Universidade Federal do Rio Grande do Sul

Seleção e Caracterização de Rizobactérias Promotoras de Crescimento de Milho Cultivadas no Rio Grande do Sul

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Abreviaturas

ADP	*adenosine diphosphate* (difosfato de adenosina)
AIA	ácido Indol Acético
Al	alumínio
Al^{3+}	íon alumínio
ATP	*adenosine triphosphate* (trifosfato de adenosina)
Ca	cálcio
Ca^{3+}	íon cálcio
DNA	*desoxyribonucleic acid* (ácido desoxirribonucléico)
FBN	fixação biológica de nitrogênio
Fe	ferro
Fe^{3+}	íon férrico
H_2	hidrogênio
HPO_4^-	ortofosfato biácido
HPO_4^{2-}	ortofosfato monoácido
N	nitrogênio
N_2	nitrogênio atmosférico
NH_3	amônia
P	fósforo
P_i	fósforo inorganico
PCR	*polymerase Chain Reaction* (reação de polimerização em cadeia)
PGPR	*plant growth promoting rhizobacteria* (rizobactéria promotora de crescimento vegetal)
pH	potencial hidrogeniônico
RFLP	*restriction fragment lenght polymorphism* (polimorfismo dos fragmentos de restrição)
RS	Rio Grande do Sul

Resumo

O uso de fertilizantes minerais nas culturas, inclusive no milho, é uma prática agrícola que provoca danos ambientais e prejuízos econômicos. No Estado do Rio Grande do Sul (RS), grandes quantidades de fertilizantes são necessárias para aumentar a produtividade dessa cultura. Uma alternativa promissora, visando melhorar a produtividade e reduzir o uso de fertilizantes, é a utilização de microrganismos benéficos associados a plantas, particularmente as rizobactérias promotoras de crescimento. Essas bactérias vivem na rizosfera e são capazes de colonizar os tecidos de diversos vegetais, beneficiando o desenvolvimento das plantas através de mecanismos de promoção de crescimento. Esse trabalho teve como objetivo isolar, caracterizar e selecionar bactérias associadas ao milho de diferentes localidades do RS, que tenham capacidade de fixar nitrogênio atmosférico, assim como outras características promotoras de crescimento vegetal, para serem usadas futuramente como inoculantes. Amostras de raiz e solo rizosférico de cinco regiões foram coletadas e de cada amostra foi realizado o isolamento das bactérias. Avaliou-se a capacidade dos isolados de produzirem ácido indol acético (AIA), sideróforos e de solubilizarem fosfatos. A caracterização molecular foi realizada através de PCR-RFLP do gene 16S rDNA para análise da diversidade microbiana e identificação dos isolados. Do total de 292 isolados obtidos, 42% foram capazes de produzir sideróforos, 53% solubilizaram fosfatos e 98% produziram AIA. A partir dos resultados obtidos, seis isolados foram selecionados para o ensaio *in vivo* para promoção de crescimento em duas cultivares de milho. Os isolados utilizados para o experimento foram: *Achromobacter* sp., *Burkholderia* sp., *Chryseobacterium* sp., *Arthrobacter* sp., *Pseudomonas* sp., *Herbaspirillum* sp., além de um inoculante comercial *Azospirillum brasilense* V6. A análise da diversidade microbiana indicou que a associação das bactérias com plantas de milho nas diferentes regiões amostradas pode sofrer influência dos fatores do solo, como o teor de argila. Os resultados estatísticos mostraram que três isolados, *Achromobacter* sp., *Burkholderia* sp. e *Arthrobacter* sp. foram eficientes como promotores de crescimento nas duas cultivares de milho testadas, aumentando a massa seca da parte aérea e da raiz do milho. O isolado *Achromobacter* aumentou o conteúdo de fósforo e nitrogênio nas plantas inoculadas. Os microrganismos identificados nesse trabalho tem potencial para serem utilizados futuramente como inoculantes.

Abstract

Mineral fertilizers use on crops, including maize, is an agricultural practice that causes environmental damage and economic losses. In the state of Rio Grande do Sul (South Brazil), large amounts of fertilizer are needed to increase productivity of this culture. A promising alternative in order to improve productivity and reduce fertilizer utilization is the use of beneficial microorganisms associated with plants, particularly growth promoting rhizobacteria. These bacteria live in the rhizosphere and are able to colonize tissues of many types of plants, and plant development can benefit through mechanisms of growth promotion. This study aimed to isolate, characterize and select bacteria associated with maize from different regions of RS which are capable of fixing atmospheric nitrogen and have other characteristics that promote plant growth, in order to be utilized as inoculant in the future. Root and rhizosphere soil samples were collected from five regions and bacteria were isolated from each sample. Assays were performed for the evaluation of the ability of isolates to produce indole acetic acid (IAA), siderophores, and also solubilize phosphates. A molecular analysis was performed by PCR-RFLP of 16S rDNA gene to examine microbial diversity and to identify the isolates. Of all 292 isolates, 42% were able to produce siderophores, 53% solubilized phosphates and 98% produced IAA. From these results, six isolates were selected for *in vivo* assays for growth promotion in two maize cultivars. The strains used in the experiment were: *Achromobacter* sp., *Burkholderia* sp., *Chryseobacterium* sp., *Arthrobacter* sp., *Pseudomonas* sp., *Herbaspirillum* sp., and commercial inoculant *Azospirillum brasilense* V6. The results obtained for microbial diversity suggest that bacterial association with maize plants in the sampled regions may be influenced by soil characteristics, such as clay content. Statistical results show that three strains - *Achromobacter* sp., *Burkholderia* sp. and *Arthrobacter* sp. - were effective as growth promoters in maize cultivars tested. These isolates increased the dry weight of shoot and root of maize. The isolate *Achromobacter* sp. increased content of phosphorus and nitrogen in inoculated plants. Concluding, the microorganisms identified in this study showed potential to be used as inoculants in the future.

Sumário

1 Introdução

O milho é um dos cereais mais antigos em todo o mundo. Essa cultura é utilizada na alimentação humana e animal, bem como para a geração de matérias-primas para a indústria. No Brasil, possui grande importância econômica e social, tanto na agricultura familiar quanto nos agronegócios. É uma cultura de grande utilização na sociedade moderna, e um dos produtos agrícolas de mais ampla distribuição mundial, seja na produção, seja no consumo. Para obtenção de rendimentos elevados em diversas culturas, em especial na cultura do milho, é necessária a aplicação de fertilizantes minerais ao solo. Porém, a aplicação desses insumos agrícolas, principalmente em gramíneas, além de ser uma das práticas mais caras na agricultura, provoca desequilíbrio nos ecossistemas naturais. O impacto do uso de fertilizantes minerais envolve a acidificação e erosão do solo, aumento da concentração de nitrato na água doce de superfície e do lençol freático, entre outros danos ambientais.

A utilização de microrganismos, aliados ou não a ferramentas genéticas e moleculares, para possíveis melhorias no cultivo e na produtividade de plantas de interesse agronômico tem sido alvo de muitas pesquisas. O uso desses microrganismos na forma de inoculantes biológicos pode ajudar o mercado agrícola, pois é uma das tecnologias mais eficientes em substituir os métodos tradicionais de adubação com fertilizantes, e atualmente é bastante utilizado em culturas leguminosas.

Recentemente, inoculantes comerciais direcionados para a cultura do milho já estão disponíveis no mercado brasileiro. Entretanto, devido ao processo de Fixação Biológica de Nitrogênio (FBN) não ser tão eficiente como para as leguminosas e existindo resultados variáveis relacionados à influência do genótipo da planta, ainda existe a necessidade de pesquisas sobre novos microrganismos que possam ser utilizados como produtos agrícolas, principalmente em gramíneas. O sucesso na procura de novas estirpes deve estar relacionado à capacidade de selecionar, incorporar e manter a sobrevivência de populações benéficas no campo.

1.1 A cultura do milho no RS e o uso de fertilizantes minerais

O milho (*Zea mays* L.), uma planta da família Poaceae, é uma das culturas mais antigas do mundo, havendo indícios de que é cultivado há pelo menos 6200 anos (Piperno e Flannery, 2001). Esse cereal é cultivado em diversas regiões do mundo, sendo bastante utilizado na agricultura familiar e na agroindústria. Na evolução mundial da produção do milho, o Brasil se destaca como o terceiro maior produtor, sendo superado apenas pelos Estados Unidos e China. No período de 2010/11 sua produção mundial ficou em torno de 821 milhões de toneladas, sendo que desses, 39% foram produzidos pelos Estados Unidos, 21% pela China e 6,7% pelo Brasil (USDA, 2012). No âmbito nacional, os Estados do Paraná e Mato Grosso são líderes na produção dessa cultura. Considerando-se que a produtividade média nacional da cultura do milho é cerca de 5.000 $kg.ha^{-1}$, vemos que o Estado do Rio Grande do Sul ainda apresenta uma baixa produtividade média deste cereal, em torno de 4.000 $kg.ha^{-1}$ (CONAB, 2012).

Para obter rendimentos elevados em diversas culturas, especialmente no milho, é necessária a aplicação de nitrogênio (N) ao solo na forma de fertilizantes nitrogenados. As doses de nitrogênio indicadas para essa cultura, segundo Amado *et al*. (2002), podem alcançar valores superiores a 150 $kg.ha^{-1}$, podendo esses valores variarem conforme a quantidade de matéria orgânica presente no solo. Um parâmetro importante a estimar é a eficiência de aproveitamento dos fertilizantes nitrogenados pelas plantas, ou seja, a quantidade de N absorvido na planta proveniente dos fertilizantes. Dados de pesquisa indicaram que, em média, 50% do N aplicado como uréia foram aproveitados pelas plantas (Garabet *et al*., 1998; Halvorson *et al*., 2002). Essa baixa eficiência do uso é principalmente devido a perdas de nitrato por lixiviação, volatilização de amônia e emissões de óxido nitroso, durante os processos de desnitrificação (Bijay-Singh *et al*., 1995; Kennedy *et al*., 2004). Todas estas formas de saída do N do sistema solo-planta causam prejuízos econômicos e impactos ambientais.

Além do N, o fósforo (P) é um importante nutriente necessário para o crescimento das plantas. As doses requeridas desse nutriente para a cultura do milho no RS, na forma de fertilizante fosfatado, variam de 35 a 115 $kg.ha^{-1}$ dependendo do teor de fósforo presente no solo (SBCS, 2004). Mesmo em solos ricos em fósforo, a maior parte deste elemento se encontra na forma insolúvel e apenas uma pequena porção está disponível às

plantas (Kirkby e Johnston, 2008). As plantas absorvem a maior parte do P nas formas de HPO_4^- e HPO_4^{2-}, e suas concentrações são dependentes do valor do pH do solo. Entretanto, quando ocorre a aplicação de adubos fosfatados, o fósforo inorgânico na forma solúvel é rapidamente absorvido, por elementos presentes no solo, como Fe, Al e Ca e, portanto, torna-se indisponível para a planta (Rodriguez e Fraga, 1999), interferindo na eficiência dos fertilizantes fosfatados.

Para reverter esse quadro, a busca por alternativas que possam diminuir a utilização de adubos sem comprometer a produtividade das culturas vem crescendo ao longo dos anos. A formulação de adubos biológicos para diversas culturas, inclusive para a cultura do milho, utilizando microrganismos que se encontram no solo e no interior de tecidos de diversos vegetais, pode auxiliar as plantas na obtenção desses nutrientes essenciais, promovendo o crescimento e aumentando a produtividade da cultura. Dessa forma, tem-se uma alternativa menos agressiva ao meio ambiente diminuindo o uso de fertilizantes.

1.2 Rizobactérias promotoras de crescimento em gramíneas

A rizosfera, local onde o solo está em contato direto com as raízes das plantas, é a região onde ocorre a maior parte das interações entre microrganismos e plantas (Luster *et al*., 2009). Uma grande variedade de bactérias que vivem próximas ou associadas às raízes, sendo estimuladas por vários exudados radiculares, são chamadas de rizobactérias. Alguns desses microrganismos são capazes de promover uma maior absorção de nutrientes e os transferir para as plantas por diversos mecanismos, sendo denominadas de Rizobactérias Promotoras de Crescimento em Plantas (PGPR, do inglês *Plant Growth Promotion Rhizobacteria*). Esse efeito é atribuído a mecanismos como: FBN, produção de substâncias reguladoras de crescimento, produção de antibióticos e sideróforos e solubilização de nutrientes como o fósforo (Hayat *et al*., 2010).

Algumas PGPR são capazes de se associarem aos tecidos vegetais, como caules, folhas e raízes, e essas são chamadas de endofíticas. Um grupo de PGPR endofíticas presentes em diferentes espécies vegetais é formado por bactérias diazotróficas, que são capazes de fixar N_2 atmosférico. Esses microrganismos tem um enorme potencial de uso por sua capacidade de colonizar o interior da planta e estabelecer uma proteção para eles próprios de fatores inibitórios, como o oxigênio. Sendo assim, o interior das plantas

representa um habitat rico em fontes de carbono e livre de diversos fatores adversos que limitam as populações de bactérias de vida livre do solo. Os diazotróficos endofíticos não sofrem limitação de substâncias ricas em carbono, pois estão livres de competição com outros microrganismos e podem transferir muito mais eficientemente os compostos essenciais para o desenvolvimento da planta (Dobbelaere *et al*., 2003). Além disso, eles colonizam nichos específicos no interior das plantas que apresentam reduzida tensão de oxigênio, necessária para a expressão da nitrogenase, enzima responsável pela FBN (James e Olivares, 1997).

Segundo Baldani *et al*. (1997), os microrganismos diazotróficos podem ser divididos em três categorias: (1) endofíticos obrigatórios, que colonizam o interior das raízes e a parte aérea das plantas; (2) endofíticos facultativos, que são capazes de colonizar raízes interna e externamente e (3) microrganismos rizosféricos, são aquelas espécies que colonizam as raízes superficialmente. Nos últimos anos, vem sendo estudado e demonstrado que várias culturas podem ser colonizadas por bactérias diazotróficas, como por exemplo, plantas de milho associadas com *Bulkhoderia unamae* (Caballero-Mellado *et al*., 2004), arroz, com *Serratia marcescens* (Gyaneshwar *et al*., 2001), trigo, com *Achromobacter insolitus* e *Zoogloea ramigera* (Sala *et al*., 2008) e sorgo e cana-de-acúcar, com *Gluconacetobacter diazotrophicus* (Medeiros *et al*., 2006; Luna *et al*., 2010).

O gênero *Azospirillum*, um organismo aeróbico, capaz de fixar nitrogênio atmosférico, tem sido encontrado na rizosfera de muitas gramíneas (Kennedy *et al*., 1997). Entretanto, o padrão de colonização das raízes das gramíneas pelas espécies de *Azospirillum* mostra uma tendência de especificidade entre plantas hospedeiras e as bactérias. As espécies *A. lipoferum e A.brasilense* tem sido isoladas das raízes e folhas de arroz, milho e trigo (Kim *et al*., 2005), enquanto que *A. amazonense* tem sido mais frequentemente detectada nas raízes de cana-de-açúcar (Oliveira *et al*., 2002).

Outro gênero bastante estudado entre as PGPR, porém de ocorrência mais restrita do que os demais, é o gênero *Herbaspirillum*. Espécies de *H. seropedicae* foram isoladas no Brasil por Baldani *et al*. (1986), em associação com raízes de arroz, milho e sorgo. Os autores viram que essa espécie apresenta baixa sobrevivência em solos sem cultivo. *H. seropedicae* também foi encontrada como uma bactéria endofítica, colonizando o interior das raízes de plantas de trigo (Sala *et al*., 2005). Outros autores também constataram que

esse gênero é capaz de colonizar tecidos de plantas de milho, sorgo e arroz (James *et al*., 1997; Monteiro *et al*., 2008).

O gênero *Burkholderia* apresenta ampla distribuição e é encontrado em associação com diversas plantas não-leguminosas. A capacidade de algumas estirpes de *B. cepacia*, isoladas da rizosfera, de fixarem N_2 atmosférico, foi relatada por Bevenino *et al*. (1994). Porém, a primeira espécie de *Burkholderia* diazotrófica descrita foi *B. viatnamiensis*, isolada da rizosfera de plantas de arroz cultivadas no Vietnã (Gillis *et al*., 1995). Estudos posteriores mostraram o surgimento desse gênero em associação com o milho cultivado em diferentes regiões do Brasil e México (Estrada de los Santos *et al*., 2001).

Diversos gêneros pertencentes à Enterobacteriaceae isolados do solo são considerados PGPR. Alguns exemplos são *Enterobacter*, *Klebsiella*, *Citrobacter*, *Pantoea* e *Serratia*. *E. orizae*, uma espécie diazotrófica, foi isolada da superfície de raízes de arroz selvagem *Oryza latifólia* (Peng *et al*., 2009). Além da FBN, foi verificado que espécies de *Klebsiella* também sintetizam fitormônios, como o AIA, promovendo um maior desenvolvimento radicular em culturas como milho e arroz (Chaiharn e Lumyong, 2011) e também em cana-de-açúcar (Govindarajan *et al*., 2007). As raízes de gramíneas, como o milho, possuem uma diversa comunidade de rizobacterias pertencentes à família Enterobacteriaceae, incluindo o gênero *Serratia*. Prieschmann *et al*. (2008) isolaram estirpes desse gênero de raízes do milho e constataram que estas bactérias, além de possuírem características promotoras de crescimento, também podem atuar como antagonistas contra fungos fitopatogênicos.

1.3 Características promotoras de crescimento

As PGPR, tanto de vida livre quanto endofíticas, podem beneficiar as culturas por meio de mecanismos de promoção de crescimento de plantas, e algumas dessas são capazes de apresentarem mais de um mecanismo (Ahmad *et al*., 2008). Gêneros como *Azoarcus*, *Arthrobacter*, *Gluconacetobacter*, *Pseudomonas*, *Serratia*, *Azotobacter*, *Azospirillum*, *Burkholderia, Klebsiella, Herbaspirillum* e *Paenibacillus* são alguns entre uma variedade que podem atuar como promotores de crescimento.

A promoção do crescimento vegetal pode ocorrer direta ou indiretamente (Glick, 1995). A promoção direta envolve a produção de compostos que nutrem ou facilitam a

entrada de certos nutrientes do ambiente para as plantas. Os mecanismos de ação direta incluem: FBN, solubilização de fosfatos, produção de sideróforos ou, ainda, a produção de reguladores de crescimento vegetal (fitormônios), como as auxinas.

O N é o elemento mais limitante para o desenvolvimento das plantas, e a forma como esse elemento se encontra na atmosfera não é assimilável pelas plantas. O processo da FBN, realizado pelas bactérias diazotróficas pode fornecer uma grande quantidade desse nutriente. A FBN, processo de conversão de N_2 atmosférico em NH_3, pelos microrganismos diazotróficos, é catalisada pela enzima nitrogenase pela seguinte reação:

$$N_2 + 8H + 8\,e^- + 16\,ATP \longrightarrow 2\,NH_3 + H_2 + 16\,ADP + 16\,P_i$$

Para realizar esse processo, a bactéria precisa de um complexo enzimático, denominado de nitrogenase, o qual é ativado pela expressão de um conjunto de genes, denominados genes *nif* (*nitrogen fixation*), os quais codificam proteínas envolvidas diretamente neste processo (Arnold *et al*., 1988). Estima-se que a exploração da FBN contribua com 30% do nitrogênio necessário ao desenvolvimento das culturas (Galloway *et al*., 2003), constituindo-se em uma importante via de incorporação de N, contribuindo, assim, para a redução da aplicação de compostos nitrogenados.

O complexo nitrogenase depende do Fe para a sua formação. O Fe é um nutriente essencial para as plantas, mas a forma como ele se encontra no solo é relativamente insolúvel. Como estratégia para a obtenção do Fe, algumas PGPR são capazes de produzir sideróforos. Sideróforos são moléculas transportadoras que são capazes de sequestrar Fe^{+3} presente no solo e transportá-lo para dentro da célula bacteriana tornando-o disponível para as plantas na forma do complexo sideróforo-Fe^{+3} (Benite e Machado, 2002). Com isso, o mecanismo de produção de sideróforos disponibiliza o Fe essencial para o processo da FBN. Entretanto, a produção de sideróforos pelas PGPR somente é ativada sob condições de baixas concentrações de Fe no solo (Crowley *et al*., 1991). Além disso, a produção de sideróforos pode atuar inibindo microrganismos que diminuam o crescimento da planta, pela indisponibilidade de Fe essencial para a proliferação de fitopatógenos (Solano *et al*., 2008). O gênero *Pseudomonas* tem se destacado como produtor de sideróforos, os quais são utilizados para sustentar a sobrevivência e o crescimento das células bacterianas em condições limitantes de Fe (Mercado-Blanco e Bakker, 2007). A inoculação com estirpes

de *Pseudomonas* pode ocasionar o crescimento da planta, devido à supressão de microrganismos fitopatogênicos.

Depois do nitrogênio, o P é o segundo mineral limitante do crescimento vegetal. No solo, há grandes reservas deste elemento nas formas insolúveis, pois é altamente reativo com elementos como Al, Fe e Ca, tornando-o indisponível para as plantas. Entre as bactérias presentes na rizosfera, algumas são capazes de secretar ácidos orgânicos e fosfatases que facilitam a conversão das formas insolúveis em solúveis, disponibilizando esse nutriente para as plantas (Chen *et al*., 2006). De acordo com Venieraki *et al*. (2010), as bactérias solubilizadoras de fosfatos dissolvem o fosfato insolúvel pela produção de ácidos orgânicos no meio em que o microrganismo se desenvolve cuja ação tem sido atribuída às suas propriedades quelantes, possibilitando a formação de complexos estáveis com os íons Ca^{+2}, Fe^{+3} e Al^{+3}. O ácido mais frequentemente observado entre os solubilizadores de fosfatos é o ácido glucônico, mas foram observados também ácidos cetoglucônico, lático, isovalérico, isobutírico, acético, glicólico, malônico e succínico em diferentes espécies de PGPR (Rodríguez e Fraga, 1999).

As populações de solubilizadores são consideravelmente altas em solos rizosféricos, incluindo bactérias dos gêneros *Rhizobium*, *Enterobacter*, *Serratia*, *Citrobacter*, *Klebsiella*, *Pseudomonas*, *Burkholderia*, *Achromobacter*, entre outras (Rodriguez *et al*., 2006). A utilização desses microrganismos como inoculantes em culturas agronomicamente importantes, como o milho, é uma alternativa viável ao uso da adubação convencional, promovendo um melhor aproveitamento do fósforo já existente no solo (Silva Filho e Vidor, 2001).

Outro mecanismo utilizado pelas PGPR é a produção de fitormônios, como as auxinas, citocinas e giberelinas. As auxinas são os fitormônios mais importantes das plantas e são produzidas principalmente na gema apical do caule e transportadas através das células do parênquima até as raízes. Seu principal efeito é promover o crescimento de raízes e caules, através do alongamento das células recém-formadas nos meristemas. Esse efeito depende, no entanto, da concentração do hormônio (Barazani e Friedman, 1999). A principal auxina encontrada nas plantas é o ácido indol-acético (AIA) e as PGPR também podem produzi-lo, através da enzima indolpiruvato descarboxilase na presença do aminoácido triptofano (Prinsen *et al*., 1993).

O AIA, uma auxina mais comumente produzida pelas PGPR, é sintetizada principalmente em rotas bioquímicas dependentes do triptofano, embora alguns autores já tenham demonstrado algumas vias independentes deste aminoácido (Prinsen *et al*., 1993; Cohen *et al*., 2003). Muitas PGPR são capazes de sintetizar AIA, e tem sido estimado que 80% das bactérias isoladas da rizosfera podem produzir esse regulador de crescimento (Patten e Glick, 1996). Entre as várias espécies capazes de sintetizar esse hormônio, os gêneros *Azospirillum* e *Klebsiella* tem mostrado potencial para produção de AIA em condições de laboratório.

Os efeitos da colonização desses gêneros sobre a morfologia e fisiologia das raízes das plantas com as quais se associam são marcantes. Tem sido observadas alterações na densidade e no comprimento dos pelos radiculares, resultando no aumento da superfície do sistema radicular e permitindo melhor exploração dos nutrientes e água do solo (Gutiérrez-Manero *et al*., 1996; Solano *et al*., 2008). Tais efeitos estão associados à capacidade destes e muitos outros microrganismos rizosféricos liberarem substâncias reguladoras do crescimento. Porém, sabe-se que esse estímulo é dependente da dosagem do hormônio, pois o excesso dele pode retardar ou até inibir o crescimento do vegetal (Ahmad *et al*., 2005).

1.4 Biofertilizantes para a cultura do milho

Microrganismos utilizados como inoculantes para gramíneas, podem ser uma fonte de N eficiente que pode parcialmente substituir o N da uréia no cultivo de milho e de outros cereais. Para as espécies vegetais como o milho, a FBN proporciona um acréscimo de 20 a 30% na aquisição de N (Moreira *et al*., 2010). Tais resultados demonstram a contribuição das PGPR em fornecer N para o metabolismo e proporcionar benefícios para o crescimento da planta.

Em plantas de milho, a maioria dos experimentos tem sido conduzida com a inoculação de *Azospirillum*. Algumas espécies como, *A. lipoferum*, *A. indigens* e *A. brasilense* são capazes de aumentar a produtividade do milho. No Brasil, atualmente, *A. brasilense* está sendo utilizado como inoculante para gramíneas como trigo, arroz e o milho, e a sua contribuição para essas culturas pode chegar a 25% de aumento na produtividade (Hungria *et al*., 2010). Entretanto, o conteúdo de N fixado pode variar entre

as espécies de *Azospirillum* e a cultivar do milho. El-Komy *et al*. (1998) em experimentos com solo em casa de vegetação, demonstraram que com o genótipo Giza215, a contribuição variou de 26 a 31% com *A. lipoferum*, mas apenas 17% com a espécie *A. brasilense*. Esses autores demonstraram que esta variação também ocorreu com outro genótipo de milho (Giza310).

Espécies de *Burkholderia* e *Herbaspirillum* tem sido isoladas da rizosfera e do interior de tecidos vegetais de várias culturas no Brasil. Em um estudo de inoculação com *Burkholderia* em plantas de arroz, Baldani *et al*. (2000), relataram que a biomassa da planta aumentou 69%, e que 31% foi devido ao processo da FBN. Alves (2007) verificou que a inoculação de estirpes dos gêneros *Burkholderia* e *Herbaspirillum* contribuiu com até 34% do N absorvido em plantas de milho. Espécies de *Herbaspirillum*, como *H. seropedicae*, tem sido alvo de estudos em plantas de milho. Riggs *et al*. (2001) em um experimento em casa de vegetação, evidenciaram que a inoculação de sementes de milho com essa estirpe proporcionou aumentos significativos (50%) sobre o desenvolvimento da cultura. Entretanto, quando testadas em condições de campo, essa porcentagem caiu para 20%. Atualmente, mais interesse tem sido dado ao estudo dessas bactérias, pois sua ocorrência nos tecidos das plantas de milho tornou estas bactérias promissoras para o suprimento de nitrogênio nos sistemas agrícolas.

Embora exista uma ampla evidência de efeitos positivos na produtividade de diferentes culturas com a inoculação de PGPR, o mecanismo de promoção de crescimento vegetal é um processo complexo que pode ser influenciado por fatores bióticos e abióticos (Shishido e Chanway, 1998; Johri *et al*., 2003; Grover *et al*., 2011). De acordo com Pillay e Nowak (1997), a densidade do inóculo, as condições meteorológicas e o genótipo da planta são alguns dos fatores que podem influenciar a atuação dessas bactérias como promotoras de crescimento. Sendo assim, é necessária, a seleção de espécies e estirpes mais eficientes na promoção de crescimento da cultura do milho que possam ser utilizadas futuramente como biofertilizantes.

2 Objetivos

Isolar, caracterizar e selecionar bactérias associadas ao milho de diferentes localidades do Estado do Rio Grande do Sul, que tenham capacidade de fixar nitrogênio atmosférico, assim como outras características promotoras de crescimento vegetal, para serem usadas futuramente como inoculante.

Os objetivos específicos foram:

a) Avaliar, dentre os isolados bacterianos, a presença de características de promoção de crescimento em plantas, tais como: produção de compostos indólicos, solubilização de fosfatos e produção de sideróforos.

b) Caracterizar molecularmente todas as linhagens bacterianas através de PCR-RFLP do gene 16S rDNA e realizar o sequenciamento parcial deste gene de pelo menos dois isolados que pertençam a um mesmo agrupamento obtido pela técnica de PCR-RFLP.

c) Avaliar a diversidade genética dos isolados nas cinco regiões produtoras da cultura do RS, correlacionando a diversidade com parâmetros do solo de cada região amostrada.

d) Testar *in vivo* as linhagens bacterianas mais eficientes e avaliar o potencial destas no aumento da produção do milho, visando a sua posterior utilização como inoculantes.

Capítulo I

Screening of rhizobacteria isolated from maize (*Zea mays* L.) in Rio Grande do Sul State (South Brazil) and analysis of their potential to improve plant growth

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Screening of rhizobacteria isolated from maize (*Zea mays* L.) in Rio Grande do Sul State (South Brazil) and analysis of their potential to improve plant growth

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Abstract

Plant growth-promoting rhizobacteria (PGPR) are considered to have a beneficial effect on host plants and may facilitate plant growth by different mechanisms. In this work, the influence of different soil types on the bacterial diversity and the stimulatory effects of selected PGPR on two cultivars of maize were investigated. A set of 292 strains was isolated from the roots and rhizosphere soil of maize cultivated in five different areas of

the Rio Grande do Sul State in Brazil. 16S rDNA-PCR-RFLP and 16S rDNA partial sequencing were used for identification, and the Shannon-Weaver index was used to evaluate bacterial diversity. We evaluated the ability of each isolate to produce indole acetic acid (IAA), siderophores and solubilize phosphates. On the basis of multiple PGP traits, six isolates were selected to test their potential as plant growth-promoting rhizobacteria on maize plants. In both the roots and the rhizospheric soil of maize, the dominant bacterial genera identified were *Klebsiella* and *Burkholderia*. IAA producers were distributed widely among isolates, regardless of the sampling site. Approximately 42% of the isolates exhibited at least two attributes, and 24% showed all three PGP traits. Three strains, identified as *Achromobacter*, *Burkholderia*, and *Arthrobacter,* were effective as PGPR in both of the cultivars evaluated.

Keywords: maize; PGPR; IAA; siderophore; phosphate solubilization

1. Introduction

Maize (*Zea mays* L.) is one of the oldest crops and is cultivated in many areas of the world. This grain is used in human food and in animal feed and for generating raw industrial materials. In Brazil, it has economic and social importance in both family farming and agribusiness. It is a crop of great use in modern society and is one of the agricultural products of the greatest worldwide distribution in both production and consumption. To obtain high yields in most crops, as is particularly true in maize, it is necessary to apply mineral fertilizers to the soil. However, the application of agricultural inputs, especially in non-leguminous crops, causes imbalance in natural ecosystems in addition to being one of the most expensive practices in agriculture. The environmental damage caused by the use of fertilizers involves soil erosion, increased concentrations of

nitrate in surface freshwater and groundwater, loss of nitrate by leaching, ammonia volatilization and emissions of nitrous oxide during denitrification (Bijay-Singh et al., 1995; Kennedy et al., 2004). Therefore, although this agricultural practice is necessary, it represents a major economic loss and has an environmental impact.

Research about how soil microorganisms that may play a role in plant fertility may provide a means of reducing the quantities of fertilizers needed has been performed for several decades. Free-living soil bacteria that are beneficial to plants are referred to as plant growth-promoting rhizobacteria (PGPR), and when associated with roots and other tissues, these bacteria improve the supply of nutrients to crop plants by several mechanisms (Kloepper et al., 1989; Saharan and Nehra, 2011). PGPR can promote plant growth directly or indirectly (Glick, 1995). The direct promotion involves mechanisms in which the production of compounds by microorganisms facilitates the uptake of nutrients from the environment into the plants. These mechanisms include the following: biological nitrogen fixation (BNF), solubilization of minerals such as phosphorus (P), siderophore production and the synthesis of growth-promoting substances, including phytohormones. On the contrary, the indirect promotion occurs when the rhizobacteria decrease or prevent the deleterious effects of one or more pathogenic microorganisms (Penrose and Glick, 2003). This process is accomplished by the production of antagonistic substances, leading to better protection of the plants and resultantly increased plant growth.

Rhizobacteria that have these PGP attributes are desirable for possible applications in the field aimed at increasing agricultural production. Many bacteria, including *Klebsiella, Bacillus, Azospirillum, Herbaspirillum, Burkholderia* and *Pseudomonas* spp., have been found to be associated with cereals including maize and other crops. The inoculation of maize with such bacteria has been shown to enhance crop yields (Wu et al.,

2005; Mehnaz et al., 2010). Over the years, many studies have demonstrated the beneficial effect of *Azospirillum* sp. to the plant through the stimulation of root development through the synthesis of phytohormones (Perrig et al., 2007; Spaepen et al., 2008). In Brazil, a commercial inoculant for maize with *Azospirillum brasilense* strains is currently available; however, its performance may be influenced by factors including adaptation to different soil types (de Oliveira et al., 2006), different weather conditions (Saharan and Nehra, 2011) and variability in response to genotypes (Han and New, 1998).

Rio Grande do Sul is the southernmost state in Brazil and is characterized by a wide diversity of soil types and climates, which differs from the rest of the country. This diversity necessitates the selection and screening of rhizobacterial strains adapted to different soil conditions, weather conditions and maize cultivars for possible commercial applications. Therefore, the purpose of this work was to select and characterize growth-promoting rhizobacteria associated with maize plants from different sites in Rio Grande do Sul and to assess their potential to increase the growth of two maize cultivars. The diversity of rhizobacteria in each soil sample was evaluated through 16S rDNA restriction analysis.

2. Materials and Methods

2.1. Soil sampling and rhizobacteria isolation

Samples of rhizosphere soil (soil adhered to root) and the roots of maize plants were collected from the following five regions in Rio Grande do Sul State, Brazil: Júlio de Castilhos, Porto Alegre, Rio Grande, Vacaria and Veranópolis. Soil samples were taken for the determination of pH, clay percentage, P, K, Al, Ca, Mg and total soil organic matter (Table 1). Root samples were surface-sterilized and macerated prior to rhizobacterial

isolation. Ten-gram samples of roots or rhizosphere soil were suspended in sterile saline solution (0.85% NaCl) and were incubated at 28ºC with shaking for 16 h. Aliquots (0.1 ml) from serially diluted samples (up to 10^{-3}) were added to three different semi-solid nitrogen (N)-free culture media, NFB, LGI and LGI-P. The procedures for the isolation of bacteria were performed according to Döbereiner et al. (1995). Pure-culture isolates were preserved in 50% glycerol and were stored at -20ºC.

2.2. DNA extraction

The boiling method was adapted from Leuko et al. (2008) for the extraction of total DNA. The strains were grown in vials containing 5 ml of Luria-Bertani (LB, Sambrook and Russel, 2001) culture medium for 48 h with shaking. After this period, 100 µl was collected from the cultures, transferred to sterile microtubes and then incubated at 99ºC in a dry bath (*LGC* Biotecnologia) for 5 min. Next, the samples were centrifuged for 5 min at 10,000 rpm, and the tubes were placed directly on ice. Finally, 5 µl of the supernatant were collected for the PCR amplification reactions of the 16S rDNA gene.

2.3. 16S rDNA gene amplification and restriction fragment length polymorphism (RFLP) analysis

Bacterial isolates were subjected to polymerase chain reaction (PCR) for amplification of the 16S rDNA gene. The primers U968 (AACGCGAAGAACCTTAC) and L1401 (CGGTGTGTACAAGACCC) (Felske et al., 1999) were used to amplify fragments of approximately 450 bp. The PCR reactions contained 50 ng of template DNA, 1X reaction buffer, 1U *Taq* DNA polymerase (Invitrogen®), 100 µM of each deoxynucleotide, 1 µM of each primer, 50 mM $MgCl_2$ and ultrapure sterile water to a final

121 volume of 25 µl. The amplification was performed by an initial denaturation at 94ºC for 5
122 min, followed by 30 amplifications cycles of denaturation at 94ºC for 1 min, annealing at
123 56ºC for 1 min, and extension at 72ºC for 1 min, and a final extension step at 72ºC for 5
124 min. The PCR products were visualized by electrophoresis in 1% agarose gel stained with
125 Blue Green Loading Dye I (*LGC* Biotecnologia).

126 The restriction procedure adapted from Widmer et al. (1999) was performed with
127 the addition of 6 µl of PCR product to a mixture of ultrapure water, 2 U of restriction
128 enzyme and its respective buffer. The restriction enzymes used were *Hae*III at 37ºC and
129 *Taq*I at 65ºC, and the digestions were performed to completion overnight. The products of
130 digestion were separated by electrophoresis in 10% polyacrylamide gel at a current of
131 200V for 3 h, and the gels were then stained with silver nitrate (Sambrook and Russel,
132 2001).

133

134 *2.4. DNA fingerprint analysis and 16S rDNA gene sequencing*

135 The software Gel-Pro Analyzer 3.1 was used for the analysis of the 16S rDNA-
136 RFLP through the construction of a binary matrix comprising the total of the bands of the
137 profiles generated by the two enzymes, in which 0 indicated the absence of a band and 1 its
138 presence. The genetic similarity/dissimilarity between the isolates was measured by the
139 Jaccard coefficient (i, j), which does not consider any negative similarities. The matrices
140 were analyzed with the software Paleontological Statistics (PAST) with dendrograms
141 obtained by the UPGMA (*Unweighted Pair Group Method with Arithmetic Averages*)
142 method within the Multivar Cluster Analysis application of PAST (Hammer et al., 2007).
143 From each resulting cluster, one or two isolates were selected for 16S rDNA sequencing.
144 The PCR products of partial 16S rDNA genes were sequenced in the ACT-Gene

145 Laboratory (Centro de Biotecnologia, UFRGS, RS, Brazil) using the automatic sequencer
146 *ABI-PRISM 3100 Genetic Analyzer* 50 cm capillary (Applied Biosystems). Sequence
147 analyses of 16S rDNA were performed with the program BLASTN (NCBI BLAST®
148 homepage). The nucleotide sequences of the partial 16S rDNA gene have been deposited
149 in the GenBank database under the accession numbers JQ736362-JQ736463.
150
151 *2.5. Diversity index*
152 The diversity index (*H'*, Shannon and Weaver, 1949) was estimated based on the
153 number of isolates belonging to each cluster of profiles of the 16S-PCR-RFLP. Principal
154 coordinate analysis (PCA) was used to determine the statistical correlation between soil
155 properties and population diversity (Rico et al., 2004).
156
157 *2.6. In vitro indole acetic acid production*
158 The production of indole acetic acid was evaluated according to Asghar et al.
159 (2002). Bacterial cultures were grown in King's B medium supplemented with L-
160 Tryptophan (500 $\mu g.ml^{-1}$) and incubated at 28ºC for 5 days. The cultures were centrifuged
161 at 7,000 rpm for 5 min, and the supernatant was quantified spectrophotometrically at 535
162 nm with Salkowski's reagent (2 ml 0.5 M $FeCl_3.6H_2O$; 98 ml 35% $HClO_4$). The level of
163 IAA produced was estimated from a standard IAA curve and was expressed as micrograms
164 per milliliter (Sarwar and Kremer, 1992).
165
166 *2.7. Siderophore production*
167 To test for the production of siderophores, the bacterial isolates were first grown for
168 48 h at 28ºC in LB medium. One drop of each isolate was inoculated onto Petri dishes

containing King's B medium supplemented with a complex chrome azurol S (CAS/Fe^{+3}/hexadecyltrimethyl ammonium bromide) as described by Lavaca et al. (2008). The plates were incubated at 28ºC for 2 days, and the ability of isolates to convert the blue color of the medium surrounding the bacterial colonies to yellow/red indicated production of siderophore.

2.8. Phosphate solubilization

All bacterial isolates were screened for inorganic phosphate solubilization according to the method of Sylvester-Bradley et al. (1982). Bacteria were inoculated onto GL medium containing inorganic phosphate, and the plates were incubated at 28ºC for 5 days. A clear halo around the bacterial colony was considered positive for phosphate solubilization.

2.9. Plant growth promotion assay

Strains were selected based on their previously evaluated PGP traits for growth promotion testing (Table 2). The experiment comprised nine treatments (seven strains and two controls, negative and positive) applied to two cultivars of maize, the hybrid Pioneer 30R50 and the varietal Fepagro 35, with four replicates, in a 9 x 2 factorial design. Maize seeds of each cultivar were surface-sterilized with 70% ethanol for 2 min and in 1% sodium hypochlorite, followed by ten washes in sterile distilled water. Bacterial cultures were grown in LB medium at 28ºC and diluted in sterile distilled water to a final concentration of 10^9 cfu.ml^{-1}. Seed inoculation was performed with the strains AGR27 (*Pseudomonas* sp.), VC36 (*Achromobacter* sp.), VC50 (*Chryseobacterium* sp.), VN50 (*Herbaspirillum* sp.), RG38 (*Burkholderia* sp.), TU39 (*Arthrobacter* sp.) and the

commercial strain *Azospirillum brasilense* V6 by soaking the seeds in bacterial suspensions at room temperature for 1 h. The seeds were sown in Leonard jars containing a mixture of vermiculite and sand (2:1, v/v) and 25% Hoagland nutrient solution. Maize seeds were then planted at a depth of 2 cm. Three seeds were sown per pot, with subsequent thinning after emergence to establish one plant per pot. After a period of 7 days from sowing, a total of 100 mg of N in the form of NO_3NH_4 was added to the positive control, while negative control remained without N fertilizer and bacterial inoculation. The experiment was conducted for 21 days. In this plant assay the following characteristics were evaluated: shoots and roots dry matter production and amounts of the nutrients N, P and K accumulated in the shoots of maize plants. The amounts of NPK were analyzed according to Tedesco et al. (1995). Statistical analyses of plant biomass and NPK levels were analyzed with ANOVA, and comparisons between treatment means were calculated using the Scott-Knott test (Sisvar 5.1 Build 72) at the 0.05 significance level (Ferreira, 2011).

3. Results

3.1. Isolation, identification, and diversity analysis of microbes within the rhizospheric soils and roots of maize

A selective medium was used for the isolation and selection of growth-promoting rhizobacteria. We obtained a total of 292 isolates, with 143 from roots and 149 from rhizosphere soil. Of these 292 isolates, 5.82% could not be identified at the genus/species level. A dendrogram showing the genetic relationships of isolates was constructed for each sampling site (Fig.1). One representative bacteria belonging to each cluster, obtained with the 16S rDNA-PCR-RFLP, was chosen for partial sequencing of the 16S rDNA and the

sequences obtained were BLASTN-aligned with the sequences of different bacterial genera from the GenBank database, as shown in Table 3. The identification of the genera through analyses of the sequences of 16S rDNA classified the isolates to the following six bacterial divisions: *gammaproteobacteria* (66.44%), *betaproteobacteria* (20.55%), *alphaproteobacteria* (2.40%), *Firmicutes* (1.37%), *Actinobacteria* (2.05%) and *Bacteroidetes* (1.37%) (Fig. 2). According to their 16S rDNA sequences, most of the bacteria found in association with the maize roots and rhizospheric soil belonged to the division *gammaproteobacteria*, including several species of the genera *Klebsiella*, *Pseudomonas*, *Stenotrophomonas*, *Enterobacter*, *Serratia*, *Pantoea* and *Citrobacter*. *Burkholderia* was the most abundantly represented genus belonging to the division *betaproteobacteria*. The genera *Bosea*, *Microbacterium* and *Arthrobacter* were found only in rhizospheric soil.

Dendrograms were also used to estimate the microbial diversity of the maize plant rhizobacteria in each sampling site (Fig. 1). A high Shannon-Weaver diversity index, calculated from the number of groups and number of individuals per group, was found at all five sampling sites. The diversity indices were as follows: Vacaria (*H'* = 3.431, n=59), Julio de Castilhos (*H'* = 3.233, n=57), Veranópolis (*H'* = 2.968, n=57), Rio Grande (*H'* = 2.835, n=48) and Porto Alegre (*H'* = 2.746, n=57). PCA analysis was used to investigate the relationships between bacterial diversity (*H'*) and soil factors. The principal components of PCA (PCA1 and PCA2) explained 86.1%, with component 1 accounting for 72.1% and component 2 for 14.0% of the total variation. Clay content had a direct correlation with bacterial diversity as well, whereas P and Al content had an inverse correlation (Fig. 3).

241 *3.2. PGP traits*

242 All 292 rhizobacterial strains were screened for PGP traits. A total of 72 isolates
243 demonstrated all of the characteristics evaluated, 76 were able to produce indole-acetic
244 acid and solubilize phosphates, and 47 were able to produce indole-acetic acid and
245 siderophores. None of the isolates showed simultaneous phosphate solubilization and
246 siderophore production. A total of 154 strains (53%) were able to solubilize phosphates.
247 Porto Alegre had the highest number of phosphate solubilizers, whereas Veranópolis had
248 the smallest number (3.3%) (Fig. 4). Siderophore production was observed in 122 strains,
249 among which, Júlio de Castilhos was the most highly represented region. The genera
250 *Burkholderia*, *Enterobacter* and *Pseudomonas* were the most common siderophore
251 producers in all five regions (Fig. 5). Most of the strains (98%) produced IAA in the
252 presence of tryptophan, considering production positive above 0.1 $\mu g.ml^{-1}$. The IAA
253 production ranged from 0.1 to 130.27 $\mu g.ml^{-1}$. In the Porto Alegre site, *Klebsiella* sp. were
254 the most numerous among IAA producers, followed by the genera *Pseudomonas*,
255 *Burkholderia*, *Acinetobacter* and *Enterobacter*. The genus *Burkholderia* were predominant
256 as IAA producers at the Julio de Castilhos site, whereas the genus *Enterobacter* were
257 predominant at the Vacaria site. The most efficient producers of IAA, with productions
258 above 100 $\mu g.ml^{-1}$, belonged to the genus *Klebsiella* and were isolated at the Rio Grande
259 site. *Pseudomonas* sp. were the most numerous IAA producers isolated at the Veranópolis
260 site.

261

262 *3.3. Plant growth promoting assay*

263 The inoculation with different bacterial strains significantly promoted the growth of
264 maize plants. The results were not affected by the choice of cultivars because there were no

significant interactions between bacteria and cultivars for any analyzed parameter. The isolates VC36 (*Achromobacter* sp.), RG38 (*Burkholderia* sp.), AGR27 (*Pseudomonas* sp.) and TU39 (*Arthrobacter* sp.) had significantly increased root dry matter (50-68%) compared to the negative control and were equal to the positive control. Shoot dry matter was also increased (25-54%) in comparison to the negative control after inoculation with strains VC36 (*Achromobacter* sp.), RG38 (*Burkholderia* sp.) and TU39 (*Arthrobacter* sp.). These strains had no significant differences compared to the positive control. These strains outperformed the commercial strain *Azospirillum brasilense* V6, which was no different from the negative control (Table 4).

The bacterial inoculations also increased the total N and P content of maize shoots. K content showed no significant difference between treatments. Inoculation with the strains RG38 (*Burkholderia* sp.) and VC36 (*Achromobacter* sp.) enhanced P uptake by maize plants by 27% and 55%, respectively, while only the isolate VC36 (*Achromobacter* sp.) increased the N content, by approximately 55% (Table 4).

4. Discussion

Many researchers around the world are studying PGPR in different plant species to understand how to use them to benefit agriculture. Numerous genera, including *Achromobacter*, *Arthrobacter*, *Azospirillum*, *Bacillus*, *Paenibacillus*, *Burkholderia*, *Chryseobacterium*, *Enterobacter*, *Citrobacter*, *Klebsiella*, *Herbaspirillum*, *Pantoea*, *Pseudomonas*, *Rhizobium* and others, have been found in the rhizosphere of gramineous plants (Malik et al., 1997; Mirza et al., 2006; Perin et al., 2006; Beneduzi et al., 2008; Jha and Kumar, 2009). In our study, 21 different genera of rhizobacteria were found in association with the roots and rhizosphere soil of maize plants in Rio Grande do Sul. Our

289 results showed that *Burkholderia* (*betaproteobacteria*) and *Klebsiella*
290 (*gammaproteobacteria*) were the only genera found at all of the sampling sites. In studies
291 of the diversity of the bacterial community of maize, Mclnrey and Kloepper (1995) also
292 reported that the most commonly isolated genera belonged to the *gammaproteobacteria*
293 and *betaproteobacteria* divisions. *Burkholderia* sp. has been isolated from different
294 locations and niches, and strains of *Burkholderia tropica* have been identified in the
295 rhizosphere of maize and sugarcane in Brazil (Reis et al., 2004). Additionally, Roesch et
296 al. (2007) reported that *Klebsiella* was one of the genera most commonly associated with
297 maize in different soils in Rio Grande do Sul. This demonstrates that some genera of
298 rhizobacteria may be preferentially associated with maize regardless of the soil type and
299 the weather conditions of the sampling area.

300 With the construction of the dendrograms, it was possible to confirm and visually
301 represent the high genetic diversity present in all of the samples. The differences in
302 diversity among the bacterial communities may have resulted from the combined effects of
303 the weather and the edaphic factors. This study showed a correlation between clay content
304 and bacterial diversity (*H*'). Zhang et al. (2007), studying bacterial communities in paddy
305 soils, observed that a higher bacterial diversity may be influenced by fine particulates such
306 as clay, as well as the total content of soil organic carbon. Some authors have also
307 suggested that soil texture and clay are parameters that might affect the richness and
308 diversity of bacterial communities in the soil (Bashan et al., 1995; Sessitsch et al., 2002).
309 Our findings showed that bacterial diversity (*H*') was higher in soils with low P and Al
310 levels. Similar results were found by Oliveira et al. (2009). In a study of bacterial diversity
311 in the rhizosphere of maize genotypes, those authors found higher bacterial diversity
312 among plants cultivated in soils with lower P contents.

313 Rhizobacteria that have characteristics that promote plant growth, including
314 phosphate solubilization, indole and siderophore production, nitrogen fixation, among
315 others, have a potential for use as PGP inoculants to improve crops. Most soils contain
316 high amounts of P, but only a small proportion of it becomes available to the plants. It is
317 well known that several rhizobacteria are capable of solubilizing inorganic phosphate in
318 soil, and this activity is determined by their ability to produce organic acids, whose
319 carboxyl groups chelate cations, especially Ca-bound P, and thus release soluble P
320 available for plant assimilation (Venieraki et al., 2010;). In the present study, more than
321 half of the isolates were able to solubilize phosphates and the greatest number of phosphate
322 solubilizing strains was isolated from rhizosphere soil. It has been reported that higher
323 concentrations of phosphate-solubilizing bacteria are commonly found in the rhizosphere
324 soil compared to bulk soil (Keswani et al., 1977). Most of our phosphate-solubilizing
325 bacteria were identified as *Burkholderia*. *In vitro* phosphate solubilization activity has been
326 documented for *Burkholderia* sp. strains (Park et al., 2010). Song et al. (2008) isolated
327 *Burkholderia cepacia* from cultivated soils in Korea, and this strain showed an intense
328 activity of phosphate solubilization by the production of organic acids, especially gluconic
329 acid.

330 Siderophores are iron chelators synthesized by several PGPR and play an important
331 role in promoting plant growth either directly, by Fe supply to the plant, or indirectly, by
332 limiting the Fe availability to pathogens (Glick, 1995). We found several siderophore-
333 producing rhizobacteria, most of which belonged to the genera *Pseudomonas* and
334 *Burkholderia*. In fact, many studies have reported that *Pseudomonas* sp. are potent
335 siderophore producers and thus may promote plant growth and act as plant pathogen
336 antagonists (Cornelis and Matthijs, 2007; Fischer et al., 2010). The ability to produce

337 siderophores was observed in many of our P-solubilizing isolates and IAA producers. The
338 simultaneous production of siderophores and solubilization of phosphate by soil
339 microorganisms has been reported by several authors (Vassilev et al., 2006; Jha et al.,
340 2009). IAA production was the most common PGP trait, with almost all of the
341 rhizobacterial isolates from the different locations able to produce IAA in the presence of
342 tryptophan, although much variability was observed in their capacities. The differences in
343 the performance of IAA-producing PGPR may be attributed to the inherent properties of
344 the individual bacteria (Sarwar et al., 1992). Similarly, El-Azeem et al. (2007) reported that
345 all 81 of their strains isolated from different plants, including maize, were able to
346 synthesize phytohormones. IAA is the most common growth regulator produced by PGPR
347 and is important for improved plant growth, including root development. It has been
348 estimated that 80% of bacteria isolated from rhizosphere soil may produce growth-
349 regulating substances (Patten and Glick, 1996).

350 Bacterial strains selected by their PGP traits were tested in a greenhouse assay with
351 two maize cultivars. The inoculation of maize seeds with strains VC36 (*Achromobacter*
352 sp.), RG38 (*Burkholderia* sp.) and TU39 (*Arthrobacter* sp.) caused a significant increase in
353 the shoot and root dry matter of both cultivars. Additionally, two of these isolates had
354 significantly increased either P (RG38) content or both N and P (VC36) contents in
355 inoculated maize plants. These findings demonstrate that BNF did not seem to be a
356 significant mechanism of growth promotion because the concentration of N (or the
357 percentage thereof) was not altered by inoculation with different bacterial strains. These
358 strains had shown high IAA production that may have affected the development of the
359 roots of the maize plants. The plant growth promotion may be attributed to the ability of
360 the isolates to produce IAA because IAA positively influences root growth and

development and therefore enhances nutrient uptake (Khalid el al., 2004). In our investigation, *Achromobacter* sp., that under *in vitro* conditions showed a lower IAA production, was the isolate that most effectively increased biomass weight and nutrient uptake. Similar to our results, Bertrand et al. (2000) isolated *Achromobacter* sp. from the canola rhizosphere to investigate the growth potential of this strain and found that root growth was stimulated by the ionic transport system providing more nitrate uptake and biomass production. These findings show that PGPR might stimulate root and shoot growth by an alternative mechanism leading to increased nutrient uptake.

In the present study, it was observed that members of the genera *Burkholderia* and *Arthrobacter* had the ability to promote plant growth. The results obtained from inoculation of *B. vietnamiensis* on rice (Trân Van et al., 2000) and *B. amfibaria* on maize (Ciccillo et al., 2002) have already shown the very high potential of *Burkholderia* as plant growth-promoting rhizobacteria. The positive effects exerted by *Burkholderia* sp. are not only restricted to the mechanisms that improve the nutrient availability to plants but also exhibit a considerable potential for biocontrol (Caballero-Mellado et al., 2007; Hernandez et al., 2008). *Arthrobacter* sp. are found in the rhizosphere of some plants, and some studies have reported the ability of this genus to produce indole-acetic acid and other biologically active metabolites (Grappelli and Rossi, 1981). Sachdev et al. (2009) found *A. globiformis* associated with the rhizosphere of wheat, and this species demonstrated its efficiency as PGPR through IAA production as well as siderophore production.

Although many reports have shown a contribution to the growth of maize plants by *Azospirillum brasilense*, this commercial strain had no positive effect on growth in the present study. Some studies showed that the extent of positive bacterial effects on plant growth may vary between the species or on different genotypes of the same crop (Chanway

et al., 1988; Montanez et al., 2009). De Salmone and Dobereiner (1996) evaluating the efficiency of *A. brasilense* strains isolated from maize and found that among fifteen maize genotypes, only six showed a positive response to inoculation with these strains. Variations in the growth responses to inoculation with rhizobacteria are reported in other crops, including rice (Alam et al., 2003), wheat (Sala et al., 2007) and sugarcane (Moutia et al., 2010). Nevertheless, our bacterial inocula showed positive responses in two maize cultivars, Pioneer 30R50 and Fepagro 35, in greenhouse conditions. This result demonstrated that the bacterial strains were not plant-specific, and they stimulated the plant growth of maize regardless of the cultivars.

5. Conclusion

The present research shows a high bacterial diversity in the roots and rhizosphere soil of the maize plants cultivated in different regions of Rio Grande do Sul and that soil parameters, such as clay content, had an influence on the rhizobacterial community. Among the six bacterial isolates evaluated for plant growth promotion, this study demonstrates the potential of *Achromobacter* sp., *Burkholderia* sp., and *Arthrobacter* sp. strains. The positive effects of these strains on shoot and root weight and nutrient uptake of maize plants shows the beneficial role of these PGPR, which might be attributed to IAA production, phosphorus solubilization, or even other non-evaluated PGPR traits that stimulate plant growth. Screening of rhizobacteria that show multiple PGP traits suggests that they have better potential for greenhouse and field testing and applications in improving the yield of maize. Therefore, future investigations include the application of maize plants inoculated with these most promising PGPR in field conditions.

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591
592

593 Table 1 Soil properties at the sampling sites

Sampled site	Clay	OMC	pH	P_{exc}	K_{exc}	Ca_{exc}	Mg_{exc}	Al_{exc}
	%	%	H_2O	(mg/dm^3)	(mg/dm^3)	($cmol_c/dm^3$)	($cmol_c/dm^3$)	($cmol/_cdm^3$)
Júlio de Castilhos	48	2.9	5.3	10.8	440	4.5	1.8	0.2
Porto Alegre	19	2.8	4.8	22.2	80	2.8	0.7	0.8
Rio Grande	5	1.0	4.5	167.2	62	0.3	0.1	0.8
Vacaria	64	5.6	6.5	4.0	68	9.2	5.9	0
Veranópolis	56	3.0	7.0	8.2	170	11.9	5.1	0

594 *OMC*: organic matter content, exc: exchangeable

595

596 Table 2 *In vitro* results of PGP traits of the strains used in the plant experiment

Isolates[a]	16S rDNA sequence	PGP traits		
		Phosphate solubilization	Siderophore production	IAA production ($\mu g.ml^{-1}$)
VN50	*Herbaspirillum* sp.	-	+	80.48
VC50	*Chryseobacterium* sp.	+	+	78.38
VC36	*Achromobacter* sp.	-	-	17.35
RG38	*Burkholderia* sp.	+	+	130.27
AGR27	*Pseudomonas* sp.	+	+	49.25
TU39	*Arthrobacter* sp.	-	-	21.85
AbV6	*Azospirillum brasilense*	nd	nd	nd

597 nd: not determined; IAA: Indole Acetic Acid
598 [a] Bacteria isolated from: VN50 (Veranópolis); VC50 and VC36 (Vacaria);
599 RG38 (Rio Grande); AGR27 (Porto Alegre); TU39 (Júlio de Castilhos). All
600 bacteria were isolated from rhizospheric soil, except AGR27, which was
601 isolated from roots. AbV6 is a commercial inoculant strain.
602

603 Table 3. Representative isolates from each dendrogram cluster and respective 16S DNA
604 sequence

Sampling site	Representatives isolates	Number of isolates		16S DNA sequence
		root	rhizosphere	
	TU30	1	0	*Herbaspirillum* sp.
	TU51, TU56, TU11	0	3	*Azospirillum* sp.
	TU1, TU17, TU7, TU4	6	2	*Stenotrophomonas* sp.
	TU12, TU6, TU21, TU15, TU19, TU14	10	1	*Enterobacter* sp.
	TU34, TU37	0	3	*Pantoea* sp.
	TU54, TU59, TU48, TU43, TU57	0	15	*Burkholderia* sp.
Júlio de	TU2, TU22	4	0	*Klebsiella* sp.
Castilhos	TU44	0	1	*Bosea* sp.
	TU42	0	1	*Rhizobium* sp.
	TU10	3	0	*Serratia* sp.
	TU53	0	1	*Microbacterium* sp.
	TU39	0	3	*Arthrobacter* sp.
	TU58, TU18	2	1	*Citrobacter* sp.
	TU31	0	1	*Pseudomonas* sp.
	AGR48, AGR34, AGR42, AGR49	0	4	*Enterobacter* sp.
	AGR12	1	0	*Rhizobium* sp.
	AGR50	0	1	*Stenotrophomonas* sp.
	AGR19, AGR57, AGR10	4	2	*Burkholderia* sp.
Porto	AGR9	1	0	*Bacillus* sp.
Alegre	AGR17, AGR39, AGR37, AGR54	9	15	*Klebsiella* sp.
	AGR2	3	0	*Serratia* sp.
	AGR56, AGR29, AGR26, AGR31	8	3	*Pseudomonas* sp.
	AGR22	2	0	*Azospirillum* sp.
	AGR60, AGR58	0	4	*Acinetobacter* sp.
	RG59	0	1	*Paenibacillus* sp.
	RG53, RG24, RG54, RG1, RG39, RG26, RG10, RG12, RG23	5	4	*Klebsiella* sp.
	RG58	0	2	*Microbacterium* sp.
Rio Grande	RG11, RG46, RG47, RG45, RG34, RG40, RG25, RG38	8	15	*Burkholderia* sp.
	RG19, RG29, RG21	3	0	*Stenotrophomonas* sp.
	RG42	0	1	*Acinetobacter* sp.
	RG14	1	0	*Agrobacterium* sp.
	VC56, VC59, VC41, VC33	0	4	*Stenotrophomonas* sp.
	VC21, VC17, VC16, VC34, VC26	4	1	*Klebsiella* sp.
	VC12, VC1, VC37, VC46, VC49, VC40	8	8	*Enterobacter* sp.
	VC3, VC30	3	0	*Serratia* sp.
	VC2	1	0	*Pantoea* sp.
	VC15, VC52, VC50	1	2	*Chryseobacterium* sp.
Vacaria	VC22, VC20	5	0	*Citrobacter* sp.
	VC42, VC58, VC45, VC5, VC23, VC43	2	6	*Pseudomonas* sp.
	VC8, VC6	2	0	*Acinetobacter* sp.
	VC13, VC36	2	3	*Achromobacter* sp.
	VC53, VC38	0	5	*Burkholderia* sp.
	VC29	1	0	*Paenibacillus* sp.
	VC32	0	1	*Bacillus* sp.
	VN50, VN32, VN53	0	4	*Herbaspirillum* sp.
	VN31	0	1	*Burkholderia* sp.
	VN43	0	1	*Rhizobium* sp.
	VN45, VN26, VN28, VN12, VN40, VN29	11	10	*Pseudomonas* sp.
	VN55, VN4	5	1	*Enterobacter* sp.
	VN35, VN54, VN8, VN57, VN24, VN1, VN59	9	8	*Klebsiella* sp.
Veranópolis	VN52	0	1	*Azospirillum* sp.
	VN42	0	1	*Rhizobium* sp.
	VN51	0	1	*Stenotrophomonas* sp.
	VN19	2	0	*Pantoea* sp.
	VN22	1	0	*Flavobacterium* sp.
	VN38	0	1	*Bacillus* sp.

605
606

607 Table 4 Influence of inoculation with different bacterial strains on growth and
608 nutrient uptake in maize plants (means of two cultivars and four replicates)
609

Treatments	Root	Shoot			
	Dry weight (mg)	Dry weight (mg)	Plant N content (mgN/plant)	Plant P content (mgP/plant)	Plant K content (mgK/plant)
Negative control	182.50 b	355.00 b	6.67 b	0.47 b	2.29 a
Herbaspirillum sp.	205.00 b	333.75 b	6.07 b	0.45 b	1.26 a
Chryseobacterium sp.	191.25 b	375.50 b	6.29 b	0.41 b	1.36 a
Achromobacter sp.	307.50 a	548.75 a	10.34 a	0.73 a	2.83 a
Burkholderia sp.	275.00 a	446.25 a	7.61 b	0.60 a	1.88 a
Pseudomonas sp.	278.75 a	397.50 b	6.88 b	0.46 b	1.57 a
Arthrobacter sp.	276.25 a	443.75 a	7.11 b	0.50 b	2.42 a
Azospirillum brasilense V6	210.00 b	302.50 b	5.16 b	0.41 b	1.14 a
Positive control	286.25 a	463.75 a	9.03 a	0.44 b	1.56 a

610 Means followed by the same letter do not differ by the Scott-Knott test at a 5% error
611 probability.
612

Figures

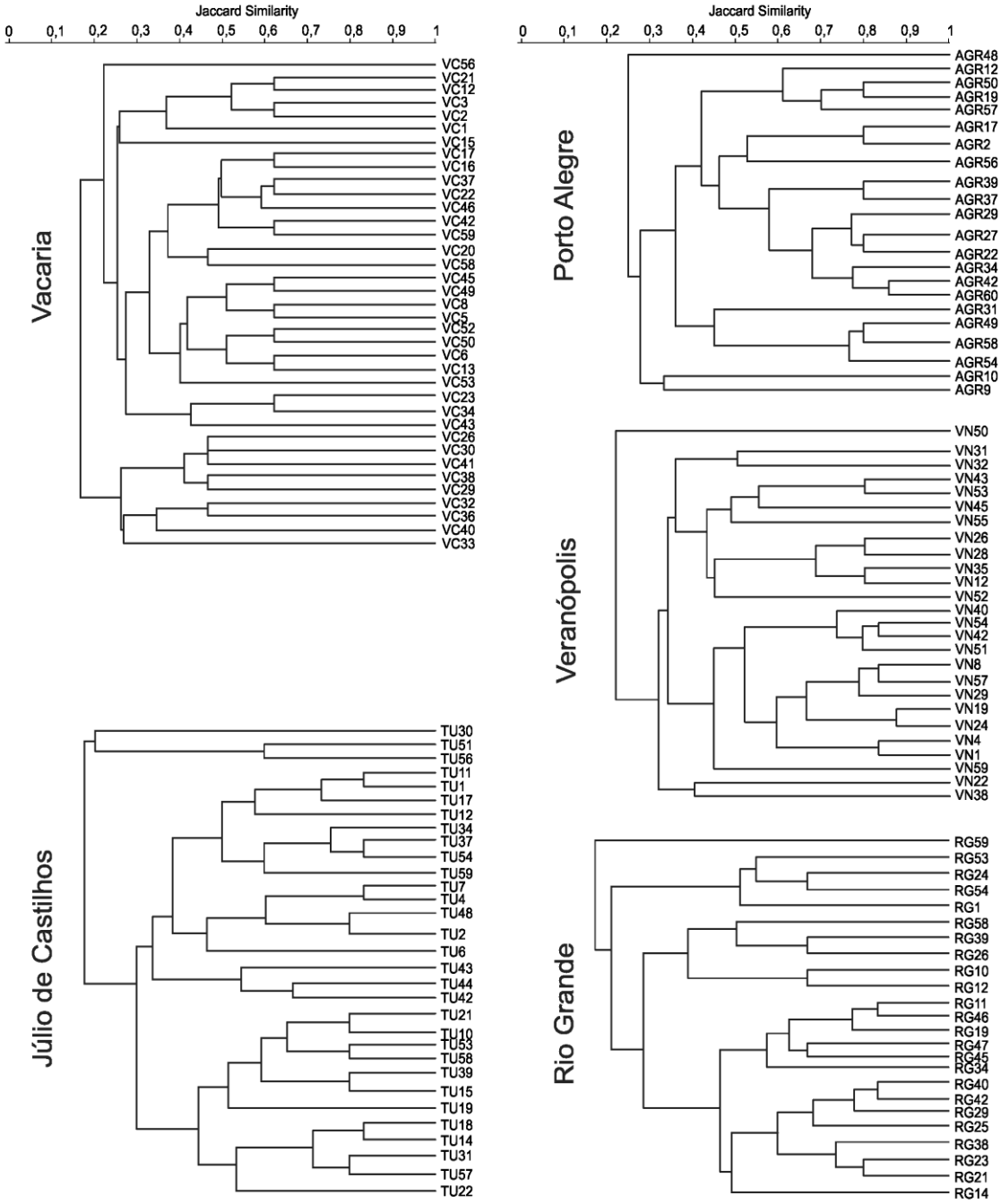
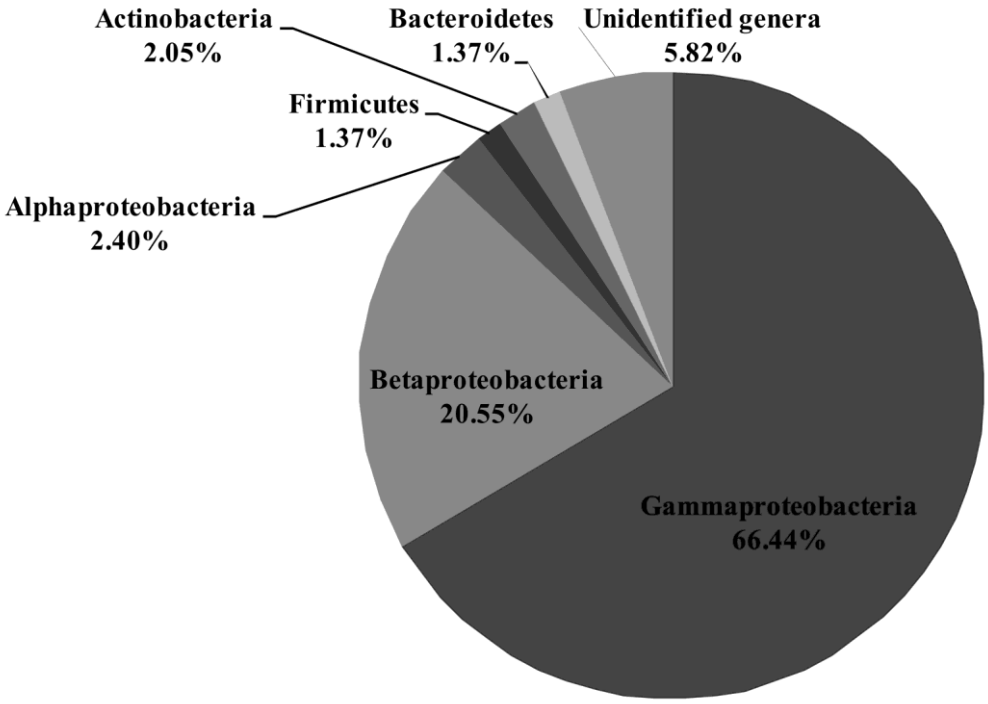


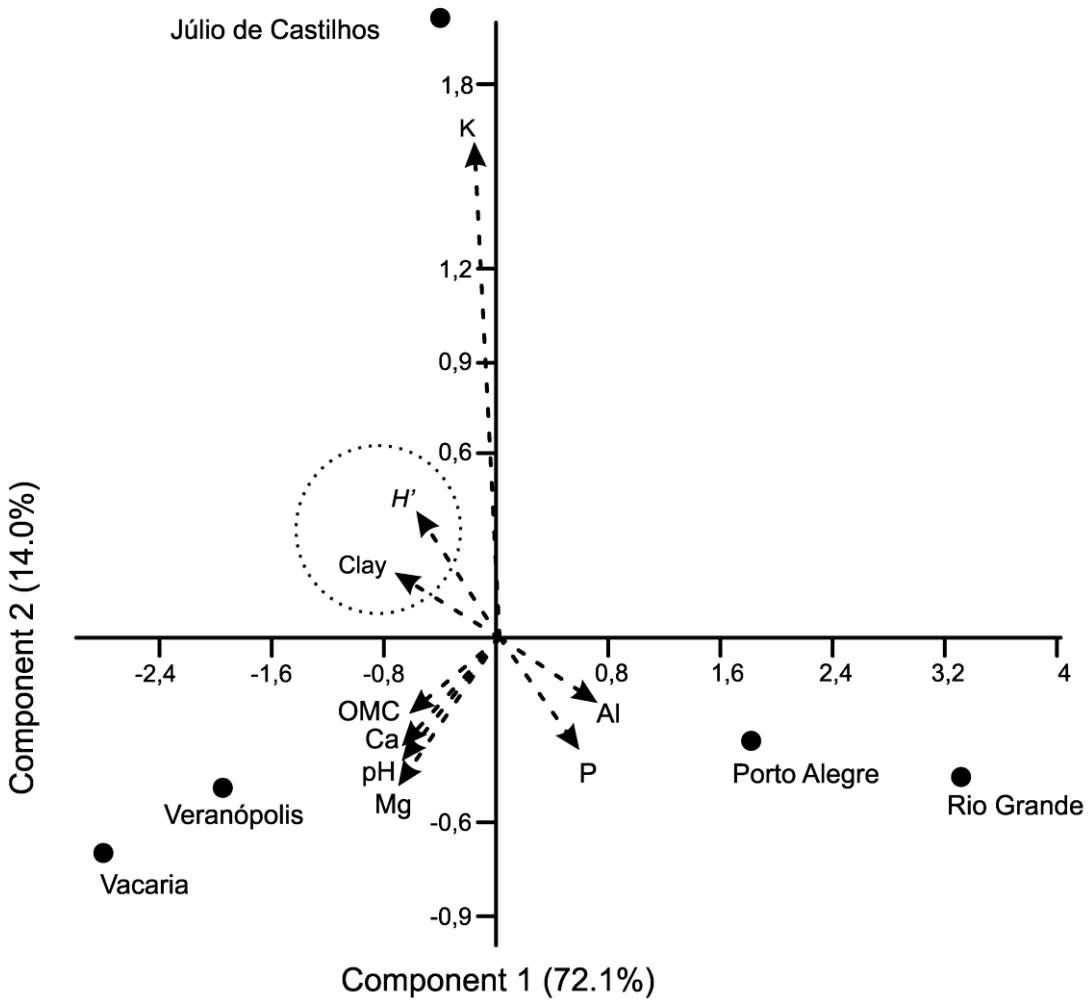
Fig. 1 Dendrograms based on UPGMA cluster analysis (PAST Software 1.07) using the PCR-RFLP 16S rDNA data obtained from isolates of each sampled site. Each dendrogram was summed up and only one in each cluster (the representative isolate) is represented. Each isolate is denoted according to the site at which it was sampled (TU: Júlio de Castilhos, AGR: Porto Alegre, RG: Rio Grande, VC: Vacaria and VN: Veranópolis)**.**



621

622 **Fig. 2** Taxonomic affiliations for roots and rhizosphere soil through analysis of 16S rDNA
623 sequences.

624



625

626 **Fig. 3** Principal coordinate analysis of sampled sites related to soil properties (OMC, Clay,
627 pH, Ca, Mg, P and Al) that have shown some statistical correlation with diversity index
628 (*H'*)
629

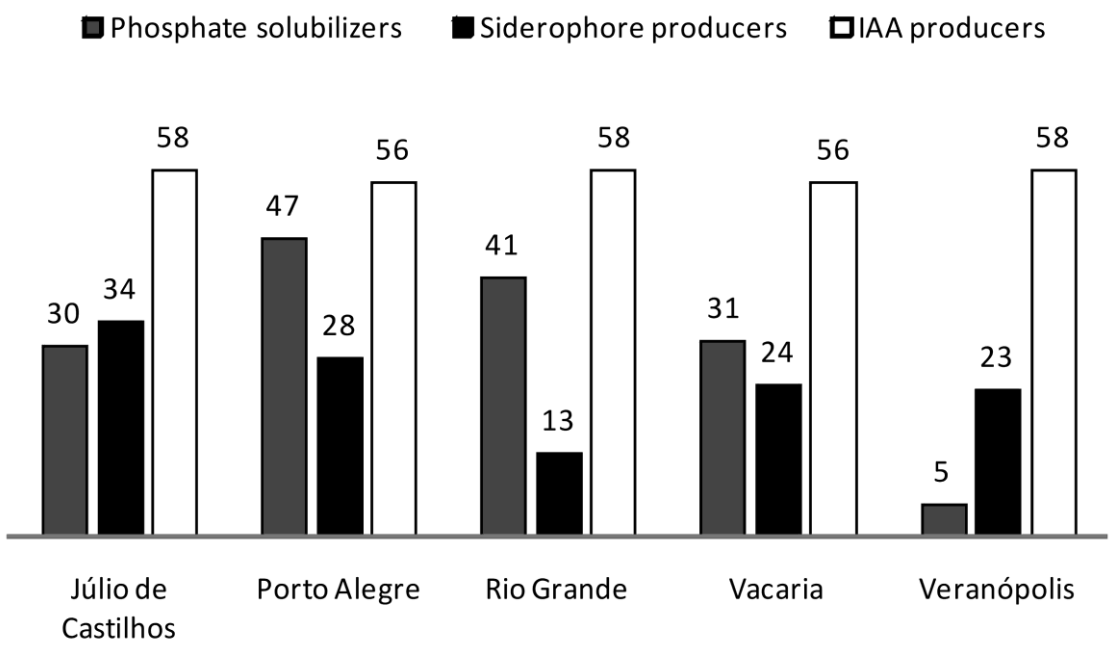


Fig. 4 Number of phosphate solubilizers, siderophores and IAA producers by bacterial isolates in each sampling site.

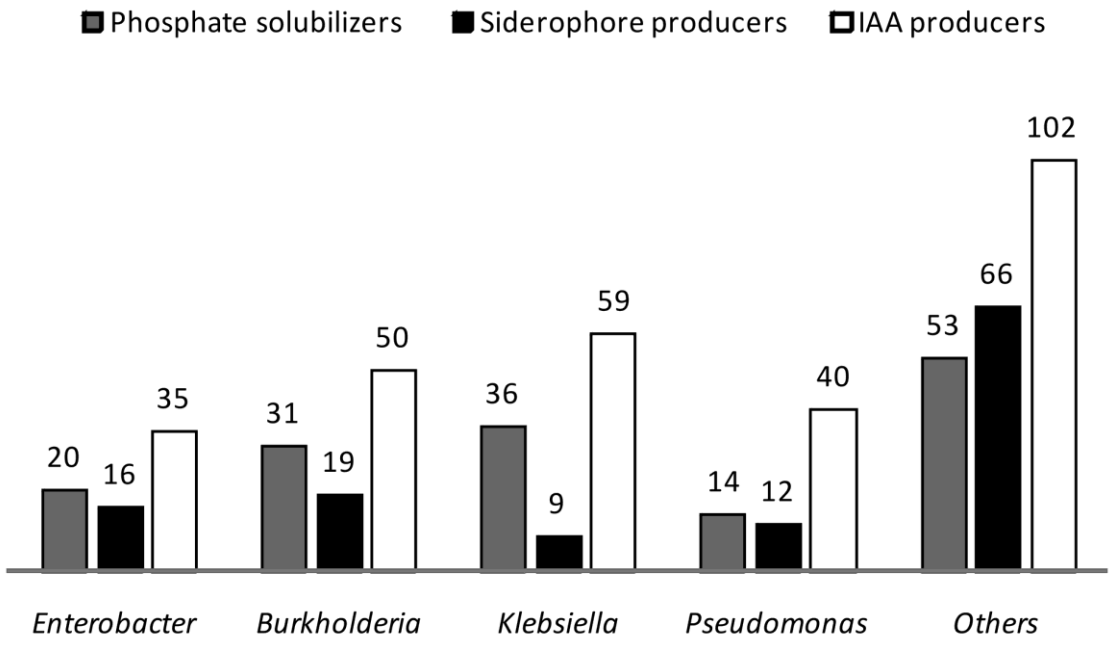


Fig. 5 Most abundant genera in terms of PGP traits. The number of isolates found for each trait and their respective genera are shown.

4. Conclusões gerais

As análises dos resultados permitiram as seguintes conclusões:

1) Baseado nos ensaios *in vitro*, uma alta porcentagem de bactérias tem a capacidade de produzir AIA, e essa característica demonstrou ser independente do local e região de amostragem.

2) Esse estudo mostrou que o teor de argila teve uma correlação direta com a diversidade bacteriana, enquanto que os teores de P e Al tiveram uma correlação inversa. Isso demonstra que fatores do solo podem influenciar na comunidade microbiana.

3) Embora tenha sido observada uma grande diversidade de bactérias associadas ao milho, nas cinco regiões amostradas, alguns gêneros tais como: *Klebsiella* e *Burkholderia* foram dominantes, sugerindo que a presença desses gêneros independe do ambiente.

4) Os isolados utilizados no ensaio *in vivo*, *Achromobacter* sp., *Burkholderia* sp. e *Arthrobacter* sp. promoveram o aumento de peso de raiz e da parte aérea de plantas de milho nas duas cultivares testadas e demonstraram ter potencial para serem utilizados como inoculantes.

5) Somente o isolado *Achromobacter* sp. mostrou diferença no teor de N, sugerindo que os isolados promoveram o crescimento das plantas de algum outro mecanismo que não a FBN.

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