UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

INSTITUTO DE BIOCIÊNCIAS

PROGRAMA DE PÓS GRADUAÇÃO EM GENÉTICA E BIOLOGIA MOLECULAR

Caracterização de bactérias promotoras do crescimento vegetal associadas à cultura da canola (*Brassica napus* L.)

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Dissertação submetida ao Programa de Pós-Graduação em Genética e Biologia Molecular da UFRGS como requisito parcial para a obtenção do grau de Mestre em Genética e Biologia Molecular

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SUMÁRIO

LISTA DE ABREVIATURAS

ACC- 1-aminociclopropano-1-carboxilato

AIA- ácido indol acético

ANI- identidade média de nucleotídeos

ARA- ensaio de redução do acetileno

BGC- grupamento gênico biossintético

DDH- hibridização DNA:DNA

FBN- fixação biológica do nitrogênio

ISR- indução de resistência sistêmica

MPS- solubilização de fosfato mineral

NRPS- peptídeo sintetase não-ribossomal

P-fósforo

PGP- promoção ou promotor de crescimento vegetal

PGPB- bactérias promotoras de crescimento vegetal

PKS- sintetases de policetídeos

PSB- bactéria solubilizadora de fosfato

RP- rocha fosfática

SAR-resistência sistêmica adquirida

UFC- unidades formadoras de colônia

VOC- composto orgânico volátil

RESUMO

O sistema radicular abriga uma ampla diversidade de bactérias que vivem no solo rizosférico ou colonizando endofiticamente o interior dos tecidos vegetais. Estes micro-organismos podem promover o crescimento das plantas, principalmente pela modulação dos níveis de fitohormônios e por favorecerem a aquisição de nutrientes, especialmente nitrogênio e fósforo (P). Também, como mecanismo indireto, inibem o crescimento de fitopatógenos. Por essas características, as PGPB (*plant growth promoting bacteria*) são agronomicamente empregadas como biofertilizantes ou agentes de biocontrole. A aplicação destes produtos biológicos tem um potencial elevado para aumentar a produção agrícola e reduzir o uso de fertilizantes químicos ambientalmente prejudiciais. O objetivo deste trabalho foi caracterizar as habilidades de promoção do crescimento vegetal e de biocontrole de bactérias provenientes de raízes de canola. Foram avaliadas trinta bactérias gram-positivas formadoras de esporos, pertencentes a quatro gêneros: *Bacillus* (24), *Paenibacillus* (4), *Lysinibacillus* (1) e *Microbacterium* (1). Esses micro-organismos foram capazes de fixar nitrogênio, sintetizar auxinas, solubilizar fosfato, produzir enzimas hidrolíticas e sideróforos. Cinco isolados apresentaram atividade antifúngica *in vitro* contra o patógeno de canola *Sclerotinia sclerotiorum*, e quatro desses isolados suprimiram o crescimento do fungo pela produção de compostos orgânicos voláteis (VOC). Os genomas das bactérias antagonistas foram sequenciados e três isolados (01TAZ, 08TAZ, 32PB) foram identificados como *Bacillus safensis*, um (7PB) como *Bacillus pumilus* e um (16PB) como *Bacillus megaterium*, usando métricas genômicas. Vários *clusters* gênicos de antimicrobianos e metabólitos secundários foram encontrados em comum no genoma dos isolados, usando a ferramenta de mineração de genoma antiSMASH. Esses grupos gênicos incluíram agrupamentos biossintéticos de lipopeptídeos, bacteriocinas e sideróforos. No entanto, nenhum tipo de lipopeptídeo antimicrobiano foi identificado por espectrometria de massas nos extratos das culturas bacterianas. Apesar dos resultados obtidos *in vitro* e do potencial genômico para o biocontrole, estes isolados foram incapazes de proteger a canola contra a infecção por *S. sclerotiorum* em condições de câmara de crescimento. A solubilização de P foi uma das características de promoção de crescimento vegetal mais comum entre os isolados. Essas bactérias foram triadas quanto à solubilização de diferentes fosfatos insolúveis: hidroxiapatita ($Ca_5(PO_4)_3(OH)$), $FePO_4$, $AlPO_4$, e fosfato de rocha (RP). Seis isolados foram capazes de solubilizar hidroxiapatita e RP. Os três melhores (6PB, 20PB, 27PB) foram selecionados e melhor caracterizados. Essas bactérias foram identificadas como *Paenibacillus graminis* pelo sequenciamento do gene 16S rRNA e uso de métricas genômicas. Foi verificada a influência de fatores nutricionais, tais como a fonte de carbono, nitrogênio e P solúvel, sobre a eficiência de solubilização. As bactérias produziram exopolissacarídeos utilizando P insolúvel e também formaram biofilme sob condições de deficiência de P. Todos os isolados selecionados reduziram o pH do meio de cultura e produziram vários ácidos orgânicos, detectados por espectrometria de massas. Essas características constituem um possível mecanismo de solubilização de P. A análise do genoma dos isolados 6PB e 20PB revelou a presença de diferentes genes relacionados ao metabolismo e homeostase do P. Todas estas características destacam as bactérias avaliadas como potenciais candidatos a agentes de promoção do crescimento de plantas.

Palavras-chave: bactéria solubilizadora de fosfato, atividade antifúngica, *Sclerotinia sclerortiorum*; *Brassica napus.*

ABSTRACT

Plant root systems harbor a wide diversity of bacteria living in the rhizospheric soil or colonizing endophytically the inner tissues of plants. These microorganisms can promote plant growth, mainly by modulating phytohormone levels, and by favoring the acquisition of nutrients, especially nitrogen and phosphorus (P). They can also inhibit the growth of phytopathogens as an indirect mechanism of plant growth promotion. Due to these features, PGPB (plant growth promoting bacteria) are agronomically used as biofertilizers or biocontrol agents. The application of these biological products has an elevated potential to increase crop yields and reduce the use of environmentally harmful chemical fertilizers. The aim of this work was to characterize the plant growth promotion and biocontrol abilities of bacterial isolates from canola roots. The thirty evaluated gram-positive spore-forming bacteria belong to four genera: *Bacillus* (24), *Paenibacillus* (4), *Lysinibacillus* (1) and *Microbacterium* (1). These microorganisms were able to fix nitrogen, synthesize auxins, solubilize phosphate, produce hydrolytic enzymes and siderophores. Five isolates displayed antifungal activity *in vitro* against the canola pathogen *Sclerotinia sclerotiorum*, and four of these isolates were able to suppress fungal growth by volatile organic compounds (VOCs) production. The genomes of these bacteria were sequenced and three isolates (01TAZ, 08TAZ and 32PB) were identified by genomic metrics as *Bacillus safensis*, one isolate (7PB) as *Bacillus pumilus* and one (16PB) as *Bacillus megaterium*. Several antimicrobial gene clusters were found in common among the genome of the isolates using the genome mining tool antiSMASH. These gene clusters included lipopeptides, bacteriocins and siderophores biosynthetic clusters. However, no antimicrobial lipopeptide compound was identified by mass spectrometry in bacterial culture extracts. In spite of the *in vitro* results and the genomic potential for biocontrol, these isolates were unable to protect canola plants against *S. sclerotiorum* in growth chamber conditions. One of the most spread plant growth promotion traits among the isolates was phosphate solubilization. The microorganisms were screened for solubilization of different insoluble P sources: Hydroxyapatite ($Ca_5(PO_4)_3(OH)$), $FePO_4$, $AlPO_4$ and Rock phosphate (RP). Five isolates were able to solubilize Hydroxyapatite and RP. The three best isolates (6PB, 20PB, and 27PB) were selected and better characterized. These bacteria were identified as *Paenibacillus graminis* by 16S rRNA gene sequencing and genomic metrics. The influence of nutritional factors such as carbon and nitrogen sources or soluble P on their solubilization efficiency was evaluated. The isolates produced exopolysaccharide using P insoluble source and formed biofilm under P deficiency. All selected isolates reduced the pH of the culture medium and produced several organic acids, detected by mass spectrometry. These characteristics constitute a possible mechanism of phosphate solubilization. The genomic analyses of 6PB and 20PB isolates revealed the presence of different genes related to phosphate metabolism and homeostasis. All these features highlight the characterized bacteria as potential candidates for plant growth promotion agents.

Key-words: Phosphate solubilizing bacteria; antifungal activity; *Sclerotinia sclerotiorum*; *Brassica napus*.

1 INTRODUÇÃO

1.1. Bactérias promotoras do crescimento vegetal

A rizosfera compreende a porção de solo sob influência direta do sistema radicular. Essa região sustenta uma exuberante diversidade microbiana, que se difere tanto constitutivamente, quanto funcionalmente da comunidade microbiana presente nos solos adjacentes (Philippot et al., 2013; Kuzyakov e Blagodatskaya, 2015; Lugtenberg, 2015). Diversas bactérias mutualísticas se estabelecem na rizosfera, beneficiando-se nutricionalmente dos compostos orgânicos liberados no exsudato vegetal. Em contrapartida, esses organismos estão associados a diversos mecanismos de promoção do crescimento vegetal, sendo antropicamente explorados na prática agrícola (Prathap e Bd 2015; Venturi e Keel, 2016).

As bactérias promotoras do crescimento vegetal ou PGPB (*Plant Growth Promoting Bacteria*) podem se estabelecer no solo rizosférico como organismos de vida livre, assim como colonizar a superfície das raízes das plantas (rizoplano). Ocasionalmente, diversas bactérias rizosféricas também apresentam a capacidade de penetrar e colonizar endofiticamente os vegetais (Bulgarelli et al., 2013; Vejan et al., 2016; Gosal et al., 2017). Os ditos micro-organismos endofíticos são, por definição, aqueles que se estabelecem no interior dos tecidos vegetais sem, no entanto, provocar qualquer dano aparente à planta hospedeira ou levar à formação de estruturas externas perceptíveis (Podolich et al., 2015; Santoyo et al., 2016).

A capacidade das PGPB em promover o crescimento vegetal está associada tanto a mecanismos de atuação direta, quanto indireta. Diretamente, esses micro-organismos atuam no aumento da disponibilização de nutrientes do solo, no fornecimento de nitrogênio, pelo processo de fixação biológica desse composto, e na produção e regulação dos níveis de hormônios vegetais (Kaur et al., 2016; Ahemad e Kibret, 2014). Os mecanismos indiretos relacionam-se à capacidade dessas bactérias de participarem na proteção do vegetal contra patógenos e, também, na redução de danos provocados por estresses abióticos (Dey et al., 2014; Hassen et al., 2016).

A interação benéfica entre plantas e PGPB encontra uma ampla aplicabilidade biotecnológica, sendo visada excepcionalmente no desenvolvimento de inoculantes, em substituição ao uso convencional de agroquímicos. Os inoculantes envolvem combinações

entre linhagens de micro-organismos, aditivos, substâncias carreadoras e protetoras, em formulações diversas e adaptadas à diferentes propósitos agronômicos (Bashan et al., 2014; Preininger et al., 2018). Podem ser empregados como biofertilizantes, fitoestimulantes, agentes de controle biológico ou mesmo com propósitos de biorremediação. Essas formulações são de grande importância, uma vez que proporcionam ganhos de produtividade com reduzidos impactos sobre o ambiente (Baez-Rogelio et al., 2017; Alori e Babalola, 2018).

1.2. Disponibilização de nutrientes e outros mecanismos diretos de promoção do crescimento vegetal: importância no contexto agronômico e ambiental

O nitrogênio é o macronutriente mais limitante para o crescimento vegetal. Apesar de em sua composição gasosa (N_2) representar o maior constituinte atmosférico (78%), essa forma não pode ser diretamente assimilada pela maioria dos organismos, incluindo vegetais. Isso torna o processo biológico de fixação de nitrogênio, realizado por bactérias diazotróficas, extremamente importante para o desenvolvimento vegetal (Chanway et al., 2014). Essas bactérias possuem um complexo enzimático conhecido como nitrogenase, que é capaz de reduzir o nitrogênio atmosférico (N_2) à amônia (NH_3). Essa pode ser diretamente absorvida pelas plantas ou posteriormente convertida em demais compostos assimiláveis pelos vegetais (Hoffmann, 2007; Pii et al., 2015).

A elevada demanda nutricional de nitrogênio torna a prática agrícola fortemente dependente de um uso extensivo de fertilizantes nitrogenados. As formas nitrogenadas incorporadas nesses agroquímicos são provenientes, principalmente, do processo de Haber–Bosch, realizado sob condições de altas temperaturas e pressão (Galloway et al., 2013; Jez et al., 2016). Os elevados gastos energéticos inerentes a essa produção industrial, somados a alta demanda desse nutriente, fazem com que a aplicação de nitrogênio assuma os maiores custos no processo de fertilização para a maioria das culturas agrícolas (Gellings e Parmenter, 2004).

Além dos aspectos econômicos, o uso de fertilizantes nitrogenados esbarra na problemática ambiental. A produção e utilização desses adubos são responsáveis por grandes emissões de gases do efeito estufa (Garcia et al., 2013; Zahoo et al., 2014). Além disso, parte do nitrogênio aplicado nos solos pode ser perdida pela volatilização da amônia, sendo depositado no ambiente junto à precipitação pluvial. O metabolismo microbiano desses

compostos é responsável pela emissão de óxido nitroso, potente gás do efeito estufa que também atua na depleção da camada de ozônio (Cameron et al., 2013; Fowler et al., 2013). Convém também ressaltar que parte do nitrogênio introduzido nos solos pode ser lixiviado, contribuindo para o processo de eutrofização dos corpos hídricos (Erisman et al., 2013).

Uma alternativa em substituição, ou ao menos à redução, no uso de fertilizantes nitrogenados é a adoção de inoculantes agrícolas a base de bactérias diazotróficas (Lesueur et al., 2016). Para leguminosas, um grupo bacteriano genericamente conhecido como rizóbios é capaz de estabelecer uma relação simbiótica com o vegetal, suprindo a necessidade nutricional da planta por nitrogênio. Ao colonizar as raízes, essas bactérias são capazes de se estabelecer em nódulos que proporcionam um ambiente de alta eficiência para o processo de fixação (Laranjo et al., 2014; Abd-Alla et al., 2014). Em culturas como a soja, o uso de inoculantes a base de rizóbios pode chegar a dispensar completamente a aplicação de adubos nitrogenados, sem perdas de produtividade (Hungria e Mendes, 2015).

Diversas espécies vegetais de interesse agronômico, como, por exemplo, as pertencentes aos gêneros *Brassica* e *Poacea*, são incapazes de estabelecerem relações simbióticas similares à da soja com os rizóbios e, portanto, incapazes de formar nódulos. Sabe-se, no entanto, que esses grupos taxonômicos também se beneficiam, em diferentes níveis, da interação com demais grupos de bactérias diazotróficas. Essas podem se associar endofiticamente ou se estabelecer na rizosfera (Santi et al., 2013; Carvalho et al., 2014).

Depois do nitrogênio, o fósforo (P) é o macronutriente mais limitante para o crescimento vegetal. Nos solos, esse elemento encontra-se sob formas pouco acessíveis à captação direta pelas plantas (Alori et al., 2017). O P normalmente faz parte da constituição de compostos minerais de baixa solubilidade ou encontra-se retido na matéria orgânica (Barea e Richardson, 2015). O papel desempenhado por PGPB na disponibilização desse elemento relaciona-se, especialmente, à capacidade de mineralizar o fósforo orgânico, utilizando-se de um amplo arsenal enzimático, ou solubilizar os compostos minerais. O último processo está relacionado, principalmente, à produção de ácidos, entre outros mecanismos cogitados (Shen et al. 2011; Gupta e Sahu, 2017)

O fornecimento de alguns micronutrientes também pode ser facilitado pela atuação de micro-organismos rizosféricos. As PGPB estão relacionadas à solubilização de zinco a partir de compostos como ZnO e $ZnCO_3$ presentes no solo. Acredita-se que os mecanismos

de solubilização desses compostos se assemelham aos descritos para a solubilização de fosfato mineral (Saravanan et al., 2011; Gandhi e Muralidharan, 2016).

Quanto ao fornecimento de ferro, bactérias rizosféricas estão envolvidas, principalmente, na produção de moléculas conhecidas como sideróforos (Saha et al., 2016). Tratam-se de compostos orgânicos de baixo peso molecular, secretados no meio extracelular e que possuem a capacidade de complexar íons Fe^{+3}. Esse elemento, muitas vezes presente em baixas concentrações, é adquirido pelas células bacterianas ao reincorporar os sideróforos que a ele se complexaram (Ali e Vidhale, 2013). Plantas também são caracterizadas pela produção de um tipo análogo de quelante, conhecido como fitosideróforo. Os receptores vegetais para essas moléculas podem se ligar e incorporar sideróforos bacterianos. Desse modo, PGPB produtoras desses metabólitos conseguem atuar no suprimento nutricional desse elemento para plantas (Saharan e Nehra, 2011; Ahmed e Holmström, 2014).

PGPB são também descritas por sua capacidade de sintetizar e / ou regular os níveis de algumas das principais classes de hormônios vegetais (giberelinas, auxinas, citocininas, etileno, entre outros). A produção de auxinas, em especial do ácido indol-3-acético, seu principal representante, é extensivamente caracterizada em bactérias associativas de plantas (Spaepen, 2015; Egamberdieva et al., 2017). As auxinas estão relacionadas a vários processos fisiológicos, participando desde processos celulares básicos, até importantes alterações morfológicas, como o desenvolvimento de raízes, diferenciação de tecidos, entre outros (Sauer et al., 2013). A produção de AIA por micro-organismos afeta o balanço de auxinas nas plantas hospedeiras, podendo promover um maior alongamento radicular. Isso impacta diretamente na captação de nutrientes, favorecendo o crescimento vegetal (Spaepen e Vanderleyden, 2011).

Outro mecanismo relacionado à regulação de hormônios vegetais é a produção bacteriana da enzima ACC deaminase. Essa enzima é capaz de degradar o ácido 1-carboxílico-1-aminociclopropano (ACC), percursor metabólico na síntese do etileno. Esse hormônio está envolvido principalmente na resposta do vegetal a condições ambientais de estresse e relaciona-se a processos de senescência e abscisão (Glick, 2014; Gontia-Mishra et al., 2014). A capacidade bacteriana de reduzir os níveis de etileno promove uma maior

tolerância dos vegetais àquelas condições nas quais as altas concentrações do hormônio provocam efeitos fisiológicos danosos (Glick, 2015).

1.2.1 Disponibilização de fósforo solúvel por PGPB

Apesar de estar presente em muitos tipos de solo em concentrações relativamente altas (200 a >1000 mg kg^{-1}), em média, a concentração de fósforo solúvel ($H_2PO_4^-$ e HPO_4^{2-}) é 0,05 mg kg^{-1}, menos de 0,1% da concentração total desse elemento (Hopkins, 2015). Em solos com alto teor de matéria orgânica, o fósforo está principalmente imobilizado em compostos como inositol fosfato (fitato do solo), que podem compreender de 30 a 50% do fósforo total (Ahmed e Shahab, 2009).

A fertilização a base de fósforo tem uma baixa eficiência de aproveitamento. Estima-se que 75 – 90% do fósforo introduzido no solo via adubação química seja precipitado junto a íons de cálcio, ferro e alumínio, sob a forma de compostos altamente insolúveis (Sindhu et al., 2014). A maioria das áreas agricultáveis no mundo compreende solos com alta capacidade de retenção de fósforo (Kochian, 2012). Em solos ácidos, há o predomínio de fosfatos de ferro e alumínio, que compreendem os mais baixos índices de solubilidade, enquanto que em solos básicos, os fosfatos de cálcio são as formas insolúveis prevalentes (Bashan et al., 2013).

A principal matéria prima para produção de fertilizantes fosfatados são as rochas fosfáticas, que constituem um recurso mineral limitado. Há grandes divergências quanto à durabilidade das atuais reservas, mas algumas estimativas apontam que uma exploração intensiva poderia levar à completa depleção desse recurso em algumas décadas (Cooper et al., 2011; Reijnders, 2014; Baveye, 2015). Além dessa problemática controversa, há uma limitação ambiental em relação ao uso de fertilizantes fosfatados. A lixiviação do fósforo em solos cultivados também representa um grande contribuinte para o processo de eutrofização (Dodds e Smith, 2016).

Muitas PGPB têm sido extensivamente caracterizadas por sua capacidade de auxiliar no fornecimento de fósforo aos vegetais. Esses micro-organismos podem atuar tanto na mineralização de fósforo orgânico, quanto na solubilização do fosfato mineral. Essa habilidade é observada para grupos bacterianos taxonômicamente diversos, tais como *Bacillus, Paenibacillus, Pseudomonas, Rhizobium, Bradyrhyzobium, Enterobacter, Pantoea*, entre outros (Barea e Richardson, 2015; Oteino et al., 2015). A utilização dessas

bactérias como inoculantes agrícolas é apontada como uma importante estratégia para a redução do uso de fertilizantes fosfatados (Owen et al. 2015)

Embora o fitato seja o constituinte majoritário do fósforo orgânico presente no solo, outras formas orgânicas incluem ácidos nucleicos, fosfolipídeos, fosfomonoésteres, fosfodiésteres, fosfonatos, entre outros (Rodríguez e Fraga, 1999; Turner e Blackwell, 2013). O processo de mineralização ocorre principalmente pela produção de três grupos de enzimas: (1) fosfatases não específicas; (2) fitases e (3) fosfonatases e C-P liases, essas últimas relacionadas a degradação de compostos mais recalcitrantes, como fosfonatos que envolvem ligações do tipo P-C (Ingle e Padole, 2017)

As fosfatases inespecíficas (fosfohidrolases) atuam na quebra de ligações fosfoéster e fosfoanídricas e, conforme o pH ótimo de atividade, podem ser classificadas em fosfatases ácidas ou alcalinas, sendo as primeiras o grupo mais frequente (Khan et al., 2009; Behera et al., 2014). Já as fitases são fosfatases específicas que clivam as ligações entre o inositol e os resíduos de ácido fosfórico presentes no fitato. Entre os tipos mais comuns de fitases, 6-fitase e 3-fitase, a última é principal forma produzida por micro-organismos (Mukhametzyanova et al. 2012). A inoculação em campo com bactérias produtoras de fitases tem sido associada a um aumento na aquisição de fósforo e a um maior estímulo no crescimento vegetal (Singh e Satyanarayana, 2011).

O principal mecanismo descrito para a solubilização de fosfato mineral é a produção de ácidos orgânicos, como o acetato, lactato, malato, oxalato, citrato, glucanato e α-cetoglutarato, que representam alguns dos compostos mais proeminentes (Vassilev et al., 2014). Esses ácidos atuam deslocando os cátions (Fe^{3+}, Al^{3+}, Ca^{+2}) associados ao fosfato, permitindo, então, sua liberação sob a forma aniônica solúvel (Khan et al., 2014). A produção desses ácidos está condicionada, principalmente, à oxidação direta de açúcares de baixo peso molecular, como a glicose (Krishnaraj e Dahale, 2014).

O primeiro gene relacionado ao fenótipo de solubilização de fosfato mineral (MPS+) foi clonado por Goldstein e Liu (1987) a partir de *Erwinia herbicola*. Atualmente, os mecanismos bioquímicos e moleculares mais bem caracterizados em relação a essa característica envolvem a produção de ácido glucônico e seus derivados, pela via de oxidação direta da glicose (Sashidhar e Podile, 2010). A síntese desse composto ocorre através da enzima glucose desidrogenase (GDH), expressa no espaço periplasmático das

bactérias, e é dependente da pirroloquinolina quinona (PQQ) como co-fator. Sequencialmente, o ácido 2-cetoglucônico pode ser produzido a partir do ácido glucônico pela enzima gluconato desidrogenase (GADH) (Goldstein, 1995). Embora seja um mecanismo bastante comum e bem caracterizado em organismos gram-negativos, também tem sido descrito para estirpes solubilizadoras gram-positivas (Farhat et al., 2015).

1.3 Mecanismos indiretos de promoção do crescimento vegetal: biocontrole

Indiretamente, as bactérias endofíticas e rizosféricas também promovem o crescimento vegetal inibindo o crescimento de organismos invasores, suprimindo processos infecciosos e minimizando os efeitos deletérios provocados por fitopatógenos (Enebe e Babalola, 2019). Essa capacidade atribui a essas bactérias uma posição estratégica como agentes de controle biológico (Eljounaidi et al. 2016). Sua efetividade é demonstrada não apenas contra patógenos microbianos, já que as PGPB têm sido satisfatoriamente utilizadas no controle de insetos e nematoides (Ramjegathesh et al., 2013; Mhatre et al., 2019).

Os principais mecanismos relacionados à capacidade protetiva desses organismos envolvem: competição, antibiose, modulação dos mecanismos de defesa vegetal, produção de enzimas hidrolíticas e outros metabólitos (Prashar et al., 2013). Tanto PGPB quanto patógenos microbianos disputam os mesmos sítios de colonização nos vegetais hospedeiros. Ao ocupar esse ambiente, consumindo os nutrientes essenciais disponíveis, essas bactérias benéficas restringem o desenvolvimento dos patógenos competidores (Hassani et al., 2018). A já mencionada síntese de sideróforos por PGPB é um importante fator, também relacionado a esse processo, uma vez que a produção dessas substâncias limita a disponibilidade de ferro para o crescimento de micro-organismos eventualmente prejudiciais (Saha et al., 2016).

A antibiose se refere à atividade antagônica mediada pela produção de metabólitos microbianos com efeito inibitório direto sobre demais organismos susceptíveis (Fravel, 1988). O arsenal de biomoléculas relacionadas a esse processo em PGPB é vasto, envolvendo diversas classes de metabólitos (Raaijmakers e Mazzola, 2012). Diferentes *clusters* de genes biossintéticos (*Biosynthetic gene cluster* - BGC) de antimicrobianos estão presentes no genoma de espécies com capacidade de biocontrole, sendo os BGC relacionados à produção de policetídeos e peptídeos antimicrobianos os mais frequentes. Os últimos compostos podendo ser, ou não, de síntese ribossomal (Olanrewaju et al., 2017)

Os policetídeos englobam uma das mais extensas e numerosas classes de compostos naturais, incluindo poliéteres, polifenóis, polienos e macrolídeos. São sintetizados por complexos enzimáticos conhecidos como policetídeo sintase (PKS), que utilizam Acil-CoA como moléculas percursoras básicas (Hertweck, 2009; Miyanaga, 2017). Entre os peptídeos antimicrobianos produzidos por síntese ribossomal, as bacteriocinas constituem a principal classe e estão igualmente envolvidas em processos de biocontrole por PGPB. (Subramanian e Smith, 2015). Essas substâncias têm ação inibitória principalmente sobre bactérias taxonômicamente relacionadas. As bacteriocinas, positivamente carregadas, possuem alta afinidade pelas membranas citoplasmáticas, com cargas opostas, provocando a formação de poros letais, com a perda de controle celular sobre a difusão de substâncias. Sendo que esses antimicrobianos podem, também, atuar na inibição da síntese de parede celular bacteriana (Hassan et al., 2012; Martínez et al., 2016).

Os peptídeos não ribossomais são sintetizados por complexos enzimáticos conhecidos como NRPS (*nonribosomal peptide synthetases*) (Finking e Marahiel, 2004). Essas enzimas possuem uma organização distribuída em módulos, contendo os domínios catalíticos básicos (adenilação, tiolação e condensação) para incorporação sequencial de cada aminoácido específico na estrutura. Tipicamente, a síntese dessas substâncias pode envolver a inclusão de vários aminoácidos não proteinogênicos (Strieker et al., 2010; Süssmuth e Mainz, 2017).

Os lipopeptídeos são uma das classes mais importantes de derivados de peptídeos não ribossomais. Formados pela ligação de uma cadeia lipídica à um peptídeo cíclico ou linear, diferenciam-se tanto pela composição das sequências de aminoácidos, quanto nas características da porção hidrofóbica (Cochrane e Vederas, 2016). Algumas classes mais comuns são surfactinas, pumilacidinas, liquenisina, bacilomicina, micosubtilisina, iturinas e fengicinas. A atividade biológica de muitos lipopeptídeos é atribuída à capacidade desses compostos em provocar disfunções na membrana citoplasmática. O espectro de ação pode ser amplo, inibindo agentes patogênicos bacterianos, fúngicos e também virais (Meena e Kanwar, 2015; Mnif e Ghribi, 2015).

Outra classe de biomoléculas relacionadas ao antagonismo bacteriano são enzimas líticas, que têm um papel excepcionalmente importante na inibição de fungos patogênicos. As principais hidrolases envolvidas, quitinases (endo e exo - quitinases), β-1,3-glucanase e

proteases, atuam na degradação dos componentes majoritários da parede celular desses organismos (Brzezinska et al., 2014; Olanrewaju et al., 2017). Algumas PGPB são ainda capazes de colonizar extensivamente as hifas de fungos fitopatogênicos, formando micro-colônias e se estabelecendo como biofilme nessas estruturas. Ao parasitá-los, afetam diretamente a sobrevivência, germinação e esporulação desses micro-organismos, restringindo a colonização de plantas susceptíveis (Lugtenberg et al., 2013).

As PGPB também podem agir como importantes moduladores do sistema de defesa vegetal, induzindo um importante mecanismo de proteção que é a indução de resistência sistêmica (*Induced Systemic Resistance* - ISR). Esse estado torna as plantas menos susceptíveis a infecções subsequentes (Choudhary et al., 2007). Como resultado da ISR, ocorre uma maior produção de metabólitos envolvidos no processo de defesa, fortificação das paredes celulares e outras alterações fisiológicas responsáveis pela proteção a eventuais exposições a patógenos (Dey et al., 2014).

A produção de compostos orgânicos voláteis (*Volatile organic compound* - VOC) constitui outra importante via de biocontrole. Essas substâncias são compostos à base de carbono, de baixo peso molecular, e elevada pressão de vapor, que podem ter acentuada atividade antimicrobiana, mesmo em baixas concentrações (Kanchiswamy et al., 2015; Selim et al., 2017). São produzidos tanto por micro-organismos rizosféricos, como por endofíticos, se difundindo entre partículas do solo ou no interior de tecidos vegetais. O espectro de ação dos VOC é vasto e confere proteção a diferentes tipos de patógenos (Schulz-Bohm et al., 2017). Os VOC têm uma atuação especial sobre a fungistase, que trata-se da redução do crescimento ou germinação de propágulos fúngicos presentes no solo (Garbeva et al., 2011).

A proteção proporcionada pelos VOC envolve tanto uma atividade antimicrobiana direta, como indiretamente são capazes de modular a expressão de genes de defesa e incitar resistência sistêmica induzida (ISR) nos vegetais (Farag et al., 2013). São bastante diversificados os voláteis produzidos por bactérias, sendo que as principais classes envolvem: derivados de ácidos graxos (hidrocarbonetos, cetonas e álcoois), ácidos, compostos sulfatados, compostos nitrogenados e terpenos (Audrain et al., 2015). Os mecanismos bioquímicos e moleculares de ação dessas substâncias sobre os patógenos são diversificados e dependentes da natureza química da substância envolvida. Algumas classes

de compostos podem provocar coagulação citoplasmática, disfunção da membrana celular, afetar a atividade de proteínas ou enzimas específicas, entre outros (Gabriel et al., 2018)

Outra forma de biocontrole é ação de PGPB degradando agentes de virulência e patogenicidade (Saraf et al., 2014). Um clássico exemplo é a capacidade de bactérias biocontroladoras de *Sclerotinia sclerotiorum* e *Botrytis cinerea* em metabolizar o ácido oxálico, intensivamente produzido por esses fungos durante o processo de infecção dos hospedeiros, e que é apontado como importante fator de patogênese (Schoonbeek et al., 2007). Já em bactérias, a produção de moléculas de *quorum sensing* é uma importante via relacionada à expressão de genes de virulência e disseminação por fitopatógenos. Um importante modo de controle desses organismos por PGPB é a interferência no sinal de *quorum sensing* pela degradação das moléculas de autoindutores (Molina et al., 2003; LaSarre e Federle, 2013)

1.4 A cultura da canola: aspectos gerais e perspectivas

O gênero *Brassica*, família *Brassicacea*, envolve mais de 100 espécies vegetais já caracterizadas, incluindo algumas de grande interesse agronômico. As brássicas são fonte de uma extensa variedade de sementes, tubérculos, flores, frutos e folhagens destinados ao consumo humano e animal (Rakow, 2004; OGTR, 2008). Como destaque, esse grupo taxonômico inclui também diversas espécies, em especial a colza (*Brassica napus* L.), que são tradicionalmente cultivadas para extração de óleo vegetal, tanto para fins alimentícios como industriais (Sun, 2015).

A origem da colza é inicialmente proposta em 1935 dentro de um sistema posteriormente reconhecido como Triângulo U. Esse modelo estabelece que os três cruzamentos interespecíficos possíveis entre os diploides *Brassica rapa* (genoma A, n=10); *Brassica nigra* (genoma B, n=8) e *Brassica oleracea* (genoma C, n=9) supostamente deram origem aos três híbridos anfidiploides: *Brassica juncea* (genoma AB, n=18), *Brassica carinata* (genoma BC, n=17) e *Brassica napus* (genoma AC, n=19) (U, 1935; Cheng et al., 2014). Dentre essas espécies, além da colza, *B. juncea* e *B. rapa* também constituem importantes oleaginosas atualmente cultivadas (Brown et al., 2008).

Nas sementes de colza, o conteúdo de óleo pode corresponder de 40 – 50% da massa para a maioria das variedades comerciais (Jiang et al., 2014). No entanto, uma característica bastante difundida entre as espécies do gênero *Brassica* é o elevado teor de ácido erúcico

presente no óleo extraído. Essa substância de comprovada toxicidade, quando em altas concentrações, limita a utilização do óleo de colza para o consumo humano (Rahman et al., 2013; Knutsen et al., 2016). Além do ácido erúcico, as sementes de algumas espécies de *Brassica* apresentam também uma alta concentração de glucosinolatos. Essas substâncias constituem uma classe de metabólitos secundários envolvidos na resposta protetiva do vegetal à infecção por organismos patogênicos, mas que compromete sensivelmente a palatabilidade dos derivados da fração sólida dos grãos (Cartea e Velasco, 2008).

Como resultado do melhoramento genético da colza, surgiram no Canadá, durante a década de 1970, as primeiras variedades de *Brassica* com baixo teor de ácido erúcico e glucosinolatos. Essas novas variedades viriam a constituir o que é definido como canola. O termo foi originalmente registrado pelo *Western Canadian Oil Seed Crushers* e corresponde à abreviação da expressão *Canadian Oil Low Acid* (Boyles et al., 2012).

Por canola, atualmente compreendem-se variedades do gênero *Brassica* pertencentes às espécies *Brassica napus*, *Brassica rapa* e, mais recentemente, *Brassica juncea*. Essa denominação, no entanto, é, por definição, restrita apenas às variedades cujo óleo apresenta um perfil de ácidos graxos com menos de 2% de ácido erúcico, além de uma quantidade inferior a 30 micromoles de glucosinolatos por grama de massa seca das sementes (Knodel e Kandel, 2011).

O óleo de canola é considerado um dos óleos vegetais mais saudáveis entre os que são largamente produzidos para o consumo humano. A qualidade diferencial desse óleo é atribuída ao elevado teor de ácidos graxos monoinsaturados e às altas concentrações de ácido oleico, linoleico e alfa-linoleico, entre outras substâncias cardioprotetoras (Loganes et al., 2016). O consumo do óleo de canola é altamente recomendado pela Organização Mundial de Saúde, principalmente por estar associado ao controle dos níveis plasmáticos de colesterol e à consequente redução no risco de doença arterial coronariana (Lin et al., 2013).

Propriedades adicionais, como a alta concentração de ácidos graxos de cadeias longas (acima de 18 carbonos), atribuem ao óleo de canola um padrão singular de viscosidade, estabilidade e lubricidade. Essas características o tornam altamente apropriado para diversas aplicações industriais (Brown et al., 2008). Na Europa, o óleo de canola corresponde à principal matéria prima na geração de biodiesel, representando 57 – 70% da produção total desse biocombustível (Zentkovà e Cvengrosova, 2013). Além do aproveitamento comercial

do óleo, como co-produto do processo de extração, obtém-se também o farelo de canola a partir da fração sólida das sementes. O farelo de canola apresenta um elevado teor proteico, sendo amplamente utilizado na produção de ração animal (Campbell et al., 2016).

A canola constitui a segunda maior oleaginosa cultivada no mundo, atrás apenas da cultura da soja. Para o período de 2018/2019 a produção global foi de 70,91 milhões de toneladas, sendo o Canadá, União Europeia e China os maiores produtores (USDA, 2019). A produção brasileira de canola, em relação ao contexto internacional, é ainda bastante modesta. Com 35,5 mil hectares de área plantada, foi obtida uma produção nacional de 49,5 mil toneladas em 2018. Os maiores produtores são os estados do Rio Grande do Sul e Paraná (CONAB, 2019).

No país, o cultivo da canola tem se restringido à espécie *Brassica napus* L. var. oleifera, tendo sido introduzida como cultura de inverno (Tomm, 2007). Além dos rendimentos obtidos a partir da produção dos grãos, o cultivo da canola demonstra grande potencial para solução de problemas fitossanitários. Uma vez que as brássicas não apresentam susceptibilidade a diversos patógenos comuns a gramíneas (milho e trigo) e a leguminosas (feijão e soja), a plantação de canola em sistema de rotatividade com essas culturas reduz significativamente a incidência de doenças nas plantações subsequentes (Norton et al., 1999; Harker et al., 2014). Desse modo, a plantação de canola tem sido proposta no país como uma alternativa à cobertura de solos ociosos durante o inverno, na entressafra do cultivo de milho e soja (Tomm, 2007).

O potencial do Brasil para o cultivo da canola é bem maior que a atual área plantada. Embora essa cultura se encontre em modesta expansão, ainda permanece uma série de desafios que tem restringido uma exploração máxima da capacidade produtiva (Estevez et al. 2014). Uma tendência atual é a extensão das áreas de cultivo para regiões de baixa latitude. Esse processo, conhecido como tropicalização, tem demandado um intensivo programa de pesquisas agronômicas visando a seleção de genótipos mais bem adaptados e o aperfeiçoamento e introdução de novas práticas de cultivo (Tomm et al., 2008; Tomm et al., 2010).

Mesmo em regiões tradicionalmente produtoras, como o Rio Grande do Sul, a produtividade média atual é de 1398 kg ha^{-1}, valor ainda muito inferior ao rendimento preconizado para o país (2500 kg ha^{-1}). Em alguns países, como o Canadá, pode-se atingir

uma produtividade média de até 4500 kg ha^{-1} (Tomm et al., 2010; CONAB, 2019). Entre os principais gargalos para a consolidação da cultura da canola no Brasil, no que tange as necessidades de pesquisa agronômica, tem se destacado: a) Ausência do estabelecimento de condições de fertilização específicas para cada região; b) Controle de pragas do solo e da parte aérea; c) Falta de estudos que embasem o aumento de rendimentos, redução de perdas e ampliação da distribuição de cultivo pelo território brasileiro (Mori et al., 2014).

O cultivo da canola tem sido recomendado para solos de alta fertilidade e requer adubação intensiva para assegurar uma elevada produtividade, sendo a fertilização a principal demanda no plantio (Tomm et al., 2009; Norton, 2016). No mundo, a produção e uso de fertilizantes no plantio da canola são apontados como os responsáveis pelos maiores impactos ambientais associados a essa cultura (MacWilliam et al., 2016). O uso de adubos industriais também é indicado como um dos principais fatores que impactam negativamente o balanço energético da cultura, dentro de estimativas que avaliam o potencial uso da canola para produção de biodiesel no Brasil (Silva et al., 2017).

A cultura da canola é susceptível a diferentes doenças fúngicas e bacterianas, responsáveis por perdas econômicas severas em todo o mundo. No Brasil, o primeiro e mais amplo levantamento fitossanitário relacionado a essa cultura no país foi realizado por Cardoso et al. na década de 1990. Entre os agentes fitopatogênicos encontrados foram identificados os fungos *Sclerotinia sclerotiorum*, *Alternaria brassicae*, *A. raphani* e *A. alternata*, *Erysiphe polygoni*, *Rhizoctonia solani*, *Albugo cândida* e *Phoma* sp. Sendo também identificado *Xanthomonas campestris* pv. *campestris* como o principal patógeno bacteriano, e já conhecido agente etiológico da podridão negra das crucíferas (Cardoso et al., 1996).

No mundo, a canela-preta (*Black leg*) e a podridão das hastes – SSR (*Sclerotinia stem rot*), também conhecida por mofo-branco, causadas, respectivamente pelos fungos *Leptosphaeria maculans* e *Sclerotinia sclerotiorum*, constituem as mais proeminentes e devastadoras doenças da canola. No Brasil, o fungo *Leptosphaeria maculans* foi responsável por prejuízos acentuados no início dos anos 2000, com destruição parcial ou total de lavouras no Rio Grande do Sul (Tomm et al., 2009). A estirpe de ocorrência no Brasil e demais países sul americanos foi identificada como pertencente ao mesmo grupo de patogenicidade de ocorrência na Austrália (Fernando et al., 2003).

A implementação de variedades com resistência poligênica a *Leptosphaeria maculans*, a partir de híbridos australianos, proporcionou maior segurança no cultivo da canola no Brasil em relação a ocorrência da canela preta (Tomm et al., 2009). No entanto, entre as principais variedades atualmente cultivadas no Brasil, a susceptibilidade a *Sclerotinia sclerotiorum* é ainda bastante variável, não havendo nenhuma cultivar com completa resistência ao patógeno (Silveira et al., 2016). De fato, o mofo-branco causado por *S. sclerotiorum* e a podridão negra das crucíferas são, recentemente, as doenças mais relatadas entre os produtores no país. Sendo ainda a primeira, a que mais demandou por métodos de controle e uso de fungicidas (Mori et al., 2017)

A introdução de inoculantes agrícolas tem promovido ganhos significativos em associação a algumas culturas de interesse econômico, sendo bastante cogitados como uma possibilidade para o aumento da produtividade de *Brassica* (Hunter et al., 2014). Para a cultura da canola, têm sido obtidos resultados experimentais satisfatórios envolvendo a inoculação de PGPB, tanto para promoção direta de crescimento vegetal, como biocontrole (Bertrand et al., 2001; El-Howeity e Asfour, 2012; Ahmadi-Rad et al., 2016; Sun et al., 2017). Isso tem ascendido o interesse na busca por estirpes microbianas com potencial para uso nas variedades localmente empregadas, dentro das condições de cultivo brasileiras.

1.5 *Sclerotinia sclerotiorum* e a podridão das hastes em canola

S*clerotinia sclerotiorum* (Lib.) de Bary é um fungo filamentoso necrotrófico capaz de infectar mais 400 espécies de plantas, de 278 gêneros distintos e 75 famílias, constituindo um dos mais importantes e cosmopolitas fitopatógenos do mundo. Além da canola, alguns de seus principais hospedeiros agregam espécies de grande importância agronômica, incluindo demais oleaginosas, como a soja e o girassol (Boland,1994; McCaghey et al., 2018; Na et al., 2018).

S. sclerotiorum apresenta uma importante vantagem adaptativa, que é a formação de estruturas de resistência conhecidas como escleródios. Essas estruturas tornam a permanência do fungo bastante persistente nos campos, dificultando enormemente os métodos de controle da doença. Os escleródios são aglomerados de hifas, recobertas por uma rígida e mielinizada camada de proteção. A região interna da estrutura, também conhecida como medula, por sua vez, constitui-se de células fúngicas envoltas em uma matriz rica em β-glucana e proteínas (Ordóñez-Valencia, 2015; Sharma et al., 2015). Uma vez no solo, em

profundidades maiores que 5 centímetros, os escleródios podem se manter viáveis por 3-5 anos, podendo mesmo sobreviver por períodos superiores de até 10 anos (Saharan e Mehta, 2008)

Os escleródios podem germinar de duas maneiras distintas, relacionadas às formas reprodutivas do fungo, seja sexuadamente (germinação carpogênica de escleródios) ou assexuadamente (germinação misceliogênica de escleródios) (Bolton et al., 2006). No primeiro caso, há a formação de corpos de frutificação conhecidos como apotécios. Nessas estruturas ocorre a formação de ascósporos, por reprodução sexuada, e esses se disseminam entre os hospedeiros através do ar. No segundo caso, há emissão de hifas a partir do escleródio, essas se desenvolvem até atingir diretamente a base das plantas susceptíveis, dando início ao processo de infecção (Rakesh et al., 2016).

Os ascóscoporos conseguem se manter nos locais em que se depositam, mas por períodos relativamente curtos, desde que havendo condições ambientais favoráveis. Eles apresentam, no entanto, uma capacidade limitada de crescer sobre estruturas vegetais íntegras (Jamaux et al., 1995). Normalmente, infectam os hospedeiros durante a etapa de florescimento, utilizando as flores como fonte nutricional primária. Uma vez infectadas, as pétalas se desprendem, depositando-se sobre folhas e ramificações caulinares, permitindo, assim, a dispersão do fungo no hospedeiro (Jamaux et al., 1995; Saharan e Mehta, 2008).

As regiões infectadas desenvolvem lesões iniciais (*water-soaked lesions*), que são posteriormente tomadas pelo aspecto branco característico do desenvolvimento micelial de *S. sclerotiorum*. A parte aérea da planta pode ser amplamente tomada pela doença. Os caules, quando infectados, sofrem as lesões características da podridão das hastes. Nesse estágio, devido à fragilização da estrutura, podem ocorrer quebras. Nos estágios finais, há aglomeração de hifas e formação de escleródios, que, posteriormente, são dispersos no solo com a morte da planta ou colheita (Purdy, 1979; Kamal et al., 2016). A perda causada pela SSR (*Sclerotinia stem rot* - SSR) varia, principalmente, em função do estágio de desenvolvimento em que as plantas são afetadas. Se a infecção ocorre antes ou nos estágios iniciais de florescimento, a perda é elevada, podendo em casos extremos, atingir 100% da produção. Quando a doença se manifesta após o florescimento, as perdas são minimizadas, mas podem acometer 50% da geração de grãos (Shukla, 2005)

Os principais fatores de patogênese da doença se relacionam à liberação de enzimas hidrolíticas e à produção de ácido oxálico. Como fungo necrotrófico, *S. sclerotiorum* nutre-se, principalmente, da degradação tecidual de seus hospedeiros. Durante o processo de infecção, são liberadas algumas enzimas extracelulares, tais como celulases, pectinases e xilanase, que atacam a parede celular vegetal (Huang et al., 2008). A produção de ácido oxálico é, também, um fator fundamental nesse processo, uma vez que a redução do pH resultante de sua liberação favorece a atividade dessas hidrolases. Além disso, esse composto está relacionado ao sequestro de íons cálcio (Ca^{+2}), favorecendo a desestabilização da parede celular (Dutton e Evans, 1996; Liang e Rollins, 2018). Ainda, atua também como um fator de proteção ao fungo contra a resposta oxidativa desencadeada pelo mecanismo de defesa da planta afetada (Cessna et al., 2000).

O controle de *S. sclerotiorum* associado à cultura da canola envolve diferentes estratégias de manejo. O processo é bastante dificultado pela alta persistência do fungo no solo, pela baixa eficiência e custos elevados na aplicação de antifúngicos, além da inexistência de variedades com resistência total ao patógeno. Isso torna o biocontrole uma das alternativas mais apreciáveis no manejo do mofo branco (Smolińska e Kowalska, 2018). Resultados positivos têm sido obtidos com a utilização de fungos antagonistas, como *Coniothyrium minitans,* já disponível comercialmente (Li et al., 2006). Da mesma forma, tem sido bastante satisfatória a utilização de algumas estirpes bacterianas, especialmente do gênero *Bacillus* (Kamal et al., 2015).

2 OBJETIVOS

2.1 Objetivos gerais

Caracterizar isolados bacterianos de raízes de canola quanto a diferentes aspectos fenotípicos e genômicos relacionados à promoção do crescimento vegetal e à capacidade de biocontrole.

2.2 Objetivos específicos

- Realizar a identificação molecular e análises filogenéticas de bactérias associadas a raízes de canola;
- Promover a triagem *in vitro* dos isolados quanto a diferentes características de promoção do crescimento vegetal;
- Avaliar os isolados bacterianos quanto ao potencial de inibição do crescimento do fungo *Sclerotinia sclerotiorum* (Lib.) de Bary;
- Caracterizar as bactérias associativas de canola quanto à capacidade de solubilização de fosfatos minerais;
- Investigar aspectos genômicos relacionados às propriedades de biocontrole e promoção do crescimento vegetal.

3 CAPÍTULOS

3.1 PRIMEIRO CAPÍTULO

Characterization of antifungal activity against *Sclerotinia sclerotiorum* (Lib.) de Bary and plant growth promoting abilities by bacterial isolates from canola (*Brassica napus* L.) roots.

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Abstract

Endophytic bacteria show important abilities in promoting plant growth and suppressing phytopathogens. Therefore, these microorganisms are largely explored in agriculture as biofertilizers or biocontrol agents of important crop diseases. Here we isolated spore-forming bacteria from canola roots and screened them for different plant growth promotion (PGP) traits and biocontrol of *Sclerotinia sclerotiorum*. Thirty isolates were obtained belonging to four different genera: *Bacillus, Paenibacillus*, *Lysinibacillus,* and *Microbacterium*. Several isolates produced auxin, siderophores, hydrolytic enzymes, and also fixed nitrogen and solubilized phosphate. Five isolates presented antifungal activity against *S. sclerotiorum* by the dual culture assay and 4 isolates also inhibited fungal growth by VOCs production. All antagonistic isolates belonged to the *Bacillus* genus, based on 16S rRNA gene analysis. The genomes of these isolates were sequenced for the search of gene clusters related to antimicrobial metabolites using the antiSMASH tool. Three isolates were identified as *Bacillus safensis*, one as *Bacillus pumilus* and another as *Bacillus megaterium*, using the genomic metrics ANI and dDDH. Most strains showed several common gene clusters, including bacteriocin, PKS and NRPS clusters, related to pumilacidin, bacillibactin, bacilysin, and other antimicrobial compounds. However, in the evaluated conditions, no lipopeptides were detected on solid culture extracts by MALDI-TOF analysis. The ability of the isolates to protect canola from soil-borne *S. sclerotiorum* were evaluated in growth-chamber conditions and no isolate displayed biocontrol or PGP activity. The genomic features demonstrated the potential of these isolates in the suppression of plant pathogens; however, some aspects of plant-bacterial interactions remain to be elucidated.

Key-words: *Bacillus;* non-ribosomal peptides; endophytic; white mold

1 Introduction

Canola is one of the major oilseed crops produced in the world. Despite being first introduced in Brazil in in the 1970s as a winter crop, Brazilian production is still modest if compared with the world's largest canola producers, such as China, India, Canada, and EU (De Mori et al. 2014). Several efforts have been employed to increase Brazilian canola yields and enlarge the cropping areas to non-traditional producing regions. However, many challenges remain to consolidate canola's production in Brazil, including technical difficulties, the need of proper management practices for different Brazilian environmental conditions, and the control of canola diseases (Tomm et al. 2009). One of the most important canola diseases is the white mold or Sclerotinia stem

rot, caused by the necrotrophic fungus *Sclerotinia sclerotiorum*. This disease is also responsible for several losses in different cropping areas around the world (Sharma et al. 2015). *S. sclerotiorum* is one of the most relevant, devastating, and cosmopolitan plant pathogens, infecting more than 400 plant species (Boland and Hall 1994). In Brazil, the susceptibility of canola to *S. sclerotiorum* is indicated as an important limiting factor for canola cropping expansion to new cultivable areas (Mainardes et al. 2018). Canola can either be infected by air-borne ascospores, released by apothecia produced via carpogenic germination of sclerotia, or directly by myceliogenic germination of soil-borne sclerotia. The sclerotia is resistant to diverse environmental and chemical factors and allows *S. sclerotiorum* to survive in soils for many years, which constitutes the most difficult factor involved in disease management (Kamal et al. 2016). The absence of safe and effective synthetic products for complete eradication of *S. sclerotiorum* from soils highlights the importance of biological control agents. These methods are more environmentally secure than traditional chemical control and have been successfully employed for *S. sclerotiorum* management (Smolińska and Kowalska 2018).

Not only the rhizobacteria community that live in the soil region surrounding roots, but also the endophytes that are present inside plant tissues, are a plentiful source of plant beneficial micro-organisms displaying inhibitory activity against phytopathogens (Elshahat et al. 2016; Afzal et al. 2019). In addition to protecting plants, these microorganisms can promote plant growth by several mechanisms, including phytohormone production, solubilization of nutrients from the soil, nitrogen fixation, and also reduction of plant stress in different conditions (Vejan et al. 2016). Plant growth promoting bacteria (PGPB) can produce several inhibitory metabolites, such as hydrolytic enzymes that degrade the cell-wall of phytopathogenic fungi, volatile organic compounds (VOCs), siderophores, and different classes of antibiotics and related compounds (Saraf et al. 2014). These beneficial bacteria can also trigger induced systemic resistance (ISR) in plants, preventing their host from subsequent infections by harmful microorganisms (Choudhary et al. 2007).

Bacillus and *Paenibacillus* are gram-positive spore-forming bacteria, that include different species of PGPB and efficient biocontrol agents (McSpadden Gardener 2004). Species from both genera are able to produce a wide variety of bioactive metabolites with antifungal properties, especially non-ribosomal peptides, such as lipopeptides (LPs) (Cochrane and Vederas 2016; Olishevska et al. 2019). The main classes of *Bacillus* LPs comprise the surfactant and antimicrobial surfactin, iturin, and fengycin. Most importantly, iturin and fengycin are promising antifungal compounds (Ongena and Jacques 2008). LPs are composed of a peptide segment linked to a fatty acid chain and differ by the size of the fatty acid tail or by the amino acid sequence (Mnif and Ghribi 2015). The peptide component of these metabolites is produced by an enzymatic complex, called NRPS (non-ribosomal peptide synthetase). NRPS biosynthetic gene clusters (BGC) are highly diverse and allow the production of a wide variety of LPs homologs (Bloudoff and Schmeing 2017). LPs production can be the main mechanism of plant protection conferred by several antagonistic *Bacillus* strains against phytopathogenic fungi (Shafi et al. 2017). The aim of this work is to isolate and characterize bacteria from canola roots and evaluate their *in vitro* plant growth promoting abilities and biocontrol potential against *S. sclerotiorum* (Lib.) de Bary.

2 Materials and methods

2.1 Isolation

Roots of *Brassica napus* (Hyola 61) at the flowering stage were collected from a canola-producing region, in Vacaria, Rio Grande do Sul State, Brazil (28º 30' 44" S, 50º 56' 02" W). The roots were washed in running tap water and superficially disinfected by immersion in 70% ethanol (1 min) and 4% hypochlorite solution (2 min), followed by rinsing five times with sterile distilled water. After disinfection, the roots were sliced and 10 g were placed into Erlenmeyers flasks containing 90 ml of sterile saline solution (0.85% NaCl). Flasks were shaken for 24 h at 4°C and the suspensions were pasteurized (80°C, 10 min) to eliminate non-sporulating bacteria.

Serial dilutions were performed and inoculated on thiamine-biotin agar (TB N-free medium, Seldin et al. 1983) and Starch Casein Agar (SCA, Küster and Williams 1964). The plates were incubated in anaerobic jars for 7 days at 28 ºC. Bacterial colonies showing distinct morphological traits were inoculated and isolated on King B medium (KB, Glickmann and Dessaux 1995). Bacterial cultures were stained using the Gram method to certify their purity and afterward stored in 20% sterile glycerol at −20°C.

2.2 PGP characteristics

The isolates were screened for auxin and siderophore production, phosphate solubilization, nitrogen fixation, and for the production of different hydrolytic enzymes related to rhizosphere competence. Indoleacetic acid (IAA) production was evaluated by colorimetric assay, according to the methodology described by Glickmann and Dessaux (1995) and adapted by Ambrosini et al. (2012). The microorganisms were cultivated for 48 h in KB medium supplemented with L-tryptophan. After that, the culture supernatant was collected and mixed with an equal volume of Salkowski reagent (12 g L^{-1} $FeCl_3$ + 7.9 M H_2SO_4). IAA concentration was determined by spectrophotometry at 530 nm after calibration with a standard curve.

For the phosphate solubilization assay, isolates were spot inoculated on NBRIP agar plates (Nautiyal 1999). After 7 days of incubation, the presence of solubilization haloes around the colonies was considered a positive result. The method described by Schwyn and Neilands (1987) was used to evaluate siderophore production. The isolates were inoculated on Chromo Azurol S agar plates. After 48-72 h of incubation, those isolates that formed a yellow halo around their colonies were considered to be siderophore producers.

The nitrogen fixation ability was verified using the acetylene reduction assay (ARA), according to Boddey and Knowles (1987) and adapted by Fernandes et al. (2014). Isolates were cultivated for 48 h in vials containing TBNR medium (Seldin et al. 1983). Then, flasks were closed with a rubber septum and acetylene gas (C_2H_2) was added to 10% of the air phase. After 24 h of incubation, ethylene (C_2H_4) was measured by gas-chromatography (Clarus 600, Perkin Elmer) with a Col-Elite-Alumina column (50 m× 0.53 mm ID× 10 µm). The cultivated medium was sampled, and bacterial cells were lysed with 0.2 M NaOH solution. Afterward, protein concentration was measured by the Bradford method and results were expressed as nmol C_2H_4 mg $protein^{-1}$ h^{-1}.

The ACC deaminase activity was verified using the DF salt medium supplemented with 1-aminocyclopropane-1-carboxylic acid (0.5 M) as sole nitrogen source (Penrose and Glick 2003). Isolates were previously cultivated in KB medium and bacterial cells were centrifuged, washed three times with saline

solution, and spot inoculated on agar plates. Bacterial growth after 5 days was an indication of a positive result. Inoculated plates without ACC were used as a negative control.

Bacteria were evaluated for hydrolytic enzyme production using enzymatic plate assay, according to Bach et al. (2016). Isolates were spot inoculated on different media containing starch, carboxymethyl cellulose, xanthan, and skim milk, for amylolytic, cellulolytic, xanthanase, and proteolytic activity, respectively. When appropriate, reagents were added to reveal haloes (Cadmus et al. 1982; Blanco and Pastor 1993; Mac Faddin 2000; Bach et al. 2011). The presence of a clear zone around the isolates indicating substrate degradation was considered as hydrolytic enzyme production.

2.3 Bacterial identification and phylogenetic analysis

Genomic DNA was extracted by the phenol-chloroform method, according to Sambrook and Russell (2001) and Alippi and Aguilar (1998). For bacterial identification, partial 16S rRNA gene sequence was amplified (Ambrosini et al. 2012). Complete gene sequences of selected isolates were also analyzed. The PCR reactions and sequencing were performed according to Bach et al (2011). The approximately 1500- bp complete sequences of 16S rRNA gene were assembled and analyzed with the Codon code Aligner® (CodonCode Corporation). Partial and complete 16S rRNA sequences were submitted to a Basic Local Alignment Search Tool (BLAST) at the NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and EzBioCloud (www.ezbiocloud.net/) databases for bacterial identification. The type strain sequences of the most closely related species were obtained from GenBank and used for phylogenetic analysis. The sequences were aligned using the SINA Software from Silva platform (https://www.arb-silva.de/aligner/) and phylogenetic trees were generated by MEGA 7 Software (Kumar et al. 2016) using the maximum-likelihood method and a bootstrap method with 1000 replications. The nucleotide sequences of the partial and complete 16S rRNA genes determined in this study were deposited in the GenBank database.

2.4 *Sclerotinia sclerotiorum* growth inhibition on plate assay

Antifungal activity of the isolates was assessed on Potato Dextrose Agar (PDA, KASVI®) plates using a dual culture technique, according to Vinodkumar et al. (2017) with adaptations. Five-day-old fungal discs (5 mm diameter) of previously PDA cultured *S. sclerotiorum* (Lib) de Bary were placed on the edge of 9-cm-diameter Petri dishes while bacterial isolates were streaked on the opposite side. Mycelial growth diameter was measured after incubation at 28°C for 7 days. Plates without bacterial inoculum were used as control. The percentage of fungal inhibition (FI) was calculated according to Kumar et al. (2012): $FI = 100 \times C - T/C$ (T, fungal colony diameter of treatment; C, fungal colony diameter of control). The assay was performed with five replicates.

2.5 Volatile Organic Compounds (VOCs) production

VOCs production was evaluated by dual Petri dish assay (Raza et al. 2015). Bacterial inoculum from an exponential growth culture was spread onto PDA plates. A five-day-old fungal disc (5 mm diameter) of previously cultivated *S. sclerotiorum* was placed in the center of other PDA plate. Plates inoculated with

bacteria constituted the lid of plates containing fungal discs. All plates were sealed with parafilm and were incubated for 5 days. Plates without bacterial inoculum were considered the control. The percentage of inhibition of the mycelial growth was determined considering the difference between treatment and control, according to the formula described by Gotor-Vila et al. (2017): (C-T/C) X 100, where C is the fungal colony diameter of the control, and T is the measurement of the fungus with bacterial treatment.

2.6 Inhibition of other filamentous fungi on plate assay

Antifungal activities against other filamentous fungi were verified according to Bach et al. (2016). Approximately, 10^4 spores of *Aspergillus flavus*, *Aspergillus niger*, *Penicillium herquei*, *Penicillium chrysogenum*, and *Bipolaris sorokiniana* were seeded on PDA plates followed by inoculation of 20 µL of each bacteria in the exponential growth phase. Plates were incubated and evaluated after 48 h. Fungal inhibition zones around the bacterial colonies were checked and considered as the positive result of the antagonistic activity.

2.7 Genome sequencing and analysis

Genomic DNAs from selected isolates were sequenced in the MiSeq Illumina platform using the MiSeq Reagent kit v3 (2 x 300). The genomes were assembled using A5 (Coil et al. 2014) and SPAdes softwares (Bankevich et al. 2012). Best quality assemblies were chosen using CheckM (Parks et al. 2015) and QUAST (Gurevich et al. 2013) softwares. Draft genomes were submitted to the Rast server (Aziz et al. 2008) for an automatic annotation and to antiSMASH (Weber et al. 2015) for antimicrobial biosynthetic gene clusters searching. For taxonomic comparisons using genome metrics, the genomes of the most similar species according to the 16S rRNA gene sequence analysis were obtained from the NCBI database. Identification of isolates was corroborated by Average Nucleotide Identity (ANI) and digital DNA:DNA hybridization (dDDH), considering species cutoff of 95 and 70%, respectively (Sant'Anna et al. 2019). ANI values based on BLAST and MUMmer alignments were calculated at JSpeciesWS (http://jspecies.ribohost.com/jspeciesws). Estimations of dDDH were performed at http://ggdc.dsmz.de/distcalc2.php based on alignment BLAST+ and recommended formula 2 (Bach et al. 2017). All taxonomic analyses were carried out comparing the sequences of our canola strains with the type strain of each species available in the NCBI.

2.8 Lipopeptides detection by MALDI-TOF

Detection of lipopeptides synthesis on solid agar culture was performed according to Torres et al. (2016). First, bacterial isolates were submitted to dual culture technique as described in section 2.4. Then, agar discs (5 mm) removed from the inhibition zone between bacterial streak and fungal colony edge were suspended in 0.5 mL of 100 % acetonitrile (ACN). This suspension was vigorously shaken for 30 s and incubated overnight at 4ºC before being centrifuged and concentrated in a vacuum centrifuge (SpeedVac). Agar discs excised from plates with bacteria growing alone were used as the control.

For each sample, three agar plugs were extracted with 100% ACN and analyzed by MALDI-TOF/TOF mass spectrometer (MS). Aliquots of 1 µl of each sample were mixed with 1 µl of 10 mg mL^{-1} α-cyano-4-hydroxycinnamic acid (CHCA) (Sigma-Aldrich) solution prepared in TA30 (30:70 v/v ACN: 0.1%

Trifluoroacetic Acid in water). Then, 1 µl of this mixture was spotted onto a stainless steel MALDI sample plate and allowed to dry at room temperature. The snapshots were acquired in a range of m/z 500-2500 by a MALDI-TOF/TOF MS (Autoflex Speed, Bruker) operating in linear positive mode. Each spectrum was the sum of at least 2000 laser shots. All the spectra were externally calibrated using a commercial mixture of Peptide Calib Standard mono (Bruker).

2.9 Growth chamber biological control assay

Canola seeds, variety Hyola 61, were surface-sterilized by treating with 70% ethanol and 4% hypochlorite solution and pre-germinated on filter paper (germitest) for 3 days. After that, seedlings were transplanted to the center of 500 mL pots containing a nutritive substrate for plants (Carolina Soil®). The fungal inoculum was prepared as described by Ávila et al. (2005). Fungal discs of *S. sclerotiorum* were inoculated in 250 mL Erlenmeyrs flasks containing rice grains hydrated with 60% distilled water (w/v), and incubated for 2 weeks. Four rice grains with mycelial growth of *S. sclerotiorum* were equidistantly inoculated around the canola seedlings at 1 cm depth. Afterward, 5 mL of bacterial cultures grown in King B medium were diluted to a concentration of 10^8 cell mL^{-1} for inoculation on each pot. Two treatments without bacterial inoculum were used: while one of them was inoculated with *S. sclerotiorum*, the other received no fungal inoculum. Both treatments received 5 mL of sterile culture medium. Plants were cultivated for 20 days with 12-h photoperiod at 24ºC± 3ºC. The percentage of plant's survival was verified. Shoot and root sizes, as well as dry weight were also measured.

2.10 Statistical analysis

Both the morphological parameters measured on the biological control assay and the percentage values of fungal inhibition on the dual culture challenge were submitted to one-way analysis of variance and the means were compared by the Tukey test at 5% error probability. Analyses were performed using the software Statistica 7.0 (StatSoft).

3 Results

3.1 Isolation, identification and PGP traits

A total of thirty bacterial isolates, belonging to four different genera of gram-positive bacteria, were obtained using a selective isolation procedure for spore-forming bacteria (Table 1). The genus *Bacillus* was the predominating taxonomical group, representing 80% of total isolates, followed by *Paenibacillus*, with approximately 14% of the rhizobacteria. *Lysinibacillus* and *Microbacterium* were the least abundant genera, since only one isolate belonging to each group was obtained. These isolates were screened for different PGP traits. All of them were capable to produce at least one hydrolytic enzyme, revealing an important ability related to rhizosphere competence (Supplementary Table 1).

With regards to the other PGP characteristics, phosphate solubilization was the most widespread feature between isolates, followed by siderophore production, auxin synthesis and antifungal activity. Only three *Paenibacillus* strains were able to fix nitrogen by ARA, while ACC deaminase production was not

detected. Phylogenetic analysis revealed that most of the *Bacillus* isolates were very closely related to the *Bacillus cereus* complex (Supplementary Figure 1). Since this group includes well-characterized human pathogenic species, all related isolates (10) were excluded from further biocontrol experiments.

Table 1 Abundance of canola roots isolates showing each PGP trait

Genus	Nº isolates	P solub.	Sider.	Hydrolytic Enzymes*	IAA	N fixation	ACC deaminase	Antifungal Activity†
Bacillus	24	22	18	24	10	0	0	9
Paenibacillus	4	4	1	4	2	3	0	0
Lysinibacillus	1	1	0	1	1	0	0	1
Microbacterium	1	1	1	1	0	0	0	0
Total	30	28	20	30	13	3	0	10

P solub.: phosphate solubilization; Sider.: siderophores production; IAA: indoleacetic acid production; N fixation: Nitrogen fixation. * Isolate producing at least one of the tested enzymes (Protease; cellulase; xanthanase and amylase).† Antifungal activity against at least one of the tested filamentous fungi (*Aspergillus flavus*, *Aspergillus niger*, *Penicillium herquei*, *Penicillium chrysogenum*, and *Bipolaris sorokiniana*)

3.2 Antifungal activity against *S. sclerotiorum*

Five isolates showed antifungal activity against *S. sclerotiorum* by dual culture assay. Fungal growth inhibition ranged from 20 to 70%. The highest value was obtained to isolate 32PB. The other isolates did not differ statistically among them on the percentage of inhibition (Figure 1). These isolates were submitted to VOCs production assay by dual plate technique and all of them presented positive results, except for isolate 7PB. The percentage of fungal inhibition by VOCs production did not differ statistically between isolates (Figure 2).

The five antagonistic isolates were identified as belonging to the *Bacillus* genus. Phylogenetic analysis based on the complete sequence of 16S rRNA gene revealed that three isolates (1TAZ, 8TAZ, and 32PB) are related to *Bacillus safensis*. 7PB was clustered with *Bacillus pumilus* and *Bacillus zhangzhouensis*, while 16PB was related to *Bacillus aryabhattai* and *Bacillus qingshengii* (Figure 3). The general PGP features of these isolates are shown in Supplementary Table 1. All of them solubilized phosphate and produced protease. Three isolates (01TAZ, 08TAZ, and 7PB) were also able to produce siderophores and showed antifungal activity against other tested filamentous fungi (*A. niger*, *A. flavus*, and *P. herquei*), two of them (01TAZ and 08TAZ) also inhibited the growth of *P. chrysogenum.*

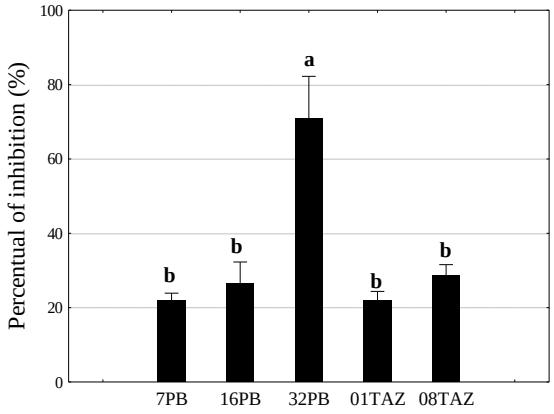


Figure 1 *S. sclerotiorum* inhibition by dual culture assay**.** Mean values followed by the same letter don't differ statistically among themselves by Tukey's test at a 5% significance level.

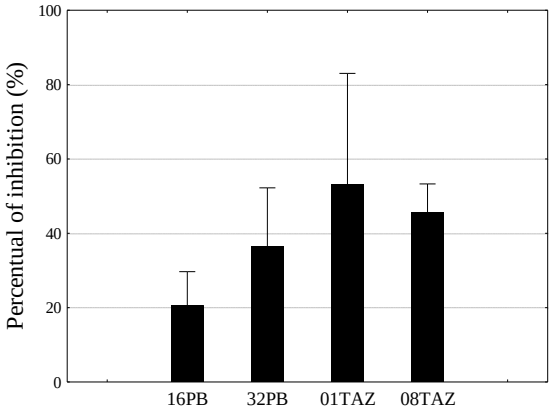


Figure 2 *S. sclerotiorum* inhibition by VOCs production. Bars represent means ± SE. Mean values don't differ statistically among themselves by Tukey's test at a 5% significance level

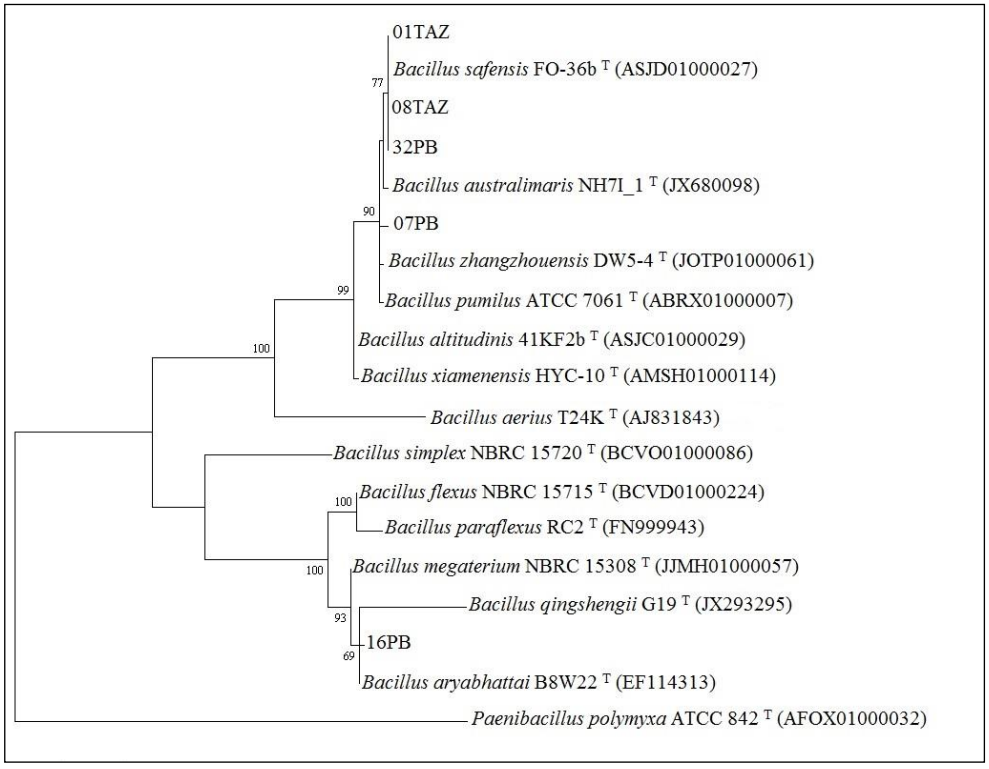


Figure 3 Bacterial phylogenetic tree based on the complete 16S rRNA gene sequences. The phylogenetic analysis was performed using the Maximum Likelihood method based on the Jukes-Cantor model. *Paenibacillus polymyxa* was considered the outgroup. Bootstrap confidence values above 60 are shown near each branch.

3.3 Genome sequencing and analysis

The genome sizes of antagonistic isolates ranged from 3.66 to 5.9 Mb and presented the general features as shown in Supplementary Table 2. Genomic metrics were used to confirm the identity of these

bacteria. The isolates 01TAZ, 08TAZ, and 32PB were identified as *Bacillus safensi*s strains, with ANI values of 98.84, 98.85, 98.84%, respectively, and dDDH values of 96.26, 96.27 and 96.28%, respectively (Supplementary Table 3). The isolate 16PB was identified as *Bacillus megaterium*, with ANI and dDDH values of 95.77 and 81,13%, respectively, while the isolate 7PB was identified as *Bacillus pumilus* with ANI value of 95.28 %. However, the dDDH value (65.09%) for this isolate was below the taxonomic threshold (70%).

Genome mining using antiSMASH predicted a total of 11 BGCs for *B. safensis* strains (1TAZ and 8TAZ), 12 for *B. pumilus* 7PB and *B. safensis* 32PB, and 9 for *B. megaterium* 16PB (Table 2). Most BGC were common for all *B. safensis* and *B. pumilus* strains. However, the 32PB isolate showed an extra bacteriocin (plantazolicin) biosynthetic cluster and the isolate 7PB presented an NRPS-Type 1 Polyketide Synthase (PKS) hybrid cluster, displaying low levels of similarity (18%) with the antifungal antibiotic zwittermycin A (He et al. 1994). Besides that, all these four closely related genomes harbored 4 NRPS clusters, 3 of them were predicted to be related to the antimicrobial compounds bacilysin, the surfactin-related lichenysin, and fengycin. The fourth NRPS biosynthesizes the siderophore bacillibactin (100% of similarity). Additionally, another siderophore BGC was found in all five genomes; although related to the production of an NRPS- independent siderophore of the lucA_lucC family.

For *B. pumilus* and *B. safensis* strains, other ribosomal BGCs are putatively related to the synthesis of 2 bacteriocins (a sactipeptide- head-to-tail and an unknown circular compound), 2 terpenes (related to carotenoid biosynthesis and a terpene-cyclase), and an unknown betalactone. There was also an unknown T3PKS putatively having a chalcone and stilbene synthase domain protein. For *B. megaterium* 16PB no NRPS gene cluster was found. However, this isolate displayed 4 unique BCGs, which was not found in other genomes. These BCGs include a terpene gene cluster (phytoene-surfactin-related), one bacteriocin (lanthipeptide) cluster, and two unknown gene clusters related to siderophores and phosphonate production. Interestingly, most canola strains' BGC were annotated with low similarity values and could actually be producing new homologs of known molecules.

The predicted amino acid and acyl-CoA monomers that comprise the backbone of metabolites produced by NRPS and PKS clusters were the same for all *B safensis* and *B. pumilus* isolates. AntiSMASH could only predict substrate and modules of bacillibactin, zwittermicin A, and surfactin-related BGC (Supplementary Table 4). The annotation of bacillibactin and the surfactin-related compound were corroborated by the monomer composition fingerprint analysis of the NORINE database.

The surfactin class of antibiotics comprises various similar LP compounds, such as lichenysin produced by *Bacillus licheniformis*, and pumilacidin, produced by *B. pumilus* and *B. safensis* (Jacques 2011). Comparative analyses of *Bacillus* strains show that the main difference between pumilacidin and lichenysin and surfactin BGC is the presence of two genes, ORFy and ORFx, which code for two peptide synthases (Figure 4a). These enzymes have 7 and 8 catalytic domains, respectively (Domingos et al. 2015; Saggese et al. 2018). Similar genes were found in NRPS clusters of *B. pumilus* and *B. safensis* strains but were absent in the known most similar cluster suggested by antiSMASH (lichenysin, Figure 4b).

Table 2 Putative gene clusters coding for antimicrobial or secondary metabolites in the genomes of antagonistic bacteria as predicted by antiSMASH

antiSMASH predicted gene cluster	*B. safensis* 01TAZ		*B. safensis* 08TAZ		*B. pumilus* 7PB		*B. megaterium* 16PB		*B. safensis* 32PB		most probable function
	Size (Kb)	Sim. (%)	Size (Kb)	Sim. (%)	Size (Kb)	Sim. (%)	Size (Kb)	Sim. (%)	Size (Kb)	Sim. (%)	
NRPS											
bacilysin	6.1	85	6.1	85	6.1	85	ND	ND	6.1	85	bacilysin biosynthesis
lichenysin	51.44	85	edge	edge	45.55	85	ND	ND	51.44	85	pumilacidin biosynthesis
betalactone	11.27	53	11.27	53	11.28	53	ND	ND	11.27	53	unknown- fengycin-related
bacillibactin	11.72	100	11.72	100	11.72	100	ND	ND	11.72	100	bacillibactin biosynthesis
NRPS-T1PKS	ND	ND	ND	ND	58.71	18	ND	ND	ND	ND	zwittermycin A- related
T3PKS	3.03	-	3.03	-	2.97	-	4.23	-	3.03	-	chalcone and stilbene synthase domain protein
BACTERIOCIN											
unknown bacteriocin	3.28	-	3.28	-	4.99	-	6.27	-	3.28	-	circular- IId superfamily
head-to-tail-sactipeptide-RiPP	6.06	85	6.06	85	6.05	100	ND	ND	6.06	85	sporulation killing factor skfA
lanthipeptide- RiPP	ND	ND	ND	ND	ND	ND	7.52	-	ND	ND	putative class I
linearazol(in)e-containing peptides LAP	ND	ND	ND	ND	ND	ND	ND	ND	9.78	100	plantazolicin
Terpene											
terpene I	7.93	50	7.93	50	7.93	50	7.17	50	7.93	50	phytoene- carotenoid biosynthesis
terpene II	ND	ND	ND	ND	ND	ND	0.8	-	ND	ND	phytoene- surfactin-related
terpene III	3.46	-	3.46	-	3.46	-	2.7	-	3.46	-	terpene-cyclase
OTHERS											
unknown betalactone	12.25	-	12.25	-	12.25	-	ND	ND	12.25	-	unknown
siderophore	5.48	-	5.48	-	5.48	-	6.57	-	5.48	-	lucA-lucC family of NRPS- independent siderophore
siderophore	ND	ND	ND	ND	ND	ND	14.24	-	ND	ND	unknown
phosphonate	ND	ND	ND	ND	ND	ND	1.51	-	ND	ND	unknown

*Size was determined according to the region predicted as related to biosynthetic genes by antiSMASH. When most similar known gene clusters were suggested by antiSMASH, those BGC were used to estimate size. Estimation was not performed when BGC was on the edge of contigs. Sim. (%): Similarity (%); ND: not detected.

The predicted composition and organization of catalytic domains were similar to previously described pumilacidin ORFy and ORFx proteins, the only exception was the presence of a CAL (Co-A ligase) domain instead of one A (adenylation) domain. This evidence suggests that this gene cluster may actually be a pumilacidin biosynthetic cluster.

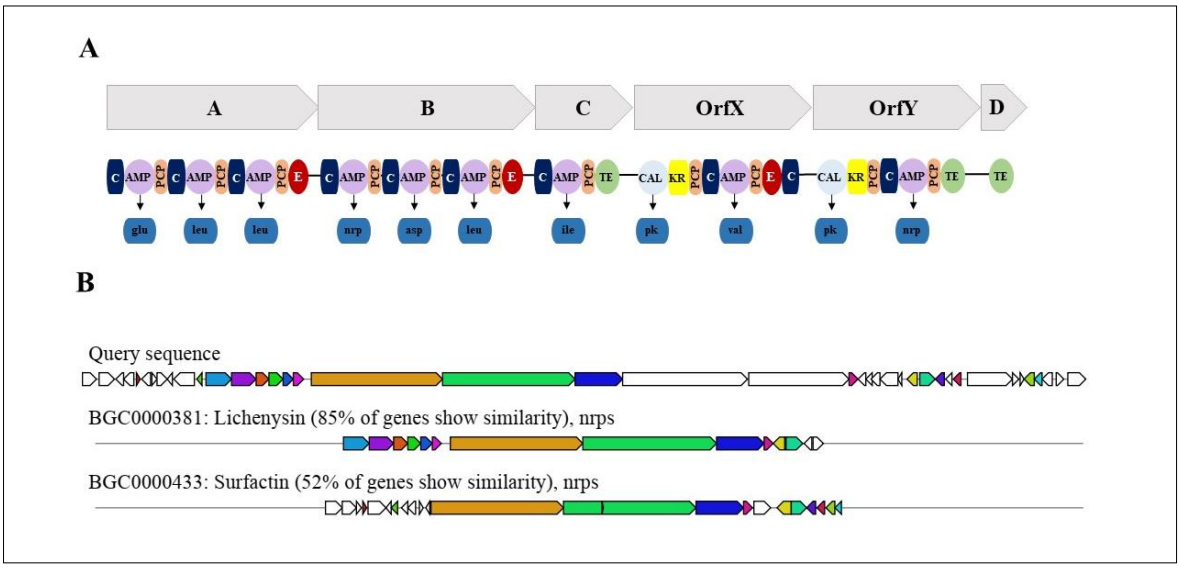


Figure 4 Pumilacidin biosynthetic gene cluster (BGC) module prediction (A) and comparison with other surfactin-related antimicrobial compounds (B). C: condensation domain; AMP: AMP-binding domain; PCP: peptidyl carrier protein; E: epimerization domain; TE: thioesterase domain; CAL: Co-A ligase domain; KR: ketoreductase domain.

3.4 MALDI -TOF analysis of metabolites

Spectrometry analysis of acetonitrile extracts from the inhibition zone of solid culture of canola strains grown near fungi was performed for the identification of lipopeptides, acquiring the snapshots in a range of m/z 500-2500. Several peak masses were detected in the range of typical lipopeptide masses as m/z= 1,000-1,150 (surfactin-related) and m/z= 1,450-1,550 (fengycin-related; Supplementary Figure 3). However, no mass value corresponded with the expected masses from the surfactin, iturin, or fengycin classes, even considering the formation of adducts (Dimkić et al. 2017). Additionally, no group of peaks was observed presenting the typical mass difference of 14 Da of lipopeptide homologs. Therefore, we could not detect the production of lipopeptides by these antagonistic canola strains in the conditions tested.

3.5 Growth chamber biological control of *S. sclerotiorum*

All antagonistic isolates were submitted to biocontrol assay in growth chamber conditions. The control 1, without bacterial inoculum and challenged with *S. sclerotiorum*, showed a reduced percentage of surviving plants if compared with unchallenged control 2. Although for surviving plants of control 1, dry mass, root size, and shoot size values did not differ statistically from measured parameters of control 2. Treatments that received bacterial inoculum did not increase plant survival or morphological parameters values if compared

with control 1 (Table 3). Thus, the necessary conditions for these strains act as biocontrol and plant growth promoter need further investigations.

Table 3 Biocontrol of *Sclerotinia sclerotiorum* infecting canola plants grown in growth chamber by *Bacillus* strains

Treatments	Survival (%)	Shoot size (cm)	Root size (cm)	Dry mass (mg)
Control 1	35	4.81[a]	5.47[a]	7.35[a]
Control 2	90	5.82[a]	5.19[a]	9.06[a]
Bacillus safensis 01TAZ	35	5.64[a]	4.77[a]	8.60[a]
Bacillus safensis 08TAZ	35	5.77[a]	5.90[a]	8.54[a]
Bacillus pumilus 7PB	25	5.70[a]	6.30[a]	9.65[a]
Bacillus safensis 32PB	45	5.31[a]	5.67[a]	7.85[a]
Bacillus megaterium 16PB	20	6.60[a]	5.05[a]	8.30[a]

Control 1: control with fungal inoculum without bacterial treatment; Control 2: control without bacterial or fungal inoculum. Mean values followed by the same letter in the same column do not differ among themselves by a Tukey's test at a 5% significance level

4 Discussion

Thirty isolates from Brazilian canola roots were obtained using a selective isolation for gram-positive spore-forming bacteria. Gram-positive constitutes the less studied and explored group of plant-associated bacteria if compared with gram-negative bacteria (Emmert 1999; Francis et al. 2010). Several taxonomical groups of spore-forming gram-positive bacteria include important PGPB and biocontrol strains, especially *Bacillus* and related genera (Selvakumar et al. 2016). In addition to an important adaptative advantage, the ability to form spores make these microorganisms suitable for inoculant formulations, once these resistant structures allow great stability and bacterial viability for long periods in different environmental conditions (Rahman 2016).

Bacillus was the predominant genus among the isolates. Even not using a direct methodological approach to isolate spore-forming bacteria, some studies reported *Bacillus* as one of the predominating genera in the cultivable bacterial communities associated with *B. napus* roots (Germida et al. 1998; Croes et al. 2013). On the other hand, this was not observed by Farina et al. (2012) when they isolated bacteria from canola (Hyola 60) cultivated in the same producing region considered in this study (Vacaria, Rio Grande do Sul State, Brazil). Using N-free media, none of the bacteria isolated by these authors belonged to the genera obtained in the present work.

Our isolates were screened for different biochemical features that are relevant in beneficial plant-bacteria interactions. All isolates were able to produce at least one hydrolytic enzyme, revealing an important ability related to rhizosphere competence (Bach et al. 2016). This feature is related to the capacity of PGPB

colonize and survive in the rhizosphere or plant roots and is an important factor for the efficiency of biocontrol strains in natural conditions (Prashar et al. 2014). The soil zone surrounding the roots harbors a huge microbial community, therefore it constitutes a highly competitive environment (Raaijmakers et al. 2009). The ability to assimilate or metabolize diverse carbon sources, polymers, and other substances exudated by plant roots is a strategy that offers a selective advantage in the rhizosphere (Barret et al. 2011).

With regards to the other PGP characteristics, phosphate solubilization was the most spread feature among the isolates. Interestingly, IAA synthesis or siderophore production is usually the most widespread PGP trait reported for bacterial isolates associated with canola in normal field conditions (Farina et al. 2012; Croes et al. 2013; Etesami and Alikhani 2016). These divergences could be explained by the fact that different environmental conditions can modulate plant-bacteria interactions. The occurrence of bacteria with specific PGP traits is associated with factors such as soil richness, for example. Besides that, plant interactions with nutrient solubilizers or hormone producers are favored under different environmental conditions (Costa et al. 2014).

Several commonly isolated environmental microorganisms can be opportunistic human pathogens, including beneficial plant-interacting bacteria. Thus, the risks associated with the use of novel PGP strains is of utmost concern when developing inoculant products (Selvakumar G et al. 2014). Here, 11 bacteria were closely related with the *Bacillus cereus* group, which includes pathogenic and non-pathogenic species for humans (Messelhäußer and Ehling-Schulz 2018; Rossi et al. 2018). However, the identification of bacterial species related to *B. cereus* is hampered mainly since the traditional approach based on 16S rRNA gene sequence is insufficient to differentiate bacteria within this group (Liu et al. 2015). Therefore, the *B. cereus*-related isolates were excluded from subsequent procedures.

Five isolates were able to inhibit *S. sclerotiorum* growth by plate assay. For these bacteria, phylogenetic analysis based on the complete sequences of 16S rRNA gene revealed three strains (1TAZ, 8TAZ, and 32PB) very similar to *Bacillus safensis*, one strain (7PB) closely related with *Bacillus zhangzhouensis* and *Bacillus pumilus*, and another isolate (16PB) taxonomically close to *Bacillus aryabhattai* and *Bacillus qingshengii.* As is the case of *B. cereus* strains, *B. pumilus*-related bacteria cannot be efficiently distinguished at species level by 16S rRNA gene analysis, therefore additional approaches have been recommended (Wang et al. 2013). For better identification, analysis based on genomic metrics were carried out. The identity of 01TAZ, 08TAZ, and 32PB was confirmed as *B. safensis*, 7PB was identified as *B. pumilus*, and 16PB was identified as *B. megaterium*.

B. safensis, *B. pumilus* and *B. megaterium* are important species for the biocontrol of different phytopathogens and plant growth promotion (Kildea et al. 2008, Lateef et al. 2015; Lopes et al. 2018). For instance, the *Bacillus pumilus* strain QST 2808 is commercially available in Brazil and other countries for the control of white-mold caused *by S. sclerotiorum* on oilseed crops (Bettiol et al. 2012). Several other *B. pumilus* strains, such as GB34, BU F-33, and GHA 181 registered on EPA (U.S. Environmental Protection Agency) are agriculturally employed as biofungicide or plant growth promoter agents (Borriss 2015).

The canola isolates showed mycelial inhibition range of 20 to 70% on plate assay. Considering other bioprospecting studies, Sharma et al. (2019) isolated *B. pumilus* and *B. safensis* strains from chickpea rhizosphere that inhibited *S. sclerotiorum* growth by 56.84 and 62.41%, respectively. Forchetti et al. (2007) obtained seven *B. pumilus* endophytic strains from sunflower roots showing an *S. sclerotiorum* suppression ranging from 35 to 87.5%. For *B. megaterium*, the TV-103b strain was able to reduce 48.5% of *S. sclerotiorum* growth by plate assay (Tozlu et al. 2016). In the context of *S. sclerotiorum* biocontrol in the *Brassica* genus, *B. pumilus* strain YSPMK11 suppressed disease severity of Sclerotinia stem rot by 93% when inoculated on *Brassica oleracea* var. *botrytis* L. *In vitro*, this strain showed maximum fungal-growth inhibition of 81.50% and was able to produce the lipopeptides iturin A and surfactin (Kaushal et al. 2017). The *B. megaterium* strain A6 is an efficient plant growth promoter of oilseed rape and can successfully protect this crop from *S. sclerotiorum* under field conditions (Hu et al. 2013)

Four isolates were able to inhibit *S. sclerotiorum* growth by VOCs production. With regards to the *Bacillus* genus, the *Bacillus amyloliquefaciens* strain NJZJSB3 displayed effective protection of canola against *S. sclerotiorum* and 5 different VOCs were detected being produced by this strain (Wu et al. 2014). Among the antagonistic strains, *B. pumilus* 7PB was the only isolate that showed a negative result in the VOCs test. In spite of this negative result, other *B. pumilus* strains isolated from cucumber rhizosphere have already been reported suppressing the development of *S. sclerotiorum* and other phytopathogenic fungi by VOCs producing (Liu et al. 2008).

Several gene clusters related to secondary metabolism and antibiotics production were found in the genome of the canola isolates. In fact, most plant-associated *Bacillus* and related firmicutes harbor LP and PK gene clusters if compared with non-plant associated strains, indicating an ecological role of these clusters in plant-beneficial bacteria adaptations (Aleti et al. 2015). Two clusters related to LP production were present in genomes of *B. pumilus* and *B. safensis* strains, including BGC similar to pumilacidin and fengycin. Pumilacidin was already described as an antifungal metabolite and has inhibitory activity against *Rhizoctonia solani*, *Pythium aphanidermatum*, and *Sclerotium rolfsii* as described for the endophytic *B. pumilus* MAIIIM4a (Melo et al. 2009).

Fengycin A and B are involved in the antifungal activity of *Bacillus subtilis* against *S. sclerotiorum* and other three filamentous pathogenic fungi of canola (Hou et al. 2006). However, no fengycin related compound was found in culture extracts of our canola isolates. The putative gene cluster predicted for the canola strains' genomes showed a very low similarity with the most closely related known fengycin cluster. Likewise, in a comparative genomic study of Bacilalles, Zhao and Kuipers (2016) did not found any fengycin cluster in eight available genomes of *B. pumilus* strains.

Unexpectedly, neither pumilacidin or any other LP compound was detected by mass spectrometry on solid culture or supernatant extracts (data not shown). This may be attributed to a low concentration of these substances in the analyzed samples or these compounds may not be expressed under the experimental conditions tested. Different nutritional, physicochemical, and physiological factors can significantly affect LP

production *in vitro* and the optimal conditions for LP production may require adjustments for each strain (Kumar and Johri 2012)

A gene cluster for bacilysin was also found in most genomes. This metabolite is a dipeptide composed of L-alanine and the unusual amino acid L-anticapsin, that possesses antibacterial and antifungal activity (Sansinenea and Ortiz 2011). Bacilysin was found in the culture supernatant of different biocontrol strains, but the antifungal potential is mainly described against yeast (Loeffler et al. 1986; Chung et al. 2008). The probable mechanism of action involves the hydrolyzation of bacilysin by a fungal proteinase and then the released product, anticapsin, inhibits the enzymatic activity of aminotransferase of glucosamine-6-phosphate synthase (GFA) intracellularly (Wang et al. 2018)

BGCs related to siderophore production were found in all analyzed genomes. In fact, 01TAZ, 08TAZ, and 7PB also showed positive results for siderophore production on plate assay. The best-characterized region was an NRPS cluster for the synthesis of bacillibactin. This siderophore belongs to the catechol family and is commonly produced by *Bacillus* strains (Raza et al. 2008). These substances have an important role in biocontrol process, once siderophores capture Fe in the rhizosphere environment and thus limits the amount of iron available for the growth of pathogens (Saha et al. 2016).

Another mechanism of fungal growth inhibition by antagonistic bacteria involves the production of hydrolytic enzymes related to the degradation of cell-wall components of pathogens (Swiontek Brzezinska et al. 2014). Two purified chitinases (ChiS and ChiL) from *B. pumilus* SG2 displayed antifungal activity against *S. sclerotiorum* (Ghasemi et al. 2010) and this enzymatic mechanism must be investigated for our canola isolates. All five antagonistic strains were positive for protease production. Supposedly, the activity of proteases on fungal-wall increase their susceptibility to lysis by chitinases and glucanases (Haran et al. 1996). This enzyme was also related to direct inhibitory activity of a rhizobacteria against the plant pathogen *Macrophomina phaseolina* (Illakkiam et al. 2013). The biocontrol strain *B. pumilus* M3-16 produces a highly stable protease induced by the presence of phytopathogenic fungus *Botrytis cinerea* (Essghaier et al. 2009). Therefore, the role of ribosomally produced compounds on the antifungal activity of our Brazilian canola strains should be further investigated.

No antagonistic isolate suppressed *S. sclerotiorum* disease in pot experiment or promoted plant growth. Evaluating *Bacillus* strains, Akinrinlola et al. (2018) demonstrated that no set of *in vitro* PGP traits is a predictor of growth promotion efficiency. Growth conditions can intensively affect canola-bacteria interactions, while rhizobacteria that present multiple PGP abilities *in vitro* can result in either a beneficial or a deleterious effect depending on different environmental situations (Hudek et al. 2018). Despite the dual culture plate test having a strong correlation with antifungal activity on plant assay, some *in vitro* antagonistic isolates fail to protect plants (Bach et al. 2016; Shehata et al. 2016). Besides that, here we employed a soil-borne fungal inoculum, which could be a possible via for canola infection by *S. sclerotiorum*. However, canola is most frequently infected by air-borne ascospores deposited on petals (Kamal et al. 2016). The direct activity of our canola isolates on ascospores germination was not evaluated and the potential of these bacteria in air-borne pathogen control remains to be elucidated.

5 References

Afzal I, Shinwari ZK, Sikandar S, Shahzad S (2019) Plant beneficial endophytic bacteria: Mechanisms, diversity, host range and genetic determinants. Microbiol Res 221:36–49. doi: 10.1016/J.MICRES.2019.02.001

Akinrinlola RJ, Yuen GY, Drijber RA, Adesemoye AO (2018) Evaluation of Bacillus Strains for Plant Growth Promotion and Predictability of Efficacy by In Vitro Physiological Traits. Int J Microbiol 2018:1–11. doi: 10.1155/2018/5686874

Aleti G, Sessitsch A, Brader G (2015) Genome mining: Prediction of lipopeptides and polyketides from Bacillus and related Firmicutes. Comput Struct Biotechnol J 13:192–203. doi: 10.1016/j.csbj.2015.03.003

Alippi AM, Aguilar OM (1998) Characterization of Isolates ofPaenibacillus larvaesubsp.larvaefrom Diverse Geographical Origin by the Polymerase Chain Reaction and BOX Primers. J Invertebr Pathol 72:21–27. doi: 10.1006/jipa.1998.4748

Ambrosini A, Beneduzi A, Stefanski T, et al (2012) Screening of plant growth promoting Rhizobacteria isolated from sunflower (Helianthus annuus L.). Plant Soil 356:245–264. doi: 10.1007/s11104-011-1079-1

Ávila ZR de, Carvalho SS, Braúna LM, et al (2005) Seleção de isolados de Trichoderma spp. antagônicos a Sclerotium rolfsii e Sclerotinia sclerotiorum

Aziz R, Bartels D, Best A, et al (2008) The RAST Server: Rapid Annotations using Subsystems Technology. BMC Genomics 9:75

Bach E, Cannavan FS, Duarte FRS, et al (2011) Characterization of feather-degrading bacteria from Brazilian soils. Int Biodeterior Biodegradation 65:102–107. doi: 10.1016/j.ibiod.2010.07.005

Bach E, Sant'Anna FH, Magrich dos Passos JF, et al (2017) Detection of misidentifications of species from the *Burkholderia cepacia* complex and description of a new member, the soil bacterium *Burkholderia catarinensis* sp. nov. Pathog Dis 75:ftx076

Bach E, Seger GD dos S, Fernandes G de C, et al (2016) Evaluation of biological control and rhizosphere competence of plant growth promoting bacteria. Appl Soil Ecol 99:141–149. doi: 10.1016/j.apsoil.2015.11.002

Bankevich A, Nurk S, Antipov D, et al (2012) SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. J Comput Biol 19:455–477

Barret M, Morrissey JP, O'Gara F (2011) Functional genomics analysis of plant growth-promoting rhizobacterial traits involved in rhizosphere competence. Biol Fertil Soils 47:729–743. doi: 10.1007/s00374-011-0605-x

Bettiol W, Morandi MAB, Pinto ZV, et al (2012) Produtos Comerciais à Base de Agentes de Biocontrole de Doenças de Plantas. Jaguariúna

Blanco a., Pastor FIJ (1993) Characterization of cellulase-free xylanases from the newly isolated Bacillus sp. strain BP-23. Can J Microbiol 39:1162–1166. doi: 10.1139/m93-175

Bloudoff K, Schmeing TM (2017) Structural and functional aspects of the nonribosomal peptide synthetase condensation domain superfamily: discovery, dissection and diversity. Biochim Biophys Acta - Proteins Proteomics 1865:1587–1604. doi: 10.1016/J.BBAPAP.2017.05.010

Boddey RM, Knowles R (1987) Methods for quantification of nitrogen fixation associated with gramineae. CRC Crit Rev Plant Sci 6:209–266. doi: 10.1080/07352688709382251

Boland GJ, Hall R (1994) Index of plant hosts of Sclerotinia sclerotiorum. Can J Plant Pathol 16:93–108. doi: 10.1080/07060669409500766

Borriss R (2015) Bacillus, A Plant-Beneficial Bacterium. In: Lugtenberg B (ed) Principles of Plant-Microbe Interactions. Springer International Publishing, Cham, pp 379–391

Cadmus MC, Jackson LK, Burton KA, et al (1982) Biodegradation of Xanthan Gum by Bacillus sp

Choudhary DK, Prakash A, Johri · B N (2007) Induced systemic resistance (ISR) in plants: mechanism of action

Chung S, Kong H, Buyer JS, et al (2008) Isolation and partial characterization of Bacillus subtilis ME488 for suppression of soilborne pathogens of cucumber and pepper. Appl Microbiol Biotechnol 80:115–123. doi: 10.1007/s00253-008-1520-4

Cochrane SA, Vederas JC (2016) Lipopeptides from Bacillus and Paenibacillus spp.: A Gold Mine of Antibiotic Candidates. Med Res Rev 36:4–31. doi: 10.1002/med.21321

Coil D, Jospin G, Darling AE (2014) A5-miseq: an updated pipeline to assemble microbial genomes from Illumina MiSeq data. Bioinformatics btu661

Costa PB da, Granada CE, Ambrosini A, et al (2014) A Model to Explain Plant Growth Promotion Traits: A Multivariate Analysis of 2,211 Bacterial Isolates. PLoS One 9:e116020. doi: 10.1371/journal.pone.0116020

Croes S, Weyens N, Janssen J, et al (2013) Bacterial communities associated with Brassica napus L. grown on trace element-contaminated and non-contaminated fields: a genotypic and phenotypic comparison. Microb Biotechnol 6:371. doi: 10.1111/1751-7915.12057

De Mori C, Tomm GO, Ernani P, Ferreira P (2014) Aspectos econômicos e conjunturais da cultura da canola no mundo e no Brasil. Embrapa Trigo, Passo Fundo

Dimkić I, Stanković S, Nišavić M, et al (2017) The Profile and Antimicrobial Activity of Bacillus Lipopeptide Extracts of Five Potential Biocontrol Strains. Front Microbiol 8:1–12. doi: 10.3389/fmicb.2017.00925

Domingos DF, de Faria AF, de Souza Galaverna R, et al (2015) Genomic and chemical insights into biosurfactant production by the mangrove-derived strain Bacillus safensis CCMA-560. Appl Microbiol Biotechnol 99:3155–3167. doi: 10.1007/s00253-015-6377-8

Elshahat MR, Ahmed AA, Enas AH, Fekria MS (2016) Plant growth promoting rhizobacteria and their potential for biocontrol of phytopathogens. African J Microbiol Res 10:486–504. doi: 10.5897/AJMR2015.7714

Emmert E (1999) Biocontrol of plant disease: a (Gram-) positive perspective. FEMS Microbiol Lett 171:1–9. doi: 10.1016/S0378-1097(98)00570-9

Essghaier B, Bejji M, Jijakli H, et al (2009) High salt-tolerant protease from a potential biocontrol agent Bacillus pumilus M3-16

Etesami H, Alikhani HA (2016) Rhizosphere and endorhiza of oilseed rape (Brassica napus L.) plant harbor bacteria with multifaceted beneficial effects. Biol Control 94:11–24. doi: 10.1016/j.biocontrol.2015.12.003

Farina R, Beneduzi A, Ambrosini A, et al (2012) Diversity of plant growth-promoting rhizobacteria communities associated with the stages of canola growth. Appl Soil Ecol 55:44–52. doi: 10.1016/J.APSOIL.2011.12.011

Fernandes G de C, Trarbach LJ, de Campos SB, et al (2014) Alternative nitrogenase and pseudogenes: unique features of the Paenibacillus riograndensis nitrogen fixation system. Res Microbiol 165:571–580. doi: 10.1016/j.resmic.2014.06.002

Forchetti G, Masciarelli O, Alemano S, et al (2007) Endophytic bacteria in sunflower (Helianthus annuus L.): isolation, characterization, and production of jasmonates and abscisic acid in culture medium. Appl Microbiol Biotechnol 76:1145–1152. doi: 10.1007/s00253-007-1077-7

Francis I, Holsters M, Vereecke D (2010) The Gram-positive side of plant-microbe interactions: Minireview. Environ Microbiol 12:1–12. doi: 10.1111/j.1462-2920.2009.01989.x

Germida JJ, Siciliano SD, Renato de Freitas J, Seib AM (1998) Diversity of root-associated bacteria associated with field-grown canola (Brassica napus L.) and wheat (Triticum aestivum L.). FEMS Microbiol Ecol 26:43–50. doi: 10.1111/j.1574-6941.1998.tb01560.x

Ghasemi S, Ahmadian G, Sadeghi M, et al (2010) First report of a bifunctional chitinase/lysozyme produced by Bacillus pumilus SG2. Enzyme Microb Technol 48:225–231. doi: 10.1016/j.enzmictec.2010.11.001

Glickmann E, Dessaux Y (1995) A critical examination of the specificity of the Salkowski reagent for indolic compounds produced by phytopathogenic bacteria. Appl Environ Microbiol 61:793–796

Gotor-Vila A, Teixidó N, Di Francesco A, et al (2017) Antifungal effect of volatile organic compounds produced by Bacillus amyloliquefaciens CPA-8 against fruit pathogen decays of cherry. Food Microbiol 64:219–225. doi: 10.1016/j.fm.2017.01.006

Gurevich A, Saveliev V, Vyahhi N, Tesler G (2013) QUAST: quality assessment tool for genome assemblies. Bioinformatics 29:1072–1075

Haran S, Schickler H, Chet I (1996) Molecular mechanisms of lytic enzymes involved in the biocontrol activity of Trichoderma harzianum. Microbiology 142:2321–2331

He H, Silo-Suh LA, Handelsman J, Clardy J (1994) Zwittermicin A, an antifungal and plant protection agent from Bacillus cereus. Tetrahedron Lett 35:2499–2502. doi: 10.1016/S0040-4039(00)77154-1

Hou X, Boyetchko SM, Brkic M, et al (2006) Characterization of the anti-fungal activity of a Bacillus spp. associated with sclerotia from Sclerotinia sclerotiorum. Appl Microbiol Biotechnol 72:644–653. doi: 10.1007/s00253-006-0315-8

Hu X, Roberts DP, Xie L, et al (2013) Bacillus megaterium A6 suppresses Sclerotinia sclerotiorum on oilseed

rape in the field and promotes oilseed rape growth. Crop Prot 52:151–158. doi: 10.1016/j.cropro.2013.05.018

Hudek L, Enez A, Webster WAJ, et al (2018) Inoculation of Brassica napus L. (canola) with Pseudomonas fluorescens DUS1-27 leads to inhibition of plant growth due to accumulation of hydrogen peroxide. Plant Soil 430:361–379. doi: 10.1007/s11104-018-3740-4

Illakkiam D, Anuj NL, Ponraj P, et al (2013) Proteolytic enzyme mediated antagonistic potential of Pseudomonas aeruginosa against Macrophomina phaseolina. Indian J Exp Biol 51:1024–31

Jacques P (2011) Surfactin and Other Lipopeptides from Bacillus spp. Springer, Berlin, Heidelberg, pp 57–91

Kamal MM, Savocchia S, Lindbeck KD, Ash GJ (2016) Biology and biocontrol of Sclerotinia sclerotiorum (Lib.) de Bary in oilseed Brassicas. Australas Plant Pathol 45:1–14. doi: 10.1007/s13313-015-0391-2

Kaushal M, Kumar A, Kaushal R (2017) Bacillus pumilus strain YSPMK11 as plant growth promoter and bicontrol agent against Sclerotinia sclerotiorum. 3 Biotech 7:90. doi: 10.1007/s13205-017-0732-7

Kildea S, Ransbotyn V, Khan MR, et al (2008) Bacillus megaterium shows potential for the biocontrol of septoria tritici blotch of wheat. Biol Control 47:37–45. doi: 10.1016/J.BIOCONTROL.2008.07.001

Kumar A e Johri BN (2012) Antimicrobial Lipopeptides of Bacillus: Natural Weapons for Biocontrol of Plant Pathogens. In: Satyanarayana T, Johri BN, Prakash A (eds) Microorganisms in Sustainable Agriculture and Biotechnology. Springer Netherlands, Dordrecht, pp 91–111

Kumar P, Dubey RC, Maheshwari DK (2012) Bacillus strains isolated from rhizosphere showed plant growth promoting and antagonistic activity against phytopathogens. Microbiol Res 167:493–499. doi: 10.1016/j.micres.2012.05.002

Kumar S, Stecher G, Tamura K (2016) MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. Mol Biol Evol 33:1870–1874. doi: 10.1093/molbev/msw054

Küster E e Williams ST (1964) Selection of Media for Isolation of Streptomycetes. Nature 202:928–929. doi: 10.1038/202928a0

Lateef A, Adelere IA, Gueguim-Kana EB (2015) The biology and potential biotechnological applications of Bacillus safensis. Biologia (Bratisl) 70:411–419. doi: 10.1515/biolog-2015-0062

Liu W, Mu W, Zhu B, et al (2008) Antagonistic Activities of Volatiles from Four Strains of Bacillus spp. and Paenibacillus spp. Against Soil-Borne Plant Pathogens. Agric Sci China 7:1104–1114. doi: 10.1016/S1671-2927(08)60153-4

Liu Y, Lai Q, Göker M, et al (2015) Genomic insights into the taxonomic status of the Bacillus cereus group. Sci Rep 5:14082. doi: 10.1038/srep14082

Loeffler W, Tschen JS-M, Vanittanakom N, et al (1986) Antifungal Effects of Bacilysin and Fengymycin from Bacillus subtilis F-29-3 A Comparison with Activities of Other Bacillus Antibiotics. J Phytopathol 115:204–213. doi: 10.1111/j.1439-0434.1986.tb00878.x

Lopes R, Tsui S, Gonçalves PJRO, de Queiroz MV (2018) A look into a multifunctional toolbox: endophytic Bacillus species provide broad and underexploited benefits for plants. World J Microbiol Biotechnol 34:94. doi: 10.1007/s11274-018-2479-7

Mac Faddin JF (2000) Biochemical tests for identification of medical bacteria. Lippincott Williams & Wilkins

Mainardes EL, Garcia LC, Weirich Neto PH, et al (2018) Economic Feasibility of Canola Production in the Region of Campos Gerais, Paraná, Brazil. Am J Plant Sci 09:958–965. doi: 10.4236/ajps.2018.95073

McSpadden Gardener BB (2004) Ecology of Bacillus and Paenibacillus spp. in Agricultural Systems. Phytopathology 94:1252–1258. doi: 10.1094/PHYTO.2004.94.11.1252

Melo FMP de, Fiore MF, Moraes LAB de, et al (2009) Antifungal compound produced by the cassava endophyte Bacillus pumilus MAIIIM4a. Sci Agric 66:583–592. doi: 10.1590/S0103-90162009000500002

Messelhäußer U, Ehling-Schulz M (2018) Bacillus cereus—a Multifaceted Opportunistic Pathogen. Curr Clin Microbiol Reports 5:120–125. doi: 10.1007/s40588-018-0095-9

Mnif I, Ghribi D (2015) Review lipopeptides biosurfactants: Mean classes and new insights for industrial, biomedical, and environmental applications. Biopolymers 104:129–147. doi: 10.1002/bip.22630

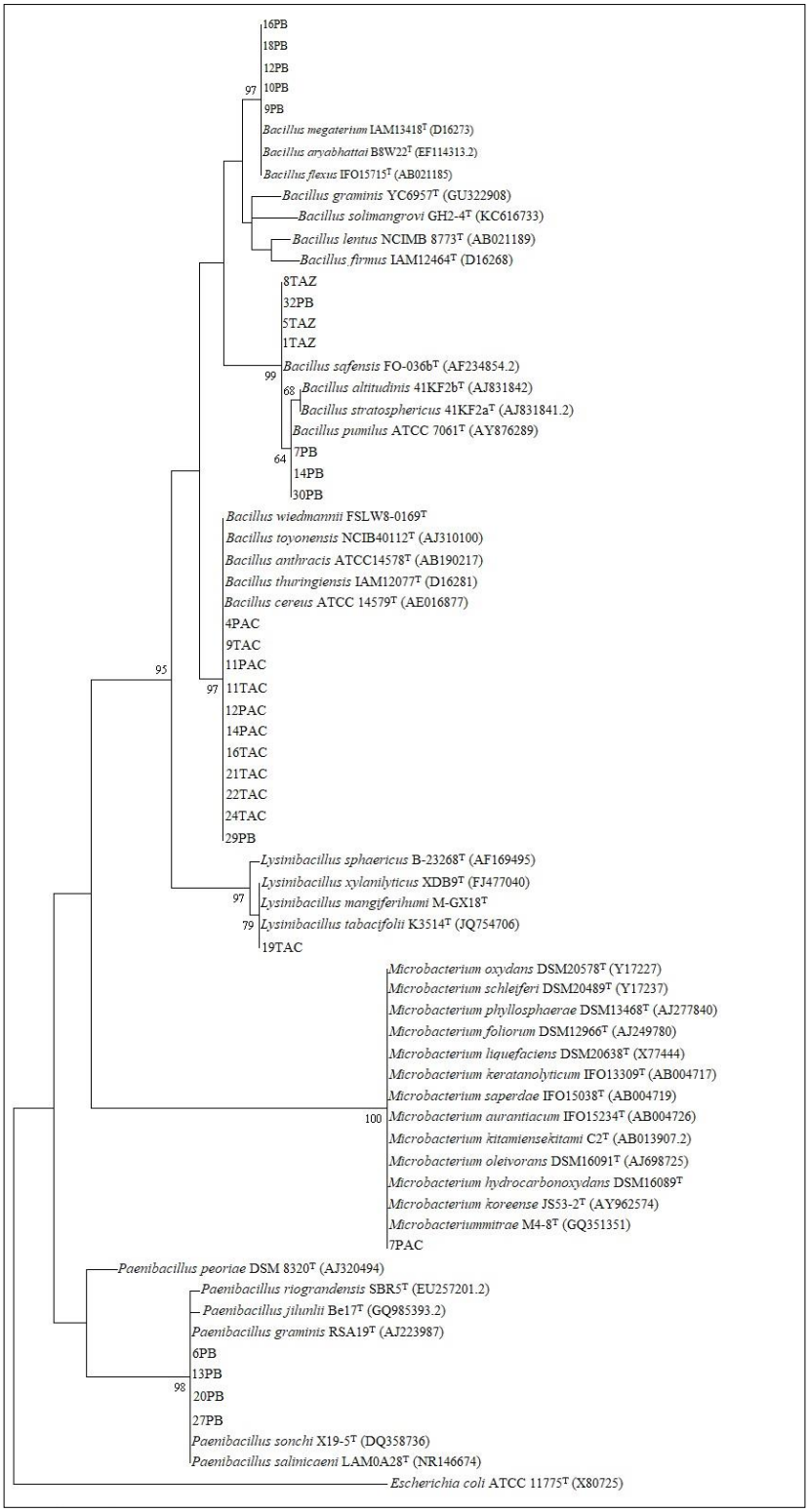
Nautiyal CS (1999) An efficient microbiological growth medium for screening phosphate solubilizing microorganisms. FEMS Microbiol Lett 170:265–270. doi: 10.1111/j.1574-6968.1999.tb13383.x

Olishevska S, Nickzad A, Déziel E (2019) Bacillus and Paenibacillus secreted polyketides and peptides involved in controlling human and plant pathogens. Appl Microbiol Biotechnol 103:1189–1215. doi: 10.1007/s00253-018-9541-0

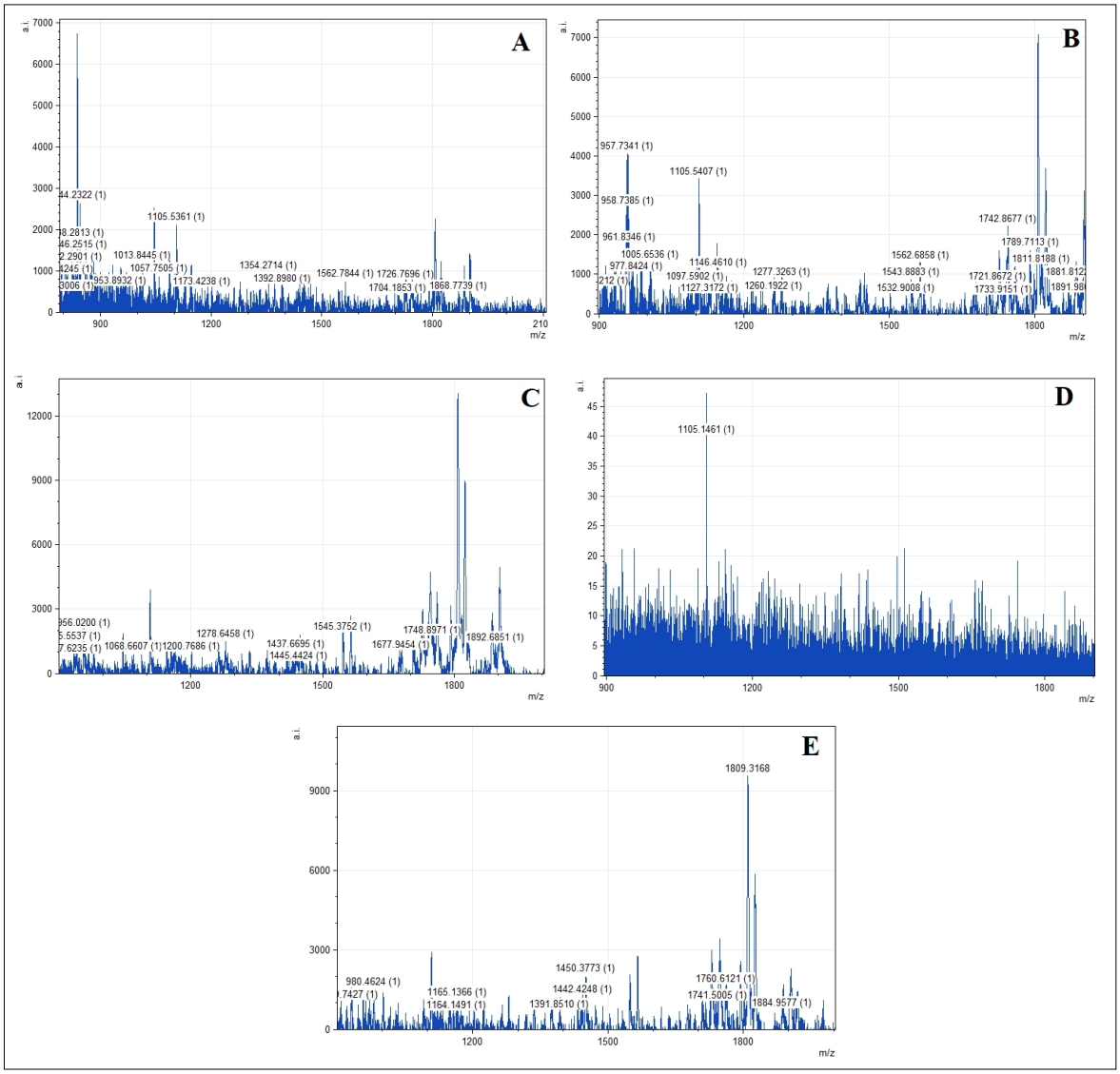
Ongena M, Jacques P (2008) Bacillus lipopeptides: versatile weapons for plant disease biocontrol. Trends

Microbiol 16:115–125. doi: 10.1016/j.tim.2007.12.009
Parks DH, Imelfort M, Skennerton CT, et al (2015) CheckM: assessing the quality of microbial genomes recovered from isolates, single cells, and metagenomes. Genome Res 25:1043–1055
Penrose DM, Glick BR (2003) Methods for isolating and characterizing ACC deaminase-containing plant growth-promoting rhizobacteria. Physiol Plant 118:10–15. doi: 10.1034/j.1399-3054.2003.00086.x
Prashar P, Kapoor N, Sachdeva S (2014) Rhizosphere: its structure, bacterial diversity and significance. Rev Environ Sci Bio/Technology 13:63–77. doi: 10.1007/s11157-013-9317-z
Raaijmakers JM, Paulitz TC, Steinberg C, et al (2009) The rhizosphere: a playground and battlefield for soilborne pathogens and beneficial microorganisms. Plant Soil 321:341–361. doi: 10.1007/s11104-008-9568-6
Rahman M (2016) Bacillus spp.: A Promising Biocontrol Agent of Root, Foliar, and Postharvest Diseases of Plants. In: Islam MT, Rahman M, Pandey P, et al. (eds) Bacilli and Agrobiotechnology. Springer International Publishing, Cham, pp 113–141
Raza W, Wu H, Ali M, et al (2008) A catechol type siderophore, bacillibactin: biosynthesis, regulation and transport in Bacillus subtilis. J Basic Microbiol 48:1–12. doi: 10.1002/jobm.200800097
Raza W, Yuan J, Ling N, et al (2015) Production of volatile organic compounds by an antagonistic strain Paenibacillus polymyxa WR-2 in the presence of root exudates and organic fertilizer and their antifungal activity against Fusarium oxysporum f. sp. niveum. Biol Control 80:89–95. doi: 10.1016/j.biocontrol.2014.09.004
Rossi GAM, Aguilar CEG, Silva HO, et al (2018) Bacillus cereus group: genetic aspects related to food safety and dairy processing. Arq Inst Biol (Sao Paulo) 85:. doi: 10.1590/1808-1657000232017
Saggese A, Culurciello R, Casillo A, et al (2018) A Marine Isolate of Bacillus pumilus Secretes a Pumilacidin Active against Staphylococcus aureus. Mar Drugs 16:180. doi: 10.3390/md16060180
Saha M, Sarkar S, Sarkar B, et al (2016) Microbial siderophores and their potential applications: a review. Environ Sci Pollut Res 23:3984–3999. doi: 10.1007/s11356-015-4294-0
Sambrook J, Russell D (2001) Molecular cloning: a laboratory manual. Cold spring harbor laboratory press, New York
Sansinenea E, Ortiz A (2011) Secondary metabolites of soil Bacillus spp. Biotechnol Lett 33:1523–1538. doi: 10.1007/s10529-011-0617-5
Sant'Anna FH, Bach E, Porto RZ, et al (2019) Genomic metrics made easy: what to do and where to go in the new era of bacterial taxonomy. Crit Rev Microbiol 0:1–19. doi: 10.1080/1040841X.2019.1569587
Saraf M, Pandya U, Thakkar A (2014) Role of allelochemicals in plant growth promoting rhizobacteria for biocontrol of phytopathogens. Microbiol Res 169:18–29. doi: 10.1016/J.MICRES.2013.08.009
Schwyn B, Neilands JB (1987) Universal chemical assay for the detection and determination of siderophores. Anal Biochem 160:47–56. doi: 10.1016/0003-2697(87)90612-9
Seldin L, Van Elsas JD, Penido EGC (1983) Bacillus nitrogen fixers from Brazilian soils. Plant Soil 70:243–255. doi: 10.1007/BF02374784
Selvakumar G, Bindu GH, Panneerselvam P, Ganeshamurthy AN (2016) Potential and Prospects of Aerobic Endospore-Forming Bacteria (AEFB) in Crop Production. In: Islam MT, Rahman MM, Pandey P, et al. (eds) Bacilli and Agrobiotechnology. Springer International Publishing, Cham, pp 213–236
Selvakumar G, Panneerselvam P, An G (2014) Biosafety of Novel Bioinoculants. doi: 10.4172/2155-6202.1000145
Shafi J, Tian H, Ji M (2017) Bacillus species as versatile weapons for plant pathogens: a review. Biotechnol Biotechnol Equip 31:446–459. doi: 10.1080/13102818.2017.1286950
Sharma A, Kashyap PL, Srivastava AK, et al (2019) Isolation and characterization of halotolerant bacilli from chickpea (Cicer arietinum L.) rhizosphere for plant growth promotion and biocontrol traits. Eur J Plant Pathol 153:787–800. doi: 10.1007/s10658-018-1592-7
Sharma P, Meena PD, Verma PR, et al (2015) Sclerotinia sclerotiorum (Lib.) de Bary causing Sclerotinia rot in oilseed Brassicas: A review. J Oilseed Brassica 6:1–44
Shehata HR, Lyons EM, Jordan KS, Raizada MN (2016) Relevance of in vitro agar based screens to characterize the anti-fungal activities of bacterial endophyte communities. BMC Microbiol 16:8. doi: 10.1186/s12866-016-0623-9
Smolińska U, Kowalska B (2018) Biological control of the soil-borne fungal pathogen Sclerotinia sclerotiorum — a review. J Plant Pathol 100:1–12. doi: 10.1007/s42161-018-0023-0
Swiontek Brzezinska M, Jankiewicz U, Burkowska A, Walczak M (2014) Chitinolytic Microorganisms and Their Possible Application in Environmental Protection. Curr Microbiol 68:71–81. doi: 10.1007/s00284-

013-0440-4
Tomm GO, Wiethölter S, Dalmago GA, Santos HP Dos (2009) Tecnologia para produção de canola no Rio Grande do Sul. Embrapa trigo 88
Torres MJ, Brandan CP, Petroselli G, et al (2016) Antagonistic effects of Bacillus subtilis subsp. subtilis and B. amyloliquefaciens against Macrophomina phaseolina: SEM study of fungal changes and UV-MALDI-TOF MS analysis of their bioactive compounds. Microbiol Res 182:31–39. doi: 10.1016/j.micres.2015.09.005
Tozlu E, Parisa M, Merve Senol K, et al (2016) Biological control of Sclerotinia sclerotiorum (Lib.) de Bary, the causal agent of white mould disease in red cabbage, by some bacteria. Plant Prot Sci 52:188–198. doi: 10.17221/96/2015-PPS
Vejan P, Abdullah R, Khadiran T, et al (2016) Role of Plant Growth Promoting Rhizobacteria in Agricultural Sustainability—A Review. Molecules 21:573. doi: 10.3390/molecules21050573
Vinodkumar S, Nakkeeran S, Renukadevi P, Malathi VG (2017) Biocontrol Potentials of Antimicrobial Peptide Producing Bacillus Species: Multifaceted Antagonists for the Management of Stem Rot of Carnation Caused by Sclerotinia sclerotiorum. Front Microbiol 8:1–13. doi: 10.3389/fmicb.2017.00446
Wang L, Sun F, Shao Z, et al (2013) Phylogenetic Diversity of the Bacillus pumilus Group and the Marine Ecotype Revealed by Multilocus Sequence Analysis. PLoS One 8:e80097. doi: 10.1371/journal.pone.0080097
Wang T, Liu X-H, Wu M-B, Ge S (2018) Molecular insights into the antifungal mechanism of bacilysin. J Mol Model 24:118. doi: 10.1007/s00894-018-3645-4
Weber T, Blin K, Duddela S, et al (2015) antiSMASH 3.0- a comprehensive resource for the genome mining of biosynthetic gene clusters. Nucleic Acids Res 43:W237–W243
Wu Y, Yuan J, Raza W, et al (2014) Biocontrol traits and antagonistic potential of Bacillus amyloliquefaciens strain NJZJSB3 against Sclerotinia sclerotiorum, a causal agent of canola stem rot. J Microbiol Biotechnol 24:1327–1336. doi: 10.4014/jmb.1402.02061
Zhao X, Kuipers OP (2016) Identification and classification of known and putative antimicrobial compounds produced by a wide variety of Bacillales species. BMC Genomics 17:882. doi: 10.1186/s12864-016-3224-y



Supplementary Figure 1 Phylogenic tree based on partial sequence of 16S rRNA gene**.** The phylogenetic analysis was performed using the Maximum Likelihood method based on the Jukes-Cantor model. *Escherichia coli* was considered the outgroup. Bootstrap confidence values above 60 are shown near each branch. The GenBank accession numbers of sequences used in this tree are between parenthesis.



Supplementary Figure 2 MALDI-TOF mass spectra of strains (A) *B. safensis* 01TAZ; (B) *B. safensis* 08TAZ; (C) *B. pumilus* 7PB; (D) *B. megaterium* 16PB and (E) *B. safensis* 32PB. Samples were obtained from acetonitrile extraction of agar plugs from the fungal inhibition zone produced by each bacterial isolate.

Supplementary Table 1 General PGP characteristics of bacterial isolates from canola roots

Isolate	Identification*	Auxin	Hydrolytic enzymes				Sid	N fixation	P Solub	Antifungal activity				
			Prot	Xan	Am	Cel				*A. flavus*	*A. niger*	*B. sorokiniana*	*P. chrysogenum*	*P. herquei*
1 TAZ	***Bacillus* sp.**	+	+	-	-	-	+	-	+	+	+	-	+	+
5 TAZ	*Bacillus* sp.	-	+	-	-	-	+	-	+	+	-	-	+	-
8 TAZ	***Bacillus* sp.**	-	+	-	-	-	+	-	+	+	+	-	+	+
6 PB	*Paenibacillus* sp.	+	+	+	-	+	-	+	+	-	-	-	-	-
7 PB	***Bacillus* sp.**	-	+	-	-	+	+	-	+	-	-	-	-	-
9 PB	*Bacillus* sp.	-	+	+	-	+	-	-	+	+	-	-	-	+
10 PB	*Bacillus* sp.	+	+	+	-	+	-	-	+	+	-	-	-	-
12 PB	*Bacillus* sp.	-	+	+	-	+	+	-	+	-	-	-	-	-
13 PB	*Paenibacillus* sp.	-	+	+	-	+	+	-	+	-	-	-	-	-
14 PB	*Bacillus* sp.	-	+	+	+	+	+	-	+	-	-	-	-	-
16 PB	***Bacillus* sp.**	-	+	+	+	+	-	-	+	-	-	-	-	-
18 PB	*Bacillus* sp.	+	+	+	-	+	+	-	+	-	-	-	-	-
20 PB	*Paenibacillus* sp.	+	+	+	+	+	-	+	+	-	-	-	-	-
27 PB	*Paenibacillus* sp.	-	+	+	+	+	-	+	+	-	-	-	-	-
29 PB	*Bacillus* sp.	+	+	-	+	+	+	-	+	-	-	-	-	+
30 PB	*Bacillus* sp.	-	+	-	-	-	+	-	+	-	-	-	-	-
32 PB	***Bacillus* sp.**	+	+	+	-	+	-	-	+	+	+	-	-	+
19 TAC	*Lysinibacillus* sp.	+	+	-	-	-	-	-	+	-	-	-	+	+
4 PAC	*Bacillus* sp.	-	+	-	-	-	+	-	-	-	-	-	+	+
6 PAC	*Bacillus* sp.	-	+	-	-	+	+	-	+	-	-	-	-	-
7 PAC	*Microbacterium* sp.	-	+	-	+	+	+	-	+	-	-	-	-	-
11 PAC	*Bacillus* sp.	+	+	-	+	+	+	-	+	-	-	-	-	-
12 PAC	*Bacillus* sp.	+	+	-	+	+	+	-	+	-	-	-	-	-
14 PAC	*Bacillus* sp.	+	+	-	+	+	+	-	+	-	-	-	-	-
9 TAC	*Bacillus* sp.	+	+	-	+	+	+	-	+	-		-	-	-
11 TAC	*Bacillus* sp.	-	+	-	+	+	+	-	+	+	+	+	+	+
16 TAC	*Bacillus* sp.	-	+	-	+	+	+	-	-	-	-	-	-	-
21 TAC	*Bacillus* sp.	+	+	-	+	+	-	-	+	-	-	-	-	-
22 TAC	*Bacillus* sp.	-	+	-	+	+	-	-	+	-	-	-	-	-
24 TAC	*Bacillus* sp.	-	+	-	+	+	+	-	+	-	-	-	-	-

(+): positive results; (-): not detected; Prot: protease; Xan: xanthanase; Am: amylase; Cel: cellulase. Sid: siderophore production; N fixation: nitrogen fixation; P solub: phosphate solubilization. * Identification based on partial sequence of 16S rRNA gene. Antagonistic isolates against *S. sclerotiorum* are displayed in bold.

Supplementary Table 2 General features of canola bacterial genomes

	B. safensis **01TAZ**	***B. safensis*** **08TAZ**	***B. pumilus*** **7PB**	***B. safensis*** **32PB**	***B. megaterium*** **16PB**
Genome size (bp)	3,667,127	3,664,256	3,802,776	3,662,671	5,993,068
G + C content (%)	41.7	41.7	41.4	41.7	37.4
Number of contigs	22	37	35	16	99
N50	873,159	239,501	343,779	833,674	369,454
L50	2	7	5	3	5
Number of CDSs	3844	3849	4014	3828	6542
Number of RNAs	84	81	83	76	137
Completeness	99.59	99.59	99.59	99.60	99.43
Contamination	0.62	0.62	0.21	0.62	0.13
Best assembler	A5	Spades	Spades	Spades	Spades

Supplementary Table 3 Comparison of selected antagonistic canola isolates with its close relative type strains through genome metrics

ANIb(ANIm)\ dDDH (%)*	**01TAZ**	**08TAZ**	**7PB**	**16PB**	**32PB**	***Bacillus safensis* FO 36b**	***Bacillus pumilus* ATCC7061**	***Bacillus aryabhattai* B8W22**	***Bacillus megaterium* NBRC15308**	***Bacillus australimaris* NH7_1**	***Bacillus zhangzhouensis* DW5-4**	***Bacillus altitudinis* 41KF2b**
01TAZ	*	**98.22**	4.93	0	**98.18**	**96.26**	7.67	0	0.01	25.15	4.53	0.99
08TAZ	**99.99 (99.99)**	*	4.95	0	**98.29**	**96.27**	7.64	0	0.01	25.14	4.53	0.99
7PB	90.72 (91.10)	90.72 (91.10)	*	0	4.94	5.21	65.09	0	0.01	3.93	2.37	0.64
16PB	67.63 (84.70)	67.61 (84.56)	67.63 (84.95)	*	0	0.01	0.01	65.74	**81.13**	0	0	0
32PB	**100 (99.99)**	**100 (99.99)**	90.73 (91.11)	67.54 (84.66)	*	**96.28**	7.65	0	0.01	25.14	4.53	0.99
B. safensis FO 36b (NZ CP010405.1)	**98.84 (99.03)**	**98.85 (99.03)**	90.77 (91.13)	67.72 (84.85)	**98.84 (99.03)**	*	8.04	0	0.02	25.12	4.68	1.02
B. pumilus ATCC7061 (NZ ABRX01000001.1)	91.31 (91.64)	91.31 (91.64)	**95.28 (95.76)**	67.79 (86.94)	91.31 (91.64)	91.39 (91.67)	*	0	0.02	5.93	4.08	0.92
B. aryabhattai B8W22 (NZ JYOO01000001.1)	67.6 (84.48)	67.51 (84.47)	67.58 (84.10)	94.75 (95.69)	67.49 (84.54)	67.75 (84.78)	67.6 (84.11)	*	68.62	0	0	0
B. megaterium NBRC15308 (NZ CP009920.1)	67.96 (85.08)	67.87 (84.16)	68 (84.46)	**95.77 (96.78)**	67.88 (84.26)	68.11 (85.85)	68.07 (84.87)	95.16 (95.82)	*	0	0	0,01
B. australimaris NH71 (NZ LGYN01000001.1)	93.22 (93.41)	93.21 (93.41)	90.39 (90.77)	67.58 (86.41)	93.23 (93.41)	93.11 (93.42)	91 (91.30)	67.46 (84.14)	67.75 (83.99)	*	4	0.85
B. zhangzhouensis DW54 (NZ JOTP01000001.1)	90.55 (90.98)	90.57 (90.98)	89.7 (90.23)	67.41 (84.38)	90.58 (90.98)	90.58 (91.00)	90.37 (90.81)	67.37 (83.66)	67.69 (84.19)	90.41 (90.84)	*	2.24
B. altitudinis 41KF2b (NZ ASJC01000001.1)	88.62 (89.01)	88.64 (89.01)	88 (88.59)	67.6 (84.51)	88.65 (89.01)	88.65 (89.01)	88.42 (88.93)	67.57 (84.41)	67.94 (85.30)	88.45 (88.86)	89.62 (90.10)	*

ANI, Average Nucleotide Identity; ANIb is based on BLAST and ANIm on MUMmer alignments; dDDH, Digital DNA:DNA hybridization. dDDH values are shown in the upper triangle and ANI values are shown in the lower triangle *Bold values were above the expected taxonomic threshold and indicate these strains belong to the same species (95% for ANI and 70% for dDDH)

Supplementary Table 4 Predicted core amino acid and acyl-CoA monomers recognized by NRPS and PKS related biosynthetic gene cluster (BGC) found in the genome of antagonistic canola strains

Bacterial strain	BGC	Predicted amino acid backbone structure	Most similar compound*
Bacillus safensis 01TAZ	NRPS	(glu - leu - leu) + (nrp - asp - leu) + (ile) + (pk - val) + (pk - nrp)	Surfactin related
	NRPS	(dhb) + (gly - thr)	Bacillibactin
Bacillus safensis 08TAZ	NRPS	(glu - leu - leu) + (nrp - asp - leu) + (ile) + (pk - val) + (pk - nrp)	Surfactin related
	NRPS	(dhb) + (gly - thr)	Bacillibactin
Bacillus safensis 32PB	NRPS	(glu - leu - leu) + (nrp - asp - leu) + (ile) + (pk - val) + (pk - nrp)	Surfactin related
	NRPS	(dhb) + (gly - thr)	Bacillibactin
Bacillus pumilus 7PB	NRPS	(glu - leu - leu) + (nrp - asp - leu) + (ile) + (pk - val) + (pk - nrp)	Surfactin related
	NRPS	(dhb) + (gly - thr)	Bacillibactin
	NRPS/PKS	(asn) + (asn - ohmal) + (glu) + (ohmal) + (ohmal) + (mal)	-

*based on monomer composition fingerprint analysis using NORINE database

3.2 SEGUNDO CAPÍTULO

Mineral phosphate solubilization by *Paenibacillus graminis* strains isolated from canola (*Brassica napus* L.) roots

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Mineral phosphate solubilization by *Paenibacillus graminis* strains isolated from canola (*Brassica napus* L.) roots

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Abstract

Phosphorus (P) availability is a limiting factor for plant growth. The inorganic forms of this element are found in soils mainly as hardly-soluble mineral compounds. However, plant-associated bacteria are capable to solubilize these compounds, promoting plant growth. In this work, P solubilizing ability of bacterial isolates from Brazilian canola rhizosphere was characterized. Fourteen isolates were screened for solubilization of hydroxyapatite, $FePO_4$, $AlPO_4$ and rock phosphate (RP). These isolates displayed low or median efficiency of hydroxyapatite solubilization on plate assays and released soluble P in a concentration ranging from 22.17 to 75.2 µg/mL in liquid culture. Interestingly, six isolates were also able to solubilize RP and no isolate solubilized $FePO_4$ and $AlPO_4$. The three best solubilizing isolates that yielded a medium with more than 50 µg/mL of soluble P from RP were selected for better characterization. These isolates were identified as *Paenibacillus graminis* by 16S rRNA gene sequence analysis and genome metrics. The best solubilization performances were obtained using ammonium as nitrogen source and glucose or sucrose as carbon sources. The *P. graminis* strains were able to produce exopolysaccharides and form biofilm under low P conditions and the addition of exogenous soluble P induced an inhibitory effect on hydroxyapatite solubilization. Several organic acids were identified in the culture supernatants by mass spectrometry, suggesting a role of these compounds in the solubilization mechanism of these isolates. The genomes of *P. graminis* strains were sequenced and various genes related to P uptake and metabolism were identified, including genes involved in organic P mineralization. To the best of our knowledge, this study is the first characterization of phosphate solubilizing mechanisms related to *Paenibacillus graminis* species.

Keywords: Hydroxyapatite; phosphorus solubilizing mechanisms; rock phosphate; genome features

1 Introduction

Phosphorus (P) is the second most important element for crop production. This nutrient can be found in diverse organic and inorganic compounds so that more than 170 phosphate minerals have already been identified (Holford 1997). However, P soluble forms, such as $H_2PO_4^-$ and HPO_4^{-2}, which are available for plant

uptake, constitute only a small fraction of total P present in the soil. Phosphate usually associates with ions, oxides, or clay particles, forming compounds of low solubility not uptaken by plants (Arai and Sparks 2007). Many agricultural soils in the world are located in regions of elevated P retention potential (Kochian 2012). Calcium phosphates predominate in alkaline/calcareous soils, while iron and aluminum phosphates are the main forms found in acid soils. Considering the low P solubility and the elevated demand for this nutrient by plants, P fertilization is usually required to reach high crop production (Hopkins 2015, Shen et al. 2011). Rock phosphate (RP) is a non-renewable resource that constitutes the main raw material to P fertilizers production. The shortage of P sources is of utmost concern, considering the estimative that current global RP reserves might be depleted in 50-100 years (Cordell et al 2009). However, these predictions are controversial. Geopolitical aspects concerning P production, mainly account to unequal distributions of RP reserves around the world (Chowdhury et al. 2017). Besides that, the pollution associated with the use of P fertilizers is also problematic. When applied in soils, P can be leached and deposited in water bodies, favoring eutrophication processes (Daneshgar et al. 2018). These issues highlight the importance of reducing or more efficient using of P fertilizers, minimizing the impact to the environment and loss of natural resources (Menezes-Blackburn et al. 2018).

Beneficial micro-organisms associated with plants can support P nutrition of the host by different mechanisms. They can solubilize phosphate from mineral compounds or mineralize P from organic matter, as well as directly mobilize soluble phosphate from soil to plant roots, increasing the uptake of this element (Barea and Richardson 2015). The latter is the main mechanism related to the widespread symbiotic association between plants and mycorrhizal fungi (Gupta and Sahu 2017). However, a reduced group of plants is incapable to establish mycorrhizal symbiosis, these include some species of the *Brassicaceae* family, like canola (*Brassica napus* L.). Notwithstanding, these plants have been reported to benefit from the association with diverse phosphate solubilizing bacteria (Cosme et al 2018, Dabrowska et al. 2014, Valetti et al 2018). Microbial inoculants based on phosphate solubilizing bacteria (PSB) stand out as an important alternative for the replacement or for reducing the use of P fertilizers (Granada et al. 2018). These microorganisms can promote mineral phosphate solubilization (MPS) using different strategies, such as proton (H+) extrusion or organic and inorganic acid production. Organic acids can act as chelator agents, forming a complex with metal cations (Ca^{+2}, Fe^{+3}, or Al^{+}) present in soil and consequently, releasing P (Alori et al. 2017).

Frequently, gram-negative bacteria are more efficient at dissolving mineral phosphates when compared to gram-positive bacteria. They achieve higher P solubilization by the secretion of a wide diversity of organic acids through the metabolism of sugars, mainly glucose (Sashidhar and Podile 2010). For these organisms, the best characterized MPS mechanism involves extracellular oxidation of glucose to gluconic acid and 2-ketogluconic acid via the direct oxidation pathway. This process is provided by the expression of the periplasmatic enzyme glucose dehydrogenase (GDH) and gluconate dehydrogenase (GADH) (Goldstein 1995; Krishnaraj and Dahale 2014). However, several gram-positive bacteria, such as spore-forming *Bacillus* and *Paenibacillus* strains, show important MPS ability and plant growth promotion characteristics; therefore, they have been proposed as potential inoculants (Kang et al. 2014, Li et al. 2017, Saeid et al. 2018). The aim of this work is to characterize the MPS ability of Brazilian gram-positive sporulating strains isolated from canola

rhizosphere. The mechanisms involved in MPS were investigated and the impact of different factors on the efficiency of MPS was also evaluated.

2 Methodology

2.1 Bacterial strains and culture conditions

Bacterial strains were previously isolated from Brazilian canola rhizosphere employing a selective protocol for sporulating bacteria (Ribeiro et al., CAPÍTULO I). These isolates were characterized for their plant growth promotion features and stored in 20% glycerol at -20ºC. For experimental procedures, strains with calcium phosphate solubilization abilities were selected, reactivated, and subcultured in King B medium (KB; Glickmann and Dessaux 1995), which contains in g/L: peptone 20, K_2HPO_4 1.15, $MgSO_4$ 1.5, glycerol 15. Bacterial cultures were incubated at 28°C at 120 rpm for at least 24 h.

2.2 Phosphate solubilization efficiency by plate assay

Phosphate solubilizing ability was assessed by plate assay using a modified NBRIP medium (Nautiyal 1999), which contains in g/L: glucose, 10.0; $MgCl_2.6\ H_2O$, 5.0; $MgSO_4.7\ H_2O$, 0.25; KCl, 0.2; $(NH_4)_2SO_4$, 0.1. One g/L of hydroxyapatite ($Ca_5(PO_4)_3(OH)$), iron phosphate ($FePO_4$), or aluminum phosphate ($AlPO_4$) was added as an insoluble P source. Bacterial isolates were spot inoculated on plates and incubated for 7 and 14 days, at 28ºC. After incubation, solubilization halo (H) and bacterial colony diameter (C) were measured. Solubilization Index (SI) was determined by the following formula: SI= H/C. The efficiency of P solubilization was classified as low (SI< 2.0), median (2.0 ≤SI≥ 3.0) or high (SI> 3.0) (Silva Filho and Vidor 2000).

2.3 Phosphate solubilization in liquid culture

Bacterial strains were cultivated in KB broth for 48 h, at 30ºC, and cells were collected by centrifugation, washed, and suspended with saline solution (0.85% NaCl, w/v). The inoculum was adjusted to a bacterial concentration of 0.5 of Optical Density (OD) at 600 nm. The cell suspension was inoculated into tubes containing modified NBRIP broth, supplemented with 1 g/L of $Ca_5(PO_4)_3(OH)$, $FePO_4$, or $AlPO_4$ as P sources. For RP solubilization test, isolates were cultivated in 100 mL Erlenmeyer flasks, containing NBRIP medium supplemented with 1 g/L of macerated RP. For medium containing hydroxyapatite, pH was adjusted to 7.0, while for other P sources, pH was adjusted to 5.0. Tubes and flasks were maintained in agitation (120 rpm), at 30ºC, and culture broth was evaluated after 7 days. Bacterial cells were collected and lysed by boiling, after that, the protein concentration of the lysates was determined by the Bradford method (Bradford 1976). P concentration in the culture supernatant was quantified by the molybdenum blue method (Murphy and Riley 1962) and standardized by protein concentration as an estimative of bacterial growth. The P concentrations values were submitted to one-way analysis of variance and the means were compared by the Tukey test at 5% error probability. Analyses were performed using the software Statistica 7.0 (StatSoft).

2.4 Phosphate solubilization kinetics

Selected P solubilizer isolates had their hydroxyapatite and RP solubilizing activity monitored for 10 days in liquid culture at 30ºC. Bacterial strains were cultivated in 250 mL Erlenmeyer flasks containing NBRIP

broth with each P source and maintained in agitation (120 rpm). The culture broth was evaluated for soluble P and protein concentration after 24, 48, 72, 120, 168, and 240 h, as described above. Also, culture supernatant pH was estimated using pH indicator strips (MColorpHast™).

2.5 Effect of different carbon and nitrogen sources on phosphate solubilization ability

Bacterial cultivation and parameters measurements were performed as described in section 2.3. To assess the effect of the carbon source on hydroxyapatite solubilization, the glucose of NBRIP medium was replaced by galactose, maltose, mannitol or sucrose (1% w/v). Whereas, to investigate the nitrogen source effect, $(NH_4)_2SO_4$ was replaced by the following nitrogen sources: $(NH_4)_2NO_3$, KNO_3, $Ca(NO_3)_2$, or NH_4Cl, all in the final concentration of 0.015 % (w/v)

2.6 Effect of soluble P on phosphate solubilization ability

To assess the effect of soluble P on solubilization processes, NBRIP agar plates with 1 g/L hydroxyapatite containing additional six concentrations of exogenous soluble phosphate (0, 0.5, 1, 5, 10, and 20 mM KH_2PO_4) were prepared. Potassium concentration was equally adjusted in all media using KCl. Then, bacterial isolates were inoculated and plates were evaluated as described in section 2.2.

2.7 Biofilm formation under different concentrations of soluble P

Biofilm assay was performed using a modified crystal violet (CV) method with a 96-well microtiter plate. For inoculum preparation, the isolates were previously grown in KB broth. Then, bacterial cells were collected by centrifugation, washed, and suspended in saline solution (0.85% NaCl, w/v). Each well was filled with 150 µL of modified NBRIP medium inoculated with 20 µL of bacterial suspension. NBRIP medium was supplemented with either 0.1 or 0.001% KH_2PO_4 solution. After 7 days of static incubation, the plates were washed with saline solution, air dried, and biofilm formed was stained adding 150 µL of CV solution (0.1% w/v) in each well. After 30 min, plates were washed again and 150 µL of acetic acid (30% v/v) were added into each well. The optical density of this solution was spectrophotometrically measured at 560 nm using a microplate reader (SpectraMax® M4). Procedures were performed according to O'Toole et al. (2011) with modifications. Wells without bacterial inoculum were used as control.

2.8 Exopolysaccharide production

Exopolysaccharide (EPS) production under phosphate solubilizing condition was evaluated. Bacterial strains were cultivated for 7 days, at 30 ºC, in NBRIP medium containing 1 g/L hydroxyapatite as P source. For EPS precipitation, the culture supernatant was collected and mixed with 95% ethanol in a proportion of 1:3, respectively. The mixture was incubated overnight at -20 ºC and centrifuged at 13,000 rpm for 25 minutes at -1ºC. The precipitated EPS was dried and suspended with ultrapure water (MiliQ®). The suspension was precipitated with ethanol twice, according to the procedure described above. The final precipitate was dried and suspended with ultrapure water (MiliQ®). EPS concentration was determined spectrophotometrically (485 nm) by the phenol-sulfuric acid method, using a glucose standard solution for the construction of a calibration curve (DuBois et al. 1956).

2.9 Molecular identification and phylogenetic analysis

Genomic DNA of the best solubilizing isolates was extracted by the phenol-chloroform method, according to Sambrook and Russel (2001) and Alippi and Aguilar (1998). For bacterial identification, complete 16S rRNA gene sequence was analyzed. The PCR reactions and sequencing were performed according to Bach et al. (2011). The approximately 1500- bp sequences of 16S rRNA gene were assembled and analyzed with the Codon code Aligner® (CodonCode Corporation). Afterward, sequences were compared using the Basic Local Alignment Search Tool (BLAST) in the NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and EzBioCloud (www.ezbiocloud.net/) databases. The type strain sequences of the most closely related species were downloaded from GenBank and used for phylogenetic analysis. Sequences were aligned using the SINA Software from Silva platform (https://www.arb-silva.de/aligner/) and phylogenetic trees were generated by MEGA 7 Software (Kumar et al. 2016) using the maximum-likelihood method and a bootstrap method with 1000 replications. The nucleotide sequences of the almost complete 16S rRNA genes determined in this study were deposited in the GenBank database.

2.10 Genome sequencing and characterization

Genomic DNA of the selected isolates was sequenced in the MiSeq Illumina platform using the MiSeq Reagent kit v3 (2 x 300). The genome was assembled using A5 (Coil et al. 2014) and SPAdes softwares (Bankevich et al. 2012). Best quality assemblies were chosen using CheckM (Parks et al. 2015) and QUAST (Gurevich et al. 2013) softwares. Draft genome was submitted to the Rast server (Aziz et al. 2008) for automatic annotation. For taxonomic comparisons using genome metrics, the genomes of the most similar species according to the 16S rRNA gene sequence analysis were obtained from the NCBI database. Identification of the isolate was corroborated by Average Nucleotide Identity (ANI) and digital DNA:DNA hybridization (dDDH), considering species cutoff of 95 and 70%, respectively (Sant'Anna et al. 2019). ANI values based on BLAST and MUMmer alignments were calculated at JspeciesWS (http://jspecies.ribohost.com/jspeciesws). Estimations of dDDH were performed at (http://ggdc.dsmz.de/distcalc2.php) based on alignment BLAST+ and recommended formula 2 (Bach et al. 2017). All taxonomic analyses were carried out comparing the sequence of the canola strains with the type strain of each species available in the NCBI.

2.11 Organic acids identification by mass spectrometry

Bacterial isolates were cultivated in NBRIP medium with hydroxyapatite as described in section 2.4. The supernatant was collected by centrifugation after 120 h and filtered using a 0.22 µm membrane (Millipore®). Cell-free supernatant was ultrafiltered by an ultra-0.5 centrifugal filter device (Amicon® Ultra 3K device) and desalinated using the solid phase extraction column Oasis PRiME HLB, Waters. Samples were analyzed by direct infusion through electrospray ionization mass spectrometry (ESI-MS) with an Impact II QTOF™ (Bruker). Data were collected in the positive (ESI+) and negative (ESI-) ion mode, under the following conditions: capillary voltage 4500 V, for positive mode, and 2500V for negative mode, at 200ºC. Data acquisition was performed along the m/z 50- 600 range. Lactic, citric, maleic, malic, succinic, and gluconic acids were used as standards.

3 Results

3.1 Solubilization of different phosphate sources

Fourteen Brazilian canola rhizosphere isolates were used in this study, 10 belonging to the *Bacillus* and 4 to the *Paenibacillus* genera, as identified by partial 16S rRNA gene sequencing. All of them displayed calcium phosphate solubilization on a previous qualitative test (Ribeiro et al., CAPÍTULO I). The ability to solubilize more recalcitrant P sources as hydroxyapatite, aluminum P and iron P were evaluated by plate assay and liquid culture. After 14 days of cultivation, most of the isolates showed low efficiency of hydroxyapatite solubilization (SI≤ 2.0) on solid medium. Two isolates demonstrated median efficiencies (2.0≤ SI< 3.0) after 7 days for isolate 32PB and after 7 and 14 days for 7PB (Table 1).

Table 1 Hydroxyapatite solubilization by canola rhizosphere isolates on solid and liquid culture assays as determined by using the Solubilization Index (SI) or quantifying supernatant's soluble phosphate (PO_4^{-3}), respectively

Isolates	Identification	SI (7days)	SI (14 days)	$[PO_4^{-3}]$ (µg/mL)	$[PO_4^{-3}]$/ [Protein]
6PB	*Paenibacillus* sp.	+	+	75.2 ^{a}	1.04 ^{bc}
7PB	*Bacillus* sp.	++	++	43.35 ^{abc}	1.60 ^{ab}
9PB	*Bacillus* sp.	+	+	40.80 ^{abc}	1.53 ^{abc}
10PB	*Bacillus* sp.	+	+	43.98 ^{abc}	1.02 ^{bc}
12PB	*Bacillus* sp.	+	+	32.93 ^{bc}	0.69 ^{c}
13PB	*Paenibacillus* sp.	+	+	67.73 ^{a}	1.09 ^{abc}
14PB	*Bacillus* sp.	+	+	63.38 ^{ab}	1.13 ^{abc}
16PB	*Bacillus* sp.	+	+	67.39 ^{ab}	1.89 ^{a}
18PB	*Bacillus* sp.	+	+	45.75 ^{abc}	1.18 ^{abc}
20PB	*Paenibacillus* sp.	+	+	59.52 ^{ab}	1.68 ^{ab}
27PB	*Paenibacillus* sp.	+	+	68.88 ^{a}	1.10 ^{abc}
32PB	*Bacillus* sp.	++	+	22,17 ^{c}	1.16 ^{abc}
01TAZ	*Bacillus* sp.	-	+	27.72 ^{c}	0.97 ^{bc}
08TAZ	*Bacillus* sp.	+	+	24.53 ^{c}	0.81 ^{c}

+ SI < 2.0 (Low efficiency); ++ 2.0 ≤ SI ≥ 3.0 (Median efficiency); +++ SI > 3.0 (High efficiency). Mean values followed by the same letter were not statistically different among themselves by a Tukey's test at a 5% significance level.

The concentration of soluble P in culture supernatant ranged from 22.17 to 75.2 µg/mL. The highest concentrations were obtained for 6PB (75.2 µg/mL), 27PB (68.88 µg/mL), and 13PB (67.73 µg/mL); despite their mean values not differing significantly from other isolates, except for 32PB, 01TAZ, and 08TAZ, which showed the lowest soluble P concentration. When P solubilization data were normalized by protein concentration of cell lysates, most isolates showed similar hydroxyapatite solubilizing abilities (Table 1). No isolate could solubilize aluminum and iron P on the plate or liquid culture assays.

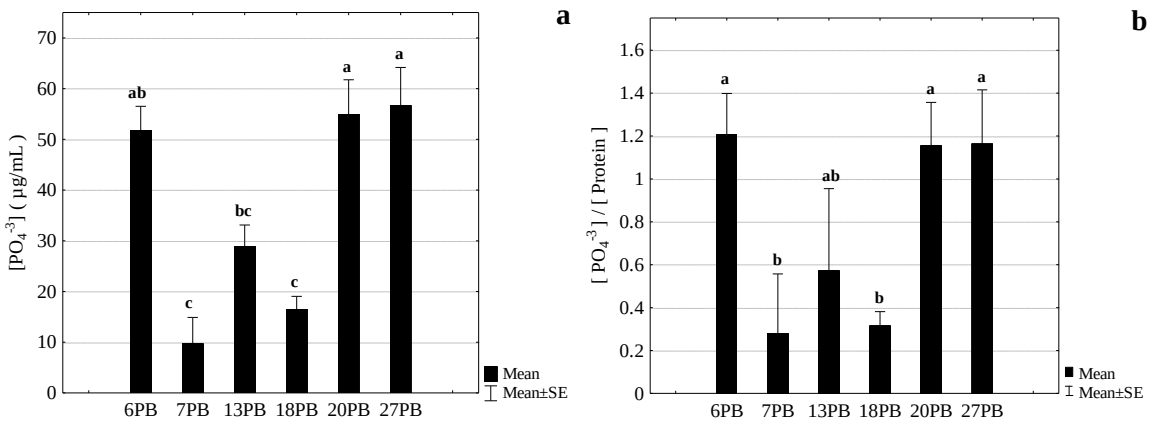


Figure 1 Rock phosphate solubilization by bacterial isolates from canola rhizosphere. (a): PO_4^{-3} concentration in the culture supernatant. (b): PO_4^{-3} concentration standardized by protein concentration of bacterial cell lysates. P solubilization activity evaluated after 7 days of growth in NBRIP medium. Mean values followed by the same letter didn't differ statistically among themselves by a Tukey's test at a 5% significance level.

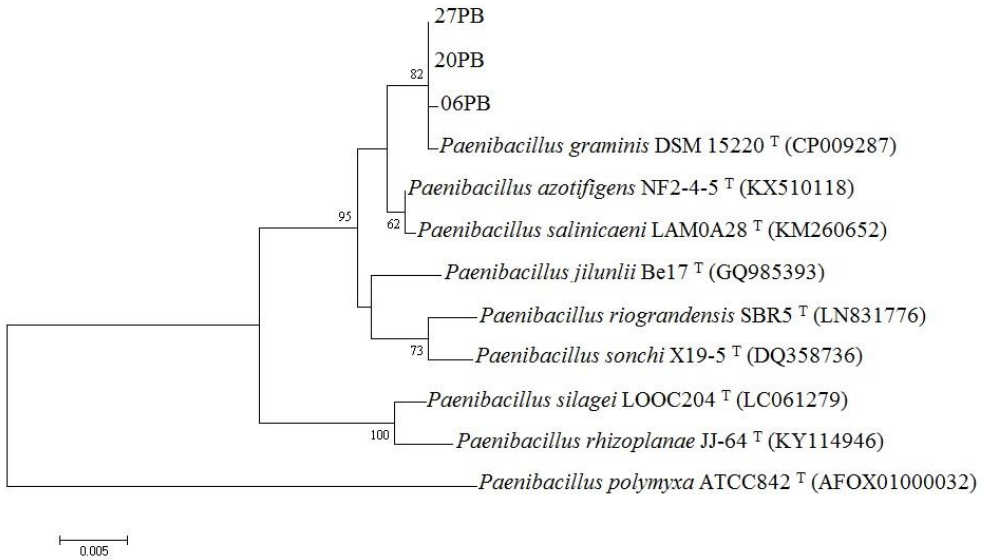


Figure 2 Phylogenetic tree based on 16S rRNA gene sequences of selected PSB**.** The phylogenetic analysis was performed using the Maximum Likelihood method based on the Jukes-Cantor model. *Paenibacillus polymyxa* was considered the outgroup. Bootstrap confidence values above 60 are shown near each branch.

Interestingly, six isolates exhibited the capacity of solubilizing the insoluble P source RP (Figure 1a and 1b). The highest results were obtained for isolates 6PB, 20PB, and 27PB, considering P concentration in culture supernatant and the same data normalized by protein concentration of bacterial lysates. The mean values of soluble P concentration in supernatant ranged from 9.68 to 56.65 µg/mL, all three more efficient solubilizing strains yielded P concentrations over 50 µg/mL. These isolates were selected for further investigation, aiming a better characterization of their phosphate solubilizing abilities under different nutritional conditions. The sequencing of the complete 16S rRNA gene showed that all three selected *Paenibacillus* isolates belong to the *Paenibacillus graminis* species (Figure 2).

3.2 Effect of different factors on phosphate solubilization

Nitrogen effect on phosphate solubilization was evaluated using two ammonium sources (NH_4Cl and NH_4SO_4), two nitrate sources (KNO_3 and $Ca(NO_3)_2$), and NH_4NO_3 (Supplementary Table 1). Considering soluble P in the culture supernatant of 6PB and 27PB, nitrogen sources containing only ammonium resulted in better hydroxyapatite solubilization than compounds containing solely nitrate. However, when P concentration was normalized by protein concentration, the only statistically significant difference among nitrogen sources was found for isolate 27PB, for which NH_4Cl resulted in a higher soluble P releasing than compounds containing only nitrate. Glucose and sucrose were the best carbon sources for phosphate solubilization for all three selected *Paenibacillus* isolates, considering total P soluble in culture supernatant and standardized values by protein concentration (Supplementary Table 2). For isolate 27PB, sucrose was a better carbon source than glucose; although, there was no statistically significant difference in the hydroxyapatite solubilization among these sugars for 6PB and 20PB isolates.

The effect of soluble P in hydroxyapatite solubilization was assessed by plate assay (Supplementary Table 3). Despite not observing any statistically significant difference among SI values for P concentrations ranging from 0 to 1 mM, SI values obtained for treatments with 5 mM of soluble P were lower than treatments without K_2HPO_4 addition, considering 6PB and 7PB strains. Supplementations of soluble P higher than 10 mM were enough to completely inhibit the formation of solubilizing halos.

3.3 Biofilm formation and EPS production

Biofilm formation was verified by CV assay in microplates. All three selected *Paenibacillus* isolates were able to form biofilm when grown in NBRIP medium and this ability increased under low soluble P availability, compared with higher K_2HPO_4 supplementation (Figure 3).

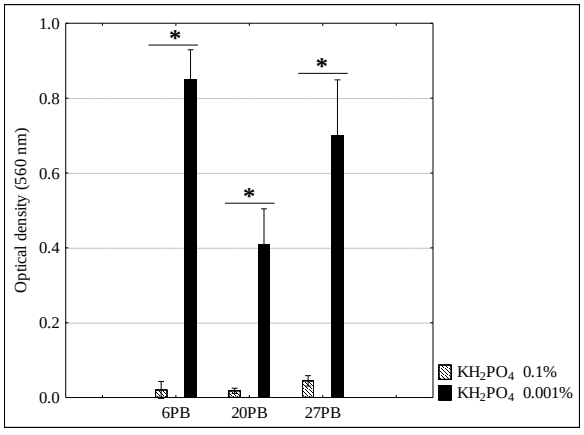


Figure 3 Biofilm formation under different soluble phosphate concentrations**.** *Statistically different by t-test at a 5% significance level. Bars represent mean values ± SE**.**

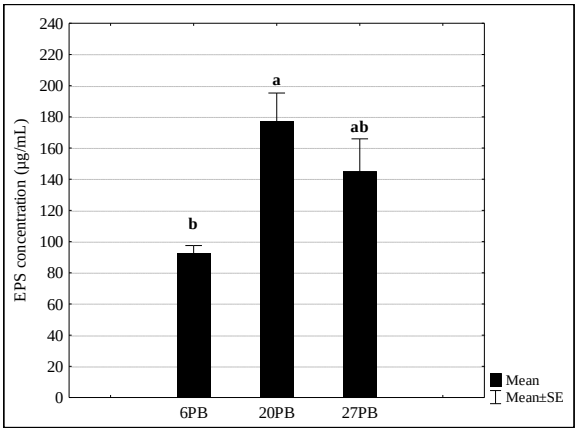


Figure 4 EPS production using hydroxyapatite as P source**.** Columns showing the same letter don't differ statistically among themselves by a Tukey's test at a 5% significance level.

EPS production was also verified in culture supernatant under hydroxyapatite solubilizing condition. All three selected isolates showed positive results and EPS production ranged from 92.30 to 176.89 µg/mL (Figure 4). The highest concentration value was obtained for isolate 20PB.

3.3 Organic acid production and genome analysis

Solubilization process of hydroxyapatite and RP of the selected *Paenibacillus* strains were assessed for 240 h (Supplementary Figure 1). Phosphate concentration increased rapidly within the first 72 h, while pH reduced. Concentration values of soluble P and protein were higher when solubilizing hydroxyapatite than using RP as an insoluble P source. For all three isolates, in all conditions, the culture media were acidified and pH values were reduced to below 5. Media acidification could be a result of organic acids production. The presence of organic acids in the culture supernatant of NBRIP medium was evaluated by mass spectrometry. Indeed, several organic acids as lactic, succinic, α-ketoglutaric, citric, acetic, glutamic, gluconic, and maleic acid were identified with high confidence (Table 2). The isolate 20PB produced the most diverse organic acid profile.

Table 2 Organic acids detected in the bacterial culture supernatant by direct infusion through QTOF-MS in positive and negative ionization modes

Isolate	Meas. m/z	Ion Formula	m/z	error [ppm]	mSigma	rdb	Tentative assignment
6PB	117.0192	C4H5O4	117.0182	-7.9	688.5	2.5	succinic acid
	119.0343	C4H7O4	119.0339	-3.1	714	1.5	acetic acid
	148.0603	C5H10NO4	148.0604	1	554.5	1.5	glutamic acid
	195.0515	C6H11O7	195.0499	-7.9	624.7	1.5	gluconic acid
20PB	89,0242	$C_3H_5O_3$	89,0244	3.0	n.a.	1.5	lactic acid
	117,0194	$C_4H_5O_4$	117,0193	-1.0	7.5	2.5	succinic acid
	145,0144	$C_5H_5O_5$	145,0142	-1.4	10.3	3.5	α-ketoglutaric acid
	191,0192	$C_6H_7O_7$	191,0197	3.0	83.2	3.5	citric acid
	195,0502	$C_6H_{11}O_7$	195,0510	4.1	n.a.	1.5	gluconic acid
	115,0037	$C_4H_3O_4$	115,0037	0.2	n.a.	3.5	maleic acid
27PB	148.0595	C5H10NO4	148.0604	6.1	646.9	1.5	glutamic acid
	89.0235	C3H5O3	89.0244	10.4	n.a.	1.5	lactic acid

The genomes of the three selected isolates were sequenced and analyzed to understand the phosphate solubilizing features of these bacteria. Genome sizes and features were as expected for *Paenibacillus graminis* strains (7.17 Mb) (Al-Bayssari, 2018), as shown in Supplementary Table 4. The genomic metrics confirmed the identity of these isolates as *Paenibacillus graminis*. In spite of the low quality of 27PB draft genome, genome completeness higher than 20% is enough to perform ANI and dDDH analyses (Sant'Anna et al. 2019). The ANI and dDDH values for 6PB were 98.61 and 95.65%, respectively, while for 20PB and 27PB, the ANI

values were 98.59 and 95.69%, respectively, and dDDH values were 95.57 and 94.94%, respectively. All these values are above the species circumscription thresholds (Supplementary Table 5).

The presence of several genes related to bacterial phosphate metabolism was verified. However, considering the low quality of 27PB genome, this isolate was excluded from this analysis (Figure 5). *P. graminis* 6PB and 20PB harbor the Pho regulon, which includes genes for alkaline phosphatase (AP) activity, the PhoP-PhoR proteins, PstABCS (responsible for inorganic phosphate uptake under P starvation conditions), and PhoU proteins. Genes related to P availability from organic matter were also investigated. We found *phn*A and *phn*B genes, which are related to phosphonate metabolism, and a gene encoding 3-phytase. However, these bacterial strains did not display *phnCDEWXM* genes, involved in C-P lyase complex expression. More interestingly, genes for glucose 1 dehydrogenase (*gcd*) and gluconate dehydrogenase (*gad*), that are involved with gluconic and 2-ketogluconic acid production by direct oxidation of glucose, were also not found.

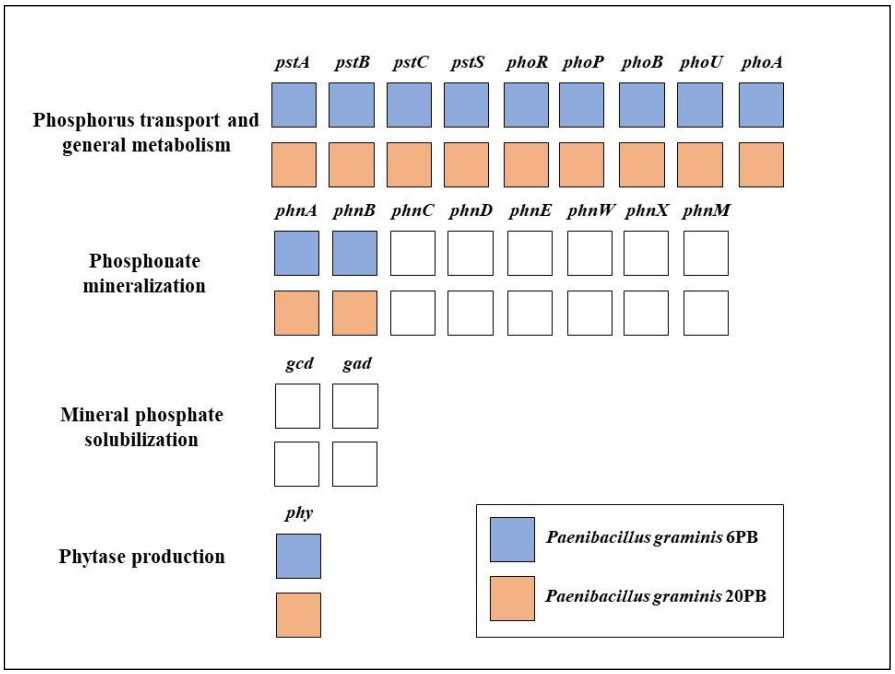


Figure 5 Presence of genes related to P homeostasis and metabolism in the genome of *Paenibacillus graminis* canola isolates. Colorful boxes represent the presence of the gene, while the white boxes represent its absence.

4 Discussion

The best studied plant growth promoting bacteria (PGPB) are mostly gram-negative organisms. However, gram-positive microorganisms include diverse taxonomical groups that comprise important plant-beneficial bacteria, such as *Bacillus* and *Paenibacillus* genera, both characterized by forming endospores (Govindasamy et al. 2010). Sporulating bacteria with PGP traits are promising for the production of inoculants, once their resistance structures allow bacterial viability for prolonged periods in different conditions (Francis

et al. 2010; Grady et al. 2016). In this study, we evaluated the phosphate solubilization ability of 14 spore-forming bacterial strains from a collection of Brazilian canola rhizosphere isolates.

We employed hydroxyapatite, RF, $AlPO_4$, and $FePO_4$ as insoluble mineral P for solubilization tests. The use of only one P source for the evaluation of solubilization activity may be insufficient to characterize a bacterial strain as PSB; therefore, testing different insoluble phosphate sources is the most recommended approach (Bashan et al. 2013a). Furthermore, soils comprise distinct P minerals and the predominating forms constantly change, especially in different soil pH (Bashan et al. 2013b). Six canola rhizosphere isolates (43%) displayed the ability to solubilize more than one P source (hydroxyapatite and RP) and no isolate was able to solubilize $AlPO_4$ and $FePO_4$. In fact, both latter constitute the most insoluble mineral phosphate sources among the tested compounds. Several studies reported broad isolation of calcium phosphates solubilizers that are incapable to solubilize both $AlPO_4$ and $FePO_4$ (Pérez et al. 2007; Dinic et al. 2014; Matos et al. 2017).

The three best RF solubilizing isolates were selected to better characterize their solubilizing abilities. Rock phosphate can be directly applied on the soil as fertilizer. This is usually a more economically and eco-friendly process than the traditional use of chemical fertilizers, mainly by acting as a slow release fertilizer and reducing eutrophication occurrence (Fayiga and Nwoke 2016). The concomitant employment of RP and PSB is more effective, enhancing P availability for plant uptake. For instance, satisfactory results have already been reported for canola crop. The simultaneous inoculation of a P- solubilizing strain (*Bacillus* sp.) with RF addition increased P nutrition and promoted plant growth (Salimpour et al. 2012).

All three selected isolates were identified as *Paenibacillus graminis* by 16S rRNA gene sequence analysis and genomic metrics. This bacterial species was first isolated from maize rhizosphere and has been also described in association with wheat and sorghum (Berge et al. 2002; Vollú et al. 2006; Beneduzi et al. 2008). Phosphate solubilization ability of this species is poorly reported in the literature. Interestingly, *P. graminis* strain R200 was described as an efficient growth promoter of wheat, soybean, and maize, but was unable to solubilize phosphate and did not display another plant-growth promotion trait *in vitro* (Akinrinlola et al. 2018). Another *P. graminis* strain, isolated from the Asteraceae *Aster gymnocephalus* growing on mine tailing, was reported as able to solubilize calcium phosphate (Navarro-Noya et al. 2012).

Nutrients needed for bacterial growth are important factors in the mechanism of mineral phosphate solubilization (Nahas 2007). The influence of nitrogen source on the solubilization process was performed comparing nitrates with ammonium sources. All tested isolates were able to solubilize P using nitrate, but the highest amount of soluble P was obtained with the addition of ammonium, for all *P. graminis* strains. Similar results were found by Prabhu et al. (2018) for P-solubilizing strain *Bacillus marisflavi* FA7. The acidifying process associated with proton extrusion during ammonium assimilation is a proposed mechanism of calcium phosphates solubilization (Illmer and Schinner 1995) and could also be an explanation for the hydroxyapatite solubilization shown in this work.

P. graminis isolates solubilized hydroxyapatite using all tested carbon sources, but glucose and sucrose yielded the highest amount of soluble P in culture supernatants. These results are according to other studies, for which glucose is reported as the best carbon source for diverse PSB, such as *Enterobacter* sp.,

Acinetobacter sp., *Bacterium* sp. and *Pseudomonas* sp. (Mardad et al. 2014; Suleman et al. 2018). In a rhizosphere context, plants secrete plenty of organic compounds in root exudates, including sugars and complex polymers that support nutritionally associated rhizobacteria (Sasse et al. 2018). Glucose has been found as the major sugar in the root exudates of different plant species (Kamilova et al. 2006). However, plant rhizodeposition, especially from *Brassica napus* L., is very variable and influenced by different environmental factors such as P availability (Wang et al. 2015). Deubel et al. (2007) demonstrated that solubilization of tricalcium P by *Pseudomonas* sp. and *Pantoea* sp. strains was enhanced when bacteria were grown using the same C sources predominantly found in the exudates produced by pea plants growing under P deficiency. A better investigation is necessary to verify if a similar process occurs with *P. graminis* isolates.

The concentration of soluble P available in the environment may also influence bacterial solubilization abilities of insoluble sources. Hydroxyapatite solubilization was completely inhibited in concentrations of soluble P higher than 10 mM. This effect of soluble P on solubilization process has been observed for other PSB (Nahas 2007). For *Burkholderia multivorans* WS-FJ9, solubilization of hydroxyapatite is inhibited with soluble P concentrations over 20 mM. Transcriptional analysis of this organism reveals that genes involved in glucose metabolism were down-regulated in this condition. A production of pyruvic acid, possibly involved in P solubilization, was also reduced (Zeng et al. 2017). The transcriptional profile of *Paenibacillus riograndensis*, which is taxonomically closely related to *P. graminis*, was analyzed in the presence of hydroxyapatite or soluble P. In these conditions, the expression of genes related to carbon metabolism was modified and organic acid production was highly expressed when grown with the insoluble P source (Brito 2017).

The effect of exogenous soluble P on biofilm formation was also evaluated by the CV method. All selected isolates formed biofilm and this ability decreased with high soluble P availability. Nutritional deficiency, including of a P source, is a known factor that stimulates bacterial biofilm formation (Velmourougane et al. 2017). Considering other PSB, the strains *Rhizobium miluonense* Rm3 and *Burkholderia anthina* Ba8 increase biofilm formation using RP and hydroxyapatite as P source (Taktek et al. 2017). In these biofilms, they solubilize P reducing the pH and releasing gluconic acid. Electron microscopy also revealed a strong cell adherence of these strains on RF surface. Indeed, we could also observe the formation of aggregates involving the insoluble P source precipitated in the liquid cultures of our *P. graminis* strains (data not shown). In MPS context, biofilm formation by P-solubilizer bacteria *Bradyrhizobium elkanii* associated with P-solubilizing fungi raises their solubilization efficiencies when compared to the planktonic microorganisms (Jayasinghearachchi and Seneviratne 2006). With regards to the *Paenibacillus* genus in plant-bacteria interaction, biofilm formation was reported as an important process to plant roots colonization by the PGPR *P. polymyxa* (Timmusk et al. 2005).

For many bacterial species, biofilm formation under P deficiency is controlled by the Pho regulon. This system is a global mechanism associated with P metabolism and involves the two-component regulatory system which comprises an inner-membrane histidine kinase sensor protein and a cytoplasmic transcriptional response regulator (Santos-Beneit 2015). PhoP and PhoR genes were found in 6PB and 20PB genome;

however, the effective role of this system on the biofilm formation process, as well as the function of biofilm on phosphate solubilizing process of *P. graminis* strains still need further elucidation.

EPS production was detected in all *P. graminis* strains investigated in this work. Mucous colony formation, resulting from EPS secretion, is a known characteristic of *P. graminis* growing on agar plates supplemented with glucose or sucrose (Berge et al. 2002). In our study, bacterial strains produced over 80 µg/mL of EPS in the broth containing hydroxyapatite as the P source. The composition of these polymeric substances are widely varied among *Paenibacillus* species and are involved in diverse biological roles and applications (Liang and Wang 2015). Moreover, Yi et al. (2008) proposed that EPS production may constitute an additional mechanism of phosphate solubilization. These substances are able to induce changes in P homeostasis affecting P solubilization, whereas they keep medium free of P, and consequently, a higher P amount is released from the insoluble-P source.

During the assessment of the solubilizing process using both P sources, media pH reduced dramatically below 5 followed by an increase of soluble P in the first 72 h. Similar results were observed for *B. megaterium*, *B. flexus* and *P. riograndensis* under hydroxyapatite solubilizing condition (Brito 2017; Ibarra-Galeana et al. 2017). Reduction of pH can be attributed to organic acid production. Supernatant analysis by mass spectrometry revealed the presence of several low-molecular-weight organic acids. The secretion of these substances is the main mechanism of phosphate solubilization (Rodríguez et al. 2006). Organic acids act by lowering the pH and forming complexes with Fe and Al ions, consequently releasing soluble P into the medium. The efficiency of the solubilization process depends on the amount and characteristic of the organic acids. For instance, tricarboxylic is usually more efficient than mono- or dicarboxylic acids (Kaur et al. 2016). All acids found in culture supernatants of our canola *P. graminis* strains were previously reported being produced by PSB (Alori et al. 2017) and could be related to their P solubilization process.

With regards to the *Paenibacillus* genus, the release of 2-ketogluconic, tartaric, and propionic acids were observed for *P. kribbensis* during solubilization of $Ca_3(PO_4)_2$ (Marra et al. 2015). *P. riograndensis* produces succinic, oxoglutaric, acetic, gluconic, and citric acids when grown with a soluble P source. However, the concentration of gluconic and acetic acids significantly increased in the presence of hydroxyapatite as the only P source, indicating these compounds as main acids in the solubilizing process of this bacterium (Brito 2017). Gluconic acid production by direct oxidative pathway constitutes one of the best characterized P-solubilizing mechanisms. Although this acid was found in the culture supernatant of our *P. graminis* strains, the glucose dehydrogenase (*acd*) and gluconic acid dehydrogenase (*gad*) genes were not identified in genome of 6PB and 20PB. This fact indicates that the production of gluconic acid might be occurring by another metabolic pathway. These genes can also be important for the P solubilization ability of some gram-positive bacteria, such as *Streptomyces* sp. CTM396 (Farhat et al. 2015). A comparative genomic study carried out by Xie et al. (2016) appointed that several *Paenibacillus* species have these genes, suggesting a role in phosphate solubilization.

The Pst (phosphate-specific transport) system and PhoP-PhoR system are also present in *P. graminis* (6PB and 20PB) genome, as found in other *Paenibacillus* strains genomes (Xie et al. 2016). Acting as an

important regulator of phosphorus metabolism, PhoP-PhoR controls phosphate uptake by bacteria and secretion of phosphatases enzymes under P limitations (Santos-Beneit 2015). Little is known about the role of this system on the control of phosphate solubilization mechanism. *phoA* and *phoR* are also present in *P. riograndensis* genome, but the expression of these genes was not altered when grown in different P sources (Brito 2017).

Despite this work being focused on the MPS process, genes related to phosphatases and phytase production were also found in analyzed genomes, also indicating a potential of this isolate on soluble-P releasing from organic matter. Phytate is an important P storage form in plant seeds and constitutes the main organic P form in soils (Gerke 2015). One gene related to 3-phytase was found in *P. graminis* genomes, suggesting a possible role of this isolate on the degradation of this substance. Another P source is phosphonate, an organophosphorus compound that presents very stable C-P binding. Phosphonate compounds can be enzymatically degraded by phosphonate hydrolases and C-P lyases (Kamat and Raushel 2013). Since the phosphonate hydrolase gene (*phn*A) was found in 6PB genome, these bacteria may have the potential to degrade phosphonoacetate.

In summary, our work described and characterized the solubilization abilities of insoluble P sources by three Brazilian *P. graminis* strains isolated from canola rhizosphere. We could also relate other physiological processes as biofilm formation and EPS production with their phosphate metabolism. Mass spectrometry revealed the presence of organic acids in the supernatant of these bacteria that are probably related to P solubilization. The genome of two strains was successfully sequenced and we could find genes related to its P homeostasis. These results expand the understanding of P solubilization mechanisms and highlight the potential of the selected isolates to act beneficially in plant nutrition. Further studies must be carried out to elucidate the effectiveness of these strains as inoculants. To the best of our knowledge, this study is the first characterization of phosphate solubilizing mechanisms related to *Paenibacillus graminis* species.

5 References

Akinrinlola RJ, Yuen GY, Drijber RA, Adesemoye AO (2018) Evaluation of Bacillus Strains for Plant Growth Promotion and Predictability of Efficacy by In Vitro Physiological Traits. 2018:. doi: 10.1155/2018/5686874

Alippi AM, Aguilar OM (1998) Characterization of Isolates ofPaenibacillus larvaesubsp.larvaefrom Diverse Geographical Origin by the Polymerase Chain Reaction and BOX Primers. J Invertebr Pathol 72:21–27. doi: 10.1006/jipa.1998.4748

Alori ET, Glick BR, Babalola OO (2017) Microbial phosphorus solubilization and its potential for use in sustainable agriculture. Front Microbiol 8:1–8. doi: 10.3389/fmicb.2017.00971

Al-Bayssari C, Dubourg G, Cimmino T, et al (2018) Genome sequence and description of Paenibacillus ihuae strain GD6 sp. nov., isolated from the stool of a 62-year-old Frenchman. New Microbes New Infect 23:77–82. doi: 10.1016/j.nmni.2018.02.010

Arai Y, Sparks DL (2007) Phosphate Reaction Dynamics in Soils and Soil Components: A Multiscale Approach. In: Donald L. Sparks (ed) Advances in Agronomy. Academic Press, pp 135–179

Aziz R, Bartels D, Best A, et al (2008) The RAST Server: Rapid Annotations using Subsystems Technology. BMC Genomics 9:75

Bach E, Cannavan FS, Duarte FRS, et al (2011) Characterization of feather-degrading bacteria from Brazilian soils. Int Biodeterior Biodegradation 65:102–107. doi: 10.1016/j.ibiod.2010.07.005

Bach E, Sant'Anna FH, Magrich dos Passos JF, et al (2017) Detection of misidentifications of species from the Burkholderia cepacia complex and description of a new member, the soil bacterium Burkholderia catarinensis sp. nov. Pathog Dis 75:ftx076

Bankevich A, Nurk S, Antipov D, et al (2012) SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. J Comput Biol 19:455–477

Barea J, Richardson AE (2015) Phosphate Mobilisation by Soil Microorganisms. In: Lugtenberg B (ed) Principles of Plant-Microbe Interactions. Springer International Publishing, Cham, pp 225–234

Bashan Y, Kamnev AA, De-Bashan LE (2013a) A proposal for isolating and testing phosphate-solubilizing bacteria that enhance plant growth. Biol Fertil Soils 49:1–2. doi: 10.1007/s00374-012-0756-4

Bashan Y, Kamnev AA, De-Bashan LE (2013b) Tricalcium phosphate is inappropriate as a universal selection factor for isolating and testing phosphate-solubilizing bacteria that enhance plant growth: A proposal for an alternative procedure. Biol Fertil Soils 49:465–479. doi: 10.1007/s00374-012-0737-7

Beneduzi A, Peres D, da Costa PB, et al (2008) Genetic and phenotypic diversity of plant-growth-promoting bacilli isolated from wheat fields in southern Brazil. Res Microbiol 159:244–250. doi: 10.1016/j.resmic.2008.03.003

Berge O, Achouak W, Normand P, et al (2002) Paenibacillus graminis sp. nov. and Paenibacillus odorifer sp. nov ., isolated from plantroots, soil, and food. Int J Syst Evol Microbiol 4:607–616. doi: 10.1099/ijs.0.01883-0

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254. doi: 10.1016/0003-2697(76)90527-3

Brito LF De (2017) Genomic, transcriptomic and phenotypic analysis of the plant growth promoting and phosphate solubilizing Paenibacillus riograndensis SBR5. Bielefeld University

Chowdhury RB, Moore GA, Weatherley AJ, Arora M (2017) Key sustainability challenges for the global phosphorus resource, their implications for global food security, and options for mitigation. J Clean Prod 140:945–963. doi: 10.1016/j.jclepro.2016.07.012

Coil D, Jospin G, Darling AE (2014) A5-miseq: an updated pipeline to assemble microbial genomes from Illumina MiSeq data. Bioinformatics btu661

Cordell, D., Drangert, J. O., & White, S. (2009). The story of phosphorus: global food security and food for thought. Global environmental change, 19(2), 292-305.

Cosme, M., Fernández, I., Van der Heijden, M. G., & Pieterse, C. M. (2018). Non-mycorrhizal plants: the exceptions that prove the rule. Trends in plant science. DOI: 10.1016/j.tplants.2018.04.004

Dabrowska G, Baum C, Trejgell A, Hrynkiewicz K (2014) Impact of arbuscular mycorrhizal fungi on the growth and expression of gene encoding stress protein - metallothionein BnMT2 in the non-host crop Brassica napus L. J Plant Nutr Soil Sci 177:459–467. doi: 10.1002/jpln.201300115

Dąbrowska, G., Baum, C., Trejgell, A., & Hrynkiewicz, K. (2014). Impact of arbuscular mycorrhizal fungi on the growth and expression of gene encoding stress protein–metallothionein BnMT2 in the non-host crop Brassica napus L. Journal of Plant Nutrition and Soil Science, 177(3), 459-467. 10.1002/jpln.201300115

Daneshgar S, Callegari A, Capodaglio A, Vaccari D (2018) The Potential Phosphorus Crisis: Resource Conservation and Possible Escape Technologies: A Review. Resources 7:37. doi: 10.3390/resources7020037

Deubel A, Gransee A, Merbach W (2007) Tricalcium-phosphate solubilizing efficiency of rhizosphere bacteria depending on the P-nutritional status of the host plant. In: First International Meeting on Microbial Phosphate Solubilization. Springer Netherlands, Dordrecht, pp 257–260

Dinic Z, Ugrinovic M, Bosnic P, et al (2014) Solubilization of inorganic phosphate by endophytic Pseudomonas sp. from French bean nodules. Ratar i Povrt 51:100–105. doi: 10.5937/ratpov51-6222

DuBois M, Gilles KA, Hamilton JK, et al (1956) Colorimetric Method for Determination of Sugars and Related Substances. Anal Chem 28:350–356. doi: 10.1021/ac60111a017

Farhat M Ben, Boukhris I, Chouayekh H (2015) Mineral phosphate solubilization by Streptomyces sp. CTM396 involves the excretion of gluconic acid and is stimulated by humic acids. FEMS Microbiol Lett 362:1–8. doi: 10.1093/femsle/fnv008

Fayiga AO, Nwoke OCC (2016) Phosphate rock: origin, importance, environmental impacts, and future roles. NRC Res Press 24:403–415. doi: 10.1139/er-2016-0003

Francis I, Holsters M, Vereecke D (2010) The Gram-positive side of plant-microbe interactions: Minireview. Environ Microbiol 12:1–12. doi: 10.1111/j.1462-2920.2009.01989.x

Gerke J (2015) Phytate (Inositol Hexakisphosphate) in Soil and Phosphate Acquisition from Inositol Phosphates by Higher Plants. A Review. Plants 4:253–266. doi: 10.3390/plants4020253

Glickmann E, Dessaux Y (1995) A critical examination of the specificity of the Salkowski reagent for indolic compounds produced by phytopathogenic bacteria. Appl Environ Microbiol 61:793–796

Goldstein AH (1995) Recent progress in understanding the molecular genetics and biochemistry of calcium phosphate solubilization by gram negative bacteria. Biol Agric Hortic 12:185–193. doi: 10.1080/01448765.1995.9754736

Govindasamy V, Senthilkumar M, Magheshwaran V, et al (2010) Bacillus and Paenibacillus spp.: Potential PGPR for Sustainable Agriculture. In: Maheshwari DK (ed) Microbiology Monographs. Springer Berlin Heidelberg, Berlin, Heidelberg, pp 333–364

Grady EN, MacDonald J, Liu L, et al (2016) Current knowledge and perspectives of Paenibacillus: A review. Microb Cell Fact 15:1–18. doi: 10.1186/s12934-016-0603-7

Granada CE, Passaglia LMP, de Souza EM, Sperotto RA (2018) Is phosphate solubilization the forgotten child of plant growth-promoting rhizobacteria? Front Microbiol 9:2016–2019. doi: 10.3389/fmicb.2018.02054

Gupta A, Sahu PK (2017) Phosphorus Nutrition of Plants: A Microbial Perspective. MOJ Eco Environ Sci 2(6): 00043. DOI: 10.15406/mojes.2017.02.00043

Gurevich A, Saveliev V, Vyahhi N, Tesler G (2013) QUAST: quality assessment tool for genome assemblies. Bioinformatics 29:1072–1075

Holford, I. C. R. (1997). Soil phosphorus: its measurement, and its uptake by plants. Soil Research, 35(2), 227-240. DOI: 10.1071/S96047

Hopkins, B.G. 2015. Phosphorus. In Barker, A.V. and D.J. Pilbeam (eds.) Handbook of plant nutrition, second edition. p. 65-126. CRC Press, Boca Raton, Florida

Ibarra-Galeana JA, Castro-Martínez C, Fierro-Coronado RA, et al (2017) Characterization of phosphate-solubilizing bacteria exhibiting the potential for growth promotion and phosphorus nutrition improvement in maize (Zea mays L.) in calcareous soils of Sinaloa, Mexico. Ann Microbiol 67:801–811. doi: 10.1007/s13213-017-1308-9

Illmer P, Schinner F (1995) Solubilization of inorganic calcium phosphates—Solubilization mechanisms. Soil Biol Biochem 27:257–263. doi: 10.1016/0038-0717(94)00190-C

Jayasinghearachchi HS, Seneviratne G (2006) Fungal solubilization of rock phosphate is enhanced by forming fungal–rhizobial biofilms. Soil Biol Biochem 38:405–408. doi: 10.1016/j.soilbio.2005.06.004

Kamat SS, Raushel FM (2013) The enzymatic conversion of phosphonates to phosphate by bacteria. Curr Opin Chem Biol 17:589–596. doi: 10.1016/j.cbpa.2013.06.006

Kamilova F, Kravchenko L V., Shaposhnikov AI, et al (2006) Organic Acids, Sugars, and Tryptophane in Exudates of Vegetables Growing on Stonewool and Their Effects on Activities of Rhizosphere Bacteria. Mol Plant-Microbe Interact 19:250–256. doi: 10.1094/MPMI-19-0250

Kang, S. M., Radhakrishnan, R., You, Y. H., Joo, G. J., Lee, I. J., Lee, K. E., & Kim, J. H. (2014). Phosphate solubilizing Bacillus megaterium mj1212 regulates endogenous plant carbohydrates and amino acids contents to promote mustard plant growth. Indian journal of microbiology, 54(4), 427-433.DOI: 10.1007/s12088-014-0476-6

Kaur C, Selvakumar G, Ganeshamurthy AN (2016) Organic Acids in the Rhizosphere: Their Role in Phosphate Dissolution. In: Microbial Inoculants in Sustainable Agricultural Productivity. Springer India, New Delhi, pp 165–177

Kochian L V. (2012) Rooting for more phosphorus. Nature 488:466–467. doi: 10.1038/488466a

Krishnaraj PU, Dahale S (2014) Mineral Phosphate Solubilization: Concepts and Prospects in Sustainable Agriculture. Proc Indian Natl Sci Acad 80:389. doi: 10.16943/ptinsa/2014/v80i2/55116

Kumar S, Stecher G, Tamura K (2016) MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. Mol Biol Evol 33:1870–1874. doi: 10.1093/molbev/msw054

Li, Y., Liu, X., Hao, T., & Chen, S. (2017). Colonization and maize growth promotion induced by phosphate solubilizing bacterial isolates. International journal of molecular sciences, 18(7), 1253. DOI: 10.3390/ijms18071253

Liang TW, Wang SL (2015) Recent advances in exopolysaccharides from Paenibacillus spp.: Production, isolation, structure, and bioactivities. Mar Drugs 13:1847–1863. doi: 10.3390/md13041847

Mardad I, Serrano A, Soukri1 A (2014) Effect of Carbon, Nitrogen Sources and Abiotic Stress on Phosphate Solubilization by Bacterial Strains Isolated from a Moroccan Rock Phosphate Deposit. J Adv Chem Eng 4:. doi: 10.4172/2090-4568.1000102

Marra LM, de Oliveira-Longatti SM, Soares CRFS, et al (2015) Initial pH of medium affects organic acids production but do not affect phosphate solubilization. Brazilian J Microbiol 46:367–375. doi: 10.1590/S1517-838246246220131102

Matos ADM, Gomes ICP, Nietsche S, et al (2017) Phosphate solubilization by endophytic bacteria isolated from banana trees. An Acad Bras Cienc 89:2945–2954. doi: 10.1590/0001-3765201720160111

Menezes-Blackburn D, Giles C, Darch T, et al (2018) Opportunities for mobilizing recalcitrant phosphorus from agricultural soils: a review. Plant Soil 427:5–16. doi: 10.1007/s11104-017-3362-2

Murphy J, Riley JP (1962) A modified single solution method for the determination of phosphate in natural waters. Anal Chim Acta 27:31–36. doi: 10.1016/S0003-2670(00)88444-5

Nahas, E. (2007). Phosphate solubilizing microorganisms: Effect of carbon, nitrogen, and phosphorus sources. In First International Meeting on Microbial Phosphate Solubilization(pp. 111-115). Springer, Dordrecht.

Nautiyal CS (1999) An efficient microbiological growth medium for screening phosphate solubilizing microorganisms. FEMS Microbiol Lett 170:265–270. doi: 10.1111/j.1574-6968.1999.tb13383.x

Navarro-Noya YE, Hernández-Mendoza E, Morales-Jiménez J, et al (2012) Isolation and characterization of nitrogen fixing heterotrophic bacteria from the rhizosphere of pioneer plants growing on mine tailings. Appl Soil Ecol 62:52–60. doi: 10.1016/j.apsoil.2012.07.011

O'Toole GA (2011) Microtiter Dish Biofilm Formation Assay. J Vis Exp 10–11. doi: 10.3791/2437

Parks DH, Imelfort M, Skennerton CT, et al (2015) CheckM: assessing the quality of microbial genomes recovered from isolates, single cells, and metagenomes. Genome Res 25:1043–1055

Pérez E, Sulbarán M, Ball MM, Yarzábal LA (2007) Isolation and characterization of mineral phosphate-solubilizing bacteria naturally colonizing a limonitic crust in the south-eastern Venezuelan region. Soil Biol Biochem 39:2905–2914. doi: 10.1016/j.soilbio.2007.06.017

Prabhu N, Borkar S, Garg S (2018) Phosphate solubilization mechanisms in alkaliphilic bacterium Bacillus marisflavi FA7. Curr Sci 114:845–853. doi: 10.18520/cs/v114/i04/845-853

Rodríguez H, Fraga R, Gonzalez T, Bashan Y (2006) Genetics of phosphate solubilization and its potential applications for improving plant. Plant Soil 287:15–21

Saeid, A., Prochownik, E., & Dobrowolska-Iwanek, J. (2018). Phosphorus solubilization by Bacillus species. Molecules, 23(11), 2897.DOI: 10.3390/molecules23112897

Salimpour S, Khavazi K, Nadian H, et al (2012) Canola Oil Production and Nutrient Uptake As Affected By Phosphate Solubilizing and Sulfur Oxidizing Bacteria. J Plant Nutr 35:1997–2008. doi: 10.1080/01904167.2012.716892

Sambrook J, Russel DW (2001) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Nova York.

Sant'Anna FH, Bach E, Porto RZ, et al (2019) Genomic metrics made easy: what to do and where to go in the new era of bacterial taxonomy. Crit Rev Microbiol. *In press*. doi: 10.1080/1040841X.2019.1569587

Santos-Beneit F (2015) The Pho regulon: A huge regulatory network in bacteria. Front Microbiol 6:1–13. doi: 10.3389/fmicb.2015.00402

Sashidhar, B., & Podile, A. R. (2010). Mineral phosphate solubilization by rhizosphere bacteria and scope for manipulation of the direct oxidation pathway involving glucose dehydrogenase. Journal of applied microbiology, 109(1), 1-12.

Sasse J, Martinoia E, Northen T (2018) Feed Your Friends: Do Plant Exudates Shape the Root Microbiome? Trends Plant Sci 23:25–41. doi: 10.1016/j.tplants.2017.09.003

Shen J, Yuan L, Zhang J, et al (2011) Phosphorus Dynamics: From Soil to Plant. PLANT Physiol 156:997–1005. doi: 10.1104/pp.111.175232

Silva Filho GN, Vidor C (2000) Phosphate solubilization by microorganisms in the presence of different carbon sources. Rev Bras Ciência do Solo 24:311–319. doi: 10.1590/S0100-06832000000200008

Suleman M, Yasmin S, Rasul M, et al (2018) Phosphate solubilizing bacteria with glucose dehydrogenase gene for phosphorus uptake and beneficial effects on wheat. PLoS One 13:e0204408. doi: 10.1371/journal.pone.0204408

Taktek S, St-Arnaud M, Piché Y, et al (2017) Igneous phosphate rock solubilization by biofilm-forming mycorrhizobacteria and hyphobacteria associated with Rhizoglomus irregulare DAOM 197198. Mycorrhiza 27:13–22. doi: 10.1007/s00572-016-0726-z

Timmusk S, Grantcharova N, Gerhart EH, Wagner EGH (2005) Paenibacillus polymyxa Invades Plant Roots and Forms Biofilms Paenibacillus polymyxa Invades Plant Roots and Forms Biofilms. Appl Environ Microbiol 71:274–302. doi: 10.1128/AEM.71.11.7292

Valetti, L., Iriarte, L., & Fabra, A. (2018). Growth promotion of rapeseed (Brassica napus) associated with the inoculation of phosphate solubilizing bacteria. Applied Soil Ecology, 132, 1-10. DOI: 10.1016/j.apsoil.2018.08.017

Velmourougane K, Prasanna R, Saxena AK (2017) Agriculturally important microbial biofilms: Present status and future prospects. J Basic Microbiol 57:548–573. doi: 10.1002/jobm.201700046

Vollú RE, Fogel R, dos Santos SCC, et al (2006) Evaluation of the diversity of cyclodextrin-producing Paenibacillus graminis strains isolated from roots and rhizospheres of different plants by molecular methods. J Microbiol 44:591–9. doi: 2469 [pii]

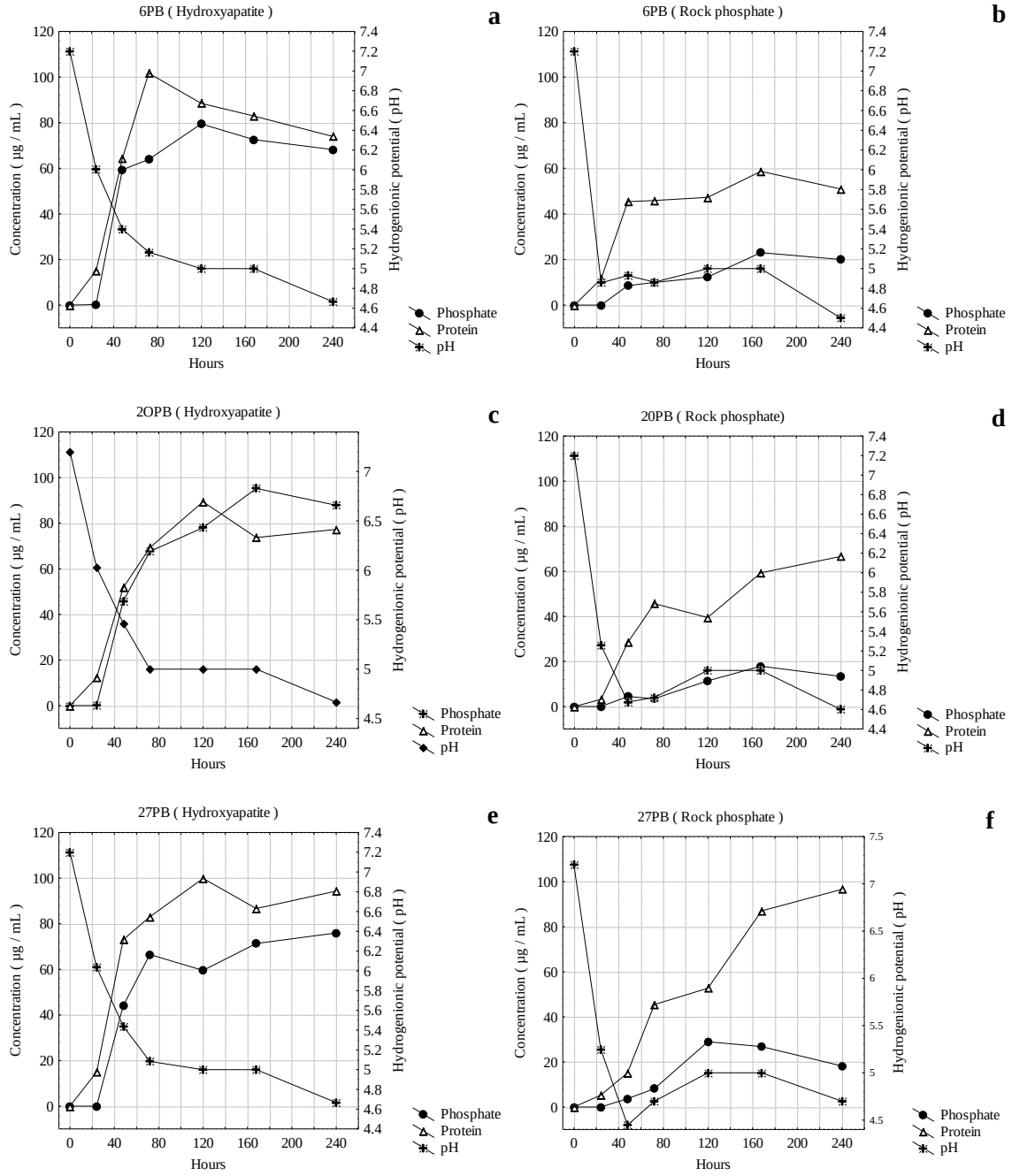
Wang Y-L, Almvik M, Clarke N, et al (2015) Contrasting responses of root morphology and root-exuded organic acids to low phosphorus availability in three important food crops with divergent root traits. AoB Plants 7:plv097. doi: 10.1093/aobpla/plv097

Xie J, Shi H, Du Z, et al (2016) Comparative genomic and functional analysis reveal conservation of plant growth promoting traits in Paenibacillus polymyxa and its closely related species. Sci Rep 6:21329. doi: 10.1038/srep21329

Yi Y, Huang W, Ge Y (2008) Exopolysaccharide: A novel important factor in the microbial dissolution of tricalcium phosphate. World J Microbiol Biotechnol 24:1059–1065. doi: 10.1007/s11274-007-9575-4

Zeng Q, Wu X, Wang J, Ding X (2017) Phosphate solubilization and gene expression of phosphate-solubilizing bacterium Burkholderia multivorans WS-FJ9 under different levels of soluble phosphate. J Microbiol Biotechnol 27:844–855. doi: 10.4014/jmb.1611.11057

Supplementary material



Supplementary Figure 1 Phosphate solubilization kinetics of canola *Paenibacilus graminis* isolates. Phosphate solubilization activity evaluated using NBRIP medium supplemented with 5 g/L of hydroxyapatite or Rock phosphate. A and B: isolate 6PB; C and D: isolate 20PB; E and F: isolate 27PB.

Supplementary Table 1 Effect of nitrogen source in the hydroxyapatite solubilization ability of selected *Paenibacillus* strains isolated from canola rhizosphere

Nitrogen source	$[PO_4^{-3}]$ (µg/mL)			$[PO_4^{-3}]$ / [Protein]		
	6PB	20PB	27PB	6PB	20PB	27PB
NH_4Cl	65.42^{a}	69.72^{a}	80.22^{a}	1.56^{a}	1.25^{a}	1.85^{a}
NH_4SO_4	56.58^{a}	29.22^{b}	53.21^{a}	1.12^{a}	3.71^{a}	0.79^{ab}
NH_4NO_3	42.46^{ab}	31.48^{b}	55.33^{a}	3.07^{a}	2.67^{a}	1.16^{ab}
KNO_3	9.57^{c}	8.12^{b}	15.87^{b}	0.20^{a}	0.23^{a}	0.67^{b}
$Ca(NO_3)_2$	21.28^{bc}	15.00^{b}	11.44^{b}	0.88^{a}	0.99^{a}	0.24^{b}

P solubilization activity evaluated after 7 days of growth in NBRIP medium. For the same column, mean values followed by the same letter don't differ statistically among themselves by a Tukey's test at a 5% significance level.

Supplementary Table 2 Effect of carbon source in hydroxyapatite solubilization ability of selected *Paenibacillus* strains isolated from canola rhizosphere

Carbon source	$[PO_4^{-3}]$ (µg/mL)			$[PO_4^{-3}]$ / [Protein]		
	6PB	20PB	27PB	6PB	20PB	27PB
Glucose	94.54^{a}	81.86^{a}	62.83^{b}	1.95^{b}	1.68^{a}	2.24^{b}
Sucrose	108.51^{a}	100.91^{a}	86.35^{a}	2.97^{a}	1.88^{a}	3.14^{a}
Maltose	43.00^{b}	27.25^{b}	41.04^{c}	0.84^{c}	0.64^{bc}	1.32^{c}
Mannitol	15.98^{c}	10.58^{c}	16.49^{d}	0.32^{c}	0.21^{c}	0.47^{d}
Galactose	42.27^{b}	46.11^{b}	44.59^{bc}	0.80^{c}	1.04^{b}	1.35^{c}

P solubilization activity evaluated after 7 days of growth in NBRIP medium. For the same column, mean values followed by the same letter don't differ statistically among themselves by a Tukey's test at a 5% significance level

Supplementary Table 3 Effect of soluble P in hydroxyapatite solubilization of selected *Paenibacillus* strains isolated from canola rhizosphere on plate assay

[Phosphate] (mM)	6PB	20PB	27PB
0	1.53[a]	1.44[a]	1.48[a]
0.5	1.42[ab]	1.45[a]	1.33[ab]
1	1.23[ab]	1.15[a]	1.16[ab]
5	1.12[b]	1.17[a]	1.12[b]
10	-	-	-
20	-	-	-

P solubilization activity evaluated after 7 days of growth in NBRIP medium. Mean values followed by the same letter don't differ statistically among themselves by a Tukey's test at a 5% significance level. (-): absence of solubilization halo

Supplementary Table 4 General features of the draft genome

	***P. graminis* 6PB**	***P. graminis* 20PB**	***P. graminis* 27PB***
Genome size (bp)	7,175,973	7,175,733	5,991,435
G + C content (%)	50.1	50.1	50.2
Number of contigs	78	113	3597
N50	202,067	133,357	2,118
L50	9	15	37
Number of CDSs	6960	7018	415
Number of RNAs	96	94	6
Completeness	99.8	99.9	88.46
Contamination	2.03	1.96	4.11
Best assembler	Spades	Spades	Spades

*Genome of low quality

Supplementary Table 5 Comparison of selected P-solubilizing 6PB isolate with its close relative type strains through genome metrics

ANIb(ANIm)\ dDDH (%)*	***P. graminis* 27PB**	***P. graminis* 20PB**	***P. graminis* 6PB**	***P. graminis* DSM15220**	***P. jilunlii* DSM23019**	***P. riograndensis* SBR5**	***P. sonchi* X19-5**	***P. polymyxa* ATCC842**
P. graminis 27PB	*	**80.04**	**78.71**	**94.94**	25.83	22.18	18.41	12.36
P. graminis 20PB	**97.58 (99.80)**	*	**98.24**	**95.57**	24.49	13.11	16.49	0
P. graminis 6PB	**97.5 (99.83)**	**99.96 (99.96)**	*	**95.65**	24.85	13.34	16.75	0
P. graminis DSM15220 (NZ CP009287.1)	**95.69 (98.56)**	**98.59 (98.72)**	**98.61 (98.74)**	*	23.9	52.25	16.3	0.01
P. jilunlii DSM23019 (NZ LIPY01000124.1)	89.91 (93.56)	93.03 (93.40)	93.06 (93.42)	92.99 (93.34)	*	22.7	28.65	0
P. riograndensis SBR5 (NZ LN831776.1)	89.89 (93.02)	91.62 (92.48)	91.66 (92.52)	91.59 (92.49)	92.59 (93.31)	*	**83.45**	0
P. sonchi X19-5 (NZ AJTY01000067.1)	89.69 (93.11)	92.1 (92.78)	92.12 (92.81)	91.88 (92.75)	93.16 (93.69)	96.19 (96.95)	*	0
P. polymyxa ATCC842 (NZ GL905390.1)	69.81 (95.05)	68.7 (85.72)	68.68 (85.78)	68.8 (85.83)	68.83 (85.84)	68.7 (85.45)	68.86 (85.94)	*

ANI, Average Nucleotide Identity; ANIb is based on BLAST and ANIm on MUMmer alignments; dDDH, Digital DNA:DNA hybridization. dDDH values are shown in the upper triangle and ANI values are shown in the lower triangle *Bold values were above the expected taxonomic threshold and indicate these strains belong to the same species (95% for ANI and 70% for dDDH)

4 CONSIDERAÇÕES FINAIS

Foram avaliadas trinta bactérias gram-positivas, esporulantes, que apresentaram múltiplas características de promoção do crescimento vegetal. Cinco destes isolados revelaram atividade antagônica à *S. sclerotiorum* em placas, sendo que a capacidade inibitória de fungos filamentosos de gêneros distintos também foi observada. Isso demonstra que o potencial como agentes de biocontrole desses isolados pode ser explorado em relação a demais micro-organismos fitopatogênicos, tanto de canola como de outras espécies vegetais de interesse agronômico.

Os genomas dos isolados com atividade antagonista foram sequenciados a fim de se elucidar possíveis mecanismos relacionados ao processo de inibição de *S. sclerotiorum*. As análises genômicas revelaram *clusters* putativos para produção de bacteriocinas, alguns deles não apresentando, no entanto, qualquer similaridade com *clusters* gênicos específicos, já caracterizados para essas substâncias. Considerando as propriedades antimicrobianas dessa classe de compostos e seu protagonismo na supressão de algumas doenças vegetais por PGPB (Subramanian e Smith, 2015), a capacidade inibitória de patógenos bacterianos pelos isolados obtidos pode ser investigada de modo mais esclarecedor. No que se refere à cultura da canola, a podridão negra da crucíferas, causada por *Xanthomonas campestris* pv. *campestris* é uma doença bacteriana recorrente (Tomm et al., 2009) e um possível alvo de controle a ser testado com esses isolados.

Não foi observada a capacidade de inibição do desenvolvimento de *S. sclerotiorum* pelo sobrenadante das culturas bacterianas. Procedeu-se com a tentativa de obtenção de possíveis lipopeptídeos presentes nesses sobrenadantes, através de extração com butanol seguida de precipitação ácida, mas nenhuma amostra obtida apresentou atividade (dados não apresentados). Esses fatos direcionaram os esforços para a tentativa de se caracterizar os lipopeptídeos, se presentes, em recortes de ágar da zona de inibição do fungo confrontado com as bactérias antagonistas crescidas de modo pareado. No entanto, não se obteve a detecção de massas relativas aos compostos mais comuns das classes de surfactina, iturina ou fengicina e nem de derivados de pumilacidinas, embora *clusters* gênicos biosintéticos para esses últimos tenham sido encontrados nos genomas analisados. A otimização das condições de cultivo bacteriano, do processo de extração e dos métodos analíticos poderá fornecer melhores resultados na confirmação da produção putativa desses compostos.

Outros mecanismos bioquímicos podem também ser os responsáveis pela atividade antifúngica observada *in vitro*. Isso pode envolver uma grande diversidade de enzimas hidrolíticas com ação sobre a parede celular fúngica e, também, a produção de compostos orgânicos voláteis (VOC) (Beneduzi et al., 2012). Quatro isolados foram capazes de inibir o crescimento de *S. sclerotiorum* através da produção dessas substâncias. Análises do *headspace* das culturas microbianas por GC-MS (Timm et al., 2018) poderão ser empregadas a fim de se identificar potenciais compostos voláteis antimicrobianos relacionados a esse processo.

Dentro das condições experimentais utilizadas, não foi possível observar nenhum efeito da supressão da *S. sclerotiorum* em canola. Outras formas de infecção, como a germinação de ascósporos na parte aérea do vegetal durante a fase de florescimento, são de ocorrência natural muito mais frequentes no cultivo da canola (Kamal et al., 2016). Portanto, resultados satisfatórios ainda podem ser obtidos considerando outras vias de infecção pelo fungo, outras formas de inoculação bacteriana (aspersão da parte aérea) e outros estágios de desenvolvimento do vegetal. As respostas dos vegetais à inoculação com PGPB são bastante variáveis em diferentes cultivares. Outras variedades de canola também podem ser consideradas. A escolha da variedade da Hyola 61 se baseou no fato de atualmente esse híbrido ser o mais utilizado no Brasil, dada sua resistência poligênica à canela preta (De Mori et al., 2014).

Os isolados apresentaram diversas características *in vitro* de promoção de crescimento vegetal e competência rizosférica. Essa última habilidade é considerada importante no sucesso de muitos inoculantes e explica a falha de estirpes de elevada potencialidade *in vitro* na tentativa de promoverem o crescimento vegetal em campo (Prashar et al., 2014). Vários outros aspectos não considerados estão envolvidos nesse processo, tais como a produção de *quorum sensing*, a resistência a condições de estresse oxidativo, modulação da resposta de defesa vegetal, formação de biofilme, expressão de moléculas de adesão, quimiotaxia, entre outros (Barret et al., 2011). Esses são, portanto, aspectos ainda a serem elucidados no que diz respeito à interação planta-bactéria pelos isolados obtidos.

O nitrogênio e fósforo constituem os macronutrientes mais limitantes para o crescimento vegetal e a obtenção de ambos elementos pelas plantas pode ser facilitada pela

interação mutualística com PGPB (Goswami et al., 2016). Apenas três isolados apresentaram a capacidade de fixar nitrogênio, avaliada pela técnica de ARA, mas a solubilização de fosfato foi uma característica bastante disseminada. Esse fato conduziu a uma melhor caracterização dessa habilidade no segundo capítulo da dissertação. Coincidentemente, os três isolados fixadores de nitrogênio obtidos foram os isolados de destaque no estudo sobre solubilização de fosfato, reforçando o potencial desses micro-organismos como agentes promotores de crescimento vegetal.

Os genomas dos três isolados de *P. graminis* que se destacaram em termos de solubilização de fosfato também foram sequenciados. No entanto, as sequencias obtidas para o isolado 27PB apresentaram baixa qualidade. Um novo sequenciamento desse genoma poderá fornecer resultados mais conclusivos em relação às análises genômicas empregadas. A partir dos genomas anotados e do perfil de metabólitos encontrados por espectrometria de massas, pretende-se identificar as possíveis vias metabólicas relacionadas à produção dos ácidos orgânicos encontrados.

Até o momento, não foi demonstrada a capacidade de promoção do crescimento vegetal por aumento no fornecimento de fósforo aos vegetais, como consequência da inoculação das estirpes solubilizadoras identificadas. No entanto, pretende-se otimizar as condições experimentais para essa averiguação e estender a verificação dessa capacidade também para demais culturas de relevância pertencentes a outros grupos taxonômicos de plantas.

5 REFERÊNCIAS BIBLIOGRÁFICAS

Abd-Alla MH e Ohyam T (2014) Impact of Harsh Environmental Conditions on Nodule Formation and Dinitrogen Fixation of Legumes. Advances in Biology and Ecology of Nitrogen Fixation. InTech, p 131–193

Ahemad M and Kibret M (2014) Mechanisms and applications of plant growth promoting rhizobacteria: Current perspective. J King Saud Univ - Sci 26:1–20. doi: 10.1016/J.JKSUS.2013.05.001

Ahmadi-Rad S, Gholamhoseini M, Ghalavand A, Asgharzadeh A e Dolatabadian A (2016) Foliar application of nitrogen fixing bacteria increases growth and yield of canola grown under different nitrogen regimes. Rhizosphere 2:34–37. doi: 10.1016/j.rhisph.2016.08.006

Ahmed E e Holmström SJM (2014) Siderophores in environmental research: Roles and applications. Microb Biotechnol 7:196–208. doi: 10.1111/1751-7915.12117

Ahmed N, Shahab S (2009) Phosphate solubilization: their mechanism genetics and application. Int J Microbiol 9: 1-19

Ali SS e Vidhale NN (2013) Review Article Bacterial Siderophore and their Application: A review. Int J Curr Microbiol Appl Sci 2:303–312.

Alori ET e Babalola OO. (2018). Microbial inoculants for improve crop quality and human health. Front microbiol 9: 2213.

Alori ET, Glick BR and Babalola OO (2017) Microbial phosphorus solubilization and its potential for use in sustainable agriculture. Front Microbiol 8:1–8. doi: 10.3389/fmicb.2017.00971

Audrain B, Farag MA, Ryu C-M and Ghigo J-M (2015) Role of bacterial volatile compounds in bacterial biology. FEMS Microbiol Rev 39:222–233. doi: 10.1093/femsre/fuu013

Baez-Rogelio A, Morales-García YE, Quintero-Hernández V e Muñoz-Rojas J. (2017). Next generation of microbial inoculants for agriculture and bioremediation. Microb Biotechnol 10: 19-21.

Barea J and Richardson AE (2015) Phosphate Mobilisation by Soil Microorganisms. In: Lugtenberg B (ed) Principles of Plant-Microbe Interactions. Springer International Publishing, Cham, pp 225–234

Barret M, Morrissey JP and O'Gara F (2011) Functional genomics analysis of plant growth-promoting rhizobacterial traits involved in rhizosphere competence. Biol Fertil Soils 47:729–743. doi: 10.1007/s00374-011-0605-x

Bashan Y, de-Bashan LE, Prabhu SR e Hernandez JP. (2014). Advances in plant growth-promoting bacterial inoculant technology: formulations and practical perspectives (1998–2013). Plant Soil 378: 1-33.

Bashan Y, Kamnev AA e De-Bashan LE (2013) Tricalcium phosphate is inappropriate as a universal selection factor for isolating and testing phosphate-solubilizing bacteria that enhance plant growth: a proposal for an alternative procedure. Biol Fertil Soils 49:465–479. doi: 10.1007/s00374-012-0737-7

Baveye PC (2015) Looming Scarcity of Phosphate Rock and Intensification of Soil Phosphorus Research. Rev Bras Ciência do Solo 39:637–642. doi: 10.1590/01000683rbcs20140819

Behera BC, Singdevsachan SK, Mishra RR, Dutta SK e Thatoi HN (2014) Diversity, mechanism and biotechnology of phosphate solubilising microorganism in mangrove—A review. Biocatal Agric Biotechnol 3:97–110. doi: 10.1016/j.bcab.2013.09.008

Beneduzi A, Ambrosini A and Passaglia LMP (2012) Plant growth-promoting rhizobacteria (PGPR): Their potential as antagonists and biocontrol agents. Genet Mol Biol 35:1044–51.

Bertrand H, Nalin R, Bally R e Cleyet-Marel J-C (2001) Isolation and identification of the most efficient plant growth-promoting bacteria associated with canola (Brassica napus). Biol Fertil Soils 33:152–156. doi: 10.1007/s003740000305

Boland GJ and Hall R (1994) Index of plant hosts of Sclerotinia sclerotiorum. Canadian Journal of Plant Pathology 16: 93-108.

Bolton MD, Thomma BP e Nelson BD. (2006). Sclerotinia sclerotiorum (Lib.) de Bary: biology and molecular traits of a cosmopolitan pathogen. Mol Plant Pathol 7: 1-16.

Boyles M, Peeper T e Stamm M (2012) Great Plains Canola Production Handbook. Kansas State University

Brown J, Davis JB, Lauver M e Wysocki D (2008) Canola Growers ' Manual. 71.

Bulgarelli D, Schlaeppi K, Spaepen S, van Themaat EVL e Schulze-Lefert P (2013) Structure and Functions of the Bacterial Microbiota of Plants. Annu Rev Plant Biol 64:807–838. doi: 10.1146/annurev-arplant-050312-120106

Cameron KC, Di HJ e Moir JL (2013) Nitrogen losses from the soil/plant system: a review. Ann Appl Biol 162:145–173. doi: 10.1111/aab.12014

Campbell L, Rempel C e Wanasundara J (2016) Canola/Rapeseed Protein: Future Opportunities and Directions—Workshop Proceedings of IRC 2015. Plants 5:17. doi: 10.3390/plants5020017

Cardoso RML, Oliveira MAR, Campos RMVB, Barbosa LCJ and Balbino LC. Doenças de Canola no Paraná (1996) IAPAR, Cascavel, 32 p.

Cartea ME e Velasco P (2008) Glucosinolates in Brassica foods: bioavailability in food and significance for human health. Phytochem Rev 7:213–229. doi: 10.1007/s11101-007-9072-2

Carvalho TLG, Saraiva RM, Ferreira PCG e Hemerly AS (2014) Nitrogen signalling in plant interactions with associative and endophytic diazotrophic bacteria. J Exp Bot. doi: 10.1093/jxb/eru319

Cessna SG, Sears VE, Dickman MB e Low PS. (2000). Oxalic acid, a pathogenicity factor for Sclerotinia sclerotiorum, suppresses the oxidative burst of the host plant. The Plant Cell, 12: 2191-2199.

Chanway CP, Anand R e Yang H (2014) Nitrogen Fixation Outside and Inside Plant Tissues. Advances in Biology and Ecology of Nitrogen Fixation. InTech, p 3–22

Cheng F, Wu J e Wang X (2014) Genome triplication drove the diversification of Brassica plants. Hortic Res 1:14024. doi: 10.1038/hortres.2014.24

Choudhary DK, Prakash A e Johri BN. (2007). Induced systemic resistance (ISR) in plants: mechanism of action. Indian J Microbiol 47: 289-297.

Cochrane SA and Vederas JC (2016) Lipopeptides from Bacillus and Paenibacillus spp.: A Gold Mine of Antibiotic Candidates. Med Res Rev 36:4–31. doi: 10.1002/med.21321

CONAB (2019) Acompanhamento da Safra Brasileira de Grãos/ safra 2018/19. v 6, n. 4. Brasilia, 126 p.

Cooper J, Lombardi R, Boardman D e Carliell-Marquet C (2011) The future distribution and production of global phosphate rock reserves. Resour Conserv Recycl 57:78–86. doi: 10.1016/j.resconrec.2011.09.009

De Mori C, Tomm GO, Ernani P and Ferreira P (2014) Aspectos econômicos e conjunturais da cultura da canola no mundo e no Brasil. Embrapa Trigo, Passo Fundo

Dey R, Pal KK and Tilak KVBR (2014) Plant Growth Promoting Rhizobacteria in Crop Protection and Challenges. In: Goyal A and Manoharachary C (eds) Future Challenges in Crop Protection Against Fungal Pathogens. Springer, New York, pp 31–58

Dey R, Pal KK e Tilak KVBR (2014) Future Challenges in Crop Protection Against Fungal Pathogens. doi: 10.1007/978-1-4939-1188-2

Dodds W e Smith V (2016) Nitrogen, phosphorus, and eutrophication in streams. Inl Waters 6:155–164. doi: 10.5268/IW-6.2.909

Dutton MV e Evans CS. (1996). Oxalate production by fungi: its role in pathogenicity and ecology in the soil environment. Can J Microbiol 42: 881-895.

Egamberdieva D, Wirth SJ, Alqarawi AA, Abd Allah EF e Hashem A (2017). Phytohormones and beneficial microbes: essential components for plants to balance stress and fitness. Front microbiol 8

El-Howeity MA e Asfour MM (2012) Response of some varieties of canola plant (Brassica napus L.) cultivated in a newly reclaimed desert to plant growth promoting rhizobacteria and mineral nitrogen fertilizer. Ann Agric Sci 57:129–136. doi: 10.1016/j.aoas.2012.08.006

Eljounaidi K, Lee SK and Bae H (2016) Bacterial endophytes as potential biocontrol agents of vascular wilt diseases – Review and future prospects. Biol Control 103:62–68. doi: 10.1016/j.biocontrol.2016.07.013

Enebe MC and Babalola OO (2019) The impact of microbes in the orchestration of plants' resistance to biotic stress: a disease management approach. Appl Microbiol Biotechnol 103:9–25. doi: 10.1007/s00253-018-9433-3

Erisman JW, Galloway JN, Seitzinger S, Bleeker A, Dise NB, Petrescu AMR, Leach AM e de Vries W (2013) Consequences of human modification of the global nitrogen cycle. Philos Trans R Soc B Biol Sci 368:20130116–20130116. doi: 10.1098/rstb.2013.0116

Estevez RL, Duarte JB, Chambo APS e Cruz MIF (2014) A Cultura da Canola (Brassica napus var. oleifera). Sci Agrar Parana 13:1–9. doi: 10.18188/1983-

1471/sap.v13n1p1-9

Farag MA, Zhang H and Ryu C-M (2013) Dynamic Chemical Communication between Plants and Bacteria through Airborne Signals: Induced Resistance by Bacterial Volatiles. J Chem Ecol 39:1007–1018. doi: 10.1007/s10886-013-0317-9

Farhat M Ben, Boukhris I and Chouayekh H (2015) Mineral phosphate solubilization by Streptomyces sp. CTM396 involves the excretion of gluconic acid and is stimulated by humic acids. FEMS Microbiol Lett 362:1–8. doi: 10.1093/femsle/fnv008

Fernando, WGD, Parks PS, Tomm G, Viau LV e Jurke C (2003) First Report of Blackleg Disease Caused by Leptosphaeria maculans on Canola in Brazil. Plant disease, 87: 314-314.

Finking R and Marahiel MA (2004) Biosynthesis of Nonribosomal Peptides. Annu Rev Microbiol 58:453–488. doi: 10.1146/annurev.micro.58.030603.123615

Fowler D, Coyle M, Skiba U, Sutton MA, Cape JN, Reis S, Sheppard LJ, Jenkins A, Grizzetti B, Galloway JN et al. (2013) The global nitrogen cycle in the twenty-first century. Philos Trans R Soc B Biol Sci 368:20130164–20130164. doi: 10.1098/rstb.2013.0164

Fravel DR (1988) Role of Antibiosis in the Biocontrol of Plant Diseases*. Annu Rev Phytopathol 26:75–91. doi: 10.1146/annurev.py.26.090188.000451

Gabriel KT, Joseph Sexton D and Cornelison CT (2018) Biomimicry of volatile-based microbial control for managing emerging fungal pathogens. J Appl Microbiol 124:1024–1031. doi: 10.1111/jam.13667

Galloway JN, Leach AM, Bleeker A e Erisman JW (2013) A chronology of human understanding of the nitrogen cycle. Philos Trans R Soc B Biol Sci 368:20130120–20130120. doi: 10.1098/rstb.2013.0120

Gandhi A e Muralidharan G (2016) Assessment of zinc solubilizing potentiality of Acinetobacter sp. isolated from rice rhizosphere. Eur J Soil Biol 76:1–8. doi: 10.1016/j.ejsobi.2016.06.006

Garbeva P, Hol WHG, Termorshuizen AJ, Kowalchuk GA and de Boer W (2011) Fungistasis and general soil biostasis – A new synthesis. Soil Biol Biochem 43:469–477. doi: 10.1016/j.soilbio.2010.11.020

Garcia G, Cardoso AA e Dos Santos OAM (2013) Da escassez ao estresse do planeta: Um século de mudanças no ciclo do nitrogênio. Quim Nova 36:1468–1476. doi: 10.1590/S0100-40422013000900032

Gellings C e Parmenter K (2004) Energy efficiency in fertilizer production and use. In: Clark W. Gellings e Kornelis Blok (orgs) Encyclopedia of Life Support Systems (EOLSS). Eolss Publishers, Oxford, p 1–15

Glick BR (2014) Bacteria with ACC deaminase can promote plant growth and help to feed the world. Microbiol Res 169:30–39. doi: 10.1016/j.micres.2013.09.009

Glick BR (2015) Beneficial Plant-Bacterial Interactions. Benef Plant-Bacterial Interact. doi: 10.1007/978-3-319-13921-0

Goldstein AH (1995) Recent progress in understanding the molecular genetics and biochemistry of calcium phosphate solubilization by gram negative bacteria. Biol

Agric Hortic 12:185–193. doi: 10.1080/01448765.1995.9754736

Goldstein AH and Liu ST (1987) Molecular Cloning and Regulation of a Mineral Phosphate Solubilizing Gene from Erwinia Herbicola. Nat Biotechnol 5:72–74. doi: 10.1038/nbt0187-72

Gontia-Mishra I, Sasidharan S e Tiwari S (2014) Recent developments in use of 1-aminocyclopropane-1-carboxylate (ACC) deaminase for conferring tolerance to biotic and abiotic stress. Biotechnol Lett 36:889–898. doi: 10.1007/s10529-014-1458-9

Goswami D, Thakker JN and Dhandhukia PC (2016) Portraying mechanics of plant growth promoting rhizobacteria (PGPR): A review. Cogent Food Agric 2:1127500. doi: 10.1080/23311932.2015.1127500

Gupta A and Kumar Sahu P (2017) Phosphorus Nutrition of Plants: A Microbial Perspective. MOJ Ecol Environ Sci 2:6–8. doi: 10.15406/mojes.2017.02.00043

Harker KN, O'Donovan JT, Turkington TK, Blackshaw RE, Lupwayi NZ, Smith EG, Peng G (2014). Canola rotation frequency impacts canola yield and associated pest species.Can. J Plant Sci 95: 9-20.

Hassan M, Kjos M, Nes IF, Diep DB and Lotfipour F (2012) Natural antimicrobial peptides from bacteria: characteristics and potential applications to fight against antibiotic resistance. J Appl Microbiol 113:723–736. doi: 10.1111/j.1365-2672.2012.05338.x

Hassani MA, Durán P and Hacquard S (2018) Microbial interactions within the plant holobiont. Microbiome 6:58. doi: 10.1186/s40168-018-0445-0

Hassen AI, Bopape FL e Sanger LK (2016) Microbial Inoculants as Agents of Growth Promotion and Abiotic Stress Tolerance in Plants. Microbial Inoculants in Sustainable Agricultural Productivity. Springer India, New Delhi, p 23–36

Hertweck C (2009) The Biosynthetic Logic of Polyketide Diversity. Angew Chemie Int Ed 48:4688–4716. doi: 10.1002/anie.200806121

Hoffmann LV (2007) Biologia Molecular da Fixação Biológica do Nitrogênio. In: Silveira APD da e Freitas S dos S (orgs) Microbiota do solo e qualidade ambiental. Omnipax, Campinas, p 153–312

Hopkins BG (2015) Phosphorus. In:Barker AV, Pilbeam DJ (eds) Handbook of plant nutrition. CRC press, pp 65-126.

Huang L, Buchenauer H, Han Q, Zhang X and Kang Z. (2008). Ultrastructural and cytochemical studies on the infection process of Sclerotinia sclerotiorum in oilseed rape. Journal of Plant Diseases and Protection, 115: 9-16.

Hungria M e Mendes IC (2015) Nitrogen Fixation with Soybean: The Perfect Symbiosis? Biological Nitrogen Fixation. John Wiley & Sons, Inc, Hoboken, NJ, USA, p 1009–1024

Hunter PJ, Teakle GR e Bending GD (2014) Root traits and microbial community interactions in relation to phosphorus availability and acquisition, with particular reference to Brassica. Front Plant Sci 5:1–18. doi: 10.3389/fpls.2014.00027

Ingle KP and Padole DA (2017) Phosphate Solubilizing Microbes: An Overview. Int J Curr Microbiol Appl Sci 6:844–852. doi: 10.20546/ijcmas.2017.601.099

Jamaux I, Gelie B e Lamarque C. (1995). Early stages of infection of rapeseed petals and leaves by Sclerotinia sclerotiorum revealed by scanning electron microscopy. Plant Pathol 44: 22-30.

Jez JM, Lee SG e Sherp AM (2016) The next green movement: Plant biology for the environment and sustainability. Science (80-) 353:1241–1244. doi: 10.1126/science.aag1698

Jiang C, Shi J, Li R, Long Y, Wang H, Li D, Zhao J e Meng J (2014) Quantitative trait loci that control the oil content variation of rapeseed (Brassica napus L.). Theor Appl Genet 127:957–968. doi: 10.1007/s00122-014-2271-5

Kamal MM, Lindbeck KD, Savocchia S e Ash GJ. (2015). Biological control of sclerotinia stem rot of canola using antagonistic bacteria. Plant Pathol 64: 1375-1384.

Kamal MM, Savocchia S, Lindbeck KD and Ash GJ (2016) Biology and biocontrol of Sclerotinia sclerotiorum (Lib.) de Bary in oilseed Brassicas. Australas Plant Pathol 45:1–14. doi: 10.1007/s13313-015-0391-2

Kanchiswamy CN, Malnoy M and Maffei ME (2015) Chemical diversity of microbial volatiles and their potential for plant growth and productivity. Front Plant Sci 6:151. doi: 10.3389/fpls.2015.00151

Kaur H, Kaur J e Gera R (2016) Plant Growth Promoting Rhizobacteria: A Boon to Agriculture. Int J Cell Sci Biotechnol 5:17–22.

Khan AA, Jilani G, Akhtar MS, Saqlan SM e Rasheed M (2009) Phosphorus Solubilizing Bacteria: Occurrence, Mechanisms and their Role in Crop Production. J Agric Biol Sci 1:48–58. doi: 10.5923/j.re.20120201.10

Khan MS, Zaidi A e Ahmad E (2014) Mechanism of Phosphate Solubilization and Physiological Functions of Phosphate-Solubilizing Microorganisms. In: Khan MS, Zaidi A e Musarrat J (orgs) Phosphate Solubilizing Microorganisms. Springer International Publishing, Cham, p 31–62

Knodel JJ e Kandel H (2011) Canola production field guide. North Dakota State University

Knutsen HK, Alexander J, Barregård L, Bignami M, Brüschweiler B, Ceccatelli S, Dinovi M, Edler L, Grasl-Kraupp B, Hogstrand C et al. (2016) Erucic acid in feed and food. EFSA J. doi: 10.2903/j.efsa.2016.4593

Kochian L V. (2012) Rooting for more phosphorus. Nature 488:466–467. doi: 10.1038/488466a

Krishnaraj PU e Dahale S (2014) Mineral Phosphate Solubilization: Concepts and Prospects in Sustainable Agriculture. Proc Indian Natl Sci Acad 80:389. doi: 10.16943/ptinsa/2014/v80i2/55116

Kuzyakov Y e Blagodatskaya E (2015) Microbial hotspots and hot moments in soil: Concept & review. Soil Biol Biochem 83:184–199. doi: 10.1016/j.soilbio.2015.01.025

Laranjo M, Alexandre A e Oliveira S (2014) Legume growth-promoting rhizobia: An overview on the Mesorhizobium genus. Microbiol Res 169:2–17. doi: 10.1016/j.micres.2013.09.012

LaSarre B and Federle MJ (2013) Exploiting Quorum Sensing to Confuse Bacterial Pathogens. Microbiol Mol Biol Rev 77:73–111. doi: 10.1128/MMBR.00046-12

Lesueur D, Deaker R, Herrmann L, Bräu L e Jansa J (2016) The Production and Potential of Biofertilizers to Improve Crop Yields. Bioformulations: for Sustainable Agriculture. Springer India, New Delhi, p 71–92

Liang X e Rollins JA (2018) Mechanisms of broad host range necrotrophic pathogenesis in Sclerotinia sclerotiorum. Phytopathology 108: 1128-1140.

Li GQ, Huang HC, Miao HJ, Erickson RS, Jiang DH e Xiao YN. (2006). Biological control of sclerotinia diseases of rapeseed by aerial applications of the mycoparasite Coniothyrium minitans. Eur J Plant Pathol 114: 345-355.

Lin L, Allemekinders H, Dansby A, Campbell L, Durance-Tod S, Berger A e Jones PJ (2013) Evidence of health benefits of canola oil. Nutr Rev 71:370–385. doi: 10.1111/nure.12033

Loganes C, Ballali S e Minto C (2016) Main Properties of Canola Oil Components: A Descriptive Review of Current Knowledge. Open Agric J 10:69–74. doi: 10.2174/1874331501610010069

Lugtenberg B (2015) Principles of Plant-Microbe Interactions. doi: 10.1007/978-3-319-08575-3

Lugtenberg B, Malfanova N, Kamilova F, Berg G (2013) Microbial control of plant diseases. In: Molecular Microbial Ecology of the Rhizosphere. John Wiley & Sons, Hoboken, pp 575 - 586

MacWilliam S, Sanscartier D, Lemke R, Wismer M e Baron V (2016) Environmental benefits of canola production in 2010 compared to 1990: A life cycle perspective. Agric Syst 145:106–115. doi: 10.1016/j.agsy.2016.03.006

Martínez B, Rodríguez A and Suárez E (2016) Antimicrobial Peptides Produced by Bacteria: The Bacteriocins. In: Villa TG and Vinas M (eds) New Weapons to Control Bacterial Growth. Springer International Publishing, Cham, pp 15–38

McCaghey M, Willbur J, Smith DL and Kabbage M (2018). The complexity of the Sclerotinia sclerotiorum pathosystem in soybean: virulence factors, resistance mechanisms, and their exploitation to control Sclerotinia stem rot. Tropical Plant Pathology: 1-11.

Meena KR and Kanwar SS (2015) Lipopeptides as the antifungal and antibacterial agents: Applications in food safety and therapeutics. Biomed Res Int. doi: 10.1155/2015/473050

Mhatre PH, Karthik C, Kadirvelu K, Divya KL, Venkatasalam EP, Srinivasan S, Ramkumar G, Saranya C and Shanmuganathan R (2019) Plant growth promoting rhizobacteria (PGPR): A potential alternative tool for nematodes bio-control. Biocatal Agric Biotechnol 17:119–128. doi: 10.1016/j.bcab.2018.11.009

Miyanaga A (2017) Structure and function of polyketide biosynthetic enzymes: various strategies for production of structurally diverse polyketides. Biosci Biotechnol Biochem 81:2227–2236. doi: 10.1080/09168451.2017.1391687

Mnif I and Ghribi D (2015) Review lipopeptides biosurfactants: Mean classes and new insights for industrial, biomedical, and environmental applications. Biopolymers 104:129–147. doi: 10.1002/bip.22630

Molina L, Constantinescu F, Michel L, Reimmann C, Duffy B and Daofago G (2003) Degradation of pathogen quorum-sensing molecules by soil bacteria: a preventive and curative biological control mechanism. FEMS Microbiol Ecol 45:71–81. doi: 10.1016/S0168-6496(03)00125-9

Mori C, Costamilan LM, Marsaro-Júnior AL and Ferreira PEP (2017) Levantamento de ações de controle de doenças de canola utilizadas por produtores no sul do Brasil. Embrapa, Passo Fundo

Mori C, Tomm GO e Ferreira PEP (2014) Aspectos econômicos e conjunturais da cultura da canola no mundo e no Brasil. 36.

Mukhametzyanova AD, Akhmetova AI and Sharipova MR (2012) Microorganisms as phytase producers. Microbiology. doi: 10.1134/s0026261712030095

Na R, Luo Y, Bo H, Jia R, Meng Q, Zhou H and Zhao J (2018) Responses of sunflower induced by Sclerotinia sclerotiorum infection. Physiological and Molecular Plant Pathology, 102: 113-121.

Norton R, Kirkegaard J, Angus J, Potter T (1999) Canola in rotations. In: Salisbury P, Potter, McDonald TG, Green A (eds) Canola in Australia: the first thirty years. Organising Committee of the 10th International Rapeseed Congress, pp 23-28.

Norton RM (2016) Nitrogen management to optimise canola production in Australia. Crop Pasture Sci 67:419. doi: 10.1071/CP15297

Office of the Gene Technology Regulator - OGTR (2008) The Biology of Brassica napus L .(Canola/Rapeseed). 2:1–62.

Olanrewaju OS, Glick BR and Babalola OO (2017) Mechanisms of action of plant growth promoting bacteria. World J Microbiol Biotechnol 33:197. doi: 10.1007/s11274-017-2364-9

Ordóñez-Valencia C, Ferrera-Cerrato R, Quintanar-Zúñiga RE, Flores-Ortiz C M, Guzmán GJM, Alarcón A, García-Barradas O. (2015). Morphological development of sclerotia by Sclerotinia sclerotiorum: a view from light and scanning electron microscopy. Annals of microbiology, 65: 765-770

Oteino N, Lally RD, Kiwanuka S, Lloyd A, Ryan D, Germaine KJ e Dowling DN (2015) Plant growth promotion induced by phosphate solubilizing endophytic Pseudomonas isolates. Front Microbiol 6:1–9. doi: 10.3389/fmicb.2015.00745

Owen D, Williams AP, Griffith GW and Withers PJA (2015) Use of commercial bio-inoculants to increase agricultural production through improved phosphrous acquisition. Appl Soil Ecol 86:41–54. doi: 10.1016/j.apsoil.2014.09.012

Philippot L, Raaijmakers JM, Lemanceau P e van der Putten WH (2013) Going back to the roots: the microbial ecology of the rhizosphere. Nat Rev Microbiol 11:789–99. doi: 10.1038/nrmicro3109

Pii Y, Mimmo T, Tomasi N, Terzano R, Cesco S e Crecchio C (2015) Microbial interactions in the rhizosphere: beneficial influences of plant growth-promoting rhizobacteria on nutrient acquisition process. A review. Biol Fertil Soils 51:403–415. doi: 10.1007/s00374-015-0996-1

Podolich O, Ardanov P, Zaets I, Pirttilä AM e Kozyrovska N (2015) Reviving of the

endophytic bacterial community as a putative mechanism of plant resistance. Plant Soil 388:367–377. doi: 10.1007/s11104-014-2235-1

Prashar P, Kapoor N and Sachdeva S (2013) Biocontrol of Plant Pathogens Using Plant Growth Promoting Bacteria. In: Lichtfouse E (ed) Sustainable Agriculture Reviews. Springer Netherlands, Dordrecht, pp 319–360

Prashar P, Kapoor N and Sachdeva S (2014) Rhizosphere: its structure, bacterial diversity and significance. Rev Environ Sci Bio/Technology 13:63–77. doi: 10.1007/s11157-013-9317-z

Prathap M e Bd RK (2015) A Critical Review on Plant Growth Promoting Rhizobacteria. J Plant Pathol Microb 6:4–7. doi: 10.4172/2157-7471.1000266

Preininger C, Sauer U, Bejarano A e Berninger T. (2018). Concepts and applications of foliar spray for microbial inoculants. Appl microbiol biotechnol 102: 7265-7282.

Purdy L. (1979). Sclerotinia sclerotiorum: history, diseases and symptomatology, host range, geographic distribution, and impact. Phytopathology 69: 875-880.

Raaijmakers JM and Mazzola M (2012) Diversity and Natural Functions of Antibiotics Produced by Beneficial and Plant Pathogenic Bacteria. Annu Rev Phytopathol 50:403–424. doi: 10.1146/annurev-phyto-081211-172908

Rahman MH, Akter S, Begum T, Khan MSI, Islam MS e Begum MR (2013) Effects of Mild Ingestion of Used Fried Rapeseed Oil (Mustard Oil) on Hepatic Tissues in Long-Evans Rats. J Food Sci Technol Nepal 8:78–80.

Rakesh R, Rathi AS, Kumar P, Kumar e Kumari P. (2016). Sclerotinia rot of rapeseed mustard: A comprehensive review. Journal of Applied and Natural Science, 8: 2325-2336.

Rakow GFW (2004) Species Origin and Economic Importance of Brassica. In: Pua E-C e Douglas CJ (orgs) Biotechnology in Agriculture and Forestry: Brassica. Springer Berlin Heidelberg, Berlin, Heidelberg, p 3–11

Ramjegathesh R, Samiyappan R, Raguchander T, Prabakar K and Saravanakumar D (2013) Plant–PGPR Interactions for Pest and Disease Resistance in Sustainable Agriculture. Bacteria in Agrobiology: Disease Management. Springer Berlin Heidelberg, Berlin, Heidelberg, pp 293–320

Reijnders L (2014) Phosphorus resources, their depletion and conservation, a review. Resour Conserv Recycl 93:32–49. doi: 10.1016/j.resconrec.2014.09.006

Rodríguez H and Fraga R (1999) Phosphate solubilizing bacteria and their role in plant growth promotion. Biotechnol Adv 17:319–339. doi: 10.1016/S0734-9750(99)00014-2

S.K. Gosal, Jupinder Kaur and JK (2017) Probiotics and Plant Health. Probiotics Plant Heal. doi: 10.1007/978-981-10-3473-2

Saha M, Sarkar S, Sarkar B, Sharma BK, Bhattacharjee S e Tribedi P (2016) Microbial siderophores and their potential applications: a review. Environ Sci Pollut Res 23:3984–3999. doi: 10.1007/s11356-015-4294

Saharan BS e Nehra V (2011) Plant Growth Promoting Rhizobacteria : A Critical Review. Life Sci Med Res 2011:1–30.

Saharan GS e Mehta N. (2008). Sclerotinia diseases of crop plants: biology, ecology and disease management. Springer.

Santi C, Bogusz D e Franche C (2013) Biological nitrogen fixation in non-legume plants. Ann Bot 111:743–767. doi: 10.1093/aob/mct048

Santoyo G, Moreno-Hagelsieb G, del Carmen Orozco-Mosqueda M e Glick BR (2016) Plant growth-promoting bacterial endophytes. Microbiol Res 183:92–99. doi: 10.1016/j.micres.2015.11.008

Saraf M, Pandya U and Thakkar A (2014) Role of allelochemicals in plant growth promoting rhizobacteria for biocontrol of phytopathogens. Microbiol Res 169:18–29. doi: 10.1016/J.MICRES.2013.08.009

Saravanan VS, Kumar MR e Sa TM (2011) Microbial Zinc Solubilization and Their Role on Plants. Bacteria in Agrobiology: Plant Nutrient Management. Springer Berlin Heidelberg, Berlin, Heidelberg, p 47–63

Sashidhar B and Podile AR (2010) Mineral phosphate solubilization by rhizosphere bacteria and scope for manipulation of the direct oxidation pathway involving glucose dehydrogenase. J Appl Microbiol 109:1–12. doi: 10.1111/j.1365-2672.2009.04654.x

Sauer M, Robert S e Kleine-Vehn J. (2013). Auxin: simply complicated. J Exp Bot 64: 2565-2577.

Schoonbeek H, Jacquat-Bovet A-C, Mascher F and Métraux J-P (2007) Oxalate-Degrading Bacteria Can Protect Arabidopsis thaliana and Crop Plants Against Botrytis cinerea. Mol Plant-Microbe Interact 20:1535–1544. doi: 10.1094/MPMI-20-12-1535

Schulz-Bohm K, Martín-Sánchez L and Garbeva P (2017) Microbial Volatiles: Small Molecules with an Important Role in Intra- and Inter-Kingdom Interactions. Front Microbiol 8:2484. doi: 10.3389/fmicb.2017.02484

Selim KA, El Ghwas DE, Selim RM and Abdelwahab Hassan MI (2017) Microbial Volatile in Defense. In: Choudhary DK, Sharma AK, Agarwal P, Varma A and Tuteja N (eds) Volatiles and Food Security. Springer Singapore, Singapore, pp 135–170

Shen J, Yuan L, Zhang J, Li H, Bai Z, Chen X, Zhang W and Zhang F (2011) Phosphorus Dynamics: From Soil to Plant. PLANT Physiol 156:997–1005. doi: 10.1104/pp.111.175232

Shukla, A. K. (2005). Estimation of yield losses to Indian mustard (Brassica juncea) due to Sclerotinia stem rot. Journal of Phytological Research, 18: 267-268.

Silva LFL e, Gonçalves WM, Maluf WR, Resende LV, Sarmiento CM, Licursi V e Moretto P (2017) Energy balance of biodiesel production from canola. Ciência Rural 47:1–6. doi: 10.1590/0103-8478cr20151084

Silveira, CH (2016). Reação de cultivares de canola a clerotinia sclerotiorum. Universidade Federal de Uberlândia

Sindhu SS, Phour M, Choudhary SR e Chaudhary D (2014) Phosphorus Cycling: Prospects of Using Rhizosphere Microorganisms for Improving Phosphorus Nutrition of Plants. In: Parmar N e Singh A (orgs) Geomicrobiology and Biogeochemistry. Springer Berlin Heidelberg, Berlin, Heidelberg, p 199–237

Singh B and Satyanarayana T (2011) Microbial phytases in phosphorus acquisition and plant

growth promotion. Physiol Mol Biol Plants 17:93–103. doi: 10.1007/s12298-011-0062-x

Smolińska U, Kowalska B (2018) Biological control of the soil-borne fungal pathogen Sclerotinia sclerotiorum — a review. J Plant Pathol 100:1–12. doi: 10.1007/s42161-018-0023-0

Spaepen S e Vanderleyden J (2011) Auxin and plant-microbe interactions. Cold Spring Harb Perspect Biol 3:1–13. doi: 10.1101/cshperspect.a001438

Spaepen S. (2015) Plant hormones produced by microbes. In: Lugtenberg (eds) Principles of Plant-Microbe Interactions. Springer, pp 247-256.

Strieker M, Tanovicánd A and Marahiel MA (2010) Nonribosomal peptide synthetases: structures and dynamics. doi: 10.1016/j.sbi.2010.01.009

Subramanian S and Smith DL (2015) Bacteriocins from the rhizosphere microbiome – from an agriculture perspective. Front Plant Sci 6:909. doi: 10.3389/fpls.2015.00909

Sun G, Yao T, Feng C, Chen L, Li J e Wang L. (2017). Identification and biocontrol potential of antagonistic bacteria strains against Sclerotinia sclerotiorum and their growth-promoting effects on Brassica napus. Biol control 104: 35-43

Sun R (2015) Economic/Academic Importance of Brassica rapa. In: Wang X e Kole C (orgs) The Brassica rapa Genome. Springer-Verlag, Berlin, Heidelberg, p 1–15

Süssmuth RD and Mainz A (2017) Nonribosomal Peptide Synthesis-Principles and Prospects. Angew Chemie Int Ed 56:3770–3821. doi: 10.1002/anie.201609079

Swiontek Brzezinska M, Jankiewicz U, Burkowska A and Walczak M (2014) Chitinolytic Microorganisms and Their Possible Application in Environmental Protection. Curr Microbiol 68:71–81. doi: 10.1007/s00284-013-0440-4

Tariq M, Noman M, Ahmed T, Manzoor N e Zafar M (2017) Antagonistic features displayed by Plant Growth Promoting Rhizobacteria (PGPR): A Review. J Plant Sci Phytopathol 1:38–43.

Timm CM, Lloyd EP, Egan A, Mariner R and Karig D (2018) Direct Growth of Bacteria in Headspace Vials Allows for Screening of Volatiles by Gas Chromatography Mass Spectrometry. Front Microbiol 9:491. doi: 10.3389/fmicb.2018.00491

Tomm GO (2007) Indicativos tecnológicos para produção de canola no Rio Grande do Sul. Embrapa Trigo, Passo Fundo - RS

Tomm GO, Ferreira PEP, Aguiar JLP de, Castro AMG de, Lima SMV e Mori C de (2010) Panorama atual e indicações para aumento de eficiência da produção de canola no Brasil, 1o ed. Embrapa Trigo, Passo Fundo.

Tomm GO, Raposo RWC, Souza TAF de, Oliveira JT de L, Raposo EHS, Neto CP da S, Arthur, Brito C, Nascimento R de S, Raposo AWS et al. (2008) Desempenho de genótipos de canola (Brassica napus L.) no Nordeste do estado da Paraíba, Nordeste do Brasil. 11.

Tomm GO, Wiethölter S, Dalmago GA and Santos HP Dos (2009) Tecnologia para produção de canola no Rio Grande do Sul. Embrapa trigo 88.

Turner BL and Blackwell MSA (2013) Isolating the influence of pH on the amounts and

forms of soil organic phosphorus. Eur J Soil Sci 64:249–259. doi: 10.1111/ejss.12026

U. Nagaharu (1935) Genome analysis in Brassica with special reference to the experimental formation of B. napus and peculiar mode of fertilization. Jpn J Bot 7:389–452

USDA- United States Department of Agriculture (2019) Oilseeds: World markets and trades. (https://apps.fas.usda.gov/psdonline/circulars/oilseeds.pdf)

Vassilev N, Mendes G, Costa M e Vassileva M (2014) Biotechnological Tools for Enhancing Microbial Solubilization of Insoluble Inorganic Phosphates. Geomicrobiol J 31:751–763. doi: 10.1080/01490451.2013.822615

Vejan P, Abdullah R, Khadiran T, Ismail S e Nasrulhaq Boyce A (2016) Role of Plant Growth Promoting Rhizobacteria in Agricultural Sustainability—A Review. Molecules 21:573. doi: 10.3390/molecules21050573

Venturi V e Keel C (2016) Signaling in the Rhizosphere. Trends Plant Sci 21:187–198. doi: 10.1016/j.tplants.2016.01.005

Zahoo, Ahmad W, Hira K, Ullah B, Khan A, Shah Z, Khan FA e Raja Mohib Muazzam Naz (2014) Role of Nitrogen Fertilizer in Crop Productivity and Environmental Pollution. Int J Agric For 4:201–206. doi: 10.5923/j.ijaf.20140403.09

Zentkovà I e Cvengrosova E (2013) The utilization of rapeseed for biofuels production in the EU. Visegr J Bioeconomy Sustain Dev 2:11–14.