

Fig. 1 Dendrogram based on UPGMA cluster analysis with the NTSYS-PC program using the *nifH*-PCR-RFLP data obtained from 40 representative isolates. The profiles obtained with restriction endonucleases *Hae*III and *Taq*I are shown. Each isolate is identified by the sampled site (CS = Cachoeira do Sul, Es = Espumoso, PF = Passo Fundo, SB = São Borja, SLG = São Luiz Gonzaga, and Va = Vacaria) and the place of sampling (S = soil; R = rhizosphere). All these isolates had their 16S rRNA gene partially sequenced and were grouped according to its genus or species designation.

Capítulo IV

***Paenibacillus riograndensis*, a new nitrogen-fixing *Paenibacillus* species isolated from rhizosphere of *Triticum aestivum* in South Brazil**

Submetido ao período *International Journal of Systematic and Evolutionary Microbiology*

Paenibacillus riograndensis, a new nitrogen-fixing *Paenibacillus* species isolated from rhizosphere of *Triticum aestivum* in South Brazil

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ABSTRACT

A bacterial strain designated SBR5^T was isolated from the rhizosphere of *Triticum aestivum*. A phylogenetic analysis based on the 16S rRNA gene sequence placed the isolate within the genus *Paenibacillus*, being most closely related to *P. graminis* species. The isolate is a Gram-variable, motile, facultatively anaerobic bacterium, with spores in terminal position in cells. Starch is utilized and dihydroxyacetone and catalase are produced. Strain SBR5^T displays plant growth promoting rhizobacteria characteristics: ability to fix nitrogen, to produce siderophores, and indole-3-acetic acid. The DNA G + C content was 55.1 mol%. The main fatty acid is anteiso-$C_{15:0}$. DNA relatedness data and the results of phylogenetic and phenotypic analyses showed that strain SBR5^T should be considered as the type strain of a novel species of the genus *Paenibacillus*, for which the name *Paenibacillus riograndensis* sp. nov. is proposed. The type strain is SBR5^T (= CCGB 1313 = CECT 7330).

Bacteria belonging to *Paenibacillus* genus are among the most widely distributed microorganisms and play significant roles in microbial communities (Reva *et al*., 1995). They can be found associated with plants or freely in soils, with potential application in different fields of agricultural biotechnology. In particular, the nitrogen-fixing bacteria related to this genus are promising candidates to crop inoculation, not only for its nitrogen-fixing ability, but

also for its capacity to promote plant growth through the production of phytohormones (auxins and cytokines) and antimicrobial substances (Rosado *et al.*, 1996). Although several species of plant growth promoting rhizobacteria (PGPR) have been already described (Lebuhn *et al*., 1997; Timmusk & Wagner, 1999; Timmusk *et al*., 1999; Helbig, 2001; von der Weid *et al*., 2003), the vast majority of rhizospheric bacterial species present in many soils remains unknown, and their identification could be useful in the formulation of new inoculants to improve crop production.

In the present report, we describe the morphological, phylogenetic and physiological characteristics of a novel PGPR bacterium, SBR5^T, isolated from the rhizosphere of *Triticum aestivum* cultivated in Rio Grande do Sul State, Brazil.

Aliquots of serially diluted pasteurized (10 min, 80°C) rhizosphere suspensions of wheat (*Triticum aestivum*) were inoculated onto thiamine-biotin agar (TB N-free medium, Seldin *et al.*, 1983) and incubated in anaerobic jars (Permution) for 7 days at 28°C. Anaerobic bacilli colonies were transferred to fresh TB agar plates for another period of anaerobic incubation. Single colonies were then transferred to aerobic GB broth (Seldin *et al*., 1983). A bacterial strain, designated SBR5^T, was isolated and a pure culture was maintained in a glycerol suspension (20%) at -20ºC.

The morphology of cells was examined by phase-contrast microscopy. Flagellum and spore types were examined with transmission electron microscope using cells from 48-h cultures in GB broth. Cells were fixed according to Borges *et al*. (2004) and the grids were examined using a model XL-30 transmission electron microscope (Philips). Gram behaviour was ascertained by staining (Doetsch, 1981). Motility was verified by the SIM test (Mac Faddin, 2000). Cells of strain SBR5^T were Gram variable, rod shaped, sporulating and motile. The isolate produced ellipsoidal spores with a regular stripe pattern (Fig.1).

The SBR5^T isolate was also evaluated for the presence of desirable PGPR characteristics. To assess indole-3-acetic acid (IAA) production, the method described by Glickmann & Dessaux (1995) was used. SBR5^T isolate produced 213.7 and 269.4 µg IAA ml^{-1} after 72 and 144 h of incubation, respectively. The amount of *in vitro* biological nitrogen fixation produced by this strain was measured by sulfur digestion and distillation with NaOH 10-mol l^{-1}, as described by Bremner & Keeney (1966), and the value obtained was 8 µg N ml^{-1}. Strain SBR5^T was also analyzed for its siderophore production capacity in Petri dishes containing King B

medium (Glickmann & Dessaux, 1995) supplemented with a complex cromoazurol S [CAS/iron(III)/hexadeciltrimethyl ammonium bromide], as described by Schwyn & Neilands (1987). Strain SBR5^T grew producing a yellow halo in the blue-green media, which indicated its ability to produce siderophores.

For 16S rRNA gene sequencing, DNA was directly extracted from bacterial cultures by a direct-lysis method that consisted in boiling the samples for 5 min at 100°C in 200 µl 0.1 M NaCl. Amplification and sequencing of the 16S rRNA gene were performed as described by Heuer *et al*. (1997) and Garbeva *et al*. (2003). An almost-complete (1,102 bp) 16S rRNA sequence was obtained and compared with those deposited in public databases. Sequences were aligned using the CLUSTAL X software (Thompson *et al*., 1997). Evolutionary distances were calculated using the method of Kimura (1980). Phylogenetic trees were inferred using the neighbour-joining method (Saitou & Nei, 1987). Bootstrap analysis was based on 1,000 resamplings. The MEGA 2.1.0 package (Kumar *et al*., 2001) was used for all analyses.

A comparison of the 16S rRNA gene sequence of strain SBR5^T and sequences held in GenBank indicated that the organism is phylogenetically related to members of the genus *Paenibacillus*. Fig. 2 shows the phylogenetic tree obtained with the neighbour-joining method (an expanded tree is available as Supplementary Fig. A in IJSEM Online). The closest related recognized species are *Paenibacillus odorifer* TOD45^T (95 % similarity), *P. wynnii* LMG22176^T (95 %), *P. borealis* KK19^T (95 %), and *P. graminis* RSA19^T (94 %). According to Wayne *et al*. (1987) strains with approximately 70 % or greater DNA-DNA relatedness are considered to be members of the same species. However, Stackebrandt & Goebel (1994) concluded that the DNA of organisms that have less than 97.0 % 16S rDNA or rRNA similarities would not reassociate to more than 60 %, irrespective of the hybridization method used. It is therefore very likely that strain SBR5^T will not have more than 70 % DNA relatedness with other *Paenibacillus* species and thus can be considered as a new *Paenibacillus* species.

For base composition analysis, DNA was prepared according to the method of Chun & Goodfellow (1995), and the G + C content was determined using the thermal denaturation method (Mandel & Marmur, 1968). The DNA G + C content of strain SBR5^T was 55.1 mol

%, which is similar to that obtained for *Paenibacillus dendritiformis* (Tcherpakov *et al.*, 1999).

The predominant fatty acids were analyzed by GLC as described by MIS Operating Manual (2001) and the results are shown in Table 1. The predominant fatty acids in SBR5^T strain were anteiso-$C_{15:0}$ and $C_{16:0}$. According to these results, the fatty acid composition of strain SBR5^T is similar to those reported for *Paenibacillus* species (Shida *et al*., 1997).

Details of phenotypic characteristics that differentiate strain SBR5^T and phylogenetically related species are given in Table 2. Other characteristics determined are given under the species description below. Phenotypic characterization was performed according to the standard methods described by Claus & Berkeley (1986). Strain SBR5^T differs with respect to *P. graminis* in terms of growth at 40°C, gas production from D-glucose and nitrate reduction; with respect to *P. odorifer*, in terms of nitrate reduction and acid production from D-mannitol; with respect to *P. wynnii*, in terms of spore position and nitrate reduction; and with respect to *P. borealis*, in terms of casein hydrolysis and growth at pH 10. SBR5^T differs from all the above strains in terms of aesculin hydrolysis.

On the basis of the phylogenetic, chemotaxonomic and phenotypic data, we propose that isolate SBR5^T (= LFB-FIOCRUZ 1313 = CECT 7330) represents a novel species of the genus *Paenibacillus*, for which the name *Paenibacillus riograndensis* sp. nov. is proposed.

Description of *Paenibacillus riograndensis* sp. nov.

Paenibacillus riograndensis (ri.o.gran.den.sis. N. L. masc. adj. *riograndensis* referring to Rio Grande do Sul, the State located in the Southern Brazil, where the strain was isolated).

Cells are rod-shaped, measuring 4.12 x 0.75 µm, Gram-variable, motile, aerobic or facultatively anaerobic. Spores are in terminal position in cells. Colonies on GB medium are circular, convex, white, and translucent. Usually they are 1-2 mm in diameter within 24 h at 28°C. Optimal growth temperature is 28°C, optimal growth pH is 7. Could not grow in the presence of 5% NaCl. Catalase positive. This species is phylogenetically most closely related to *P. graminis*. DNA G + C content is 55.1 mol %. The main fatty acid is anteiso-$C_{15:0}$. Gas is not produced from D-glucose. Acid is produced from D-glucose, sucrose, D-mannose, lactose, raffinose, maltose, D-xylose, mannitol, L-arabinose, galactose, glycerol, D-fructose,

trehalose, D-raffinose. Dulcitol, meso-inositol, citrate do not serve as carbon sources for growth. Starch is hydrolyzed. Casein and aesculin are not hydrolyzed and acetoin is not produced. Gelatinase, urease, phenylalanine deaminase, indol, hydrogen sulfide and acetoin (in Voges-Prokauer medium) are not produced. Nitrate is not reduced to nitrite. Strain SBR5^T displays PGPR characteristics: is able to fix nitrogen, to produce siderophores and indole-3-acetic acid.

The type strain, SBR5^T (= LFB-FIOCRUZ 1313, = CECT 7330), was isolated from the rhizosphere of wheat (*Triticum aestivum*) in Rio Grande do Sul State, Southern of Brazil. GenBank accession number EU257201.

Acknowledgements

This work was supported by a grant and fellowships from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Brazil).

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232 **Table 1** Cellular fatty acid composition of strain SBR5^T and phylogenetically related species
233 of the genus *Paenibacillus*. Species: 1, *P. riograndensis*; 2, *P. odorifer*; 3, *P. wynnii*; 4, *P.*
234 *borealis*. Data for fatty acid composition of strains of *Paenibacillus* were taken from
235 Rodríguez-Dias *et al*. (2005).
236

Fatty acid	**1**	**2**	**3**	**4**
Straight-chain:				
$C_{14:0}$	7.96	4.7	6.0	18.8
$C_{16:0}$	17.61	14.7	32.2	12.5
Iso-branched:				
$C_{14:0}$	3.85	4.3	5.3	4.6
$C_{15:0}$	10.26	14.4	5.7	13.2
$C_{16:0}$	8.97	5.5	4.9	9.0
$C_{17:0}$	2.44	2.9	1.3	2.4
Anteiso-branched:				
$C_{15:0}$	45.74	49.3	33.1	37.0
$C_{17:0}$	3.16	2.3	>1	2.3

Table 2. Phenotypic characteristics that differentiate *Paenibacillus riograndensis* SBR5^{T} from its closest relatives in the genus *Paenibacillus*.

Species: 1. *Paenibacillus riograndensis* (data from this study); 2. *Paenibacillus. graminis* (Berge *et al*., 2002); 3. *Paenibacillus odorifer* (Berge *et al*., 2002); 4. *Paenibacillus wynnii* (Rodríguez-Díaz *et al*., 2005); 5. *Paenibacillus borealis* (Elo *et al*., 2001). +, Positive reaction; -, negative reaction; ND, not determined; v, variable reaction. All species are rod-shaped, motile, grow anaerobically and are positive for starch hydrolysis, catalase and acid production from D-glucose, fructose, galactose, lactose, maltose, sucrose, D-xylose and trealose. All species are negative for growth at 50°C, dulcitol and citrate.

Characteristic	**1**	**2**	**3**	**4**	**5**
Spore shape*	E	E	E	E	E
Spore position†	T	T	T	C or S	S or T
Casein hydrolysis	-	ND	ND	-	+
Gelatin hydrolysis	-	-	ND	-	-
Aesculin hydrolysis	-	+	+	+	+
Voges-Proskauer	-	ND	ND	-	-
Nitrate reduction	-	+	+	+	-
Acid from:					
L-Arabinose	+	+	+	v	+
Glycerol	+	+	v	v	+
D-Mannitol	+	+	-	+	+
Mannose	+	+	v	+	+
Raffinose	+	+	+	+	ND
Gas production from glucose	-	+	-	-	-
Growth in the presence of 5% NaCl	-	ND	ND	-	-
Growth at 40°C	-	+	-	-	-
Growth at pH 10	+	ND	ND	+	-
Hydrogen sulphide	-	ND	ND	-	-
Indole	-	ND	ND	-	-
Urease	-	ND	ND	-	ND

* E, ellipsoidal or oval.

† C, Central or paracentral; S, subterminal; T, terminal.

Figures

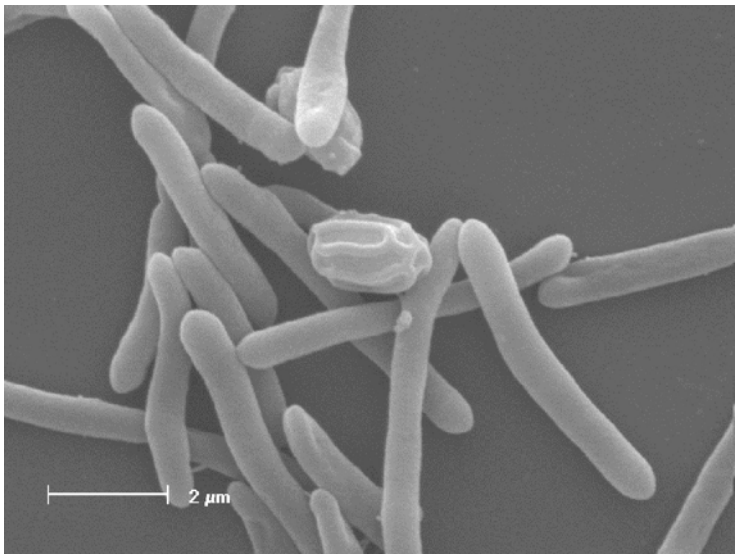

Fig. 1. Scanning electron microscopy of vegetative cells of strain SBR5^T and spores showing a regular pattern of stripes.

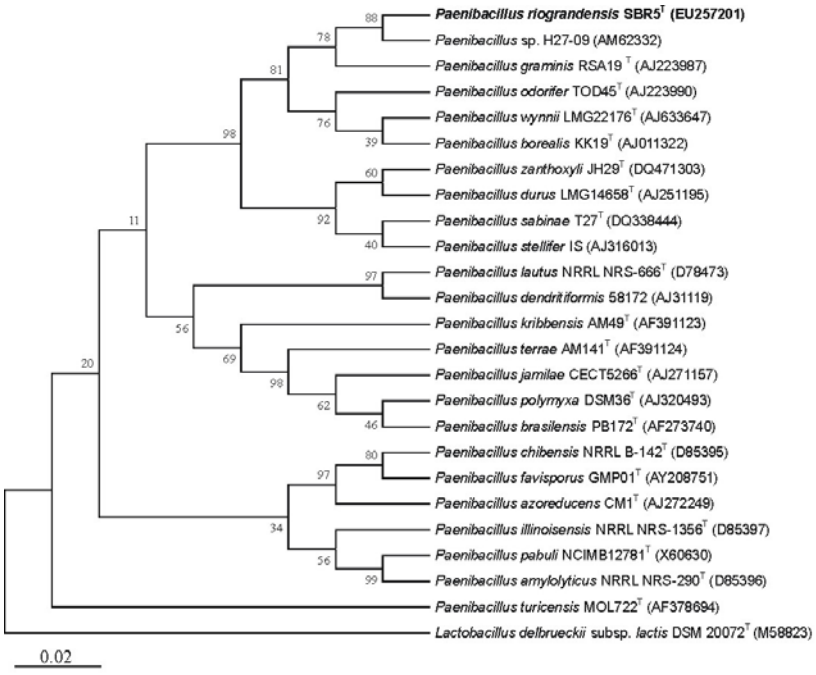

Fig. 2. Neighbour joining of 16S rDNAs showing the phylogenetic position of strain SBR5^T compared with species of the genus *Paenibacillus*. Sequence of *Lactobacillus delbrueckii* subsp. *lactis* was used to root the dendrogram. Bootstrap analyses were made with 1,000 cycles. Bar, 2 substitutions per 100 nt.

4 Considerações finais

Os microrganismos fixadores de nitrogênio são muito diversos, com representantes cultiváveis em todos os grupos filogenéticos de procariotos (Gram positivos, Gram negativos, Archaea e Cianobactérias). Apesar dessa variedade, os diazotróficos de vida livre, ao contrário dos simbiontes, não disponibilizam ou disponibilizam pouco nitrogênio fixado para as plantas. Além da fixação de nitrogênio, há várias maneiras pelas quais as rizobactérias promotoras do crescimento de plantas (PGPRs) podem afetar o crescimento vegetal. Elas podem agir diretamente, através da solubilização de minerais, tais como fósforo, da produção de sideróforos ou pela produção de reguladores de crescimento (fito-hormônios), que acentuam o crescimento vegetal em vários estágios do desenvolvimento. A promoção de crescimento vegetal também pode ser indireta, quando a PGPR diminui ou previne os efeitos deletérios de um ou mais organismos fitopatogênicos. Isto pode acontecer devido à produção de substâncias antagonistas ou através da indução de resistência a patógenos. A bactéria pode contribuir para o crescimento vegetal por um ou mais desses mecanismos e também utilizar diferentes habilidades para promover o crescimento em vários estágios do ciclo de vida da planta. As bactérias que tenham uma ou mais dessas características são potenciais candidatas a serem utilizadas na formulação de inoculantes, a fim de aumentar a produtividade de lavouras.

Mesmo que diferentes PGPRs tenham sido identificadas em diversos taxa bacterianos, muitas delas são espécies de *Bacillus*, que, sob a forma de endósporos, conferem estabilidade populacional durante a formulação e estocagem do produto inoculante. Na forma de endósporos, os bacilos também resistem no solo sob condições de estresse. Outras características comuns, importantes para a sobrevivência dos bacilos, incluem a produção de uma estrutura multicamada de parede celular, secreção de vários tipos de antibióticos, de moléculas sinalizadoras e de enzimas extracelulares. Todas essas características conferem aos bacilos um enorme potencial como inoculantes de lavouras. Há evidências de linhagens de *Paenibacillus* altamente eficientes em fixar nitrogênio em solos tropicais do nodeste brasileiro e Minas Gerais e que são o alvo de estudos na UFRJ (Rosado *et al.*, 1996, 1998a e 1998b; Seldin *et al.*, 1998). Mas, apesar disso, não existem estudos de bacilos com características de PGPRs em solos subtropicais, como os do Rio Grande do Sul. De acordo com a Secretaria de Agricultura e Abastecimento do RS, nosso Estado é o segundo estado brasileiro na produção de grãos (principalmente arroz, soja, milho e trigo), tendo 45% de seu produto interno bruto vinculado ao agronegócio (Secretaria da Agricultura e do Abastecimento, 1999-2000, p.5-10).

Anteriormente, os conhecimentos a respeito da fixação biológica de nitrogênio em bacilos estavam restritos ao gênero *Paenibacillus*, sendo as espécies *P. polymyxa* e *P. durus* (*P. azotofixans*) as mais estudadas. Atualmente se sabe que existem outras espécies de *Paenibacillus* fixadoras de nitrogênio, tais como *P. macerans*, *P. peoriae*, *P. borealis*, *P. graminis*, *P. odorifer*, *P. brasilensis*, *P. wynnii*, *P. sabinae*, *P. zanthoxyli*, *P. massiliensis*, *P. donghaensis* e *P. forsythiae*. Além desses, vários autores encontraram evidências de fixação de nitrogênio também em representantes do gênero *Bacillus* (Li *et al.*, 1992; Ding *et al.*, 2005; Xie *et al.*, 1998 e 2003; Ahmad *et al.*, *in press*; Rózycki *et al.*, 1999).

Considerando-se que não existiam estudos de bacilos com características de PGPRs na região sul do Brasil, as principais lavouras cultivadas com arroz e trigo no estado do Rio Grande do Sul foram escolhidas para a realização desse trabalho. Os objetivos principais do estudo foram a obtenção de dados referentes à diversidade dos bacilos fixadores de nitrogênio presentes no solo e na rizosfera das plantas, a determinação de quais eram as espécies mais comumente encontradas e, também, a avaliação do potencial de promoção de crescimento vegetal dessas linhagens.

Sendo o arroz a lavoura mais cultivada no mundo atual, é extremamente importante saber como usar linhagens de PGPRs que possam fixar nitrogênio biologicamente e produzir substâncias que contribuam para a melhoria do crescimento vegetal, a fim de tornar essa cultura menos dependente da aplicação de fertilizantes. Para alcançar os objetivos propostos no trabalho, foram selecionadas sete regiões do RS fortemente associadas com a cultura de arroz, as quais diferiam em características físico-químicas de solo. Essa preocupação teve o objetivo de avaliar se haveria alguma propriedade do solo que pudesse afetar significativamente a população de bacilos associados ou não às raízes de arroz. Através da análise da coordenada principal (*principal coordinate analysis*, PCA) foi possível determinar que o pH e as quantidades de argila e de matéria orgânica foram os fatores do solo que mais afetaram a diversidade da população bacteriana. Outros autores já haviam observado que estes parâmetros poderiam exercer influência na diversidade bacteriana (Palmer e Young, 2000) e que o pH pode, definitivamente, ser uma barreira para a diversidade (Brockwell *et al.*, 1991; Fierer e Jackson 2006). Normalmente, uma baixa diversidade é encontrada em solos ácidos. O presente estudo corrobora essa afirmação, já que as regiões com pH mais elevado foram as que apresentaram a maior diversidade de bactérias.

Das sete regiões orizícolas amostradas foram isolados 296 bacilos. Em relação às análises de atividade de PGPR, foi observado que os isolados da rizosfera de arroz apresentaram uma produção de AIA maior que os isolados do solo. Foram encontrados

poucos isolados capazes de solubilizar fosfatos e de produzir sideróforos. Enquanto muitas espécies de bacilos podem ser detectadas nos solos e na rizosfera (Garbeva *et al.*, 2003; Ding *et al.*, 2005; Nishijima *et al.*, 2005), não existem trabalhos relatando quais são as espécies mais comumente isoladas. A fim de identificarmos as bactérias isoladas em nível de gênero, foi realizado o seqüenciamento parcial do gene ribossomal 16S (rRNA 16S), utilizando-se um iniciador especifico para bacilos, BacF. Os resultados obtidos indicaram que os gêneros *Paenibacillus* e *Bacillus* eram os predominantes nessas regiões, e as espécies *P. graminis*, *P. borealis* e *P. rhizosphaerae* as mais freqüentes. Contudo, a maioria dos isolados não pode ser identificada em nível de espécie. Muitas dessas bactérias podem consistir de espécies ainda não descritas. Apesar disso, as análises das seqüências do rRNA 16S revelaram que elas estão fortemente relacionadas às espécies *P. borealis* e *P. graminis*. Uma das bactérias isoladas, a linhagem SVPR30, foi escolhida para testes em casa de vegetação. Esse foi justamente o isolado mais eficiente na produção de AIA, capaz de produzir sideróforos e de solubilizar fosfatos. Além disso, essa bactéria, isolada da rizosfera de arroz da região de Santa Vitória do Palmar, foi capaz de fixar nitrogênio. Os testes *in vivo* em casa de vegetação, utilizando-se a mesma variedade de arroz da qual SVPR30 foi isolada, revelaram que essa linhagem bacteriana foi bastante eficiente em promover um aumento significativo das raízes e das partes aéreas do arroz, apresentando-se como um inoculante altamente promissor para lavouras.

A linhagem SVPR30 foi analisada em maiores detalhes, uma vez que havia uma grande probabilidade de tratar-se de uma nova espécie de bacilo, ainda não descrita. Através do seqüenciamento quase total do gene do RNA ribossomal 16S e por diferentes análises fenotípicas e morfológicas, foi concluído que essa linhagem poderia realmente ser considerada como uma nova espécie do gênero *Bacillus*, para a qual foi proposto o nome de *Bacillus oryzae*. Um artigo descrevendo essa nova espécie foi submetido ao *International Journal of Systematic and Evolutionary Microbiology*. O enfoque principal desse artigo foi, então, a descrição de uma nova espécie pertencente ao gênero *Bacillus*, com característica de promotora de crescimento vegetal, isolada da rizosfera de arroz de lavoura do RS.

Um estudo bastante semelhante ao realizado em lavouras de arroz foi conduzido em lavouras de trigo do RS. Um total de 311 isolados de bacilos foi obtido de sete regiões tríticolas gaúchas. Através da técnica de PCR-RFLP do gene *nifH* foram obtidos 40 perfis diferentes e pelo seqüenciamento do rRNA 16S de um representante de cada perfil, observou-se que *Paenibacillus* e *Bacillus* eram, novamente, os gêneros predominantes nas populações bacterianas isoladas, e que as espécies *P. borealis* e *P. graminis* eram as mais abundantes.

Como no estudo anterior com o arroz, os isolados identificados como *Paenibacillus* sp. estão muito próximos filogeneticamente a *P. borealis* e *P. graminis*. Quanto às características de PGPR, os isolados da rizosfera foram produtores de AIA e de sideróforos mais eficientes. Também foram encontrados poucos isolados solubilizadores de fosfato. Através da análise de propriedades físico-químicas dos solos foi constatado que o pH destes era praticamente neutro. Sabe-se da literatura que bactérias solubilizadoras de fosfatos são mais abundantemente em solos ácidos, já que tais bactérias contribuem para a acidificação dos solos ao produzirem ácidos orgânicos, que facilitam a solubilização dos fosfatos (Chen *et al.*, 2006). De acordo com as características de PGPR, três isolados foram selecionados para experimentos *in vivo* em casa de vegetação: CSR16, ER7 e SBR5, utilizando-se a mesma espécie de trigo da qual eles foram isolados. Essas bactérias, além de produzirem sideróforos e solubilizarem fosfato, excretaram uma grande quantidade de AIA e foram identificadas como pertencentes ao gênero *Paenibacillus*. A inoculação de trigo com cada isolado separadamente resultou em um aumento significativo do peso seco e da parte aérea das plantas, mas pouco afetou o crescimento da raiz. Está bem descrito que uma grande quantidade de AIA promove o crescimento das partes aéreas, mas inibe o crescimento da raiz (Tanimoto, 2005).

Assim como SVPR30, o isolado SBR5, proveniente da rizosfera de trigo da região de São Borja, foi analisado em maiores detalhes, visto que o mesmo, também, apresentava uma alta probabilidade de ser uma nova espécie de *Paenibacillus*. Essa bactéria, além das características de PGPR mencionadas anteriormente, apresentou uma alta atividade de fixação de nitrogênio. A análise filogenética, baseada no seqüenciamento quase integral do rRNA 16S, demonstrou que essa linhagem está mais próxima filogeneticamente de *P. graminis*, que também é uma espécie fixadora de nitrogênio. Os resultados das análises, tanto genéticas quanto fenotípicas, mostraram que SBR5 poderia igualmente ser considerada uma nova espécie do gênero *Paenibacillus,* para a qual o nome de *Paenibacillus riograndensis* foi proposto. Um artigo descrevendo essa nova espécie foi encaminhado ao *International Journal of Systematic and Evolutionary Microbiology.* Nesse artigo, o enfoque principal foi a descrição de uma nova espécie de *Paenibacillus*, fixadora de nitrogênio e com características de PGPR, isolada da rizosfera de trigo de uma lavoura do Estado do Rio Grande do Sul.

Após um período de quatro anos dedicado ao estudo da diversidade dos bacilos diazotróficos em solos gaúchos, resultados preliminares importantes foram obtidos nesse trabalho. Sabe-se ainda muito pouco sobre os bacilos diazotróficos que habitam os solos do Brasil e muito estudo ainda se faz necessário. Esta tese é apenas o ponto de partida para

investigações mais abrangentes, em outras lavouras e outras regiões. Além disso, foi evidenciado o enorme potencial para a descoberta de espécies ainda não descritas e que apresentam um futuro promissor como inoculantes para uma agricultura sustentável e livre de adubos químicos. O isolamento e a identificação de bacilos com características de PGPR de solos temperados e subtropicais, que combinem a habilidade em fixar nitrogênio com a produção de substâncias capazes de promover o crescimento vegetal, poderão aumentar significativamente a produção das lavouras no Estado do Rio Grande do Sul e no Brasil.

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