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Bioprospecção de bactérias candidatas à formulação de inoculantes para o cultivo de girassol (*Helianthus annuus* L.)

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Sumário

Lista de abreviaturas

aa: aminoácidos

ACC: do inglês, *1-aminocyclopropane-1-carboxylate*, 1-aminociclopropano-1-carboxilato

AIA: ácido indol-3-acético

ARA: do inglês, *acetylene reduction assay*, ensaio da redução de acetileno

ATP: do inglês *adenosine triphosphate*, trifosfato de adenosina

BLAST: do inglês *Basic Local Alignment Search Tool*

bp: do inglês *base pairs*, pares de bases

DNA: do inglês *deoxyribonucleic acid*, ácido desoxirribonucléico

dNTP: do inglês *deoxyribonucleotide*, desorribonuleotídeo trifosfatado

EDTA: ácido etilenodiaminotetracético

FBN: fixação biológica do nitrogênio atmosférico

Gfp: do inglês *green fluorescent protein*, proteína fluorescente verde

H': índice de diversidade de Shannon and Weaver

kb: quilobases

kDa: quilodaltons

LB: meio de cultura Luria Bertani

Mb: megabase

NCBI: do inglês *National Center for Biotechnology Information*

ng: nanograma

Nif: do inglês *nitrogen fixation*, proteínas relacionadas à fixação de nitrogênio

ORF: do inglês *open reading frame*, fase de leitura aberta

PCA: do inglês, *Principal Components Analysis*, análise de componentes principais

PCR: do inglês *Polymerase Chain Reaction*, reação em cadeia da polimerase

PGPR: do inglês *Plant Growth Promoting Rhizobacteria,* bactérias promotoras do crescimento vegetal

pH: potencial de hidrogênio

rDNA: do inglês *ribosomal deoxyribonucleic acid*, ácido desoxirribonucleico ribossomal

RFLP: polimorfismo de comprimento de fragmento de restrição

RNA: do inglês *ribonucleic acid*, ácido ribonucleico

rRNA: do inglês *ribosomal ribonucleic acid*, ácido ribonucleico ribossomal

SSU rRNA: do inglês *small subunit ribosomal RNA*, subunidade menor do rRNA

TBE - Tris-borato EDTA

TGH: transferência genética horizontal

v/v: volume por volume

μg: micrograma

μL: microlitro

μM: micromolar

Resumo

A adoção de práticas sustentáveis na agricultura é um tema multidisciplinar de extrema relevância para a produção de alimentos ou vegetais destinados à produção de energia, tendo em vista o balanço entre impacto ambiental e produtividade agrícola. Entre as práticas conhecidamente danosas ao meio-ambiente encontra-se o intenso uso de fertilização química nas lavouras. As sementes do girassol possuem alto rendimento em óleo e essa cultura apresenta uma ampla gama de utilidades, sendo uma das matérias-primas para produção de biodiesel. Nas interações entre plantas e micro-organismos benéficos do solo, diversas bactérias disponibilizam nutrientes e compostos que contribuem para o desenvolvimento vegetal. A partir do estudo da diversidade de bactérias cultiváveis associadas às raízes de girassol, os gêneros *Burkholderia* e *Enterobacter* foram os Gram-negativos dominantes, enquanto que linhagens pertencentes às espécies *Bacillus arbutinivorans* e *Paenibacillus pabuli,* as Gram-positivas mais frequentes. Análises de polimorfismo de comprimento de fragmento de restrição (RFLP) de sequências do gene 16S rRNA foram utilizadas para classificação dos isolados Gram-negativos. Similarmente, análises multivariadas com base em RFLP *in silico*, juntamente com índices de identidade nucleotídica e relações filogenéticas, foram usadas para uma classificação consenso dos isolados Gram-positivos pertencentes aos gêneros *Bacillus* e *Paenibacillus*. Dos 400 isolados bacterianos obtidos no trabalho, a característica de produção de compostos indólicos foi a mais comum em ambos os grupos. A produção de sideróforos, mesmo sendo prevalente a quase 80% dos isolados Gram-negativos, foi detectada em apenas 2% dos isolados Gram-positivos. Isolados fixadores de nitrogênio identificados como *Azospirillum brasilense* (Vi22) e *B. mycoides* (B38V) foram eficientes na promoção do crescimento vegetal. Experimentos de biofertilização a campo, com um produto inoculante baseado na linhagem Vi22 de *A. brasilense*, confirmaram o potencial dessa bactéria na interação com girassol. O emprego da biofertilização em girassol por meio das estirpes analisadas nessa tese poderá auxiliar no aumento da produtividade e qualidade do cultivo dessa oleaginosa.

Abstract

Sustainable agriculture is a multidisciplinary theme with a relevant importance to food and energy production, aiming to reduce the environmental impact and increase agricultural productivity. Chemical fertilization usage is among agricultural practices that are most prejudicial to environment. Sunflower seeds present a high oil yield and this crop is broadly utilized, being one of the best raw materials for biodiesel production. During the interaction between plants and benefic soil microorganisms, several bacteria affect positively plant growth due to the availability of nutrients and different compounds. In this study focusing on the diversity of cultivable bacteria associate to sunflower roots, *Burkholderia* and *Enterobacter* genera were the Gram-negative most abundant whereas strains belonging to *Bacillus arbutinivorans* and *Paenibacillus pabuli* species were the Gram-positive most frequent. Restriction fragment length polymorphism (RFLP) analysis of 16S rRNA gene sequences was utilized to classify Gram-negative isolates. Similarly, multivariate analysis based on *in silico* RFLP, together with nucleotide identity indices and phylogenetic relationships were used to formulate a consensus identification of Gram-positive isolates belonging to *Bacillus* and *Paenibacillus* genera. From the 400 bacterial isolates obtained in this work, indolic compound production was the common characteristic to both bacterial groups. Siderophore production was the plant growth promotion (PGP) ability most prevalent in almost 80% of the Gram-negative isolates, but only 2% of the Gram-positives presented this trait. Nitrogen fixing isolates belonging to *Azospirillum brasilense* (Vi22) and *B. mycoides* (B38V) species were efficient to promote plant growth. Biofertilization field assay with an inoculant product based on *A. brasilense* Vi22 strain confirmed the bacterial potential to positively interact with sunflower. The sunflower biofertilization through the utilization of bacterial strains isolated in this work could help to increase the productivity and quality of this oleaginous crop.

1. Introdução Geral

O solo é um ambiente composto por diferentes organismos, substâncias orgânicas e minerais presentes em três fases: sólida, líquida e gasosa (Kabata-Pendias, 2004). Por ser um sistema complexo, o arranjo das partículas de diferentes tamanhos e composições químicas contribui para a sua porosidade e dinâmica espacial (Ettema e Wardle, 2002). Os procariotos são ubíquos nos solos e a composição e atividade enzimática desses micro-organismos influenciam na disponibilidade dos mais diversos nutrientes. Devido à dinâmica e complexidade do sistema, muitas variáveis podem afetar a estrutura do solo e a distribuição e o estabelecimento das diferentes populações de bactérias, tais como as condições edafoclimáticas (Fierer e Jackson, 2006; Faoro et al., 2010; Pulleman et al., 2012), os sistemas de manejo (Welbaum et al., 2004; Ding et al., 2013) e a interação com diferentes micro-organismos (Barea et al., 2005; Fernández, 2005) e espécies de plantas (Bais et al., 2006; Berg e Smalla, 2009; Eisenhauer et al., 2010; Mao et al., 2011).

As raízes vegetais contribuem de forma direta para a estabilidade dos agregados do solo com sua inerente riqueza em matéria orgânica (Watt et al., 1993) e, de forma indireta, através da produção de diversos compostos, como exemplo dos exopolissacarídeos (EPS), e a respectiva estimulação da atividade microbiana (Degens et al., 1994; Alami et al., 2000; Zhang et al., 2013). A agregação do solo também é influenciada diretamente pelo teor de argila e ciclos de umedecimento e secagem e, em um amplo contexto, os agregados determinam as propriedades mecânicas do solo, a temperatura, a aeração e a retenção e movimento de água (Dinel et al., 1992; Schmidt et al., 2011). Os diferentes tamanhos dos agregados fornecem diversos micro-habitat para a microbiota e, consequentemente, influenciam na disponibilidade de nutrientes (Zhang et al., 2013).

Decompositores, principalmente bactérias e fungos, adquirem carbono (C) a partir de fontes orgânicas que são fornecidas, principalmente, pelos produtores, tais como plantas e algas; os produtores, por sua vez, adquirem nutrientes a partir de fontes inorgânicas, que são fornecidas, principalmente, pelos decompositores (Naeem et al., 2000). A chamada co-dependência entre produtores e decompositores é acentuada na interação entre plantas e bactérias, sobretudo na rizosfera, a região do solo adjacente às raízes (Eisenhauer et al., 2010). A deposição de compostos orgânicos na rizosfera é

largamente dependente do estado nutricional da planta, como, por exemplo, a liberação de ácidos orgânicos em resposta à deficiência de fósforo e ferro, ou a liberação de fitossideróforos em resposta à escassez de ferro e zinco (Dakora e Phillips, 2002). Em plantas cultivadas sob baixa disponibilidade de fosfato ou na presença de concentrações tóxicas de alumínio, a exsudação de ácidos orgânicos, tais como ácido oxálico, málico e cítrico, é aumentada (Neumann e Römheld, 1999; Piñeros et al., 2002). Os compostos químicos secretados pelas raízes são amplamente definidos como exsudatos (Walker et al., 2003) e as plantas podem liberar uma mistura diversa e complexa de moléculas além dos ácidos orgânicos e fitossideróforos, tais como açúcares, vitaminas, aminoácidos e moléculas gasosas (i.e., CO_2 e H_2) (Dakora e Phillips, 2002).

O termo "rizosfera" foi cunhado por Lorentz Hiltner em 1904 para descrever a porção de solo onde os processos mediados por micro-organismos estão sob a influência do sistema radicular (*in* Berg e Smalla, 2009). Os exsudatos podem ser utilizados pelos micro-organismos presentes na rizosfera, resultando em um aumento da biomassa e da atividade microbiana em torno das raízes, o chamado efeito rizosfera (Bais et al., 2006; Doornbos et al., 2012). A concentração de bactérias que é encontrada em volta das raízes é geralmente muito maior do que em outras porções do solo (Glick, 1995). Entretanto, as comunidades da rizosfera são menos diversificadas que no restante do solo e supõe-se que isso seja fruto de um competitivo estabelecimento microbiano dependente do tipo de planta e dos exsudatos que ela libera (Buchan et al., 2010).

A atividade microbiana na rizosfera pode ter um efeito profundo no crescimento e na saúde dos vegetais, sendo que tais associações podem ser deletérias, benéficas ou neutras para as plantas (Lynch, 1990; Doornbos et al., 2012). A ocorrência de maior número de interações benéficas na rizosfera pode contribuir positivamente para o crescimento vegetal, uma vez que este é o ambiente circundante para a captação de água e nutrientes pelas plantas (Alami et al., 2000). Dessa forma, as bactérias da rizosfera, também chamadas de rizobactérias, podem influenciar as plantas de várias maneiras, dependendo da sua capacidade de incorporar exsudatos radiculares, excretar metabólitos ou competir com outros micro-organismos do solo (Zeller et al., 2007).

As rizobactérias promotoras de crescimento vegetal (PGPR, do inglês *Plant Growth Promoting Rhizobacteria*) constituem um grupo heterogêneo de micro-organismos que pode ser encontrado na rizosfera ou em associação com as raízes. O termo PGPR foi adotado por Schroth e Hancock em 1982 para descrever as bactérias

benéficas capazes de colonizar as raízes e estimular o crescimento vegetal (*in* Botelho, 1996). As PGPRs podem estabelecer relações do tipo endofíticas (dentro do tecido vegetal) ou associativas (na superfície radicular ou muito próxima a ela). Segundo Gray e Smith (2005) existe um gradiente de proximidade entre as rizobactérias e a raiz que pode ser classificado da seguinte forma: (i) as bactérias que vivem no solo, perto de raízes, utilizando exsudatos como fontes de C e N, (ii) as bactérias capazes de colonizar o rizoplano (superfície da raiz), (iii) as bactérias residentes no tecido vegetal, habitando espaços entre células corticais e, finalmente, (iv) as bactérias que vivem no interior das células, em estruturas especializadas da raiz ou nos nódulos (que geralmente se dividem em dois grupos: rizóbios e leguminosas; *Frankia* e plantas lenhosas).

Diversas bactérias Gram-negativas e Gram-positivas já foram cultivadas e avaliadas quanto aos efeitos benéficos sobre as plantas. PGPRs ocorrem naturalmente em todos os tipos de plantas e o nível das interações é amplamente determinado pela sinalização molecular entre as espécies (Singh et al., 2004; Eisenhauer et al., 2010). O reconhecimento dos sinais químicos e a "troca de informações" no solo fazem com que as associações entre bactérias e plantas variem de acordo com as espécies envolvidas, o que estimula a busca dos pesquisadores por linhagens que possam favorecer o desempenho das mais variadas culturas de interesse agronômico (Badri et al., 2009). A quimiotaxia bacteriana na rizosfera (capacidade de migração dos micróbios em direção aos exsudatos radiculares) também tem sido considerada um fator importante para o estabelecimento de interações benéficas e pode ser uma vantagem competitiva no processo de colonização inicial por PGPRs (Buchan et al., 2010). Todavia, as interações na rizosfera não são unicamente impulsionadas pelas raízes e o estabelecimento e resultado de tais associações também depende dos outros organismos e dos fatores edáficos locais. Dessa forma, a atuação de uma PGPR está relacionada ao estabelecimento de relações positivas com a planta – compatibilidade e competência rizosférica, à capacidade de sobrevivência da bactéria no solo e à interação com os outros micro-organismos, fazendo com que o resultado de todos esses processos possa muitas vezes ser de difícil previsão (Dodd et al., 2011).

Dentre as PGPRs de interesse agrícola, as bactérias fixadoras de nitrogênio atmosférico (N_2) são extensivamente estudadas devido à importância do nitrogênio (N) para o desenvolvimento vegetal. O N é um dos principais constituintes das biomoléculas e representa cerca de 2% da matéria seca total das plantas que entram na cadeia

alimentar (Santi et al., 2013). O N está entre os nutrientes mais limitantes para a produção agrícola, assim como o fósforo (P) e o potássio (K). Dessa forma, quando uma planta se estabelece em um solo com deficiência desses elementos, ela não é capaz de se desenvolver com alto grau de produtividade. Tendo em vista a importância desses nutrientes para o desenvolvimento de culturas agrícolas, o uso de fertilizantes químicos passou a fazer parte da cadeia de produção de alimentos a partir da segunda metade do século XX, dado o aumento de rendimento que é obtido com sua aplicação (Evenson e Gollin, 2003).

A alta produtividade agrícola está normalmente relacionada à fertilização química, mas tal prática causa efeitos negativos no meio ambiente e esses efeitos têm se intensificado na mesma medida que aumenta o cultivo de alimentos. A maior parte (60 a 90%) do fertilizante aplicado não é absorvida pelas plantas e acaba sendo lixiviada para os corpos d'água ou volatilizada para a atmosfera (Adesemoye e Kloepper, 2009). O escoamento de N e P para as águas, por exemplo, influencia no crescimento de populações de algas e cianobactérias devido ao excesso de nutrientes e pode causar a eutrofização dos corpos hídricos. As transformações internas do ciclo do N exercem forte controle sobre a disponibilidade de N para a produtividade primária líquida, lixiviação de nitrato nas águas subterrâneas e emissões de gás de efeito estufa (Silver et al., 2001). Aumentos excessivos do número de algas e cianobactérias causam a diminuição da passagem de oxigênio dissolvido na água e o aumento da mortalidade de peixes e outros seres aquáticos, o que leva a desordens entre os níveis tróficos e perda de diversidade (Tsugeki e Urabe, 2013). As manifestações biológicas ao excesso de fertilização química nas lavouras também influenciam diretamente as populações humanas, causando danos à rede pesqueira e intensificação do tratamento das redes de captação de água para consumo (Evenson e Gollin, 2003).

1.1. A fixação biológica do nitrogênio

O processo que consiste na redução do N_2 à amônia (NH_3) é chamado de fixação biológica do N_2 (FBN – Newton, 2000) e é realizado pelos micro-organismos diazotróficos. O N gasoso (N_2) é quimicamente inerte e poucos organismos são capazes de utilizá-lo, mesmo sendo ele um dos principais constituintes da atmosfera (quase 80%). Entre os diazotróficos encontram-se representantes cultiváveis e amplamente distribuídos entre diferentes procariotos, como Gram-positivos, Gram-negativos,

archaea e cianobactérias. Os diazotróficos podem ser simbióticos (quando estabelecem estrita relação com determinadas espécies vegetais) ou de vida-livre (obtêm energia a partir de compostos orgânicos do solo e podem estabelecer diferentes associações com as plantas – endofíticos ou associativos) (Gray e Smith, 2005). Embora existam muitas diferenças morfológicas, fisiológicas e genéticas entre os micro-organismos diazotróficos, assim como uma enorme variabilidade de ambientes onde eles podem ser encontrados, todos eles contêm o complexo enzimático da nitrogenase (Dixon e Kahn, 2004).

A nitrogenase é formada por duas proteínas essenciais para a redução de N_2, dinitrogenase e dinitrogenase redutase, as quais são bastante sensíveis ao oxigênio. A dinitrogenase (FeMo-proteína) é o maior componente e é composta de dois pares de subunidades ($\alpha_2\beta_2$) não idênticas, as quais são codificadas pelos genes *nifD* (α) e *nifK* (β). O componente menor é a dinitrogenase redutase (Fe-proteína) que contém duas subunidades idênticas (γ_2) codificadas pelo gene *nifH*, o qual é altamente conservado (Boucher et al., 2003;. Zehr et al., 2003). A subunidade α contém o sítio ativo para a redução do N_2, o grupo $MoFe_7S_9$ (chamado cofator-FeMo). Alguns organismos possuem nitrogenases alternativas, além da codificada pelo sistema Nif, onde Mo é substituído por Fe ou V, e a nomenclatura Anf ou Vnf, respectivamente, é usada ao invés de Nif (Raymond et al., 2004). A nitrogenase requer ATP, Mg^{2+} e uma fonte de elétrons para o processo da fixação de N_2 (Moat e Foster, 1995). Para reduzir 1 mol de N_2 são necessários 16 mols de ATP e 8 mols de elétrons, o que faz da FBN um dos processos metabolicamente mais custosos entre os seres vivos (Simpson e Burris, 1984).

Os genes estruturais *nifHDK* são dispostos contiguamente em um único operon em muitos diazotróficos, mas existem pelo menos outros 17 genes *nif* com funções reguladoras ou de apoio à fixação de N_2 (Madigan et al., 2000; Anand, 2010). A análise das sequências do gene *nifH* geralmente é congruente com a filogenia inferida pela sequência do gene que codifica o RNA da menor subunidade do ribossomo, o gene 16S rRNA (Young, 1992; Zehr et al., 2003). Entretanto, evidências de um complexo modo de herança e de ocorrências de transferência genética horizontal (TGH) dos genes *nif* têm demonstrado que algumas inferências filogenéticas podem ser distintas daquelas feitas com o gene 16S rRNA (Hirsch et al., 1995; Henson et al., 2004; Raymond et al., 2004; Kechris et al., 2006). Em um trabalho sobre a filogenia de espécies de *Paenibacillus* diazotróficos, por exemplo, *P. riograndensis* é agrupado junto a *P. sonchi*

na filogenia inferida pelo gene 16S rRNA, enquanto que para o gene *nifH*, *P. riograndensis* é mais próximo à *P. wynii*, e *P. sonchi* à *P. jilunlii* (Xie et al., 2012). A ocorrência de distribuição irregular observada nas filogenias com genes *nif* tem sido atribuída, por diferentes autores, à TGH ou à perda de função em determinadas linhagens (Kechris et al., 2006; Anand, 2010). Alguns claros exemplos da TGH de genes *nif* em bactérias existem para linhagens de *Burkholderia* spp. (Bontemps et al., 2010), *Ensifer fredii* (Barcellos et al., 2007), *Rhodopseudomonas palustris* (Cantera et al., 2004) e *Wolinella succinogenes* (Baar, 2003). Anand (2010) observou que muitos estudos filogenéticos que mostram discrepâncias entre filogenias baseadas em *nifH* e 16S rRNA são de cianobactérias, actinobactérias (*Frankia*) e outras bactérias Gram-positivas, tornando estes grupos cruciais para a resolução do debate sobre a herança genética dos genes *nif*.

Considerando a distribuição da característica de fixação de N_2, os estudos baseados na amplificação do gene *nifH* são úteis para estimativa da sua presença ou da sua atividade (Achouak et al., 1999; Poly et al., 2001; Hurek et al., 2002; Burgmann et al., 2003; Knauth et al., 2005; You et al., 2005; Saito e Minamisawa, 2006; Fürnkranz et al., 2008; Terakado-Tonooka et al., 2008; Couillerot et al., 2010; Mao et al., 2011). A obtenção de colônias oriundas de amostras ambientais em meio sem adição de N não garante que tenha ocorrido fixação de N_2 por parte das bactérias isoladas, pois oligotróficos e *scavengers* podem se beneficiar dos baixos níveis de N reduzido que é lançado na atmosfera, ou de N liberado por um 'vizinho' fixador de N_2 (Boström et al., 2007; Chowdhury et al., 2007). O ensaio da redução de acetileno (C_2H_2) é um dos métodos mais precisos para estimativas da atividade da nitrogenase devido à quantificação direta da produção de etileno (o produto da quebra da ligação tripla do C_2H_2) (Döbereiner e Day, 1976; Danyal et al., 2010; Fernández et al., 2013).

O grau de atividade da nitrogenase entre diferentes bactérias não é diretamente relacionado a um menor ou maior potencial para a promoção de crescimento vegetal. Devido à intensa quantidade de sinais moleculares envolvidos na interação entre bactéria e planta, a fixação de N_2 pode não ser determinante para o estabelecimento de relações benéficas. De todo modo, estima-se que diazotróficos capazes de fixar quantidades elevadas de N_2 possam liberar uma fração significativa de N dissolvido no ambiente e, então, favorecer a captação do nutriente pelas raízes, ou ainda, fixar N_2 nos espaços intercelulares do tecido vegetal, como ocorre com as

bactérias endofíticas. Diversos diazotróficos de vida-livre podem estabelecer diferentes relações com as plantas, como exemplo de bactérias pertencentes aos gêneros *Azospirillum*, *Burkholderia*, *Gluconacetobacter* e *Paenibacillus* (Reiter et al., 2003; Somers et al., 2004).

A fixação de N_2 por micro-organismos e o potencial desse processo para o aumento da produtividade agrícola tem como seu principal exemplo as relações simbióticas entre os rizóbios e as plantas da família Fabaceae (leguminosas) (Willems, 2007). As trocas simbióticas ocorrem dentro de estruturas radiculares especializadas, os nódulos. Dentro do nódulo o rizóbio pode realizar efetivamente a FBN devido ao adequado controle da presença de oxigênio, um inibidor da atividade da nitrogenase. Os rizóbios são capazes de fornecer compostos nitrogenados assimiláveis ao vegetal e obter, em troca, compostos fotossintetizados fornecidos pela planta (van Lonn e Bakker, 2003). Muitas espécies de micro-organismos são utilizadas agronomicamente no cultivo de monoculturas de interessse econômico. Exemplos são as diferentes estirpes de *Bradyrhizobium japonicum* e *B. elkanii* utilizadas como inoculantes para a soja, *Rhizobium leguminosarum bv. viceae* para lentilha, grão-de-bico e ervilha e *R. tropici* para feijão. Devido ao sucesso das interações entre rizóbios e leguminosas e o uso dessas bactérias na agricultura, os estudos com PGPRs foram sendo cada vez mais intensificados na área da microbiologia agrícola. A importância do N e o potencial de fixação dos diazotróficos de vida-livre fez com que as bactérias que não estabelecem associações simbióticas também merecessem atenção especial nesse campo.

A introdução natural de N nos solos depende principalmente da incorporação do N inorgânico através da FBN, mas após assimilação e incorporação a esqueletos de C para formar compostos orgânicos, o N pode ser imobilizado na biomassa ou transformado através dos processos-chave de mineralização, nitrificação ou denitrificação (Morozkina e Zvyagilskaya, 2007). Enquanto a imobilização e a mineralização mantêm o N no sistema, a nitrificação [conversão oxidativa de amônia a nitrito e a nitrato, utilizando-a como doador de elétrons do metabolismo energético] e denitrificação [respiração anaeróbica com redução sequencial de nitrato, nitrito, óxido nítrico, óxido nitroso e N_2] estão envolvidas com a remoção desse nutriente do ambiente (Heylen e Keltjens, 2012).

Devido aos diferentes estados de oxidação do N (N^{5+} à N^{3-}) e a ampla capacidade biológica de conversão entre nove diferentes formas nitrogenadas, outros

processos de transformação de N são fundamentais para o dinamismo do ciclo devido à ampla complexidade químico-físico-biológica dos solos (Robertson e Groffman, 2007). Os processos de redução anaeróbica de nitrato a nitrito, redução de nitrito a óxido nítrico, óxido nitroso e N_2, redução de nitrito a amônia, oxidação aeróbica e anaeróbica do amônio e a produção de N_2 e oxigênio (O_2) através da óxido-nítrico dismutase, são todos pertencentes a diferentes modos de metabolismo de energia respiratória que conduz à geração de ATP em diversas bactérias (Simon e Klotz, 2013).

1.2. Mecanismos relacionados à promoção de crescimento vegetal

A atuação benéfica de micro-organismos na rizosfera deve-se ao resultado de diferentes mecanismos, mas pode ser amplamente classificada como promoção direta ou indireta do crescimento, dependendo do tipo de efeito que a bactéria exerce sobre a planta (Glick, 1995). As PGPRs podem afetar diretamente o crescimento das plantas quando são hábeis em exercer características como: 1) FBN (citado acima); 2) produção de sideróforos (quelantes de íon férrico); 3) solubilização de minerais, tais como fósforo; 4) produção de reguladores de crescimento vegetal (hormônios vegetais ou fito-hormônios); 5) atividade da enzima ACC deaminase (Selosse et al., 2004; Glick, 2005; Vega, 2007). A promoção indireta de crescimento pode ser exemplificada pela capacidade que uma determinada bactéria possui em impedir ou minimizar os efeitos deletérios causados por micro-organismos fitopatogênicos, o que pode ocorrer pela indução de resistência a patógenos ou pela produção de substâncias antagonistas (Glick, 1995; Glick et al., 1999).

As influências das PGPRs sobre diferentes plantas variam de espécie para espécie e também entre linhagens de uma mesma espécie. Inúmeras linhagens são PGPRs que atuam diretamente sobre o crescimento vegetal, mas também podem ser utilizadas como agentes de controle biológico de fitopatógenos, contribuindo indiretamente para a saúde da planta (Lacey et al., 2001; Siddiqui e Shakeel, 2009). PGPRs que apresentem além dos mecanismos diretos a capacidade de produzir diferentes antibióticos, por exemplo, podem ocasionar o decréscimo das atividades de micro-organismos deletérios, colaborando ainda mais para o crescimento das plantas. Além disso, os elicitores de resistência sistêmica induzida (ISR, do inglês *induced systemic resistance*) podem beneficiar as plantas através do desencadeamento de uma resposta nos tecidos, o que pode resultar na redução da incidência ou gravidade de

doenças causadas por agentes patogênicos (Kloepper et al., 2004). Quanto à produção de toxinas, um dos melhores exemplos é a proteína *cry* de *B. thuringiensis* e sua atuação sobre o sistema digestivo de larvas de determinados insetos (Pardo-Lopez et al., 2012).

Em relação à disponibilidade de ferro no ambiente e a importância da produção de sideróforos, pode se admitir que essa característica cause efeitos do tipo diretos ou indiretos nas plantas. Embora o Fe seja o segundo metal (após o alumínio) e o quarto elemento mais abundante da crosta terrestre, apenas uma pequena fração está disponível para ser utilizada pelos seres vivos. Em solos aerados e com pH neutro sua solubilidade é muito baixa (~1,4 x 10^{-9} M) devido à predominância de íon férrico encontrado na forma de hidróxido insolúvel de composição geral FeOOH (Chipperfield e Ratledge, 2000; Ratledge e Dover, 2000; Benite et al., 2002). Devido à alta capacidade de aceitar e doar elétrons prontamente, interconvertendo-se entre a forma férrica (Fe^{3+}) e ferrosa (Fe^{2+}), o Fe integra metaloproteínas e metaloenzimas centrais em diversos processos celulares vitais, tais como transporte de elétrons, redução de oxigênio durante a síntese de ATP, síntese de aminoácidos e nucleosídeos, síntese de DNA, fotossíntese e FBN. Dessa forma, o Fe é um elemento limitante para o crescimento de praticamente todas as formas de vida, salvo raras exceções, como os lactobacilos, que desenvolveram outros mecanismos para suprir essa carência nutricional (Guerinot et al., 1990; Benite et al., 2002; Andrews et al., 2003; Wandersman e Delepelaire, 2004).

A escassez de Fe é superada por muitos micro-organismos através da produção de sideróforos, metabólitos secundários que possuem alta afinidade pelo íon férrico, sendo produzidos sob condições de indisponibilidade desse metal no ambiente (Benite et al., 2002; Wandersman e Delepelaire, 2004). Após quelar o Fe^{3+} no ambiente externo o sideróforo é transportado de volta à célula microbiana para, finalmente, disponibilizar o metal à bactéria (Dobbelaere et al., 2003). Como benefício aos vegetais, a ligação Fe^{3+}-sideróforo pode impedir a proliferação de patógenos ao redor das raízes devido ao sequestro de Fe do meio ambiente. Ao contrário dos fitopatógenos microbianos, as plantas não são prejudicadas com a depleção de Fe pelas PGPRs e algumas podem até mesmo capturar e utilizar o complexo F^{3+}-sideróforo bacteriano (Dimkpa et al., 2009).

A habilidade de solubilizar fosfato pode ser encontrada entre diferentes espécies de PGPRs, as quais podem acidificar o solo e solubilizar fosfato mineral

insolúvel, liberando íons ortofosfato solúveis que podem ser captados pelas plantas (Jones, 1998). Os solubilizadores de fosfato secretam diferentes tipos de ácidos orgânicos, reduzindo o pH na rizosfera e, consequentemente, dissociando as formas de fosfato, como $Ca_3(PO_4)_2$ em solos calcários (Deubel e Merbach, 2005). Adicionalmente, tais micro-organismos são capazes de mineralizar compostos orgânicos fosfatados pela produção de enzimas fosfatases, atividade exercida especialmente pela microbiota e substancialmente aumentada na rizosfera (Garcia et al., 1992; Rodríguez e Fraga, 1999). Estima-se que bactérias solubilizadoras de fosfato na rizosfera de diferentes plantas constituam cerca de 20 a 40% dos micro-organismos cultiváveis do solo (Chabot et al., 1993; Rodríguez e Fraga, 1999).

A adição de fertilizantes fosfatados é uma prática comum na agricultura, entretanto, uma grande porção do fosfato inorgânico solúvel aplicado ao solo como fertilizante é rapidamente imobilizada logo após a aplicação (pelo Fe e Al em solos ácidos, e por Ca em solos calcáreos), tornando-se, assim, indisponível para as plantas (Holford, 1997). O ciclo de P no solo é um processo dinâmico e envolve sua transformação por processos geoquímicos e biológicos. O P existe no solo em uma variedade de formas orgânicas (como as derivadas de micro-organismos e plantas) e inorgânicas (como aquelas de fertilizantes fosfatados) (Paul e Clarck, 1989). As formas inorgânicas e orgânicas de P são lábeis e fracamente solúveis no solo, e, como resultado, apenas 1 a 5% permanecem disponíveis para as plantas (Dobbelaere et al., 2003). Em geral, o P disponível para as plantas ocorre na solução do solo na forma de ânions ortofosfato, predominantemente H_2PO_{4-} e HPO_4^{2-} (Arcand e Schneider, 2006).

A influência microbiana na rizosfera das plantas é grande parte devida à produção de fito-hormônios por micro-organismos do solo. Os fito-hormônios são pequenas moléculas derivadas do metabolismo secundário que atuam como reguladores naturais, influenciando grandemente os processos fisiológicos e desempenhando um importante papel no crescimento e desenvolvimento vegetal (Santner e Estelle, 2009). As citocininas podem atuar, por exemplo, no controle do crescimento vegetal através da regulação da divisão e diferenciação celular, agindo de forma antagônica às auxinas no controle das atividades dos meristemas (Jaillais e Chory, 2010). Ácido jasmônico está bastante envolvido com a defesa da planta contra patógenos (Santner e Estelle, 2009; Forchetti et al., 2010). A intensa regulação da atividade hormonal – e os mais variados

efeitos que ela acarreta, ilustra a complexidade do papel dos fito-hormônios nas plantas (Santner e Estelle, 2009; Baudoin et al., 2010; Jaillais e Chory, 2010).

A produção de determinados fito-hormônios é amplamente distribuída entre os micro-organismos, sobretudo do ácido indol-3-acético, comumente chamado de auxina ou AIA, e outros compostos indólicos. Estima-se que cerca de 80% das bactérias associadas a plantas são capazes de produzir diferentes compostos indólicos, sobretudo o AIA (Zakharova et al., 1999). As interações entre bactérias produtoras de AIA e plantas variam da patogênese à fito-estimulação e isso parece estar estritamente relacionado à rota de biossíntese do AIA que é utilizada pela bactéria (Spaepen et al., 2007). Em bactérias fito-patogênicas, tais como *Agrobacterium tumefaciens* e *Pseudomonas syringae*, o AIA é produzido a partir do aminoácido triptofano via o intermediário indol-acetoamida. As bactérias benéficas sintetizam AIA predominantemente por um caminho alternativo também dependente de triptofano, através do ácido indol-pirúvico. Pesquisas realizadas com bactérias mutantes deficientes na síntese de AIA demonstraram a existência de mais de uma rota biossintética dependente de triptofano para a produção de AIA em um mesmo micro-organismo (Patten e Glick, 2002; Baudoin et al., 2010).

As plantas podem ser beneficiadas por bactérias produtoras de AIA devido à influência desse fito-hormônio no crescimento radicular. O AIA estimula o estabelecimento do alongamento das raízes primárias ou a proliferação de raízes laterais e adventícias e é especialmente vantajoso para plantas jovens (Patten e Glick, 2002). Devido à importância das raízes para ancoragem no solo e obtenção de água e nutrientes do ambiente, o estímulo fornecido pelo AIA produzido por PGPRs pode contribuir para o sucesso no estabelecimento das plantas. O AIA bacteriano foi tido por muito tempo como uma molécula de importância relacionada apenas à relação bactéria-planta, mas alguns trabalhos tem relatado que ele pode ser um modulador dos níveis de determinadas moléculas sob condições de estresse das células bacterianas (Braeken et al., 2006). A quantidade de AIA produzido por uma bactéria pode afetar positiva ou negativamente a planta, visto que muitos micro-organismos fito-patogênicos liberam altas concentrações durante o estabelecimento da infecção (Kazan e Manners, 2009). Dessa forma, a produção de AIA é tida como uma característica benéfica, mas o papel do AIA bacteriano na promoção do crescimento vegetal ainda não está totalmente esclarecido (Patten e Glick , 2002; Kazan e Manners, 2009).

Muitas plantas inoculadas com PGPRs exibem aumento de resistência ao estresse ambiental, incluindo o estresse hídrico (Forchetti et al., 2007). Um dos mecanismos propostos para esse efeito é a atividade da enzima ACC deaminase bacteriana (Glick, 2005; Forchetti et al., 2007; Jalili et al., 2009; Farajzadeh et al., 2010), que age na redução dos níveis de etileno, fito-hormônio que tem sua síntese aumentada sob diversas situações de estresse. Condições de estresse geradas por salinidade, seca, metais pesados e patogenicidade, por exemplo, elevam a produção endógena de etileno pelas plantas, afetando de forma negativa o crescimento das raízes e, consequentemente, o desenvolvimento vegetal como um todo (Saleem et al., 2007). A ACC deaminase bacteriana regula a produção de etileno vegetal através da metabolização de ACC (precursor imediato da síntese de etileno em plantas superiores) a ácido α-cetobutírico e amônia (Arshad et al., 2007; Saleem et al., 2007). Plantas inoculadas com bactérias que possuam atividade de ACC deaminase apresentam crescimento radicular aumentado devido à diminuição de etileno ao redor das raízes, assim como uma atenuação do nível de estresse em resposta ao hormônio (Burd et al., 2000; Glick, 2005; Safronova et al., 2006).

1.3. Inoculantes biológicos e identificação bacteriana

A presença de diferentes habilidades em uma mesma bactéria (tais como aquelas características descritas acima) pode se tornar uma dificuldade no estudo da determinação do efeito benéfico de um único traço no desenvolvimento vegetal. De todo modo, diversos estudos demonstraram que PGPRs com diferentes habilidades são mais eficientes em promover o crescimento de plantas sob condições experimentais ou em ambientes naturais. Indiferente de ser uma bactéria Gram-negativa ou Gram-positiva, por exemplo, a PGPR necessita ser isolada em meio de cultura e analisada sobre os mais variados aspectos genotípicos e fenotípicos, assim como avaliada em relação a sua interação benéfica com a planta. Entretanto, muitas bactérias se destacam dependendo do tipo de planta ou característica estudado, como no caso dos rizóbios para leguminosas e *Gluconacetobacter diazotrophicus* para cana-de-açúcar, enquanto que na defesa contra patógenos o genêro *Bacillus* é bem explorado (Bloemberg e Lugtenberg, 2001; Vega, 2007). Entre os bacilos mais estudados encontram-se *Bacillus cereus* (Handelsman et al., 1990; Ryder et al., 1999; Silva et al., 2004; Wang et al., 2013), *B. licheniformis* (Chen et al., 1996; Probanza et al., 2002), *B. subtilis* (Turner e Blackman,

1991; Zhang e Smith, 1996; Chen et al., 2013) e *B. thurigiensis* (Bai et al., 2002; Hyakumachi et al., 2013).

Azospirillum brasilense também é uma espécie bastante estudada devido à capacidade de promover o crescimento de variadas plantas, sobretudo gramíneas. Variadas linhagens de *Azospirillum* têm sido isoladas dos solos e apresentam, em geral, atividades relacionadas à produção de hormônios vegetais e FBN (Fibach-Paldi et al., 2011; Farina et al., 2012; Souza et al., 2012). No entanto, análises quantitativas da fixação de N_2 em casa de vegetação e a campo, bem como a caracterização de mutantes para os genes da fixação de N_2 (como *nifH*), indicam que a contribuição de N fixado por *A. brasilense* não desempenha um papel fundamental na promoção do crescimento de plantas (Spaepen et al., 2009; Fibach-Paldi et al., 2011). Outras bactérias fixadoras de N_2 também se destacam pela promoção de crescimento vegetal, além dos rizóbios e *Azospirillum* spp., tais como *Azoarcus* sp., *Burkholderia* sp., *Herbaspirillum* sp., *Azotobacter* sp. e *Paenibacillus polymyxa*, as quais já foram isoladas das raízes ou solo rizosférico de uma série de plantas, tais como arroz, cana de açúcar, milho, sorgo e outros cereais (Barriuso et al., 2008).

Solubilizadores de fosfato como *B. megaterium* e *P. polymyxa* foram capazes de incrementar o crescimento e a produção, mas não a captação de P em canola, indicando que a solubilização de fosfato não é o principal mecanismo responsável pela resposta positiva de crescimento (de Freitas et al., 1997). *Pseudomonas putida* inibe o crescimento de *Fusarium oxysporum*, assim como *P. aeruginosa* inibe o crescimento de *Pythium* (fungos patogênicos de tomate), através da produção de sideróforos (Vandendergh e Gonzalez, 1984; Buysens et al., 1995). *P. putida* secretando altos níveis de AIA foi capaz de promover um aumento de 35 a 50% no crescimento primário das raízes de canola (Patten e Glick, 2002). Um mutante de *A. brasilense* Sp245 para produção de AIA foi menos eficiente na nodulação e fixação de N em raízes de feijão co-inoculadas com rizóbios, quando em comparação com a linhagem selvagem, o que indica a resposta diferencial da planta à ausência de auxina produzida pelas bactérias (Remans et al., 2008). Experimentos de inoculação com diferentes PGPR com atividade positiva da ACC deaminase têm sido analisados em diversos estudos: alongamento das raízes em canola (Madhaiyan et al., 2006) e milho (Shaharoona et al., 2006), aumento no comprimento das raízes e folhas também em canola (Saleh e Glick, 2001) e maiores taxas de crescimento em soja (Cattelan et al., 1999).

A adoção de técnicas de microbiologia e biotecnologia variadas pode contribuir grandemente para o conhecimento das interações entre micro-organismos e plantas e, sobretudo, favorecer a produção agrícola através da adoção de práticas que não agridam o meio ambiente. Devido à grande demanda por "tecnologias limpas" e a viabilização da agricultura sustentável, estima-se que futuramente um maior número dessas bactérias sejam utilizadas na produção de alimentos (Kaur et al., 2013). O uso de inoculantes – também chamados de biofertilizantes, além de evitar ou diminuir a contaminação ambiental ocasionada pelas adubações, pode contribuir para o aumento e estabilidade da produtividade vegetal. Além dos ganhos em produtividade (ou manejo de doenças no caso de micro-organismos de biocontrole), esses produtos também são adequados aos sistemas de produção em que a legislação não permite o uso de agroquímicos, como em sistemas orgânicos.

As pesquisas na área da microbiologia agrícola geram conhecimento a respeito da distribuição das características entre os diferentes isolados, diversidade bacteriana e interações planta-bactéria. A busca por mecanismos de interesse, como a fixação do N_2, pode direcionar o pesquisador a uma área específica de estudo e gerar um maior conhecimento a cerca das espécies isoladas. O isolamento e estudo de diversas rizobactérias presentes na rizosfera ou intimamente associadas às raízes de determinada planta permitem a seleção de espécies e estirpes com potencial para utilização agrícola. Outra análise essencial diz respeito à adequada identificação dos isolados que serão usados a campo, tendo em vista a necessidade de serem selecionadas bactérias que não apresentem toxicidade aos seres humanos, animais e plantas (não patogênicas em geral). Para determinadas espécies, como *A. brasilense*, características morfológicas da colônia e a fixação de N_2, além da identificação baseada em uma porção do gene 16S rRNA, são consideradas suficientes para a classificação (Lin et al., 2011). Entretanto, muitas espécies de bacilos, por exemplo, podem ser bastante semelhantes genética e fenotipicamente e, consequentemente, mais difíceis de serem classificadas (Priest et al., 1994; Joung e Côté, 2001; Heyrman et al., 2004; Wu et al., 2006)

A identificação bacteriana baseada no 16S rDNA permite grande acurácia devido às propriedades do gene, que é bastante conservado para permitir a amplificação, mas suficientemente divergente para proporcionar um sinal filogenético (Fraher, 2012). Além de um número menor de ocorrências relatadas de TGH em relação a diversos outros genes *housekeeping*, o 16S rDNA apresenta ampla distribuição, estabilidade

funcional e presença de regiões conservadas e variáveis (Wang e Qian, 2009; Schloss et al., 2011; Harish e Caetano-Anollés, 2012). As relações filogenéticas baseadas na sequência do 16S rDNA podem ser realizadas por meio de métodos baseados em caracteres como, por exemplo, a máxima parcimônia e máxima verossimilhança, ou por meio de métodos baseados em distância, como Neighbor-Joining e UPGMA (*unweighted pair-group average*). Os métodos baseados em caracteres, principalmente os probabilísticos como a máxima verossimilhança, são tidos como mais precisos devido às análises dos dados brutos (caracteres) e não de dados reduzidos a um único valor, como ocorre por computação de matriz de distância (Bhargava e Sharma, 2013). Por outro lado, a análise de dados fenéticos por matrizes binárias pode fornecer uma estimativa precisa da similaridade genética quando a reprodutibilidade não é um problema recorrente na técnica (Osborn, 2005). As técnicas de *fingerprinting* têm sido usadas na classificação de linhagens ou espécies em estudos clínicos e ambientais e a análise do polimorfismo de comprimento de fragmento de restrição (RFLP, do inglês *restriction fragment length polymorphism*) está entre as mais utilizadas (Shangkuan et al., 2000; Jang et al., 2002; Vardhan et al., 2011).

Após a adequada seleção de estirpes e experimentos em câmaras de crescimento ou casas de vegetação, é desejável que as bactérias sejam analisadas a campo para a realização de ensaios de promoção de crescimento vegetal em condições reais de cultivo. Os estudos a campo envolvem a adoção de normas técnicas do Ministério da Agricultura, Pecuária e Abastecimento (MAPA), as quais correspondem às práticas agrícolas empregadas para uma correta avaliação da produtividade vegetal. A etapa da produção do inoculante – em um veículo líquido ou turfoso, por exemplo, é fundamental para que o produto apresente condições viáveis de sobrevivência ao micro-organismo por um período de pelo menos seis meses a partir da data de fabricação (MAPA). Nesse sentido, a capacidade das bactérias em produzir estruturas de resistência ao estresse – como os endósporos de bactérias Gram-positivas pertencentes aos gêneros *Bacillus* e *Paenibacillus*, é um aspecto interessante por contribuir para a manutenção de células viáveis (na forma de endósporos) e adequada estabilidade populacional das bactérias no veículo utilizado para a produção do inoculante (McSpadden Gardener, 2004). Entre as Gram-negativas, como citado acima, diversas bactérias já são comumente utilizadas na agricultura, como os inoculantes a base de

Bradyrhizobium elkanii e *B. japonicum* para soja e *A. brasilense* para trigo e milho (Hungria et al., 2010).

1.4. Girassol (*Helianthus annuus* L.)

O gênero *Helianthus* compreende 49 espécies e 19 subespécies, com 12 espécies anuais e 37 perenes, todas nativas das Américas (Ungaro,1990). O girassol (*Helianthus annuus* L.) é uma dicotiledônea anual da família Asteraceae, originária do continente norte-americano (Brighenti et al., 2004). Por ser uma cultura que apresenta características desejáveis sob o ponto de vista agronômico, tais como ciclo curto, ampla capacidade de adaptação a diferentes ambientes e elevado rendimento em óleo, o cultivo de girassol é uma boa opção aos produtores brasileiros (Castro, 2007; Pires et al., 2007; Silva et al., 2007; Amorim et al., 2008; Backes et al., 2008). O girassol é indicado à alimentação devido à alta qualidade nutricional das sementes (24% proteínas: 47% óleo: vitaminas E, B1 e B5) e do óleo (alto teor de ômega 6) (Gazzola et al., 2012). Sua importância econômica está relacionada ao mercado de óleos comestíveis nobres, apicultura, alimentação animal, utilização ornamental e, mais recentemente, à produção de biodiesel (Castro, 2007; Gazzola et al., 2012).

A tolerância a baixas temperaturas na fase inicial de desenvolvimento faz do girassol uma importante alternativa de cultivo em diferentes regiões agrícolas do Brasil (Amorim et al., 2008; Cadorin, 2012). No sistema de sucessão de culturas, onde é interessante que pelo menos uma das espécies utilizadas apresente cultivares de ciclo precoce, o girassol também é uma boa alternativa por ter um ciclo vegetativo que varia de aproximadamente 90 a 130 dias (Silva e Dalbem, 1989; Leite et al., 2005; Gazzola et al., 2012). Seu cultivo ocorre, predominantemente, após a colheita da safra de verão em sucessão à soja ou milho, podendo também ser utilizado como primeira cultura (Rizzardi et al., 2000; Amorim et al., 2008). No Estado do Rio Grande do Sul ele tem sido cultivado no período que antecede as culturas de soja e de milho (Pires et al., 2007).

O cultivo do girassol ainda necessita de uma adequação aos diferentes sistemas de produção das culturas tradicionais, como milho, soja, cana-de-açúcar, arroz e outras, assim como carece de incrementos à produtividade e bons rendimentos da cultura (Castro, 2007; Smiderle e Costa, 2012). Amorim e colaboradores (2008) salientam a importância da umidade do solo no desenvolvimento da cultura do girassol,

ressaltando que o rendimento máximo é alcançado em capacidade de campo. A produtividade de grãos é um caráter complexo e resultante da expressão e associação de diferentes componentes considerados pelo melhorista no processo de seleção de novos genótipos (Amorim et al., 2008). Nas análises de rendimento da cultura, os caracteres diâmetro do capítulo e peso dos grãos, por exemplo, estão correlacionados de forma positiva e significativa com a produtividade (Amorim et al. 2008; Backes et al. 2008).

As variações edafoclimáticas interferem de forma direta sobre o potencial produtivo e de forma indireta, por exemplo, na proliferação de doenças como a podridão branca (*Sclerotinia sclerotiorum*), a qual é favorecida por temperaturas amenas (19 a 20°C) e umidade relativa alta (Backes et al., 2008). Desta forma, a temperatura, a umidade relativa do ar e a luminosidade, além da disponibilidade hídrica já citada, afetam a produtividade e o rendimento da cultura (Backes et al., 2008). Segundo Castro e Farias (2005), temperaturas baixas, pouca luminosidade e alta umidade prolongam o ciclo da cultura, atrasando a floração e a maturação fisiológica. Além disso, em uma mesma cultura, diferentes genótipos podem distinguir-se quanto à produtividade de grãos e ciclo vegetativo, entre outras características (Heckler, 2002). No estudo de Pires e colaboradores (2007) no Planalto Médio do Rio Grande do Sul, detectou-se variação entre os genótipos estudados para o rendimento de grãos, estádios de desenvolvimento e teor de óleo, entre outras características.

A interação entre genótipos e ambiente e a variação do comportamento de cultivares em função da região e época de plantio pode interferir enormemente na produção de girassol (Porto et al., 2007). As condições físicas e químicas do solo, sobretudo, são parâmetros de forte impacto sobre o rendimento das culturas (Alami et al., 2000). A variação da produtividade pode ser exemplificada pela comparação entre diferentes regiões do Rio Grande do Sul, onde a produtividade variou de cerca de 1.000 a 2.400 kg/ha na safra 2006/2007 (IBGE, 2007). Os programas de melhoramento genético procuram desenvolver genótipos de girassol que tenham, concomitantemente, alto teor de óleo, ciclo precoce, porte baixo, resistência a fatores bióticos e abióticos e alta produtividade (Amorim et al., 2008). A Embrapa tem desenvolvido, desde 1989, estudos que incluem o melhoramento genético do girassol, obtenção de maior potencial de rendimento na cultura, resistência a doenças, ampla adaptação e elevado teor de óleo. A obtenção de informações por meio da pesquisa tem sido decisiva para dar suporte

tecnológico ao desenvolvimento da cultura, garantindo uma melhor produtividade e um retorno econômico competitivo (Porto et al., 2007).

A multiplicidade de fatores associados à produtividade e rendimento desta cultura influencia grandemente na produção e cultivo. Segundo Castro (2007), a baixa produtividade dos grãos é, entre outros critérios, um entrave para a produção de girassol no Brasil. Entre os estudos já realizados com girassol, como exemplos dos benefícios causados ao crescimento vegetal por PGPR, destacam-se os seguintes. Em 2007, Forchetti e colaboradores isolaram 29 linhagens bacterianas endofíticas de girassol cultivado sob condições de seca e boa irrigação. Destas, quatro linhagens de bacilos exibiram altas atividades de solubilização de fosfato, atividades positivas para catalase, oxidase e fixação de N e produção de jasmonatos, ácido oxo-fitodienóico e ácido-abscísico. Além destas características, houve forte inibição do crescimento dos patógenos *Verticillum orense* e *S. sclerotiorum* sob condições de seca. Alternativamente, outros dois bacilos foram capazes de inibir a proliferação de *S. sclerotiorum* e *Alternaria* sp. sob condições de boa irrigação. Exemplos de bactérias endofíticas de girassol isoladas neste estudo foram: *Bacillus pumilus, Achromobacter xiloxidans* e *Alcaligenes* sp.

Uma linhagem de *Rhizobium* sp. isolada da região associada às raízes em girassol (Alami et al., 2000) apresentou alta produção de exopolissacarídeos (EPS), rápida colonização e habilidade de persistir em alta densidade por até duas semanas na rizosfera. Após inoculação com esta bactéria, o aumento populacional e de EPS produzidos ampliou significativamente a massa de solo aderido à raiz e o peso do tecido radicular, independentemente das condições hídricas. Tal benefício está relacionado ao acréscimo de solo ao redor das raízes ou à maior estabilidade dos agregados de solo, ambos efeitos possivelmente influenciados pela produção de EPS. A inoculação também beneficiou o aumento de peso seco das folhas, além de oferecer limitação aos efeitos negativos gerados sob condição de seca: a planta sob estresse, mas inoculada com rizóbio, apresentou melhor razão entre massa de solo aderido à raiz/peso do tecido radicular do que a planta controle, mantida sob adequada condição hídrica e não inoculada.

No trabalho de Akbari e colaboradores (2011), a inoculação de girassol com *Azosporillum* e *Azotobacter*, em conjunto com fertilizante nitrogenado ou esterco, foi capaz de promover grandes rendimentos no cultivo a campo. O biofertilizante melhorou

a produtividade da planta e a qualidade das sementes de girassol e, interessantemente, diminuiu os ácidos graxos saturados (palmítico e esteárico) e aumentou o teor de ácidos graxos insaturados (ácido linoléico e ácido oléico) e teor de óleo, comparado com as plantas não tratadas.

2.1. Objetivo geral

Tendo em vista a obtenção de maiores conhecimentos quanto às diferentes comunidades de bactérias cultiváveis naturalmente associadas a girassol, o objetivo principal desse estudo foi identificar e selecionar estirpes com potencial para a fixação de nitrogênio, além de outros mecanismos de promoção de crescimento vegetal, em prol do uso de biofertilização no cultivo dessa oleaginosa.

2.2. Objetivos específicos

I. Obter diferentes isolados bacterianos e caracterizar as linhagens quanto a habilidades relativas à promoção de crescimento vegetal, tais como a fixação de N_2, produção de compostos indólicos, produção de sideróforos, solubilização de fosfato, atividade de ACC deaminase e degradação de ácido oxálico;

II. Analisar os dados obtidos por PCR-RFLP *in vitro* e *in silico*, identidade de sequência nucleotídica e filogenia baseados no 16S rDNA, para uma identificação mais acurada dos isolados;

III. Contribuir para o conhecimento da distribuição de comunidades de PGPR associadas à rizosfera de girassol, para ambos os grupos de bactérias Gram-negativas e Gram-positivas;

IV. Submeter os isolados bacterianos promissores a ensaios de inoculação de sementes e experimentos em câmara de crescimento, a fim de avaliar a promoção efetiva desses ao desenvolvimento das plantas;

V. Avaliar a eficiência agronômica da estirpe selecionada na forma de um produto inoculante para experimentos de biofertilização a campo, visando a posterior utilização da bactéria em biofertilizantes para as lavouras de girassol;

VI. Incentivar a pesquisa em prol do cultivo de girassol a partir da utilização de inoculantes que possam contribuir para o aumento da produtividade e a redução do uso de fertilizantes minerais na cultura dessa oleaginosa.

Capítulo I

Screening of plant growth promoting Rhizobacteria isolated from sunflower (*Helianthus annuus* L.)

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REGULAR ARTICLE

Screening of plant growth promoting Rhizobacteria isolated from sunflower (*Helianthus annuus* L.)

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Abstract
Background and Aims This study was aimed at assessing the diversity of putatively diazotrophic rhizobacteria associated with sunflower (*Helianthus annuus* L.) cropped in the south of Brazil, and to examine key plant growth promotion (PGP) characteristics of the isolates for the purposes of increasing plant productivity.
Methods 299 strains were isolated from the roots and rhizosphere of sunflower cultivated in five different areas using N-free media. 16S rDNA PCR-RFLP and 16S rRNA partial sequencing were used for identification and the Shannon index was used to evaluate bacterial diversity. Production of siderophores and indolic compounds (ICs), as well phosphate solubilization activities of each isolate were also evaluated in vitro. On the basis of multiple PGP activities, eight isolates were selected and tested for their N-fixation ability, and their capacity as potential PGPR on sunflower plants was also assessed.
Results All except three Gram-positive strains (phylum Actinobacteria) belonged to the Gram-negative Proteobacteria subgroups [Gamma (167), Beta (78), and Alpha (50)] and the family Flavobacteriaceae (1)]. Shannon indexes ranged from 0.96 to 2.13 between the five sampling sites. *Enterobacter* and *Burkholderia* were the predominant genera isolated from roots and rhizosphere, respectively. Producers of siderophores and ICs were widely found amongst the isolates, but only 19.8% of them solubilized phosphate. About 8% of the isolates exhibited all three PGP traits, and these mostly belonged to the genus *Burkholderia*. Four isolates were able to stimulate the growth of sunflower plants under gnotobiotic conditions.
Conclusions *Enterobacter* and *Burkholderia* were the dominant rhizospheric bacterial genera associated with sunflower plants. Inoculation with isolates belonging to the genera *Achromobacter*, *Chryseobacterium*, *Azospirillum*, and *Burkholderia* had a stimulatory effect on plant growth.

Keywords Sunflower · PGPR · Diversity · *Burkholderia* · *Enterobacter*

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Introduction

Microorganisms in the soil are essential for the maintenance and sustainability of plant and animal communities

by enabling the transformation of nutrients and by performing many key biological processes. The organic matter released by plant roots increases the microbial activity around the roots, where a large number of microscopical organisms, such as bacteria, fungi, protozoa and algae coexist. The 'rhizosphere' is a term first used by Hiltner in 1904 to describe this locality as 'the compartment influenced by the roots' in which intense interactions between beneficial and pathogenic microorganisms occur at all times (Hinsinger and Marschner 2006; Barriuso et al. 2008). While the bacteria utilize the nutrients that are released from the host for their growth, they also secrete a wide variety of metabolites into the rhizosphere (van Loon 2007). Root-colonizing plant-beneficial bacteria are called plant growth-promoting rhizobacteria (PGPR) and are characterized by at least two of the three following criteria: they competitively colonize roots, stimulate growth and/or reduce the incidence of disease (Haas and Défago 2005). Moreover, some root-colonizing bacteria increase tolerance against abiotic stresses, such as drought, salinity and metal toxicity (Dimpka et al. 2009). Generally, about 2–5% of rhizospheric bacteria are PGPR (Antoun and Prévost 2005).

Plant-beneficial bacteria can also be found on leaves (phyllosphere bacteria) or they may exist within plant tissues (endophytic bacteria) (Glick et al. 2007). In root endophytic bacterial relationships there is no formation of differentiated structures such as nodules, and the bacteria reside within apoplastic spaces, dead cells and xylem vessels inside the host plant (James 2000; Vessey 2003). Changes to the physical, chemical and biological properties of rhizospheric soil have a significant influence on the subsequent growth and health of plants (Richardson et al. 2009). The complex web of interactions that takes place among plants and PGPR may affect plant growth directly or indirectly (Barriuso et al. 2008). Among the processes that contribute to increasing nutrient availability to plant roots, nitrogen fixation is the most widespread due to the importance of nitrogen as a limiting factor for crop productivity (James 2000). However, many other processes, such as solubilization of unavailable forms of nutrients (as phosphate), siderophore production which helps in the transport of certain compounds (notably ferric iron), production of phytohormones (such as auxin and cytokines) and volatile growth stimulants (such as ethylene and 2,3-butanediol) (Haas and Défago 2005), are recognized mechanisms of plant growth promotion (PGP). For some PGPR, termed biofertilizers (biological fertilizers), PGP dominates through the mechanisms cited previously. However, not all PGPR can be considered biofertilizers; bacteria that promote plant growth by control of deleterious organisms are biocontrol agents, but not biofertilizers (Vessey 2003). Free-living bacteria or endophytes that have been found to display beneficial effects on various plants include species of the genera *Arthrobacter*, *Azotobacter*, *Azospirillum*, *Bacillus*, *Burkholderia*, *Enterobacter*, *Herbaspirillum*, *Pseudomonas*, *Rhizobium,* and *Serratia* (Gray and Smith 2005).

Plants affect the indigenous microbial populations in soil, and each plant species is thought to select specific microbial populations that contribute most to their fitness, creating a very selective environment and limited diversity (Berg and Smalla 2009). There are indications that some rhizobacterial strains show strong host-plant selectivity and colonize a single plant species or variety (Zeller et al. 2007). Several studies and reviews have also reported stringent host-plant specificity of rhizospheric bacteria (Wieland et al. 2001; Singh et al. 2004; Bergsma-Vlami et al. 2005; Berg and Smalla 2009; Raaijmakers et al. 2009; Buchan et al. 2010). In this context, culture-based techniques can allow for the selection of strains better adapted to the rhizospheric environment as well as for the characterization of microbial diversity and information about which microorganism is capable of establishing a relationship with the studied plant.

Soil nutrients are transferred towards the root surface through the rhizosphere and thus the functional and structural diversity of bacteria has a significant implication for plant growth and nutrition. (Simon and Daniel 2009). Sunflower (*Helianthus annuus* L.) is a plant with high adaptability to different environmental conditions, and is a good alternative crop within crop rotations in different agricultural regions of Brazil (Amorim et al. 2008; Backes et al. 2008). This oleaginous plant is one of the four most important oilseed crops in the world (Škorić et al. 2008), and its economic importance is due to the nutritional quality of its oil, beekeeping, animal feed and biofuel production. However, increases in productivity and yield per cultivated area are still needed in Brazil in order to meet demand. There have been very few studies of the microbial diversity associated with sunflower, and

hence the bacterial diversity of sunflower in Brazilian soils remains largely unknown. As the selection of microbial strains displaying the capability to stimulate plant growth and productivity for potential application in sustainable agriculture depends on traditional culture-based methods, the aim of this work was to assess the diversity of putatively diazotrophic rhizobacteria (either root or rhizospheric bacteria) associated with sunflower cropped in different areas in the south of Brazil. Some isolates were also tested for their abilities to stimulate sunflower growth. Further studies will clarify the potential use of these bacteria as biofertilizers.

Materials and methods

Sample processing

Samples of roots and rhizospheric soil from sunflower (cv. Aguará 6) were collected between November 2009 and April 2010 (summer months) from five different sunflower producer regions of Rio Grande do Sul (RS) State, Brazil: Encruzilhada do Sul [E (30°32′38″S, 52°31′19″W)], São Borja [SB (28°39′39″S, 56°00′14″W)], São Gabriel [SG (30°20′09″S, 54°19′12″W)], Vacaria [Vac (28°30′43″S, 50°56′02″W)], and Viamão [Vi (30°04′51″S, 51°01′22″W)]. This region presents a relatively balanced distribution of rainfall throughout the year, but the average rainfall lies between 1299 and 1500 mm in the south and 1800 and 1500 mm in the north, with higher intensity rainfall in the northeast region of the State. The climate of RS is humid subtropical (Cfa by Köppen's classification) and the average temperatures range between 15 and 18°C, with large variations between a minimum of −10°C and a maximum of 40°C.

The samples were collected at the flowering stage from fields of annual crops under a no-tillage system. Moreover, although the sites had a different cropping history they followed a standard fertilization regime. Ten samples each of 0.5 kg of fresh weight soil were taken at a depth of 15 cm and bulked to obtain a representative composite sample. Sub-samples (0.5 kg fresh weight) were analyzed for clay, organic matter content (OMC), pH, P, K, Fe, Ca, Mg and Al by standard methods (Table 1).

Isolation of putative diazotrophic root and rhizospheric bacteria

Bacteria were isolated according to Döbereiner (1988). Roots were separated from the rest of the sunflower plant, and rhizospheric bacteria were isolated from soil adhered to the roots. Root bacteria were isolated after surface disinfection, which was performed by washing the roots in running tap water, and then by immersion in 70% ethanol for 1 min and in sodium hypochlorite solution (4%, v/v) for 2 min, followed by five serial rinses in sterilized distilled water. Immediately after disinfection, the roots were sliced with a sterile scalpel. A total of 10 g of soil (for rhizospheric bacteria) and 10 g of root segments (for root bacteria) were placed in individual sterile 500 ml Erlenmeyer flasks containing 90 ml of sterile saline solution (0.85% NaCl). Samples were incubated at 28° C under agitation (200 rpm) for 16 h. Aliquots of 0.1 ml of three-fold serial dilutions were inoculated, in triplicate, into vials containing 4 ml of semi-solid N-free medium (0.18% agar-agar), either NFb, LGI or LGI-P (Döbereiner 1988). Five days after incubation at 28°C, those vials showing a veil-like pellicle near the surface of the medium were considered to be positive for bacterial growth, and were used for reinoculation into others vials containing the same semi-solid N-free medium previously utilized. The cultures from the positive vials were subjected to further purification steps by streaking them onto specific agar plates of the same medium as was used in the semi-solid vials, but containing 20 mg l^{-1} of yeast extract, and incubated at 28°C for 2 d. After incubation, distinct colonies were grown in liquid LB medium (Sambrook and Russel 2001) at 28°C under agitation (200 rpm); approximately 60 colonies were isolated from each sunflower cropping region (about 30 colonies each for both root and rhizospheric bacteria). After Gram testing, each pure culture was suspended in 50% sterilized glycerol solution and stored at −18°C.

Genomic DNA extraction

Genomic DNA was extracted from bacteria using the following protocol: a 1.5 ml sample of a bacterial culture grown in LB was centrifuged for 5 min at 12,000*g*. The bacterial pellet was rinsed with 700 µl of TES buffer (10 mM Tris pH 8.0, 25 mM EDTA,

Table 1 Abiotic characteristics of the soils at the sampling sites

Sampling sites [a]	Clay (%)	OMC (%)	pH (H_2O)	P (mg/dm^3)	K (mg/dm^3)	Fe (g/dm^3)	Ca $_{exc}$ ($cmol_c/dm^3$)	Mg $_{exc}$ ($cmol_c/dm^3$)	Al $_{exc}$ ($cmol_c/dm^3$)
1. Encruzilhada do Sul	25	2.1	5.2	1.6	71	0.17	1.7	1.2	0.4
2. São Borja	26	4.8	5.3	4.6	73	3.5	7.0	3.3	0.2
3. São Gabriel	28	3.1	5.6	24	205	1.5	6.8	3.4	0.0
4. Vacaria	64	5.6	6.5	12	149	2.8	8.5	5.2	0.0
5. Viamão	19	1.3	5.4	5.8	121	0.7	2.2	1.6	0.4

OMC organic matter content

exc: exchangeable

[a] Soil classification: Encruzilhada do Sul and Viamão (sandy clay loam Paleudult), São Borja (Ferralic Nitisol), São Grabriel (Rhodic Palleudult) and Vacaria (Haplumbrept)

150 mM NaCl), recentrifuged and resuspended in 500 µl of TE buffer (10 mM Tris pH 8.0, 25 mM EDTA) plus 25 µl of lysozyme (20 mg ml^{-1}) with incubation at 37°C for 30 min. A 108 µl volume of sodium dodecyl sulfate (20% SDS) and a 5 µl volume of proteinase K (20 mg ml^{-1}) were added. The samples were homogenized for 30 s in a vortex and incubated at 56°C for 15 min. A 600 µl volume of ammonium acetate (8 M pH 8.0) was added and the samples were kept on ice for 30 min and subsequently centrifuged for 20 min at 12,000*g* for precipitation of cellular waste. Extractions with phenol-chloroform and ethanol precipitation were performed as described by Sambrook and Russel (2001). The pellet was washed with 70% ethanol and subsequently air dried then resuspended in 50 µl of TE buffer. The DNA quality and integrity were checked by electrophoresis in 0.8% agarose gels in 1 X Tris-borate-EDTA buffer (Sambrook and Russel 2001) with ethidium bromide and visualized by UV light.

Polymerase chain reaction (PCR) amplification, restriction fragment length polymorphism (RFLP) analysis and partial sequencing of bacterial 16S rRNA gene

Selected primers U968 (AACGCGAAGAACCT TAC) and L1401 (CGGTGTGTACAAGACCC, Felske et al. 1997) flanking a region of about 500 base pairs between nucleotides 968 and 1401 of the *Escherichia coli* 16S rRNA gene, including variable regions V6 to V8 (Brosius et al. 1978), were used. Amplification of 16S rDNA was performed using 50 ng of DNA as template in 25 µl reactions containing 20 µM each deoxynucleoside triphosphates, 0.3 µM of each primers U968 and L1401, 2 mM $MgCl_2$ and 2 U Taq polymerase (Invitrogen®) in 1 X Taq buffer. The amplifications were performed in a PCR Express Temperature Cycling System (Thermo Hybrid) as follows: an initial denaturation step at 94°C for 5 min followed by 30 cycles at 94°C for 45 s, 52°C for 45 s, 72°C for 45 s and one cycle at 72°C for 10 min for final elongation. PCR products were analyzed by electrophoresis in 1% agarose gels in TBE buffer with ethidium bromide and visualized by UV light. Subsamples of about 5 µl from each PCR product were digested with restriction endonucleases HaeIII and TaqI (Promega®) according to the manufacturer's specifications. Digestions were performed overnight to ensure complete fragmentation. RFLP analysis was performed by electrophoresis in a 10% polyacrylamide gel for 4 h at 180 V in 1 X 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 entire restriction profile obtained by RFLP was used to discriminate each isolate, by constructing a binary matrix for each region. Data were subjected to diversity analysis using PAST software (Hammer et al. 2001; available on http://folk.uio.no/ohammer/past/), by UPGMA algorithm and Jaccard coefficient. A dendrogram showing the genetic relationships of isolates (roots and rhizopheric bacteria were summed up) was constructed for each sampled site. One representative bacteria belonging to each cluster obtained with the 16S rDNA-PCR-RFLP was chosen for partial sequencing of the 16S rRNA gene. Sequencing was performed on an ABI PRISM 3100 Genetic Analyzer automatic sequencer (Applied Biosystems) in the

ACTGene Laboratory (Centro de Biotecnologia, UFRGS, RS, Brazil) using forward and reverse primers, as described by the manufacturer. Sequence analyses were performed with BioEdit version 7.0.9.0 software (Hall 1999) in order to verify their quality and to check for possible chimeric origins. DNA sequences (approximately 500 bp) were compared with sequences from EzTaxon Server version 2.1 (Chun et al. 2007; available on http://www.eztaxon.org/) and GenBank database using BLASTN software (Altschul et al. 1997; available on http://blast.ncbi.nlm.nih.gov/). The nucleotide sequences of the 74 partial 16S rRNA gene segments determined in this study have been deposited in GenBank. Their accession numbers are presented in Fig. 1.

Diversity index

The diversity index H' (Shannon and Weaver 1949) was estimated based on dendrograms of each sampled site by counting the number of clusters at the 70% similarity level and the number of taxa within each cluster (Borges et al. 2003; Kaschuk et al. 2006). Principal component analysis (PCA) was used to verify the statistical correlation between soil properties and bacterial diversity (Rico et al. 2004). Pair-wise squared Euclidean distances based on different soil properties were calculated for the five analyzed soils in order to obtain a double-centered distance matrix for factoring (Rohlf 1990). The H' as well as PCA were computed using PAST software (Hammer et al. 2001).

PGP characteristics

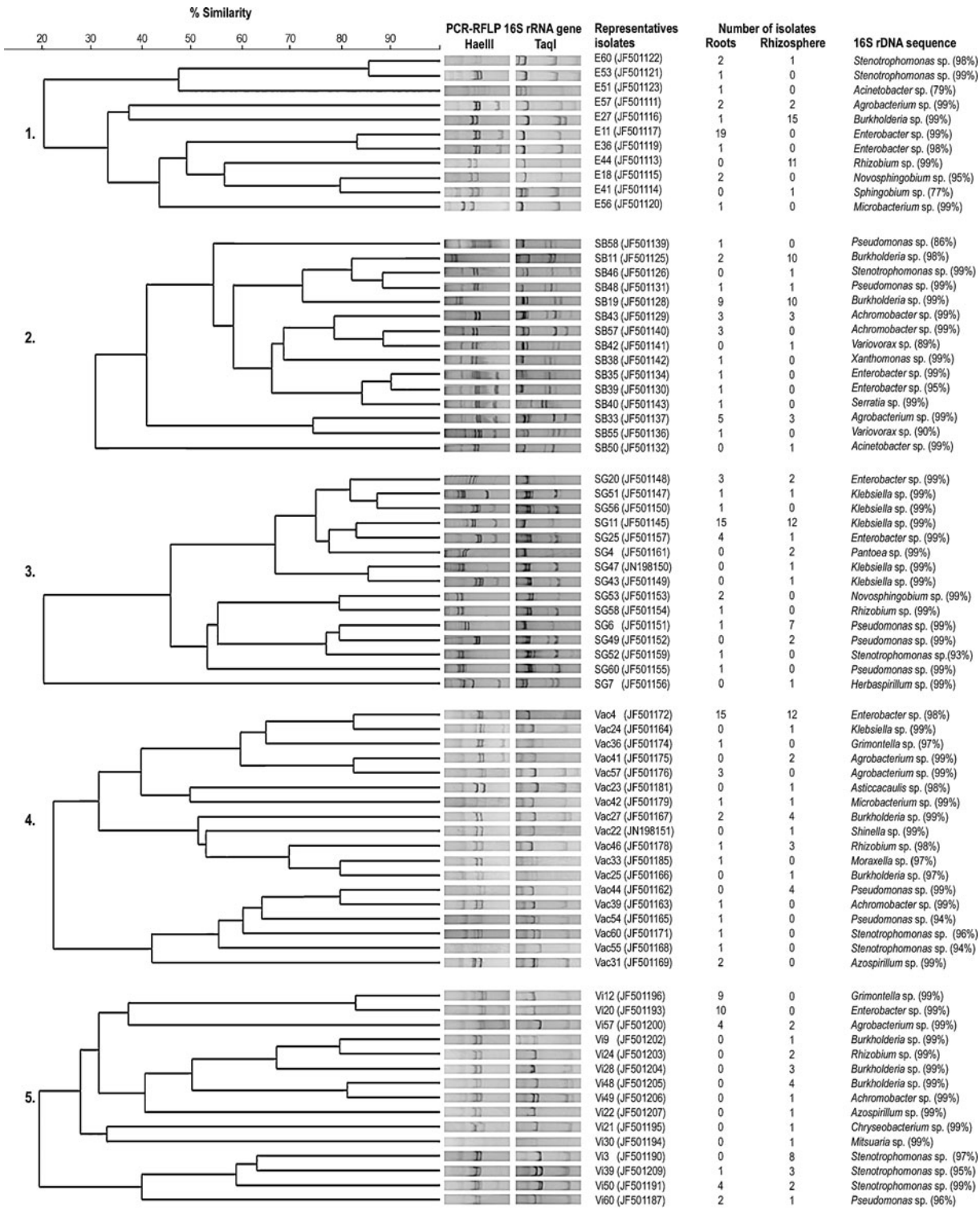
Analysis of production of indolic compounds and siderophores, as well as phosphate solubilization activities were carried out for all bacterial isolates and in vitro biological nitrogen fixation assays were performed on eight selected isolates. Bacterial suspensions (10 μl of 10^6 CFU ml^{-1}) of isolates grown in LB medium at 28°C under agitation (200 rpm) for 48 h were used as inocula in the four PGP assays.

The in vitro indolic compounds production assay was performed in Kings B medium with tryptophan (Glickmann and Dessaux 1995) by incubation the isolates at 28°C under agitation (200 rpm) for 72 h. About 1.5 ml of each culture was then centrifuged at 12,000*g* for 6 min, after which the supernatant (500 μl) was mixed with an equal volume of Salkowski reagent (12 gl^{-1} $FeCl_3$+7.9 M H_2SO_4) in test tubes, and the mixture was kept in the dark for 30 min to allow for color development. The pink to red color produced after exposure to Salkowski reagent was considered to be indicative of bacterial production of indolic compounds. The samples were measured spectrophotometrically at 550 nm using a standard curve for calibration (Sarwar and Kremer 1992).

Siderophore production was assayed according to Schwyn and Neilands (1987) using Kings B medium (Glickmann and Dessaux 1995) without tryptophan which was diluted fivefold. The isolates were spot inoculated onto Chrome azurol S agar plates divided into equal sectors, and the plates were incubated at 28°C for 48–72 h. Development of a yellow, orange or violet halo around the bacterial colony was considered to be positive for siderophore production.

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) 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 of distilled water, and the other containing 10 g $CaCl_2$ in 100 ml of distilled water. These solutions were added to one liter of GY medium just before pouring onto Petri dishes, and together they formed an insoluble layer of calcium phosphate that made the medium opaque. The plates were inoculated with the bacterial isolates, and then incubated for seven days at 28°C. Those isolates that formed visibly clear halos around their colonies were considered to be phosphate solubilizers.

The nitrogen-fixing ability of isolates was tested using the acetylene reduction assay (ARA), as described by Boddey (1987). The nitrogenase activity was measured after growth into 10 ml vials containing 4 ml of semi-solid (0.18% agar-agar) N-free media, either NFb, LGI or LGI-P. After 72 h incubation at 28°C in the dark the vials were sealed with rubber septa and 10% (v/v) of the air phase was replaced with acetylene (Burris 1972). The cultures were then incubated for 1 h in acetylene, after which the amount of C_2H_4 produced was measured for three vials for each isolate using a Clarus 600 gas chromatograph (Perkin Elmer) with a Col-Elite-Alumina column (50 m×0.53 mm ID×


% Similarity
20 30 40 50 60 70 80 90
PCR-RFLP 16S rRNA gene
HaeIII TaqI
Representatives isolates
Number of isolates
Roots Rhizosphere
16S rDNA sequence
1.
E60 (JF501122) 2 1 Stenotrophomonas sp. (98%)
E53 (JF501121) 1 0 Stenotrophomonas sp. (99%)
E51 (JF501123) 1 0 Acinetobacter sp. (79%)
E57 (JF501111) 2 2 Agrobacterium sp. (99%)
E27 (JF501116) 1 15 Burkholderia sp. (99%)
E11 (JF501117) 19 0 Enterobacter sp. (99%)
E36 (JF501119) 1 0 Enterobacter sp. (98%)
E44 (JF501113) 0 11 Rhizobium sp. (99%)
E18 (JF501115) 2 0 Novosphingobium sp. (95%)
E41 (JF501114) 0 1 Sphingobium sp. (77%)
E56 (JF501120) 1 0 Microbacterium sp. (99%)
2.
SB58 (JF501139) 1 0 Pseudomonas sp. (86%)
SB11 (JF501125) 2 10 Burkholderia sp. (98%)
SB46 (JF501126) 0 1 Stenotrophomonas sp. (99%)
SB48 (JF501131) 1 1 Pseudomonas sp. (99%)
SB19 (JF501128) 9 10 Burkholderia sp. (99%)
SB43 (JF501129) 3 3 Achromobacter sp. (99%)
SB57 (JF501140) 3 0 Achromobacter sp. (99%)
SB42 (JF501141) 0 1 Variovorax sp. (89%)
SB38 (JF501142) 1 0 Xanthomonas sp. (99%)
SB35 (JF501134) 1 0 Enterobacter sp. (99%)
SB39 (JF501130) 1 0 Enterobacter sp. (95%)
SB40 (JF501143) 1 0 Serratia sp. (99%)
SB33 (JF501137) 5 3 Agrobacterium sp. (99%)
SB55 (JF501136) 1 0 Variovorax sp. (90%)
SB50 (JF501132) 0 1 Acinetobacter sp. (99%)
3.
SG20 (JF501148) 3 2 Enterobacter sp. (99%)
SG51 (JF501147) 1 1 Klebsiella sp. (99%)
SG56 (JF501150) 1 0 Klebsiella sp. (99%)
SG11 (JF501145) 15 12 Klebsiella sp. (99%)
SG25 (JF501157) 4 1 Enterobacter sp. (99%)
SG4 (JF501161) 0 2 Pantoea sp. (99%)
SG47 (JN198150) 0 1 Klebsiella sp. (99%)
SG43 (JF501149) 0 1 Klebsiella sp. (99%)
SG53 (JF501153) 2 0 Novosphingobium sp. (99%)
SG58 (JF501154) 1 0 Rhizobium sp. (99%)
SG6 (JF501151) 1 7 Pseudomonas sp. (99%)
SG49 (JF501152) 0 2 Pseudomonas sp. (99%)
SG52 (JF501159) 1 0 Stenotrophomonas sp.(93%)
SG60 (JF501155) 1 0 Pseudomonas sp. (99%)
SG7 (JF501156) 0 1 Herbaspirillum sp. (99%)
4.
Vac4 (JF501172) 15 12 Enterobacter sp. (98%)
Vac24 (JF501164) 0 1 Klebsiella sp. (99%)
Vac36 (JF501174) 1 0 Grimontella sp. (97%)
Vac41 (JF501175) 0 2 Agrobacterium sp. (99%)
Vac57 (JF501176) 3 0 Agrobacterium sp. (99%)
Vac23 (JF501181) 0 1 Asticcacaulis sp. (98%)
Vac42 (JF501179) 1 1 Microbacterium sp. (99%)
Vac27 (JF501167) 2 4 Burkholderia sp. (99%)
Vac22 (JN198151) 0 1 Shinella sp. (99%)
Vac46 (JF501178) 1 3 Rhizobium sp. (98%)
Vac33 (JF501185) 1 0 Moraxella sp. (97%)
Vac25 (JF501166) 0 1 Burkholderia sp. (97%)
Vac44 (JF501162) 0 4 Pseudomonas sp. (99%)
Vac39 (JF501163) 1 0 Achromobacter sp. (99%)
Vac54 (JF501165) 1 0 Pseudomonas sp. (94%)
Vac60 (JF501171) 1 0 Stenotrophomonas sp. (96%)
Vac55 (JF501168) 1 0 Stenotrophomonas sp. (94%)
Vac31 (JF501169) 2 0 Azospirillum sp. (99%)
5.
Vi12 (JF501196) 9 0 Grimontella sp. (99%)
Vi20 (JF501193) 10 0 Enterobacter sp. (99%)
Vi57 (JF501200) 4 2 Agrobacterium sp. (99%)
Vi9 (JF501202) 0 1 Burkholderia sp. (99%)
Vi24 (JF501203) 0 2 Rhizobium sp. (99%)
Vi28 (JF501204) 0 3 Burkholderia sp. (99%)
Vi48 (JF501205) 0 4 Burkholderia sp. (99%)
Vi49 (JF501206) 0 1 Achromobacter sp. (99%)
Vi22 (JF501207) 0 1 Azospirillum sp. (99%)
Vi21 (JF501195) 0 1 Chryseobacterium sp. (99%)
Vi30 (JF501194) 0 1 Mitsuaria sp. (99%)
Vi3 (JF501190) 0 8 Stenotrophomonas sp. (97%)
Vi39 (JF501209) 1 3 Stenotrophomonas sp. (95%)
Vi50 (JF501191) 4 2 Stenotrophomonas sp. (99%)
Vi60 (JF501187) 2 1 Pseudomonas sp. (96%)

◀ **Fig. 1** Dendrograms based on UPGMA cluster analysis (PAST software 2.09) using the 16S rDNA PCR-RFLP data obtained from isolates of the each sampled site: 1. Encruzilhada do Sul, 2. São Borja, 3. São Gabriel, 4. Vacaria, and 5. Viamão. Each dendrogram was summed up and only one isolate in each cluster (the representative isolate) is represented. Each isolate is denoted according to the site at which it was sampled (E: Encruzilhada do Sul, SB: São Borja, SG: São Gabriel, Vac: Vacaria, and Vi: Viamão) and the source of sampling (i.e. roots or rhizosphere). The percentages identities (in parenthesis) are based upon comparisons with the Genbank database using the BLASTN program. These representative isolates had their 16S rRNA gene partially sequenced (access number in parenthesis) and were grouped according to their generic or species designation

10 um) and a flame ionization detector connected to a chromatography data computer system. Cells were collected and broken by lysing with 0.2 M NaOH for 12 h at room temperature. Protein concentration in the resulting mixture was determined by a standard method (Bradford 1976). *Azospirillum brasilense* Sp7 (ATCC29145) was used as a positive control.

In vivo plant growth promotion assay

A plant growth experiment with sunflower inoculated with previously selected native bacterial strains was carried out in a controlled environment chamber with a photoperiod of 16 h light (28°C) and 8 h dark (20°C). Seeds of sunflower cv. Aguará 6 (obtained from FEPAGRO) were surface-disinfected by washing them in 70% ethanol for 1 min, followed by a sodium hypochlorite solution (4%, v/v) with three drops of Tween 80 wash for 5 min, and five serial rinses in sterilized distilled water. The treated seeds (one seed per pot) were planted 2.0 cm below the soil surface in pots (15 cm× 20 cm) containing sterile vermiculite and Hoagland's nutrient solution diluted to 25%. The stock nutrient solution (100%) was prepared as follows: (1) Macronutrients (g l^{-1}): $Ca(NO_3)_2.4H_2O$ (0.227), KNO_3 (0.098), $MgSO_4.7H_2O$ (0.089), KH_2PO_4 (0.024); (2) Micronutrients (mg l^{-1}): H_3BO_3 (0.108), $MnCl_2.4H_2O$ (0.072), $ZnSO_4.7H_2O$ (0.025), $CuSO_4.5H_2O$ (0.009), $Na_2MoO_4.2H_2O$ (0.004); (3) FeEDTA (mg l^{-1}): Na_2EDTA (1.42) and $FeSO_4.7H_2O$ (1.07). Eight bacterial isolates were grown in LB medium under agitation (200 rpm) for 48 h at 28°C. Pure bacterial cultures were centrifuged and diluted to a final concentration of 10^8 cfu ml^{-1} in sterile 25% Hoagland's solution. Plants were inoculated with 5 ml aliquots of cell suspensions by directly irrigating the substrate just after seeding, and the treatments were arranged in a randomized design with five replicates. The following treatments were investigated: (1) negative control, (2) uninoculated control, (3–10) inoculated with isolates (3) E8, (4) E37, (5) SB28, (6) Vac22, (7) Vac30, (8) Vi21, (9) Vi22, and (10) Vi49. Plants from the negative control treatment were irrigated only with water, and plants from the uninoculated control were irrigated once with 100 ml of 25% Hoagland's solution, and water thereafter. Treatments 3–10 received 100 ml of 25% Hoagland's solution before inoculation. Plants were harvested at 30 days after sprouting, at which time their height was recorded. Roots and shoots were dried at 65° C to constant weight to evaluate their dry matter, and then the dry matter of all the shoots from each treatment was combined for elemental analysis. Shoot nutrient content was determined by the Kjeldahl method (detection limit 0.01%) for N and nitric-perchloric wet digestion/ICP-OES (Inductively Coupled Plasma—Optical Emission Spectrometry at detection limit 0.01%) for P and K. The nutrient content of plants was estimated per treatment through uptake per gram of plant tissue multiplied by total yield per treatment (i.e. yield × percent nutrient per gram of plant tissue) (Adesemoye et al. 2009). Data obtained from the different treatments were statistically analyzed using the one-way ANOVA and the means were compared by the Tukey test for multiple comparisons. Differences were considered significant at the 0.05 level. Statistical analyses were done with IBM SPSS Statistics version 19 (SPSS 2010).

Results

Isolation, identification, and diversity of putative diazotrophic root and rhizospheric bacteria

A total of 299 bacterial strains were selectively isolated from the rhizosphere or roots of sunflower based on their growth in one of three semi-selective semi-solid media without nitrogen i.e. NFb, LGI or LGI-P (Table 2). These N-free media were used as part of a strategy to select putative nitrogen-fixing and plant growth-promoting rhizobacteria.

Only three isolates were characterized as Gram-positive bacteria, and these strains were subsequently

Table 2 Bacterial genera (identified through partial 16S rRNA sequencing) found in association with the roots and in the rhizosphere of sunflower plants in the five different sampling sites

TOTAL	Sampling sites [a]					GENERA	Sampling sites [a]					TOTAL
	1	2	3	4	5		1	2	3	4	5	
	Root bacteria						Rhizospheric bacteria					
7	-	6	–	1	–	*Achromobacter*	–	3	–	–	1	4
1	1	–	–	–	–	*Acinetobacter*	–	1	–	–	–	1
14	2	5	–	3	4	*Agrobacterium*	2	3	–	2	2	9
0	–	–	–	–	–	*Asticcacaulis*	–	–	–	1	–	1
2	–	–	–	2	–	*Azospirillum*	–	–	–	–	1	1
14	1	11	–	2	–	*Burkholderia*	15	20	–	5	8	48
0	–	–	–	–	–	*Chryseobacterium*	–	–	–	–	1	1
54	20	2	7	15	10	*Enterobacter*	–	–	3	12	–	15
10	–	–	–	1	9	*Grimontella*	–	–	–	–	–	0
0	–	–	–	–	–	*Herbaspirillum*	–	–	1	–	–	1
17	–	–	17	–	–	*Klebsiella*	–	–	15	1	–	16
2	1	–	–	1	–	*Microbacterium*	–	–	–	1	–	1
0	–	–	–	–	–	*Mitsuaria*	–	–	–	–	1	1
1	–	–	–	1	–	*Moraxella*	–	–	–	–	–	0
4	2	–	2	–	–	*Novosphingobium*	–	–	–	–	–	0
0	–	–	–	–	–	*Pantoea*	–	–	2	–	–	2
7	–	2	2	1	2	*Pseudomonas*	–	1	9	4	1	15
2	–	–	1	1	–	*Rhizobium*	11	–	–	3	2	16
1	–	1	–	–	–	*Serratia*	–	–	–	–	–	0
0	–	–	–	–	–	*Shinella*	–	–	–	1	–	1
0	–	–	–	–	–	*Sphingobium*	1	–	–	–	–	1
11	3	–	1	2	5	*Stenotrophomonas*	1	1	–	–	13	15
1	–	1	–	–	–	*Variovorax*	–	1	–	–	–	1
1	–	1	–	–	–	*Xanthomonas*	–	–	–	–	–	0
149												150

- = not found

[a] Sampling sites: 1: Encruzilhada do Sul; 2: São Borja; 3: São Gabriel; 4: Vacaria, and 5: Viamão

identified as belonging to the genus *Microbacterium.* Root and rhizospheric bacteria belonging to different species were discriminated by PCR-RFLP of the 16S rRNA gene by using two selected restriction enzymes, HaeIII and TaqI. The RFLP patterns obtained allowed for the construction of a dendrogram for each sampled site (Fig. 1), and a representative strain from each cluster was chosen for the 16S rRNA gene sequence analysis. Twenty-four different genera were identified from partial sequencing of the 16S rRNA gene of representative isolates. The most abundant genera found in association with the roots of sunflower were *Enterobacter* (54 isolates), *Klebsiella* (17), and *Burkholderia* (14), whereas the predominant rhizospheric bacterial strains also belonged to the genera *Burkholderia* (48), *Klebsiella* (16), and *Enterobacter* (15). These results indicate that bacteria belonging to the genera *Enterobacter*, *Burkholderia* and *Klebsiella* corresponded to approximately 23%, 21% and 11% of all isolates, respectively. *Enterobacter* was found in the five sampling sites whereas *Burkholderia* was identified in four (Table 2). These genera could be considered dominant in their association with sunflower regardless of the soil type or geographic region in which the plants were cultivated. In addition to the above genera, a few isolates of microorganisms

commonly isolated from the rhizosphere of various other plants were found, such as *Stenotrophomonas* (8.7%), *Agrobacterium* (7.7%), *Pseudomonas* (7.4%), and *Rhizobium* (6%).

High genetic diversity was found within the different populations of bacteria from Viamão (*H'*=2.13) and Vacaria (*H'*=1.78) localities. São Borja (*H'*=1.26) and Encruzilhada do Sul (*H'*=1.68) had intermediary diversity and São Gabriel (*H'*=0.96) the lowest diversity index. Principal component analysis (PCA) was used to investigate the relationships between abiotic soil parameters and bacterial diversity (*H'*) at the 70% similarity level (Fig. 2). The first two dimensions of PCA explained 83% of the total variation, with principal component 1 (PC1) accounting for 63% and principal component 2 (PC2) for 20% of the variance. The PC1 was loaded positively with clay, pH, OMC, Mg, Fe, Ca, K and P, and negatively loaded with Al and *H'*. The PC2 was positively loaded with Al, *H'*, clay, pH, OMC, Mg, Fe and Ca, and negatively loaded with P and K.

PGP traits of bacterial isolates

Table 3 shows the grouping of PGP characteristics analyzed for each isolate from the roots and rhizosphere of sunflower according to each sampled region. About 78% of the strains were capable of producing siderophores (Fig. 3a). Only 59 of the 299 isolates were identified as phosphate solubilizers. Rhizospheric soils from São Borja and Encruzilhada do Sul localities had the highest number of phosphate solubilizers compared to the other sites, and isolates from the genus *Burkholderia* were predominant in terms of solubilizing phosphate (Fig. 3b). About

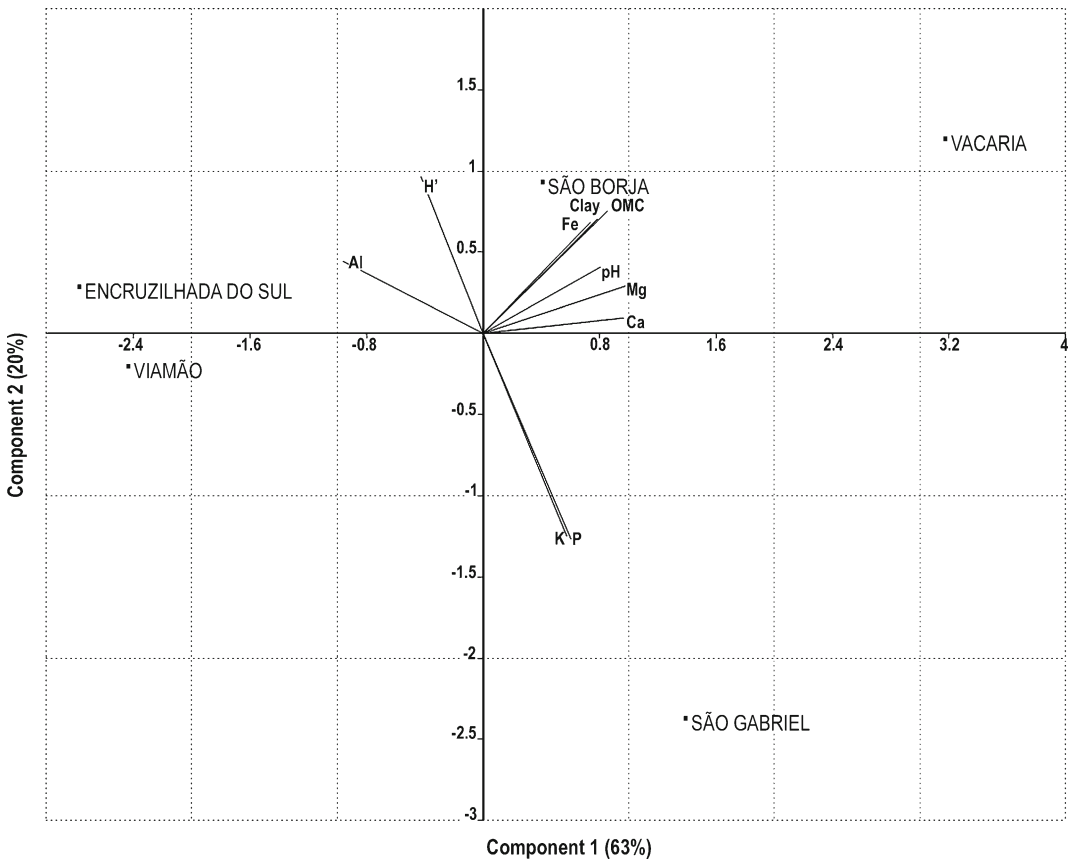


Fig. 2 Principal component analysis (PCA) of the diversity indexes (*H'*) of the five sampling sites (Encruzilhada do Sul, São Borja, São Gabriel, Vacaria, and Viamão) in relation to different soil properties [clay, organic matter content (OMC), pH, P, K, Fe, Ca, Mg and Al]. Principal component 1 and component 2 accounted for 63% and for 20% of the total variation, respectively

Table 3 Siderophore production, phosphate solubilization and production of indolic compounds (ICs) by bacterial isolates. Data are grouped in each sampling site according to the number of isolates from the roots and the rhizosphere

Sampling sites	Source	Number of isolates	Siderophore producers	Phosphate solubilizers	ICs producers (μg ml^{-1})	
					0.1–49.9	>50
1. Encruzilhada do Sul	Roots	30	26	1	5	21
	Rhizosphere	30	28	16	15	2
2. São Borja	Roots	29	12	10	13	8
	Rhizosphere	30	17	19	13	3
3. São Gabriel	Roots	30	26	3	16	3
	Rhizosphere	30	26	1	13	14
4. Vacaria	Roots	30	26	0	7	17
	Rhizosphere	30	25	1	10	12
5. Viamão	Roots	30	28	1	3	18
	Rhizosphere	30	20	7	5	1
TOTAL		299	234	59	100	99

66% of all isolates were capable of producing indolic compounds. The production of indolic compounds ranged from 0.2 to 347 μg ml^{-1} for all isolates, and 51% belonged to the Enterobacteriaceae (Fig. 3c). Analysis of those isolates that had more than one PGP characteristic showed that 132 were able to produce indolic compounds and siderophores, 27 produced siderophores and solubilized phosphates, six produced indolic compounds and solubilized phosphates, and 23 isolates were able to produce indolic compounds, siderophores and solubilize phosphates. The genus *Burkholderia* represented 74% (17) of those that had the capability for all the three PGP activities.

Based on their taxonomic identification and their PGP characteristics, eight isolates were selected to inoculate the seeds of sunflower plants in a growth experiment. The isolates chosen were E8, E37, SB28, Vac22, Vac30, Vi21, Vi22, and Vi49 (Table 4). The PCR-RFLP profiles of the 16S rRNA genes of all eight selected isolates can be seen in Fig. 1 i.e. E8 (the same as E27), E37 (the same as E18), SB28 (the same as SB19), Vac22, Vac30 (the same as Vac27), Vi21, Vi22, and Vi49. All were able to produce siderophores, but only three of them were phosphate solubilizers (E8, SB28, Vi49). The production of indolic compounds was high for the isolates Vi22, Vac22, SB28, E37, and Vi21. All eight isolates exhibited nitrogenase activity, but most of them had low levels of activity (values between 0.26 and 1.26 nmol C_2H_4 mg protein min^{-1}) as compared with the positive control strain *A. brasilense* Sp7 (141.1 nmol C_2H_4 mg protein min^{-1}). The exception was the isolate Vi22 which had the highest nitrogenase activity at 81.85 C_2H_4 mg protein min^{-1}.

Plant assay

The inoculation of sunflower plants with eight selected isolates resulted in different effects on the dry biomass production and nutrient contents of the plants. The results of inoculation of rhizobacteria on shoot and root growth under gnotobiotic conditions is shown in Table 5. The dry weight of roots treated with isolates Vac30 (*Burkholderia* sp.) and Vi22 (*Azospirillum* sp.) was significantly higher than the uninoculated control plants, with an increase of 58 and 77%, respectivelly. Isolates Vi21 (*Chryseobacterium* sp.) and Vi49 (*Achromobacter* sp.) significantly increased the dry weight of both shoots and roots by approximately 70% compared to uninoculated controls. However, even though shoot and root lengths were increased by inoculation with each of the isolates, these increases were not significantly different from controls.

With regard to plant nutrient content, inoculation with isolates E37 (*Novosphingobium* sp.) and Vi49 (*Achromobacter* sp.) enhanced N uptake by sunflower plants (by 62 and 140%, respectively) (Fig. 4). Since the N content of the uninoculated control plants was not determined due to the small amount of material

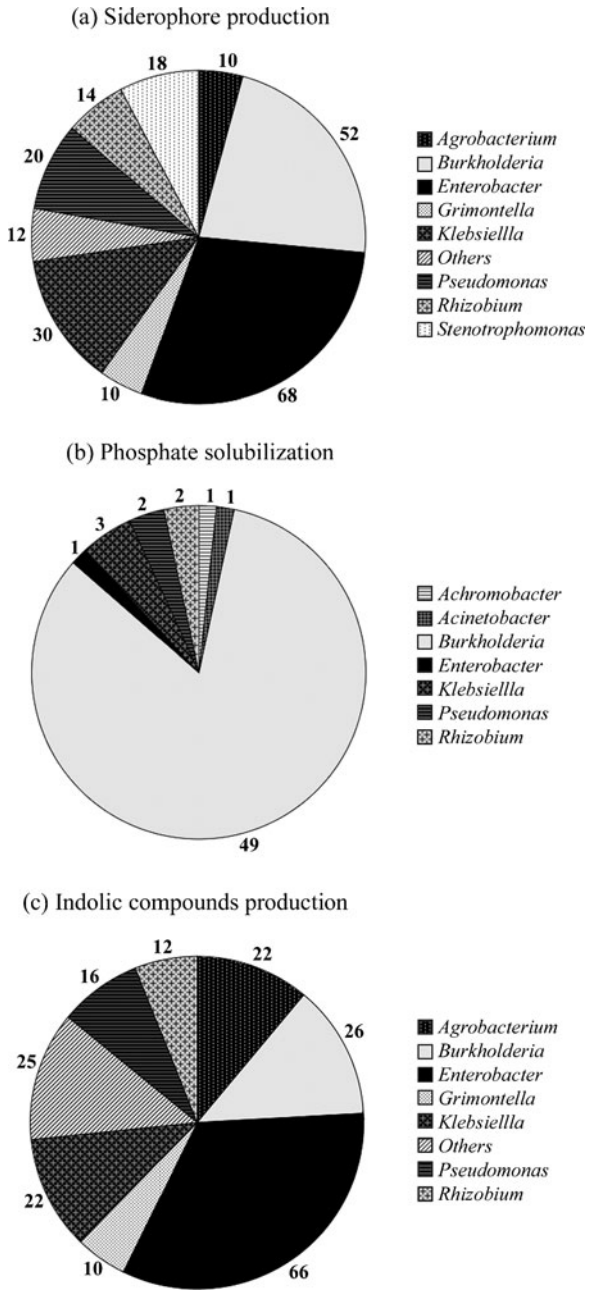


Fig. 3 Most abundant genera in terms of (**a**) siderophore production, (**b**), phosphate solubilization (**c**) production of indolic compounds. The number of isolates found for each trait and their respective genera are shown

obtained, data from the negative control plants were used for this statistical analysis. Isolate Vi49 also stimulated the P uptake of plants by 64% relative to uninoculated controls. Similar data were achieved for strains Vi22 (*Azospirillum* sp.) (68%) and Vac22 (*Shinella* sp.) (60%). Compared to uninoculated plants the plant K content was not significantly affected by inoculation with any of the strains, although all differed significantly from the negative control plants (data not shown). The effect of inoculation by the eight isolates on sunflower growth promotion in comparison to negative and uninoculated controls can be seen in Fig. 5.

Discussion

Bacteria associated with the rhizosphere of several plants have been isolated and identified by many researchers, and the beneficial effects promoted by different strains are already well-established (Andrews et al. 2003; Vessey 2003). Although strains of various bacteria, such as *Rhizobium radiobacter*, *Bacillus*, *B. cereus, B. licheniformis*, *B. pumilus, Pseudomonas*, *P. fluorescens*, *P. putida, P. vesicularis*, *Burkholderia cepacia*, *Flavobacterium odoratum*, *Stenotrophomonas maltophilia*, *Rhizobium*, *Achromobacter xilosoxidans, Azospirillum*, *Methylobacterium*, and *Dyella thiooxydans* were found to be associated with sunflower in other countries (Fages and Lux 1991; Hebbar et al. 1991; Kong et al. 1997; Alami et al. 2000; Forchetti et al. 2007; Kamal and Bano 2008; Schauer and Kutschera 2008; Qihui et al. 2009; Anandham et al. 2010), until the present study the rhizobacterial diversity of sunflower in Brazilian soils remained largely unknown. In the present study root and rhizospheric soil samples of sunflower cultivated in southern Brazil were screened for diversity and presence of putative nitrogen-fixing and plant growth-promoting rhizobacteria. Twenty-four different bacterial genera were identified, and among these, only one Gram-positive genus, *Microbacterium*, comprising one isolate from the rhizosphere and two from roots, was found. Strains of *Microbacterium* have been found associated with many plants, such as wheat (*Triticum aestivum*) (Conn and Franco 2004), rice (*Oryza sativa*) (Tilak et al. 2005), maize (*Zea mays*) (Rijavec et al. 2007), cassava (*Manihot esculenta*) (Teixeira et al. 2007) and tomato (*Solanum lycopersicum*) (Marquez-Santacruz et al. 2010). Most studies on bacteria interacting with plants have selected mostly Gram-negative strains, and some researchers have suggested that the rhizosphere tends to select for Gram-negative bacteria over Gram-positive ones (Kloepper et al. 1992; Gilbert et al. 1993). According to Gray and Smith (2005), plant

Table 4 Identification and PGP abilities of selected isolates

Isolates [a]	16S rRNA sequence [b]	Siderophore production	Phosphate solubilization	Production of ICs (μg ml^{-1}) [c]	ARA (nmol C_2H_4 mg protein min^{-1}) [c d]
E8	*Burkholderia* sp. (99%)	+	+	–	0.61
E37	*Novosphingobium* sp. (95%)	+	–	10.16	0.59
SB28	*Burkholderia* sp. (99%)	+	+	22.87	1.26
Vac22	*Shinella* sp. (99%)	+	–	32.32	0.31
Vac30	*Burkholderia* sp. (99%)	+	–	–	0.26
Vi21	*Chryseobacterium* sp. (99%)	+	–	9.89	0.66
Vi22	*Azospirillum* sp. (99%)	+	–	204.22	81.85
Vi49	*Achromobacter* sp. (99%)	+	+	–	0.44
Control	*A. brasilense* Sp7	nd	nd	nd	141.1

nd not determined; *ICs* indolic compounds; *ARA* acetylene reduction assay

[a] Bacteria isolated from: E8 (rhizospheric soil / Encruzilhada do Sul); E37 (roots / Encruzilhada do Sul); SB28 (rhizospheric soil / São Borja); Vac22 and Vac30 (rhizospheric soil / Vacaria); Vi21, Vi22 and Vi49 (rhizospheric soil / Viamão)

[b] Identities (in parentheses) are based on comparisons with the Genbank database using the BLASTN program (Altschul et al. 1997)

[c] Results are means of three experiments conducted separately under identical conditions

[d] Nitrogenase activity was measured after growth in three semi-solid (0.18% agar-agar) N-free media i.e. NFb (SB28, Vi22, Vi49 and *A. brasilense* Sp7) LGI (Vac22 and Vac30) or LGI-P (E8, E37 and Vi21)

growth-promoting rhizobacteria are mostly Gram-negative and rod-shaped, with a lower proportion being Gram-positive rods, cocci and pleomorphic forms. Beattie (2006) also reported that among the cultivated isolates from leaves, Gram negative bacteria are more common than Gram positive.

Isolates from the genera *Stenotrophomonas*, *Agrobacterium*, *Pseudomonas* and *Rhizobium* encompassed about 30% of the bacteria encountered in sunflower roots and rhizosphere soil. *Stenotrophomonas* strains have been detected as endophytes in dune grasses (*Ammophila arenaria* and *Elymus mollis,* Dalton et al. 2004), rice (Sun et al. 2008), sugarcane (*Saccharum* sp.) (Mehnaz et al. 2010) and tomato (Marquez-Santacruz et al. 2010). Pseudomonads are among the most important rhizospheric bacteria, as

Table 5 Effect of inoculation by various isolates on root length, shoot length and dry matter of sunflower plants at 30 d after sprouting

Treatment	Shoot growth		Root growth	
	Length (cm)	Dry matter (mg)	Length (cm)	Dry matter (mg)
Negative control	3.64 (± 1.07) a	103.80 (± 40.30) a	17.50 (± 5.63) a	45.20 (± 18.85) a
Uninoculated	7.56 (± 5.00) a,b	208.80 (± 90.28) a,b	15.38 (± 1.83) a	68.80 (± 30.12) a,b
E8	10.86 (± 4.56) a,b	318.40 (± 24.34) b,c	18.78 (± 4.50) a	101.80 (± 13.16) b,c
E37	10.84 (± 2.95) a,b	294.40 (± 57.60) b,c	25.40 (± 9.72) a	104.60 (± 10.14) b,c
SB28	10.56 (± 1.37) a,b	255.60 (± 19.71) b,c	19.54 (± 4.09) a	87.20 (± 19.72) b,c
Vac22	9.46 (± 2.69) a,b	291.40 (± 45.67) b,c	23.08 (± 11.03) a	101.40 (± 11.50) b,c
Vac30	12.30 (± 4.18) b	318.40 (± 38.37) b,c	16.40 (± 4.05) a	122.40 (± 6.88) c
Vi21	11.26 (± 4.65) b	360.40 (± 95.31) c	17.70 (± 3.13) a	109.80 (± 26.74) c
Vi22	11.14 (± 2.65) b	306.00 (± 55.21) b,c	20.10 (± 5.38) a	113.80 (± 12.15) c
Vi49	10.96 (± 2.41) b	343.00 (± 37.40) c	20.12 (± 2.93) a	123.00 (± 17.61) c

Data are mean (± SD) of five replicates of plants grown in vermiculite in a photoperiod chamber

Values in the same column followed by the same letter did not differ significantly at $p>0.05$ (Tukey test)

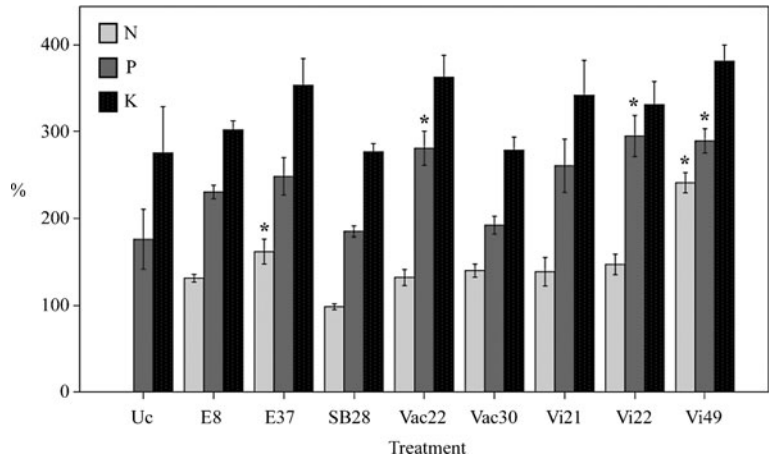


Fig. 4 Influence of isolates E8, E37, SB28, Vac22, Vac30, Vi21, Vi22, and Vi49 on uptake of N, P and K by sunflower growing in vermiculite (30 d after sprouting in a pot experiment) compared to uninoculated control plants (Uc). The values are given relative to the negative control treatment which was considered to be 100%. The nutrient content of plants from the negative control was (mg/g of shoots): 7 mg of N, 0.8 mg of P and 6.5 mg of K. The * above bars means values are significantly different from the uninoculated control (P and K content) and the negative control (N content) at $p>0.05$ (Tukey test). The N content of the uninoculated control was not determined (nd) due to the small amount of material obtained

they are aggressive colonizers and utilize a wide range of substrates as carbon sources (Misko and Germida 2002). Shilev et al. (2006) studied the role of *P. fluorescens* in alleviating abiotic stress in sunflower in the presence of arsenic and under saline stress (Shilev et al., 2010), and they reported the stimulation of plant growth by the inoculation of seeds in both cases. In another study on sunflower, *B. cepacia* and *P. aeruginosa* were able to reduce infection by *Sclerotinia sclerotiorum* (McLoughlin et al. 1992). On the other hand, bacteria belonging to the genus *Rhizobium* are well-known for their capacity to establish symbiosis with legumes and by their direct contribution to nutrient availability through nitrogen fixation. However, many reports also indicate that these symbiotic bacteria can be found in association with non-legumes and have the potential to be used as PGPR (Sessitsch et al. 2002; Vessey 2003). For example, an exopolysaccharide-producing *Rhizobium* was able to induce a significant increase in the amount of soil adhered to the roots of sunflower plants under both normal and water stress conditions by modifications to the soil

Fig. 5 Effect of inoculation by eight PGP strains on sunflower plants at 30 d after sprouting in a pot experiment under gnotobiotic conditions in a photoperiod chamber. (1) Negative control; (2) Uninoculated control, (3) E8, (4) E37, (5) SB28, (6) Vac22, (7) Vac30, (8) Vi21, (9) Vi22, and (10) Vi49

structure around the root system and by decreasing the negative effect of water deficit on growth (Alami et al. 2000). Moreover, inoculation made the use of fertilizer [^{15}N] nitrate more effective by increasing nitrogen uptake by the sunflower plantlets.

Enterobacter and *Burkholderia* were the predominant culturable bacterial genera found in association with sunflower in the present study, regardless of the soil type or geographical region in which the plants were cultivated. The preferential colonization and the occurrence of dominant bacterial types have been detected in other plant species. For example, about 50% of the bacteria associated with sweet potato roots were grouped according to their *nifH* sequences and shown to belong to the genus *Rhizobium* (Reiter et al. 2003), and in rapeseed (*Brassica napus*), 35 and 23% of the bacteria isolated from the roots belonged, respectively, to the genera *Pseudomonas* and *Stenotrophomonas* (Misko and Germida 2002). According to Zak et al. (2003), because plant species differ in their biochemical composition, both the quantity and quality of root exudates will vary according to plant species, thus controlling the composition and function of soil microbial communities.

The plant may confer advantages upon bacteria that colonize the rhizosphere, but endophytes or root bacteria may gain other benefits, as once inside the plant the bacteria can be protected from the the more extreme conditions found in the soil (Lodewyckx et al. 2002). The best studied diazotrophic endophytes are *Gluconacetobacter diazotrophicus* in sugarcane (Baldani et al. 1997; Oliveira et al. 2002), *Azoarcus* in Kallar grass (*Leptochloa fusca*), and *Alcaligenes*, *Azospirillum*, *Bacillus*, *Enterobacter*, *Herbaspirillum*, *Klebsiella*, *Pseudomonas* and *Rhizobium* in rice and maize (James 2000; Andrews et al. 2003). Our results suggest that Enterobacteriaceae are the most dominant bacteria within sunflower roots, although, surprisingly, this is the first study to report the association between these organisms. Enterobacteria have been found in association with a large number of plant species, such as citrus (*Citrus aurantium*) (Araujo et al. 2001), maize (Hinton and Bacon 1995; McInroy and Kloepper 1995), rice (Engelhard et al. 2000; Gyaneshwar et al. 2001; Rosenblueth et al. 2004; Verma et al. 2004), sweet potato (*Ipomoea batatas*) (Asis and Adachi 2003; Reiter et al. 2003), soybean (*Glycine max*) (Kuklinsky-Sobral et al. 2004) and wheat (Iniguez et al. 2005). In a study by Roberts et al. (1999), *Enterobacter cloacae* mutants deficient in the utilization of certain carbohydrates and the corresponding wild type strain were able to colonize sunflower, pea (*Pisum sativum*) and soybean, but the numbers of the mutant strain colonizing cucumber (*Cucumis sativus*) and radish (*Raphanus sativus*) seeds were significantly reduced compared with the WT strain. The difference between the plant species was related to the total amount of carbohydrates and amino acids released by them e.g. the soybean and sunflower seeds released significantly greater amounts of fructose, total carbohydrates and amino acids than the cucumber or radish seeds.

This study showed no correlation between pH and other soil parameters with bacterial diversity (*H'*). This may be due to a similar value of pH and clay, which together with soil texture are the soil parameters that most influence microbial community structure (Bashan et al. 1995; Fierer and Jackson 2006; Beneduzi et al. 2008; Lauber et al. 2009; Chu et al. 2010). Similar soil types tend to select similar bacterial communities (Gelsomino et al. 1999). Although a standard suite of soil characteristics were measured and analyzed statistically in our study, it is entirely possible that other soil variables that were not measured could be correlated with the microbial diversity. Germida et al. (1998) also showed that crop type plays a major role in controlling the diversity of root-associated bacteria and several studies on many plant species in different locations, using both culturing and non-culturing (molecular) methods, have indicated that plant type, genotype, root zone, plant age and plant community composition are, indeed, more important factors influencing the diversity of microbial communities than soil (Germida et al. 1998; Grayston et al. 1998; Kaiser et al. 2001; Marschner et al. 2001; Smalla et al. 2001; Kowalchuk et al. 2002; Mitchell et al. 2010).

Associative and free-living microorganisms may contribute to the nutrition of plants through a variety of mechanisms, including direct effects on nutrient availability, providing the plant with plant growth-promoting substances that are synthesized by the bacterium or by facilitating the uptake of certain plant nutrients from the environment (Andrews et al. 2003; Ahmad et al. 2006; Raaijmakers et al. 2009). A particular bacterium may affect plant growth using any one, or more, of these mechanisms. Moreover, a bacterium may provide different benefits at various times during the life cycle of the plant (Glick 2005). Three plant growth promotion characteristics were evaluated for all isolates in this study. Siderophore production was the most common trait amongst the isolates, as

about 78% of them displayed this ability; about 66% of the strains were capable of producing indolic compounds, while only 59 of the 299 isolates were identified as phosphate solubilizers. Similarly low numbers of phosphate-solubilizing bacteria have been documented by other studies. Beneduzi et al. (2008), in their study to evaluate the diversity of plant growth-promoting bacilli isolated from wheat, found only nine phosphate-solubilizing strains out of 311 bacterial isolates analyzed. Hameeda et al. (2008) isolated 207 PGPR from maize in India, but of these, only five isolates had phosphate-solubilizing ability.

The occurrence and growth of bacteria with the formation of a typical subsurface pellicle in N-free semi solid medium, but without being accompanied by any evident acetylene reduction activity has been reported earlier (Tripathi et al. 2002), and, indeed, such pellicles have revealed the presence of non-diazotrophic bacteria together with diazotrophs. Approximately 90% of bacteria isolated from surface-sterilized rice plants using N-free media have been shown to be non-diazotrophs (James 2000). Apparently, such non-diazotrophs could be nitrogen scavengers or oligotrophs, growing on the nitrogen fixed and released by diazotrophs during their growth. Therefore, with this in consideration, eight selected isolates from the present study were submitted to an in vitro nitrogen fixation assay (the ARA). All the isolates exhibited nitrogenase activity, but only one of them (Vi22 isolate identified as *Azospirillum* sp.) exhibited values that were similar or higher than those of *A. brasilense* Sp7, a strain well known for its ability to fix nitrogen. Many strains of *Azospirillum* are able to improve crop yields due to such widely documented plant-beneficial properties as associative nitrogen fixation, production of phytohormones, and production of nitric oxide and ACC deaminase activity (Andrews et al. 2003; Bashan *et al*. 2004; Blaha *et al*. 2006). Indeed, *A. lipoferum* isolated from maize roots promoted sunflower growth in a 6-day germination test and in pot experiments (Fages and Arsac 1991).

In order to evaluate the potential of native rhizobacteria to promote sunflower growth, eight isolates were selected for an in vivo inoculation experiment. Significant increases of growth were obtained with isolates Vi49 (*Achromobacter* sp.), Vi21 (*Chryseobacterium* sp.), Vi22 (*Azospirillum* sp.), and Vac30 (*Burkholderia* sp.). Some of these isolates also significantly increased either the P (Vi22), or both N and P (Vi49), contents of the inoculated plants. The efficient uptake of nutrients from soil by roots is a critical issue for plants given that in many environments the poor availability of nutrients may be deficient for optimal growth (Richardson et al. 2009). An important regulator directly influencing plant development is the auxin hormone. According to Jaillais and Chory (2010), auxin acts as a molecule that integrates the activities of multiple phytohormones to control plant growth in response to the environment. However it is not possible to explain the intrinsic network of bacterial-plant interactions in the present study of sunflower based upon these characteristics, as the effects caused by certain strains can result from either the single or joint action of bacterial metabolites and regulators on the roots. For example, the isolates Vac30 and Vi49 were not able to produce auxin in our in vitro experiment, but were effective at growth promotion under gnotobiotic conditions. On the other hand, the isolate Vi22 was able to produce high amounts of fixed nitrogen in vitro and was also the highest producer of indolic compounds amongst all the isolates tested. Strains of *Azospirillum* and *Burkholderia* are well known for their abilities to promote plant growth. *Azospirillum* strains have been widely studied due to the versatility of their metabolism which allows for their high competitive ability and hence improves their ability to promote growth of more than one hundred species of cereals and legumes (Bashan et al. 2004). *Azospirillum* species are also known for their capacity to interact with many different plants, to fix nitrogen, to produce phytohormones and nitric oxide, as well as for their ACC deaminase activity (Bashan et al. 2004; Blaha et al. 2006). *Burkholderia* species are able to synthesize a remarkable array of metabolites, including siderophores, antibiotics, and phytohormones (Vial et al. 2007; Suárez-Moreno et al. 2011), and they are also known for the ability to improve plant growth, as been showed with maize (Ciccillo et al. 2002), onion (*Allium cepa*) (Sessitsch et al. 2005), and rice (Ait Barka et al. 2006). Caballero-Mellado et al. (2007) isolated *Burkholderia* strains from the rhizosphere and rhizoplane of tomato plants, and many of these exhibited activities involved in bioremediation, PGP or biological control in vitro. Indeed, owing to their high metabolic diversity and multiple PGP characteristics, plant beneficial and diazotrophic Burkholderias are now seen as a rapidly emerging class of potential biofertilizers (Suárez-Moreno et al. 2011).

Strains isolated from a particular host plant (i.e. "homologous strains") can be more efficient in providing

benefits to that plant than "non-homologous" strains isolated from another plant species, and this has been demonstrated in many previous studies (Bhattarai and Hess 1993; Germida and Siciliano 2001; Bergsma-Vlami et al. 2005; Zeller et al. 2007; Buchan et al. 2010; Schnitzer et al. 2011). The capacity to establish good root colonization is an important first step to protect the plant against the infection by soil-borne pathogens and for the formation of beneficial associations with microorganisms (Barassi et al. 2007). An endophytic strain of *Achromobacter* isolated from sunflower was able to enhance the growth of seedlings under water stress and to inhibit the growth of pathogenic fungi (Forchetti et al. 2010). However, depending on the stage of plant development and the bacterial characteristics, some strains are also able to produce positive effects on non-homologous plant species as well. For example, a strain of *Chryseobacterium balustinum*, isolated from the rhizosphere of *Lupinus albus*, was able to reduce infection against *P. syringae* on *Arabidopsis thaliana* (Solano et al. 2008). In addition, *A. lipoferum* isolated from maize roots promoted sunflower growth in a 6 d germination test and in pot experiments (Fages and Arsac 1991).

Conclusion

There are many factors influencing the maintenance and activity of bacteria in the rhizosphere of a plant, but soil characteristics and the type of plant can be highlighted as some of the primary determining factors for the establishment and survival of populations of microorganisms. Culture-based and molecular approaches were used in this study which was designed to evaluate the distribution and PGP characteristics of putatively diazotrophic bacteria from the rhizosphere and roots of sunflower. To the best of our knowledge, this study is the first to report the occurrence of the genera *Enterobacter, Klebsiella, Grimontella, Novosphingobium, Microbacterium, Acinetobacter, Pantoea, Variovorax, Asticcacaulis, Chryseobacterium, Herbaspirillum, Mitsuaria, Moraxella, Serratia, Shinella, Sphingobium* and *Xanthomonas* associated with the rhizosphere of this crop.

The mechanisms of plant growth stimulation by PGPR are strongly dependent on the bacterial and host plant genotype (Montesinos et al. 2002). It is likely that strains that are better adapted to a specific rhizosphere environment are more competitive than strains that were isolated from a different environment. These strains could be used as biofertilizers to stimulate root growth, to promote rhizoremediation or to ameliorate plant biotic and abiotic stresses. In this study, all the genera identified were microorganisms already known for their biological nitrogen fixation ability, and the majority of isolates displayed more than one of the PGP characteristics analyzed. Sunflower is an important crop which produces good quality oil for human consumption, and has high potential as a new source of energy for renewable fuels. Although non-culturing DNA/RNA-based methods are now commonly used to examine bacterial diversity associated with plants, the isolation and culturing methods used in the present study are particularly suited for obtaining microbial populations capable of multiplication in vitro for the purposes of the eventual production of biofertilizers. Indeed, further studies are now being undertaken to assess these PGPR isolates under field conditions with the aim of developing viable biofertilizers for sunflower crops in Brazil (and elsewhere).

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Capítulo II

Relatório descritivo de patente de invenção: "Método de melhoramento da produção de girassol utilizando biofertilizante e composição compreendendo biofertilizante a base de *Azospirillum brasilense* Vi22"

RELATÓRIO DESCRITIVO DE PATENTE DE INVENÇÃO

MÉTODO DE MELHORAMENTO DA PRODUÇÃO DE GIRASSOL UTILIZANDO BIOFERTILIZANTE E COMPOSIÇÃO COMPREENDENDO BIOFERTILIZANTE A BASE DE *AZOSPIRILLUM BRASILENSE* VI22.

Campo da Invenção

A solução técnica da presente proposta apresentada através de tal invenção se situa no campo da agricultura e biotecnologia do solo. Mais especificamente, a presente invenção proporciona um método de melhoramento da produção de planta através da utilização de biofertilizante, assim como das composições compreendendo o mesmo. O biofertilizante da invenção compreende uma nova linhagem da bactéria *A. brasilense*.

Antecedentes da Invenção

Fertilização, biofertilização e o beneficiamento do solo e plantas cultivadas

A adição de fertilizantes às lavouras é prática comum na agricultura, sendo que os elementos N e P são os mais requisitados pelas espécies cultivadas em geral. A ausência de fertilização, principalmente a nitrogenada, causa grandes decréscimos de produtividade, como os exemplos de limitações de crescimento da planta ou rendimento dos grãos. Entretanto, a aplicação de fertilizantes químicos a base de elementos únicos é conhecidamente realizada de forma demasiada, o que intensifica a preocupação com a intensa perda do conteúdo do fertilizante adicionado através dos processos de lixiviação. O excesso de fertilização não pode garantir o aumento da produtividade, mas, geralmente, promove a exaustão acelerada de outros nutrientes principais ou minoritários e a redução da fertilidade do solo como um todo. Sobretudo, a prática da fertilização contribui diretamente para diversos desequilíbrios ambientais, tais como a eutrofização das águas, mortandade de peixes e emissão de gases de efeito estufa. A dinâmica do processo de fertilização é complexa devido ao grande número de constituintes do solo, como organismos e as substâncias orgânicas e minerais, além de todas as influências edafoclimáticas do sistema.

Dentre os organismos do solo, as bactérias constituem grande parte da ampla diversidade biológica presente nesse sistema. Os solos agrícolas são especialmente interessantes, dada a necessidade da constante busca por uma produção sustentável de alimentos ou vegetais destinados à produção de energia. As bactérias benéficas do solo são comumente conhecidas como rizobactérias promotoras de crescimento de plantas, ou PGPR (*plant growth promoting rhizobacteria*). O nome rizobactéria está relacionado às bactérias capazes de estabelecer relações com a planta no ambiente da rizosfera (porção de solo intimamente ligada às raízes). A PGPR pode ser qualquer bactéria benéfica que se estabeleça na rizosfera e afete positivamente o crescimento de uma planta. A promoção de crescimento vegetal por uma PGPR pode ocorrer de forma direta, como através da disponibilização de compostos ou nutrientes. Os diferentes compostos ou nutrientes disponibilizados serão característicos da linhagem analisada e das condições para a interação bactéria-planta. PGPRs com a capacidade de fixar N atmosférico (N_2) ou produzir hormônios vegetais são bastante interessantes, dada à relevância dessas características na fisiologia vegetal.

Os micro-organismos fixadores de N_2 são chamados de diazotróficos e são os únicos seres conhecidos em apresentar a habilidade de utilizar diretamente o N_2. A redução de N_2 a um composto que pode ser facilmente absorvido pelas raízes vegetais, a amônia, pode ser uma contribuição importante da PGPR para a fertilização da planta. Os diazotróficos que fixam N_2 dentro de estruturas especializadas de plantas, tais como os nódulos das leguminosas, são chamados de diazotróficos simbióticos. Os diazotróficos que fixam N_2 em vida-livre no solo podem estabelecer relações diversas com as plantas e podem ser bactérias diazotróficas associativas em diferentes níveis de interações com a planta. Uma bactéria associativa pode ser encontrada na rizosfera, na superfície das raízes (rizoplano) ou dentro do tecido vegetal, quando são chamados de diazotróficos endofíticos.

A produção de hormônios vegetais, tais como o ácido indol-3-acético (auxina ou AIA) é realizada pela maioria das bactérias do solo, mas utilizada de forma distinta, dependendo do tipo de relação entre bactéria e planta. Fitopatógenos podem utilizar e se beneficiar da produção de AIA em alguma etapa durante o processo de infecção, enquanto que a produção de AIA por PGPR está voltada à interação positiva com as plantas. Como todo hormônio e seu papel regulatório, o AIA faz parte de complexas respostas na fisiologia vegetal, mas, de forma bastante sintetizada, ele está

relacionado ao alongamento das raízes primárias e secundárias. O alongamento ou aumento da massa seca da raiz é uma característica de extrema importância para a saúde do vegetal, uma vez que esse é o órgão responsável pela ancoragem do organismo e, também, pela captação de água e nutrientes no solo. Estudos demonstraram que PGPRs (independente do seu nível de relação com a planta, se simbiótica ou associativa) são hábeis em promover a melhora do crescimento vegetal através da produção de AIA.

Outra forma de promoção de crescimento vegetal é a indireta, o que pode ocorrer quando a PGPR previne os efeitos deletérios de um ou mais organismos fitopatogênicos, por exemplo. O benefício indireto está relacionado à produção e/ou secreção de substâncias que atuem negativamente sobre o fitopatógeno, como antibióticos e moléculas quelantes de íon férrico, os sideróforos. Uma bactéria com capacidade de produzir sideróforo pode captar mais ferro que o patógeno e, dessa forma, inibir ou impedir sua proliferação ao redor das raízes. Outra característica relacionada à promoção indireta do crescimento é a degradação de ácido oxálico, um ácido orgânico que é amplamente produzido e utilizado durante o processo de infecção vegetal por determinados fungos fitopatogênicos. Dessa forma, bactérias que são capazes de degradar ácido oxálico podem contribuir indiretamente para a defesa vegetal contra o fitopatógeno.

Biofertilizantes a base de bactérias benéficas podem contribuir para a fertilização do solo e de plantas cultivadas através de diferentes mecanismos. A união de diversas características benéficas em um único micro-organismo é, portanto, um bom indicativo de seu potencial como PGPR. Ademais, a pesquisa com micro-organismos que já são conhecidos pelo potencial de promoção de crescimento em plantas variadas também se constitui em um suporte para a hipótese de "eficiência" da PGPR no solo. *A. brasilense* é uma bactéria Gram-negativa que tem sido cada vez mais relacionada à habilidade de favorecer positivamente o cultivo de diversas plantas, notadamente gramíneas e leguminosas (nesse último caso, quando biofertilizada em conjunto com os rizóbios). Diversas espécies de *Azospirillum* são fixadoras de N_2, produtoras de auxina, óxido nítrico, carotenoides e uma gama de componentes da superfície celular.

É estimado que cerca de 175 milhões de toneladas de N são adicionadas ao solo por ano através da fixação biológica do N_2. A fertilização química, além do dano em potencial ao meio ambiente, é muito custosa para o produtor. A utilização de biofertilizantes é uma inovação mediante o uso de uma tecnologia de baixo custo e

impacto ambiental. Além disso, a biofertilização é adequada aos sistemas de cultivo orgânico. Mesmo que múltiplas espécies de *Azospirillum* possam ser detectadas no solo e rizosfera de diferentes plantas, o estudo de uma linhagem isolada diretamente de uma determinada planta é fundamental, dada a importância das relações evolutivas que levaram ao reconhecimento e troca de sinais moleculares entre os organismos envolvidos. Um exemplo bastante estabelecido é a intensa "comunicação molecular" que ocorre durante a simbiose entre rizóbios e leguminosas. Esse reconhecimento molecular entre os organismos é tido como fator essencial para o sucesso das interações.

No âmbito patentário, alguns documentos descrevem métodos de fixação de nitrogênio e de crescimento de plantas.

O documento WO 2008/097501 descreve um método para aperfeiçoar o crescimento de plantas aplicando um fertilizante especialmente livre de hormônios e sarcosina, a fim de aumentar a biomassa da planta durante o crescimento e retardar o crescimento de fungos e bactérias. Além desse método, também é descrita uma composição para o crescimento de plantas. A presente invenção difere desse documento por não utilizar a sarcosina, mas, sim, somente organismos vivos, comumente encontrados no solo.

O documento WO 2006/098225 descreve um método de construção de plantas com nódulos de elevada atividade de fixação de nitrogênio. Esse método compreende a super-expressão do gene da globina não-simbiótica. A presente invenção difere da desse documento por não utilizar super-expressão de genes, mas, sim, organismos capazes de melhorar as condições do solo e da planta para que a mesma tenha seu crescimento aumentado.

O documento WO 2005/062899 descreve métodos e composições que fornecem efeitos agronomicamente benéficos em leguminosas e não-leguminosas. Em especial, esse método compreende o uso de um fungicida e/ou um inseticida. A presente invenção difere desse documento por não necessitar de fungicida e/ou um inseticida sendo, portanto, menos agressiva ao meio ambiente.

O documento WO 05/110068 descreve composições e métodos para controlar a infestação de pestes compreendendo a aplicação de determinadas sequências de RNA e, adicionalmente, proteínas de *Bacillus* como agentes pesticidas. A presente invenção difere desse documento por não compreender as referidas sequências.

Do que se depreende da literatura pesquisada, não foram encontrados documentos antecipando ou sugerindo os ensinamentos da presente invenção, de forma que a solução aqui proposta possui novidade e atividade inventiva frente ao estado da técnica.

Sumário da Invenção

Em um aspecto, a presente invenção proporciona um processo de melhoramento da produção de girassol (*Helianthus annuus* L.) compreendendo a biofertilização com uma linhagem da bactéria *Azospirillum brasilense*. Em uma realização preferencial, o melhoramento da produção de girassol pela presente invenção compreende a modulação da fixação de nitrogênio e/ou de fatores promotores de crescimento.

São dadas, portanto, em referência ao desenvolvimento geral do método de melhoramento da produção de girassol utilizando esse biofertilizante, as seguintes etapas sumarizadas:

a) selecionar a planta de interesse e coletar raízes de amostras de diferentes locais, de forma a obter uma amostragem significativa para o isolamento das bactérias;

c) determinar os mecanismos da promoção de crescimento que sejam mais adequados às necessidades da planta em questão e avaliar a presença de tais características entre os isolados obtidos;

d) selecionar isolados sem potencial à patogenicidade de forma quantitativa (nº de características) e qualitativa (maior ou menor grau de atividade);

e) investigar a capacidade de promoção do crescimento à planta de interesse por bactérias selecionadas, através de diversos processos de biofertilização em câmara de crescimento com condições controladas;

f) realizar ensaios a campo com biofertilização das sementes, plantio e avaliação do melhoramento vegetal.

É outro objeto da presente invenção, composições para inoculantes agrícolas compreendendo:

a) o material biológico da linhagem da espécie que compreende *A. brasilense*;

b) veículo aceitável e bem adequado a esse tipo de prática agrícola.

Estes e outros objetos da invenção são descritos em detalhes suficientes para sua reprodução na descrição a seguir.

Descrição Detalhada da Invenção

Os exemplos aqui mostrados têm o intuito somente de exemplificar uma das inúmeras maneiras de se realizar a invenção, sem limitar, contudo, o escopo da mesma.

Métodos para melhoramento da produção de plantas

O método que utiliza biofertilizante e visa o aumento da produtividade de girassol em conjunto com a redução da quantidade de fertilizantes químicos aplicados às lavouras compreende as etapas de:

> Em uma realização preferencial, o melhoramento da presente invenção compreende a modulação da fixação de nitrogênio e/ou de fatores promotores de crescimento.

Linhagem promotora do crescimento vegetal

a) emprego de metodologias que favoreçam a obtenção da bactéria diazotrófica de interesse durante o processo, como o cultivo em meio de cultura apropriado ao seu crescimento e sem adição de N;

b) isolamento de linhagens a partir de solos rizosféricos de girassol (v. Aguará 6) de diferentes regiões.

Material Biológico

O material biológico útil na presente invenção inclui, mas não se limita aos elementos como DNA, RNAs e/ou proteínas, inteiros ou parciais, isolados de *A. brasilense* Vi22.

Em especial, o material biológico da presente invenção compreende células portadoras da sequência da região do gene 16S ribossomal, depositada no Genbank (Altschul et al., 1997) como a sequência de número de acesso JF501207.

Material Biológico Vegetal

O material biológico útil na presente invenção inclui, mas não se limita aos elementos como DNA, RNAs e/ou proteínas, inteiros ou parciais, células, órgãos, tecidos vegetais, incluindo plantas e/ou sementes em formação ou completamente formadas.

Células trabalhadas

As células trabalhadas na presente invenção compreendem pelo menos um material biológico da linhagem da espécie escolhida que compreende *A. brasilense* Vi22.

Composições

As composições da presente invenção compreendem:

a) pelo menos um material biológico da linhagem da espécie que compreende *A. brasilense* Vi22;

b) veículo aceitável e bem adequado a esse tipo de prática agrícola

Veículo Aceitável na Agricultura

O veículo aceitável na agricultura da presente invenção pode ser escolhido do grupo que compreende excipientes e carreadores, doses e tratamentos convenientes para uso em composições particulares que podem ser descritas em uma série de regimes.

<u>Exemplo 1. Obtenção e caracterização da bactéria de melhoramento</u>

Amostragem e preparação de amostra

Amostras do solo de rizosfera foram coletadas a partir da mesma variedade de girassol (v. Aguará 6) na região de Viamão, Rio Grande do Sul (RS), Brasil [Vi (30°04′ 51″S, 51°01′22″W)] (Estação da Fundação Estadual de Pesquisa Agropecuária do RS – Fepagro). Dez sub-amostras do solo (0-15 cm de camada) foram obtidas e misturadas para obter uma amostra representativa.

A linhagem Vi22 de *Azospirillum brasilense* foi isolada de acordo com Döbereiner (1995). Dez gramas de solo aderido às raízes, considerado como solo de rizosfera, foram misturadas com 90 ml de solução salina (NaCl 0,85%) e usadas para o procedimento de isolamento da bactéria. A suspensão do solo foi incubada a 4ºC e mantida sob agitação constante por 16 h. Foram realizadas diluições sucessivas e inoculações em meio de cultura semi-sólido sem N – NFb. A colônia da bactéria obtida foi re-inoculada nas mesmas condições e posteriormente isolada em meio líquido LB (Sambrook e Russel, 2001) para verificação da pureza do inóculo, o qual foi analisado em microscopia ótica pela coloração de Gram. A coloração e as características morfológicas da colônia também foram avaliadas. Culturas puras foram estocadas a -18ºC em 50% de glicerol.

Isolamento de DNA

DNA foi diretamente extraído da cultura bacteriana pelo método descrito no trabalho de Ambrosini e colaboradores (2012) e consiste na utilização de lisozima (20 mg ml^{-1}) e dodecil sulfato de sódio (SDS 20%) para lise das células. Após recuperação do DNA com acetato de amônio (8 M pH 8.0), foi realizada uma extração com fenol-clorofórmio e precipitação com etanol. As amostras de DNA foram visualizadas em gel de agarose 0,8% e avaliadas visualmente quanto à integridade e quantificação das moléculas.

PCR e análise de RFLP do gene 16S rRNA

Cerca de 50 nanogramas de DNA foram usadas como molde em procedimentos de PCR. Os oligo-iniciadores específicos selecionados [U968 e L1401 (Felske et al., 1997)] foram usados para amplificar uma região de aproximadamente 450 pares de bases (pb) do gene, juntamente com os demais constituintes da reação (Promega®). Amplificações em cadeia da polimerase (PCR) foram feitas conforme descrito por Ambrosini e colaboradores (2012).

O produto de PCR foi avaliado por análise do polimorfismo de comprimento de fragmento de restrição (RFLP, do inglês *restriction fragment length polymorphism*) com a utilização de duas endonucleases de restrição [HaeIII e TaqI (Promega ®)]. As etapas do emprego da técnica de RFLP foram realizadas de acordo

com Sambrook e Russel (2001). O perfil de bandas foi analisado em gel de poli-acrilamida 10%.

O produto de PCR foi sequenciado em um sequenciador automático [ABI PRISM 3100 Genetic Analyzer (Applied Biosystems)] e avaliado nos bancos de dados de sequência Ez-Taxon (Chun et al., 2007) e GenBank – BLASTN (Altschul et al., 1997). A sequência foi depositada no GenBank sob o número de acesso: JF501207.

Produção in vitro de compostos indólicos

O isolado foi cultivado em meio King B a 28ºC sob agitação constante por 48 h e 100 µl da suspensão bacteriana (10^6 CFU ml^{-1}) foram re-inoculados em meio King-B com o aminoácido triptofano, estimulador da síntese de compostos indólicos (Glickmann e Dessaux, 1995). Sobrenadantes de cultivo foram adicionados a igual volume do reagente de Salkowski (12 gL^{-1} $FeCl_3$ + 7,9 M H_2SO_4) e quantificados em um espectrofotômetro para a produção de ácido indol-3-acético (AIA), ácido indolpirúvico (IPyA) e indolacetamida (IAM), os quais serão referidos nesse texto coletivamente como compostos indólicos.

Produção de Sideróforo

O isolado foi cultivado em meio LB (conforme acima) e 2 µl da suspensão bacteriana (10^6 CFU ml^{-1}) foram inoculados em meio apropriado para a produção de sideróforos. Foram usadas placas de Petri contendo meio King-B diluído 5 vezes e suplementado com o complexo cromo-azurol S (CAS), como descrito por Schwyn e Neilands (1987). As placas foram incubadas a 28ºC por 48–72 h e a produção de sideróforo foi avaliada através da formação de um halo ao redor da bactéria.

Degradação de ácido oxálico

A capacidade de degradar ácido oxálico foi avaliada através da inoculação de 2 µl [do isolado cultivado em meio LB (10^6 CFU ml^{-1})] em meio mineral de Schlegel contendo 4g L^{-1} de oxalato de cálcio (Braissant et al., 2002). A formação de halo ao redor da colônia foi o indicativo da degradação de oxalato, o que ocorreu normalmente, após 3 semanas de incubação a 28ºC.

Fixação de nitrogênio

A habilidade de fixar N_2 foi testada usando a técnica de redução de acetileno *in vitro* (ARA, do inglês *acetylene reduction assay*), como descrito por Boddey (1987). A atividade da enzima nitrogenase, essencial para a redução de N_2 à amônia, foi avaliada pela inoculação da bactéria em vidros contendo 4 mL de meio NFb semi-sólido e incubação a 28ºC por 72 h. Os vidros foram selados com uma tampa de borracha e gás acetileno foi introduzido [10% (v/v)]. Após 1 h de incubação a 28ºC a quantidade de etileno produzida foi avaliada através da utilização de um cromatógrafo a gás [Clarus 600 gas chromatograph (Perkin Elmer)].

Experimento de promoção de crescimento de girassol em câmara de crescimento

O experimento de inoculação das sementes e cultivo de girassol foi realizado em uma câmara de crescimento com ambiente controlado e fotoperíodo de 16 h de luz (28°C) e 8 h de escuro (20°C). Sementes de girassol (v. Aguará 6) foram desinfetadas lavando-as em etanol 70% durante 1 min, seguido de lavagens de solução de hipoclorito de sódio (4% v / v) com três gotas de Tween 80 durante 5 min e, finalmente, cinco lavagens em série com água destilada esterilizada. As sementes tratadas foram plantadas 2,0 centímetros abaixo da superfície de vermiculita esterilizada (1 semente por copo) em copos (15 cm × 20 cm), contendo 100 mL de solução nutritiva de Hoagland diluída a 25% (Ambrosini et al., 2012). O isolado bacteriano foi cultivado em meio LB sob agitação (200 rpm) durante 48 h a 28°C e o precipitado celular foi suspendido na solução nutritiva (acima), até obtenção de uma concentração final de 10^8 UFC ml^{-1}. As plantas foram inoculadas com alíquotas de 5 ml de células diretamente sobre as sementes e o experimento consistiu de tratamentos inoculados e não inoculados, além do controle negativo que não recebeu nem solução nutritiva nem bactéria. As plantas foram colhidas aos 30 dias após a germinação e foram coletados dados quanto ao comprimento e peso seco da parte aérea e da raiz, além do conteúdo de NPK, a partir da massa seca da parte aérea (Tedesco et al., 1995). Cada tratamento consistiu de 5 replicatas e os dados foram estatisticamente analisados usando-se one-way ANOVA e comparações múltiplas das médias pelo teste de Tukey. As diferenças foram consideradas significativas ao nível 0,05. As análises estatísticas foram feitas com IBM SPSS Statistics versão 19 (Norusis; SPSS Inc., 2010).

Produção de biofertilizante

O biofertilizante contendo a linhagem Vi22 de *A. brasilense* foi desenvolvido na empresa Simbiose Indústria e Comércio de Fertilizantes e Insumos Microbiológicos – Cruz Alta (RS), através de tecnologia industrial de fermentação. Foram adotados adequados controles da curva de crescimento bacteriano (câmara de Petroff Hausser), dos meios de culturas em diferentes situações de aeração, agitação, pH, PO^2 e temperatura em um fermentador piloto B-Braun C e da elaboração de multiplicação em escala industrial, adaptando o processo aos fermentadores de 150, 1.500 e 15.000 L.

As formulações foram avaliadas quanto a diversas metodologias a fim de avaliar o mais adequado modo de conservação; análise do aspecto físico da formulação relacionado à solubilidade e dispersibilidade junto à calda; análise da compatibilidade das formulações com outros insumos agrícolas químicos e biológicos; desenvolvimento de sistema de envase e embalagens adequadas ao produto; avaliação periódica da estabilidade físico-química e biológica da vida de prateleira das formulações desenvolvidas; estabelecimento de condições e indicações de armazenamento e transporte do produto final.

A adequação de todas as etapas durante o processo seguiram as normas das legislações regulamentares vigentes, perante o MAPA (Ministério da Agricultura, Pecuária e Abastecimento), FEPAM (Fundação Estadual de Proteção Ambiental Henrique Luiz Roessler – RS), IBAMA (Instituto Brasileiro de Meio Ambiente e dos Recursos Naturais Renováveis) e outros órgãos fiscalizadores. O biofertilizante foi inicialmente produzido em formulação líquida à concentração celular na casa de 10^8 células mL^{-1}, com validade de 3 meses.

Experimento de biofertilização a campo

O experimento a campo foi realizado nas dependências da Fundação Estadual de Pesquisa Agropecuária do RS (Fepagro), no município de Viamão. A análise do solo foi realizada para caracterização química e física. P, K e B, além das correções de pH, foram realizadas conforme indicado para a cultura. O biofertilizante obtido foi utilizado para biofertilização de sementes de girassol plantadas em campo e da adição de 250 mL de biofertilizante (1 x 10^8 células/mL) para 50 kg de sementes (v. BRS321). As parcelas experimentais consistiram de 4,2 x 6,0 m (distanciadas em 1,0 m) e o delineamento experimental utilizado foi o de blocos ao acaso com quatro repetições

e o total de 20 parcelas. Os tratamentos (T) foram diferenciados pela biofertilização (B) e doses de ureia (N), a qual foi aplicada 50% no plantio e 50% na floração, conforme a dose estipulada para a cultura: T1 (controle negativo: ausência de ureia e de biofertilizante); T2 (controle com metade da dose de ureia recomendada para a cultura); T3 (dose de ureia recomendada para a cultura); T4 (biofertilizante e ausência de ureia); T5 (biofertilizante com metade da dose de ureia recomendada para a cultura).

Amostras de folhas foram colhidas de 10 plantas da área útil de cada parcela para determinação do conteúdo de NPK (Tedesco et al., 1995) aos 60 dias após o plantio. A colheita de 10 plantas da área útil das parcelas ocorreu aos 120 dias após o plantio e foram avaliados massa seca da parte aérea, diâmetro do capítulo, produção de grãos e produtividade (Kg ha^{-1}). Os dados foram estatisticamente analisados usando ANOVA para experimentos em blocos ao acaso e as médias foram comparadas pelo teste de Skott-Knott para comparações múltiplas. As diferenças foram consideradas significativas ao nível de 0,05 e as análises estatísticas foram feitas com Assistat (Silva, 1996; versão 7.6 beta, 2013).

Azospirillum brasilense a partir de Helianthus annuus L.

Na presente invenção, a linhagem Vi22 foi identificada como pertencente à espécie *A. brasilense* (Tarrand et al., 1978), uma bactéria Gram-negativa, flagelada e fixadora de N_2. O gênero *Azospirillum* é conhecido pela característica de acúmulo de poli-hidroxibutirato, um tipo de substância que pode auxiliar em situações de estresse por atuar como fonte de C e energia (Tal e Okon, 1985). A promoção de crescimento vegetal por bactérias desse gênero, sobretudo *A. brasilense*, tem sido associada à fixação de N_2 e produção de AIA, mas diversos outros processos podem estar envolvidos no sistema de interação, como a quimiotaxia, produção de carotenoides e de exopolissacarídeos (EPS) da superfície celular (Fibalch-Paldi et al., 2011). A pesquisa com o isolado do solo mostrou suas habilidades de fixação de N_2 (81,85 nmol C_2H_4 mg protein min^{-1}), produção de compostos indólicos (204,22 μg ml^{-1}), produção de sideróforo e degradação de oxalato.

A união de várias características de interesse é um ponto importante para a seleção de PGPRs na agricultura, assim como a adequada utilização dos métodos é essencial para determinação das atividades relacionadas à bactéria. A linhagem isolada fixa altos níveis de N_2, característica marcante da espécie (Hungria et al., 2010, Fibalch-

Paldi et al., 2011). A produção de compostos indólicos também já é bem caracterizada para essa espécie (Bothe et al. 1992; Bashan and Holguin 1997; Steenhoudt and van der Leyden 2000), assim como a produção de sideróforos (Bachhawat e Ghosh 1987; Tapia-Hernández et al. 1990). Quanto à degradação de oxalato, *A. brasilense* ($Sp7^T$) degrada o composto em maior grau que *A. lipoferum* ($Br17^T$), enquanto *A. amazonense* ($Y6_T$) não apresenta essa atividade (Weber et al., 1999). O maior ganho no produto do presente processo consiste na biofertilização com uma linhagem de *A. brasilense* com alto potencial agrícola e isolada da rizosfera da planta de interesse, o girassol.

Na inoculação em câmara de crescimento com a linhagem Vi22 de *A. brasilense*, o peso seco das raízes inoculadas foi significativamente mais alto do que as plantas controles não inoculadas, com um acréscimo de 77% (Tabela 1). A bactéria também estimulou a captação de P pela planta em torno de 68% de aumento em relação ao controle não inoculado. Tais dados corroboram os da literatura sobre o estímulo de linhagens de *A. brasilense* sobre o desenvolvimento das raízes (Barassi et al., 2007; Fibalch-Paldi et al., 2011).

Na biofertilização a campo a linhagem Vi22 de *A. brasilense* apresentou resultados tão positivos quanto o controle que recebeu a totalidade da dose recomendada de N para a cultura (T3). Quanto às plantas colhidas aos 60 dias após o plantio, o teor de N das folhas foi significativamente mais alto nos tratamentos que receberam adubação nitrogenada (T2, T3 e T5), mas não houve diferença estatística entre as doses 50 e 100% de N (Tabela 2). A presença da bactéria foi relacionada à diminuição do teor de K na planta, mas os resultados são de difícil interpretação dado mesmo teor do nutriente para os tratamentos sem N (T1) e com a dose recomendada de N (T3) (Tabela 2).

A massa seca das plantas colhidas aos 120 após o plantio também não apresentou diferença estatística entre os tratamentos, embora os valores tenham sido mais altos para T3 e T5 (biofertilizante com metade da dose de ureia recomendada para a cultura) (Tabela 3). O diâmetro do capítulo também foi uma característica que não apresentou diferenças estatísticas entre os dados, e, novamente, os valores foram maiores para os tratamentos que receberam a dose máxima de N (T3) ou a metade da dose mais o biofertilizante (T5) (ambos com 14,9 cm). A característica de maior interesse, isto é, o aumento da produtividade dos grãos, foi significativamente mais alto para os tratamentos T3 e T5. Em síntese, os dados mostram que o girassol atingiu uma alta produtividade de grãos quando fertilizado com 100% da dose de N recomendada

para a cultura ou quando fertilizado com 50% da dose de ureia recomendada mais o biofertilizante a base da linhagem Vi22 de *A. brasilense*.

Tabela 1: Efeito da inoculação da linhagem Vi22 de *A. brasilense* sob plântulas de girassol (v. Aguará 6) cultivadas por 30 dias em câmara de crescimento.

	Crescimento da parte aérea		**Crescimento da raiz**	
Tratamento	**Comprimento (cm)**	**Peso seco (mg)**	**Comprimento (cm)**	**Peso seco (mg)**
Controle negativo	3,64 (± 1,07) b	103,80 (± 40,30) c	17,50 (± 5,63) a	45,20 (± 18,85) c
Controle não-inoculado	7,56 (± 5,00) a,b	208,80 (± 90,28) b,c	15,38 (± 1,83) a	68,80 (± 30,12) b,c
Vi22	11,14 (± 2,65) a	306,00 (± 55,21) a,b	20,10 (± 5,38) a	113,80 (± 12,15) a

Dados são médias (± DP) de cinco replicatas de plantas crescidas em vermiculita estéril em uma câmara com fotoperíodo controlado. Valores de uma mesma coluna, seguidos da mesma letra, não diferem significativamente (p>0,05) (teste de Tukey)

Tabela 2: Efeito da biofertilização de *A. brasilense* Vi22 no teor de NPK de folhas de girassol (v. BRS321) cultivado por 60 dias a campo.

Tratamento	**N (%)**	**P (%)**	**K (%)**
T1 (0% N)	3,18 (± 0,47) b	0,25 (± 0,03) a	3,70 (± 0,22) a
T2 (50% N)	3,61 (± 0,11) a	0,24 (± 0,02) a	3,80 (± 0,37) a
T3 (100% N)	3,80 (± 0,13) a	0,22 (± 0,03) b	3,70 (± 0,03) a
T4 (B 0% N)	3,37 (± 0,21) b	0,21 (± 0,03) b	3,30 (± 0,14) b
T5 (B 50% N)	3,60 (± 0,19) a	0,26 (± 0,02) a	3,50 (± 0,29) b
Coeficiente de Variação	**6,81%**	**9,27%**	**4,38%**

B (bactéria); T1 (controle negativo: ausência de ureia e de biofertilizante); T2 (controle com metade da dose de ureia recomendada para a cultura); T3 (controle com a dose de ureia recomendada para a cultura); T4 (biofertilizante e ausência de ureia); T5 (biofertilizante com metade da dose de ureia recomendada para a cultura).

Dados são médias (± DP) de 10 plantas da área útil de cada uma das 4 parcelas de cada tratamento. Valores de uma mesma coluna, seguidos da mesma letra, não diferem significativamente (p>0,05) (Skott-Knott).

Tabela 3: Efeito da biofertilização de *A. brasilense* Vi22 na massa seca, diâmetro do capítulo e produtividade dos grãos de girassol (v. BRS321) cultivado por 120 dias a campo.

Tratamento	**Massa seca (Kg)**	**Diâmetro capítulo (cm)**	**Produtividade grãos (Kg ha^{-1})**
T1 (0% N)	1,05 (± 0,19) a	13,24 (± 2,21) a	2910,00 (± 186,55) b
T2 (50% N)	1,12 (± 0,14) a	13,59 (± 0,77) a	3412,50 (± 345,87) b
T3 (100% N)	1,32 (± 0,12) a	14,90 (± 0,52) a	4065,00 (± 426,03) a
T4 (B 0% N)	1,18 (± 0,28) a	13,38 (± 1,37) a	3142,00 (± 350,18) b
T5 (B 50% N)	1,24 (± 0,13) a	14,90 (± 1,22) a	3787,50 (± 317,84) a
Coeficiente de Variação	**12,49%**	**9,43%**	**10,40%**

B (bactéria); T1 (controle negativo: ausência de ureia e de biofertilizante); T2 (controle com metade da dose de ureia recomendada para a cultura); T3 (controle com a dose de ureia recomendada para a cultura); T4 (biofertilizante e ausência de ureia); T5 (biofertilizante com metade da dose de ureia recomendada para a cultura).

Dados são médias (± DP) de 10 plantas da área útil de cada uma das 4 parcelas de cada tratamento. Valores de uma mesma coluna, seguidos da mesma letra, não diferem significativamente (p>0,05) (Skott-Knott).

REIVINDICAÇÕES

MÉTODO DE MELHORAMENTO DA PRODUÇÃO DE GIRASSOL UTILIZANDO BIOFERTILIZANTE E COMPOSIÇÃO COMPREENDENDO BIOFERTILIZANTE A BASE DE *AZOSPIRILLUM BRASILENSE* VI22.

1. Método de melhoramento da produção de girassol caracterizado pela aplicação de uma composição biofertilizante compreendendo a linhagem Vi22 de *A. brasilense*;

2. Método, de acordo com a reivindicação 1, caracterizado pelo fato de que a referida composição biofertilizante modula fatores diversos da promoção de crescimento vegetal.

3. Composição biofertilizante caracterizada por compreender:

a) pelo menos um material biológico da linhagem da espécie que compreende *A. brasilense*: Vi22;

b) veículo aceitável e bem adequado a esse tipo de prática agrícola.

RESUMO

MÉTODO DE MELHORAMENTO DA PRODUÇÃO DE GIRASSOL UTILIZANDO BIOFERTILIZANTE E COMPOSIÇÃO COMPREENDENDO BIOFERTILIZANTE A BASE DE *AZOSPIRILLUM BRASILENSE* VI22.

A presente invenção descreve um método de melhoramento da produção de girassol utilizando biofertilizante a base de uma bactéria promotora do crescimento vegetal caracterizada como *A. brasilense* Vi22, bem como composições compreendendo os mesmos. O biofertilizante da presente invenção mostrou-se eficiente no processo de melhoramento da produção de girassol em conjunto com a metade da dose de nitrogênio recomendada para a cultura, correspondendo à alta produtividade alcançada quando a dose total foi adicionada. A presença do biofertilizante permitiu que metade da dose de nitrogênio pudesse ser utilizada no plantio sem perdas de produtividade, o que consiste em redução de gastos com fertilizantes e adição de 50% menos compostos químicos no solo. Os dados apresentados aqui se encontram parcialmente publicados (Ambrosini et al., 2012) e experimentos adicionais a campo estão sendo realizados.

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Capítulo III

Assessing diazotrophic bacilli communities using multivariate methods based on 16S rDNA

Assessing diazotrophic bacilli communities using multivariate methods based on 16S rDNA

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ABSTRACT

A consensus bacterial classification is suitable to an accurate identification of operational taxonomic unit (OTU). The standard methods, such as nucleotide identity and phylogenetic assays, have been questioned because of factors that influence its robustness. The phylotyping is an alternative based on genetic markers useful to bacterial discrimination, but the technique reproducibility can be a problem. The high similarity of 16S rDNA sequences among close species of some bacilli makes it difficult to classify most strains at species level by this marker. In this study, we compared sequences of bacilli strains isolated from sunflower rhizospheres and type-species from databases by standard approaches and phylotyping based on *in silico* restriction fragment length polymorphisms via cluster and principal component analyses. Through the application of the *in silico* enzymatic digestion of 16S rDNA sequences generated by 16 restriction endonuclease profiles, we used the power of this molecular marker based on both the sequencing and positioning of restriction sites, with a large amount of data generated by computer analysis. One hundred and three different bacilli were isolated and the species of *B. arbutinivorans* and *P. pabuli* were the most

abundant. The most effective nitrogen (N) fixing isolates were tested in a plant assay in different N contents and a strain of *B. mycoides* showed positive interaction with sunflower. The data generated in this study provided information related to the ecology of diazotrophic bacilli and additional support to identify strains based on 16S rDNA sequences, contributing to the phylogeny and functional diversity of *Bacillus* and *Paenibacillus* species.

Keywords: *Bacillus, Paenibacillus*, PGPR, sunflower, *in silico* RFLP, phylotyping.

INTRODUCTION

The universal function of ribosomal genes has not changed over time, thus assuring the integrity of the protein translation system to maintain cell metabolic requests. Studies employing the 16S rRNA (small-subunit of ribosomal RNA) as a phylogenetic marker are widely accepted because it meets most of the requirements for a proper phylogeny (Harish and Caetano-Anollés, 2012; Das and Dash, 2013). The variable regions of the 16S rRNA gene contribute widely to its interspecific variation and are used for species identification (Gray et al., 1984; Jonasson et al., 2002; Coenye and Vandamme, 2003). For certain groups of Gram-positive bacteria, such as the *Bacillus* species, the 16S rDNA V1-V2 regions (approximately the first 200 nucleotides) are the most variable and useful regions for identification (Giffel et al., 1997; Goto et al., 2000; Chen and Tsen, 2002).

However, bacterial identification based on standard methods, such as nucleotide similarity and phylogenetic analysis, has been questioned because of factors that influence its robustness, such as sequence alignments and the treatment of gaps (Dwivedi and Gadagkar, 2009; Schloss and Westcott, 2011; Warnow, 2012). Moreover, little has been discussed regarding bacterial identification based on sequence similarity and the possibility of identity mistakes due to sequencing errors (Dickie, 2010; Kuninet al., 2010). Some researchers have concluded that OTU-based methods are more sensitive to sequencing errors than phylotype-based methods, because phylotyping methods operate on a broader level than that of OTU-based methods, leading to an overall silencing of sequencing errors (Kunin et al., 2010; Schloss and Westcott, 2011).

Gram-positive bacteria show surprising functional and morphological diversity. Members of *Bacillaceae* and *Paenibacillaceae* have a variety of applications on diverse

microbiological areas, such as biocontrol and biofertilization. Several strains of *Bacillus* (Cohn, 1872) and *Paenibacillus* (Ash et al., 1994) are able to associate with many crops and can display plant growth promotion traits, including the ability to fix atmospheric nitrogen (N_2), produce indolic compounds (IC) and siderophores (McSpadden Gardener, 2004; Beneduzi et al., 2008a; Zawadzka et al., 2009; Farina et al., 2012; Souza et al., 2012). Crop productivity depends greatly on N and, because of this, the diazotrophic are among the most suitable bacteria for biofertilization assays (Olivares et al., 2013; Santi et al., 2013). However, Gram-positive diazotrophic have been mostly related to species of the *Clostridium* and *Paenibacillus* genera (Achouak et al., 1999; Minamisawa et al., 2004; Beneduzi et al., 2008a and 2008b; Kumar et al., 2012; Xie et al., 2012).

In an evaluation of diazotrophic bacilli communities on rhizospheric soil of *Helianthus annuus* L. (sunflower), an oilseed plant of great economic and agricultural importance, we found several isolates whose taxonomic positions were variable when using 16S rRNA gene sequencing and databases. Moreover, according to the phylogenetic trees, some clusters containing isolates and type-species were not consistent. Indeed, the high similarity of the 16S rDNA sequences among the closest species of some bacilli make it difficult to classify the majority of strains through those approaches (Bavykin et al., 2004; Alcaraz et al., 2010; Økstad and Kolstø, 2011). One example is the *B. cereus* group (e.g., *B. anthracis*, *B. cereus*, *B. mycoides*, *B. pseudomycoides*, *B. thuringiensis*, and *B. weihenstephanensis*), which possesses strikingly similar genetic content including the 16S rDNA sequences (Han et al., 2006; Schmidt et al., 2011).

A combination of a variety of methods can be employed to produce consistent and accurate results for proper taxonomic characterization of closely related bacteria (Slabbinck et al., 2010; Schloss et al., 2011).Therefore, we compared sequences of 16S rDNA of bacilli communities and several type-species by standard approaches and phylotyping based on *in silico* restriction fragment length polymorphisms (RFLP) via cluster and principal component analyses. By applying this methodology, we avoided the variable reproducibility of fingerprinting techniques, which are biased by other factors, such as enzymatic cleavage efficiency and the visualization of bands on a gel (McGregor et al., 2000; Osborn et al., 2000; Torsvik and Øvreås, 2002, Berkeley et al., 2008). In addition for proper discrimination of strains associated with the rhizosphere,

the most effective N_2-fixing isolates were evaluated in a plant assay with different N content to test the potential of positive interactions between these microorganisms and sunflower. The data of this study provided additional information concerning the ecology of diazotrophic *Bacillus* and *Paenibacillus* and further support strain identification based on the 16S rDNA sequencing of these communities. Furthermore, the *in silico* RFLP technique contributed to a consensus bacterial identification based also on phylogeny and nucleotide sequence similarity, being suitable mainly to species with highly similar 16S rRNA gene sequences.

MATERIALS AND METHODS

Bacterial assays

Roots of *Helianthus annuus* L. (cv. Aguará 6) were sampled at the flowering stage from fields on Paleudult [Encruzilhada do Sul 'E' (30°32′38″S, 52°31′19″W)], Rhodic Palleudult [São Gabriel 'S' (30°20′09″S, 54°19′12″W)] and Haplumbrept [Vacaria 'V' (28°30′43″S, 50°56′02″W)] soils in Rio Grande do Sul, Brazil. Soil characteristics were statistically correlated to bacterial diversity (below) using Past software (Hammer et al., 2001) and Principal Component Analysis (PCA).

Rhizospheric soils of three plants of the same region were mixed and 10 g of each mixture was added to 90 ml of 0.85% saline solution. The suspensions were vigorously shaken at 4ºC for 24 h and posteriorly maintained at 4ºC for 120 days. Dilutions (10^{-1}) were pasteurized (80°C, 10 min) to eliminate non-sporulated forms, inoculated onto N-free thiamine-biotin agar (TB; Seldin et al. 1983) and anaerobically incubated in anaerobic jars at 28°C for 7 days. The colonies obtained were transferred to fresh TB agar plates for another period of anaerobic incubation. The distinct colonies were isolated on King B medium (Glickmann and Dessaux, 1995) and incubated at 28°C for 48 h. These conditions were routinely used for the bacterial growth in all experiments. All isolates were microscopically checked and subsequently stored in 50% glycerol at -18°C.

16S rDNA sequence based taxonomy

Molecular analyses

The genomic DNA of each isolate was extracted (Ambrosini et al., 2012), visually quantified on a 0.8% agarose gel and used for a polymerase chain reaction (PCR) of the

16S rRNA gene, using primers pA forward (5' AGAGTTTGATCCTGGCTCAG 3'; Stackebrandt and Liesack, 1993) and 1542R reverse (5' AGAAAG GAGGTGATCCAGCC 3'; Edwards et al., 1989). These primers annealed on positions 8-28 and 1525-1542 of the *Escherichia coli* 16S rRNA gene were used to amplify Gram-positive bacteria 16S rDNA (Ntougias and Russell, 2001; Coton and Coton, 2005; Gontang et al., 2007). PCR reactions were performed according to the manufacturer's recommendations (Invitrogen®) and subjected to one cycle of denaturation at 94°C for 5 min and 30 cycles of 94°C for 45 sec, 55ºC for 45 sec and 72ºC for 1 min, followed by one cycle at 72°C for 5 min. Sequencing was performed on an ABI PRISM 3100 Genetic Analyzer automatic sequencer (Applied Biosystems) and sequence analyses were performed using the BioEdit version 7.0.9.0 software (Hall, 1999). Isolates were grouped according to the similarity of their 16S rRNA gene sequences (>99.80%), and only one representative strain of each cluster was considered for further analysis.

Multiple alignments were performed with sequences of representative strains and their closest species (> 97% of similarity) with the ClustalX 2.1 program, using default settings (Larkin et al., 2007). Two signatures of 12 nucleotides dictated the cutoff that defined what region of the 16S rDNA sequences would be analyzed, which included the initial 5' TGCAAGTCGAGC 3', and at the end of sequence, 5' GAAGTCGTAACA 3' (all sequences were cut at same positions along the 16S rRNA gene and the resulting average length was of 1,455 nucleotides). The indexes of similarity were assigned according to identification with GenBank/BLASTN (Altschul et al. 1997), EzTaxon-e/BioCloud.net server (Kim et al., 2012), and RDP (Ribosomal Database Project; Cole et al., 2009). The 16S rDNA sequences of 31 representative strains were deposited in the GenBank database (accession numbers KF010780 to KF010810). Twenty type-species were chosen for further analysis for each *Bacillus* and *Paenibacillus* genera (Table 1).

Phylogenetic assay

The character-based maximum likelihood (ML; Felsenstein, 1981) and maximum parsimony (MP; Eck and Dayhoff, 1966) methods were employed to reconstruct phylogenetic relationships. The 16S rDNA sequences of 31 representative isolates, 40 type-species, and the outgroup *Azospirillum brasilense* Sp245 (accession number

HE577328) were used to build trees from the alignment using default parameters without manual editing by Clustal W (Larkin et al., 2007), as implemented in MEGA5 (Molecular Evolutionary Genetics Analysis version 5; Tamura et al., 2011). For the ML method, the evolutionary distances were determined using the Jukes-Cantor model and "Data Subset to Use" was tested considering each of three options: 'use all sites', 'complete deletion', and 'partial deletion'; other "Tree Inference Options" were the 'Nearest-Neighbor-Interchange (NNI)' (ML heuristic method) and 'Make initial tree automatically (MP)' (initial tree for ML). For the MP method, the options were 'use all sites' (to "Data Subset to Use") and 'Subtree-Pruning-Regrafting (SPR) (values of 10, 1, and 100)' to the "Tree Inference Options". The bootstrap confidence values were generated using 1,000 permutations to ML trees.

In silico RFLP and multivariate methods

In silico restriction fragment length polymorphism (RFLP) assays were carried out with 16 informative Type II restriction endonucleases (REs), which were analyzed independently of methylase sites (AluI, DdeI, HaeIII, Hpy188I, HpyCH4V, MaeIII, MseI, MspI, NdeII, NlaIII, RsaI, Sau96I, TaqI, TauI, TseI, and TspeI). *In silico* digestions were performed by using REBASE version1.20130228 (Roberts et al., 2010) and Microsoft Excel (Microsoft Office Home and Student, 2010). To infer what enzymes would be more efficient in the classification of the 40 type-species, a value was given for the ability of one RE to distinguish one species from another. This value was called resolving power (RP) and a value of 100% was given to enzymes that were able to distinguish all sequences. The average number of enzymatic cutoffs was calculated for all isolates and type-species (71 sequences in total).

Enzyme-specific and general (all 16 REs) matrices were established so that each isolate or type-species received a 0 or 1 for the absence or presence of the resulting DNA fragments, respectively. Data were subjected to cluster analyses by distance-based method unweighted pair-group average (UPGMA) and the similarity indices of the Jaccard coefficient. Three general binary-matrices (all sequences, *Bacillus* spp., and *Paenibacillus* spp.) were also analyzed using principal component analyses (PCA). All data concerning multivariate methods were assayed using PAST software (Hammer et al., 2001). Bootstrap confidence levels were generated by 1,000 replicates. A virtual gel plot was constructed from the matrices of the RE profiles, where the number 1

(presence of DNA fragment of certain length) was replaced by the symbol '–' (Microsoft Excel).

The diversity indices (*H';* Shannon and Weaver, 1949) were estimated based on the 16-RE profiles of isolates of each sampled locality. The UPGMA dendrograms were analyzed by counting the number of clusters at the 96% similarity level and the number of taxa within each cluster (Borges et al., 2003; Kaschuk et al., 2006).

Evaluation of nitrogenase activity and other plant growth promoting traits

The Acetylene Reduction Assay (ARA) was employed to assess the N_2-fixation ability of the isolates, according to the procedure described by Boddey (1987). Briefly, the isolates were inoculated into 10 ml vials containing 4 ml of semi-solid (0.18% agar) TB N-free medium (Seldin et al. 1983). After 48 h incubation at 28°C in the dark, the vials were sealed with rubber septa and 10% (v/v) of the air phase was replaced with acetylene (Burris, 1972). The cultures were incubated for an additional 24 h and the amount of ethylene produced from the acetylene was measured using a Clarus 600 gas chromatograph (Perkin Elmer) with a Col-Elite-Alumina column (50 m×0.53 mm ID×10 um) and a flame ionization detector connected to a chromatography data computer system. Cells were collected and lysed with NaOH for 12 h at room temperature. The protein concentration was determined by the Bradford assay (Bradford, 1976). *Paenibacillus riograndensis* $SBR5^T$ (EU257201) was used as a positive control. Measurements were carried out with at least three biological replicates.

The production of indolic compounds in tryptophan-enriched King-B medium was measured by colorimetric and spectrophotometric assays (Glickmann and Dessaux, 1995). The production of siderophores was evaluated by chrome azurol S assay (Schwyn and Neilands, 1987). Phosphate solubilization ability was assessed on medium amended with 10% K_2HPO_4 and $CaCl_2$, according to Sylvester-Bradley et al. (1982). The activity of the ACC deaminase was estimated by the bacterial ability to grow in ACC supplied minimal medium (Penrose and Glick, 2003).

Plant assay

Sunflower seeds (cv. BRS321) were surface-disinfected by washing in 70% ethanol for 1 min followed by a wash in sodium hypochlorite solution (4%, v/v) with three drops of Tween 80 for 5 min and six rinses in sterilized distilled water. The seeds

were pre-germinated under axenic conditions in a Petri dish kept at 22°C in the dark on two sheets of filter paper moistened periodically with sterile water. The pre-germinated seeds were sown at 2 cm below the surface in cups (15 cm × 20 cm) containing vermiculite sterilized by autoclaving twice (1 h for both sterilization and drying), with four replicates per treatment.

To reproduce the alterations in the N content in the growing substrates of the plantlets, Hoagland's half-strength solution (Adesemoye et al., 2009) was utilized, supplemented or not with different doses of N as $Ca(NO_3)_2 \cdot 4H_2O$. The treatments with bacteria (the five most efficient isolates with N_2-fixing ability) received a 1 ml inoculation of bacterial suspension obtained as follow: pure cultures of each isolate was grew in King-B medium until reach a $OD_{600\ nm}$ of 0.50; bacterial cells were then washed with a fresh N-free nutritive solution after centrifugation at 6000 *g* / 15 min, and ressuspended in a volume of N-free solution adequate to reach the initial OD. The plants were maintained in a growth chamber with a photoperiod of 16 h of light (28°C) and 8 h of darkness (20°C).

To evaluate the interactions between sunflowers and each one of the five selected diazotrophic bacteria as well as the increment on plant fitness in the presence of bacteria, the seeds were submitted to 0 and 3.75 mM of N in different treatments. Twenty-four days post sowing (dps) shoots were separated from roots, measured and kept in bag filter paper at 65°C for five days. Following this, the shoot dry weight and NPK contents were obtained (Tedesco et al., 1995). The nutrient content was determined by the ratio between nutrient (%) and dry weight (mg nutrient/total dry weight). The statistical analyses were performed by a one-way ANOVA and Tukey test by Assistat 7.6 Beta Software (Silva, 1996).

RESULTS

The framework, based on functional diversity, 16S rRNA gene sequences similarity, phylogenetic relationships, and *in silico* RFLP-based fingerprinting, was employed to assess and more accurately classify diazotrophic bacilli, focusing on 101 bacterial strains isolated from sunflower rhizospheric soils. Heat-treating ensured endospore-forming bacteria were obtained because cells were resistant to a long storage time and pasteurization treatments. All bacilli isolates were evaluated for their ability to fix nitrogen, as determined by the acetylene reduction assay (ARA). To estimate the

taxonomic positioning of the diazotrophic bacilli communities, the isolates were compared to the closest type-species of *Bacillales*, which were represented by 20 species of each *Bacillus* and *Paenibacillus* genera.

Classification based on 16S rDNA sequences

The 101 isolates were classified according to the identity of their 16S rRNA gene sequences and were grouped into 31 representative strains, totalizing 59 strains of *Paenibacillus* (named P isolates) and 42 of *Bacillus* (B isolates). The 31 16S rDNA representative sequences were compared to sequences from the Ez-Taxon-e, GenBank, and RDP databases and the outcomes were variables to 15 of them, even at the genus level (for example, P22V strain) (Table 2). Moreover, some related species were found only in the GenBank (*B. arbutinivorans*) or Ez-Taxon-e (*P. typhae*) databases. Differences smaller than 0.5% of similarity between the first and second best hits were also found in most of the isolates (data not shown).

The alignment between the 31 16S rDNA representative sequences and the 40 16S rDNA of the closest type-species showed only one exception to the 5' TGCAAGTCGAGC 3' signature relative to the *P. riograndensis* (5' TGCTGTTCGAGC 3', underlined nucleotides) sequence. The end, 5' GAAGTCGTAACA 3', matched with all of the sequences with four exceptions, which were incomplete sequences of type-species and included *P. glycanilyticus*, *P. jilunlii*, *P. odorifer*, and *P. taichungensis*. The alignment analysis showed that the first 180 nucleotides (on average) were the most variable, which is consistent with previously published data (Giffel et al., 1997; Goto et al., 2000; Chen and Tsen, 2002). The average length of all 71 sequences was approximately 1,455 nucleotides, which includes almost the complete sequence of the 16S rRNA gene. The 16S rDNA sequence of outgroup *A. brasilense* Sp245 between 5' TGCAAGTCGA 3' and 5' GAAGTCGTAACA 3' was 1,390 nucleotides in length.

Phylogenetic assay

The topologies of the three Maximum Likelihood (ML) trees and the Maximum Parsimony (MP) tree were similar, with adequate separation between isolates and type-species along the two main genera. Two large clusters separating *Bacillus* and *Paenibacillus* were well defined in the ML trees, which were different only with respect

to the gaps. The topologies of ML ‘partial deletion’ (Figure S1) and ML ‘complete deletion’ (Figure S2) trees were very similar, and only a few differences were observed in the bootstrap values. The ML ‘use all sites’ tree (Figure 1) was chosen as the standard tree because the gaps were included in the analysis and the loss of information regarding the sites that were deleted could be minimized.

Although the distribution of *Bacillus* spp. was similar to the three ML trees, two representative isolates grouped differently among them. The B53S isolate grouped either with *B. drentensis* (ML ‘use all sites’) or *B. senegalensis* (the others two ML trees); B3V was expected to near the *B. cereus*, but it grouped closer to the *B. pseudomycoides* in the trees with deletion of gaps. In general, the ML ‘use all sites’ tree grouped better the members of the *B. cereus* group (Figure 1). However, the *Paenibacillus* spp. showed a higher variation of topology, mainly regarding isolates related to the *P. sonchi*, *P. riograndensis*, *P. graminis*, and *P. jilunlli* type-species, which were not well resolved. The MP tree (Figure S3) did not show the two large groups that were expected to form distinguishing both genera. However, the distribution of sub-clusters was similar to the ML trees and there was a consensus in several branches.

In silico RFLP and multivariate methods

Concerning the restriction profiles and their discriminatory abilities, 16 Type II-REs were able to discriminate the sequences for most type-species of *Bacillus* and *Paenibacillus*. A smaller number of REs were able to separate the sequences showing a high percentage of identity. The HpyCH4V RE was the most efficient at discriminating sequences of species from *Bacillus* and differentiating 98.95% of all combinations between the two species. HpyCH4V was only not able to separate *B. anthracis* from *B. cereus* and *B. thuringiensis* from *B. mycoides*, but it was the one to distinguish *B. safensis* from *B. pumilus* (Figure 2A). AluI, MseI, TaqI, and MspI were additional REs with greater efficiency for *Bacillus* species, showing a resolving power (RP) of 97.90%, 95.80%, 94.20%, and 93.70%, respectively. AluI and HpyCH4V were also useful to differentiate *B. aryabhattai* from *B. megaterium* and the three type-species, *B. drentensis*, *B. senegalensis*, and *B. niacini*. MseI was able to discriminate species with almost identical sequences, such as sequences from *B. cereus*, *B. anthracis*, and *B.*

thuringiensis in the *B. cereus* group. The REs with the lowest RP (89.50%) for the *Bacillus* species were HaeIII, Hpy188I, Sau96I, and TseI.

Regarding the *Paenibacillus* species, Hpy188I was the most efficient enzyme, discriminating 100% of the type-species, whereas Sau96I and TaqI were less effective for this discrimination (both RP of 95.80%) (Figure 2B).Type-species that had very similar 16S rRNA sequences, such as *P. pabuli* and *P. xylanilyticus*, were differentiated by the AluI and Hpy188I REs. For *P. amylolyticus* and *P. xylanexedens*, DdeI and Hpy188I Res were useful. *P. illinoisensis* and *P. xylanilyticus* were differentiated by RsaI, along with MspI and Hpy188I. RsaI was the second best RE which resolved the *Paenibacillus* sequence and displayed 98.40% of RP for all sequences. In addition to Hpy188I and RsaI, AluI is among the more efficient REs to *Paenibacillus* sequences (RP of 97.90%). DdeI, MspI, HpyCH4V, and TspEI showed RP of 97.40%, and HaeIII, MaeIII, NlaIII, TauI, and TseI, displayed a RP of 96.80% each one.

For the 16 REs analyzed, MaeIII and MspI had the highest average number of cleavage sites, 10.93 and 9.14, respectively, followed by DdeI and TauI, when considering all of the sequences of the isolate and type-species (Table 3). The REs Hpy188I, TseI, NdeII, and TaqI exhibited less than five cleavage sites each. The REs HpyCH4V, AluI, MseI, Sau96I, RsaI, HaeIII, TspEI, and NlaIII showed an intermediate number of cleavage sites, approximately 5-6. The *in silico* restriction analysis of the 16S rDNA sequences of all 31 representative isolates and the 40 type-species was performed, and a virtual plot of the DNA fragments was deduced from binary matrices based on the length created by the RFLP approach (Figure 3). The REs MaeIII and MspI, which presented a higher number of cleavage sites and supplied a greater amount of different length fragments, did not provide a clear discrimination among all strains. The Hpy188I and HpyCH4V profiles were more informative to differentiate the *Bacillus* and *Paenibacillus* strains, respectively.

In addition to the 16 Type II REs employed on the *in silico* RFLP, other enzymes were useful for the identification of some of the species analyzed. SpeI (A↓CTAGT) cleaved once in the 16S rDNA sequences of *B. acidiceler* and *B. luciferensis*, and correlated isolates (B18E and B34V). StuI (AGG↓CCT) also cleaved at one site in the 16S rDNA sequences of *B. acidiceler* group as well as those from *B. drentensis*, *B. niacini*, *B. senegalensis*, *B. arbutinivorans*, and the B10S isolate, as well as *P. durus*. The 16S rDNA sequence from the B53S isolate presented two sites for StuI cleavage.

NheI (G↓CTAGC) and BmtI (GCTAG↓C) REs cleaved once in the 16s rDNA sequences from *B. aryabhattai* and from the isolate B40E. SacI (GAGCT↓C) and EcoICRI (GAG↓CTC) REs cleaved once the sequences from *P. catalpae* and *P. glycanilyticus*. The SspI (AAT↓ATT) RE cleaved the sequences of *B. weihenstephanensis, B. mycoides,* and the correlated B38V isolate. The only 16S rDNA sequence cleaved by PasI (CC↓CWGGG) was from *P. sonchi*.

The cluster analysis generated from the binary-matrix of the digestion profiles of the 16 Type II REs employed on the *in silico* RFLPs showed two large groups separating *Paenibacillus* and *Bacillus*. The major clusters, Cluster P and Cluster B, were about 10% similar in the UPGMA dendrogram (Figure 4) with bootstrap value of 98. All sub-clusters at 47% of similarity in the UPGMA dendrogram for both species of *Paenibacillus* (I-P, II-P, III-P, IV-P) and *Bacillus* (I-B, II-B, III-B, IV-B, V-B, VI-B) were distributed similarly to ML and MP methods, with the exception of II-P sub-cluster. In fact, the UPGMA method has ability to identify potential outliers (Blackwood et al., 2003). The lack of a more consistent grouping for the type-species of *P. odorifer* most likely occurred due to missing 78 nucleotides (in relationship to the *P. wynnii* sequence, which was the nearest sequence in the alignment) at the 3' end of the 16S rDNA sequence (data not shown). This is an important question for *in silico* RFLP analysis, because if one sequence has missing nucleotides among the two signatures dictated, this sequence can be grouped with bias. Moreover, the outgroup *A. brasilense* Sp245 was well clustered in the UPGMA dendrogram and ML method.

According to the UPGMA analysis, the *Bacillus* and *Paenibacillus* species were distributed into two major clusters at an approximate similarity of 22% each (Figure 4). Sub-clusters I-P and IV-P presented lower indices of similarity, 47 and 35% (bootstrap of 74 and 99), respectively. Sub-clusters II-P and III-P exhibited a similarity about 55% (bootstrap of 78 and 99), respectively. The *Bacillus* species of sub-clusters I-B, II-B, and III-B were distributed with a 42% level of similarity (bootstrap of 100). Sub-cluster I-B corresponded to the *B. aryabhattai*, *B. megaterium*, and *B. flexus* species at a 63% similarity (bootstrap of 99); sub-cluster II-B was composed of the *B. drentensis*, *B. senegalensis*, *B. niacini*, and *B. arbutinivorans* species at a 67% similarity (bootstrap of 100); and sub-cluster III-B was composed of the *B. circulans* and *B. nealsonii* species at a 54% similarity (bootstrap of 97). The sub-clusters IV-B, V-B, and VI-B were also separated into three major groups at an approximately 30% similarity, with sub-cluster

IV-B comprising the *B. acidiceler* and *B. luciferensis* species at an approximately 68% similarity (bootstrap of 100); sub-cluster V-B comprised the *B. cereus* group at a 76% similarity (bootstrap of 100); and sub-cluster VI-B contained the *B. subtilis*, *B. safensis*, and *B. pumilus* species at an approximately 60% similarity (bootstrap of 100).

Regarding the REs' resolving power (RP) based on the 16S rDNA sequences from the different type-species, only three REs were sufficient to separate all type-species by automated restriction profile, including HpyCH4V and MseI REs for *Bacillus* and the Hpy188I RE for the *Paenibacillus* species (Figure 2). Accordingly, a dendrogram containing representative isolates and type-species was created from restriction profiles obtained by *in silico* DNA cleavage, using the Hpy188I, HpyCH4V, and MseI REs (Figure S4). This dendrogram was less robust than that obtained with all 16 REs (Figure 4), but most *Bacillus* species were similarly grouped, with the exception of the B53S isolate, which grouped next to *B. senegalensis* (similarity about 80%; bootstrap of 87), and the B29S isolate, which grouped close to *B. nealsonii* at about 50% similarity (bootstrap of 61). Concerning *Paenibacillus* species, many isolates were poorly grouped close to unexpected species, such as P9S with *P. xylanilyticus* (bootstrap of 89), P7S with *P. illinoisensis* (bootstrap of 52), and P15E with *P. sonchi* (bootstrap of 28). A dendrogram based on the profiles of the five highest RP values for the *Bacillus* and *Paenibacillus* species (HpyCH4V, MseI, and TaqI for *Bacillus* species; DdeI, Hpy188I, and RsaI for *Paenibacillus* species; and AluI and MspI for both) (Figure S5) was also evaluated and showed a better resolution than the dendrogram obtained by using only the best three REs. The results confirmed the need for a large number of REs to obtain a more accurate species classification. Furthermore, the outgroup *A. brasilense* Sp245 was not well clustered in the UPGMA dendrograms with less than 16 REs. The median number of potential restriction fragments assessed by using the 16 REs was 110, whereas with 8 REs the median was 55, and with 3 REs the median was 20.

The individual profiles of strains were also investigated by principal component analysis (PCA), and a pattern very similar to that obtained by the UPGMA method was observed with a pronounced differentiation between individuals from different genera. This pattern was summarized as an approximately 40% variation of the first two principal components (PC1 and PC2) (Figure 5A). Moreover, using PCA, the *Bacillus* species (Figure 5A, right side) were positioned closer to each other and showed a more concentrated distribution compared with the *Paenibacillus* species (Figure 5A, left

side). However, when only the *Bacillus* species was plotted (Figure 5B), the PC1 summarized 37% of the total variance, and a clear distinction was observed between sub-clusters IV-B, V-B, and VI-B (as they were classified by the UPGMA). In relationship to sub-clusters I-B, II-B and III-B, the members were positioned in a scattered manner, mainly the members of sub-cluster III-B. For the species of *Paenibacillus* the sub-clusters were clearly grouped and PC 1 and 2 explained 33and 11% of the total variance of each dataset, respectively (Figure 5C). Due to the multidimensional reduction of data, isolates that grouped in sub-cluster I-P in the UPGMA analysis (P3E, P14V, P46E, P32E, and P26E) were grouped apart from the closest type-species, providing evidence for their genetic difference. PCA analysis also separated the P28E isolate from *P. catalpae*, *P. glycanilyticus*, and the P6E isolate, which were grouped together in the sub-cluster IV-P by the UPGMA analysis. Members of sub-cluster III-P were positioned very close by PCA, which was corroborated by their high nucleotide similarity.

Taxonomic analysis

Comparisons between *in silico* RFLP (assessed by UPGMA and PCA) and character-based methods (ML and MP) were more distinct for sub-cluster II-P, which discriminated P5E and P22V isolates. The bootstrap value was highest (98) in the UPGMA clustering that grouped the P5E with the P30E isolate and *P. typhae* at an 80% similarity, than for ML (53) and MP (72) methods. The P5E isolate that was related to *P. borealis* in the three databases (Table 2) was also related to *P. borealis* by ML and MP methods (Figure 1 and Figure S3), but the lack of 10 nucleotides positioned it nearest to *P. typhae* in the multivariate methods (sub-cluster II-P, Figure 4 and Figure 5C). The *P. typhae* sequence also contained the10-nucleotide deletion (as a "missing data").The P22V isolate, which was positioned nearest to *P. borealis* by UPGMA method (bootstrap of 54) and PCA, showed even higher discrepancies among the similarity results (Table 2) and it was grouped next to *P. wynnii* in ML tree (bootstrap of 31), but nearest to *P. odorifer* in MP tree (bootstrap of 59).

All of the members of sub-cluster I-P were not well classified either by sequence-based (ML and MP) or multivariate methods (UPGMA and PCA), with the exception of the P43V isolate, which was positioned closer to *P. graminis* by using UPGMA method (bootstrap 100) and PCA, as well as through GenBank identification (Table 2). In this

regard, *in silico* RFLP was able to produce a more accurate classification of the strains in sub-cluster I-P. The representative isolates P3E, P15E, P26E, P32E, P46E, and P14V, showed an intermediary percentage of identity (i.e. < 99%), with *P. sonchi* appearing in the three databases (Table 2) and demonstrating an inconsistent grouping in the phylogenetic trees. The multivariate methods resulted in a cluster of these isolates close to *P. riograndensis*, mainly in the UPGMA. The type-species of *P. riograndensis* was the second best hit for the majority of them when compared to the databases (data not showed). In the cluster analyses of each RE (data not shown), the P3E, P26E, P32E, P46E, and P14V isolates tended to cluster preferentially with *P. riograndensis*, according to the restriction profile of nine REs, with approximately 50 to 90% similarity, whereas for *P. jilunlii* the values were approximately 35 to 75% similar for four REs. P15E was similar to *P. riograndensis*, according to the patterns MaeIII (90%) and TaqI (60%), and more similar to *P. sonchi* with nine REs varying between 50 to 90% similarity (Jaccard coefficient). Moreover, the PCA results clearly showed a scattered group with the P3E, P26E, P32E, P46E, and P14V isolates; all data related to those isolates suggested that they could possibly be a new species of *Paenibacillus*. Finally, the representative P17E isolate, which was positioned close to *P. jilunlii* by UPGMA method, showed a similarity index of approximately 97.60% with *P. jilunlii* in the ExTaxon-e database (second hit), and for this reason it could not be classified at the species level.

The representative isolates that were grouped similarly through the character-based methods, B10S, B3V, B45V, P16E, P30E, and P9S, were also grouped nearest to the same type-species using distance-based method (Figure 4, names in pink). Indeed, the representative isolates B10S, B45V, and P16E were not differentiated from *B. arbutinivorans*, *B. thuringiensis*, and *P. amylolyticus*, respectively, after any of the restriction patterns obtained *in silico*. The P9S isolate was distinguished from *P. illinoisensis* only in the Hpy188I profile, as well as B3V and *B. cereus* in the DdeI profile. The isolate P30E was distinct of the *P. typhae* according to AluI, HpyCH4V, and TaqI patterns (Figure 3). Another similar example occurred for the isolates B18E and B34V, which were grouped nearest to *B. acidiceler* in the UPGMA and ML methods (Figure 4, names in orange). *B. acidiceler* was separated from B18E by the RsaI profile, and from B34V by RsaI and MseI profiles (Figure 3). The representative

isolates B18E, B34V, P16E, and P9S, were classified more accurately by UPGMA, which had higher values bootstrap than other methods (Table 4).

The doubtful identification of isolates P5E, P17E, and P22V (cited above), in addition to B53S, P8E, P28E, P49E, P7S, P14S, and P48S,could not be assigned at a species level by any of the analysis methods due to the presence of clusters with low bootstrap values or groupings that were not correlated to a single type-species. Representative isolates P49E, P48S, and B53S, for example, were similar to more than one highly related type-species in phylogenetic assay, 16S rDNA sequences similarity, and multivariate methods. However, the representative isolates P7S and P14S that were classified by three databases as *P. pabuli* and grouped next to *P. pabuli* or *P. pabuli* group, were identified at species level. The isolate P6E was well resolved by multivariate methods, showing a level of 87% similarity in the UPGMA analysis with *P. catalpae* (bootstrap of 100).The isolates B2S and B29S, in addition to similarity indices on databases, were grouped and better classified on UPGMA and character-based methods, respectively (Table 4). B24V was better grouped on UPGMA and MP methods, as identification in the EzTaxon-e (100% identity) and RDP (0.998 S_ab). The same occurred with B40E and B38V isolates, which showed high levels of identity with *B. aryabhattai* (sub-cluster I-B; bootstrap of 100) and *B. mycoides* (sub-cluster V-B; bootstrap of 98), respectively (Figure 4, names in green). The identification of B38V was also confirmed by its colony morphology, which displayed a rhizoidal pattern (Figure 6) characteristic of the *B mycoides* species. The final classification of the 31 representative isolates is shown in Table 4. Isolates belonging to *B. acidiceler*, *B. aryabhattai*, *B. mycoides*, *B. safensis*, *P. amylolyticus*, *P. catalpae*, and *P. graminis* were well resolved by the UPGMA method because they showed high bootstrap values in the dendrogram (Figure 4). Isolates related to *B. arbutinivorans*, *B. thuringiensis*, *P. illinoisensis*, and *P. typhae* showed groupings with high bootstrap values in the UPGMA, ML, and MP methods (Table 4).

The diversity indices (*H'*) were inferred based on the dendrograms generated by the *in silico* RFLP using the 16 REs and the representative isolates from each sample site. No correlation was found between *H'* and soil parameters by PCA (data not showed). The highest genetic diversity was found for the bacterial community from Encruzilhada do Sul (*H'* = 2.26), followed by that from Vacaria (*H'* = 1.83), and the lowest index was from São Gabriel (*H'* = 1.32).

***In vitro* acetylene reduction assay (ARA)**

The ability to N_2-fix was analyzed for all isolates, and a large variation of nitrogenase activity was observed (Table 5). Among the identified species, the most abundant in association with sunflower was *P. pabuli* and *B. arbutinivorans*, which presented few N_2-fixing isolates. Approximately 37% of all bacilli associated with sunflowers were able to produce between low (0.5 to 3 µmol) and high (500 to 1,550 µmols) ethylene per mg of protein, with the majority of the isolates presenting low values of ethylene production. However, the P26E isolate (and its related strain) was able to produce 1,550 µmol of ethylene per mg of protein, the highest value observed. Isolates related to the *B. thuringiensis* species and those belonging to I-P sub-cluster also showed a large variation of nitrogenase activity. The diazotrophic were lightly most frequent between the *Paenibacillus* species (23.30%) compared with the *Bacillus* species (29.50%). Indolic compound (IC) production was a common trait among isolates, and bacteria closest to *B. arbutinivorans* were the best IC producers (Table 5). Of all isolates, 22 were not able to produce IC, which constituted 19 strains of *Paenibacillus* and three strains of *Bacillus*. Siderophore production was observed only in two isolates of *B. aryabhattai* (B23S) and the *B. thuringiensis* (B47S) species. Phosphate solubilization and ACC deaminase activities were not detected in any isolate.

Plant assay

The five best N_2-fixers amongst the isolates [B38V (163.20 µmol C_2H_4mg protein^{-1}); B47V (103.70); P3E (89.20); P26E (1,550.90); and P27E (699.50)] were selected to inoculate sunflower seeds to study the interactions with the host plant. B47V and P3E also are producers of indolic compounds (33.40 and 34.20 µg mL^{-1}, respectively), and B47V is the only siderophore producer. Plants inoculated with the B38V isolate (*B. mycoides*), in the presence of 3.75 mM of N (intermediary dose),demonstrated the highest values for dry matter and N content, although these values were not statistically significant at $p>0.05$ (Table 6). These results suggest that isolate B38V in 3.75 mM of N contributed to plant fitness, interacting positively with sunflower plants.

DISCUSSION

Nitrogen fixing bacteria [such as *Azospirillum*, *Azotobacter*, *Gluconacetobacter*, *Herbaspirillum*, and *Rhizobium*] have been studied for a long time and different free-living and symbiotic diazotrophic bacteria are recognized by the positive interactions that benefit both the microorganism and host plant (Olivares et al., 2013; Santi et al., 2013). In a broad context, bacterial strains possessing N_2-fixing abilities occur widely amongst all classes and have highly polyphyletic distributions (Dobrindt et al., 2004; Kechris et al., 2006). Among the known diazotrophic bacilli, many species belong to the *Paenibacillus* genus, such as *P. polymyxa* (Grau and Wilson, 1962), *P. borealis* (Elo et al., 2001), *P. brasilensis* (von der Weid et al., 2002), *P. graminis* (Berge et al., 2002), *P. odorifer* (Berge et al., 2002), *P. massiliensis* (Roux and Raoult, 2004), *P. wynnii* (Rodríguez-Díaz et al., 2005), *P. forsythiae* (Ma and Chen, 2008), *P. sonchi* (Hong et al., 2009), and *P. riograndensis* (Beneduzi et al., 2010). Several strains of *Bacillus* also have nitrogenase activity, such as *B. cereus*, *B. licheniformis*, *B. megaterium*, *B. pumilus*, *B. subtilis*, and *B. rhizosphaerae* (Xie et al., 1998; Ding et al., 2005; Sorokin et al., 2008; Rau et al., 2009; Madhaiyan et al., 2011). However, the number of diazotrophic species is scarce (Achouak et al., 1999; McSpadden Gardener, 2004).

The analysis of N_2-fixing ability among isolated bacteria is related to the adequate identification of the strains. In fact, following the isolation of bacteria of any sample, it is generally needed reliable classification of species. The more recent versions of 'Bergey's Manual® of Systematic Bacteriology' include a "road map" based largely on the analysis of the nucleotide sequences of the ribosomal small-subunit RNA (Bergey, 2001; Garrity et al., 2005; Ludwig et al., 2009). Approximately 1,500 bp of the 16S rRNA gene facilitate sequencing and strain identification by nucleotide similarity scores. The standard measure for bacteria classification is an identity score of approximately 97%, which is used as a threshold for the delineation of prokaryotic species (Stackebrandt and Goebel, 1994). Higher levels, 98.7-99%, have been more recently proposed (Stackebrandt and Ebers, 2006). However, taxonomic multiple approaches [such as nucleotide sequence identity, phylogenetic relationships, and phylotyping] have been employed by many investigators that follow a robust microbial identification system (Jang et al., 2002; Wu et al., 2006; Bavykin et al., 2004; Alcaraz et al., 2010; Fraher et al., 2012; Loza et al., 2013). Phylogenetic assays are employed in bacterial identification by the use of sequences with wide distribution, functional

uniformity, no or rare horizontal genetic transfer (HGT), independent evolving positions (variable regions), universal primers, and adequate length, similar to that of the 16S rDNA gene (Woese, 1987; Jonasson et al., 2002; Wang and Qian, 2009; Schloss et al., 2011).

The genetic markers assessed by phylotyping can be used for diversity analysis (Smalla et al., 2007; Chandna et al., 2012) and the classification of isolates (Guillaume-Gentil et al., 2002; Heyrman et al., 2004; Wu et al., 2006) and can contribute to the identification of species (Rademaker et al., 2000; Jensen et al., 2005). Restriction-based molecular markers, such as restriction fragment length polymorphism (RFLP) and terminal-RFLP (T-RFLP), share a high fidelity for being recognized and cleaved by restriction endonucleases (REs) at differing sites, generating profile patterns that are able to differentiate individuals by variations in homologous DNA sequences. Many bacilli strains have been classified by a combination of several traditional phenotypic approaches, besides the fundamental research step based on their identification by 16S rDNA sequencing (Garbeva et al., 2003; Connor et al., 2010). The differentiation of the *B. cereus* group based on PCR-RFLP of the *gyrB* gene (362 bp) digested with Sau3A1, resulted in the discrimination of *B. mycoides* but not the distinction between *B. cereus* and *B. thuringiensis* (Jensen et al., 2005). *Bacillus* strains from environmental sources were distinguished by the digestion of an approximately 1,000 bp amplicon of 16S rDNA with AluI and TaqI (Wu et al., 2006). Moreover, uncultured phytoplasmas (bacteria lacking cells walls, which cause numerous plant diseases) have been identified by the analysis of different restriction profiles by using *in silico* RFLP analysis (Wei et al., 2007).

Phylotyping is largely used to screen a large number of strains in clinical and environmental studies, but the pattern of fragments can be amended and the reproducibility lost if they are not generated under the same conditions (Osborn, 2005). Moreover, the resolution of agarose gels can be affected and bands underestimated, which is important for the accuracy of these methods. However, higher reproducibility can be achieved by employing *in silico* RE digestion because fragment patterns will not change and comparisons can be made independently when the data were generated. Moreover, the isolates can be compared to various type-species without laborious laboratory methodologies, providing information based on well-characterized species. In this work, *in silico* RFLP and phylogenetic assays of 16S rRNA gene sequences were

employed to discriminate strains obtained from environmental samples, with a clear distinction between the *Paenibacillus* and *Bacillus* communities. Cluster analyses of restriction profiles allowed to differentiate isolates and closely related strains, such as the B38V isolate and *B. mycoides*, which were distinguished only by the DdeI profile. Rhizoidal colony morphology (Figure 6), similarity indices, and *in silico* RFLP analysis grouped B38V correctly with *B. mycoides*.

It is important to note that the use of different methodologies can help to accurately identify bacteria because even the standard methods, such as nucleotide identity and phylogenetic relationships, might contain bias (Holder and Lewis, 2003; Simmons et al., 2008; Dwivedi and Gadagkar, 2009; Schloss and Westcott, 2011; Warnow, 2012). The quality and length of the analyzed sequence is an important part of adequate identification because small fragments tend to provide insufficient information depending on organism proximity (Schloss, 2010). In addition, the large amount of sequences deposited in databases do not assure sequence quality, and sequencing errors may be indefinitely propagated if an incorrect identification is provided (Nilsson et al., 2006; Huse et al., 2010; Kunin et al., 2010). Moreover, the variability of the results in different databases is clear; for example, in this study, the P22V isolate showed low similarity with two type-species, *P. odorifer* and *P. borealis*, in the EzTaxon-e and RDP databases, but *P. odorifer* or *P. borealis* was the first or second best hit depending on the database examined (Table 2). Finally, according to Osborn (2005), all methods of phylogenetic inference from aligned sequences are imperfect, especially when dealing with lineages that evolve at different rates. Recent studies have addressed the inconsistence of alignments of variable sequence regions, such as those related to indels (Schloss and Westcott, 2011; Warnow, 2012). Because this type of gap is common in the variable regions of the 16S rDNA gene, indels are valuable to bacterial classifications because they constitute an important part of the difference between the 16S rDNA sequences.

The methods based on the 16S rRNA gene analysis provide essential information concerning taxonomy, but additional assays are needed to obtain information about the ecology and functional diversity of biochemical communities (Torsvik and Øvreås, 2002). In this work, bacilli with potential N_2-fixing were isolated from sunflower rhizosphere, and approximately 60% of them were classified as belonging to the *Paenibacillus* genus, whereas the remaining 40% belonged to the *Bacillus* genus. Isolate

B38V, which was identified as a *B. mycoides* species, presented a relatively high nitrogenase activity (163.20 µmol C_2H_4 mg protein^{-1}). In the *in vivo* assay conducted under gnotobiotic conditions, this strain presented satisfactory results for shoot dry matter and N content at 24 days post sowing, although the results were not statistically different. Strains of bacilli have previously been isolated from many agricultural soils (Probanza et al., 1996; Bargabus et al., 2002; Ghosh et al., 2003; Bargabus et al., 2004; Beneduzi et al., 2008a; Costa et al., 2012; Ratón et al., 2012). In a study with bacilli isolation of rhizosphere of wheat, *Paenibacillus* was the most abundant Gram-positive group, and the strains were identified by *nifH* PCR-RFLP and 16S rRNA gene sequence comparisons (Beneduzi et al., 2008b). *B. benzoevorans*, *B. coagulans*, *B. cereus*, *B. lentus*, *B. megaterium*, *B. mycoides*, *B. niacini*, *B. pumilus*, and *B. thuringiensis* were the most dominant among 128 isolates of grassland, and were identified by the use of denaturing gradient gel electrophoresis (DGGE) using 16S rDNA gene amplicons (Garbeva et al., 2003). *B. subtilis* and *B. amyloliquefaciens* have been observed in many studies related to plant growth promoting bacteria (Kokalis-Burelleet al., 2006; Romero et al., 2007; Idris et al., 2004; Cuartas, 2010).

The ability to produce indolic compounds is commonly detected among bacteria isolated from soils or from plant rhizospheres, whereas phosphate solubilization is a less pervasive characteristic (Beneduzi et al., 2008a; 2008b; da Mota et al., 2008; Cuartas, 2010). Indeed, indolic compound production is widespread among plant-isolated bacteria (Upadhyay et al., 2009; Yadav et al., 2011;Kumar et al., 2012). Another characteristic that is less frequent among bacilli is the presence of ACC deaminase, although some strains of *B. circulans*, *B. firmus*, and *B. globisporus* are able to utilize the compound 1-aminocyclopropane-1-carboxylic acid (ACC) as a sole nitrogen source and stimulate the development in canola (*Brassica campestris*; Ghosh et al., 2003). In our study, the production of indolic compounds was present in 80% of the isolates, and the *B. arbutinivorans* strains were the highest IC producers (Table 5).

Bacilli are known by their abilities of biocontrol against phytopathogens, which can occur through antibiosis, production of siderophores, as well as by processes of induced systemic resistance (ISR), and insecticidal protein production (Choudhary and Johri, 2009; Lugtenberg and Kamilova, 2009; Allen et al., 2010). Siderophores, such as Fe^{3+} chelators, can be able to inhibit the proliferation of phytopathogens by decreasing nutrient availability (Crowley, 2006). Whereas the production of antibiotics and

siderophores are prone to act more locally, the elicitors of ISR can benefit plants by triggering a response in distant tissues (Kloepper et al., 2004). *Bacillus* species are known as potential elicitors of ISR, and examples include *B. amyloliquefaciens*, *B. cereus*, *B. pumilus*, *B. mycoides*, *B. pasteurii*, *B. sphaericus*, and *B. subtilis* (Bargabus et al., 2002; Kloepper et al., 2004; Choudhary and Johri, 2009). In our screening, only two isolates, B23S, which was identified as a strain of *B. aryabhattai*, and B47V, which was identified as a strain of *B. thuringiensis*, were siderophore producers.

CONCLUSION

In the context of multivariate approaches concerning the information related to the 16S rRNA gene sequence, all data regarding the percentage of sequence identity, phylogenetic relationships, and phylotyping can be used to obtain a consensus for bacterial classification. This study employed *in silico* RFLP multivariate methods as tools for an improved taxonomic positioning of diazotrophic bacilli associated with sunflower rhizosphere, which is an oilseed plant of high economic and agricultural importance. The analysis of restriction sites and enzymes able to differentiate species from *Bacillus* and *Paenibacillus* genera can also contribute to future studies where the use of phylotyping is useful. These *in silico* analyses allowed for a more accurate classification of isolates and avoided the technical problem of reproducibility. The results presented in this study identified diverse diazotrophic and non-diazotrophic bacilli associated with sunflower roots. Diverse isolates showing positive nitrogenase activity belonged to different species, such as *B. arbutinivorans* (4 isolates), *B. aryabhattai* (1), *B. cereus* (2), *B. circulans* (2), *B. mycoides* (1), *B. safensis* (1), *B. thuringiensis* (2), *P. amylolyticus* (1), *P. catalpae* (1), *P. illinoisensis* (1), *P. pabuli* (4), and *P. typhae* (1). All isolates that belong to the *B. cereus* (4) and *B. thuringiensis* (6) species, as well as some non-identified *Paenibacillus* spp., showed a large variability in nitrogenase activity. Our results indicated that B38V isolate, which was identified as belonging to the *B. mycoides* species, has the potential to promote plant growth under experimental conditions.

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Tables

Table 1: Type-species and accession numbers of *Bacillus* sp. and *Paenibacillus* sp. used in this study. The total length of 16S rRNA nucleotide sequence used in the analysis is indicated (16S rDNA nt length).

Bacillus		**16S rDNA**	***Paenibacillus***		**16S rDNA**
Type-species	**accession nº**	**nt length**	**Type-species**	**accession nº**	**nt length**
B. acidiceler	DQ374637	1454	*P. alvei*	AMBZ01000001	1456
B. anthracis	NR_074453.1	1457	*P. amylolyticus*	D85396	1458
B. arbutinivorans	FJ527633.1	1443	*P. barcinonensis*	AJ716019	1460
B. aryabhattai	EF114313	1457	*P. borealis*	AJ011322	1463
B. cereus	AE016877	1457	*P. catalpa*	HQ657320	1450
B. circulans	AY724690	1453	*P. durus*	AB073195.1	1460
B. drentensis	DQ275176.1	1455	*P. glycanilyticus*	AB042938	1434
B. flexus	AB021185	1458	*P. graminis*	AB428571.1	1464
B. luciferensis	AJ419629	1455	*P. illinoisensis*	FN422001.1	1457
B. megaterium	D16273	1457	*P. jilunliix*	FN422001.1	1449
B. mycoides	ACMU01000002	1457	*P. odorifer*	AJ223990	1384
B. nealsonii	EU656111	1456	*P. pabuli*	AB073191	1457
B. niacini	AB021194	1455	*P. riograndensis*	EU257201	1454
B. pseudomycoides	ACMX01000133	1457	*P. sonchi*	DQ358736	1457
B. pumilus	ABRX01000007	1454	*P. taichungensis*	EU179327	1448
B. safensis	AY030327.1	1454	*P. tundrae*	EU558284	1458
B. senegalensis	AF519468.1	1455	*P. typhae*	JN256679	1453
B. subtilis subsp.subtilis	ABQL01000001	1455	*P. wynnii*	AJ633647	1463
B. thuringiensis	ACNF01000156	1457	*P. xylanexedens*	EU558281	1458
B. weihenstephanensis	Z84578	1456	*P. xylanilyticus*	AY427832	1457

Table 2: Identification of representative (*R*) isolates according to the GenBank (Max Score), EzTaxon-e (Similarity), and RDP (S_ab) databases. For each R isolate the best hit in each database is shown. The *R* isolates are organized according to the UPGMA dendrogram order. The underlined names for some *R* isolates represent the hit more relevant to classification. The clustering on UPGMA dendrogram and phylogenetic trees also account on consensus (see underlined names on Table 4).

	GenBank		**EzTaxon-e**		**RDP**	
***R* isolate**	**Species**	**Max Score**	**Species**	**Similarity**	**Species**	**S_ab**
P15E	*P. sonchi*[1]	2479	*P. sonchi*[1]	97.46	*P. sonchi*[1]	0.915
P3E	*P. sonchi*[1]	2595	*P. sonchi*[1]	98.97	*P. sonchi*[1]	0.945
P14V	*P. sonchi*[1]	2590	*P. sonchi*[1]	98.90	*P. sonchi*[1]	0.940
P46E	*P. sonchi*[1]	2601	*P. sonchi*[1]	99.04	*P. sonchi*[1]	0.949
P32E	*P. sonchi*[1]	2584	*P. sonchi*[1]	98.83	*P. sonchi*[1]	0.937
P26E	*P. sonchi*[1]	2595	*P. sonchi*[1]	98.97	*P. sonchi*[1]	0.946
P43V	*P. graminis* [2]	2593	*P. sonchi*[1]	98.49	*P. sonchi*[1]	0.936
P17E	*P. caespitis* [3]	2632	*P. borealis*[4]	97.74	*P. borealis*[4]	0.909
P22V	*B. circulans*[5]	2630	*P. odorifer*[6]	97.77	*P. borealis*[4]	0.873
P30E	*P. borealis*[7]	2481	*P. typhae*[8]	99.59	*P. borealis*[4]	0.914
P5E	*P. borealis*[7]	2547	*P. borealis*[4]	99.17	*P. borealis*[4]	0.962
P9S	*P. illinoisensis*[9]	2675	*P. illinoisensis*[10]	99.86	*P. illinoisensis*[11]	0.984
P14S	*P. pabuli* [12]	2641	*P. pabuli*[13]	99.52	*P. pabuli* [14]	0.965
P7S	*P. pabuli* [12]	2641	*P. pabuli*[13]	99.52	*P. pabuli* [14]	0.967
P49E	*P. tundrae*[15]	2643	*P. xylanexedens* [16]	99.18	*P. xylanexedens* [16]	0.958
P48S	*P. tundrae*[15]	2654	*P. xylanexedens* [16]	99.45	*P. xylanexedens* [16]	0.969
P16E	*P. amylolyticus*[17]	2656	*P. amylolyticus*[18]	99.72	*P. tundrae*[19]	0.973
P8E	*P. amylolyticus* [20]	2649	*P. xylanexedens* [16]	99.31	*P. xylanexedens* [16]	0.964
P6E	*P. glycanilyticus* [21]	2549	*P. catalpae*[22]	99.59	*P. glycanilyticus*[23]	0.960
P28E	*P. glycanilyticus* [24]	2538	*P. catalpae* [22]	99.10	*P. glycanilyticus*[23]	0.923
B40E	*B. aryabhattai*[25]	2691	*B. aryabhattai*[26]	99.86	*B. megaterium*[27]	0.984
B53S	*B. niacini* [28]	2621	*B. drentensis*[29]	99.15	*B. drentensis*[29]	0.942
B10S	*B. arbutinivorans*[30]	2649	*B. drentensis*[29]	99.58	*B. drentensis*[29]	0.972
B2S	*B. circulans*[31]	2660	*B. circulans*[32]	99.04	*B. circulans* [32]	0.960
B29S	*B. nealsonii*[33]	2682	*B. nealsonii* [34]	99.24	*B. nealsonii* [34]	0.960
B18E	*B. acidiceler*[35]	2667	*B. acidiceler*[36]	99.86	*B. acidiceler*[36]	0.983
B34V	*B. acidiceler*[35]	2667	*B. acidiceler*[36]	99.86	*B. acidiceler*[36]	0.982
B3V	*B. thuringiensis* [37]	2686	*B. cereus*[38]	99.93	*B. cereus*[38]	0.995
B45V	*B. thuringiensis*[39]	2691	*B. thuringiensis*[40]	100	*B. thuringiensis*[41]	1.000
B38V	*B. mycoides*[42]	2686	*B. mycoides*[43]	99.93	*B. mycoides*[44]	0.995
B24V	*B. pumilus* [45]	2686	*B. safensis*[46]	100	*B. safensis*[46]	0.998

Accession numbers: [1]DQ358736.1, [2]AB428571.1, [3]AM745263.1, [4]AJ011322, [5]Y13062.1, [6]AJ223990, [7]AB073364.1, [8]JN256679, [9]FN422001.1, [10]D85397, [11]AB073192, [12]FJ189794.1, [13]AB073191, [14]AB045094, [15]HF545335.1, [16]EU558281, [17]NR_025882.1,[18]D85396,[19]EU558284,[20]AM921628.1,[21]AB681087.1, [22]HQ657320,[23]AB042938, [24]KC355297.1, [25]JQ659928.1, [26]EF114313, [27]D16273, [28]JN993716.1, [29]AJ542506, [30]FJ527633.1, [31]AB680477.1, [32]AY724690,[33]KC329823.1, [34]EU656111,[35]NR_043774.1, [36]DQ374637,[37]CP004069.1, [38]AE016877,[39]CP004123.1, [40]ACNF01000156, [41]D16281, [42]AM747229.1, [43]ACMU01000002, [44]AB021192, [45]JN082265.1, [46]AF234854.

Table 3: The 16 restriction endonucleases (*REs*) used in this work, their recognition sites (*RS*), and their average number of cleavage sites (*CS*), considering all sequences of isolates and type-species, i.e., 71 sequences, and a median length of 1,455 nucleotides.

RE	***RS***	***CS***
AluI	AG↓CT	5.94
DdeI	C↓TNAG	7.32
HaeIII	GG↓CC	5.18
Hpy188I	TCN↓GA	4.37
HpyCH4V	TG↓CA	6.10
MaeIII	↓GTNAC	10.93
MseI	T↓TAA	5.38
MspI	C↓CGG	9.14
NdeII	↓GATC	3.45
NlaIII	CATG↓	5.03
RsaI	GT↓AC	5.25
Sau96I	G↓GNCC	5.55
TaqI	T↓CGA	2.70
TauI	GCSG↓C	7.08
TseI	G↓CWGC	4.13
TspEI	↓AATT	5.10

Table 4: Classification of representative (*R*) isolates according to the UPGMA dendrogram, Maximum Likelihood, and Maximum Parsimony trees. For each *R* isolate is presented the type-species more closely grouped, or the group containing more than one type-specie. The *R* isolates are organized according to the UPGMA dendrogram order. The underlined names for some *R* isolates represent the grouping more relevant (highest bootstrap) to classification.

		UPGMA dendrogram		**Maximum Likelihood tree**		**Maximum Parsimony tree**	
***R* isolate**	**Consensus**	**Closest type-species[a]**	**B[b]**	**Closest type-species[a]**	**B[b]**	**Closest type-species[a]**	**B[b]**
P15E	*Paenibacillus* sp.	*P. riograndensis*	40	*P. sonchi* group	70	*P. sonchi* group	26
P3E	*Paenibacillus* sp.	*P. riograndensis*	40	*P. sonchi* group	82	*P. sonchi* group	18
P14V	*Paenibacillus* sp.	*P. riograndensis*	40	*P. sonchi* group	82	*P. sonchi* group	18
P46E	*Paenibacillus* sp.	*P. riograndensis*	40	*P. sonchi* group	82	*P. sonchi* group	18
P32E	*Paenibacillus* sp.	*P. riograndensis*	40	*P. sonchi* group	82	*P. sonchi* group	18
P26E	*Paenibacillus* sp.	*P. riograndensis*	40	*P. sonchi* group	82	*P. sonchi* group	18
P43V	*P. graminis*	*P. graminis*	100	*P. sonchi* group	27	*P. sonchi* group	87
P17E	*Paenibacillus* sp.	*P. jilunlii*	70	*P. sonchi* group	70	*P. sonchi* group	75
P22V	*Paenibacillus* sp.	*P. borealis*	54	*P. wynnii*	31	*P. odorifer*	55
P30E	*P. typhae*	*P. typhae*	99	*P. typhae*	99	*P. typhae*	99
P5E	*Paenibacillus* sp.	*P. typhae* group	97	*P. borealis*	53	*P. borealis*	70
P9S	*P. illinoisensis*	*P. illinoisensis*	93	*P. illinoisensis*	59	*P. illinoisensis*	87
P14S	*P. pabuli*	*P. pabuli* group	100	*P. pabuli* group	73	*P. pabuli*	57
P7S	*P. pabuli*	*P. pabuli* group	100	*P. pabuli* group	73	*P. pabuli*	57
P49E	*Paenibacillus* sp.	*P. tundrae* group	100	*P. xylanexedens* group	18	*P. xylanexedens* group	61
P48S	*Paenibacillus* sp.	*P. tundrae* group	100	*P. xylanexedens*	18	*P. xylanexedens*	48
P16E	*P. amylolyticus*	*P. amylolyticus*	99	*P. amylolyticus*	34	*P. amylolyticus*	65
P8E	*Paenibacillus* sp.	*P. xylanexedens* group	55	*P. xylanexedens* group	18	*P. xylanexedens* group	61
P6E	*P. catalpae*	*P. catalpae*	100	*P. catalpae* group	63	*P. glycanilyticus*	28
P28E	*Paenibacillus* sp.	*P. catalpae* group	99	*P. catalpae*	47	*P. catalpae* group	99
B40E	*B. aryabhattai*	*B. aryabhattai*	100	*B. megaterium*	55	*B. aryabhattai*	34
B53S	*Bacillus* sp.	*B. niacini* group	93	*B. drentensis*	39	*B. drentensis* group	13
B10S	*B. arbutinivorans*	*B. arbutinivorans*	100	*B. arbutinivorans*	99	*B. arbutinivorans*	99
B2S	*B. circulans*	*B. circulans*	73	*B. nealsonii* group	60	*B. nealsonii* group	36
B29S	*B. nealsonii*	*B. circulans* group	74	*B. nealsonii*	98	*B. nealsonii*	96
B18E	*B. acidiceler*	*B. acidiceler*	100	*B. acidiceler*	72	*B. acidiceler* group	99
B34V	*B. acidiceler*	*B. acidiceler*	100	*B. acidiceler*	72	*B. acidiceler* group	99
B3V	*B. cereus*	*B. cereus*	47	*B. cereus*	12	*B. cereus*	45
B45V	*B. thuringiensis*	*B. thuringiensis*	99	*B. thuringiensis*	88	*B. thuringiensis*	97
B38V	*B. mycoides*	*B. mycoides*	98	*B. weihenstephanensis*	75	*B. mycoides*	27
B24V	*B. safensis*	*B. safensis*	100	*B. pumilus*	42	*B. safensis*	43

[a]accession number in Table 1

[b]B:bootstrap of closest grouping (one type-species or group containing more than one type-species)

The similarities on databases also account on consensus (see underlined names on Table 2).

Table 5: The production of indolic compounds (*IC*) and the N_2-fixing ability are indicated for each group of *R* isolates. The *R* isolates are organized according to the UPGMA dendrogram order.

			µmol C_2H_4 mg protein^{-1} (approximate *scale of values*)						
R	**Identification**	***IC***	**no**	**low**	**weak**	**Middle**	**high**	**very high**	**Total**
P15E	***Paenibacillus* sp.**	1d	-	-	1	-	-	-	**1**
P3E	***Paenibacillus* sp.**	1b	-	-	-	-	1	-	**1**
P14V	***Paenibacillus* sp.**	2b; 2d	2	1	-	1	-	-	**4**
P46E	***Paenibacillus* sp.**	1b	1	-	-	-	-	-	**1**
P32E	***Paenibacillus* sp.**	1d	1	-	-	-	-	-	**1**
P26E	***Paenibacillus* sp.**	2d	-	-	-	-	-	2	**2**
P43V	***P. graminis***	1d	1	-	-	-	-	-	**1**
P17E	***Paenibacillus* sp.**	3d	2	1	-	-	-	-	**3**
P22V	***Paenibacillus* sp.**	1a	-	1	-	-	-	-	**1**
P30E	***P. typhae***	1d	-	1	-	-	-	-	**1**
P5E	***Paenibacillus* sp.**	2d	2	-	-	-	-	-	**2**
P9S	***P. illinoisensis***	1b	-	1	-	-	-	-	**1**
P14S	***P. pabuli***	1a; 4b; 1d	3	2	1	-	-	-	**6**
P7S	***P. pabuli***	11a; 10b	18	1	-	-	-	-	**21**
P49E	***Paenibacillus* sp.**	1a; 1b; 1c	2	1	-	-	-	-	**3**
P48S	***Paenibacillus* sp.**	2b	2	-	-	-	-	-	**2**
P16E	***P. amylolyticus***	2b; 3d	4	1	-	-	-	-	**5**
P8E	***Paenibacillus* sp.**	1b	1	-	-	-	-	-	**1**
P6E	***P. catalpae***	1a	-	-	1	-	-	-	**1**
P28E	***Paenibacillus* sp.**	1b	-	1	-	-	-	-	**1**
B40E	***B. aryabhattai***	3a; 2b	4	1	-	-	-	-	**5**
B53S	***Bacillus* sp.**	4b; 1d	4	1	-	-	-	-	**5**
B10S	***B. arbutinivorans***	7b; 8c	11	4	-	-	-	-	**15**
B2S	***B. circulans***	2b	-	2	-	-	-	-	**2**
B29S	***B. nealsonii***	1b	1	-	-	-	-	-	**1**
B18E	***B. acidiceler***	1b	1	-	-	-	-	-	**1**
B34V	***B. acidiceler***	1b	1	-	-	-	-	-	**1**
B3V	***B. cereus***	2a; 2b	2	-	-	1	-	1	**4**
B47V	***B. thuringiensis***	3a; 3b	4	-	-	-	1	1	**6**
B38V	***B. mycoides***	1d	-	-	-	-	1	-	**1**
B24V	***B. safensis***	1d	-	-	1	-	-	-	**1**
									101

IC production: 'a' (1 to 10 µg mL^{-1}), 'b' (10 to 100), 'c' (> 100), and 'd' (non-producer). Scale of values ranging from 'no' (until IC production: 'a' (1 to 10 µg mL^{-1}), 'b' (10 to 100), 'c' (> 100), and 'd' (non-producer). Scale of values ranging from 'no' (until 0.50 µmol C_2H_4 mg protein^{-1}), 'low' (0.50 to 3), 'weak' (3 to 10), 'middle' (10 to 20), 'high' (50 to 150), and 'very high' (500 to 1550). The *P. riograndensis* SBR5T strain was used as a positive control and showed 960 µmol C_2H_4 mg protein^{-1}.

Table 6: Effect of inoculation by the five selected isolates to shoot dry matter and the NPK contents of sunflower plants at 24 days post sowing (dps).

		24 dps			
mM N	**Treatment**	**DM (mg)**	**N (mg)**	**P (mg)**	**K (mg)**
0	**B38V**	126.13 (± 19.16) b	1.29 (± 0.19) b	0.24 (± 0.04) a	3.30 (± 0.50) a
0	**B47V**	158.45 (± 59.99) ab	2.33 (± 0.88) ab	0.24 (± 0.09) a	2.84 (± 1.07) a
0	**P3E**	151.00 (± 31.00) b	1.66 (± 0.34) b	0.35 (± 0.07) a	2.96 (± 0.61) a
0	**P26E**	121.53 (± 34.57) b	1.17 (± 0.33) b	0.23 (± 0.06) a	2.34 (± 0.67) a
0	**P27E**	155.70 (± 64.39) ab	1.34 (± 0.55) b	0.31 (± 0.13) a	3.05 (± 1.26) a
0	**negative control**	107.05 (± 17.77) b	1.39 (± 0.23) b	0.28 (± 0.04) a	2.53 (± 0.42) a
3.75	**B38V**	319.78 (± 57.84) a	3.84 (± 0.69) a	0.38 (± 0.07) a	4.44 (± 0.80) a
3.75	**B47V**	195.95 (± 101.66) ab	2.21(± 1.15) ab	0.31 (± 0.16) a	2.92 (± 1.51) a
3.75	**P3E**	225.90 (± 86.84) ab	1.78 (± 0.69) b	0.43 (± 0.16) a	3.75 (± 1.44) a
3.75	**P26E**	235.38 (± 81.30) ab	2.14 (± 0.74) ab	0.33 (± 0.11) a	3.51 (± 1.21) a
3.75	**P27E**	247.85 (± 74.62) ab	2.77 (± 0.83) ab	0.27 (± 0.08) a	3.62 (± 1.09) a
3.75	**50% N control**	195.50 (± 60.96) ab	2.81 (± 0.88) ab	0.27 (± 0.08) a	3.44 (± 1.07) a
7.50	**100% N control**	200.00 (± 93.11) ab	2.88 (± 1.34) ab	0.30 (± 0.14) a	3.66 (± 1.70) a

DM: shoot dry matter; dps: days post sowing.

Data are mean (± SD) of four replicates of plants grown in vermiculite in a photoperiod chamber. Values in the same column followed by the same letter did not differ significantly at $p>0.05$ (Tukey test).

Figures

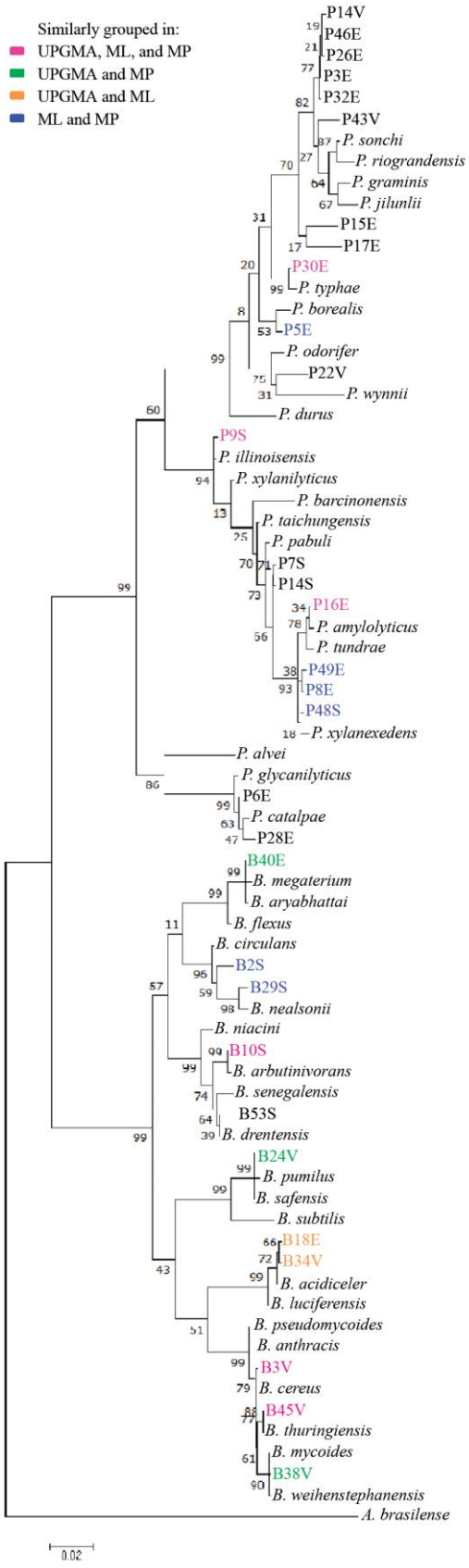
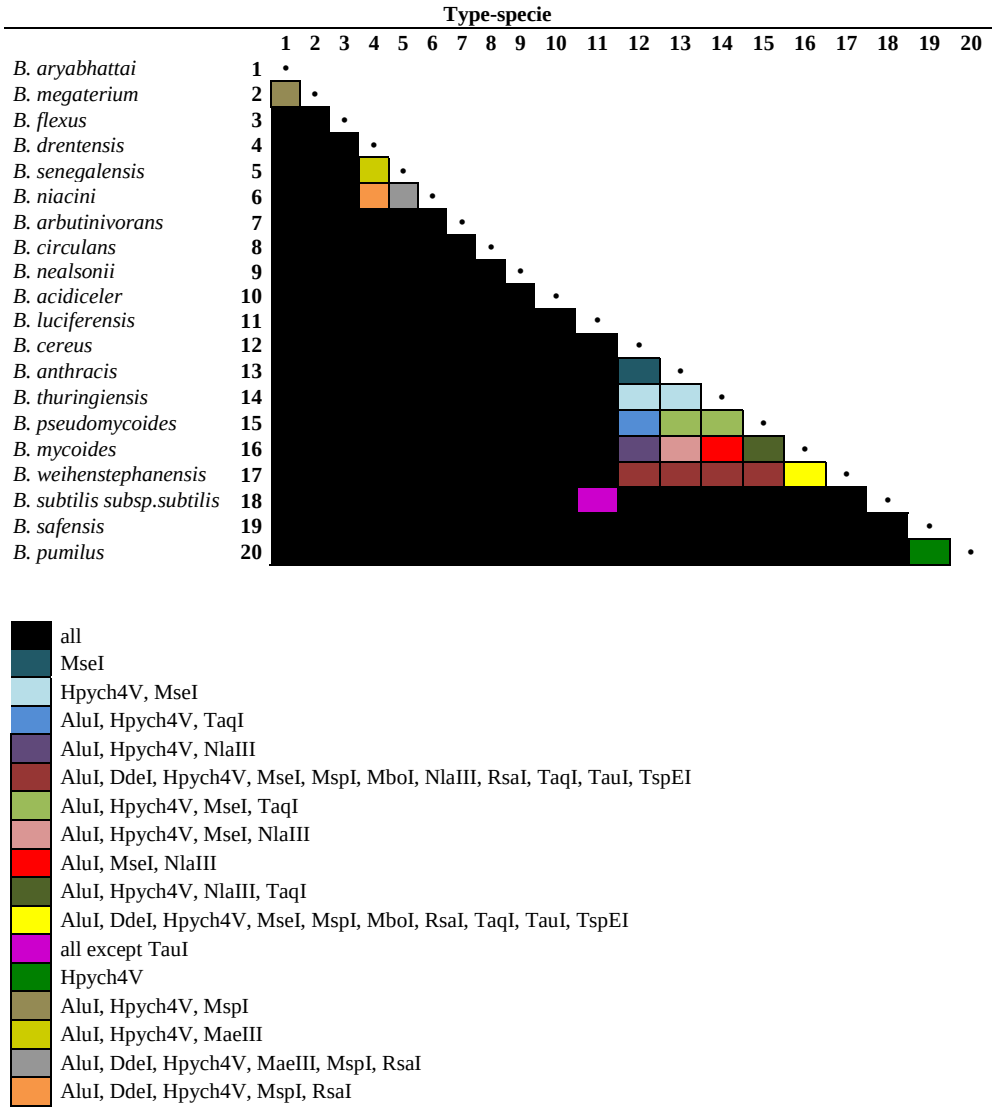

Similarly grouped in:
UPGMA, ML, and MP
UPGMA and MP
UPGMA and ML
ML and MP
P14V
P46E
P26E
P3E
P32E
P43V
P. sonchi
P. riograndensis
P. graminis
P. jilunlii
P15E
P17E
P30E
P. typhae
P. borealis
P5E
P. odorifer
P22V
P. wynnii
P. durus
P9S
P. illinoisensis
P. xylanilyticus
P. barcinonensis
P. taichungensis
P. pabuli
P7S
P14S
P16E
P. amylolyticus
P. tundrae
P49E
P8E
P48S
P. xylanexedens
P. alvei
P. glycanilyticus
P6E
P. catalpae
P28E
B40E
B. megaterium
B. aryabhattai
B. flexus
B. circulans
B2S
B29S
B. nealsonii
B. niacini
B10S
B. arbutinivorans
B. senegalensis
B53S
B. drentensis
B24V
B. pumilus
B. safensis
B. subtilis
B18E
B34V
B. acidiceler
B. luciferensis
B. pseudomycoides
B. anthracis
B3V
B. cereus
B45V
B. thuringiensis
B. mycoides
B38V
B. weihenstephanensis
A. brasilense
0.02

Figure 1: Maximum likelihood tree was generated using an alignment about 1,450 nucleotides based on 71 16S rDNA sequences (representative isolates and type-species) of *Bacillus* and *Paenibacillus* species. *Azospirillum brasilense* Sp245 (accession number HE577328) was used as outgroup. Gaps treatment was "use all sites", and the evolutionary model used was Jukes-Cantor. Bootstrap values (based on 1,000 replications) are shown at each node. The scale bar (0.02) represents evolutionary distance. The colored names (representative isolates) are related to similar grouping in UPGMA dendrogram, Maximum Likelihood tree (ML), and Maximum Parsimony tree (MP) (see legend).

(a) Type-species of *Bacillus*



(b) Type-species of *Paenibacillus*

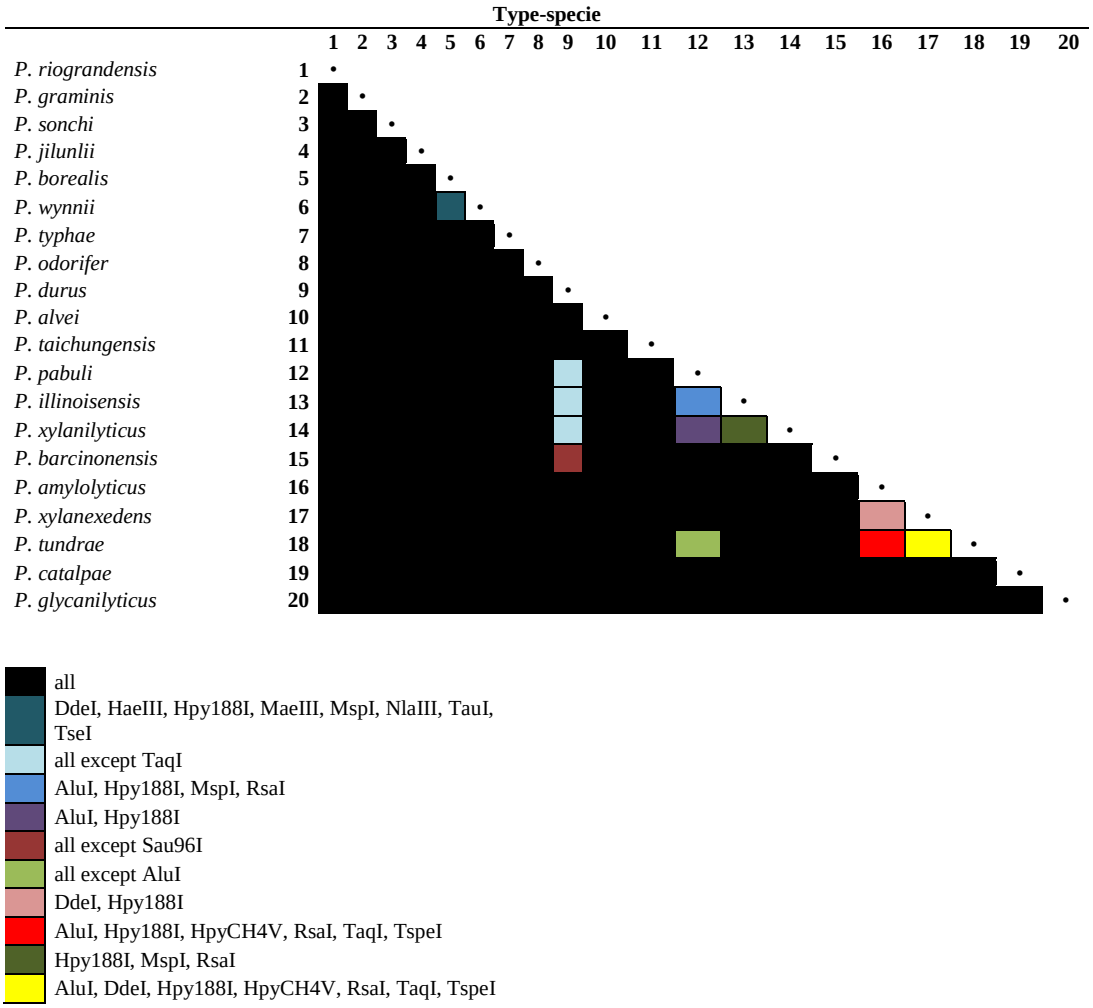


Figure 2: Type-species of *Bacillus* (a) and *Paenibacillus* (b) and the abilities of the 16 restriction endonucleases (REs) to differentiate the sequences of their 16S rRNA genes (approximately 1,450 nucleotides). The species are organized by UPGMA clustering. Colored squares represent the group of REs able to distinguish the species, with combinations between rows and columns. A black square indicates that all REs were able to differentiate the two related species, e.g., *B. aryabhattai* (number 1; in A) is differentiated from any other type-species of *Bacillus* by any RE, with the exception of *B. megaterium* (number 2): only AluI, Hpych4V, and MspI REs can distinguish the two type-species. An enzyme able to differentiate any species of *Paenibacillus*from the other 19 species of this genus received a value of 100% of resolving power (RP) and is present in all squares (as Hpy188I in B).

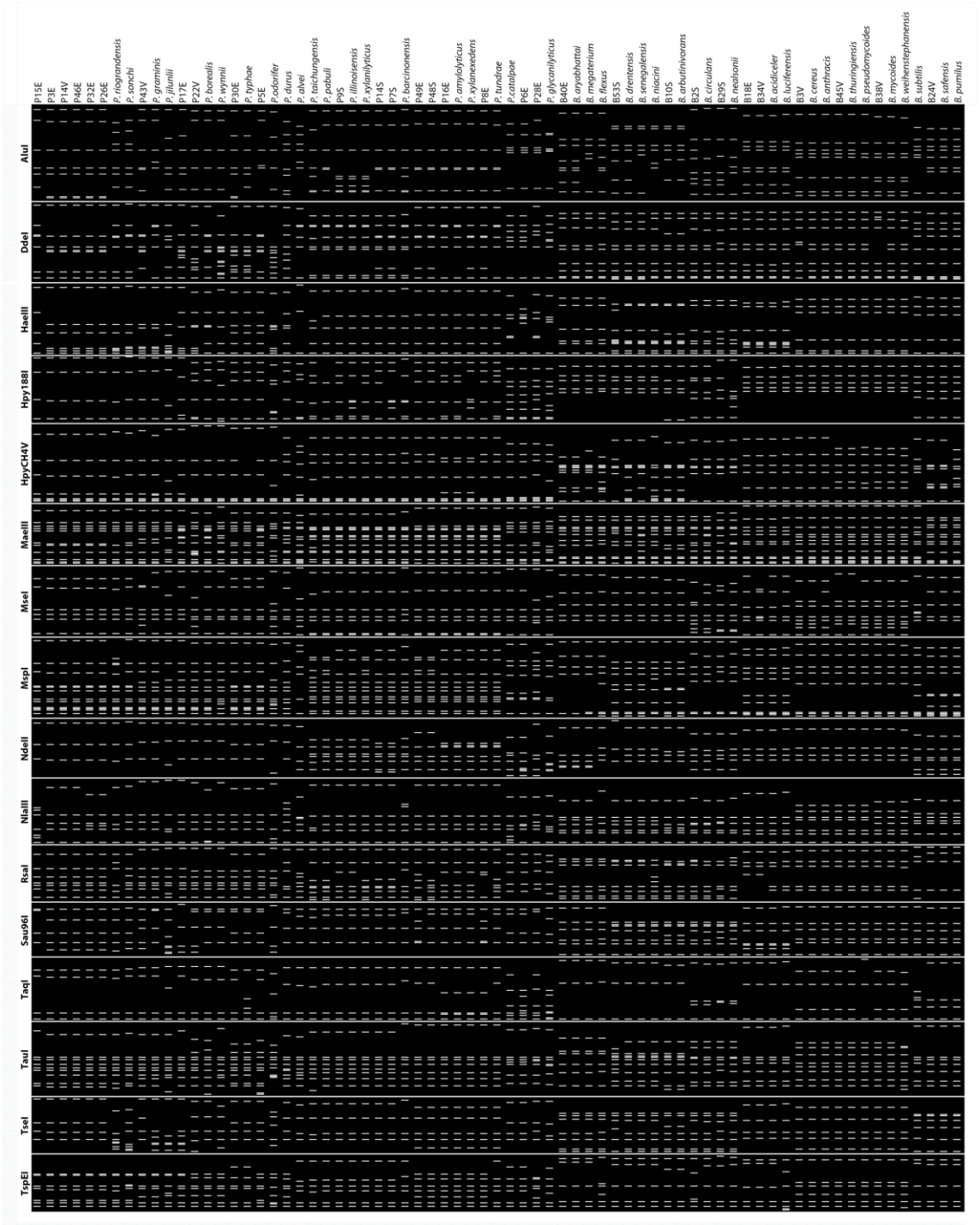


Figure 3: Automated restriction profile pattern of the 16S rRNA gene sequences (approximately1,450 nucleotides) by 16 restriction endonucleases. The representative isolates and type-species are organized by UPGMA dendrogram.

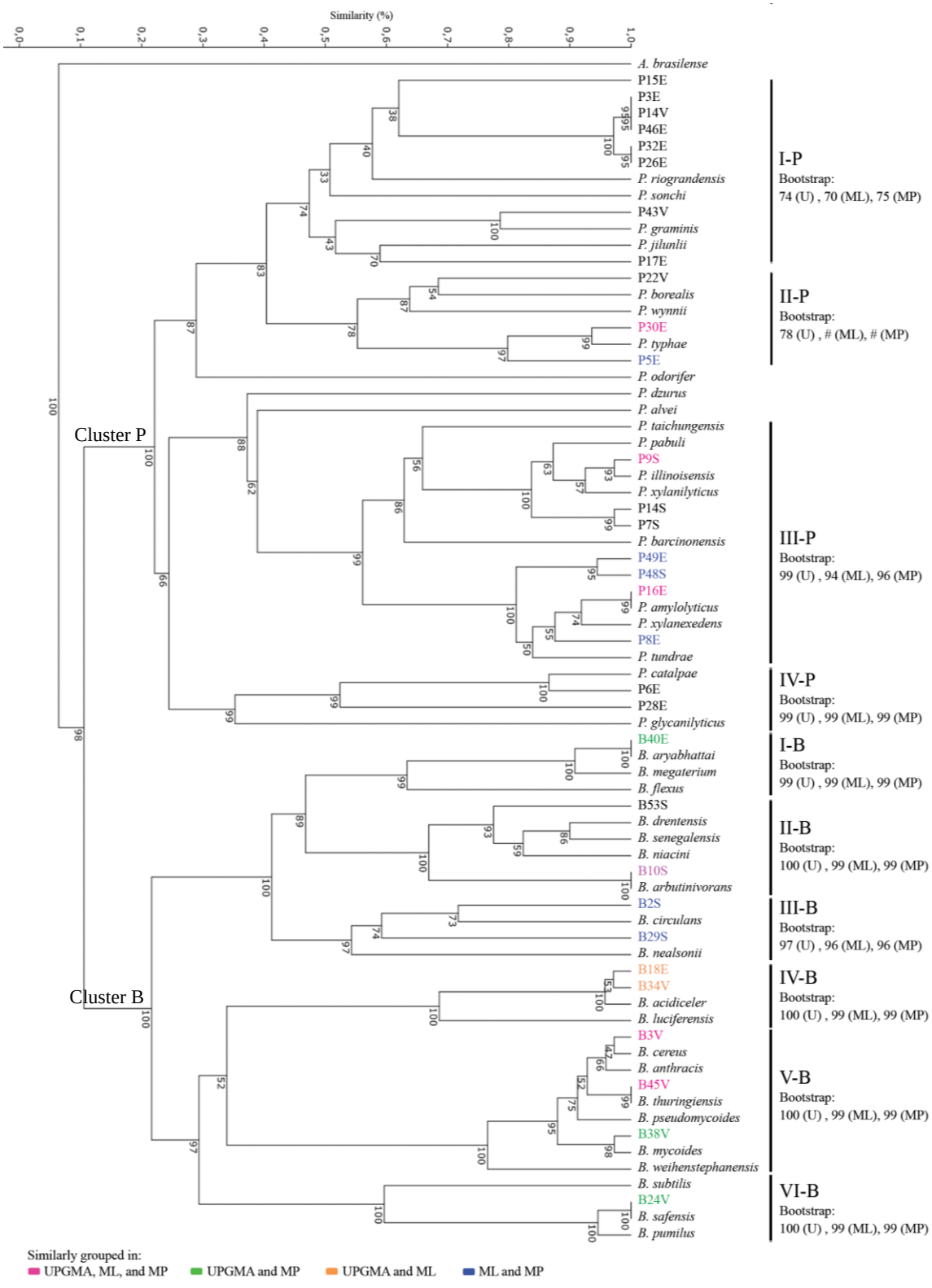
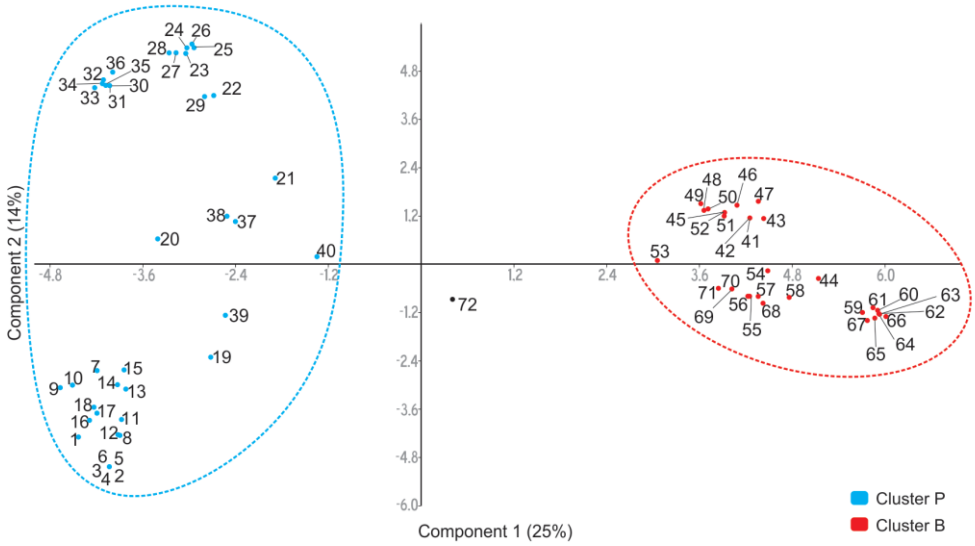

Similarity (%)
0,0
0,1
0,2
0,3
0,4
0,5
0,6
0,7
0,8
0,9
1,0
A. brasilense
P15E
P3E
P14V
P46E
P32E
P26E
P. riograndensis
P. sonchi
P43V
P. graminis
P. jilunlii
P17E
P22V
P. borealis
P. wynnii
P30E
P. typhae
P5E
P. odorifer
P. dzurus
P. alvei
P. taichungensis
P. pabuli
P9S
P. illinoisensis
P. xylanilyticus
P14S
P7S
P. barcinonensis
P49E
P48S
P16E
P. amylolyticus
P. xylanexedens
P8E
P. tundrae
P. catalpae
P6E
P28E
P. glycanilyticus
B40E
B. aryabhattai
B. megaterium
B. flexus
B53S
B. drentensis
B. senegalensis
B. niacini
B10S
B. arbutinivorans
B2S
B. circulans
B29S
B. nealsonii
B18E
B34V
B. acidiceler
B. luciferensis
B3V
B. cereus
B. anthracis
B45V
B. thuringiensis
B. pseudomycoides
B38V
B. mycoides
B. weihenstephanensis
B. subtilis
B24V
B. safensis
B. pumilus
Cluster P
Cluster B
I-P
Bootstrap:
74 (U) , 70 (ML), 75 (MP)
II-P
Bootstrap:
78 (U) , # (ML), # (MP)
III-P
Bootstrap:
99 (U) , 94 (ML), 96 (MP)
IV-P
Bootstrap:
99 (U) , 99 (ML), 99 (MP)
I-B
Bootstrap:
99 (U) , 99 (ML), 99 (MP)
II-B
Bootstrap:
100 (U) , 99 (ML), 99 (MP)
III-B
Bootstrap:
97 (U) , 96 (ML), 96 (MP)
IV-B
Bootstrap:
100 (U) , 99 (ML), 99 (MP)
V-B
Bootstrap:
100 (U) , 99 (ML), 99 (MP)
VI-B
Bootstrap:
100 (U) , 99 (ML), 99 (MP)
Similarly grouped in:
UPGMA, ML, and MP
UPGMA and MP
UPGMA and ML
ML and MP

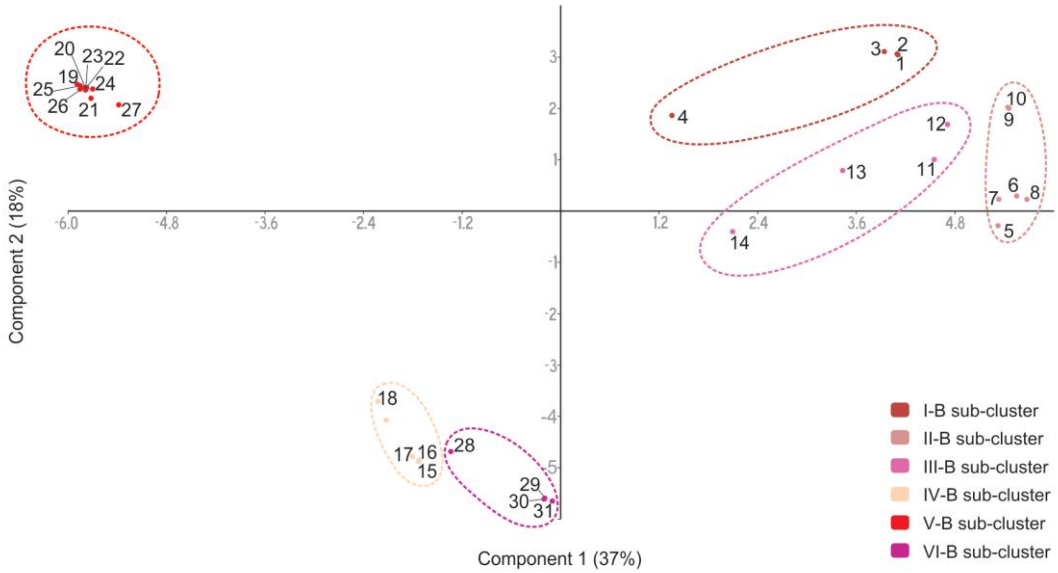
Figure 4: UPGMA dendrogram based on bands presence and absence of restriction profile (16 REs) of 71 16S rRNA gene sequences about 1,450 nucleotides (representative isolates and type-species) of *Bacillus* (Cluster B) and *Paenibacillus* (Cluster P) species. *Azospirillum brasilense* Sp245 (accession number HE577328) was used as outgroup. The similarity (%) values are relate to Jaccard coefficient. Dark vertical lines correspond to clustered at 47% similarity. Bootstrap values (based on 1,000 replications) are shown at each node. The bootstrap values below of subclusters I-P to VI-B are related to UPGMA dendrogram (U), Maximum Likelihood tree (ML), and Maximum Parsimony tree (MP). The symbol '#' indicatesdifferent grouping in the trees and in the dendrogram. The colored names (representative isolates) are related to similar grouping in UPGMA dendrogram, Maximum Likelihood tree (ML), and Maximum Parsimony tree (MP) (see legend).

(a)



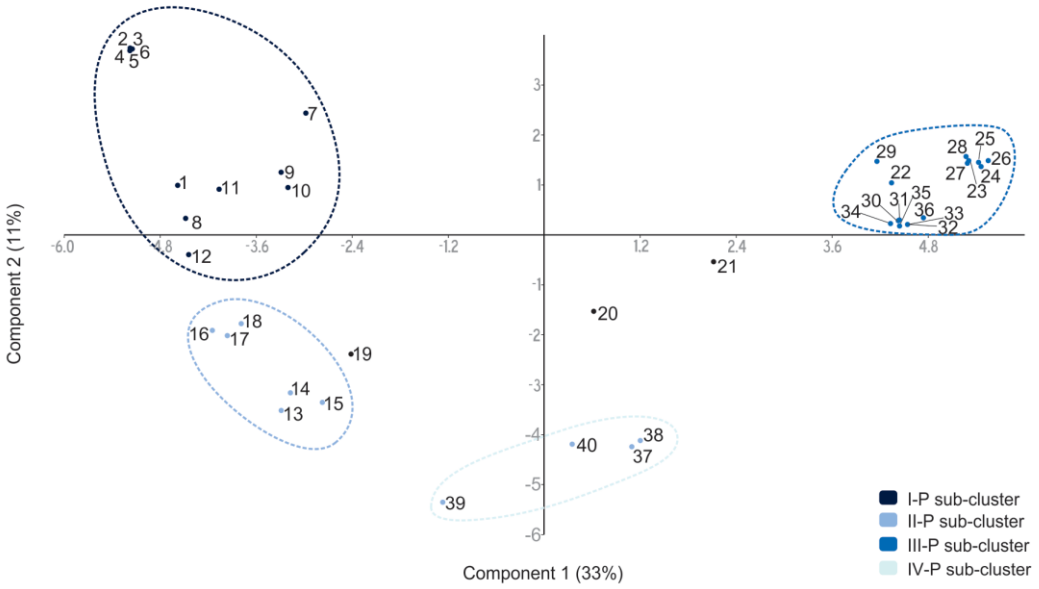
1 -P15E, 2 -P3E, 3 -P14V, 4 -P46E, 5 -P32E, 6 -P26E, 7 - *P. riograndensis*, 8 - *P. sonchi*, 9 -P43V, 10 - *P. graminis*, 11 - *P. jilunlii*, 12 -P17E, 13 -P22V, 14 - *P. borealis*, 15 - *P. wynnii*, 16 - P30E, 17 - *P. typhae*, 18 - P5E, 19 - *P. odorifer*, 20 - *P. durus*, 21 - *P. alvei*, 22 - *P. taichungensis*, 23 - *P. pabuli*, 24 - P9S, 25 - *P. illinoisensis*, 26 - *P. xylanilyticus*, 27 - P14S, 28 - P7S, 29 - *P. barcinonensis*, 30 - P49E, 31 - P48S, 32 - P16E, 33 - *P. amylolyticus*, 34 - *P. xylanexedens*, 35 - P8E, 36 - *P. tundrae*, 37 - *P. catalpae*, 38 - P6E, 39 - P28E, 40 - *P. glycanilyticus*, 41 - B40E, 42 - *B. aryabhattai*, 43 - *B. megaterium*, 44 *B. flexus*, 45 - B53S, 46 - *B. drentensis*, 47 - *B. senegalensis*, 48 - *B. niacini*, 49 - B10S, 50 - *B. arbutinivorans*, 51 - B2S, 52 - *B. circulans*, 53 - B29S, 54 - *B. nealsonii*, 55 B18E, 56 - B34V, 57 - *B. acidiceler*, 58 - *B. luciferensis*, 59 - B3V, 60 - *B. cereus*, 61 - *B. anthracis*, 62 - B45V, 63 - *B. thuringiensis*, 64 - *B. pseudomycoides*, 65 - B38V, 66 - *B. mycoides*, 67 - *B. weihenstephanensis*, 68 - *B. subtilis*, 69 - B24V, 70 - *B. safensis*, 71 - *B. pumilus*, 72 - *A. brasilense*

(b)



1 - B40E, 2 - *B. aryabhattai*, 3 - *B. megaterium*, 4 *B. flexus*, 5 - B53S, 6 - *B. drentensis*, 7 - *B. senegalensis*, 8 - *B. niacini*, 9 - B10S, 10 - *B. arbutinivorans*, 11 - B2S, 12 - *B. circulans*, 13 - B29S, 14 - *B. nealsonii*, 15 - B18E, 16 - B34V, 17 - *B. acidiceler*, 18 - *B. luciferensis*, 19 - B3V, 20 - *B. cereus*, 21 - *B. anthracis*, 22 - B45V, 23 - *B. thuringiensis*, 24 - *B. pseudomycoides*, 25 - B38V, 26 - *B. mycoides*, 27 - *B. weihenstephanensis*, 28 - *B. subtilis*, 29 - B24V, 30 - *B. safensis*, 31 - *B. pumilus*

c)



1 - P15E, 2 - P3E, 3 - P14V, 4 - P46E, 5 - P32E, 6 - P26E, 7 - *P. riograndensis,* 8 - *P. sonchi,* 9 - P43V, 10 - *P. graminis,* 11 - *P. jilunlii,* 12 - P17E, 13 - P22V, 14 - *P. borealis,* 15 - *P. wynnii,* 16 - P30E, 17 - *P. typhae,* 18 - P5E, 19 - *P. odorifer,* 20 - *P. durus,* 21 - *P. alvei,* 22 - *P. taichungensis,* 23 - *P. pabuli,* 24 - P9S, 25 - *P. illinoisensis,* 26 - *P. xylanilyticus,* 27 - P14S, 28 - P7S, 29 - *P. barcinonensis,* 30 - P49E, 31 - P48S, 32 - P16E, 33 - *P. amylolyticus,* 34 - *P. xylanexedens,* 35 - P8E, 36 - *P. tundrae,* 37 - *P. catalpae,* 38 - P6E, 39 - P28E, 40 - *P. glycanilyticus*

Figure 5: Principal component analysis (PCA) based on the restriction profiles (16 REs) of 71 sequences of the approximately 1,450 nucleotides of the 16S rRNA gene (representative isolates and type-species) inthe *Bacillus* (Cluster B) and *Paenibacillus* (Cluster P) species (a). PC1 and PC2 values show the contribution of the two principal components summarizing the total variance of each dataset. In (a) *Bacillus* and *Paenibacillus* species; (b) only *Bacillus* species; (c) only *Paenibacillus* species.Sub-clusters I-P to VI-B were grouped according to a similarity of more than 47% in UPGMA and PCA.

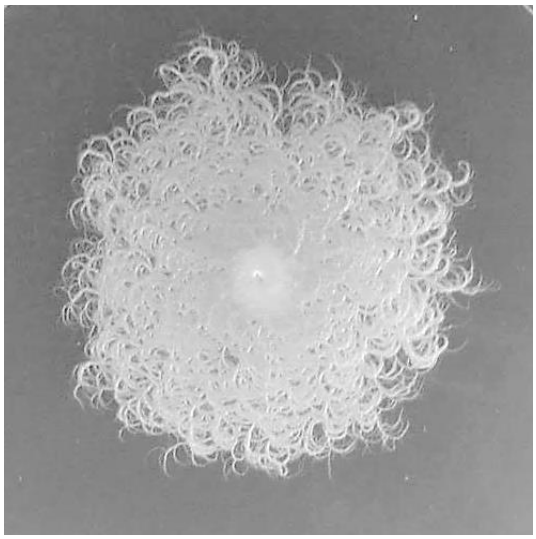


Figure 6: The morphology of a rhizoidal colony from the B38V isolate identified to belong to the *B. mycoides* species and the presence of counterclockwise curved radial filaments by this strain (SIN morphotype; Di Franco et al., 2002).

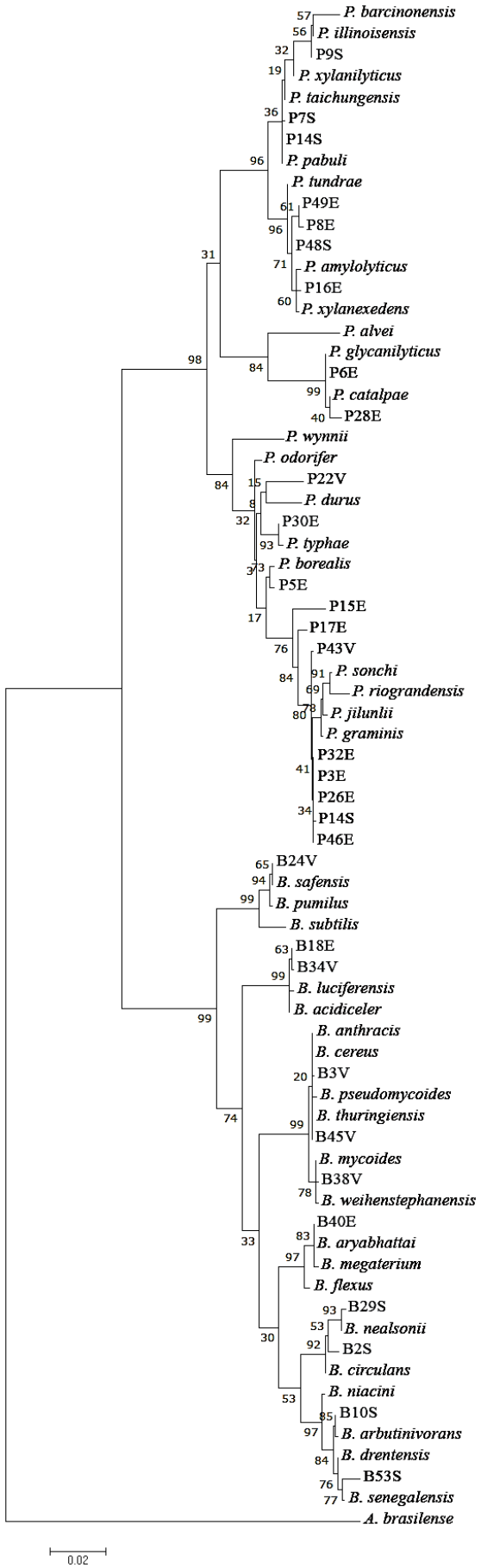

P. barcinonensis
P. illinoisensis
P9S
P. xylanilyticus
P. taichungensis
P7S
P14S
P. pabuli
P. tundrae
P49E
P8E
P48S
P. amylolyticus
P16E
P. xylanexedens
P. alvei
P. glycanilyticus
P6E
P. catalpae
P28E
P. wynnii
P. odorifer
P22V
P. durus
P30E
P. typhae
P. borealis
P5E
P15E
P17E
P43V
P. sonchi
P. riograndensis
P. jilunlii
P. graminis
P32E
P3E
P26E
P14S
P46E
B24V
B. safensis
B. pumilus
B. subtilis
B18E
B34V
B. luciferensis
B. acidiceler
B. anthracis
B. cereus
B3V
B. pseudomycoides
B. thuringiensis
B45V
B. mycoides
B38V
B. weihenstephanensis
B40E
B. aryabhattai
B. megaterium
B. flexus
B29S
B. nealsonii
B2S
B. circulans
B. niacini
B10S
B. arbutinivorans
B. drentensis
B53S
B. senegalensis
A. brasilense
0.02

Figure S1: Maximum likelihood tree was generated using an alignment about 1,450 nucleotides based on 71 16S rDNA sequences (representative isolates and type-species) of *Bacillus* and *Paenibacillus* species. *Azospirillum brasilense* Sp245 (accession number HE577328) was used as outgroup. Gaps treatment was "partial deletion", and the evolutionary model used was Jukes-Cantor. Bootstrap values (based on 1,000 replications) are shown at each node. The scale bar (0.02) represents evolutionary distance.

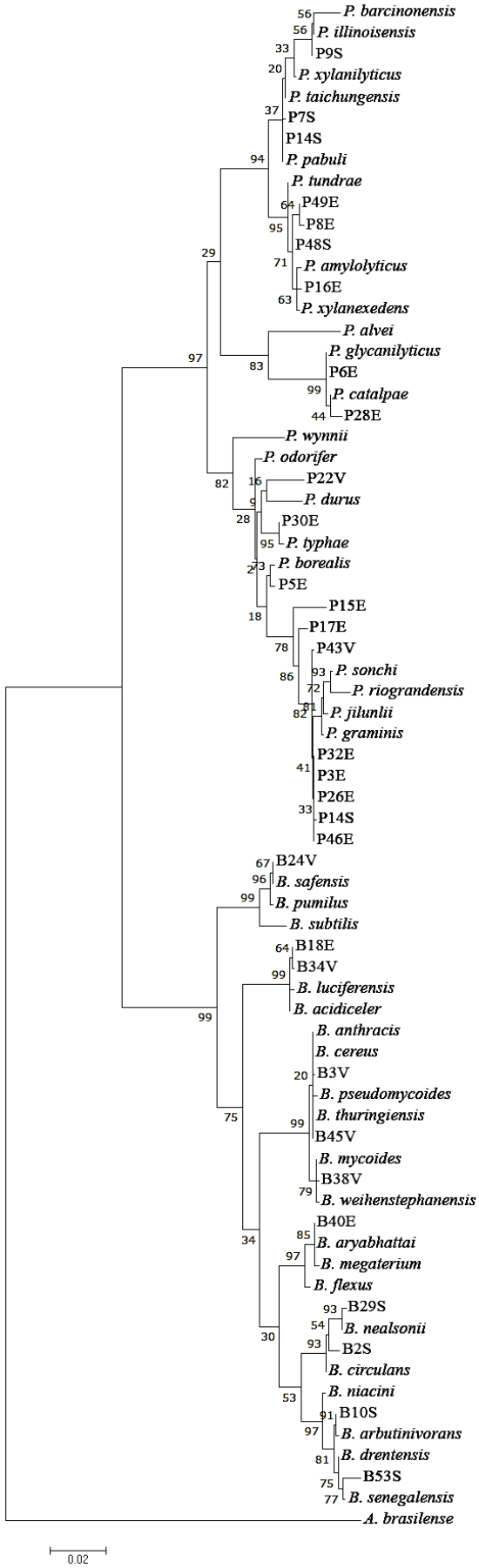

P. barcinonensis
P. illinoisensis
P9S
P. xylanilyticus
P. taichungensis
P7S
P14S
P. pabuli
P. tundrae
P49E
P8E
P48S
P. amylolyticus
P16E
P. xylanexedens
P. alvei
P. glycanilyticus
P6E
P. catalpae
P28E
P. wynnii
P. odorifer
P22V
P. durus
P30E
P. typhae
P. borealis
P5E
P15E
P17E
P43V
P. sonchi
P. riograndensis
P. jilunlii
P. graminis
P32E
P3E
P26E
P14S
P46E
B24V
B. safensis
B. pumilus
B. subtilis
B18E
B34V
B. luciferensis
B. acidiceler
B. anthracis
B. cereus
B3V
B. pseudomycoides
B. thuringiensis
B45V
B. mycoides
B38V
B. weihenstephanensis
B40E
B. aryabhattai
B. megaterium
B. flexus
B29S
B. nealsonii
B2S
B. circulans
B. niacini
B10S
B. arbutinivorans
B. drentensis
B53S
B. senegalensis
A. brasilense
0.02

Figure S2: Maximum likelihood tree was generated using an alignment about 1,450 nucleotides based on 71 16S rDNA sequences (representative isolates and type-species) of *Bacillus* and *Paenibacillus* species. *Azospirillum brasilense* Sp245 (accession number HE577328) was used as outgroup. Gaps treatment was "complete deletion", and the evolutionary model used was Jukes-Cantor.Bootstrap values (based on 1,000 replications) are shown at each node. The scale bar (0.02) represents evolutionary distance.

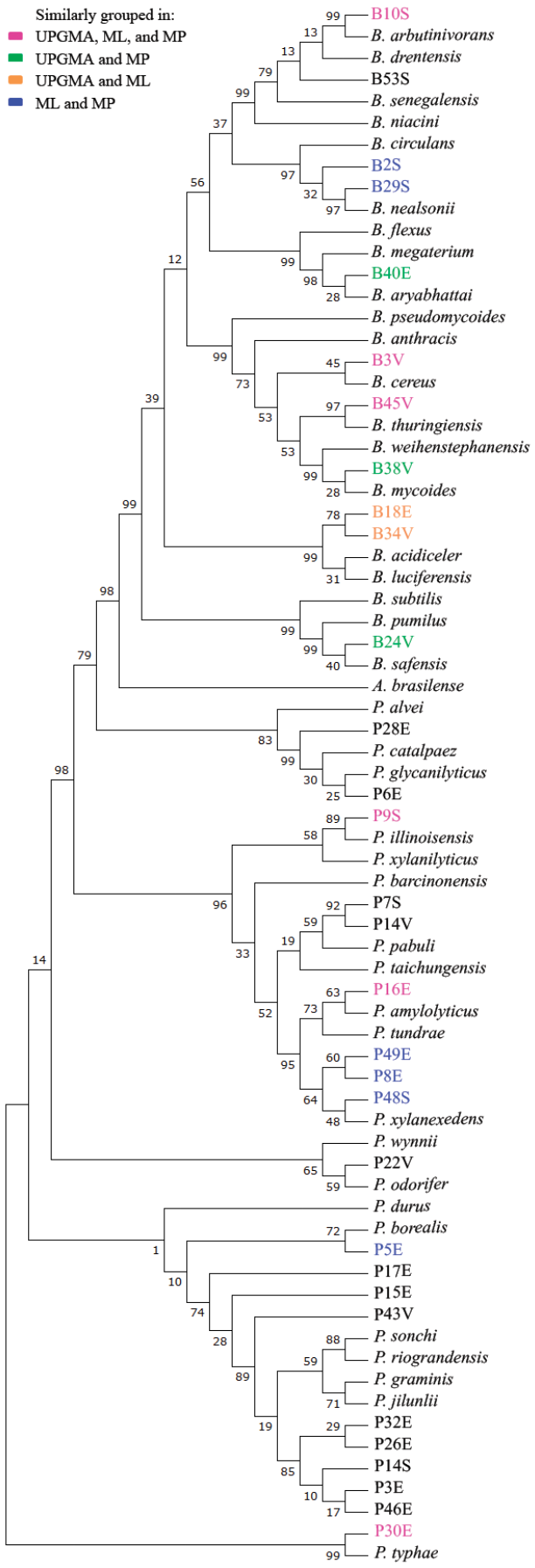

Similarly grouped in:
UPGMA, ML, and MP
UPGMA and MP
UPGMA and ML
ML and MP
B10S
B. arbutinivorans
B. drentensis
B53S
B. senegalensis
B. niacini
B. circulans
B2S
B29S
B. nealsonii
B. flexus
B. megaterium
B40E
B. aryabhattai
B. pseudomycoides
B. anthracis
B3V
B. cereus
B45V
B. thuringiensis
B. weihenstephanensis
B38V
B. mycoides
B18E
B34V
B. acidiceler
B. luciferensis
B. subtilis
B. pumilus
B24V
B. safensis
A. brasilense
P. alvei
P28E
P. catalpaez
P. glycanilyticus
P6E
P9S
P. illinoisensis
P. xylanilyticus
P. barcinonensis
P7S
P14V
P. pabuli
P. taichungensis
P16E
P. amylolyticus
P. tundrae
P49E
P8E
P48S
P. xylanexedens
P. wynnii
P22V
P. odorifer
P. durus
P. borealis
P5E
P17E
P15E
P43V
P. sonchi
P. riograndensis
P. graminis
P. jilunlii
P32E
P26E
P14S
P3E
P46E
P30E
P. typhae

Figure S3: Maximum parsimony tree was generated using an alignment about 1,450 nucleotides based on 71 16S rDNA sequences (representative isolates and type-species) of *Bacillus* (Cluster B) and *Paenibacillus* (Cluster P) species. *Azospirillum brasilense* Sp245 (accession number HE577328) was used as outgroup. Bootstrap values (based on 1,000 replications) are shown at each node. The colored names (representative isolates) are related to similar grouping in UPGMA dendrogram, Maximum Likelihood tree (ML), and Maximum Parsimony tree (MP) (see legend).

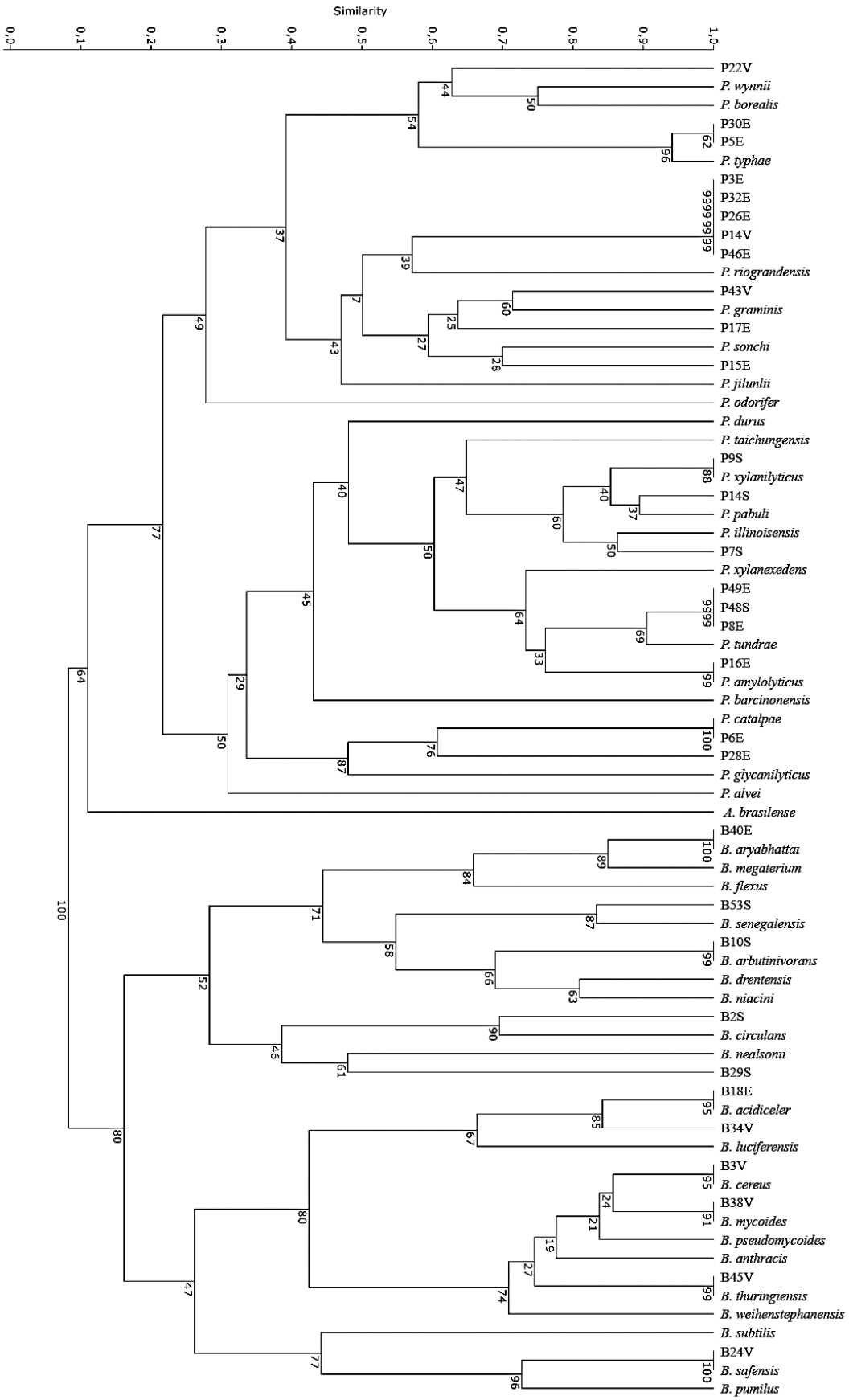

Similarity
0,0
0,1
0,2
0,3
0,4
0,5
0,6
0,7
0,8
0,9
1,0
P22V
P. wynnii
P. borealis
P30E
P5E
P. typhae
P3E
P32E
P26E
P14V
P46E
P. riograndensis
P43V
P. graminis
P17E
P. sonchi
P15E
P. jilunlii
P. odorifer
P. durus
P. taichungensis
P9S
P. xylanilyticus
P14S
P. pabuli
P. illinoisensis
P7S
P. xylanexedens
P49E
P48S
P8E
P. tundrae
P16E
P. amylolyticus
P. barcinonensis
P. catalpae
P6E
P28E
P. glycanilyticus
P. alvei
A. brasilense
B40E
B. aryabhattai
B. megaterium
B. flexus
B53S
B. senegalensis
B10S
B. arbutinivorans
B. drentensis
B. niacini
B2S
B. circulans
B. nealsonii
B29S
B18E
B. acidiceler
B34V
B. luciferensis
B3V
B. cereus
B38V
B. mycoides
B. pseudomycoides
B. anthracis
B45V
B. thuringiensis
B. weihenstephanensis
B. subtilis
B24V
B. safensis
B. pumilus

Figure S4: UPGMA dendrogram based on bands presence and absence of restriction profile (3 principal REs; Hpy188I, HpyCH4V, and MseI) of 71 16S rDNA sequences about 1,450 nucleotides (representative isolates and type-species) of *Bacillus* and *Paenibacillus* species. *Azospirillum brasilense* Sp245 (accession number HE577328) was used as outgroup. The similarity (%) values relate to Jaccard coefficient. Bootstrap values (based on 1,000 replications) are shown at each node.

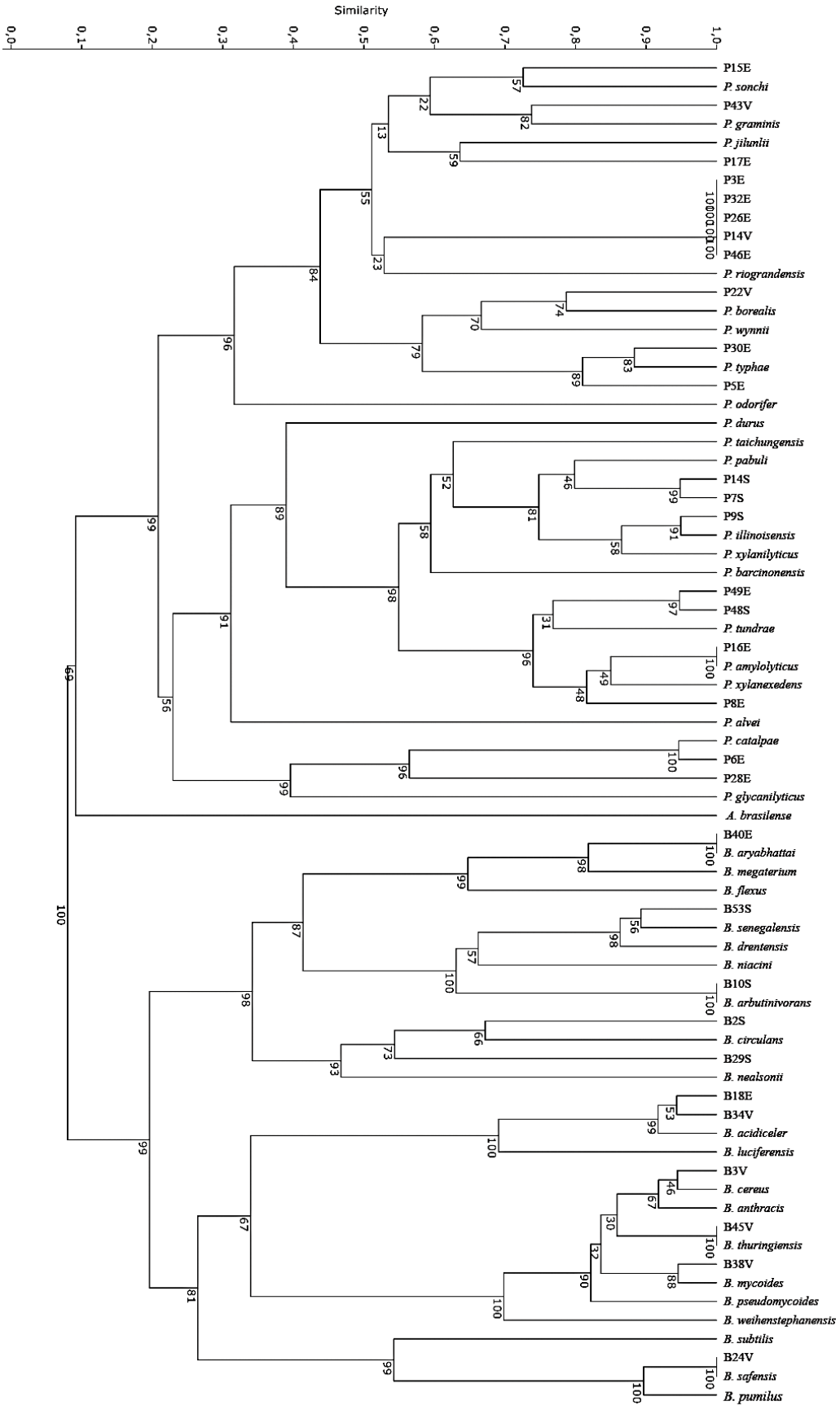

Similarity
0,0
0,1
0,2
0,3
0,4
0,5
0,6
0,7
0,8
0,9
1,0
P15E
P. sonchi
P43V
P. graminis
P. jilunlii
P17E
P3E
P32E
P26E
P14V
P46E
P. riograndensis
P22V
P. borealis
P. wynnii
P30E
P. typhae
P5E
P. odorifer
P. durus
P. taichungensis
P. pabuli
P14S
P7S
P9S
P. illinoisensis
P. xylanilyticus
P. barcinonensis
P49E
P48S
P. tundrae
P16E
P. amylolyticus
P. xylanexedens
P8E
P. alvei
P. catalpae
P6E
P28E
P. glycanilyticus
A. brasilense
B40E
B. aryabhattai
B. megaterium
B. flexus
B53S
B. senegalensis
B. drentensis
B. niacini
B10S
B. arbutinivorans
B2S
B. circulans
B29S
B. nealsonii
B18E
B34V
B. acidiceler
B. luciferensis
B3V
B. cereus
B. anthracis
B45V
B. thuringiensis
B38V
B. mycoides
B. pseudomycoides
B. weihenstephanensis
B. subtilis
B24V
B. safensis
B. pumilus

Figure S5: UPGMA dendrogram based on bands presence and absence of restriction profile of 71 16S rRNA gene sequences about 1,450 nucleotides (representative isolates and type-species) of *Bacillus* and *Paenibacillus* species. *Azospirillum brasilense* Sp245 (accession number HE577328) was used as outgroup. The restriction profiles are related to the five highest RP values to type-species of *Bacillus* and *Paenibacillus* (AluI, DdeI, Hpy188I, HpyCH4V, MseI, MspI, RsaI, and TaqI). The similarity (%) values relate to Jaccard coefficient. Bootstrap values (based on 1,000 replications) are shown at each node.

3. Considerações finais

A interação entre PGPRs e plantas é influenciada pela troca de sinais moleculares na rizosfera, um ambiente rico em nutrientes derivados dos exsudatos radiculares. A especificidade que pode ocorrer naturalmente entre os organismos estimula a busca por bactérias promotoras de crescimento e colonizadoras da espécie vegetal de interesse. As espécies vegetais liberam diferentes compostos em sua rizosfera dependendo do estado nutricional e das condições fisiológicas em que se encontram no ambiente (Singh et al., 2004). Assim, as bactérias isoladas das raízes ou rizosfera de uma determinada planta podem conter genótipos altamente adaptados ao hospedeiro e, dessa forma, para a promoção de crescimento vegetal. Em vista do potencial das PGPRs para uso na agricultura, o isolamento de bactérias e o estudo dos diferentes sistemas envolvidos na interação com as plantas podem auxiliar no conhecimento e no desenvolvimento de novos produtos inoculantes (biofertilizantes).

A união de diferentes metodologias na pesquisa com essas bactérias, como a identificação de características promotoras de crescimento, identificação do isolado, ensaios de inoculação de sementes em condições laboratoriais e experimentos de cultivo a campo, fazem parte da busca por novas opções para os cultivos agrícolas. Entretanto, muitas PGPRs, mesmo possuindo diversas habilidades favoráveis ao crescimento vegetal, também possuem alto potencial de patogenicidade, como o caso de variadas espécies de *Burkholderia* (Ronning et al., 2010; Sousa et al., 2010) e *Enterobacteriaceae* (Bach et al., 2000). No isolamento de rizobactérias das raízes e rizosfera de girassol, os gêneros *Burkholderia* e *Enterobacter* foram dominantes na associação com a planta, e os isolados desses gêneros foram os mais frequentes solubilizadores de fosfato e produtores de compostos indólicos, respectivamente. *Enterobacteriaceae* também foram muito abundantes entre os isolados das rizosferas de arroz (Costa et al., 2012; Souza et al., 2012) e canola (Farina et al., 2012). Dessa forma, quando as análises visam potenciais biofertilizantes para a adequada reintrodução dessas bactérias no meio-ambiente, muitos gêneros podem ser candidatos preferidos entre os micro-organismos obtidos durante o isolamento, tais como *Azospirillum*, *Bacillus* e *Rhizobium* (Kokalis-Burelles et al., 2006; Beneduzi et al., 2008; Molina-Favero et al., 2008; Budiharjo e Olbertz, 2011; Fibach-Paldi et al., 2011; Farina et al., 2012; Souza et al., 2012).

Na pesquisa com girassol, diferentes trabalhos têm abordado o estudo com espécies de PGPRs que se mostraram bem adaptadas à rizosfera da planta, uma vez que, por mecanismos variados, houve um aumento na promoção de crescimento vegetal ou na resposta ao estresse (Alami et al., 2000; Forchetti et al., 2007; Forchetti et al., 2010; Shilev et al., 2010). Na inoculação de girassol com uma linhagem de *Rhizobium* produtora de exopolissacarídeos (EPS), por exemplo, a bactéria modificou a estrutura do solo aderido ao sistema radicular e causou efeitos positivos, mesmo sob déficit hídrico (Alami et al., 2000). Este isolado da rizosfera de girassol também foi capaz de promover o crescimento através do aumento da absorção de nitrogênio pelas plântulas, o que pôde ser demonstrado pelo melhor aproveitamento de fertilizante a base de [^{15}N] nitrato quando a bactéria estava presente na rizosfera (Alami et al., 2000). Outros trabalhos também têm demonstrado o efeito positivo de diferentes espécies de *Azospirillum* sobre girassol (Fages e Arsac, 1991; Fages e Lux; 1991; Akbari et al., 2011) e isso suporta a busca por um biofertilizante baseado em inoculação com bactérias desse gênero.

Dentre os quatro isolados que promoveram maiores conteúdos de massa seca ou nutrientes nos experimentos do Capítulo I, isto é, Vac30 (*Burkholderia* sp.), Vi21 (*Chryseobacterium* sp.), Vi22 (*Azospirillum* sp.) e Vi49 (*Achromobacter* sp.), Vi22 foi identificado como *A. brasilense*, enquanto os outros isolados foram caracterizados como potenciais bactérias patogênicas, através de análises de todo o gene 16S rRNA (dados não mostrados). Após a produção de um biofertilizante a base de *A. brasilense* Vi22 e experimento de inoculação das sementes a campo (Capítulo II), foi possível observar que as plantas na presença da bactéria e da metade da dose de N recomendada para a cultura (60 Kg/ha) foram tão vigorosas quanto aquelas do controle contendo a dose inteira (120 Kg/ha). Se confirmados os dados em outros experimentos, tais resultados apontam para a possibilidade da redução de pelo menos 50% do conteúdo de N aplicado à produção agrícola de girassol, quando esta aplicação ocorrer em conjunto com tal biofertilizante, sem que ocorram alterações na produtividade dos grãos, massa seca e conteúdo de N.

O estudo de uma característica de interesse, assim como o exemplo da fixação de N_2, pode ser direcionado a análises da distribuição dessa habilidade entre determinados gêneros ou espécies específicos. Tendo em vista que a produção de endósporos em bactérias Gram-positivas é uma característica desejável devido à

resistência dessas estruturas, *Bacillus* e *Paenibacillus* podem ser bons candidatos para biofertilizantes. O isolamento de bacilos com potencial para a fixação de N_2 a partir da rizosfera de girassol forneceu uma estimativa da distribuição de tal característica entre diferentes linhagens, com base em ensaios de redução de acetileno *in vitro* e análises multivariadas da sequência do gene 16S rRNA. O emprego da análise do polimorfismo de comprimento de fragmento de restrição (RFLP) *in silico* permitiu alta reprodutibilidade à técnica e, juntamente com ensaios filogenéticos e de identidade nucleotídica, colaborou para uma classificação consensual e mais acurada dos isolados. Os marcadores genéticos acessados virtualmente auxiliaram na descrição de isolados, como, por exemplo, aqueles classificados como *B. acidiceler*, *B. aryabhattai*, *B. mycoides*, *B. safensis*, *P. amylolyticus*, *P. catalpae*, e *P. graminis*. As espécies de *B. arbutinivorans* e *P. pabuli* foram as mais abundantes, mas contaram com poucos isolados fixadores de N.

Entre aqueles que exibiram maiores atividades da nitrogenase estão cinco isolados de *Paenibacillus* sp. (próximos à *P. riograndensis* e *P. sonchi*), *B. mycoides* e *B. thuringiensis*. O isolado *B. mycoides* B38V foi capaz de produzir uma grande quantidade de etileno (a partir de acetileno) e também estimulou positivamente o incremento de massa seca e conteúdo de N de plantas inoculadas. Diferentes linhagens de *B. mycoides* têm sido associadas a distintos processos, tais como: (i) fixação de N_2, atividade de ACC deaminase e redução de nitrato (Karagöz et al., 2012); (ii) amonificação (Tyagny-Ryadno, 1933); (iii) produção de bacteriocinas e atividade anti-microbiana (Sharma e Gautam, 2008); (iv) resistência a alta concentração (1g L^{-1}) de metais como Cd, Pb, Zn, Ni e fito-remediação junto à girassol (Zadeh et al., 2008); (v) indução de resistência sistêmica em plantas (Choudhary e Johri, 2009).

Contudo, estudos futuros serão necessários para que possamos obter maiores conhecimentos a cerca da interação entre *B. mycoides*, assim como *A. brasilense*, com girassol. Outros ensaios a campo estão sendo conduzidos conforme as normas técnicas do MAPA. Análises da capacidade de colonização radicular, por meio da visualização de fluorescência de *gfp* na PGPR, estão sendo conduzidas com ambas as linhagens isoladas. Da mesma forma, a expressão de *nifH* em raízes de girassol inoculado com *B. mycoides* B38V será analisada por transcrição reversa e PCR quantitativo em tempo real. Essas pesquisas (entre outras relacionadas ao estudo do potencial destas bactérias para a promoção de crescimento de girassol) deverão ser

realizadas para que tais bactérias possam ser mais bem avaliadas para a indicação como biofertilizantes no cultivo dessa oleaginosa.

Em comparação a outras plantas oleaginosas, o rendimento médio do óleo de girassol é de 774 kg/ha, valores que podem ser superiores àqueles das plantações de algodão (361 kg/ha), canola (570 kg/ha), mamona (470 kg/ha) e soja (560 kg/ha) (Goes et al., 2010). O alto teor de óleo das sementes (26 a 72%), o qual é rico em ácidos graxos poli-insaturados, além do uso como farelo de alimentação animal, apicultura e utilidades ornamentais, fazem do girassol uma cultura de múltiplos benefícios (Câmara, 2003; Castro, 2007). A falta de incentivo ao cultivo ocorre porque ainda existem problemas relacionados à baixa produtividade e controle de doenças fúngicas (como *Sclerotinia sclerotiorum*), quando comparado a culturas bem estabelecidas na entressafra, como o milho (Castro, 2007). Segundo a Companhia Nacional de Abastecimento (Conab, 2012a; 2012b), a produtividade média de girassol prevista para a safra 2012/13 deverá ser de 1.255kg/ha, uma queda de 19,7%, em relação à safra anterior. Estima-se que haja diferenças entre as regiões do país, como produção média de 1.395 kg/ha no Sudeste, 1.273 kg/ha no Sul e apenas 715 kg/ha na região Nordeste.

Em relação aos ganhos do produtor na região centro sul do Brasil, o valor da saca de 60 kg de grãos de girassol tem aumentado nos últimos anos, passando de R$ 37,24 em 2011 à R$ 54,03 em 2012 (Conab, 2012a; 2012b). Em comparação a outra oleaginosa de importância econômica, como a soja, os valores são similares e têm elevado na mesmo proporção na última década, sendo que os preços médios anuais de girassol e soja em 2012 foram R$ 51,02 e R$ 58,52, respectivamente. O Rio Grande do Sul (RS) contribuiu com apenas 5% da produção nacional de grãos na safra 2012/13, enquanto que o estado de Mato Grosso (MT) com 65%, seguido de Goiás (GO) com 22% e Mato Grosso do Sul (MS) com 7% (Conab, 2012a; 2012b). É interessante observar que o RS ocupou a primeira posição em área plantada de girassol na safra de 2006/2007 (22 mil ha e produção de 31 mil t), mas, atualmente, conta com aproximadamente 3 mil ha, enquanto MT e GO com 47 e 14 mil ha, respectivamente.

Os maiores produtores mundiais de grãos de girassol são Ucrânia, Rússia, União Européia (EU-27) e Argentina (Conab, 2012a; 2012b). Em relação à produção de biodiesel no ano de 2010 os principais países produtores foram Alemanha, Brasil, França e Estados Unidos (Távora, 2012). O Brasil, entretanto, tem grande perspectivas de aumento da sua produção de biodiesel e o girassol é uma das principais oleaginosas

com potencial de expansão no país, existindo grande perspectiva de aumento da área plantada, devido ao cultivo em época distinta da semeadura das principais culturas (Castro, 2007; Porto *et al*., 2007; Backes *et al*., 2008; Petrobrás, 2011).

A busca por bactérias benéficas é importante para o desenvolvimento de novos e eficientes inoculantes na agricultura, assim como os investimentos em tecnologias que contribuam para o aumento da viabilidade do inóculo e taxa de sobrevivência de bactérias aderidas às sementes são igualmente essenciais para o sucesso da inoculação. O incentivo ao plantio de girassol é válido não apenas pela alta qualidade nutricional do óleo e a multiplicidade de usos da cultura, mas, essencialmente, pelo alto rendimento na extração de óleo das sementes e a produção sustentável de combustíveis, assim como o biodiesel (em contrapartida ao uso de combustíveis fósseis, como o petróleo) (Castro, 2007). No contexto da produção de alimentos e da demanda crescente por combustíveis alternativos, a utilização de inoculantes biológicos capazes de diminuir, ou até mesmo suspender, os efeitos nocivos da fertilização química nas lavouras, é uma alternativa viável e ainda pouco custosa para as práticas agrícolas. Os benefícios ambientais e econômicos, se levados em conta, são pontos essenciais para a adoção da tecnologia do uso de inoculantes. A introdução de bactérias no solo tende a ser menos agressiva e causar menos impactos ao meio-ambiente do que a fertilização química e, portanto, é uma prática agronômica sustentável e de incentivo à produção com redução de custos.

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