UNIVERSIDADE ESTADUAL DE LONDRINA

NATALIA CAETANO VASQUES

SELEÇÃO DE BACTÉRIAS PROMOTORAS DO CRESCIMENTO DE PLANTAS EM PROCESSOS MULTIFUNCIONAIS

Londrina
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Tese apresentada ao Programa de Pós-Graduação em Microbiologia, da Universidade Estadual de Londrina, como requisito para obtenção do título de Doutora em Microbiologia.

Orientadora: Dra. Mariangela Hungria

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SUMÁRIO

VASQUES, Natalia Caetano. **Seleção de bactérias promotoras do crescimento de plantas em processos multifuncionais.** 2025. 138 páginas. Tese (Doutorado em Microbiologia) – Universidade Estadual de Londrina, Londrina, 2025.

RESUMO

A substituição de insumos químicos por soluções biológicas sustentáveis tem ganhado destaque global, especialmente no setor agrícola, impulsionada pela crescente demanda por práticas produtivas mais seguras, com menor impacto ambiental e que contribuam para a regeneração dos ecossistemas. Essa transição reflete não apenas uma tendência de mercado, mas também uma necessidade estratégica para garantir a sustentabilidade e a competitividade da agricultura moderna. Neste contexto, métodos de avaliação *in vitro* do potencial biotecnológico de bactérias foram validados e descritos quanto à tolerância à baixa atividade de água (Aw 0,919 e 0,897) e alta temperatura (40 ± 2 °C), síntese de protease, celulase, ACC-desaminase, exopolissacarídeos, ácido indol-3-acético, biofilme, sideróforos e solubilização de fosfato de cálcio. Essas propriedades foram avaliadas para a triagem *in vitro* de 100 estirpes da "Coleção de Culturas de Bactérias Diazotróficas e Promotoras do Crescimento de Plantas da Embrapa Soja", visando identificar propriedades agronomicamente relevantes. Os ensaios em casa de vegetação, com milho inoculado com essas estirpes e submetido à restrição hídrica confirmaram a correlação entre o desempenho *in vivo* e a tolerância à baixa atividade de água e alta temperatura *in vitro*, validando o uso dessa abordagem como ferramenta eficaz para acelerar a seleção de estirpes promissoras para essas propriedades. Dentre as estirpes, destacaram-se as estirpes dos gêneros *Bacillus* e *Pseudomonas*, sendo o primeiro notável por sua multifuncionalidade. Três estirpes, *Bacillus velezensis* CNPSo 2384, *Bacillus subtilis* CNPSo 2606 e *Bacillus* sp. CNPSo 2723, foram eficientes na mitigação da restrição hídrica em milho. O potencial multifuncional presente em *Bacillus* spp., levou a uma revisão sobre suas propriedades relacionadas aos mecanismos de promoção do crescimento vegetal, controle biológico de pragas e doenças, além da contribuição para práticas de agricultura regenerativa. As bactérias promotoras do crescimento de plantas têm potencial para revolucionar a agricultura pelo aumento da produtividade das culturas e por meio de soluções biológicas para o controle de pragas e doenças, nutrição e atenuação de estresses abióticos, representando uma ferramenta essencial para uma agricultura ambientalmente responsável.

Palavras-chave: *Bacillus*; *Pseudomonas;* Tolerância à seca; Bioinsumos; Bioprospecção de microrganismos.

VASQUES, Natalia Caetano. **Selection of plant growth-promoting bacteria in multifunctional applications**. 2025. 138 pages. Thesis (Doctorate degree in Microbiology) – State University of Londrina, Londrina, 2025.

ABSTRACT

The replacement of chemical inputs with sustainable biological solutions has gained global prominence, especially in the agricultural sector, driven by the growing demand for safer production practices with lower environmental impact and contributions to ecosystem regeneration. This transition reflects not only a market trend but also a strategic need to ensure the sustainability and competitiveness of modern agriculture. In this context, *in vitro* methods for evaluating the biotechnological potential of bacteria were validated and described in terms of tolerance to low water activity (Aw 0.919 and 0.897) and high temperature (40 ± 2 °C), and for the synthesis of protease, cellulase, ACC deaminase, exopolysaccharides, indole-3-acetic acid, biofilm, siderophores, and calcium phosphate solubilization. These traits were assessed in the *in vitro* screening of 100 strains from the Collection of Diazotrophic and Plant Growth-Promoting Bacteria of Embrapa Soja, aiming to identify agronomically relevant characteristics. Greenhouse experiments with maize inoculated with these strains and subjected to water restriction confirmed the correlation between *in vivo* performance and *in vitro* tolerance to low water activity and high temperature, validating this approach as an effective tool to accelerate the selection of promising strains for these traits. Among the evaluated strains, those belonging to the genera *Bacillus* and *Pseudomonas* stood out, the former showing remarkable multifunctionality. Three strains, *Bacillus velezensis* CNPSo 2384, *Bacillus subtilis* CNPSo 2606, and *Bacillus* sp. CNPSo 2723, were effective in mitigating water restriction in maize. The multifunctional potential observed in *Bacillus* spp. led to a review of its properties related to mechanisms of plant growth promotion, biological control of pests and diseases, and contributions to regenerative agricultural practices. Plant growth-promoting bacteria have the potential to revolutionize agriculture by enhancing crop productivity and providing biological solutions for pest and disease control, nutrition, and mitigation of abiotic stresses, representing an essential tool for environmentally responsible agriculture.

Keywords: *Bacillus*; *Pseudomonas*; Drought tolerance; Biological inputs; Microbial bioprospecting.

INTRODUÇÃO

A crescente demanda global por alimentos, fibras e energia, impulsionada pelo aumento populacional e pela limitação de terras agricultáveis, representa um desafio significativo para a sustentabilidade agrícola. Para atender a essa demanda, práticas agrícolas intensivas, como o uso excessivo de fertilizantes e pesticidas, têm sido adotadas, resultando em degradação do solo e perda de biodiversidade (Barros-Rodríguez *et al*., 2021). Nesse cenário, a agricultura sustentável exige soluções que aumentem a produção sem comprometer os recursos naturais.

Entre as alternativas promissoras, destaca-se o uso de bactérias promotoras do crescimento de plantas (BPCP, ou *Plant Growth-Promoting Bacteria* – PGPB). Esses microrganismos beneficiam as plantas por diferentes mecanismos, como a produção de fitormônios, aumento da absorção de água e nutrientes, indução de resistência a estresses bióticos (como pragas e patógenos) e abióticos (como salinidade, seca, alagamento, frio, compactação do solo, deficiência nutricional e fitotoxicidade), sem comprometer a qualidade do solo e da água (Banerjee *et al*., 2020; Gupta; Pandey, 2023; He *et al*., 2024). O uso desses microrganismos ou seus metabólitos, por meio de bioinsumos, contribui para o aumento da produtividade, com maior eficiência no uso dos recursos, promovendo a sustentabilidade e atendendo de forma responsável à crescente demanda da população (Kumari *et al*., 2023; Salwan; Sharma, 2022).

Os bioinsumos podem ser agrupados em três categorias principais, de acordo com sua funcionalidade: (I) promotores do crescimento e desenvolvimento vegetal, (II) agentes de controle biológico de pragas e doenças, e (III) condicionadores ou recuperadores do solo. Cada grupo pode ser subdividido com base nos mecanismos biológicos e modos de ação específicos (Bullor *et al*., 2024). No grupo I, são incluídos os biofertilizantes, no Brasil denominados inoculantes, (como fixadores biológicos de nitrogênio e solubilizadores de nutrientes) e os bioestimulantes (outros microrganismos benéficos). O grupo II abrange os agentes de controle biológico, como biofungicidas, bioinseticidas e bionematicidas. O grupo III é formado por bioinsumos voltados ao condicionamento e recuperação do solo (Bullor *et al*., 2024) (Figura 1). É importante destacar que, em todos esses grupos, além do

uso de microrganismos vivos, existe a possibilidade de desenvolver produtos baseados apenas em seus metabólitos (como fitormônios, sideróforos, antibióticos naturais e lipopeptídeos), os quais podem atuar como ingredientes ativos sem a presença de células viáveis. Isso amplia as possibilidades de formulação, assegurando maior estabilidade, padronização e versatilidade de aplicação (Barbosa *et al*., 2025). Ademais, um mesmo bioinsumo pode exercer múltiplas funções simultaneamente, como estimular o crescimento de plantas e oferecer proteção contra fitopatógenos (Bullor *et al*., 2024).

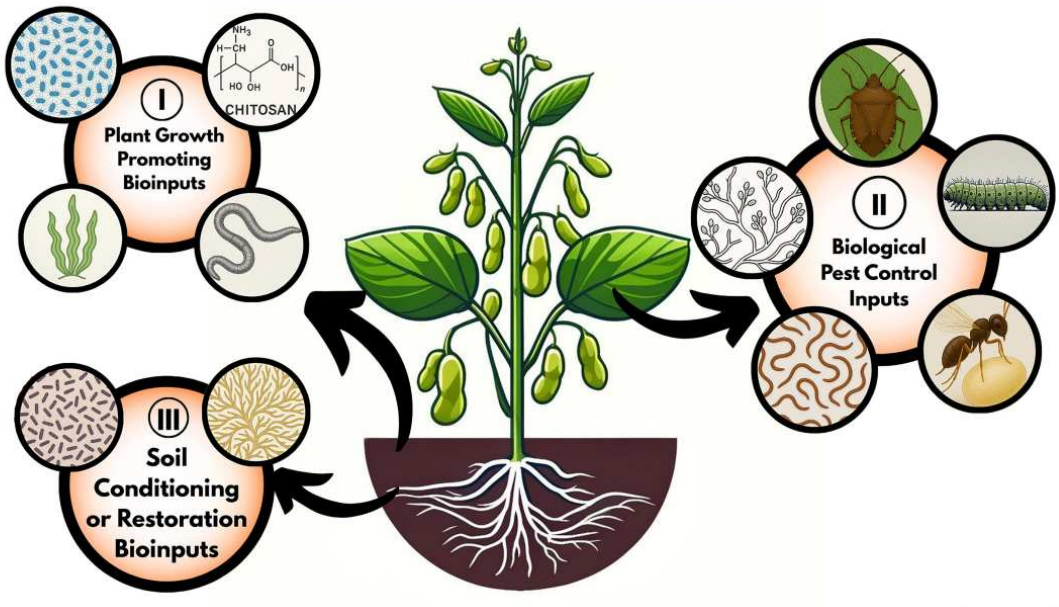

Figura 1. Categorias de bioinsumos, que podem ser classificados como: (I) promotores do crescimento vegetal, (II) para controle biológico e (III) para condicionamento ou recuperação do solo. Fonte: Barbosa *et al*. (2025).

No Brasil, o registro de bioinsumos varia de acordo com a funcionalidade: produtos do grupo I (como inoculantes e bioestimulantes) são registrados com base na cultura-alvo, ou seja, o produto deve demonstrar eficácia para a planta à qual se destina; já os bioinsumos do grupo II são registrados de acordo com o alvo biológico (pragas e patógenos); por sua vez, o grupo III é registrado de acordo com a capacidade de promover alterações benéficas específicas no solo. Essa diferenciação influencia diretamente os requisitos regulatórios, refletindo no número de registros de cada classificação e a forma de uso dos produtos no

campo. Segundo dados do aplicativo Bioinsumos do Ministério da Agricultura e Pecuária (versão de agosto de 2025), existem 636 inoculantes e 617 produtos destinados ao biocontrole registrados no país (Brasil; Empresa Brasileira de Pesquisa Agropecuária, 2024).

As BPCP pertencentes aos grupos I e II desempenham funções que já são consolidadas, como produção de ácido indol-3-acético (AIA), síntese de 1-aminociclopropano-1-carboxilato-desaminase (ACC-desaminase), produção de sideróforos, solubilização de minerais, mineralização de compostos ricos em fósforo, fixação biológica de nitrogênio, produção de exopolissacarídeos (EPS), formação de biofilmes e síntese de compostos antimicrobianos e enzimas hidrolíticas (Fanai *et al*., 2024). Além disso, podem promover resistência sistêmica nas plantas, reforçando sua defesa contra pragas e doenças. No entanto, a ampla diversidade funcional das BPCP indica um potencial ainda pouco explorado que pode contribuir significativamente para a redução do uso de insumos químicos e ampliar as possibilidades de adoção de práticas agrícolas mais sustentáveis (Kumari *et al*., 2023).

As BPCP estão distribuídas em diferentes ambientes, como solos agrícolas, rizosfera, filosfera, ambientes aquáticos e até ecossistemas com climas severos, como desertos, regiões árticas e áreas salinas. Em tais condições, muitos isolados desenvolveram adaptações metabólicas que os tornaram particularmente eficientes na promoção do crescimento vegetal sob estresse (Leontidou *et al*., 2020; Vocciante *et al*., 2022).

No contexto das mudanças climáticas, marcadas por secas prolongadas, inundações e variações térmicas, as BPCP podem aumentar a resiliência das culturas (Cao et al., 2023; Fiodor; Singh; Pranaw, 2021) por aumentar a tolerância ao estresse e aumentar a eficiência no uso de água e nutrientes. Um exemplo é a inoculação de BPCP em pastagens de braquiária (*Urochloa* spp.), em que *Azospirillum brasilense* CNPSo 2083 e CNPSo 2084, e *Pseudomonas fluorescens* CNPSo 2719 promovem o crescimento vegetal e o acúmulo de nutrientes por melhorias na arquitetura radicular, FBN, solubilização de fosfatos e síntese de fitormônios e sideróforos, além da produção de ACC-desaminase, característica diretamente relacionada à tolerância a estresses (Hungria *et al*., 2021; Mamédio et al., 2020). Em trigo (*Triticum aestivum* L.) a inoculação de BPCP obtidas de plantas tolerantes à seca, como *Alhagi pseudoalhagi* e

Chenopodium album, reduziu danos fisiológicos, aumentou o conteúdo total de clorofila, regulou os níveis de hormônios da planta e melhorou a atividade de enzimas antioxidantes, como catalase e peroxidase (Pishchik *et al*., 2024). A inoculação com *Gluconacetobacter diazotrophicus* Pal5 em arroz vermelho (*Oryza sativa* L.) atenuou os efeitos deletérios da restrição hídrica e contribuiu para o incremento da biomassa da planta e solutos osmoprotetores na parte aérea (Filgueiras *et al*., 2020). A inoculação com *Bacillus* spp. atenuou os efeitos da seca em milho (*Zea mays* L.), promovendo o crescimento, o acúmulo de pigmentos fotossintéticos e a absorção de nutrientes, além de reduzir o estresse oxidativo e a atividade de enzimas antioxidantes, demonstrando o potencial no manejo sustentável do estresse hídrico (Azeem *et al*., 2022).

O desempenho dessas bactérias no ambiente agrícola varia de acordo com sua especificidade e resulta de interações bioquímicas e fisiológicas que beneficiam tanto as plantas quanto o solo (Hartmann; Six, 2023). Diferentes gêneros bacterianos, entretanto, influenciam processos distintos, e os efeitos podem variar conforme a cultura, o ambiente e até mesmo a estirpe dentro de uma mesma espécie (Cruz-Hernández *et al*., 2022; Makar *et al*., 2023). Algumas estirpes de BPCP já são conhecidas e possuem benefícios comprovados por sua interação promotora do crescimento em culturas agrícolas de importância econômica, especialmente os gêneros *Bradyrhizobium* (Santos; Nogueira; Hungria, 2019; Zilli *et al*., 2021), *Azospirillum* (Cassán *et al*., 2020; Gómez; Mercado; Pineda, 2014; Hungria *et al*., 2010), *Pseudomonas* (David; Chandrasehar; Selvam, 2018; Yasmeen *et al*., 2021) e *Bacillus* (Tiwari; Prasad; Lata, 2019; Tsotetsi *et al*., 2022).

O gênero *Bacillus*, em particular, é notável por sua capacidade de formar endósporos, o que garante não apenas a sobrevivência em altas temperaturas, salinidade e pH do meio, mas também a estabilidade do agente biológico, assegurando sua eficiência e vida útil durante o armazenamento e aplicação no campo (Govindasamy *et al*., 2010; Russi, 2024). Além disso, bactérias desse gênero podem apresentar propriedades multifuncionais, como a produção de compostos antimicrobianos, indução de resistência sistêmica e controle biológico de fitopatógenos (Tiwari; Prasad; Lata, 2019), além de desempenharem papel ativo na degradação de substratos orgânicos por meio de enzimas hidrolíticas,

como quitinases, celulases e proteases (Etesami; Adl, 2020), que também contribuem para o controle biológico de fitopatógenos (Panicker; Sayyed, 2022).

A integração de BPCP no manejo agrícola é um caminho eficiente para enfrentar os desafios climáticos e dar suporte à intensificação sustentável da agricultura. Ao combinar promoção do crescimento, biocontrole e regeneração do solo, esses bioinsumos se consolidam como pilares para sistemas agrícolas resilientes, produtivos e ambientalmente responsáveis (Meneses *et al*., 2024).

A biodiversidade microbiana é um recurso estratégico para a agricultura moderna, oferecendo base para o desenvolvimento de novos bioinsumos. Investimentos em pesquisa e inovação têm sido impulsionados pela crescente demanda por soluções biológicas, com foco em eficiência, segurança ambiental e formulação estável (Moreno-Espíndola *et al*., 2025). As BPCP, além de reduzirem a dependência de insumos químicos, fortalecem a resiliência agrícola frente às mudanças climáticas, contribuindo para segurança alimentar global (Prasad *et al*., 2019).

O objetivo desse trabalho foi explorar a diversidade, os mecanismos de ação e o potencial biotecnológico de estirpes de BPCP da Coleção de Culturas de Bactérias Diazotróficas e Promotoras de Crescimento de Plantas da Embrapa Soja, especialmente no contexto das mudanças climáticas e da busca por uma agricultura mais sustentável. Para isso, foram desenvolvidos três capítulos. No primeiro, foram validadas e descritas metodologias para a avaliação de propriedades de interesse biotecnológico em bactérias promotoras do crescimento de plantas. No segundo, foram concluídas as avaliações iniciadas no mestrado, sobre a caracterização de propriedades *in vitro* em bactérias e a validação da correlação entre a tolerância ao estresse hídrico em condições *in vitro* e o desempenho *in vivo* de plantas de milho inoculadas. No terceiro capítulo, dada a relevância e propriedades múltiplas do gênero *Bacillus*, foi realizada uma revisão de literatura que discute o papel multifuncional desse gênero. na agricultura, com ênfase em mecanismos de promoção do crescimento vegetal, no controle biológico de pragas e doenças e em sua contribuição para práticas de agricultura regenerativa. O conjunto robusto de informações confirma o potencial de várias bactérias de compor uma nova categoria de bioinsumos multifuncionais.

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CAPÍTULO 1

BIOPROSPECÇÃO DE MICRORGANISMOS PARA O USO EM BIOINSUMOS: MÉTODOS PARA TRIAGEM INICIAL DE BIOATIVOS VISANDO À NUTRIÇÃO DE PLANTAS E À TOLERÂNCIA A ESTRESSES ABIÓTICOS E BIÓTICOS

BIOPROSPECÇÃO DE MICRORGANISMOS PARA O USO EM BIOINSUMOS: MÉTODOS PARA TRIAGEM INICIAL DE BIOATIVOS VISANDO À NUTRIÇÃO DE PLANTAS E À TOLERÂNCIA A ESTRESSES ABIÓTICOS E BIÓTICOS

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RESUMO

Como reflexo do crescente interesse do setor agrícola por insumos biológicos, observa-se um aumento expressivo na busca por soluções baseadas em microrganismos. Esse movimento reforça a necessidade de padronização de métodos que assegurem a repetibilidade e a confiabilidade na bioprospecção de novos ativos biotecnológicos, tanto em ambientes acadêmicos quanto no setor privado. Este documento foi elaborado para atender a essa demanda, reunindo e ilustrando metodologias validadas no Laboratório de Biotecnologia do Solo da Embrapa Soja, com o objetivo de facilitar a prospecção inicial de microrganismos com potencial para compor futuros bioinsumos voltados, sobretudo, à nutrição vegetal e à tolerância a estresses abióticos e bióticos. São descritos métodos *in vitro* que permitem avaliar características como o crescimento bacteriano em baixa atividade de água (Aw 0,919 e 0,897) e altas temperaturas (40 ± 2 °C), bem como a produção de protease, celulase, ácido indol-3-acético (AIA), ACC-desaminase, exopolissacarídeos (EPS), biofilme e sideróforos, além da solubilização de fosfato. O conjunto de protocolos aqui apresentados busca oferecer suporte prático e reprodutível para laboratórios e pesquisadores envolvidos na prospecção de bactérias promotoras do crescimento de plantas, destacando seu potencial biotecnológico no desenvolvimento de bioinsumos agrícolas sustentáveis. A relevância desse esforço está em fortalecer sistemas agrícolas mais resilientes, produtivos e ambientalmente responsáveis, em consonância com as demandas globais por sustentabilidade e inovação no setor.

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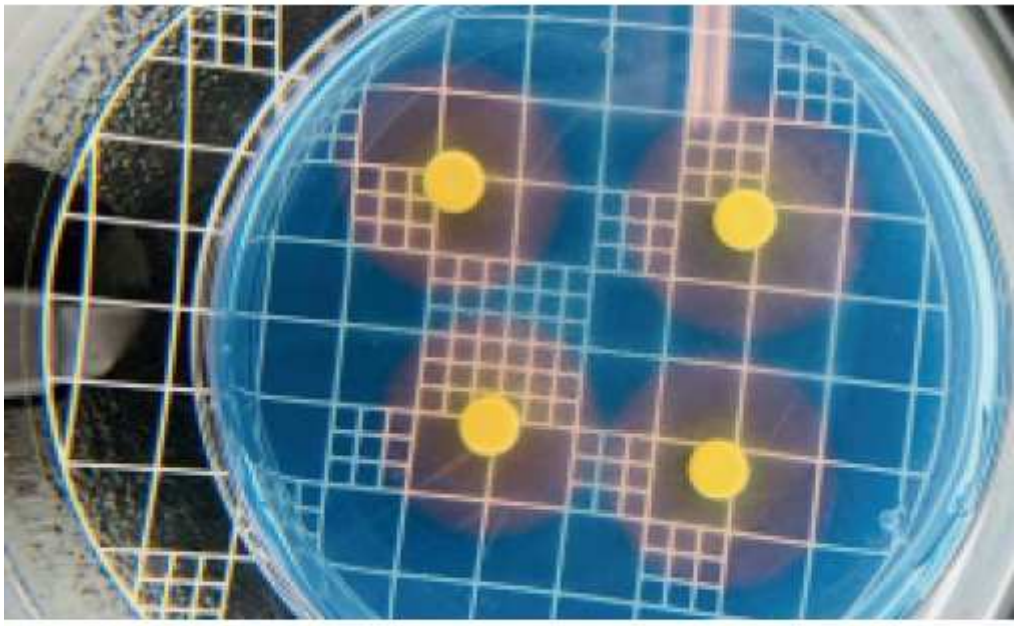

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Bioprospecção de microrganismos para o uso em bioinsumos: métodos para triagem inicial de bioativos visando à nutrição de plantas e à tolerância a estresses abióticos e bióticos

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Apresentação

É motivo de grande orgulho para a agricultura brasileira que o país tenha passado de importador de alimentos na década de 1960 para a elite dos países exportadores. O agronegócio responde, hoje, por um quarto do PIB nacional e a agricultura familiar é responsável por mais da metade da comida que vai à mesa dos brasileiros.

Uma nova percepção de agricultura, regenerativa, ou seja, indo além da sustentabilidade, com a recuperação da saúde e fertilidade do solo, passou a ganhar mais espaço nesta década. Em resposta a esse cenário, o Decreto Nº 10.375 de 26 de maio de 2020, que instituiu o Programa Nacional de Bioinsumos, veio a reforçar a vocação brasileira para o uso de insumos biológicos na agricultura. Ainda nesta década, a importância do desenvolvimento de bioinsumos com base na rica biodiversidade brasileira ganhou maior evidência com os problemas de restrições causados pela pandemia de COVID 19 e geopolíticos resultantes da guerra entre a Ucrânia e a Rússia, que evidenciaram a grande fragilidade do setor agrícola no suprimento de insumos. Como exemplo, 85% dos fertilizantes necessários para a produção agrícola nacional são importados.

Como resultado do despertar do setor agrícola para os insumos biológicos, constatou-se um incremento impactante na busca por soluções com base em microrganismos, com muitas solicitações, por parte dos setores acadêmico e privado, de indicação de métodos para a bioprospeção de novos ativos biotecnológicos.

Este Documento visa atender a essa demanda, detalhando e ilustrando métodos que foram validados no Laboratório de Biotecnologia do Solo da Embrapa Soja, para facilitar uma prospecção inicial de microrganismos que podem vir a compor futuros bioinsumos destinados, principalmente, à nutrição de plantas e à tolerância a estresses abióticos e bióticos.

Adeney de Freitas Bueno
Chefe Adjunto de Pesquisa e Desenvolvimento
Embrapa Soja

Sumário

Introdução

Bactérias Promotoras do Crescimento de Plantas (BPCP ou *Plant Growth-Promoting Bacteria*, PGPB) podem estimular o crescimento das plantas através de diferentes processos microbianos, específicos em cada estirpe bacteriana, como produção de fitormônios, a disponibilização de nutrientes, aumento da tolerância a estresses, entre outros.

Como exemplo de processos microbianos, podem-se citar: a produção de sideróforos (Crowley et al., 1991; Kraemer, 2004), a solubilização de fosfato inorgânico (Reyes et al., 1999), o estímulo à produção de substâncias reguladoras do crescimento (Arshad; Frankenberger, 1997), a produção de 1-aminociclopropano-1-carboxilato deaminase (ACC-deaminase) (Glick et al., 1998), a atividade de enzimas hidrolíticas, como a celulase e a protease (Glick, 2012; Šturšová et al., 2012; Oliveira et al., 2020), a capacidade de crescimento em condições de atividade de água reduzida (Hallsworth et al., 1998), a produção de exopolissacarídeos (EPS) e de biofilmes (Chang et al., 2007), entre outros.

Algumas BPCP podem, ainda, promover o crescimento de plantas por meio do biocontrole de doenças, devido à atividade metabólica antagônica aos fitopatógenos. Os metabólitos microbianos também podem induzir respostas de resistência sistêmica da planta, conforme observado por Naik et al. (2019), ou outras formas de suprimir o ataque de pragas e doenças (Ruiu, 2020).

É conveniente ressaltar que as BPCP estimulam o desenvolvimento das plantas; no entanto, a classificação de benefícios diretos e indiretos não se mostra consolidada, uma vez que um mesmo mecanismo pode exercer tanto influência direta, quanto indireta como, por exemplo, a produção de sideróforos (Kloepper et al., 1980; Schalk et al., 2012). Além disso, uma mesma estirpe pode ser portadora e/ou responsável por vários mecanismos simultaneamente (Mallick et al., 2018).

Estima-se que o Brasil detenha cerca de 20% da biodiversidade do mundo (Val et al., 2022), percentagem que também deve se estender aos microrganismos. Existe, portanto, um enorme potencial para bioprospecção de microrganismos da biodiversidade brasileira com propriedades de grande interesse para a agricultura. Neste contexto, neste comunicado são detalhados métodos de avaliação do potencial de promoção de crescimento de plantas por espécies bacterianas. Esses métodos permitem avaliar diferentes características promissoras dos isolados bacterianos, com potencial impacto na nutrição de plantas e na tolerância a estresses abióticos, entre outros, representando uma primeira etapa para a bioprospecção de microrganismos destinados ao uso como bioinsumos na agricultura.

Processos microbianos e sua avaliação

Enzimas hidrolíticas

Importância da síntese de enzimas hidrolíticas

Enzimas hidrolíticas são classificadas quanto à especificidade ao substrato. Dois exemplos de enzimas hidrolíticas são as proteases e as celulases, produzidas por algumas BPCP. A importância da produção dessas enzimas diz respeito à capacidade de degradação de material orgânico fibroso e como agente de biocontrole. Tendo em vista a redução dos impactos ambientais no processo produtivo, o reaproveitamento de resíduos é uma alternativa proeminente, uma vez que potencializa a utilização de recursos naturais (Schneider et al., 2013), sendo necessária a atividade de bactérias especializadas na degradação destes compostos para que os elementos ali presentes sejam ciclados. Um exemplo é a utilização da torta de filtro em sistemas de produção de alface (*Lactuca sativa*), que pode ser usada como fonte de carbono e energia pelos microrganismos, favorecendo a atividade

biológica do solo, de Santana et al. (2012). A produção das enzimas protease e celulase também pode ser efetiva no biocontrole de fitopatógenos pela degradação de β-glucanos, principal constituinte de suas paredes celulares (Ahmadzadeh; Tehrani, 2009). Algumas proteases estão envolvidas, ainda, na inativação de enzimas líticas extracelulares de fungos fitopatogênicos, diminuindo a sua capacidade de infecção (Elad; Kapat, 1999).

Métodos de avaliação da produção de enzimas hidrolíticas

Atividade proteolítica

Para a avaliação da produção de enzimas proteolíticas, os isolados devem ser cultivados em meio de cultura segundo o Manual of Methods for General Bacteriology - American Society for Microbiology de Gerhardt et al. (1981), cuja composição consta da Tabela 1.

Tabela 1. Composição de meio de cultura para avaliação da atividade proteolítica

Componente	Quantidade
Leite desnatado	100 mL
Extrato de levedura	1,5 g
NaH_2PO_4	1,0 g
NaCl	1,0 g
Ágar	15,0 g
Água deionizada	q.s.p. 1000 mL
pH	7,2

Após a esterilização em autoclave a 121 °C por 20 minutos, faz-se a distribuição do meio de cultura em placas de Petri e a inoculação de uma gota, em triplicata, da estirpe avaliada, crescida em meio líquido ou preservada em glicerol. Os resultados esperados podem ser visualizados na Figura 1.

Ilustração: Natalia Caetano Vasques.

Figura 1. Ilustração do resultado esperado com a aplicação da metodologia. À esquerda, representação de resultado negativo e à direita, resultado positivo para produção de protease.

Após incubação por três a sete dias a 28 °C, a depender do microrganismo, a hidrólise de caseína pode ser observada pela formação de halo translúcido de degradação ao redor da colônia (Figura 2), quando o teste for positivo. A ausência de halo indica que a estirpe estudada não é produtora da enzima.

Foto: Natalia Caetano Vasques.

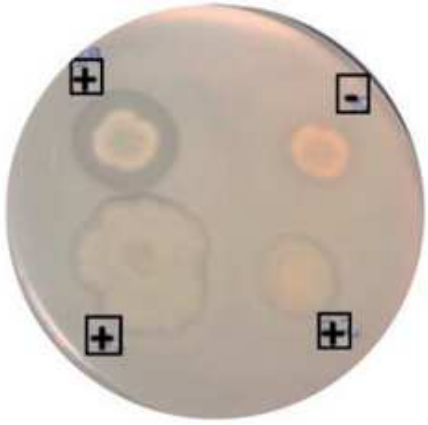

Figura 2. Resultado obtido após o crescimento de estirpes produtoras (+) e não produtoras (-) de protease.

Atividade celulolítica

Para avaliar a capacidade de degradação da celulose, o método descrito no *Manual of Methods for General Bacteriology - American Society for Microbiology* por Gerhardt et al. (1981) se mostra eficaz, sendo realizado no meio de cultura descrito na composição da Tabela 2.

Tabela 2. Composição de meio de cultura para avaliação da atividade celulolíticas.

Componente	Quantidade
K_2HPO_4	0,4 g
NaH_2PO_4	0,2 g
$MgSO_4.7H_2O$	0,2 g
NaCl	0,1 g
Extrato de levedura	0,1 g
CMC (carboximetil celulose)	5,0 g
Ágar	15,0 g
Água deionizada	1000 mL
pH	7,0

O meio deve ser submetido à autoclavagem a 121 °C por 20 minutos. Após o plaqueamento e a incubação, faz-se necessária a técnica de revelação dos halos de degradação da celulose pela adição de um pequeno volume de solução NaCl 1 mol L^{-1} à superfície da placa. Após 5 minutos, a solução salina deve ser removida e adicionada uma solução de vermelho Congo a 0,1% (em 99,9 mL de água, adicionar 0,1 g de vermelho Congo). Após 30 minutos, realiza-se a lavagem com água destilada até que seja possível a observação dos halos de degradação ao redor das colônias, no caso de atividade positiva. O resultado esperado está demonstrado na Figura 3.

Ilustração: Natália Caetano Vasques.

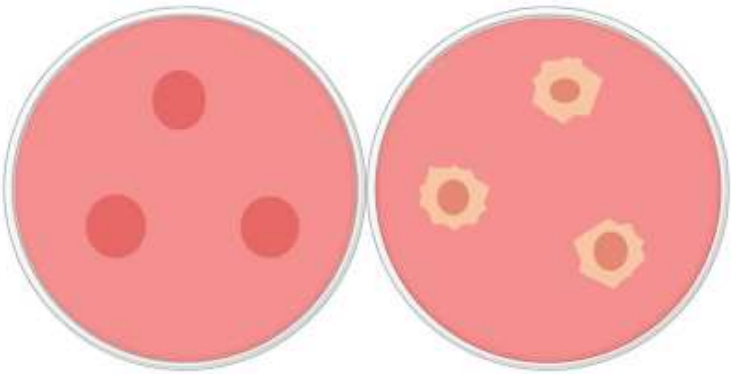

Figura 3. Ilustração do resultado esperado com a aplicação da metodologia. À esquerda, representação de resultado negativo e à direita, resultado positivo para produção de celulase.

Após a incubação por cinco dias a 28 °C e realizado o procedimento de revelação, o resultado deve ser conforme pode ser visualizado na Figura 4.

Foto: Natália Caetano Vasques.

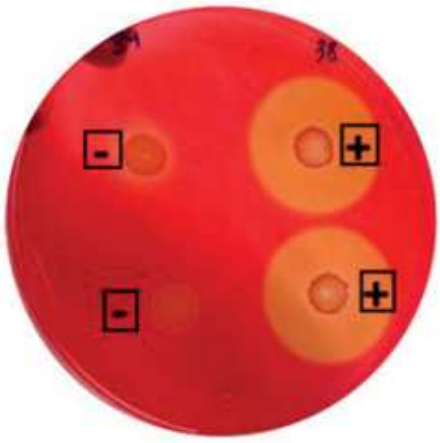

Figura 4. Resultado obtido após o crescimento de estirpes produtoras (+) e não produtoras (-) de celulase.

Crescimento em condições restritivas de água e temperatura

Importância da capacidade de crescimento em condições restritivas de água e temperatura

As condições do ambiente afetam diretamente o crescimento microbiano. A maioria das bactérias é cultivada em meio de cultura com atividade de água (Aw) em torno de 0,999 a 0,990, visto que a maioria não é capaz de crescer em meio com Aw inferior a 0,950. Desta maneira, espécies capazes de crescer em meio com Aw reduzida (< 0,950) mostram potencial para suportar variações osmóticas, seja pela alta concentração salina, seja pela restrição hídrica e, desta forma, podem trazer benefícios às plantas associadas, mesmo em condições críticas de sobrevivência para a maioria dos microrganismos (Hallsworth et al., 1998).

Método de avaliação da capacidade de crescimento microbiano em meio com atividade de água reduzida e temperatura elevada

Para avaliar o desenvolvimento das estirpes candidatas em meio com Aw reduzida, faz-se a repicagem em meio de cultura TSA (*Tryptic Soy Agar*, Tabela 3) a 10%, enriquecido com sorbitol (Tabela 4). Para Aw correspondente a 0,919, devem ser adicionados 405 g L^{-1} de sorbitol ao meio de cultura, e para Aw equivalente a 0,897 se adicionam 520 g L^{-1} de sorbitol ao meio de cultura que deve ser esterilizado em autoclave por 20 minutos a 121 °C, conforme proposto por Hallsworth et al. (1998). A Figura 5 ilustra como podem ser os resultados esperados

Tabela 3. Composição do meio de cultura TSA

Componente	Quantidade
TSB[1]	30,0 g
Ágar	15,0 g
Água deionizada	1000 mL
pH	7,1-7,5

[1] Meio de cultura comercial, em conformidade com USP (*United States Pharmacopeia*), EP (*European Pharmacopoeia*), JP (*Japanese Pharmacopeia*) e FDA/BAM (*Food and Drug Administration/ Bacteriological Analytical Guide*).

Tabela 4. Composição de meio de cultura para avaliação da capacidade de crescimento em meio com atividade de água reduzida.

Componente	0,919 Aw*	0,897 Aw
Meio TS 10%	[10%]	[10%]
Sorbitol	405,0 g	520,0 g
Ágar	15,0 g	15,0 g
Água deionizada	1000 mL	1000 mL

**Aw: Atividade de água.*

Ilustração: Natalia Caetano Vasques.

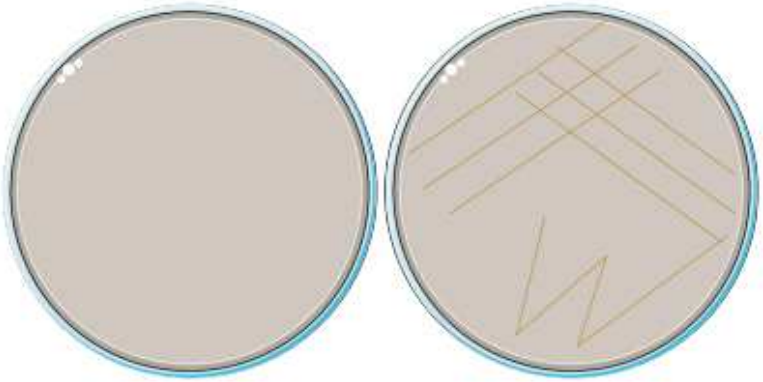

Figura 5. Ilustração do resultado esperado na avaliação da capacidade de crescimento em meio de cultura com atividade de água reduzida e temperatura elevada. À esquerda, representação de resultado negativo e à direita, resultado positivo para capacidade de crescimento.

Após incubação das culturas a 40 °C por sete dias, são consideradas positivas para tolerância a altas temperaturas as estirpes que apresentarem crescimento e negativas as que não são capazes de se desenvolverem em meio de cultura com alta concentração de soluto e incubadas em temperatura elevada (Figuras 5 e 6).

Foto: Natália Caetano Vasques.

Figura 6. Resultado obtido após a incubação de estirpes capazes de crescerem em meio com atividade de água reduzida e temperatura elevada (+) e não capazes (-).

Síntese de compostos indólicos

Importância da síntese de ácido indol-3-acético

Dentre os diversos compostos indólicos produzidos por alguns microrganismos, está o hormônio vegetal ácido indol-3-acético (AIA), que é responsável pela regulação de vários processos celulares e de desenvolvimento vegetal (Taiz et al., 2017), sendo capaz de estimular efeitos instantâneos na planta, quando em baixas concentrações, como o aumento da elongação celular, e efeitos mais prolongados, como a divisão e diferenciação celular (Dobbelaere et al., 2003). Alguns microrganismos são capazes de sintetizar esse fitormônio (Spaepen et al., 2007), auxiliando a planta em seu desenvolvimento. A síntese de AIA pode se mostrar ainda mais significativa quando as condições para produção vegetal forem desfavoráveis, uma vez que favorece a proliferação das raízes, contribuindo para a absorção de água e nutrientes pelas plantas (Lambrecht et al., 2000).

Método de avaliação da síntese de compostos indólicos

Para quantificar a produção de ácido indol-3-acético (AIA) *in vitro* (Gordon; Weber, 1951; Sarwar; Kremer,1995), cada estirpe bacteriana deve ser inoculada em 10 mL do meio de cultura líquido específico para o gênero, acrescido de triptofano (100 µg mL^{-1}), e incubada sob agitação (100 rpm) por sete dias a 28 °C, ou nas condições de crescimento especificadas para cada estirpe. A seguir, a cultura deve ser transferida para microtubos e centrifugada a 10.000 rpm, por 10 min. Em seguida, transferir 1 mL do sobrenadante para novos microtubos e adicionar 750 µL do reagente de Salkowski (Gordon; Weber, 1951) (Tabela 5). Cabe salientar que o método avalia compostos indólicos em geral, incluindo o AIA.

Tabela 5. Composição da solução reagente de Salkowski.

Componente	Quantidade
$FeCl_3.6H_2O$ (0,5 mol L^{-1})	1 mL
$HClO_4$ [35 %]	50 mL

Após 30 minutos de reação no escuro e em temperatura ambiente, a intensidade de coloração deve ser avaliada no comprimento de onda de 540 nm utilizando espectrofotômetro. Os resultados serão expressos em µg mL^{-1} de AIA no sobrenadante, de acordo com os valores obtidos em uma curva de calibração com AIA sintético (0; 50; 100; 200; 300; 400; 500; 600; 800; 1000 µg mL^{-1}), conforme apresentado na Figura 7.

Foto: Natalia Caetano Vasques.

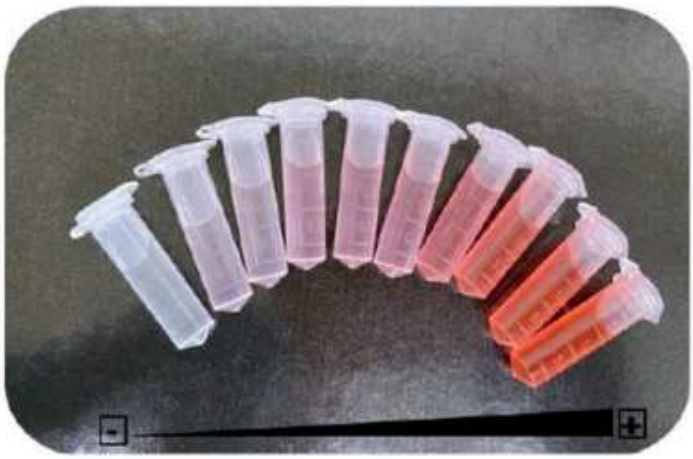

Figura 7. Curva de calibração para quantificação, por espectrofotometria, do ácido indol-3-acético produzido por bactérias promotoras do crescimento de plantas. A concentração está indicada da esquerda (-), menos concentrada, para a direita (+), ou mais concentrada.

Síntese de 1-aminociclopropano-1-carboxilato (ACC)-deaminase

Importância da produção de ACC-deaminase

O 1-aminociclopropano-1-carboxilato (ACC) é o principal precursor do etileno, uma molécula orgânica com função biológica, que atua como regulador de crescimento vegetal em baixas concentrações. No entanto, sua produção pode aumentar em situações de estresse (Glick, 2005), resultando em condições deletérias, como a inibição do crescimento radicular (Müller; Munné-Bosch, 2015; Huang et al, 2023). A enzima ACC-deaminase pode ser produzida por algumas estirpes de BPCP, sendo responsável pela degradação do ACC, transformando-o em α-cetobutirato e amônia (Glick, 2005). Além de retardar a produção de etileno, a atuação da enzima ACC-deaminase reflete no aumento do desenvolvimento radicular (Glick et al., 1998), portanto, as plantas inoculadas com bactérias produtoras dessa enzima podem tolerar melhor condições de estresse devido ao favorecimento do sistema

radicular, o que contribuirá para a formação de biomassa da planta, elevação pela taxa fotossintética, conteúdo proteico e ativação de genes responsivos ao estresse (Sapre et al., 2019). É possível que AIA e ACC-deaminase trabalhem em conjunto para estimular o alongamento das raízes. O AIA exógeno (produzido por bactérias) é conhecido por aumentar a transcrição e a atividade da ACC-sintase, que catalisa a produção de ACC em plantas. A molécula de ACC estimula a atividade da enzima ACC-deaminase em bactérias (Patten; Glick, 2002).

Método de avaliação da síntese de ACC-deaminase

A capacidade de algumas estirpes de metabolizar ACC como única fonte de N no meio de cultivo, devido à ação da enzima ACC-deaminase, foi a premissa da metodologia adaptada por Glick et al. (1995) e Lucon et al. (2008).

Preparar um meio de cultura livre de nitrogênio - LN, segundo a composição da Tabela 6 e uma solução tampão, conforme Tabela 7.

Tabela 6. Composição de meio de cultura livre de nitrogênio (LN).

Componente	Quantidade
H_3BO_4	10,0 mg
$MnSO_4.7H_2O$	10,0 mg
$MgSO_4.7H_2O$	0,2 g
$ZnSO_4$	70,0 mg
$FeSO_4.7H_2O$	1,0 mg
$CuSO_4$	50,0 mg
MoO_3	10,0 mg
Glicose	2,0 g
Ácido glucônico	2,0 g
Ácido cítrico	2,0 g
Ágar	15,0 g
Água deionizada	1000 mL

Tabela 7. Composição de solução tampão para o meio de cultura livre de nitrogênio (LN)

Componente	Quantidade
KH_2PO_4	4,0 g
Na_2HPO_4	6,0 g
Água destilada	100 mL

Autoclavar o meio de cultura por 20 minutos a 121 °C e, separadamente, a solução tampão. Quando o meio de cultura e a solução tampão atingirem uma temperatura branda (50 °C), devem ser homogeneizados em condições assépticas e a mistura enriquecida com uma solução contendo 0,3 g de ACC em 50 mL de água, previamente esterilizada em filtro bacteriológico (0,22 micra).

O mesmo meio LN deve ser preparado com a adição de tampão e sem ACC. A estirpe estudada deve ser inoculada em placas de Petri com cada um dos dois meios e incubadas a 28 °C, por oito dias. A seguir, comparar os crescimentos em ambas as condições (Figuras 8 e 9).

Ilustração: Natalia Caetano Vasques.

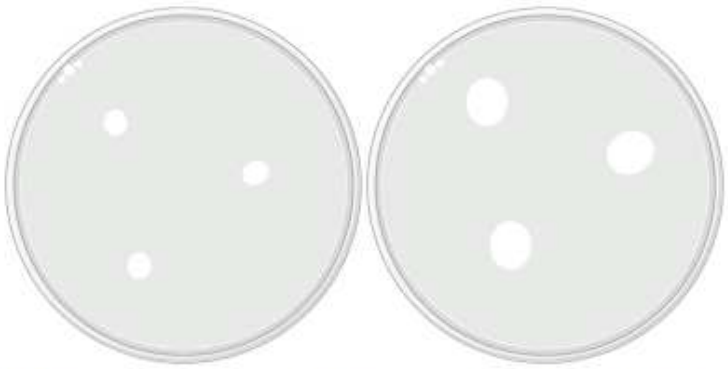

Figura 8. Ilustração do resultado esperado na avaliação da síntese de ACC-deaminase. À esquerda, representação da estirpe estudada em meio LN e à direita, em meio LN com adição de ACC, para verificação de resultado positivo.

Figura 9. Resultado obtido a partir do crescimento de estirpes capazes de produzir a enzima ACC deaminase, com crescimento destacado em meio contendo ACC (+) e imperceptível em meio LN de ACC (-).

Estirpes que mostrarem crescimento mais pronunciado no meio contendo ACC são capazes de utilizar ACC como fonte de N e, portanto, são consideradas produtoras de ACC-deaminase.

Síntese de biofilme e exopolissacarídeos

Importância da síntese de biofilme e exopolissacarídeos

Biofilmes são formados por comunidades de microrganismos cooperantes envolvidas em substâncias poliméricas extracelulares. Na natureza, os biofilmes constituem uma modalidade de crescimento protegido, permitindo que microrganismos sobrevivam em ambientes hostis (Ansari et al., 2017). Entre as principais funções dos biofilmes para a vida microbiana podem-se citar a proteção contra antimicrobianos e estresses abióticos, e o transporte facilitado de nutrientes e metabólitos, entre outros processos (Rafique et al., 2015). A composição do biofilme é variável de acordo com o ambiente em que se desenvolve mas, geralmente, o meio é constituído por água, células bacterianas e

uma matriz de polímeros extracelulares, principalmente, exopolissacarídeos (EPS) (Flemming; Wingender, 2010).

A produção de EPS por microrganismos que interagem com as plantas pode auxiliar na disponibilização de nutrientes, bem como contribuir para a sobrevivência das plantas em várias situações, como estresse hídrico e salino, entre outros (Kumar et al., 2020). Mallick et al. (2018) afirmam que a produção de substâncias poliméricas extracelulares aumenta a capacidade do microrganismo em colonizar com sucesso a raiz de uma planta.

Métodos para avaliação da síntese de biofilme e exopolissacarídeos

Síntese de biofilme

A avaliação da produção de biofilme pode ser realizada com algumas adaptações do método proposto por Lima et al. (2017), a partir do crescimento das estirpes em microtubos em meio de cultura específico para cada estirpe. É importante ressaltar que a inoculação deve ser realizada em concentração conhecida e semelhante para todas as estirpes a serem estudadas, para comparação entre estirpes (por exemplo, densidade óptica $D.O._{600}$ ~ 0,6). Após incubação a 28 °C por 96 h, o meio de cultura com as células é descartado e os tubos lavados três vezes com água destilada e, a seguir, adicionar 1 mL da solução de cristal violeta [0,1%] (em 100 mL de água, adicionar 0,1 g de cristal violeta). Após repouso por 15 minutos em temperatura ambiente, os microtubos devem ser novamente lavados por três vezes com água destilada e, então, adiciona-se 1 mL de álcool etílico [95%]. Com a adição de álcool etílico, o cristal violeta retido no biofilme é solubilizado e, dessa forma, a densidade óptica pode ser determinada por espectrofotometria a 560 nm (Figura 10).

Foto: Natalia Caetano Vasques.

Figura 10. A concentração de cristal violeta em função da produção de biofilme em meio de cultura líquido está indicada da esquerda (-), menos concentrada, para a direita (+), mais concentrada.

Nessa análise não há um padrão de concentração comparativo como uma curva de calibração, levando em consideração somente as diferenças entre as estirpes pela densidade óptica, sendo que quanto maior a leitura, maior produção de biofilme.

Síntese de exopolissacarídeos

A avaliação da produção de exopolissacarídeos (EPS) pode ser realizada com algumas adaptações do método proposto por Meneses et al. (2009) e Castellane et al. (2014). Alíquotas de 2 mL dos cultivos incubados por 72 h a 28 °C devem ser colocadas em microtubos e centrifugadas a 14.000 rpm por 12 min a 4 °C. O pélete de células deve ser descartado e uma parte do sobrenadante - 50 µL - transferida para um novo microtubo contendo 150 µL de álcool etílico [70%] gelado. Os microtubos são novamente centrifugados a 14.000 rpm a 4 °C por 10 minutos. Posteriormente, são transferidos para um concentrador a 45 °C por 1 h, ou até secar. Quando as amostras se encontrarem totalmente secas, adicionar 200 µL de água ultrapura e homogeneizar em vórtex.

A quantificação é realizada a partir do método fenol-sulfúrico para dosagem de carboidratos totais, descrito por Dubois et al. (1956). Devem ser adicionados 200 µL de solução fenol [5%] (ácido fênico) e 1 mL de H_2SO_4 concentrado e homogeneizado em vórtex. Como branco, deve ser utilizada apenas a água ultrapura, fenol [5%] e H_2SO_4. Após 15 minutos de reação em temperatura ambiente, realiza-se a análise espectrofotométrica, com leitura da D.O. a 485 nm.

Os resultados são aferidos após a obtenção de uma curva de calibração com glicose (0; 10; 20; 40; 60; 80; 100; 200 µg mL^{-1}) como substrato padrão da curva de calibração (Figura 11).

Figura 11. Curva de calibração utilizando glicose para quantificação, por espectrofotometria, da concentração de exopolissacarídeos produzidos por bactérias promotoras do crescimento de plantas. A concentração está indicada da esquerda (-), menos concentrada para a direita (+), mais concentrada.

Produção de sideróforos

Importância da síntese de sideróforos

A biodisponibilidade do ferro (Fe) é limitada no ambiente aeróbio devido à forma oxidada predominante (Fe^{3+}), contudo, alguns organismos são capazes de formar quelantes biogênicos para a complexação do Fe^{3+} presente no ambiente, nomeados sideróforos (Römheld, 1987).

Dessa forma, o nutriente que antes se apresentava indisponível pode, então, ser reduzido a Fe^{2+} e ser assimilado tanto por plantas, como por alguns microrganismos. No caso dos microrganismos, esses passarão a apresentar vantagem competitiva e, até mesmo, inibição do crescimento de patógenos, caracterizando uma estratégia de biocontrole, dado que o Fe fica indisponível para os outros microrganismos, em especial os fungos, que não possuem capacidade metabólica de assimilação do elemento por tal via (Kloepper et al., 1980; Schalk et al., 2012). A produção de sideróforos pode, ainda, estar relacionada com a solubilização de fosfato de ferro, decorrente da altíssima afinidade das moléculas por Fe, que é quelado e libera o fosfato (Batista et al., 2018).

Método de avaliação da síntese de sideróforos

Para a avaliação da produção de sideróforos, os isolados devem ser cultivados em meio de cultura ágar King B, cuja composição consta da Tabela 8 (King et al., 1954).

Tabela 8. Composição de meio de cultura ágar King B.

Componente	Quantidade
Glicerol	3,00 g
Peptona bacteriológica	4,00 g
K_2HPO_4	0,23 g
$MgSO_4.7H_2O$	0,30 g
Ágar	15,0 g
Água deionizada	1000 mL
pH	6,8

O meio deve ser esterilizado por 20 minutos a 121 °C. Ao atingir temperatura branda (50 °C), deve ser enriquecido com o corante cromoazurol S (CAS) (Schwyn; Neilands, 1987), o qual pode ser preparado a partir de quatro soluções, cujas composições constam da Tabela

9, que devem ser esterilizadas em condições similares à do meio de cultura.

Solução 1 - 7,5 mL; Solução 2 - 37,5 mL; Solução 3 - 30 mL; Solução 4 - 25 mL.

Tabela 9. Composição das quatro soluções utilizadas para formação do corante cromoazurol S (CAS).

Componente	Quantidade
Solução 1	
$FeCl.6H_2O$	13,5 mg
HCl (10 mM)	41 mL
Água deionizada	50 mL
Solução 2	
CAS (cromoazurol S)	121 mg
Água deionizada	100 mL
Solução 3	
CTAB/HDTMA (Brometo de hexadeciltrimetilamônio)	364,45 mg
Água deionizada	50 mL
Solução 4	
Piperazina anidra	4,307 g
HCl (12 M)	6,25 mL
Água destilada	100 mL

Inocular as bactérias no meio de cultura a partir de uma gota de suspensão de cultivo bacteriano em meio líquido (10 µL) e acompanhar a evolução do crescimento. Após 24 horas de incubação a 28 °C, no escuro, já é possível avaliar algumas estirpes de crescimento rápido.

A capacidade de produção de sideróforos será considerada positiva quando observada a formação de halo de coloração laranja ou rosada ao redor da colônia (Figura 12) e, então, pode ser medida sua extensão com o auxílio de um paquímetro para a obtenção da razão halo/colônia (Figura 13).

Foto: Natalia Caetano Vasques.

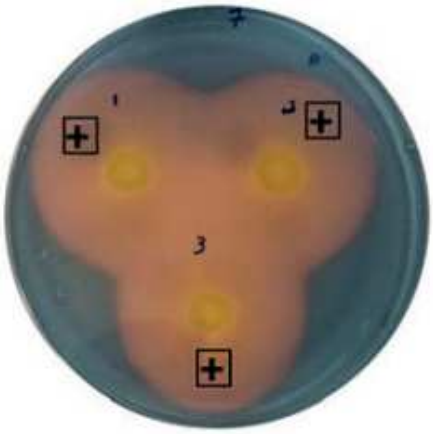

Figura 12. Resultado obtido após a incubação e crescimento de estirpes produtoras de sideróforos (+).

Ilustração: Natalia Caetano Vasques.

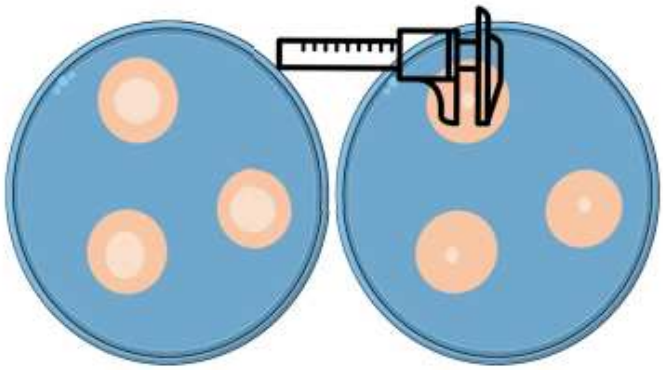

Figura 13. Ilustração do resultado esperado com a aplicação da metodologia. À esquerda, menor razão halo/colônia e à direita, maior habilidade de produção de sideróforos.

Solubilização de fontes de fósforo

Importância da capacidade de solubilização de fósfato

O fósforo (P), elemento essencial ao ciclo metabólico das plantas, é facilmente retido devido a características intrínsecas de solos tropicais. Bactérias solubilizadoras de fosfato contribuem para a ciclagem do elemento, convertendo formas pouco solúveis em formas solúveis e, portanto, assimiláveis às plantas. Tal conversão pode ocorrer por diversas vias, sendo que a principal ocorre pela produção e liberação de metabólitos (Hameeda et al., 2008; Young et al., 2013), como os ácidos orgânicos, que podem liberar fosfato solúvel pela redução do pH do meio (Salih et al., 1989; Nahas, 1991).

Método de avaliação da capacidade de solubilização de fosfatos

A avaliação da capacidade solubilizadora de fosfato pode ser realizada pelo método proposto por Sylvester-Bradley et al. (1982), em meio de cultura cuja composição consta da Tabela 10, com composição de Fe-EDTA segundo Tabela 11 e de solução de micronutrientes segundo Tabela 12.

Tabela 10. Composição de meio de cultura para avaliação da capacidade de solubilização de fosfato.

Componente	Quantidade
KNO_3	0,10 g
Glicose	10,00 g
Extrato de levedura	5,00 g
$MgSO_4.7H_2O$	0,20 g
NaCl	0,10 g
CaCl	0,02 g
Solução Fe-EDTA	4 mL
Solução de micronutrientes	2 mL
Ágar	15,0 g
Água deionizada	1000 mL

Tabela 11. Composição de solução Fe-EDTA do meio de cultura da Tabela 10.

Componente	Quantidade
Na-EDTA	6,07 g
$FeSO_4.H_2O$	6,17 g
Água deionizada	900 mL

Tabela 12. Solução de micronutrientes do meio de cultura da Tabela 10.

Componente	Quantidade
$NaMoO_4.2H_2O$	0,200 g
$MnSO_4.2H_2O$	0,235 g
H_3BO_3	0,280 g
$CuSO_4.5H_2O$	0,008 g
$ZnSO_4.7H_2O$	0,024 g
Água deionizada	200 mL

Após autoclavagem por 20 min a 121 °C, o meio de cultura pode ser enriquecido com três diferentes fontes de fosfato separadamente, de acordo com a análise desejada, para verificar a capacidade de solubilização dos fosfatos inorgânicos na forma de fostato de Ca, Fe e Al.

As soluções deverão ser preparadas a partir de 4,93 g de $CaHPO_4.2H_2O$; ou 5,35 g de $FePO_4.2H_2O$; ou 3,50 g de $AlPO_4$ suspensos em 100 mL de água destilada e, então, autoclavadas por 20 minutos a 121 °C. A mistura do meio de cultura com cada solução de fosfato deve ser realizada assepticamente, em câmara de fluxo laminar, e com o meio a, aproximadamente, 50 °C e, então, distribuída nas placas de Petri.

Após inoculação do meio de cultura a partir de uma gota de suspensão de cultivo bacteriano em meio líquido (10 µL) e incubação a 28 °C, será verificada a presença ou ausência de halo transparente ao redor da colônia (Figura 14) e, quando presente, com o auxílio de um paquímetro digital (Figura 15), pode ser medida sua extensão, bem como a extensão da colônia, para obtenção do Índice de Solubilização.

Foto: Natalia Caetano Vasques.

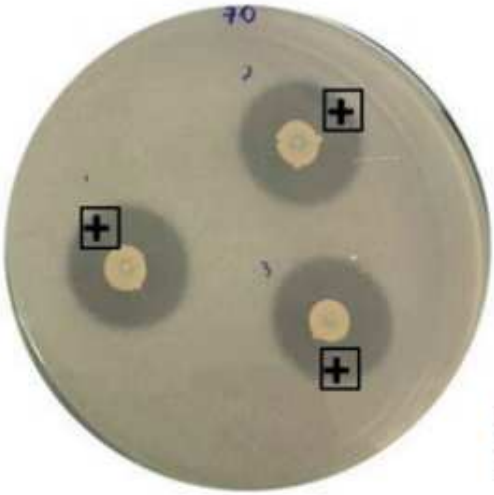

Figura 14. Resultado obtido a partir do crescimento de estirpes solubilizadoras de fósfato (+).

Ilustração: Natalia Caetano Vasques.

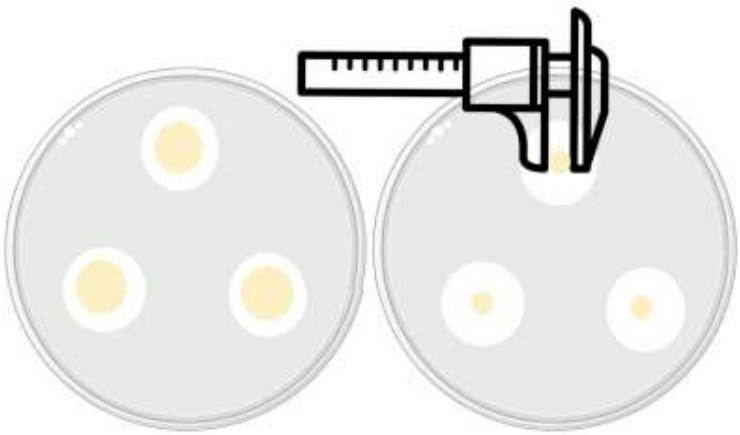

Figura 15. Ilustração do resultado esperado com a aplicação da metodologia para avaliação da capacidade de solubilização de fosfatos. À esquerda, menor razão halo/colônia ou índice de solubilização e à direita, estirpes com maior habilidade de solubilização.

Considerações finais

As BPCP desempenham um papel crucial na promoção do crescimento das plantas, influenciando uma ampla variedade de processos microbianos, com consequências positivas na qualidade do solo e na produtividade das culturas agrícolas. Os métodos apresentados neste estudo fornecem ferramentas essenciais para a identificação e avaliação de microrganismos promissores que podem ser utilizados no desenvolvimento de bioinsumos agrícolas. Esses bioinsumos têm como principal objetivo promover a nutrição das plantas e aumentar sua tolerância a estresses ambientais, tanto bióticos, quanto abióticos.

O Brasil possui uma das maiores biodiversidades do mundo, o que oferece um vasto tesouro de microrganismos ainda não explorados para uso na agricultura. Ao capitalizar essa riqueza biológica, podemos impulsionar a agricultura em direção à resiliência e à sustentabilidade, beneficiando não apenas os agricultores, mas também o meio ambiente e a sociedade como um todo. Ao explorar a biodiversidade única do Brasil, essas abordagens não apenas abrem novas oportunidades para a inovação na agricultura, mas também destacam o potencial do país para liderar o caminho em práticas agrícolas sustentáveis e adaptáveis às mudanças climáticas.

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CAPÍTULO 2

ESTRATÉGIAS PARA FACILITAR A BIOPROSPECÇÃO DE PROPRIEDADES DE INTERESSE AGRONÔMICO EM COLEÇÕES DE CULTURAS MICROBIANAS E UM CASO DE SUCESSO NA SELEÇÃO PARA MITIGAR O EFEITO DA SECA EM MILHO

ESTRATÉGIAS PARA FACILITAR A BIOPROSPECÇÃO DE PROPRIEDADES DE INTERESSE AGRONÔMICO EM COLEÇÕES DE CULTURAS MICROBIANAS E UM CASO DE SUCESSO NA SELEÇÃO PARA MITIGAR O EFEITO DA SECA EM MILHO

Artigo aprovado

RESUMO

A substituição de insumos sintéticos por soluções biológicas tem sido uma meta global de alta prioridade na agricultura. Em todo o mundo, existem diversas coleções microbianas, com elevada biodiversidade e potencial biotecnológico. Este estudo baseou-se na hipótese de que análises *in vitro* podem orientar a seleção de estirpes promissoras para posterior avaliação *in vivo*. Foram selecionadas 100 estirpes representativas da "Coleção de Culturas de Bactérias Diazotróficas e Promotoras de Crescimento de Plantas da Embrapa Soja", Brasil, para serem avaliadas *in vitro* quanto às atividades proteolítica e celulolítica, produção de 1-aminociclopropano-1-carboxilato desaminase (ACC-desaminase), sideróforos, compostos indólicos (ácido indol-3-acético, AIA), exopolissacarídeos (EPS), biofilme, solubilização de nutrientes e capacidade de crescimento em meio com atividade de água reduzida e alta temperatura (40 ± 2 °C). As 100 estirpes também foram avaliadas em casa de vegetação, em milho cultivado em substrato estéril, para verificar a capacidade de promover tolerância à seca. Atividades hidrolíticas e proteolíticas destacaram-se em *Paenibacillus*, *Pantoea* e *Bacillus*, enquanto a ACC-desaminase foi amplamente detectada em 38 estirpes de diversos gêneros. A tolerância à seca e a altas temperaturas (40 ± 2 °C) foi fortemente presente em *Bacillus*. Resultados de destaque foram obtidos com *Azospirillum* para EPS, com *Paraburkholderia*, *Pseudomonas* e *Bacillus* para biofilme, e com *Chromobacterium* para AIA. Em relação às propriedades que potencialmente auxiliam na absorção de nutrientes, 30 estirpes sintetizaram sideróforos, mas apenas sete foram capazes de solubilizar fosfato de cálcio, das quais cinco pertenciam ao gênero *Pseudomonas*. Foi observada alta correlação entre a capacidade de crescer *in vitro* em meio com baixa atividade de água e a promoção de tolerância à seca em milho *in vivo*. A partir deste experimento inicial em casa de vegetação, 15 estirpes foram

selecionadas para confirmar seu potencial em mitigar a restrição hídrica em solo não estéril. Três estirpes promissoras, *Bacillus velezensis* CNPSo 2384, *Bacillus subtilis* CNPSo 2606 e *Bacillus* sp. CNPSo 2723, foram identificadas como candidatas para compor futuros bioinsumos voltados ao aumento da tolerância das plantas à restrição hídrica. A aceleração dos programas de seleção de estirpes é altamente relevante, e certas propriedades de interesse agronômico podem ser encontradas mais facilmente em gêneros bacterianos específicos. Além disso, a prova de conceito para uma avaliação preliminar *in vitr*o foi confirmada *in vivo* quanto à tolerância das plantas à seca, incentivando, assim, a validação de outras propriedades microbianas importantes.

BMC Microbiology
Plant growth-promoting microorganisms

Strategies to facilitate the bioprospection of properties of agronomic interest in microbial culture collections and a successful case of selection to mitigate drought stress in maize

Abstract

Background: The replacement of synthetic inputs with biological solutions has been a global goal with high priority in agriculture. Worldwide, there are several well-organized microbial collections holding high biodiversity and biotechnological potential. This study was based on the hypothesis that *in vitro* analyses can guide the selection of promising strains for subsequent *in vivo* evaluation. We selected 100 strains representative of the "Diazotrophic and Plant Growth-Promoting Bacteria Culture Collection of Embrapa Soja", Brazil, to be evaluated *in vitro* for proteolytic and cellulolytic activities, production of 1-aminocyclopropane-1-carboxylate deaminase (ACC-deaminase), siderophore, indolic compounds (indole-3-acetic acid, IAA), exopolysaccharides (EPS), biofilm, solubilization of nutrients, and ability to grow in medium with reduced water activity and high temperature. The 100 strains were also evaluated in a greenhouse on maize growing in sterile substrate to assess their ability to promote tolerance to drought.

Results: Hydrolytic and proteolytic activities were highlighted in *Paenibacillus*, *Pantoea,* and *Bacillus*, and ACC-deaminase was widespread in 38 strains of several genera. Tolerance to drought and high temperature (40 ± 2 °C) was highly present in *Bacillus*. Outstanding results were obtained with *Azospirillum* for EPS, in *Paraburkholderia*, *Pseudomonas*, and *Bacillus* for biofilm, and in *Chromobacterium* for IAA. Regarding properties that could putatively help the uptake of nutrients, 30 strains synthesized siderophores, but only seven were able to solubilize calcium phosphate, five of which were classified as *Pseudomonas*. A high correlation was found between the ability to grow *in vitro* in medium with reduced water activity and tolerance to drought *in vivo*. From this

initial greenhouse experiment, 15 strains were selected to confirm their potential to mitigate drought in a greenhouse experiment with non-sterile soil. Three outstanding strains, *Bacillus velezensis* CNPSo 2384, *Bacillus subtilis* CNPSo 2606, and *Bacillus* sp. CNPSo 2723 were identified as promising candidates to compose future bio-inputs aimed at increasing plant tolerance to drought.

Conclusion: Speeding up strain selection programs is highly relevant, and certain properties of agronomic interest can be found more easily in specific bacterial genera. Additionally, the proof of concept for a preliminary *in vitro* evaluation was confirmed *in vivo* for plant tolerance to drought, thereby stimulating the validation of other important microbial properties.

Key words: Plant growth-promoting bacteria; *Bacillus*; *Pseudomonas;* IAA; EPS; biofilm; solubilization of nutrients; ACC-deaminase; siderophores.

Background

Biological collections are outstanding components of a nation's scientific and national sovereignty, being considered essential for its innovation infrastructure [1]. Therefore, microbial collections represent a rich reservoir for the survey of potential new technologies. There are important microbial culture collections (MCC) worldwide, many also known as Biological Resource Centers (BRC), acting as centers of excellence in *ex-situ* conservation and microbial taxonomy, such as ATCC (United States of America), BCCM (Belgium), DSMZ (Germany), and WDCM (China). In 2022, the World Federation for Culture Collections (WFCC) –the leading organization that synchronizes activities of the MCC– listed 820 collections worldwide [2]. In Brazil, a mega-diverse country that holds around 25% of global biodiversity [3], the existing system of collections have received increasing recognition in governmental policies as part of efforts to organize germplasms of economic interest, including microbial collections.

Microbial collections typically contain hundreds or thousands of strains or isolates. For example, our culture collection located in Londrina, State of Paraná, Brazil, "Diazotrophic and Plant Growth Promoting Bacteria Culture Collection of Embrapa Soja" (WFCC Collection # 1213, WDCM Collection # 1054), currently includes 4,800 strains obtained over more than four decades. Many of these strains were obtained from field collections of legume nodules, others from a

variety of legumes and non-legume species, from natural or agricultural areas, in studies of biodiversity [4, 5], ecology [6, 7, 8], or bioprospection for agricultural purposes [9, 10, 11].

As a result of the agricultural sector growing awareness of biological inputs (bio-inputs), there has been a significant increase in the search for solutions based on microorganisms [12]. Soil health collapses in poorly managed soils, biodiversity faces the sixth mass extinction, and crop yields have reached a plateau. Against this critical narrative, a call for regenerative agriculture emerges, one that goes beyond sustainability, focusing on the recovery of soil health and fertility [13]. In response to this scenario, a governmental program to stimulate the production and use of bio-inputs was established in Brazil in 2020 [14], reinforcing the country´s vocation for the use of bio-inputs in agriculture.

Plant growth-promoting bacteria (PGPB) have been increasingly applied in agriculture as a sustainable practice to enhance crop yields [15]. PGPB can stimulate plant growth based on several properties, including the synthesis of growth-regulating molecules [16], siderophores [17, 18], 1-aminocyclopropane-1-carboxylate deaminase (ACC-deaminase) [19], exopolysaccharides (EPS) and biofilms [20], hydrolytic enzymes such as cellulases and proteases [21, 22]. Growth promotion may also be achieved by increasing plant tolerance to abiotic stresses, such as drought [23, 24]. In addition, some bacteria have properties that can enhance the plant uptake of nutrients [25], such as the capacity for solubilize inorganic phosphate [26, 27] or associated with the biological N_2 fixation (BNF) [28]. Finally, we can cite mechanisms of growth-promotion related to the direct biocontrol of pests and diseases, including induced systemic resistance (ISR) and the synthesis of antimicrobial molecules, among others [29, 30].

In the highly dynamic microenvironment of the plant-soil system, microorganisms interact with roots, exudates, and the native microbiota, directly influencing the colonization and activity of introduced strains. Species such as *Azospirillum brasilense*, *Pseudomonas fluorescens*, and various rhizobia, stored at the Embrapa Soja's culture collection, have already demonstrated successful associations in the rhizosphere of different crops, highlighting their potential for adaptation to agricultural environments [9, 10, 11]. These bacteria not only express plant growth-promoting traits but are also influenced by factors such as

soil type, host crop, and cultivation conditions, including water deficit. Therefore, studies on the occurrence and ecological behavior of these strains in the plant-soil environment under different climatic conditions contribute to the development of formulations with greater persistence and positive interaction with plants under field conditions.

The search for biological solutions in agriculture, with an emphasis on microorganisms, has been enormous, so that the growth rate of bio-inputs use far exceeds that of chemicals, and further increases are expected over the next decades [31]. However, the search for elite microbial strains can be a laborious task, especially due to the large number of microorganisms whose functionality has not yet been fully investigated.

The objective of this study was to evaluate whether *in vitro* analyses can guide the selection of strains with agronomic potential for subsequent *in vivo* validation. For that, a careful selection of 100 representative strains based on the main genera isolation site, and indications of relevant properties from previous studies was made for the analysis of *in vitro* traits that might indicate agronomic potential (e.g., ability to increase the plant capacity for nutrient uptake, drought tolerance, production of phytohormones), and further evaluation of tolerance to drought *in vivo* in maize plants. The hypothesis tested was that traits evaluated under *in vitro* conditions would be predictive of the strains' performance in plants under water restriction. As main results, we identified genera in which certain properties might be predominant, facilitating bioprospection in culture collections, and we also confirmed a correlation between *in vitro* and *in vivo* properties conferring tolerance to drought in maize plants.

Methods

Bacterial strains

A total of 100 strains, representative of a collection containing 4,800 strains, were initially investigated *in vitro* for properties putatively indicative of their biotechnological potential of agronomic traits of interest. The strains are deposited at the “Diazotrophic and Plant Growth-Promoting Bacteria Culture Collection of Embrapa Soja” (WFCC Collection #1213, WDCM Collection # 1054) in Londrina, State of Paraná, Brazil. The 100 selected strains aimed at including representative strains of the main genera of PGPB, site of isolation, and

indications of relevant traits from previous studies. The selected strains and corresponding culture medium used for growth are shown in Supplementary Table S1 [32, 33].

Plant growth-promoting traits *in vitro*

Proteolytic activity

For the evaluation of the synthesis of proteolytic enzymes, the strains were grown in a culture medium containing skim milk, as described by Gerhardt et al. [34]. After incubation for 7 days at 28 ± 2 °C, casein hydrolysis was evaluated by the formation of a translucent haloes of degradation around the colony, as shown in Supplementary Figure S1.

Cellulolytic activity

To evaluate the cellulose degradation capacity of the strains, the method described by Gerhardt et al. [34] was employed, using a culture medium containing carboxymethyl cellulose (CMC) as carbon source. After incubation for 5 days at 28 °C, the cellulose degradation haloes were revealed by adding 2 mL of 1 Mol L^{-1} NaCl solution on the medium surface, and after 5 minutes, the saline solution was removed, and 2 mL of a 0.1% Congo red solution was added. After 30 minutes, distilled water was applied to wash off the dye solution, revealing haloes around colonies showing of positive cellulolytic activity (Figure S1).

Production of 1-aminocyclopropane-1-carboxylate (ACC) deaminase

The ability to metabolize ACC as the sole source of nitrogen (N) in the medium, due to the action of the enzyme ACC-deaminase, was evaluated by adapting the methodology of Glick et al. [35] and Lucon et al. [36]. Initially, a N-free culture medium (NF) and a buffer solution (containing KH_2PO_4 and Na_2HPO_4) were sterilized in autoclave, joined, the mixture was enriched with a 0.03% solution of ACC previously filtered through a bacteriological filter. The same NF medium was prepared without ACC, therefore without any source of N. The strains were inoculated on plates with both media and incubated at 28 °C for 8 days. Growth in both conditions was then compared. Strains that showed more pronounced growth in the ACC-containing medium compared to the NF medium were considered capable of using ACC as an ACC-deaminase producer (Figure S1).

Siderophore production

To assess siderophore production, the isolates were grown on King B agar culture medium [37] containing chromazurol S (CAS) [38]. The medium was inoculated with 10 µL of bacterial culture suspension, and after 24 and 72 h of incubation at 28ºC, in the dark, the evaluations were carried out. The ability to produce siderophores was considered positive when an orange or pink halo was observed around the colony, and its extent was then measured using a pachymeter (King Tools, São Paulo, Brazil) in both evaluations to obtain the halo/colony ratio (Figure S1).

Production of indolic compounds

To quantify the production of indole-3-acetic acid (IAA) *in vitro* [39], each bacterial strain was inoculated in the corresponding liquid culture medium for each genus (Table S1) and enriched with tryptophan (100 µg mL^{-1}), and the flasks incubated under shaking (100 rpm) for 7 days at 28 °C. The growth broth was then transferred to microtubes and centrifuged at 10,000 rpm for 10 min (Eppendorf - Centrifuge 5804 R). Following, 1 mL of the supernatant was transferred to new microtubes, and 750 µL of the Salkowski's reagent (1 mL of $FeCl_3.6H_2O$ (0.5 Mol L^{-1}), 50 mL of $HClO_4$ (35%)) was added. After 30 minutes of reaction in the dark at room temperature, the staining intensity was evaluated in a spectrophotometer (Genesys™ 10S UV-VIS spectrophotometer) at a wavelength of 540 nm. The results were expressed in µg mL^{-1} of IAA in the medium based on a calibration curve with synthetic IAA (0, 50, 100, 200, 300, 400, 500, 600, 800, 1000 µg mL^{-1}) (Figure S1).

Production of exopolysaccharides

The assessment for EPS production was based on de Meneses et al. [40] and Castellane et al. [41], with some adaptations. Aliquots of 2 mL of culture broth incubated for 72 h at 28 °C were placed in microtubes and centrifuged at 14,000 rpm (Eppendorf - Centrifuge 5804 R) for 12 min at 4 °C. The cell pellet was discarded, and 50 µL of the supernatant were transferred to a new microtube containing 150 µL of ice-cold ethanol. The microtubes were centrifuged again at 14,000 rpm at 4 °C for 10 minutes. The microtubes were then transferred to a

concentrator (Eppendorf - Concentration Plus) at 45 °C for 1 h or until dry, and then 200 µL of ultrapure water were added and homogenized in a vortex. For quantification, 200 µL of 5% phenol and 1 mL of concentrated H_2SO_4 were added and homogenized in a vortex, using ultrapure water, phenol, and H_2SO_4 as blank. After 15 minutes of reaction at room temperature, spectrophotometric analysis was carried out (Genesys™ 10S UV-VIS spectrophotometer), reading the O.D. at 485 nm. The results were obtained according to a calibration curve based on the Phenol-Sulfuric method described by DuBois et al. [42] for measuring total carbohydrates, using glucose (0, 10, 20, 40, 60, 80, 100, 200 µL mL^{-1}) as the standard substrate for the calibration curve (Figure S1).

Production of biofilm

The assessment of biofilm production was carried out according to the method proposed by Lima et al. [43], with some adaptations. Bacterial growth was assessed in microtubes (Eppendorf - 2 mL) in the respective culture medium for each strain (Table S1). In this analysis, there is no standard comparative concentration, considering only the differences among the strains based on optical density (O.D.), where higher readings indicate greater biofilm production (Figure S1).

Phosphate solubilization

The capacity for phosphate solubilization was evaluated using the Sylvester-Bradley et al. [44] method. After autoclaving (121 °C for 20 min), the medium was separately supplemented with inorganic calcium phosphate ($CaHPO_4 \cdot 2H_2O$), which was previously prepared and sterilized. The cultures were incubated at 28 °C, and the presence of a solubilization halo was assessed at 3, 7, and 12 days after inoculation (DAI). The Solubilization Index was determined by the ratio between the halo diameter and the colony diameter, using a pachymeter (King Tools, São Paulo, Brazil) (Figure S1).

Ability to grow in culture medium with reduced water activity and high temperature (40 °C)

To evaluate the development of the strains in a medium with reduced water activity (Aw), Aw was tested at 0.919 and 0.897, as proposed by Hallsworth et al.

[45]. After incubation at 40 ± 2°C for seven days in Petri dishes, the strains that exhibited visible growth were considered positive (Figure S1), while those that failed to grow in the medium with a high solute concentration and incubated at high temperatures were considered negative.

Phenotypic clustering

The *in vitro* evaluation data of 100 PGPB strains for agronomically relevant traits were used for phenotypic clustering. A binary matrix was generated based on the presence or absence of these traits, and the isolates were grouped using the UPGMA (Unweighted Pair Group Method with Arithmetic Mean) algorithm with the aid of Bionumerics® software version 7.6.3 (Applied Mathematics, Sint-Martens-Latem, Belgium) [46]. Clustering distinction was established at a 70% similarity threshold.

Numerical data were normalized to generate a heatmap in the R environment (v. 4.5.0), supported by RStudio (v. 2024.12.1) and the packages ggplot2 (v. 3.5.2), reshape2 (v. 1.4.4), and ggtext (v. 0.1.2).

Evaluation of the bacterial strains on conferring plant tolerance to water restriction under greenhouse

First experiment: Evaluation of 100 strains in maize grown in sterile substrate

Substrate preparation

Plants were grown in 1 L pots (12 cm in diameter), filled with approximately 1 kg of a substrate composed of coarse sand and ground coal in a 1:1 (v:v) ratio. The substrate was previously homogenized in trays and sterilized in an autoclave at 121 °C for 40 minutes.

Inoculum preparation

The inocula were prepared by cultivating each bacterial strain in the corresponding liquid medium (Table S1), ensuring an equivalent concentration of approximately 10^8 CFU mL^{-1} for each strain.

Conduction of the experiment

The treatments consisted of the 100 selected strains and two non-inoculated controls: one subjected to water restriction similar to the inoculated plants, and another maintained under adequate watering conditions. The experiment was laid out in a completely randomized design with three replicates. Maize (*Zea mays* L.) seeds of hybrid Pioneer 30F53 PRO 3 were surface-disinfested by immersion in 70% ethanol for 1 min, 0.4% sodium hypochlorite for 5 min, and six consecutive rinses in sterile distilled water.

The inoculation was carried out directly in the pot. The seeds were inoculated with 1 mL of the corresponding bacterial suspension cultivated in liquid medium, without removing the culture medium. This volume was applied to each seed, ensuring complete coverage of the seed by the inoculum. Immediately afterward, the seeds were covered with substrate, guaranteeing full coverage and proper contact with the soil.

The greenhouse experiment was carried out at the Experimental Station facilities of Embrapa Soja in Londrina, Paraná State, southern Brazil (23˚11′ S, 51˚11′ W). Plants were supplied with sterilized Hoagland and Arnon's nutrient solution [47] at 50% of the N concentration, in a greenhouse with forced ventilation, natural photoperiod, and a temperature of 29 ± 2 ˚C (day) and 17 ± 2 ˚C (night). Plants were submitted to water restriction at the V3 stage, 10 days after emergence (DAE), when they had three fully developed leaves, for 12 days.

Induction of water restriction

The water restriction was monitored by randomly weighing selected pots to track mass loss, ensuring the plants not to reach the permanent wilting point but keeping them stressed throughout the period of water restriction. The amount of nutrient solution for replenishment was established daily and distributed equally among the treatments. A view of the experiment is shown in Figure S2.

Data collection

Plants were collected 22 DAE, 12 days after the water restriction was imposed. Roots and shoots were separated, and the following traits were assessed: on the day before harvesting (21 DAE), plant height was measured from the soil surface

to the base of the first mature leaf. Chlorophyll was determined using a chlorophyll meter (SPAD 502, Konica Minolta Sensing, Inc., Osaka, Japan) according to the calibration described by Kaschuk et al. [48]. At harvest, fresh and dry shoot biomass, and root system volume were recorded according to Rondina et al. [49].

Second experiment: Evaluation of selected strains in maize grown in non-sterile soil

Substrate and inoculant preparations and conduction of the experiment

Fifteen bacterial strains that most promoted plant growth under water restriction in the first greenhouse experiment, as well as positive results in most *in vitro* tests, were selected for confirmation in a second greenhouse trial. This second trial followed similar inocula preparation and sowing procedures, except for the substrate, consisting of 5 kg of non-sterile soil per pot (24 cm in diameter). The soil was classified as Typic Acrudox [50] taken at 0-20 cm topsoil layer from a commercial farm located in Ponta Grossa, Paraná State, Brazil, and presenting the following characteristics: pH ($CaCl_2$) = 5.14; organic matter = 18.24 g dm^{-3}; available P= 2.85 mg dm^{-3}; exchangeable K = 0.10 $cmol_c$ dm^{-3}; Ca = 3.47 $cmol_c$ dm^{-3}; Mg = 1.10 $cmol_c$ dm^{-3}; soluble N = 2.5 mg dm^{-3}; H + Al = 4.12 $cmol_c$ dm^{-3}; Cation Exchange Capacity (CEC) = 8.79 $cmol_c$ dm^{-3}; granulometry: sand = 238 g kg^{-1}, silt = 30 g kg^{-1}, and clay = 732 g kg^{-1}.

Preparation of inoculants and procedures of inoculation were the same as described for the first experiment.

The experiment was carried out at the Experimental Station facilities of Embrapa Soja, under greenhouse conditions, with an average photoperiod of 13 h (day) and 11 h (night) ± 28 min and a temperature of 24 ± 3.1 ˚C (day) and 20 ± 2 ˚C (night). During the trial, the average day/night relative air humidity was 52.5 to 92.7%, respectively. The experiment was conducted following a completely randomized design, with five replicates.

Water stress induction

For adjustment of soil moisture, the soil water-holding capacity was determined using a tension table and Richards's extractor device, resulting in a water-retention curve that correlates the water content with the soil water potential (ψw).

During the first 22 DAE, all plants received water to maintain the ψw at 70% of the water-holding capacity. After 22 DAE, at V5 stage, when the plants had five fully developed leaves, they were submitted to water restriction. For that, the pots under water restriction were maintained at 30% of water-holding capacity, while non-inoculated control plants were kept with adequate water supply (70% water-holding capacity). Soil moisture was monitored daily by weighing each pot on an electronic scale, and adjustments of moisture were made in the morning (between 9 and 11 a.m.). We considered the fresh mass of plants at well-watered conditions at 22 DAE from extra pots to correct the effect of plant weight on the water reposition in pots containing plants subjected to water restriction. An overview of the experiment is shown in Figure S3.

Data collection

On the 3rd, 5th and 9th days of water restriction, physiological parameters were recorded in both stressed and non-stressed plants with a portable gas exchange meter, model LI-6400 (Li-Cor, Biosciences Inc., Nebraska, USA). Determinations included net photosynthetic (A) and transpiration (E) rates, stomatal conductance (gs), intercellular CO_2 concentration (Ci), and temperature of leaves. Gas exchanges were assessed in the central leaf in the morning (9–11 a.m.). Plants were collected at 35 DAE, when roots and shoots were separated. The following traits were assessed: shoot fresh weight, root mean volume, shoot dry weight, root dry weight. Plant height was evaluated before harvesting as in Experiment I, and root length was obtained according to Tennant [51].

Statistical analysis

For all experiments, the data analysis was structured to assess the significance of treatments and to explore relationships between the assessed variables. Initially, a one-way analysis of variance (ANOVA) was performed, followed by the Scott-Knott test at a 5% of significance to group similar treatments. For all ANOVA tests, the normality of residuals and the homogeneity of variances were assessed prior to analysis.

To evaluate the relationship between *in vitro* and *in vivo* results from the first greenhouse experimental trial, Pearson's correlation coefficient (PCC) was

applied using R-Statistics to determine the strength and direction of the linear relationship between the data. Statistical significance was determined at $p \leq 0.05$.

Additionally, principal component analysis (PCA) explored the variability and relationships among the *in vivo* data assessed in the first greenhouse experiment. The PCA analysis was conducted using SPSS software (version 22.0) to identify the main components that explain the variability in the dataset. This multivariate analysis allowed the summarization of the data structure and identification of key patterns associated with the treatments.

Results and Discussion

In vitro plant growth-promoting traits

The selected 100 PGPB strains were evaluated *in vitro* for ten putative plant growth-promoting traits. Protease production was confirmed in 33 strains by the formation of a typical degradation halo around the colonies on a culture medium containing skim milk (Table 1). Additionally, cellulase production was observed in 18 strains, eight of them exhibiting the production of both enzymes, belonging to the genera *Bacillus* (6), *Paenibacillus* (1), and *Pantoea* (1) (Table 1). The genus *Bacillus* has been reported as highly efficient in producing hydrolytic enzymes [52, 53, 54]. The protease activity has great potential to help in the biocontrol of pests and diseases in agriculture. For example, Khedher et al. [55] reported the effect of this enzyme produced by some species of *Bacillus* on the rupture and deformation of fungal hyphae. Proteases can impair the structure and some cellular functions of pathogenic fungi due to vacuolization, protoplast leakage, and cracking of mycellium [55]. An important advantage of biocontrol using microbial hydrolytic enzymes to replace chemical fungicides is the lack of damage to plant tissues [56].

Thirty-eight strains demonstrated ACC-deaminase activity, indicated by robust growth in medium containing ACC as the sole N source. This trait was distributed across 15 genera: *Achromobacter* (1), *Agrobacterium* (4), *Azospirillum* (2), *Bacillus* (4), *Bradyrhizobium* (4), *Chromobacterium* (3), *Delftia* (1), *Ensifer* (=*Sinorhizobium*) (1), *Methylobacterium* (1), *Neorhizobium* (1), *Paenibacillus* (2), *Pantoea* (3), *Paraburkholderia* (2), *Pseudomonas* (6), and *Rhizobium* (3) (Table 1).

The production of ACC-deaminase is related to the regulation in the levels of ethylene due to mineralization of precursor molecules, releasing ammonia, which may contribute to plant nutrition to a small extent [57, 58]. Therefore, the ACC-deaminase-producing strains help to reduce the negative response to ethylene produced under stressing conditions, such as water deficit, and also to reduce the plant senescence, a widespread physiological strategy under stress [59]. The induction of stress tolerance promoted by ACC-deaminase-producing PGPB has been broadly reported in various crops, including improvements in plant growth, nutrient content, and antioxidant properties in wheat (*Triticum aestivum* L.) associated with strains of the genera *Variovorax*, *Pseudomonas*, *Achromobacter*, and *Ochrobactrum* [60]. In maize associated with *Achromobacter xylosoxidans*, the ACC-deaminase activity increased photosynthetic rate, stomatal conductance, total chlorophyll and carotenoid contents, and grain yield [61]. In soybean (*Glycine max* (L.) Merr.), inoculation with *Curtobacterium* sp. relieved the salt stress and stimulated plant growth [62]. Interestingly, in cherry tomatoes (*Solanum lycopersicum* var. *cerasiforme*), the inoculation of an ACC-deaminase-producing strain *Leclercia adecarboxylata* promoted both growth and tolerance to salt stress [63], highlighting the biotechnological potential of ACC-deaminase-producing strains such as those identified in our study.

Siderophore production was observed in 30 strains, spanning 11 genera: *Agrobacterium* (3), *Azoarcus* (1), *Azospirillum* (1), *Bacillus* (2), *Bradyrhizobium* (2), *Chromobacterium* (3), *Delftia* (1), *Paenibacillus* (1), *Paraburkholderia* (2), *Pseudomonas* (12), and *Rhizobium* (2) (Table 1). To better assess the efficiency of siderophore production among strains with different growth rates, the assay was conducted at two distinct times (24 and 72 h of incubation). This approach was necessary because halo development was not synchronized across all strains. By including an additional reading, it was possible to capture the full expression of siderophore production in slower-growing strains and to account for differences in halo/colony ratios, which varied significantly among isolates. This metric allowed a more accurate comparison for capacity of siderophore production, besides only the presence or absence of a halo. After 72 h of incubation, *Rhizobium giardini* CNPSo 171 and *Pseudomonas* sp. CNPSo 2625

showed the highest halo-to-colony ratios, indicating high efficiency in siderophore production relative to the colony growth.

The 30 siderophore-producing strains identified in our study may be of biotechnological interest due to their ability to increase nutrient availability and inhibit pathogen growth through iron (Fe) complexation [64, 65]. Among them, *Pseudomonas* was the most prevalent genus (12 strains), as also reported Tian et al. [66]. This genus is widely recognized for enhancing Fe uptake in plants grown in Fe-deficient soils [67]. In maize, siderophore production has been associated with improved Fe transport to stalks, leaves, and seeds, which is relevant for the nutritional quality of food and feed [68]. Moreover, by restricting Fe availability in the rhizosphere, siderophores is involved in the suppression of phytopathogens, offering an eco-friendly alternative to synthetic fungicides [69].

Regarding the synthesis of phytohormones, *Chromobacterium violaceum* strain CNPSo 1954 stood out in a tryptophan-supplemented culture medium, producing the highest amount of IAA, 207 µg mL^{-1} (Table 1). Despite being quite variable, all strains were able to synthesize IAA when supplemented with tryptophan, ranging from 1.54 to 207 µg mL^{-1}; tryptophan is the precursor of IAA. *C. violaceum* is known for the synthesis of a secondary metabolite named violacein, which gives the purple color to the colonies [70]. Although violacein is not essential for bacterial growth and survival, it is involved in the synthesis of tryptophan [71]. Other genera also synthesized high concentrations of IAA, such as *Pseudomonas*, which has been widely recognized in previous studies as an important producer of indolic compounds [72, 73], and also *Pantoea*. IAA stimulates root elongation and increases the number of root hairs and roots branching, which are architectural traits crucial for nutrient uptake [74]. Additionally, depending on the concentration, IAA may increase the length of the primary root and enhance tolerance to salt and drought stress by driving the roots away from regions of elevated salinity or towards regions with more available water [75, 76].

Regarding the high biotechnological potential of *C. violaceum* CNPSo 1954 for synthesis of IAA, it is worth mentioning that when searching for strains to be used as bio-inputs, they must also be investigated for pathogenicity to humans, animals, and plants. *C. violaceum* rarely causes diseases in humans; it is widely found in Amazonian rivers, where the strain CNPSo 1954 was obtained

from [77], but as pathogenicity has been reported [78], the strains should always be investigated. If pathogenicity is confirmed, alternatives can be found, such as the use of secondary metabolites as phytostimulants [79].

Regarding EPS production, the strains *Azospirillum rugosum* CNPSo 3757 and *Azospirillum brasilense* CNPSo 2084 (=Ab-V6) stood out, with average values of 131 μg mL^{-1} and 129 μg mL^{-1}, respectively (Table 1). The production of EPS is fundamental for microbial life, as it provides an ideal environment for their survival, favoring chemical reactions and the provision of nutrients, and can also benefit plant growth by increasing soil physical conditions and facilitating the aggregation of particles, in addition to reports on mitigation of environmental stresses, such as salinity and drought [80, 81]. In this study, although two strains of *Azospirillum* stood out for EPS production, *Pseudomonas* accounted for five out of nine strains with the greatest potential. *Pseudomonas* has been reported for its plenty production of EPS under water restriction, creating a microenvironment that promotes water maintenance, thereby protecting the microorganism and the associated plant roots against dehydration [82, 83].

EPS is part of the structural matrix of biofilms; however, in this study, there was no direct relationship between the production of EPS and biofilm formation, as strains of *Paraburkholderia* had the maximum production of biofilm *in vitro*. Among the 100 strains assessed for biofilm production (SD = 17%), *Paraburkholderia nodosa* CNPSo 1299 (O.D. 1.458), *P. nodosa* CNPSo 1204 (O.D. 1.404), *Bacillus velezensis* CNPSo 2384 (O.D. 1.249), and *Pseudomonas lurida* CNPSo 2218 (O.D. 1.166) stood out, showing the highest optical density values, highlighting superior capacity to form biofilm (Table 1). This can be explained not only by the capacity of EPS synthesis but also on factors such as the structure and physiology of the microbial cell and quorum-sensing mechanisms. Biofilm production can also change during the process of association between the microorganism and the host plant, when other interfering factors may occur, such as interactions with other microorganisms, root exudates, microbial competition, physicochemical characteristics, and soil organic matter content, among others [84].

A very low number of strains was able to solubilize calcium phosphate, five of each belonging to the genus *Pseudomonas* (CNPSo 2222, CNPSo 2604, CNPSo 2719, CNPSo 2878 and CNPSo 4140), in addition to *Pantoea*

agglomerans CNPSo 2602 and *Gluconacetobacter azotocaptans* CNPSo 2783 (Table 1 and Figure 1), corroborating findings of Sánchez López et al. [85]. These strains can help plants to deal with P-limiting conditions [86], commonly found in Brazilian soils [87]. The benefits and importance of PGPB in making P available are reinforced by the finite amount of phosphate rocks mines for P-fertilizer production, increasing production costs, and the global low levels of soil available P to support agricultural production [88, 89].

Only 11 strains grew at reduced water activity in the culture medium with an Aw of 0.919 and temperature of 40 ºC, and only five had satisfactory growth under even more restrictive conditions, with Aw of 0.897 (Table 1). These five strains are species of *Bacillus*, highlighting their adaptability to environments with restricted water activity and high temperatures. This resilience is evidenced by the ability of these Gram-positive bacteria to form endospores, which makes the genus more tolerant to life-threatening abiotic conditions such as extremes of temperature, pH, and radiation [90, 91]. This combination of physiological and genetic traits allows *Bacillus* strains not only to survive but also to play important roles in challenging environments, such as those affected by water restriction and higher temperatures, a scenario ever more frequent as a result of the global warming.

Studies have shown that plants colonized by *Bacillus* spp. exhibit enhanced water uptake, which plays a crucial role in protecting them from drought-induced damage [92]. Furthermore, under water restriction, the uptake of nutrients typically decreases. However, when treated with *Bacillus* spp., plants have higher nutrient use efficiency, even under drought conditions [93]. Importantly, our results provide new evidence that selected bacterial strains can play a major role in the agriculture of the future, when water constraints will be more frequent, especially the outstanding effect of *Bacillus*.

Phenotypic clustering

The dendrogram analysis, considering potential plant growth-promoting traits among the 100 strains assessed *in vitro*, identified five clusters at a similarity level of 70% (Figure 2). Cluster 3 had the highest number of isolates, 39, with genera broadly distributed, followed by clusters 5 (formed predominantly by

Pseudomonas) and 4, with 22 and 15 isolates, respectively. Cluster 1, comprising only *Bacillus*, included nine isolates.

The heatmap analysis confirmed distinct patterns among bacterial genera regarding the quantitative expression of *in vitro* traits associated with plant growth-promotion (Figure 3). Strains of the genus *Paraburkholderia* exhibited high capacity for biofilm formation, while *Azospirillum* stood out for EPS production. IAA synthesis was particularly high in a *Chromobacterium* strain. In contrast, *Pseudomonas* strains showed consistent performance across multiple tests, indicating a multifunctional profile, while *Bacillus* displayed high variability, with results depending on the strain.

Assessment of plant tolerance to drought under greenhouse conditions

First screening for tolerance to drought

In the first experiment, assessing 100 bacterial strains on the growth of maize in sterile substrate, inoculated plants showed superior performance in some parameters compared with control, indicating promising effects of certain strains on the physiological variables assessed in this study. It is worth commenting that seeds were inoculated with 1 mL of bacterial suspensions obtained from liquid cultures with different compositions. However, we found no differences in seed germination rates and vigor that could be attributed to differences in nutrient composition and, therefore, we consider that nutrients carried with the inoculant did not affect plant growth; furthermore, plants were always supplied with nutrient solution to prevent any nutritional limitation. For plants under water restriction, 11 strains stood out: *Achromobacter* sp. CNPSo 2660, *Azoarcus indigens* CNPSo 2541, *Bacillus aryabhatai* CNPSo 2603, *B. velezensis* CNPSo 2384, *Bacillus* sp. CNPSo 2725 and CNPSo 2383, *Bradyrhizobium pachyrizi* CNPSo 2259, *C. violaceum* CNPSo 1954, *Pantoea agglomerans* CNPSo 2602, *Pantoea* sp. CNPSo 2344 and CNPSo 2493. These strains significantly increased shoot fresh and dry weight, root volume, plant size, and chlorophyll concentration compared with the non-inoculated plants (Control I) (Table 2). Inoculation with 20 strains significantly increased chlorophyll concentration, with *Bacillus velezensis* CNPSo 2657 standing out by promoting a 27% increase compared with plants grown under well-watered conditions (Control II) (Table 2). The effect of two promising strains in promoting drought tolerance in maize is shown in Figure S4.

Very important, the inoculation of 42 strains resulted in shoot dry weight of plants under water restriction statistically similar to plants kept under well-watered conditions, belonging to the genera: *Bacillus* (9), *Paraburkholderia* (6), *Pseudomonas* (7), *Chromobacterium* (5), *Pantoea* (4), *Paenibacillus* (1), *Agrobacterium* (2), *Bradyrhizobium* (2), *Rhizobium* (2), *Achromobacter* (1), *Azoarcus* (1), *Azospirillum* (1), and *Gluconacetobacter* (1) (Table 2).

The average root volume ranged from 4.12 to 13.86 mL per plant, with the greatest value observed in plants inoculated with *B. velezensis* CNPSo 2384, which was statistically different from all the other strains, in addition to the non-inoculated plants grown under well-watered conditions (Control II) (Table 2).

For 21 strains, plant size under water restriction was similar to well-watered plants (Control II). These strains included *Chromobacterium violaceum* CNPSo 1954, CNPSo 1963 and CNPSo 1958; *Psudomonas lurida* CNPSo 2218, *Pseudomonas soli* CNPSo 1987 and CNPSo 2220; *Bacillus subtilis* CNPSo 2620, *B. velezensis* CNPSo 2657, *B. aryabhatai* CNPSo 2603, *Bacillus* sp. CNPSo 2725 and CNPSo 2383; *Azoarcus indigens* CNPSo 2541; *Gluconacetobacter azotocaptans* CNPSo 2783; *Pantoea agglomerans* CNPSo 2602, *Pantoea* sp. CNPSo 2344 and CNPSo 2493; *Bradyrhizobium pachyrhizi* CNPSo 2259; *Paenibacillus polymyxa* CNPSo 2227; *Achromobacter* sp. CNPSo 2660; *Paraburkholderia franconis* CNPSo 3157, and *P. nodosa* CNPSo 1204 (Table 2).

The Pearson's correlation coefficient analysis between the traits assessed *in vitro* and the results from the first experiment in greenhouse revealed positive correlations among the traits of the strains grown *in vitro* under reduced water activity (Aw 0.919 and 0.897), at 40 °C, and the ability to promote shoot fresh weight, shoot dry weight, and chlorophyll concentration of maize (Figure 4). These results can highly speed up strain selection programs towards the development of bio-inputs aiming at the new challenges of climate changes faced by agriculture. Therefore, our initial hypothesis was confirmed for drought tolerance, while the validation of other key traits, such as phosphate solubilization, remains to be addressed.

A slightly weaker relationship was found between traits of the strains grown under reduced water activity (Aw 0.919 and 0.897), at 40°C, and biofilm production in the *in vitro* analyses (Figure 4). Although other plant growth-promoting properties, such as EPS and biofilm production, have been reported

in mitigating water stress [94], we did not observe a correlation with the *in vivo* plant performance in our study. For both root volume and chlorophyll concentration, plants inoculated with *Bacillus* spp. reached the highest averages, even higher than those of plants that were not subjected to water deficit (Table 2). The potential of *Bacillus* spp. in increasing chlorophyll concentration was also verified in cotton plants (*Gossypium hirsutum* L.) by Diaz [95]. Higher chlorophyll concentrations make plants more effective to convert light energy and accumulate biomass under stress or physiological disorders, relieving the negative effects [59].

For selection of the most promising strains in Experiment I, a principal component analysis was applied to check correlations among the variables (Figure 5A) in a factorial plan (Figure 5B). Shoot fresh and dry weights, and plant size, were related to each other, as represented by PC1, which explains 56.65% of the total variance. Considering the information gathered by PC1, the previously highlighted variables show a positive correlation; on the other hand, root volume and chlorophyll concentration were weakly related each other variables, and were best represented by the PC2.

According to the factorial plan representing the treatments, there was a great variation in the response among different strains. As an example, plants inoculated with *B. pachyrhizi* CNPSo 2259, *A. indigens* CNPSo 2541, and *P. agglomerans* CNPSo 2602 (ID 39, 44, and 45, respectively) responded differently from those inoculated with *Delftia* sp. CNPSo 3288, *Agrobacterium pusenese* CNPSo 3315, and *Agrobacterium* sp. CNPSo 4045 (ID 83, 85 and 98, respectively).

Fifteen bacterial strains that promoted more vigorous plants grown under water restriction and that were positive in most *in vitro* assessments were selected for a second greenhouse trial.

Assessment of the most promising strains in non-sterile soil

In the second experiment with 15 selected strains, plant photosynthetic rates varied over time and among strains. A general decrease in CO_2 assimilation rate was observed on the fifth day of stress, followed by partial or complete recovery by the ninth day. Control I, with plenty water supply, showed the highest values at all assessing times, while Control II, under drought stress, exhibited a marked

decrease in CO_2 assimilation, especially on the fifth and ninth days. Among the strains, *B. velezensis* CNPSo 2384 stood out for maintaining a high maize photosynthetic rate throughout the experimental period (Table 3).

Among the plants under water restriction, some strains showed potential to mitigate the stressful condition, maintaining a high photosynthetic rate, such as *C. violaceum* CNPSo 1954 and *B. velezensis* CNPSo 2384, which presented, respectively, photosynthetic rates 26 and 51% higher than the non-inoculated plants (Control I). Plants under water restriction and inoculated with *B. velezensis* CNPSo 2384 showed a reduction of only 5% in photosynthetic rate in the last evaluation compared with those irrigated throughout the whole period (Control II). Similar trends were observed for stomatal conductance and transpiration rates (Table 3), which are closely linked to photosynthetic activity, indicating a consistent physiological response among these parameters.

Shoot fresh weight ranged from 122.5 to 203.3 g per plant. The lowest value was observed in non-inoculated plants under water restriction, while the highest was recorded in plants that received plenty of water supply throughout the experiment. Inoculated plants showed superior performance compared with non-inoculated ones. For shoot dry weight, the highest average was found for well-watered plants (26.14 g), while plants under water restriction but inoculated with the *Bacillus* sp. CNPSo 2658 had the highest average (19.97 g). For plant size, the average of well-watered plants also stood out from the other treatments, reaching 1.35 m. Among the treatments under water restriction, plants inoculated with *C. violaceum* CNPSo 1954 and *G. azotocaptans* CNPSo 2783 reached 1.27 m (Table 4).

The root volume of plants inoculated with the strains of *C. violaceum* CNPSo 1954, *Pseudomonas soli* CNPSo 1987, *Pantoea agglomerans* CNPSo 2602, *Bacillus subtilis* CNPSo 2605, and *B. subtilis* CNPSo 2606 were similar to those of well-watered plants, while no differences were found for root dry weight (Table 4). The average root length ranged from 261.4 to 491.8 m per plant, with the highest value in plants inoculated with *C. violaceum* CNPSo 1954. In addition, the length of roots of plants inoculated with *Pseudomonas soli* CNPSo 1987 and *Pantoea agglomerans* CNPSo 2602 were also significantly increased (Table 4). It is worth mentioning the importance of the contact between the root system and the soil, enhancing the uptake of water and nutrients [49, 96]. We found that

plants inoculated with three strains, *C. violaceum* CNPSo 1954, *Pseudomonas soli* CNPSo 1987, and *Pantoea agglomerans* CNPSo 2602 stood out in volume, mass, and length of roots. Under field conditions, this ability could have a significant impact on drought tolerance.

Water constraints in a warmer climate concern humanity and agriculture [97]. Furthermore, global patterns of increasing crop yield [98] also enhance the demand for water. In addition, higher temperatures with more frequent hotter days, more intense radiation, and land cover/land use changes [98] may intensify the impacts of water restriction [99]. The survival capacity of plants will depend on their adaptation ability but also on the severity and duration of the restrictive period [100]. In this scenario, inoculation with PGPB should be investigated, as it can help mitigate plant damages. Indeed, it has been recently shown that strains used in our study were able to mitigate the negative impacts of a 2 °C warming on the photosynthesis, growth, and nutritional value of a tropical C4 grassland under field conditions [101].

Considering all the data analyzed, we highlight the outstanding effect of three strains, *B. velezensis* CNPSo 2384, *B. subtilis* CNPSo 2606, and *Bacillus* sp. CNPSo 2723, with high potential to be used as new bio-inputs (Figure S5). In addition, the multifunctional traits of these three selected strains confirm that *Bacillus* may have multiple uses [102, 103, 104, 30], which highly benefits agricultural sustainability.

According to Radhakrishnan et al. [92], during water restriction and the consequent increase in the concentration of toxic salts and metals in the soil solution, *Bacillus* spp. can produce EPS and siderophores. This ability can also regulate other microbial populations in the soil, including pathogens. Regarding the imbalance resulting from water restriction, the synthesis of IAA and ACC-deaminase regulates the intracellular metabolism of phytohormones, increasing the plant stress tolerance. Sivasakthi et al. [105] pointed out that *Pseudomonas* and *Bacillus* have more abilities to survive in a wide range of stressful environments than other PGPB – whether through phosphate solubilization, the production of siderophores, or the biocontrol of plant pathogens. In this study, the production of siderophores and EPS *in vitro* was mostly found in *Pseudomonas* spp. Besides the production of metabolites, the ability of *Bacillus* to form endospores may increase the cell viability under adverse conditions, even in

formulations of commercial products [106, 107]. Despite the promising results obtained under controlled conditions, further studies are needed to evaluate the behavior of selected strains under field conditions, where multiple biotic and abiotic factors affect microbial dynamics. Adaptation to the local ecosystem is essential for introduced microorganisms to establish themselves, interact with native microbiota, and promote tangible benefits to plants. According to Li et al. [108], land use plays a decisive role in shaping soil microbial communities, influencing their genomic and functional traits. These environmental factors can directly influence the success of inoculation and the effect of selected strains. Consequently, field trials are crucial for evaluating microbial inoculants under real agricultural conditions. The next step will involve verifying the agronomic potential of these strains and their consistency across diverse environments.

Conclusions

- *In vitro* assessments may accelerate the bioprospection of elite strains for bio-inputs based on high correlation between growth *in vitro* in culture medium with reduced water activity and the capacity to increase maize tolerance to water restriction;
- Searching for elite strains of agronomic interest in microbial culture collections may be facilitated by starting from specific genera, such as *Bacillus* for drought tolerance and *Pseudomonas* for phosphate solubilization.
- Microbial culture collections represent a valuable reservoir of biotechnological solutions for agricultural sustainability. Among them, plant growth-promoting bacteria stand out, offering a sustainable alternative to mitigate the impacts of abiotic and biotic stresses on plants. Many strains exhibit multiple traits of agronomic interest, and their use in agriculture should be encouraged

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Table 1 *In vitro* evaluation of protease, cellulase, growth under reduced water activity, 1-aminociclopropano-1-carboxilato deaminase(ACC)-deaminase, phosphate solubilization, biofilm and EPS production, IAA (indole-3-acetic acid) and siderophore synthesis of 100 bacterial strains of the Culture Collection of Diazotrophic and Plant Growth-Promoting Bacteria of Embrapa Soja.

Strain ID	Protease	Cellulase	Water activity		ACC deaminase	Phosphate solubilization	Biofilm (O.D)	EPS (μg mL^{-1})	IAA (μg mL^{-1})	Siderophore (Halo/colony)	
			0.919	0.897						24h	72h
***Achromobacter* sp. CNPSo 2660**					+		0.122d [a]	0.00i [a]	29.97g [a]		
***Agrobacterium deltaense*. CNPSo 2707**	+						0.337d	25.13g	88.14d	0.00i [a]	1.45i [a]
***Agrobacterium fabacearum* CNPSo 675**							0.489c	47.86e	83.01d		
***Agrobacterium pusenese* CNPSo 3315**					+		0.428d	43.0e	86.97d	1.39g	2.07g
***Agrobacterium pusense* CNPSo 3348**							0.221d	56.85d	73.87e		
***Agrobacterium* sp. CNPSo 1235**					+		0.486c	66.40d	86.83d		
***Agrobacterium* sp. CNPSo 1668**					+		0.714c	15.37h	63.22e		
***Agrobacterium* sp. CNPSo 4041**					+		0.596c	71.31c	63.40e	0.00i	1.73h
***Agrobacterium* sp. CNPSo 4045**							0.35d	74.04c	92.91d		
***Azoarcus indigens* CNPSo 2541**	+						0.515c	29.38f	15.95h	0.00i	1.73h
***Azorhizobium caulinodans* CNPSo 139**							0.297d	3.52i	29.16g		
***Azospirillum brasilense* CNPSo 2083**	+				+		0.053d	10.66h	48.53f		
***Azospirillum brasilense* CNPSo 2084**	+				+		0.078d	129.25a	30.25g		
***Azospirillum halopraeferens* CNPSo 3601**							0.049d	9.89h	23.01h		
***Azospirillum rugosum* CNPSo 3757**	+						0.173d	131.38a	14.68h		
***Azospirillum thiophilum* CNPSo 2786**							0.314d	11.93h	77.16d	2.04c	3.37d
***Bacillus aryabhatai* CNPSo 2603**			+		+		0.070d	44.30e	83.64d		
***Bacillus* sp. CNPSo 2383**							0.742c	0.68i	7.86h		

***Bacillus* sp. CNPSo 2658**		+	+			0.088d	43.93e	20.86h		
***Bacillus* sp. CNPSo 2723**	+	+	+			0.273d	41.70e	47.17f		
***Bacillus* sp. CNPSo 2725**	+	+	+			0,442d	42.16e	6.22e		
***Bacillus* sp. CNPSo 3218**	+	+	+	+		0.206d	44.77e	89.31d		
***Bacillus subtilis* CNPSo 2605**		+	+	+		0.334d	45.32e	85.47d		
***Bacillus subtilis* CNPSo 2606**	+	+	+	+		0.940b	43.30e	99.35d		
***Bacillus subtilis*. CNPSo 2620**	+	+	+	+	+	0.999b	49.19e	51.47f	1.56f	1.54h
***Bacillus velezensis* CNPSo 2384**		+	+	+	+	1.249a	2.08i	7.78h		
***Bacillus velezensis* CNPSo 2657**	+	+	+		+	0.147d	45.95e	5.76f	0.00i	4.07b
***Bradyrhizobium frederickii* CNPSo 3443**					+	0.360d	10.91h	2.41h	1.42g	1.66h
***Bradyrhizobium diazoefficiens* CNPSo 6**						0.144d	6.29i	2.00h		
***Bradyrhizobium elkanii* CNPSo 14**						0.042d	0.00i	8.74h		
***Bradyrhizobium elkanii* CNPSo 9**						0.238d	6.40i	3.09h		
***Bradyrhizobium frederickii* CNPSo 3426**					+	0.066d	13.10h	4.45h		
***Bradyrhizobium japonicum* CNPSo 7**						0.445d	0.00i	4.60h		
***Bradyrhizobium pachyrhizi* CNPSo 2259**					+	0.176d	4.11i	3.27h		
***Bradyrhizobium* sp. CNPSo 2907**	+				+	0.210d	11.86h	15.81h		
***Bradyrhizobium yuanmingense* CNPSo 3084**	+					0.075d	1.13i	1.54h	1.27h	1.31i
***Chromobacterium violaceum* CNPSO 1947**	+					0.083d	30.14f	17.25h	1.75e	2.39f
***Chromobacterium violaceum* CNPSo 1950**					+	0.139d	29.78f	22.97h	1.92d	3.27d
***Chromobacterium violaceum* CNPSo 1952**					+	0.063d	92.34b	22.97h		
***Chromobacterium violaceum* CNPSo 1954**						0.012d	37.85f	206.58a	0.00i	2.89e
***Chromobacterium violaceum* CNPSo 1958**					+	0.053d	25.69g	11.49h		
***Chromobacterium violaceum* CNPSo 1963**						0.092d	28.80f	21.33h		

***Delftia* sp. CNPSo 3288**				+		0.086d	10.92h	20.37h	1.25h	1.39i	
***Ensifer (=Sinorhizobium) mexicanus* CNPSo 2067**				+		0.116d	10.57h	44.68f			
***Enterobacter* sp. CNPSo 3867**						0.195d	35.95f	7.63h			
***Gluconacetobacter azotocaptans* CNPSo 2783**	+				+	0.006d	22.50g	9.88h			
***Methylobacterium* sp. CNPSo 989**		+		+		0.044d	1.72i	3.00h			
***Microbacterium* sp. CNPSo 3287**	+					0.051d	6.91i	40.26f			
***Microbacterium* sp. CNPSo 3855**						0.330d	30.60f	12.53h			
***Neorhizobium* (=*Rhizobium*) *huautlense* CNPSo 206**						0.066d	34.30f	77.70d			
***Neorhizobium galegae* CNPSo 2061**	+			+		0.223d	11.74h	50.42f			
***Niveispirillum irakense* CNPSo 3756**						0.579c	10.65h	52.15f			
***Ochrobactrum oryzae* CNPSo 2784**	+					0.352d	0.00i	56.79e			
***Paenibacillus polymyxa* CNPSo 2227**		+		+		0.533c	17.36h	43.20f			
***Paenibacillus* sp. CNPSo 3221**		+				0.418d	16.08h	49.81f			
***Paenibacillus* sp. CNPSo 3309**		+		+		0.060d	4.70i	79.48d	0.00i	1,21i	
***Paenibacillus* sp. CNPSo 3854**	+	+				0.265d	8.80h	28.83g			
***Pantoea agglomerans* CNPSo 2602**	+				+	0.129d	52.23e	19.05h			
***Pantoea ananatis* CNPSo 2797**		+		+		0.357d	7.54h	81.48d			
***Pantoea ananatis* CNPSo 2798**		+		+		0.568c	77.25c	114.77c			
***Pantoea ananatis* CNPSo 3282**	+	+				0.208d	19.52g	90.71d			
***Pantoea* sp. CNPSo 2344**						0.049d	12.15h	18.44h			
***Pantoea* sp. CNPSo 2493**	+		+	+		0.236d	58.11d	117.04c			
***Paraburkholderia atlantica* CNPSo 3155**		+				0.262d	10.16h	7.63h			
***Paraburkholderia franconis* CNPSo 3157**						0.142d	13.04h	7.51h			
***Paraburkholderia guartelaensis* CNPSo 2995**						0.620c	0.00i	2.17h			

***Paraburkholderia guartelaensis* CNPSo 3008**	+		+		0.548c	0.00i	2.45h	1.26h	2.14g
***Paraburkholderia nodosa* CNPSo 1204**					1.404a	19.76g	6.63h		
***Paraburkholderia nodosa* CNPSo 1213**					0.565c	3.17i	3.46h		
***Paraburkholderia nodosa* CNPSo 1294**					0.389d	10.40h	6.87h		
***Paraburkholderia nodosa* CNPSo 1299**					1.458a	0.00i	30.29g		
***Paraburkholderia nodosa* CNPSo 1301**					0.489c	0.74i	4.77h		
***Paraburkholderia nodosa* CNPSo 1307**					0.962b	3.21i	59.78e		
***Paraburkholderia sabiae* CNPSo 3136**	+		+		0.220d	11.67h	87.84d	0.00i	1.44i
***Paracocus* sp. CNPSo 3707**					0.042d	45.23e	97.18d		
***Pseudomonas fluorescens* CNPSo 2224**			+		0.547c	91.11b	58.95e	1.21h	1.32i
***Pseudomonas fluorescens* CNPSo 2799**			+		0.188d	62.55d	37.17g	1.39g	1.71h
***Pseudomonas lurida* CNPSo 2218**			+		1.166a	50.51e	75.90e	1.33g	1.91g
***Pseudomonas fluorescens* CNPSo 2719**	+			+	0.677c	47.44e	31.73g	1.79e	3.27d
***Pseudomonas soli* CNPSo 1987**	+				0.159d	45.61e	70.37e	1.25h	1.47i
***Pseudomonas* sp. CNPSo 2220**			+		0.919b	47.80e	140.27b	1.33g	1.96g
***Pseudomonas* sp. CNPSo 2222**				+	0.568c	41.36e	124.36c		
***Pseudomonas* sp. CNPSo 2604**	+			+	0.288d	62.88d	47.48f		
***Pseudomonas* sp. CNPSo 2625**	+				0.635c	52.16e	28.12g	2.62a	3.57c
***Pseudomonas* sp. CNPSo 2835**					0.252d	51.50e	44.88f	1.55f	2.92e
***Pseudomonas* sp. CNPSo 2844**	+				0.328d	78.26c	46.32f		
***Pseudomonas* sp. CNPSo 2851**	+				0.509c	88.92b	70.81e		
***Pseudomonas* sp. CNPSo 2856**					0.167d	87.60b	46.37f		
***Pseudomonas* sp. CNPSo 2864**	+				0.134d	94.05b	84.45d	1.29h	1.37i
***Pseudomonas* sp. CNPSo 2878**	+			+	0.827c	78.79c	66.12e	2.40b	3.55c

***Pseudomonas* sp. CNPSo 2887**					102.06b	47.49f		
***Pseudomonas* sp. CNPSo 4132**	+	+		0.429d	50.23e	71.22e	1.38g	1.33i
***Pseudomonas* sp. CNPSo 4140**		+	+	0.531c	31.23f	55.85e	1.42g	1.26i
***Rhizobium giardinii* CNPSo 171**		+		0.111d	10.74h	6.74h	2.69a	4.34a
***Rhizobium leucaenae* CNPSo 224**				0.057d	14.73h	63.83e		
***Rhizobium leucaenae* CNPSo 229**		+		0.299d	15.94h	18.84h		
***Rhizobium* sp. CNPSo 1627**				0.814c	96.94b	14.09h		
***Rhizobium* sp. CNPSo 3610**				0.098d	21.16g	39.55f	1.32g	1.73h
***Rhizobium tropici* CNPSo 1018**		+		0.492c	57.76d	106.54d		
***Rhizobium tropici* CNPSo 103**				0.152d	84.43c	63.87e		

Values represent the mean of three biological replicates and when followed by the same letter are not statistically different according to the Scott-Knott test (p<0.05).

Table 2 Growth parameters[a] of maize hybrid Pioneer 30F53 PRO 3 inoculated with 100 bacterial strains of the Culture Collection of Diazotrophic and Plant Growth-Promoting Bacteria of Embrapa Soja and grown in sterile substrate under water restriction.

Treatment	SFW (g pl^{-1})	SDW (g pl^{-1})	RV (mL pl^{-1})	PS (cm pl^{-1})	CC (μg cm^{-2})
Control I (non-inoculated with water restriction)	4.17d	0.720b	5.31e	33.5c	4.80d
Control II (non-inoculated without water restriction)	8.20a	1.223a	11.92b	40.8a	8.14b
Achromobacter sp. CNPSo 2660	6.08b	0.763a	5.53e	41.0a	8.10b
Agrobacterium sp. CNPSo 1235	4.89c	0.773a	5.82e	36.2c	7.67b
Agrobacterium sp. CNPSo 1668	4.93c	0.703b	6.33e	38.0b	8.68a
Agrobacterium sp. CNPSo 2707	5.64c	0.773a	6.66d	37.8b	8.95a
Agrobacterium pusenese CNPSo 3315	2.39e	0.473b	5.95e	32.7c	4.05d
Agrobacterium sp. CNPSo 3348	3.18e	0.607b	6.64d	35.0c	5.69d
Agrobacterium sp. CNPSo 4041	4.53d	0.690b	6.25e	37.3c	7.55b
Agrobacterium sp. CNPSo 4045	3.32e	0.497b	6.32e	33.3c	5.20d
Agrobacterium sp. CNPSo 675	4.04d	0.640b	5.84e	34.3c	8.52a
Azoarcus indigens CNPSo 2541	7.02b	1.037a	7.47d	44.0a	7.16b
Azorhizobium caulinodans CNPSo 139	2.76e	0.457b	5.45e	33.7c	8.58a
Azospirillum brasilense CNPSo 2083=Ab-V5	2.91e	0.403b	10.04c	32.0c	6.04c
Azospirillum brasilense CNPSo 2084=Ab-V6	3.74d	0.497b	9.85c	37.7b	5.60d
Azospirillum halopraeferens CNPSo 3601	3.17e	0.597b	7.43d	36.0c	6.43c
Azospirillum rugosum CNPSo3757	3.76d	0.633b	6.04e	33.3c	8.11b
Azospirillum thiophilum CNPSo 2786	5.17c	0.767a	9.40c	39.2b	4.43d
Bacillus velezensis CNPSo 2384	6.40b	0.913a	13.86a	38.7b	7.13b
Bacillus aryabhatai CNPSo 2603	6.11b	0.907a	11.74b	42.8a	9.71a
Bacillus sp. CNPSo 2383	6.47b	0.853a	10.70b	42.3a	7.75b
Bacillus sp. CNPSo 2658	5.76c	0.770a	5.95e	39.8b	9.19a
Bacillus sp. CNPSo 2723	5.32c	0.673b	6.97d	38.5b	7.20b
Bacillus sp. CNPSo 2725	6.24b	0.870a	7.64d	42.0a	7.32b
Bacillus sp. CNPSo 3218	5.07c	0.790a	9.11c	40.0b	6.76c
Bacillus subtilis CNPSo 2605	5.14c	0.873a	5.88e	40.5b	8.39a
Bacillus subtilis CNPSo 2606	5.55c	0.897a	5.54e	39.7b	7.44b
Bacillus subtilis CNPSo 2620	5.64c	0.903a	7.54d	44.3a	9.26a
Bacillus velezensis CNPSo 2657	5.36c	0.707b	6.87d	41.7a	10.38a
Bradyrhizobium diazoefficiens CNPSo 6	3.04e	0.600b	8.76c	35.5c	5.76d
Bradyrhizobium elkanii CNPSo 14	3.15e	0.533b	4.68e	36.8c	5.72d

Bradyrhizobium elkanii CNPSo 9	2.56e	0.467b	6.99d	32.8c	6.43c
Bradyrhizobium frederickii CNPSo 3426	3.17e	0.603b	6.44e	34.2c	5.57d
Bradyrhizobium frederickii CNPSo 3443	3.39e	0.653b	7.05d	37.2c	7.93b
Bradyrhizobium japonicum CNPSo 7	3.30e	0.563b	8.41c	34.0c	7.29b
Bradyrhizobium pachyrhizi CNPSo 2259	6.57b	0.883a	9.41c	41.8a	8.79a
Bradyrhizobium sp. CNPSo 2907	3.80d	0.567b	8.77c	34.7c	4.94d
Bradyrhizobium yuanmingense CNPSo 3084	4.95c	0.753a	8.66c	37.3c	6.20c
Chromobacterium violaceum CNPSo 1947	5.19c	0.753a	5.91e	39.7b	7.65b
Chromobacterium violaceum CNPSo 1950	5.42c	0.797a	6.24e	39.7b	7.74b
Chromobacterium violaceum CNPSo 1952	4.97c	0.727a	6.00e	39.3b	6.14c
Chromobacterium violaceum CNPSo 1954	5.96b	0.953a	5.97e	45.0a	9.29a
Chromobacterium violaceum CNPSo 1958	4.28d	0.653b	6.50e	41.7a	6.67c
Chromobacterium violaceum CNPSo 1963	4.67c	0.787a	6.53e	42.5a	6.50c
Delftia sp. CNPSo 3288	2.64e	0.510b	6.11e	32.2c	6.62c
Ensifer (=Sinorhizobium) mexicanus CNPSo 2067	3.99d	0.543b	6.48e	34.7c	6.39c
Enterobacter sp. CNPSo 3867	3.86d	0.613b	5.68e	35.0c	8.77a
Gluconacetobacter azotocaptans CNPSo 2783	5.49c	0.767a	6.98d	42.2a	6.95c
Methylobacterium sp. CNPSo 989	3.81d	0.570b	6.45e	36.2c	7.51b
Microbacterium sp. CNPSo 3287	2.50e	0.513b	6.20e	32.0c	6.58c
Microbacterium sp. CNPSo 3855	4.10d	0.690b	6.84d	36.7c	7.65b
Neorhizobium (=*Rhizobium*) *huautlense* CNPSo 206	2.86e	0.513b	5.30e	33.7c	7.72b
Neorhizobium galegae CNPSo 2061	4.11d	0.683b	6.71d	37.0c	5.69d
Niveispirillum irakense CNPSo 3756	3.46e	0.590b	6.06e	35.5c	7.51b
Ochrobactrum oryzae CNPSo 2784	3.94d	0.550b	6.14e	38.8b	4.71d
Paenibacillus polymyxa CNPSo 2227	5.54c	0.657b	10.64b	41.8a	6.62c
Paenibacillus sp. CNPSo 3221	5.09c	0.847a	8.89c	40.0b	7.93b
Paenibacillus sp. CNPSo 3309	2.73e	0.520b	7.41d	32.0c	4.61d
Paenibacillus sp. CNPSo 3854	3.78d	0.657b	5.91e	35.7c	5.24d
Pantoea agglomerans CNPSo 2602	6.10b	0.993a	9.21c	42.0a	8.81a
Pantoea ananatis CNPSo 2797	5.17c	0.717b	10.02c	39.5b	5.04d
Pantoea ananatis CNPSo 2798	3.26e	0.530b	10.81b	34.3c	4.80d
Pantoea sp. CNPSo 2344	6.63b	0.903a	10.19c	41.2a	8.00b
Pantoea sp. CNPSo 2493	6.41b	0.970a	9.39c	41.0a	7.39b
Pantoea sp. CNPSo 3282	5.03c	0.817a	10.50b	38.3b	6.64c
Paraburkholderia atlantica CNPSo 3155	5.49c	0.867a	10.19c	38.8b	8.48a
Paraburkholderia franconis CNPSo 3157	5.67c	0.920a	8.53c	40.8a	7.23b
Paraburkholderia guartelaensis CNPSo 2995	3.83d	0.670b	7.77d	32.7c	6.66c
Paraburkholderia guartelaensis CNPSo 3008	3.66d	0.610b	9.56c	31.8c	5.78d

Paraburkholderia nodosa CNPS0 1307	4.96c	0.730a	7.03d	39.2b	6.74c
Paraburkholderia nodosa CNPSo 1204	5.51c	0.847a	6.68d	40.8a	8.58a
Paraburkholderia nodosa CNPSo 1213	4.10d	0.570a	6.44e	36.3c	6.76c
Paraburkholderia nodosa CNPSo 1294	3.67d	0.553b	6.52e	35.2c	7.37b
Paraburkholderia nodosa CNPso 1299	4.43d	0.640b	6.30e	39.0b	6.29c
Paraburkholderia nodosa CNPSo 1301	4.41d	0.670b	6.79d	39.2b	9.49a
Paraburkholderia sabiae CNPSo 3136	5.45c	0.830a	10.35b	37.8b	7.90b
Paracocus sp. CNPSo 3707	3.69d	0.600b	6.38e	37.3c	8.86a
Pseudomonas fluorescens CNPSo 2224	4.92c	0.710b	11.29b	38.3b	6.18c
Pseudomonas fluorescens CNPSo 2799	3.17e	0.507b	10.43b	34.7c	6.36c
Pseudomonas lurida CNPSo 2218	4.68c	0.763a	7.36d	44.7a	8.58a
Pseudomonas soli CNPSo 1987	5.31c	0.903a	6.30e	44.0a	7.65b
Pseudomonas sp. CNPSo 2220	4.65c	0.827a	10.50b	41.7a	7.67b
Pseudomonas sp. CNPSo 2222	4.02d	0.610b	10.05c	38.0b	6.89c
Pseudomonas sp. CNPSo 2604	4.91c	0.773a	6.25e	37.0c	8.04b
Pseudomonas sp. CNPSo 2625	4.92c	0.757a	6.79d	40.3b	9.61a
Pseudomonas fluorescens CNPSo 2719	5.29c	0.683b	6.56d	37.8b	7.03c
Pseudomonas sp. CNPSo 2835	4.06d	0.677b	8.15d	39.3b	6.80c
Pseudomonas sp. CNPSo 2844	3.55d	0.623b	8.59c	37.7b	5.90c
Pseudomonas sp. CNPSo 2851	4.32d	0.697b	9.58c	38.2b	5.50d
Pseudomonas sp. CNPSo 2856	3.90d	0.573b	9.98c	35.7c	5.48d
Pseudomonas sp. CNPSo 2864	4.12d	0.617b	7.59d	36.7c	6.98c
Pseudomonas sp. CNPSo 2878	4.18d	0.733a	8.09d	35.2c	6.92c
Pseudomonas sp. CNPSo 2887	4.23d	0.680b	9.43c	33.7c	6.64c
Pseudomonas sp. CNPSo 4132	3.69d	0.557b	5.75e	36.5c	8.84a
Pseudomonas sp. CNPSo 4140	4.88c	0.803a	5.64e	40.3b	8.28b
Rhizobium giardinii CNPSo 171	2.73e	0.530b	4.12e	34.3c	7.34b
Rhizobium leucaenae CNPSo 224	3.87d	0.710b	6.59d	34.8c	7.95b
Rhizobium leucaenae CNPSo 229	3.88d	0.630b	6.75d	35.3c	6.51c
Rhizobium sp. CNPSo 1627	4.59c	0.830a	6.01e	38.0b	7.16b
Rhizobium sp. CNPSo 3610	3.16e	0.543b	5.91e	33.2c	6.32c
Rhizobium tropici CNPSo 1018	5.01c	0.837a	7.37d	37.7b	7.34b
Rhizobium tropici CNPSo 103	3.92d	0.690b	5.31e	38.3b	7.44b

Shoot Fresh Weight (SFW); Shoot Dry Weight (SDW); Root Volume (RV); Plant Size (PS); and Chlorophyll Concentration (CC) of maize plants inoculated with PGPB strains and exposed to water restriction.

Values represent the mean of three replicates and when followed by the same letter are not statistically different according to the Scott-Knott test ($p \leq 0.05$).

Table 3 Photosynthetic rate, stomatal conductance and **t**ranspiration of maize plants inoculated with the 15 selected bacterial strains grown in non-sterile soil for 3, 5, and 9 days under water restriction.

Treatment	Photosynthetic rate (μmol CO_2 m^{-2} s^{-1})			Stomatal conductance (mol H_2O m^{-2} s^{-1})			Transpiration rate (mol H_2O m^{-2} s^{-1})		
	3 days	**5 days**	**9 days**	**3 days**	**5 days**	**9 days**	**3 days**	**5 days**	**9 days**
Control I (non-inoculated under water restriction)	35.38a	9.37d	22.79b	0.17a	0.05c	0.08c	3.52a	0.99c	1.79d
Control II (non-inoculated without water restriction)	33.38a	41.35a	32.37a	0.21a	0.25a	0.23a	4.01a	4.92a	4.64a
Bacillus aryabhatai CNPSo 2603	29.61a	16.27c	23.74b	0.14b	0.10b	0.10c	3.06b	1.59c	2.26d
Bacillus subtilis CNPSo 2605	29.93a	19.83c	25.08b	0.14a	0.06b	0.10c	2.94b	1.81c	2.21d
Bacillus subtilis CNPSo 2606	24.15b	19.80c	24.96b	0.11b	0.05c	0.09c	2.56b	1.47c	2.32d
Bacillus subtilis CNPSo 2620	31.60a	22.76c	34.24a	0.16a	0.13b	0.14c	3.83a	3.25b	1.92d
Bacillus velezensis CNPSo 2384	32.15a	31.96b	34.60a	0.17a	0.05c	0.16b	3.49a	0.72c	3.71b
Bacillus velezensis CNPSo 2657	30.80a	25.69c	27.37b	0.12b	0.05c	0.11c	3.11b	1.38c	2.61c
Bacillus sp. CNPSo 2658	32.32a	22.49c	30.05a	0.10b	0.07c	0.12c	2.24b	1.89c	2.73c
Bacillus sp. CNPSo 2723	22.86b	19.46c	32.38a	0.11b	0.06c	0.14c	2.47b	1.34c	2.61c
Bacillus sp. CNPSo 2725	29.22a	21.77c	33.53a	0.15a	0.07c	0.18b	2.86b	2.07c	2.94c
Chromobacterium violaceum CNPSo 1954	31.68a	30.43b	28.83b	0.16a	0.10b	0.12c	3.56a	2.86b	2.93c
Gluconacetobacter azotocaptans CNPSo 2783	20.28b	19.36c	25.69b	0.10b	0.06c	0.10c	2.34b	1.56c	2.51c
Pantoea agglomerans CNPSo 2602	34.37a	13.73d	26.97b	0.18a	0.06c	0.13c	3.81a	1.11c	2.29d
Pantoea sp. CNPSo 3282	31.99a	9.05d	29.17b	0.15a	0.05c	0.12c	3.26a	0.91c	2.66c
Paraburkholderia franconis CNPSo 3157	32.59a	19.20c	32.23a	0.17a	0.06c	0.16b	3.73a	1.61c	2.58c
Pseudomonas soli CNPSo 1987	27.84a	19.01c	30.04a	0.12b	0.06c	0.12c	2.55b	1.39c	2.51c

Values represent the mean of five replicates and when followed by the same letter are not statistically different according to the Scott-Knott test ($p \leq 0.05$).

Table 4 Growth parameters[a] of maize hybrid ATL100 inoculated with 15 selected bacterial strains and grown in non-sterile soil under water restriction.

Treatment	**SFW** (g)	**SDW** (g)	**PS** (m)	**RV** (mL)	**RDW** (g)	**RL** (m)
Control I (non-inoculated under water restriction)	122.5c	18.10c	1.22b	65.66a	6.32a	353.2b
Control II (non-inoculated without water restriction)	203.3a	26.14a	1.35a	72.05a	7.30a	356.2b
Bacillus aryabhatai CNPSo 2603	145.2b	18.06c	1.23b	55.86b	5.98a	320.9b
Bacillus subtilis CNPSo 2605	132.6c	17.12c	1.22b	66.58a	6.10a	347.7b
Bacillus subtilis CNPSo 2606	146.8b	19.54b	1.26b	63.43a	6.96a	376.6b
Bacillus subtilis CNPSo 2620	145.1b	18.32c	1.24b	52.58b	6.22a	322.7b
Bacillus velezensis CNPSo 2384	142.2b	19.28b	1.25b	59.17b	5.93a	350.4b
Bacillus velezensis CNPSo 2657	137.3c	17.13c	1.20b	56.79b	6.47a	325.9b
Bacillus sp. CNPSo 2658	144.9b	19.97b	1.24b	61.13b	6.50a	344.3b
Bacillus sp. CNPSo 2723	145.3b	18.60b	1.25b	61.36b	6.38a	291.7b
Bacillus sp. CNPSo 2725	138.1c	17.83c	1.21b	55.28b	6.50a	261.4b
Chromobacterium violaceum CNPSo 1954	132.4c	19.01b	1.27b	67.05a	6.78a	491.8a
Gluconacetobacter azotocaptans CNPSo 2783	148.4b	18.87b	1.27b	60.50b	7.23a	384.7b
Pantoea agglomerans CNPSo 2602	142.6b	18.26c	1.26b	69.87a	6.55a	485.1a
Pantoea sp. CNPSo 3282	139.3b	19.42b	1.25b	56.75b	6.39a	310.5b
Paraburkholderia franconis CNPSo 3157	133.3c	16.52c	1.22b	62.08b	6.82a	349.8b
Pseudomonas soli CNPSo 1987	143.4b	19.22b	1.26b	69.41a	6.52a	427.3a

Shoot Fresh Weight (SFW); Shoot Dry Weight (SDW); Plant Size (PS); Root Volume (RV); Root Dry Weight (RDW); and Root Length (RL) of maize plants inoculated with PGPB strains and exposed to water restriction.

Values represent the mean of five replicates and when followed by the same letter are not statistically different according to the Scott-Knott test ($p \leq 0.05$).

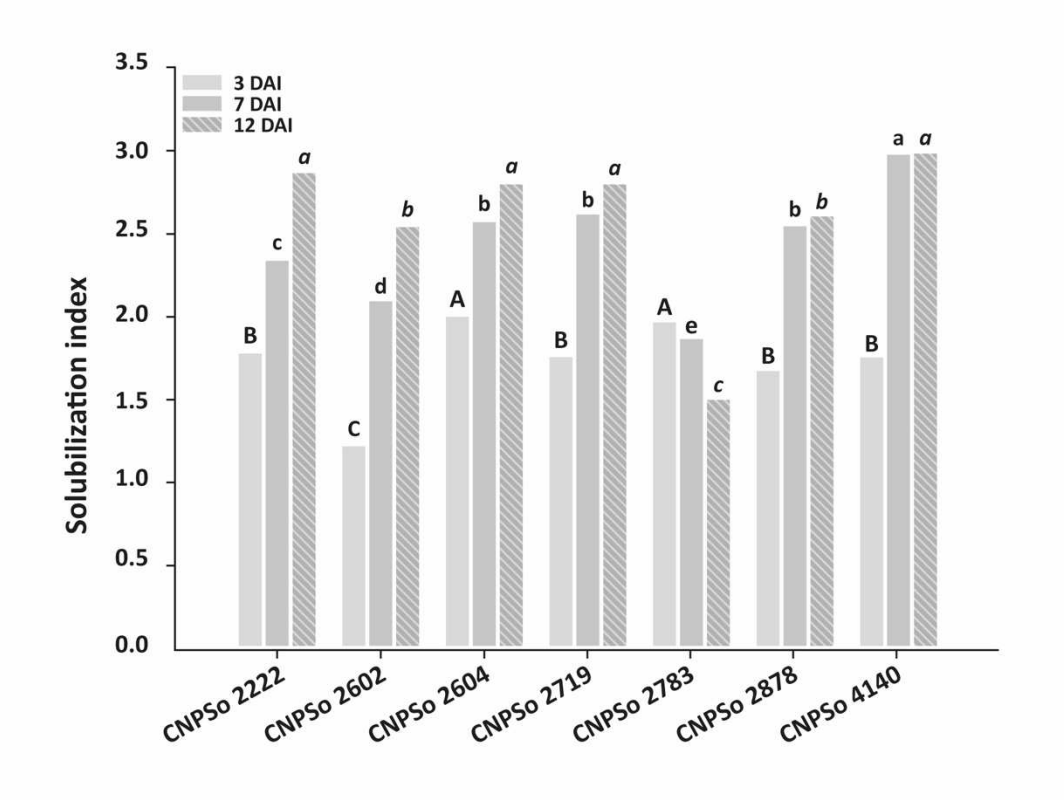


Figure 1 Solubilization index of seven strains for calcium phosphate solubilizing activity. Capital letters compare the halo/colony ratio assessed at 3 days; lower case letters compare the ratio at 7 days; italic lowercase letters compare the ratio at 12 days after inoculation. Values represent the means of three biological replicates and when followed by the same letter are not statistically different according to the Scott-Knott test ($p \leq 0.05$).

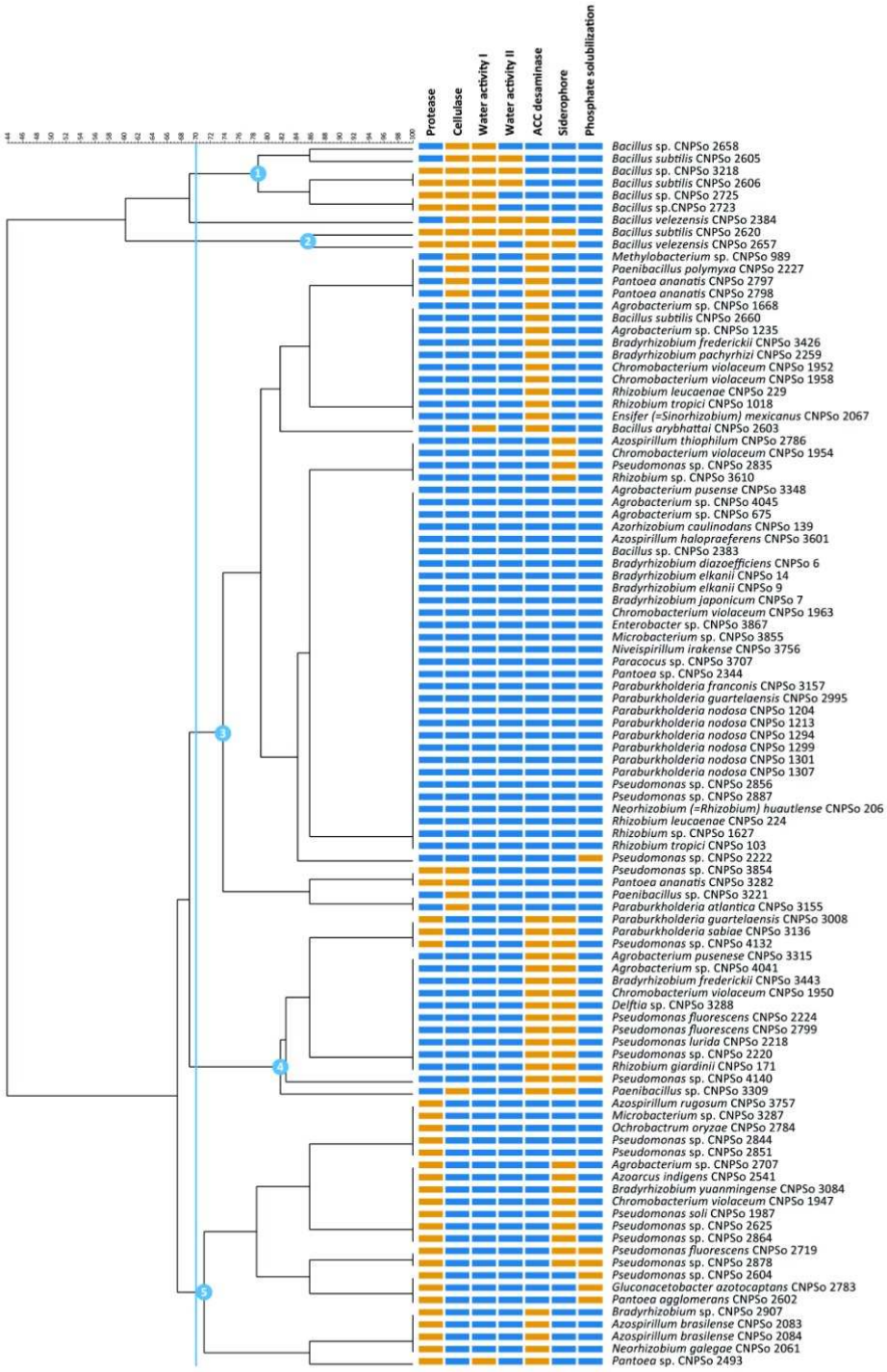


Figure 2 Phenotypic dendrogram based on putative plant growth-promoting traits of 100 bacterial strains of the Culture Collection of Diazotrophic and Plant Growth-Promoting Bacteria of Embrapa Soja. Dendrogram built on Bionumerics software (v.7.6.3) using the UPGMA algorithm. The clusters were obtained considering the similarity level of 70%. Positive traits are represented in yellow, while negative traits are shown in blue.

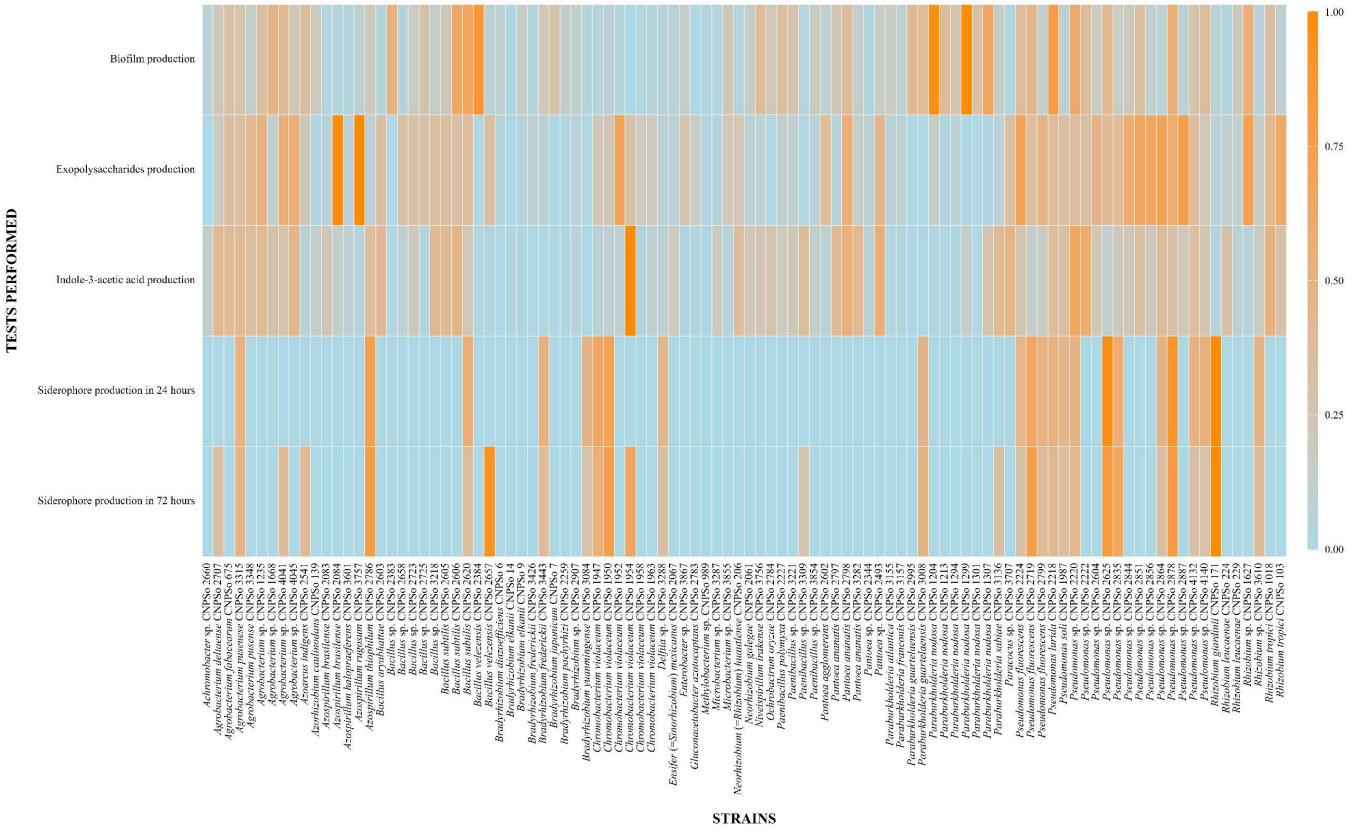


Figure 3 Heatmap of plant growth-promoting traits of 100 bacterial strains from the Culture Collection of Diazotrophic and Plant Growth-Promoting Bacteria of Embrapa Soja. Data were normalized on a scale from 0 to 1, where 1 was assigned to strains with the highest performance for the respective trait, represented by orange color. Lower values follow a gradient from lighter orange to blue, where blue indicates trait close to 0.

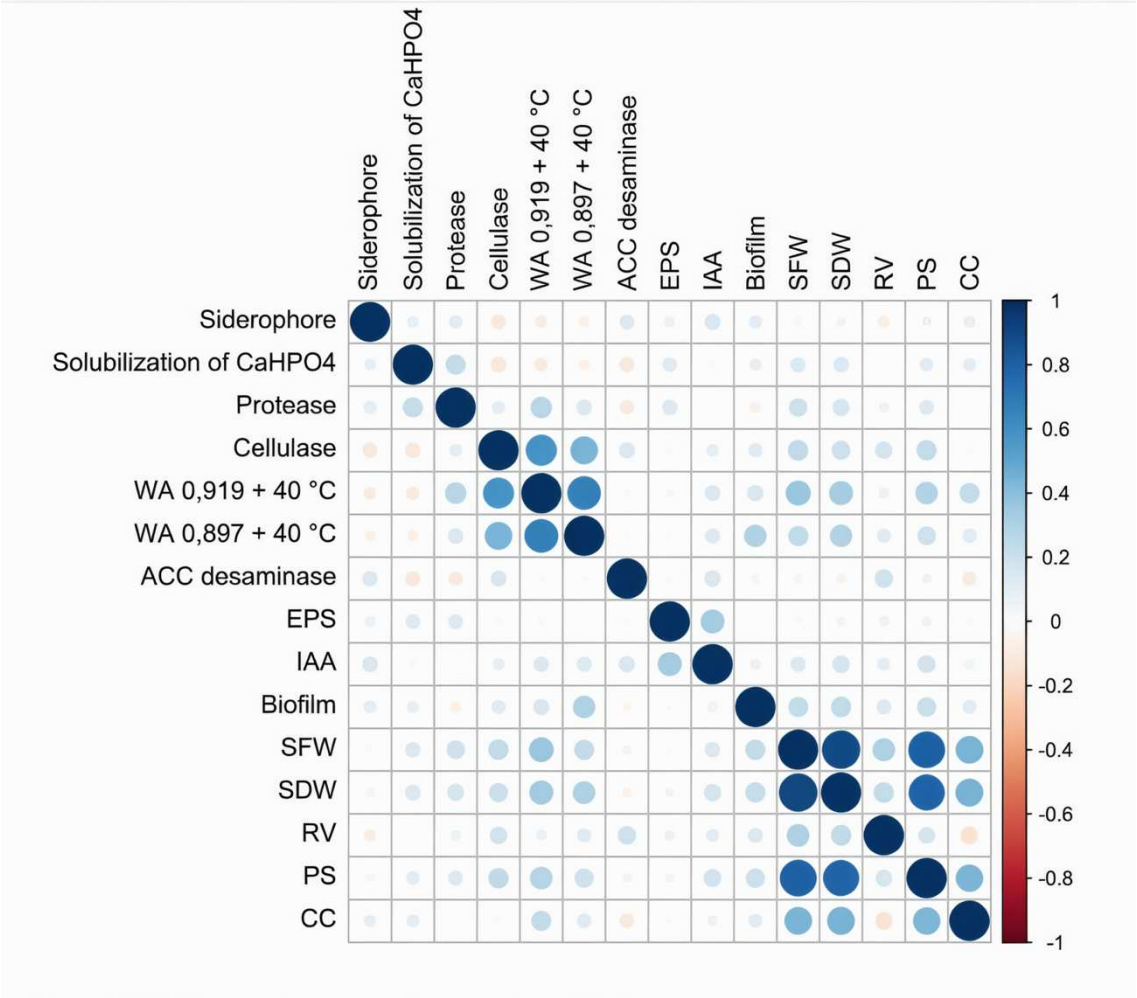
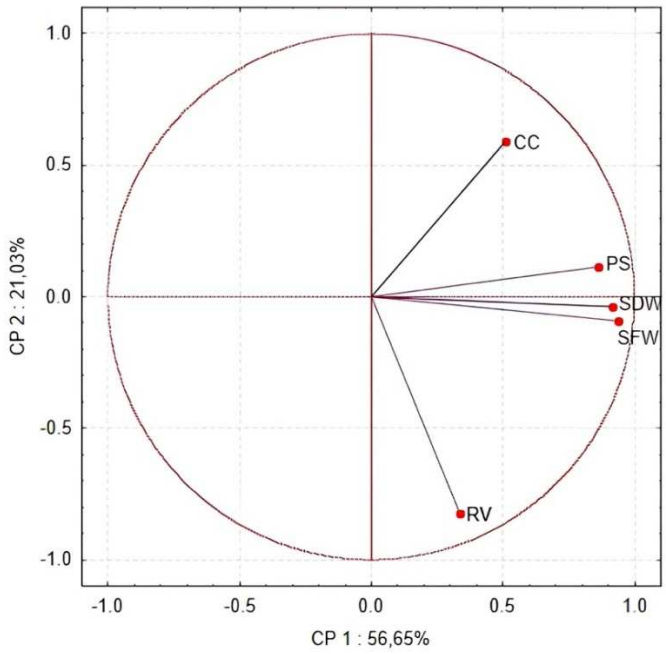


Figure 4 Pearson's correlation analysis among the *in vitro and in vivo* variables assessed for 100 bacterial strains of the Culture Collection of Diazotrophic and Plant Growth-Promoting Bacteria of Embrapa Soja. The color gradient indicates the direction and magnitude of the correlation, ranging from blue for strong positive correlations (+1 = 100%) to red for strong negative correlations (-1 = -100%), with white representing no correlation (0 = 0%). The size of the circle reflects the intensity of the correlation: larger circles indicate stronger correlations, while smaller circles indicate weaker correlations. Statistical significance was assessed at $p \leq 0.05$.

Legend: Water Activity (AW); exopolysaccharides (EPS); indole-3-acetic acid (AAI); Shoot Fresh Weight (SFW); Shoot Dry Weight (SDW); Root Volume (RV); Plant Size (PS); and Chlorophyll Concentration (CC) of maize plants inoculated with PGPB strains under water restriction.

(A)



(B)

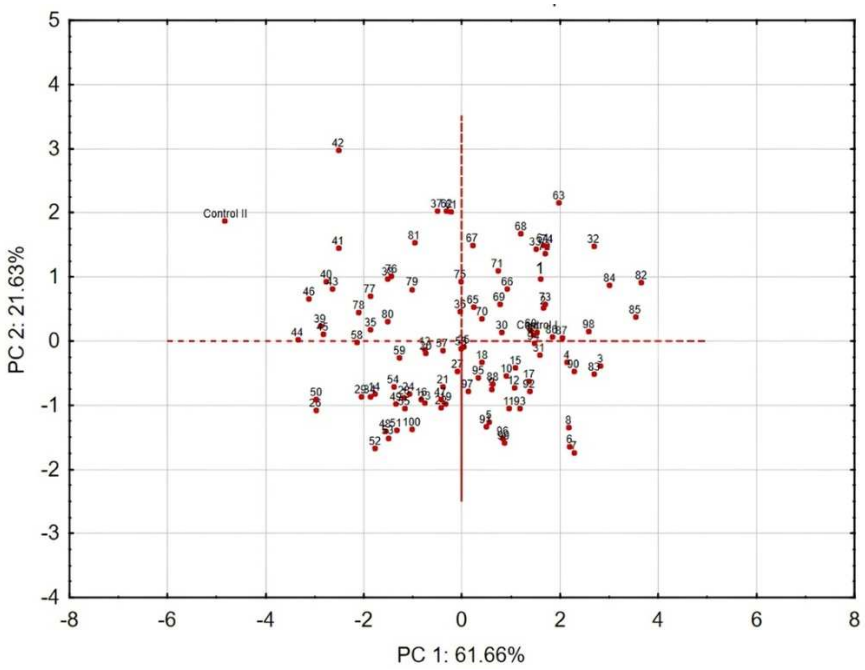


Figure 5 PCA results for variables and treatments. (A) Correlation circle among the variables: shoot fresh weight (SFW), shoot dry weight (SDW), root volume (RV), plant size (PS) and chlorophyll concentration (CC) of maize plants inoculated with 100 bacterial strains of the Culture Collection of Diazotrophic and Plant Growth-Promoting Bacteria of Embrapa Soja and grown in sterile substrate under water restriction. The proximity and direction of the variables indicate their correlation and contribution for the components. (B) Factorial plan under water restriction conditions. The numbers corresponding to each strain are shown in Table S1. The percentage of variance explained by each principal component is shown in the axis's labels.

Table S1 Bacterial strains selected from the Culture Collection of Diazotrophic and Plant Growth-Promoting Bacteria of Embrapa Soja for evaluation of *in vitro* and *in vivo* plant-growth promoting traits and culture medium used for their growth.

No.	Strain ID	Culture medium
1	*Bradyrhizobium diazoefficiens* CNPSo 6	YMA[1]
2	*Bradyrhizobium japonicum* CNPSo 7	YMA
3	*Bradyrhizobium elkanii* CNPSo 9	YMA
4	*Bradyrhizobium elkanii* CNPSo 14	YMA
5	*Rhizobium tropici* CNPSo 103	YMA
6	*Azorhizobium caulinodans* CNPSo 139	YMA
7	*Rhizobium giardinii* CNPSo 171	YMA
8	*Neorhizobium* (=*Rhizobium*) *huautlense* CNPSo 206	YMA
9	*Rhizobium leucaenae* CNPSo 224	YMA
10	*Rhizobium leucaenae* CNPSo 229	YMA
11	*Agrobacterium* sp. CNPSo 675	YMA
12	*Methylobacterium* sp. CNPSo 989	YMA
13	*Rhizobium tropici* CNPSo 1018	YMA
14	*Paraburkholderia nodosa* CNPSo 1204	YMA
15	*Paraburkholderia nodosa* CNPSo 1213	YMA
16	*Agrobacterium* sp. CNPSo 1235	YMA
17	*Paraburkholderia nodosa* CNPSo 1294	YMA
18	*Paraburkholderia nodosa* CNPSo 1299	YMA
19	*Paraburkholderia nodosa* CNPSo 1301	YMA
20	*Paraburkholderia nodosa* CNPSo 1307	YMA
21	*Rhizobium* sp. CNPSo 1627	YMA
22	*Agrobacterium* sp. CNPSo 1668	YMA
23	*Chromobacterium violaceum* CNPSo 1947	LB[2]
24	*Chromobacterium violaceum* CNPSo 1950	LB
25	*Chromobacterium violaceum* CNPSo 1952	LB
51	*Pseudomonas* sp. CNPSo 2625	TSA
52	*Bacillus velezensis* CNPSo 2657	TSA
53	*Bacillus* sp. CNPSo 2658	TSA
54	*Achromobacter* sp. CNPSo 2660	YMA
55	*Agrobacterium* sp. CNPSo 2707	YMA
56	*Pseudomonas fluorescens* CNPSo 2719	TSA
57	*Bacillus* sp. CNPSo 2723	TSA
58	*Bacillus* sp. CNPSo 2725	TSA
59	*Gluconacetobacter azotocaptans* CNPSo 2783	DYGS
60	*Ochrobactrum oryzae* CNPSo 2784	YMA
61	*Azospirillum thiophilum* CNPSo 2786	DYGS
62	*Pantoea ananatis* CNPSo 2797	YMA
63	*Pantoea ananatis* CNPSo 2798	YMA
64	*Pseudomonas fluorescens* CNPSo 2799	TSA
65	*Pseudomonas* sp. CNPSo 2835	TSA
66	*Pseudomonas* sp. CNPSo 2844	TSA
67	*Pseudomonas* sp. CNPSo 2851	TSA
68	*Pseudomonas* sp. CNPSo 2856	TSA
69	*Pseudomonas* sp. CNPSo 2864	TSA
70	*Pseudomonas* sp. CNPSo 2878	TSA
71	*Pseudomonas* sp. CNPSo 2887	TSA
72	*Bradyrhizobium* sp. CNPSo 2907	YMA
73	*Paraburkholderia guartelaensis* CNPSo 2995	YMA
74	*Paraburkholderia guartelaensis* CNPSo 3008	YMA
75	*Bradyrhizobium yuanmingense* CNPSo 3084	YMA

26	*Chromobacterium violaceum* CNPSo 1954	LB	**76**	*Paraburkholderia sabiae* CNPSo 3136	YMA
27	*Chromobacterium violaceum* CNPSo 1958	LB	**77**	*Paraburkholderia atlantica* CNPSo 3155	YMA
28	*Chromobacterium violaceum* CNPSo 1963	LB	**78**	*Paraburkholderia franconis* CNPSo 3157	YMA
29	*Pseudomonas soli* CNPSo 1987	TSA[3]	**79**	*Bacillus* sp. CNPSo 3218	TSA
30	*Neorhizobium galegae* CNPSo 2061	YMA	**80**	*Paenibacillus* sp. CNPSo 3221	TSA
31	*Sinorhizobium mexicanus* CNPSo 2067	YMA	**81**	*Pantoea* sp. CNPSo 3282	DYGS
32	*Azospirillum brasilense* CNPSo 2083	DYGS[4]	**82**	*Microbacterium* sp. CNPSo 3287	DYGS
33	*Azospirillum brasilense* CNPSo 2084	DYGS	**83**	*Delftia* sp. CNPSo 3288	DYGS
34	*Pseudomonas lurida* CNPSo 2218	TSA	**84**	*Paenibacillus* sp. CNPSo 3309	DYGS
35	*Pseudomonas* sp. CNPSo 2220	TSA	**85**	*Agrobacterium pusenese* CNPSo 3315	YMA
36	*Pseudomonas* sp. CNPSo 2222	TSA	**86**	*Agrobacterium* sp. CNPSo 3348	YMA
37	*Pseudomonas fluorescens* CNPSo 2224	TSA	**87**	*Bradyrhizobium rederickii* CNPSo 3426	YMA
38	*Paenibacillus polymyxa* CNPSo 2227	TSA	**88**	*Bradyrhizobium rederickii* CNPSo 3443	YMA
39	*Bradyrhizobium pachyrhizi* CNPSo 2259	YMA	**89**	*Azospirillum halopraeferens* CNPSo 3601	DYGS
40	*Pantoea* sp. CNPSo 2344	YMA	**90**	*Rhizobium* sp. CNPSo 3610	YMA
41	*Bacillus* sp. CNPSo 2383	TSA	**91**	*Paracocus* sp. CNPSo 3707	TSA
42	*Bacillus velezensis* CNPSo 2384	TSA	**92**	*Niveispirillum irakense* CNPSo 3756	DYGS
43	*Pantoea* sp. CNPSo 2493	YMA	**93**	*Azospirillum rugosum* CNPSo 3757	DYGS
44	*Azoarcus indigens* CNPSo 2541	DYGS	**94**	*Paenibacillus* sp. CNPSo 3854	TSA
45	*Pantoea agglomerans* CNPSo 2602	TSA	**95**	*Microbacterium* sp. CNPSo 3855	YMA
46	*Bacillus aryabhattai* CNPSo 2603	TSA	**96**	*Enterobacter* sp. CNPSo 3867	YMA
47	*Pseudomonas* sp. CNPSo 2604	TSA	**97**	*Agrobacterium* sp. CNPSo 4041	YMA
48	*Bacillus subtilis* CNPSo 2605	TSA	**98**	*Agrobacterium* sp. CNPSo 4045	YMA
49	*Bacillus subtilis* CNPSo 2606	TSA	**99**	*Pseudomonas* sp. CNPSo 4132	TSA
50	*Bacillus subtilis* CNPSo 2620	TSA	**100**	*Pseudomonas* sp. CNPSo 4140	TSA

YMA: Modified Yeast Mannitol Agar [31];

LB: Luria-Bertani [31];

TSA: Trypticase Soy Agar;

DYGS: [32].

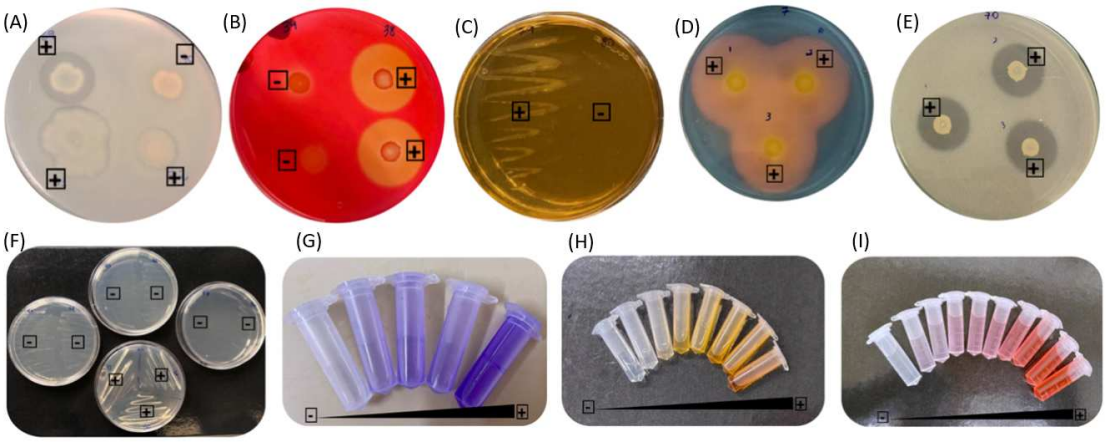


Figure S1 Examples of control and positive results, respectively, for each of the ten traits evaluated *in vitro*: (A) protease and (B) cellulase activities, (C) growth under low water activity, (D) siderophore synthesis, (E) phosphate solubilization, (F) ACC deaminase activity, (G) biofilm formation, (H) EPS (exopolysaccharides) production, and (I) IAA (indole-3-acetic acid) synthesis. Properties evaluated in 100 bacterial strains from the "Diazotrophic and Plant Growth-Promoting Bacteria Culture Collection of Embrapa Soja".

Figure S2 View of the experiment with maize inoculated with the 100 bacterial strains from the "Diazotrophic and Plant Growth-Promoting Bacteria Culture Collection of Embrapa Soja", grown on sterile substrate (Experiment I) and submitted to water restriction.

Figure S3 View of the experiment with maize inoculated with 15 bacterial strains from the Diazotrophic and Plant Growth-Promoting Bacteria Culture Collection of Embrapa Soja, selected in Experiment I. Plants grown on non-sterile soil (Experiment II) and submitted to water restriction.

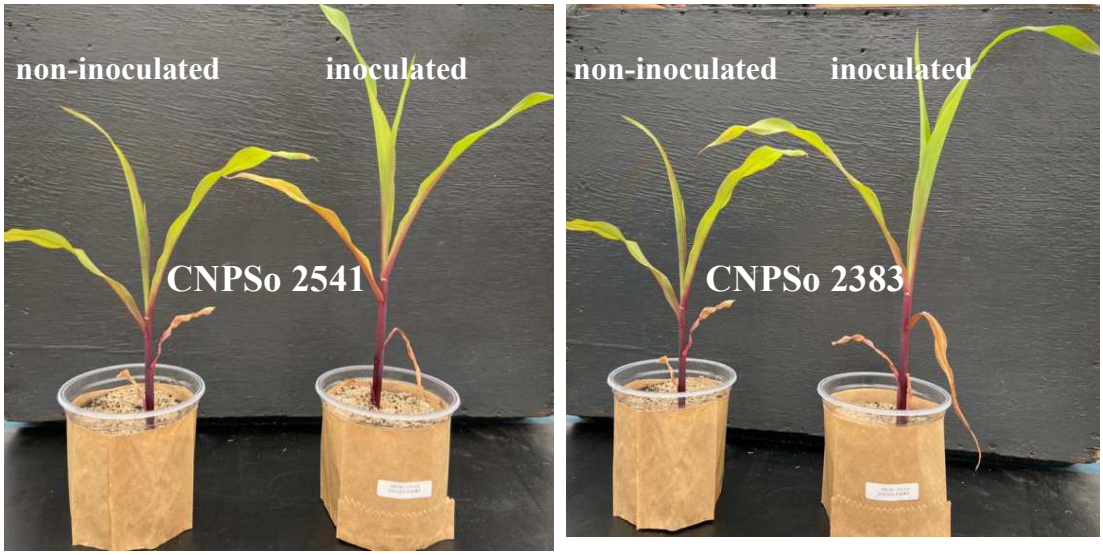


Figure S4 Growth of maize in sterile substrate under water restriction conditions (Experiment I) and inoculation with two promising strains in the mitigation of water stress. Non-inoculated and inoculated plants with *Azoarcus indigens* CNPSo 2541 and *Bacillus* sp. CNPSo 2383.

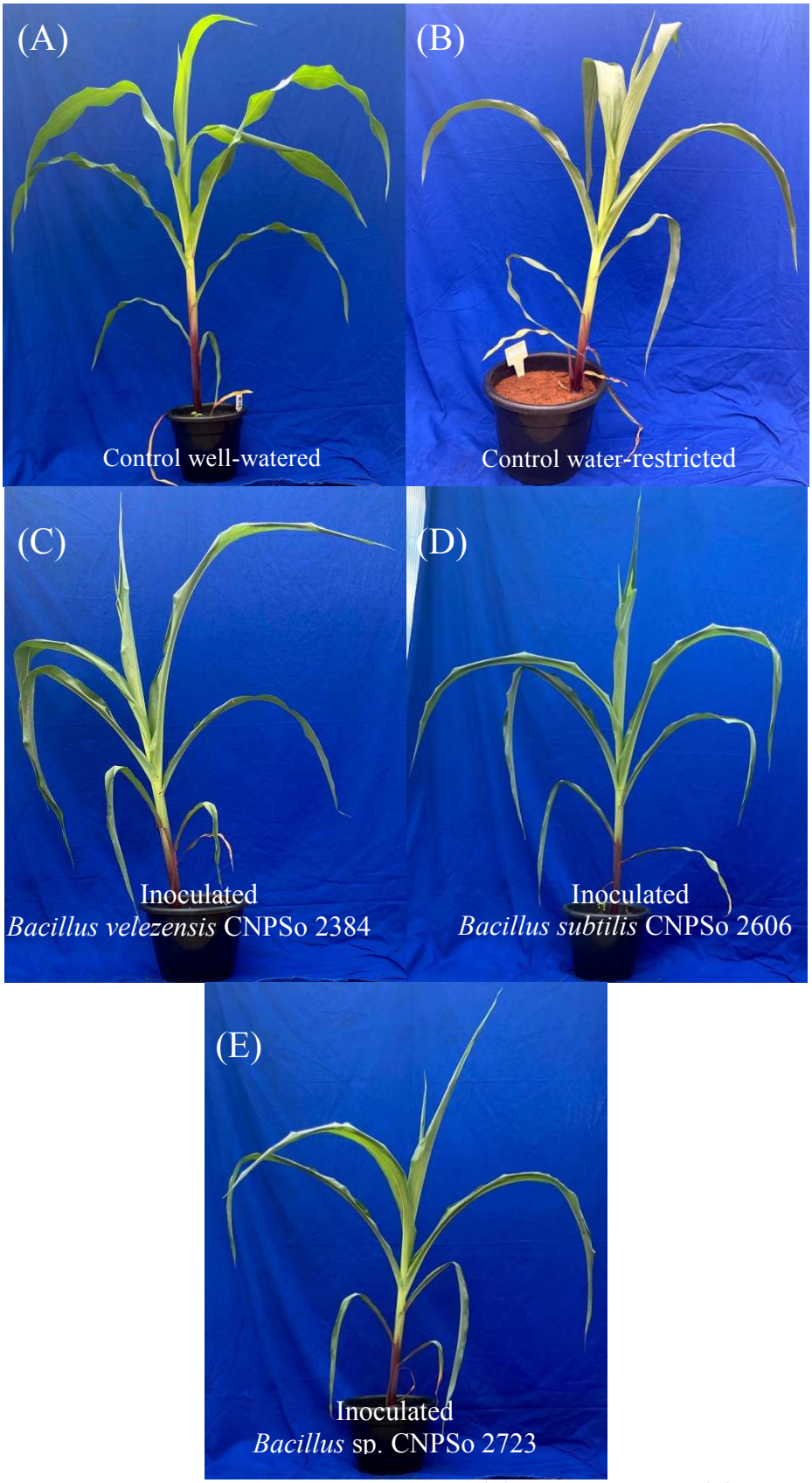


Figure S5 Maize growth in non-sterile substrate under (A) well-watered and (B) water-restricted conditions (Experiment II), inoculated with three promising strains for mitigation of water restriction: (C) *Bacillus velezensis* CNPSo 2384, (D) *Bacillus subtilis* CNPSo 2606, and (E) *Bacillus* sp. CNPSo 2723, respectively.

CAPÍTULO 3

USO CRESCENTE DE *Bacillus* MULTIFUNCIONAIS PARA O CONTROLE BIOLÓGICO DE PRAGAS E DOENÇAS E PROMOÇÃO DO CRESCIMENTO DE PLANTAS: LIÇÕES DO BRASIL

USO CRESCENTE DE *Bacillus* MULTIFUNCIONAIS PARA O CONTROLE BIOLÓGICO DE PRAGAS E DOENÇAS E A PROMOÇÃO DO CRESCIMENTO DE PLANTAS: LIÇÕES DO BRASIL

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RESUMO

O gênero microbiano *Bacillus* habita uma ampla variedade de ambientes e está amplamente distribuído em todos os biomas globais, com uma presença significativa em habitats do solo. Na agricultura, estirpes de *Bacillus* desempenham papéis multifacetados, atuando como agentes de biocontrole contra pragas e doenças e promovendo o crescimento das plantas ao facilitar a disponibilidade de nutrientes e melhorar a tolerância ao estresse. Por meio de mecanismos como solubilização de fosfato, atividade de ACC-desaminase e síntese de fitormônios e sideróforos, *Bacillus* spp. contribuem para a saúde do solo e a produtividade agrícola, em uma abordagem inovadora de agricultura regenerativa. A capacidade de *Bacillus* spp. de solubilizarem fosfatos torna esses nutrientes mais acessíveis às plantas, enquanto a atividade de ACC-desaminase ajuda as plantas a resistirem a estresses ambientais. Além disso, a síntese de fitormônios pode estimular o crescimento e o desenvolvimento das plantas, e os sideróforos podem facilitar a absorção de nutrientes, como o ferro, pelas plantas. À medida que a agroindústria adota formulações à base de *Bacillus* para o manejo de pragas e melhoria das culturas, pesquisas futuras trazem perspectivas promissoras para otimizar suas aplicações e explorar todo o seu potencial nos agroecossistemas. A exploração contínua da diversidade de *Bacillus* spp. e de suas interações com plantas e com a microbiota do solo avançará ainda mais as práticas agrícolas sustentáveis. Esta revisão contribui para o entendimento de como as estirpes de *Bacillus* podem revolucionar a agricultura ao melhorar a saúde do solo, aumentar a produtividade agrícola e fornecer soluções biológicas eficazes contra pragas e doenças. A aplicação bem-sucedida de tecnologias baseadas em *Bacillus* em milhões de hectares na agricultura brasileira demonstra a sinergia entre a necessidade de práticas agrícolas mais sustentáveis e o uso de bioinsumos.

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Review

Increasing Application of Multifunctional *Bacillus* for Biocontrol of Pests and Diseases and Plant Growth Promotion: Lessons from Brazil

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Abstract: The microbial genus *Bacillus* inhabits a diverse range of environments and is widespread across all global biomes, with a significant presence in soil habitats. In agriculture, *Bacillus* strains play multifaceted roles, serving as biocontrol agents against pests and diseases, and promoting plant growth by facilitating nutrient availability and enhancing stress tolerance. Through mechanisms such as phosphate solubilization, ACC-deaminase activity, and synthesis of phytohormones and siderophores, *Bacillus* spp. contribute to soil health and crop productivity, in a new approach of regenerative agriculture. The ability of *Bacillus* spp. to solubilize phosphate makes essential nutrients more accessible to plants, while ACC-deaminase activity helps plants withstand environmental stresses. Additionally, the synthesis of phytohormones can stimulate plant growth and development, and siderophores may facilitate the uptake of nutrients such as iron by plants. As the agricultural industry embraces *Bacillus*-based formulations for pest management and crop enhancement, future research holds promising prospects for optimizing their applications and harnessing their full potential in agroecosystems. Continued exploration of *Bacillus* spp. diversity and their interactions with plants and soil microbiota will further advance sustainable agricultural practices. This review contributes to understanding how *Bacillus* strains can revolutionize agriculture by enhancing soil health, increasing crop productivity, and providing effective biological solutions against pests and diseases. The successful application of *Bacillus*-based technologies in millions of hectares in Brazilian agriculture demonstrates the synergy between the need for more sustainable agricultural practices and the use of bio-inputs.

Keywords: agriculture; *Bacillus*; bio-inputs; biocontrol; plant growth promotion

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1. Introduction

The genus *Bacillus* consists of Gram-positive, rod-shaped, motile bacteria that are aerobic or facultatively anaerobic [1]. Under abiotic stress conditions, such as high temperature, radiation, dehydration, and exposure to certain chemicals, spore formation occurs [2]. Spore formation, or bacterial sporulation refers to the production of metabolically inactive resistance structures until they are subjected to minimal conditions for vegetative structure development [3].

In the mid-1870s, while working at the University of Breslau, Ferdinand Cohn isolated a small, motile, aerobic bacterium from hay infusions, naming it *Bacillus subtilis* (meaning "subtle rod"). From this isolate, Cohn described the life cycle of *Bacillus*, detailing spore formation, its heat resistance, and the germination process. However, the genus and the species did not gain scientific importance until 1876, when Robert Koch described a phenotypically similar organism as *Bacillus anthracis*, highlighting the importance of the genus [4].

Upon contact with a mammal, *B. anthracis*, the causative agent of anthrax, produces toxins due to the presence of genes located on plasmids, resulting in a severe disease [5]. Scientifically, this species is also important because it was the first experimental model for the development of central postulates of infectious diseases and the first to outline the role of macrophages in cellular immunity [6,7].

The genus *Bacillus* is ubiquitous, meaning that it can be found in a wide variety of habitats, including soil [8], where it may represent up to 95% of the population of Gram-positive bacteria [9]. The diversity is so great that compared to the Enterobacteriaceae family—which has dozens of genera—it is already possible to find an equivalent number of species [10]. A phylogenetic tree based on the 16S rRNA sequences highlights the outstanding diversity of *Bacillus* spp. Comparative genomic studies and complementary data have revealed the presence of genetically distinct groups that have evolved from a common ancestor. These analyses, which were conducted with hundreds of *Bacillus* genomes, aimed to clarify the evolutionary relationships and classification of species. As a result, a significant set of species has been reclassified into several new genera: *Alkalicoccus*, *Alkalihalobacillus*, *Alteribacter*, *Caldalkalibacillus*, *Caldibacillus*, *Calidifontibacillus*, *Cytobacillus*, *Domibacillus*, *Ectobacillus*, *Evansella*, *Ferdinandcohnia*, *Gottfriedia*, *Heyndrickxia*, *Lederbergia*, *Litchfieldia*, *Margalitia*, *Mesobacillus*, *Metabacillus*, *Neobacillus*, *Niallia*, *Peribacillus*, *Priestia*, *Robertmurraya*, *Rossellomorea*, *Salibacterium*, *Salisediminibacterium*, *Schinkia*, *Siminovitchia*, *Solibacillus*, *Sutcliffiella*, and *Weizmannia* [11–16]. However, they will all be treated here as *Bacillus*, and the new reclassification is included in parentheses.

The diversity of the genus encompasses species related to diseases, such as *Bacillus cereus*, which is notably associated with food poisoning [5], *B. anthracis*, and others. However, it also harbors biotechnologically promising species, such as *Bacillus safensis*, *Bacillus endophyticus*, and *B. subtilis* which have applications in the pharmaceutical industry [17], *Bacillus amyloliquefaciens* in the feed industry [18], *Bacillus licheniformis* for environmental remediation [19], and various species with significant roles in agriculture [20].

Globally, in the agricultural market, there is a tradition of using *Bacillus* for biological control of pests and diseases, owing to its ability to produce antimicrobial, nematicidal, and insecticidal compounds [21]. Brazil stands out as an important market for biocontrol, and *Bacillus* composes the large majority of the formulations—about 80% of the market, in 2023. The composition is as follows: *B. subtilis* (32.8% of the total), *B. licheniformis* (26.2%), *B. amyloliquefaciens* (8.2%), *Bacillus paralicheniformis* (6.5%), *Bacillus thuringiensis* (4.9%), and *Bacillus velezensis* (1.6%) [21,22].

In addition to their ability to synthesize biocidal molecules to protect against pests and diseases or trigger plant defense responses, there are *Bacillus* species with other plant growth-promoting properties responsible for increasing the tolerance of host plants to abiotic stress conditions, producing phytohormones, and making nutrients available to plants [21,22]. Regarding *Bacillus* spp. with prominent applicability in agriculture, a group known as plant growth-promoting bacteria (PGPB) encompasses species that play a significant role in plant development, especially in economically important crops [23].

The development of a plant's aboveground portion is highly dependent on the underground root system. In the rhizosphere, the zone of plant-microorganism communication, there is continuous interaction between roots and microbial communities established in the soil, as well as populations introduced for crop management [24]. Soil represents the primary reservoir for the potential bacterial community of the rhizosphere [25], and through root exudates, plants attract rhizospheric microorganisms to colonize the root surface and/or internal tissues, while microorganisms—via the production of phytohormones, the release of nutrients, or induction of responses to various stresses—can promote plant growth [21].

Many PGPBs, by producing phytohormones, volatile organic compounds, and secondary metabolites, play a crucial role in the architecture and growth of roots and/or root hairs [26]. With increased surface area for water and nutrient absorption by plants, there are advantages in growth and resource utilization efficiency under multiple soil

constraints [27], which characterize a significant indicator of agricultural sustainability [28]. *Bacillus* species can enhance the productivity and quality of various agricultural crops by providing nutrients in forms assimilable by plants, activating physiological and molecular processes in plants, or through the production of metabolites and compounds beneficial to plant development [23]. in addition to controlling pests and diseases [29].

The action of plant growth-promoting microorganisms can be involved in biocontrol or directly promoting plant growth (Figure 1). Examples of beneficial properties to plant growth include phosphate solubilization, 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase activity, and synthesis of phytohormones and siderophores. As an indirect property of plant development, biocontrol encompasses the production of antimicrobial metabolites and hydrolytic enzymes, quorum quenching, competition for nutrients and space, production of siderophores, and promotion of induced systemic resistance (ISR).

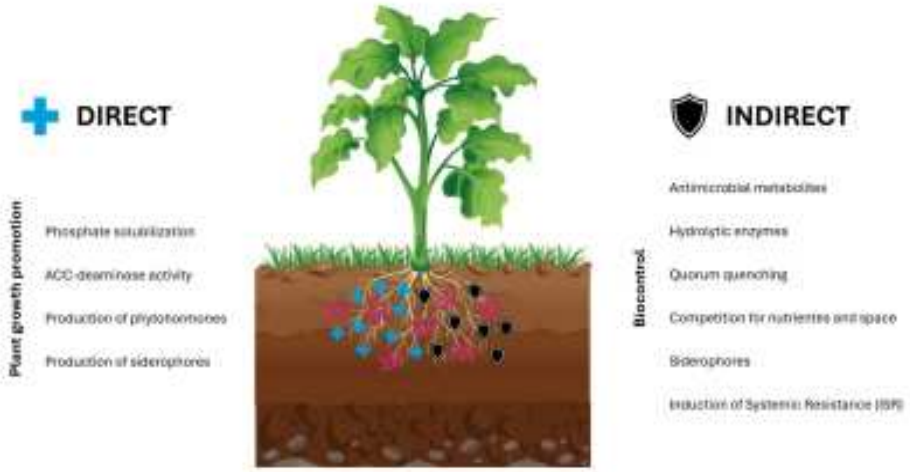

Figure 1. Mechanisms of direct or indirect action of *Bacillus* spp. with the host plant resulting in plant growth promotion. The "+" symbol refers to growth-promoting properties and the "shield" refers to biocontrol.

Traditionally, the primary application of *Bacillus* in agriculture has been as a biological control agent. However, in recent years, its use as a PGPB has been growing. Interestingly, the number of reports showing multifunctionality at the strain level is increasing. Therefore, the same strain of *Bacillus* can be used for biocontrol and to promote plant growth. Bio-inputs based on this multifunctionality can represent a very interesting approach towards a new regenerative agriculture. This review presents the main microbial processes of *Bacillus* that may improve plant growth (Figure 1), the uses of *Bacillus* in agriculture, and prospects for the future.

2. Applications of *Bacillus* spp. in Agriculture as Biocontrol

Biological control represents a sustainable strategy for combating plant pests and diseases. This process, which uses antagonists to pests and diseases, is reported as one of the most promising alternatives to chemical control [30,31]. Microorganisms-based products containing live cells or microbial metabolites have been used as biopesticides worldwide because they are generally safer for non-target organisms, including humans, have reduced persistence in the environment, and are potentially acceptable for use in organic agriculture [32].

Reports of disease control promoted by *Bacillus* are not new, as the insecticidal properties of *B. thuringiensis* (*Bt*) have long been recognized [33]. Although some reports indicate that the Egyptians were aware of the insecticidal properties of what was likely *Bt*, the microorganism was isolated by Ernst Berliner only in 1911, in Japan, from a diseased larva of the flour moth. In 1954, Thomas Angus demonstrated that the crystalline protein inclusions

produced by *B. thuringiensis* during sporulation were responsible for the insecticidal action. In Brazil, in 1994, there were also reports of products based on *B. subtilis*, responsible for controlling coffee (*Coffea* spp.) rust [34].

B. thuringiensis insecticidal products were first marketed in France in the late 1930s and are still considered safe because specific formulations only harm a narrow range of insect species. Furthermore, the incorporation of *Bt* genes related to the insecticidal crystal protein (ICP) into various agronomically important crops represented a significant advancement in the management of various agricultural pests [33]. However, any possibility of gene incorporation must be investigated for potential toxicity. While acknowledging the agronomic benefits of GMOs (Genetically Modified Organisms), it is imperative to conduct analyses of risk assessments due to potential impacts. Studies such as the one conducted by Seralini [35] have raised questions about the long-term toxicity of these organisms. Therefore, comprehensive evaluations are necessary to ensure the safety and sustainability of GMO adoption in agriculture.

An advantage of the genus is that it not only inhibits the development of the pathogen but also restores, at least in part, the microbial community altered by the presence of the disease-causing microorganism in plants [36]. For example, the strain FZB42 of *B. amyloliquefaciens* subsp. plantarum, when associated with lettuce (*Lactuca sativa* L.), did not affect the root microbiome and contributed to the restoration of the original structure of the community that was previously altered by the pathogen *Rhizoctonia solani* [36]. Therefore, *Bacillus* may highly contribute to soil health.

Bacillus species are renowned for their ability to produce a diverse array of components of significant biotechnological value [37]. The use of bio-inputs based on *Bacillus* spp. for pests and diseases control in plants has proven to be effective in various agronomically important crops due to a range of specific mechanisms associated with each species. These mechanisms include the production of antibiotic metabolites, hydrolytic enzymes, quorum quenching, competition for space and nutrients, production of siderophores, and ISR. Several examples are shown in Table 1. An interesting observation from this table is that the strains and even the species are distinct, indicating that bioprospecting for specific conditions is a path that has led to success.

Table 1. Mechanism of actions of *Bacillus* spp. as a biocontrol agent in crop protection.

Species of *Bacillus*	Crop	Mechanism	Pathogen	Study
Bacillus amyloliquefaciens JDF3; *Bacillus subtilis* RSS-1	*Glycine max*	Inhibition of ribosomal activity	Fungi *Phytophthora sojae*	[38]
Bacillus (Priestia)megaterium Sneb207	*Glycine max*	Induction of systemic resistance	Nematode *Heterodera glycines*	[39]
Bacillus sp. P12	*Phaseolus vulgaris*	Synthesis of lipopeptide isoforms: kurstakin, surfactin, iturin, polymyxin, and fengycin	Fungi *Macrophomina phaseolina*	[40]
Bacillus halotolerans QTH8	*Triticum aestivum*	Synthesis of lipopeptides: iturin, surfactin, and fengycin	Fungi *Fusarium pseudograminearum*	[41]
Bacillus velezensis BM21	*Zea mays*	Cytoplasmic necrosis and disintegration of pathogen organelles	Fungi *Fusarium verticillioides*; *Thanatephorus cucumeris*; *Typhula incarnata*; *Fusarium oxysporum*; *Pythium graminicola*; *Rhizoctonia solani*	[42]
Bacillus sp. BMH	*Oryza sativa*	Induction of systemic resistance	Fungi *Pyricularia oryzae*	[43]
Bacillus velezensis FJAT-46737	*Solanum lycopersicum*	Secretion of lipopeptides: iturins, fengycins, and surfactins	Fungi *Ralstonia solanacearum*	[44]
Bacillus subtilis K4-4; *Bacillus subtilis* GH3-8	*Citrus sinensis*	Production of HCN, siderophores, bacillomycin, iturin, fengycin	Fungi *Fusarium solani*	[45]
Bacillus pumilus	*Fragaria* × *ananassa* Duchesne	Systemic induction of resistance	Fungi *Rhizoctonia solani*; *Fusarium solani*; *Pythium* sp.	[46]
Bacillus velezensis MS20	*Zea mays*	Synthesis of surfactin	Fungi *Rhizoctonia solani*	[47]
Bacillus (Weizmannia) coagulans; *Bacillus globisporus*; *Bacillus pumilus*; *Bacillus subtilis*; *Bacillus (Niallia) circulans*; *Bacillus cereus*; *Bacillus (Weizmannia) coagulans*; *Bacillus cereus*	*Gossypium barbadense*	Systemic resistance induction and/or antibiosis	Fungi *Rhizoctonia solani*; *Macrophomina faseolina*; *Sclerotium rolfsii*; *Pythium* sp.; *Fusarium oxysporum*; *Fusarium solani*; *Fusarium moniliforme*	[48]
Bacillus velezensis ZW-10	*Oryza sativa*	Synthesis of peroxidase, protease, and cellulase	Fungi *Magnaporthe oryzae*	[49]

2.1. Antimicrobial Metabolites

Bacteriocins are ribosomal-synthesized proteins or peptides with bactericidal action against species that may be closely related to the producing bacteria and exhibit variable biochemical properties, inhibitory spectra, and mechanisms of action [50]. Most often, these molecules are targeted against competitive microorganisms; thus, providing a selective advantage to their producers [51].

Bacillus spp. is a relatively abundant source of antimicrobials, as many species of this genus synthesize antimicrobial peptides [52,53]. One example is of antibiotics of the iturin group reported in two strains of *B. subtilis*, PRBS-1 and AP-3, which inhibited the growth of five important pathogenic fungi of soybean (*Glycine max* (L.) Merr.) seeds [54]. Another example is the endophytic *Bacillus* strain DMW1, which has the ability to control *Phytophthora sojae* and *Ralstonia solanacearum* due to the presence of 12 biosynthetic gene clusters of secondary metabolites [55].

2.2. Hydrolytic Enzymes

The accumulation of hydrolytic enzymes, such as chitinases and β-1,3-glucanase, which degrade the cell walls of pathogenic fungi formed by chitin and β-1,3-glucan, represents a common defense mechanism of plants [56]. Microorganisms can also produce chitinases as an important biocontrol attribute in the rhizosphere [57]. In addition to the production of hydrolytic enzymes by the biocontrol agents themselves, PGPB can induce the production of chitinases in the host plant, accentuating the plant's defense response [58].

Strains of *Bacillus* that produce chitinases have been used as post-harvest biocontrol agents; for example, peaches (*Prunus persica* (L.) Batsch) treated with *B. cereus* AR156 showed a lower disease incidence and smaller lesion diameter compared to the control when in contact with *Rhizopus stolonifer*. In this study, it was found that the treatment with *B. cereus* AR156 notably improved chitinase and β-1,3-glucanase activities [59]. Another *Bacillus* species, *Bacillus pumilus*, including subspecies *B. pumilus* HR10, *B. pumilus* SS-10.7, *B. pumilus* MCB-7, *B. pumilus* INR7, *B. pumilus* SE52, SE34, SE49, *B. pumilus* RST25, *B. pumilus* JK-SX001, and *B. pumilus* KUDC1732 are capable of suppressing phytopathogens such as *Arthrobotrys conoides*, *Fusarium solani*, *Fusarium oxysporum*, *Sclerotinia sclerotiorum*, *R. solani*, and *Fagopyrum esculentum* [60].

2.3. Quorum Quenching

Through quorum sensing systems, bacteria change their behavior when a threshold concentration of signaling molecules is exceeded [58]; that is, it is a communication pathway between bacterial cells. Pathogenic bacteria use quorum sensing to assess the size of their population and regulate the timing of entry into the apoplast or plant cell [61,62]. However, besides cross-communication by producing the same signaling molecules, bacteria can degrade each other's signals, also known as quorum quenching.

Some strains belonging to the genus *Bacillus*, such as those studied by Caicedo and colleagues [63]—*Bacillus* sp. SJ13 and *Bacillus* sp. SJ15—can degrade the diffusible quorum signaling factor, cis-11-methyl-2-dodecenoic acid [58]. This signal is involved in regulating virulence in *Xanthomonas* spp. and *Xylella fastidiosa* [64]. *Bacillus toyonensis* AA1EC1, capable of degrading quorum signaling molecules such as N-acyl-homoserine lactones (AHLs), significantly attenuated the virulence of relevant phytopathogens, reducing symptoms of soft rot in potatoes (*Solanum tuberosum* L.) and carrots (*Daucus carota* L.) [65].

2.4. Competition for Nutrients and Space

Among the main resources necessary for microbial survival are nutrients and space, which vary between environments, so that microorganisms will compete for restricted components [66]. For example, the available carbon source may be the limiting factor for the development of a population [67]. As they grow and produce more biomass, microbial groups expand in space and compete with others to colonize areas where nutrients are more abundant [66].

The action of *B. subtilis* strains B006 and B010 in inhibiting *F. oxysporum* and *F. solani*—pathogens causing soybean root rot was studied by Guo and colleagues [68]; the control efficiency was higher than 63%, in addition to increasing soybean yield. The authors report that the rapid spread of bacterial colonies may be an important mechanism of biocontrol for these strains, due to their greater efficiency in nutrient competition. Mates and colleagues [69] evidenced that *B. velezensis* GF267 is a multisite antagonist and, among its properties of inhibiting pathogenic microorganisms, the strain stands out in competition with *Xanthomonas perforans*, for example.

2.5. Production of Siderophores

For most microorganisms, iron (Fe) is related to essential cellular processes. Some microorganisms have highly efficient Fe-acquisition systems to capture Fe from the environment under restriction conditions. In many cases, this involves the secretion and internalization of extracellular ferric chelators called siderophores [70]. Siderophores produced by a microorganism can bind to Fe with high specificity and affinity, making it unavailable to other microorganisms and thus limiting their growth [71].

This strategy may be involved in biological control against plant pathogens [72]. For example, the *B. subtilis* strain MF497446 was considered effective in siderophore production. When inoculated alone or in combination with *Pseudomonas koreensis* MG209738, it induced resistance to late wilt disease caused by *Cephalosporium maydis* in maize (*Zea mays* L.), resulting in increased plant growth and grain yield [73]. A siderophore-producing *B. subtilis* (CWTS 5) with multiple plant growth-promoting properties effectively inhibited *R. solanacearum* through secondary metabolites identified via LC-MS analysis, reducing disease severity and enhancing plant growth in *Solanum lycopersicum* L., with genomic insights supporting its use as a biocontrol agent and plant growth promoter [74].

2.6. Induction of Systemic Resistance

If defense mechanisms are triggered by a stimulus prior to pathogen infection, the disease may be less severe due to the ISR, which is a state of increased defensive capacity developed by a plant when adequately stimulated. This stimulus can be of microbial origin, and resistance is expressed throughout the plant, not just at the site of contact with the inducer [75].

Species of the genus *Bacillus* sensitize plant immune systems to increase protection without directly activating costly defenses [76]. *B. subtilis* IAGS174 is potentially a control agent for *Fusarium* wilt, and the methyl ester of phthalic acid produced by the bacterium is the determinant of ISR, which can effectively trigger defense responses in tomatoes (*Solanum lycopersicum* L.) [77]. Yadav and colleagues [78] identified that *B. subtilis* NBRI-W9 simultaneously activates systemic acquired resistance (SAR) and ISR against *Fusarium chlamydosporum* NBRI-FOL7, thereby increasing wilt resistance in tomato plants.

3. Importance of *Bacillus* spp. in Agriculture as Plant Inoculants or Biofertilizers

Unlike biocontrol, which encompasses a well-defined set of properties attributed to *Bacillus* spp., characteristics related to plant growth promotion have begun to be studied recently. Reports show that the association of strains of this genus with different crops can contribute to nutrient supply, production of phytohormones, or impart tolerance to various abiotic stresses. Some *Bacillus* strains effective in pathogen biocontrol can also benefit plants through growth-promoting properties, as demonstrated by *B. amyloliquefaciens* WS-10 in studies associated with tobacco (*Nicotiana tabacum* L.), with proven nutrient solubilization ability [79,80].

The benefits obtained from inoculation with *Bacillus* strains will depend on their compatibility with the crop and/or other associated microorganisms. For example, in soybean inoculated with *Bradyrhizobium* spp., the synthesis of phytohormones and molecules of biocontrol by *B. subtilis* strains PRBS-1 and AP-3 contributed to controlling seed-pathogenic fungi, improving root growth and nitrogen-fixation parameters, and increasing grain

yield [54,81]. However, the strains of *Bacillus* showed antibiosis to *Bradyrhizobium*, requiring the use of either formulated metabolites or mutants of *Bradyrhizobium* tolerant to *Bacillus* [81].

The use of *Bacillus* strains, either alone or in association with *Trichoderma*, has shown prominent results in biomass accumulation in soybean, rice (*Oryza sativa* L.), cowpea (*Vigna unguiculata* (L.) Walp), and maize crops, demonstrating their potential as a growth promoter, particularly efficient in the latter two crops, in terms of increased shoot and root dry mass [82]. Data from Nain et al. [83] support the results of Chagas et al. [82] regarding cowpea cultivation, adding the observed benefits in seed germination, leaf area, shoot and root length, number of pods, and grain yield per plant when cowpea seeds were inoculated with *Bacillus* sp. strain RM-2.

The benefits that *Bacillus* species can provide in the development of various agronomically important crops are diverse, through phosphate solubilization [84], ACC-deaminase production [85], phytohormones [86], and siderophores [87], which will be discussed below. Table 2 presents more examples of these contributions.

Table 2. Role of *Bacillus* spp. in augmenting the growth of economically important agricultural crops.

Species of *Bacillus*	Crop	Mechanism	Effect	Study
Bacillus amyloliquefaciens SQR9	*Zea mays*	Increase in soluble sugar content; efficiency of peroxidase/catalase activity and glutathione content; reduction in Na+ levels in the plant	Tolerance to saline stress	[88]
Bacillus (*Priestia*) *aryabhattai* SRB02	*Glycine max*	Production of abscisic acid, indole acetic acid, cytokinin, and different gibberellic acids	Tolerance to thermal, oxidative, and nitrosative stress	[89]
Bacillus subtilis BEB-ISbs	*Lycopersicon esculentum*	Not identified	Increase in fruit productivity and quality	[90]
Bacillus pumilus	*Lycopersicon. esculentum* cv Jinpeng 10	Adaptation of leaf gas exchange rates, stomatal density, and endogenous levels of ABA	Efficiency of water use under water deficiency	[91]
Bacillus sp. wp-6	*Triticum aestivum*	Alteration of alpha-linolenic acid metabolism, amino acids, and flavonoid synthesis	Increase in fresh weight of shoot and root	[92]
Bacillus velezensis JB0319	*Lactuca sativa*	Superoxide dismutase and lactoperoxidase activity; decrease in malondialdehyde accumulation and increase in osmotic regulator substance accumulation of proline	Increase in lettuce shoot biomass, root length, and alteration of rhizosphere bacterial community	[93]
Bacillus amyloliquefaciens RWL-1	*Oryza sativa*	Production of abscisic acid, glutamic acid, and proline	Increase in productivity and saline stress tolerance	[94]
Bacillus (*Priestia*) *megaterium* EGE-B-1.4.a; *Bacillus* (*Peribacillus*) *simplex* EGE-B-1.2.k; *Bacillus subtilis* EGE-B.24.4i; *Bacillus subtilis* EGE-B.26.1; *Bacillus* (*Priestia*) *megaterium* EGE-B.10.3.F; *Bacillus subtilis* EGE-B.3.P.5	*Lycopersicon lycopersicum* cv. Target F1; *Capsicum annuum* var. cv. Kekova F1; *Solanum melongena* cv. Faselis F1	P solubilization, IAA production; improvement in radicle and hypocotyl development; increase in plant growth, enhancing root and stem growth	Promotion of seed germination and vegetative development	[95]
Bacillus (*Priestia*) *aryabhattai* LAD	*Zea mays*	P solubilization	Increase in shoot length, total root length, and main root thickness	[96]
Bacillus cereus YL6	*Glycine max*; *Triticum aestivum*; *Brassica rapa* (Chinensis Group)	Solubilization of inorganic and organic P; production of indole-3-acetic acid (IAA) and gibberellin (GA)	Increase in soybean and wheat biomass in pot experiments; increased growth and yield of Chinese cabbage	[97]
Bacillus proteolyticus Cyn1; *Bacillus safensis* Cyn2	*Phaseolus vulgaris*	Production of NH_3, ACC deaminase, biofilm; P solubilization; secretion of catalase enzyme and siderophores;	Tolerance to abiotic stresses	[98]
Bacillus sp. LrM2	*Avena sativa*	Production of ACC deaminase and anticoxidant enzymes	Tolerance to saline stress, shoot growth, and root system development	[99]

3.1. Phosphate Solubilization

The process of phosphate solubilization involves making the nutrient phosphorus (P) available through the association of phosphate-solubilizing microorganisms in substrates containing poorly soluble sources of phosphate. In general, in tropical soils, although P may be present in the soil, it is practically unavailable for plant absorption due to its complexation affinity with metal ions [100]. Introducing phosphate-solubilizing microorganisms into the system facilitates the transformation of insoluble phosphates in the soil through various mechanisms, including the secretion of organic acids, enzyme production, and siderophore excretion—this process chelates metal ions and forms complexes, making phosphates available for plant uptake. However, although important, this latter pathway is not the primary mechanism responsible for phosphate solubilization [101].

The conversion of PO_4^{3-} (non-absorbable) to HPO_4^{2-} and $H_2PO_4^{-}$ (absorbable) primarily occurs through the release of metabolites, such as organic acids that lower the pH of the medium and release soluble phosphate [102–104]. This solubilization occurs through the respiratory pathway of direct oxidation, operating on the external surface of the bacterial cell membrane [105]. The organic acids produced release mineral P because of the exchange of the phosphate anion for the organic acid anion, and/or they can complex/chelate cations such as Fe, Al, and Ca in the rhizosphere [106]. The organic acids commonly released by phosphate-solubilizing microorganisms are gluconic acid [107,108], oxalic acid, citric acid [109], and lactic acid [110]. Most phosphate-solubilizing microorganisms belong to the genus *Bacillus* [111]. In the study conducted by Saeid et al. [112], the solubilizing exudates produced by *Bacillus* consisted of five organic acids: gluconic, lactic, acetic, succinic, and propionic.

Another pathway for P availability is through mineralization, which occurs by the production of phosphatases that cause dephosphorylation of organic P compounds in the soil, breaking the phosphoester or phosphoanhydride bonds. Many soil microorganisms can perform this function, including *Bacillus* [113,114]. Unlike phosphatases produced by plants, microbial-origin phosphatases have a higher affinity for P from organic compounds [115].

3.2. ACC-Deaminase Activity

The molecule ACC is the biochemical precursor of ethylene—a plant hormone responsible for fruit ripening, seed germination, cell expansion and differentiation, flowering, leaf and flower senescence, and fruit abscission [116].

ACC present in the roots or rhizosphere can be metabolized by bacteria producing the enzyme ACC-deaminase. This enzyme alters the pathway of ethylene formation, thereby reducing the level of this hormone in the roots. By lowering ethylene levels, ACC-deaminase prevents the inhibition of root growth and promotes overall plant growth. Additionally, by reducing ethylene production, the enzyme decreases the plant's susceptibility to various stresses, such as drought, salinity, and pathogens attacks [117–120]. Bacteria producing the enzyme are attracted to the ACC content produced by plants and released in exudates, establishing plant-bacteria interactions in the rhizosphere [121].

The contribution to stress tolerance by bacteria producing ACC-deaminase has been observed in various abiotic stress conditions, such as flooding [122], drought [123], salinity [124], flower senescence [125], metal pollution [126], and pathogens attacks [127]. For example, there are reports of the ability of *B. licheniformis* K11 to reduce ethylene concentration in pepper plants (*Capsicum annuum* L.), cleaving ACC under water stress and, therefore, promoting plant growth [128].

3.3. Production of Phytohormones

Many PGPBs synthesize plant growth-regulating hormones [129]. Indole-3-acetic acid (auxin or IAA) is an example of a phytohormone that controls a wide range of functions in plant development and acts as a key component in root architecture formation, through differentiation of root vascular tissue, regulation of lateral root initiation, polar positioning of root hairs, and root gravitropism [130]. However, when phytohormones are present in

high concentrations, the effects on plants can be deleterious [131], as indicated by the study of Silva et al. [132], where a few days after the application of the herbicide 2,4-D (an auxin mimetic) at the V4 and V6 stages, visual symptoms of toxicity were observed in the aerial part of soybean plants in treatments with doses equal to or greater than 20 g/ha.

Nevertheless, generally, the synthesis of IAA by PGPB does not harm the development of the associated crop. This fact can be explained due to the intimate relationship formed between the plant and the PGPB. Many PGPBs capable of synthesizing IAA are dependent on tryptophan—the precursor amino acid of IAA—which can be produced by the plant in association [133]. However, when PGPBs with a high capacity to synthesize IAA are applied in inoculation at high cellular concentrations, a decrease in plant growth can be observed, indicating the need for further investigation [134].

The production of IAA by *Bacillus* strains can contribute to the development of different crops. For example, the rooting efficiency of kiwifruit (*Actinidia deliciosa* Planch.) stem cuttings was favored in the presence of *Bacillus* sp. strain RC03 and *Bacillus* (*Peribacillus*) simplex strain RC19, due to the production of IAA, maximizing the yield of rooted clonal cuttings in nurseries and allowing the reduction in the use of synthetic/chemical rooting products [135]. Another example was reported in the cultivation of strawberries (*Fragaria* × *ananassa* Duch.) by Chebotar et al. [136], where *B. velezensis* BS89 contributed significantly to the production of IAA, reflecting on the development and yield of the plants. For soybean, the synthesis of IAA and abscisic acid (ABA) by *B. subtillis* strains PRBS-1 and AP-3 improved root growth parameters [54].

3.4. Production of Siderophores

Despite their contribution to biological control, the production of siderophores can also contribute to plant nutrition, as some organisms can form biogenic chelators to complex the predominant oxidized form of iron (Fe^{3+}) present in the aerobic environment and alter the bioavailability of Fe [137]. By this process, the nutrient that was previously unavailable can be reduced to Fe^{2+} and assimilated by both plants and some microorganisms [138].

The synthesis of siderophores such as pyoverdine, hydroxamates, and ferrioxamines by microorganisms in the rhizosphere region can contribute to up to a threefold increase in the efficiency of iron transport during root growth and plant meristem development [139].

In addition to iron availability for plants, siderophore production may also be related to the solubilization of iron phosphate, due to the affinity and formation of Fe chelators, releasing phosphate [140]. For example, *Bacillus* sp. WR12, capable of producing siderophores, significantly increased root length and dry mass, leaf chlorophyll content, and Fe content in wheat (*Triticum aestivum* L.) seedlings [141].

4. *Bacillus* spp. in Brazil: A Successful Case in Agriculture

Soybeans have played, and still play, a fundamental role in the development and advancement of the use of bio-inputs in Brazil. The biological nitrogen fixation with *Bradyrhizobium* spp. and the biological control of soybean caterpillars with *B. thuringiensis* stand out as the first successful cases [142,143].

The relationship between the soybean crop and the use of bio-inputs in Brazil is evident not only historically but also in practical terms. The demand for sustainable agricultural management has driven the search for solutions based on beneficial microorganisms, such as those of the genus *Bacillus*. These bio-inputs not only have contributed to pest and disease control but have also promoted plant growth. The application of *Bacillus*-based technologies in Brazilian agriculture exemplifies the synergy between the need for more sustainable agricultural practices and the rich history of soybean culture as a driver of innovation in the national agricultural sector [144].

Bacillus-based inoculants are particularly interesting due to their ability to form spores that can persist in fields for long periods under different climatic conditions, and they can also be produced and stored for longer periods than non-spore-forming bacteria, allowing longer marketing periods [145,146]. Despite *Bacillus* being the most abundant genus in

the rhizosphere [147], for agricultural crops, the concentration of colony-forming units (CFUs) needed to benefit yields is obtained through the annual supply of bio-inputs. Some examples of products marketed in Brazil based on *Bacillus* are listed in Table 3. It is worth emphasizing that the Brazilian market of bio-inputs based on *Bacillus* for biocontrol represents over 80% of all doses commercialized [21,22], with applications estimated in about 40 million hectares. Additionally, the new market of *Bacillus* bio-inputs for plant growth promotion has reached over 6 million hectares in only five years. Overall, the Brazilian market of bio-inputs expects an increase of 110% in the next five years.

The intimate relationship between bacteria and plants provides mutual benefits for both organisms. A single plant species can benefit from various growth promotion mechanisms, which can be expressed by different bacterial genera or species. For example, the association of different *Bacillus* species in a single formulation has shown synergy, as in the case of the composition containing *B. pumilus* CCTB05, *B. subtilis* CCTB04, and *B. amyloliquefaciens* CCTB09, registered as a plant growth-promoter inoculant [148].

Bacillus spp. were evaluated for their ability to enhance sugarcane (*Saccharum* spp.) growth by improving P availability. Selected strains, including *B. licheniformis* MGB2281, showed promising results in phosphatase activity and plant growth, highlighting its potential as an effective inoculant for sustainable agriculture [149]. Also, Castelo Souza et al. [150] highlighted the potential of *Bacillus* (*Priestia*) *aryabhattai* to enhance crop resilience in semi-arid tropical regions of Brazil. The strains promoted leaf gas exchange efficiency and plant growth, and helped to mitigate salt and water stress in maize [150].

Bacillus strains isolated from cotton (*Gossypium hirsutum* L.) roots in Brazilian fields showed potential in promoting plant growth by improving physical, phytochemical, and macronutrient parameters in cultivars FM 985 and TMG 47; the strains enhanced plant height, biomass, root volume, and various biochemical markers [151]. In addition, the use of the *B. amyloliquefaciens* strain FZB45 (Phosbac product) was found to significantly increase shoot dry mass, root dry mass, and grain yield in both maize and soybean; the increases showed a positive correlation with P uptake by the crops [152].

The most successful example of *Bacillus* spp. used as PGPB in Brazil is relatively recent. The formulation containing *B. subtilis* strain BRM 2084 and *B.* (*Priestia*) *megaterium* strain BRM 119, has a proven capacity for phosphate solubilization, synthesizing phytohormones, and other mechanisms that facilitate nutrient absorption [153]. The product has been shown to be beneficial for the growth and nutritional and physiological status of crops such as the common bean (*Phaseolus vulgaris*), maize, soybeans, and sugarcane. In addition, these strains enhance maize productivity and P uptake via seed treatment. Oliveira-Paiva and colleagues [154] evaluated their efficacy in diverse Brazilian soils, emphasizing their roles in P solubilization, indole-3-acetic acid production, biofilm formation, and enzymatic activities, while showing compatibility with other beneficial microorganisms. These results are corroborated by recent studies by Souza et al. [155], De Sousa et al. [156], Oliveira-Paiva et al. [157], and as exemplified in Figure 2, in the study by Oliveira et al. [158]. The first commercial bio-input carrying these two strains was released for the maize crop in 2019, and it has already been registered for use in maize, soybean, common bean, and sugarcane.

Due to substantial yield increases and other significant benefits driven by *Bacillus* species, important cost reductions can be experienced in various cropping systems. For example, bio-input with *B. cereus* promoted the growth of coconut (*Cocos nucifera* (L.) Arecaceae) seedlings, enhanced seedling quality, and reduced nursery time, reducing costs by 10%, and highlighting its economic efficiency [159]. The study by Oliveira et al. [158] found that the productivity gains from inoculation exceeded costs in most locations evaluated. Maize showed increased productivity in all sites, while soybeans had gains in 175 out of 181 locations, with average increases of 8.6% for maize and 6.3% for soybeans (Figure 2).

Table 3. Main bio-inputs based on *Bacillus* spp. commercialized in Brazil in 2023.

Species of *Bacillus*	Commercial Name	Mechanism	Crop	Marketed by
Bacillus subtilis CNPMS B2084 (=BRM034840) *Bacillus* (*Priestia*) *megaterium* CNPMS B119 (=BRM033112)	Biomaphos	Phosphate solubilizer	*Zea mays*; *Glycine max*	Bioma
Bacillus subtilis BRM 2084 *Bacillus* (*Priestia*) *megaterium* BRM 119	Solubphos	Phosphate solubilizer	*Zea mays*; *Glycine max*	Simbiose
Bacillus subtilis CNPMS B2084 (=BRMO34840) *Bacillus* (*Priestia*) *megaterium* CNPMS B119 (=BRMO33112)	Omsugo P	Phosphate solubilizer	*Glycine max*	Corteva
Bacillus subtilis CNPMS B2084 (-BRMO34840) *Bacillus* (*Priestia*) *megaterium* CNPMS B119 (=BRMO33112)	Omsugo Eco	Phosphate solubilizer	*Saccharum officinarum*	Corteva
Bacillus licheniformis CCTB07	Bioprince	Growth promoter	*Zea mays*	Biotrop
Bacillus pumilus CCTB05 *Bacillus subtilis* CCTB04 *Bacillus amyloliquefaciens* CCTB09	Biotrio	Growth promoter	*Zea mays* *Glycine max* *Lactuca sativa*	Biotrop
Bacillus subtilis BV09	Biobaci	Microbiological nematicide	Any crop with the following targets: *Meloidogyne incognita*, *Meloidogyne javanica*, *Meloidogyne exigua*, *Meloidogyne paranaensis*, *Pratylenchus zeae*, and *Fusarium oxysporum*	Vittia
Bacillus amyloliquefaciens UMAF6614	Veranejo	Microbiological nematicide	Any crop with the following targets: *Meloidogyne incognita*, *Meloidogyne javanica*, and *Pratylenchus zeae*	Koppert
Bacillus amyloliquefaciens SIMBI BS 10 CCT 7600	Nemacontrol	Microbiological nematicide	Any crop with the following targets: *Heterodera glycines*, *Meloidogyne exigua*, *Meloidogyne incógnita*, *Pratylenchus brachyurus*, and *Sclerotinia sclerotiorum*	Simbiose
Bacillus pumilus CNPSo 3203	Caravan	Microbiological fungicide	Any crop with the following targets: *Septoria glycines*, *Corynespora cassiicola*, and *Cercospora kikuchii*	Koppert
Bacillus amyloliquefaciens FZB45	Phosbac	Phosphate solubilizer	*Zea mays*; *Glycine max*	Andermatt
Bacillus (*Priestia*) *aryabhattai* CMAA 1363	Auras	Growth promoter (tolerance to drought)	*Zea mays*; *Glycine max*	NOOA Ciência e Tecnologia Agrícola

Another notable success is the use of *B. (Priestia) aryabhattai* (strain CMAA1363). When applied as a seed treatment to maize, this microorganism significantly enhanced plant growth and productivity, with increases ranging from 5.9% to 43.7%, proving to be an effective inoculant to the crop [160]. Noteworthy, the strain was released and announced as a *Bacillus* that increases plants' tolerance to drought.

In relation to the comparative performance of *Bacillus*-based biopesticides with other biologicals and chemical products, in studies performed outside Brazil, the application of *B. velezensis* strain BUZ-14 to grafted grapevine (*Vitis vinifera* L.) had a remarkable protective effect against vascular necrosis caused by *Neofusicoccum parvum* and *Diplodia seriata*, outperforming traditional products based on *Thichoderma harzianum* and *Trichoderma atroviride* [161]. Compared to commercial fungicides, *B. velezensis* BUZ-14 emerged as a highly effective biocontrol agent. Similarly, *B. amyloliquefaciens* Bac 28.3 exhibited comparable efficacy to commercial fungicides in controlling *Botrytis cinerea* in tomatoes, highlighting it as a promising alternative [162].

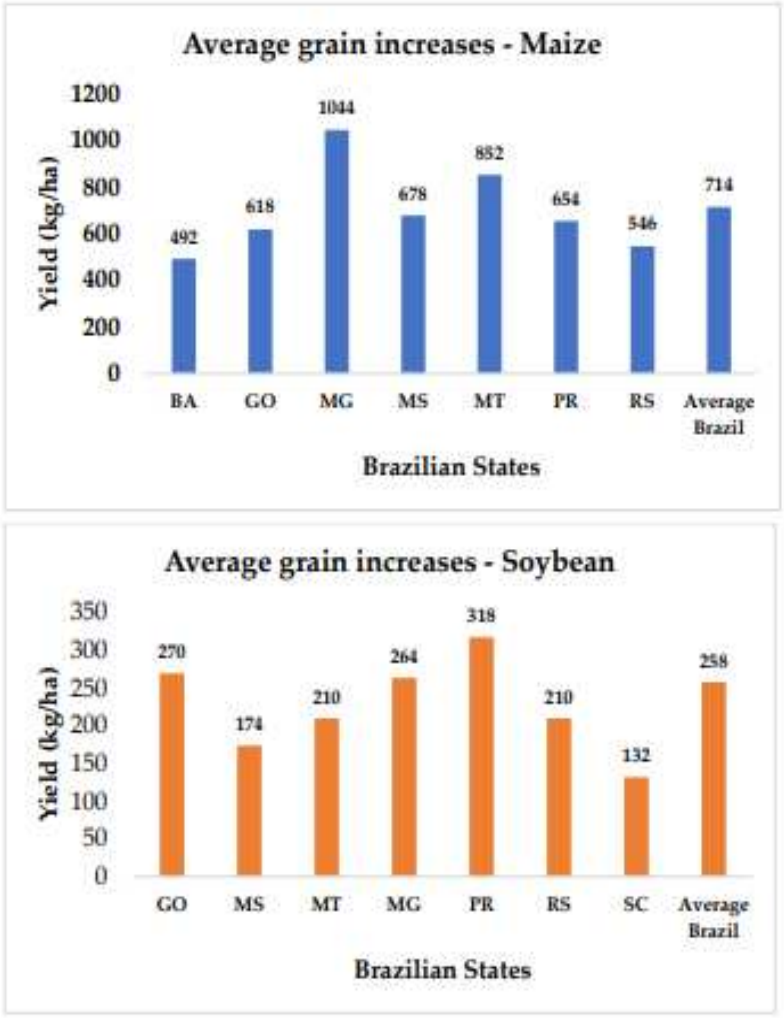

Figure 2. Average increase in maize and soybean grain yields (kg/ha) due to the inoculation with *Bacillus subtilis* strain CNPMS B2084 and *Bacillus* (*Priestia*) *megaterium* strain CNPMS B119. Trials performed in Brazilian states (BA, Bahia; GO, Goiás; MG, Minas Gerais; MS, Mato Grosso do Sul; MT, Mato Grosso; PR, Paraná; RS, Rio Grande do Sul; SC, Santa Catarina), in two crop seasons (2018/2019 and 2019/2020). Adapted from Oliveira et al. [158].

5. Future Perspectives and Biosafety Measures for the Use of *Bacillus* in Agriculture

The market value of *Bacillus*-based bio-inputs was estimated to be at least USD 18 billion in 2020, with expectations for continued growth in the coming years, particularly in the agricultural sector [163]. The use of *Bacillus* spp. in agriculture is expanding, not only for biological control of pests and diseases but also for promoting plant growth due to a variety of mechanisms, with an emphasis on plant nutrition and tolerance to drought. As research advances, new strains of *Bacillus* are expected to be discovered and developed, further enhancing their applications in various crops and environmental conditions. Biotechnology can also play a crucial role in optimizing existing strains, increasing their effectiveness and adaptability, and new biotechnological tools such as genomic editing may be promising to obtain elite strains.

The potential of *Bacillus* species is vast and continues to be explored, both in the prospecting of new species and strains and in the evaluation of their compatibility with crops grown on a large scale. This field of research offers promising prospects for the discovery of new applications and for deepening the understanding of interactions between bio-inputs based on *Bacillus* and crops of interest. However, it is essential to pay attention to the dynamics of these organisms in real environmental conditions and how species will adapt and perform their expected functions. This includes understanding how beneficial members interact with each other to establish a stable community and enhance their collective performance.

When using *Bacillus* species in agriculture, it is important to implement minimum biosafety measures to ensure safety and efficacy. First, rigorous strain identification and characterization should be conducted to confirm the absence of pathogenic or harmful traits. Second, environmental risk assessments must be performed to evaluate potential impacts on non-target organisms and ecosystems. Third, compliance with regulatory guidelines and obtaining necessary approvals from relevant authorities are essential. Additionally, proper handling, storage, and application procedures should be established to prevent contamination and ensure consistent results. Continuous monitoring and post-application assessments are recommended to track the long-term effects and efficacy of *Bacillus*-based products. Implementing these biosafety measures helps ensure that *Bacillus* applications contribute positively to sustainable agriculture without compromising environmental or human health.

It is also important to highlight that questions about the cost comparison of biological and chemical treatments are often raised. In Brazil, the costs of bio-inputs toward plant nutrition are always lower than chemical fertilizers, which are mostly imported. In general, costs are also lower for biopesticides. However, even if there were no differences in costs of applying bio-inputs based on *Bacillus* in the replacement of pesticides or chemical fertilizers, the environmental benefits should also be valued and certainly are always highly favorable to the biologicals.

As new discoveries emerge in this field, regulatory frameworks play a crucial role in promoting the use of these less harmful options for chemical fertilizers and pesticides. Studies and reviews conducted in various regions, such as the work by Nihorimbere and colleagues [164] focusing on the African context, underscore the necessity for favorable regulatory frameworks to facilitate the adoption of these technologies, particularly in underdeveloped and developing regions worldwide. These frameworks not only ensure safe and effective deployment of biocontrol agents and sustainable agricultural practices, but also foster innovation and economic development in agriculture.

6. Conclusions

Bacillus spp. can greatly contribute to agricultural production by promoting environmentally sustainable practices. The use of *Bacillus* in biocontrol to reduce the incidence of pests and diseases has been well-known for a long time, with many commercial bio-inputs available at the market. The benefits related to plant growth promotion by *Bacillus* are more recent and not yet widely spread. Considering the biotechnological potential to produce

phytohormones, improve nutrient availability, and act as a biological control offered by the genus, it is pertinent to prospect new strains and develop new bio-inputs, expanding their use to several crops. The use of *Bacillus*-based formulations as multifunctional bio-inputs, for both biological control and plant growth promotion represents an innovative approach that may have great success at the market.

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CONCLUSÕES

A disponibilização de metodologias padronizadas e reprodutíveis para a prospecção de microrganismos de interesse agrícola constitui um avanço estratégico para o desenvolvimento de novos bioinsumos. Ao consolidar técnicas que permitem avaliar características-chave relacionadas à promoção de crescimento vegetal e à resiliência frente a estresses, este documento contribui para acelerar a integração de novas bactérias em programas de inovação biotecnológica.

A seleção e o uso de microrganismos adaptados a condições adversas, como restrição hídrica e altas temperaturas, tornam-se cada vez mais estratégicos diante do cenário das mudanças climáticas. O aproveitamento do potencial funcional de coleções microbianas contribui diretamente para o desenvolvimento de novos bioinsumos capazes de promover a resiliência dos sistemas agrícolas e garantir a produtividade de forma sustentável.

A capacidade de crescimento das estirpes bacterianas em meio com baixa atividade de água e sua correlação com o desempenho de plantas de milho submetido a déficit hídrico valida o uso desse ensaios *in vitro* como um método preditivo eficiente para a seleção preliminar de microrganismos com potencial para mitigar os efeitos da restrição hídrica em condições *in vivo*.

As estirpes do gênero *Bacillus* se destacaram entre os microrganismos avaliados, com maior frequência e intensidade de características agronomicamente relevantes, como tolerância à seca e altas temperaturas e produção de enzimas hidrolíticas. Esses resultados corroboram a literatura e evidenciam o papel central do gênero *Bacillus* no desenvolvimento de tecnologias voltadas à agricultura regenerativa e ao manejo de estresses múltiplos. As estirpes do gênero *Pseudomonas* apresentaram destaque nas análises *in vitro*, com alta frequência de resultados positivos para solubilização de fosfato de cálcio, produção de sideróforos e EPS. Esse desempenho indica o potencial do gênero, assim como das estirpes estudadas para essas características.

Além das propriedades associadas à promoção de crescimento de plantas, algumas estirpes bacterianas podem demonstrar atividade antagonista contra fitopatógenos, indicando potencial para uso em estratégias de biocontrole.

Essa capacidade amplia o escopo de aplicação das BPCP, possibilitando o desenvolvimento de bioinsumos multifuncionais, que atuem tanto na indução do crescimento vegetal, por meio de diferentes mecanismos, quanto na proteção das culturas.

Ensaios em campo, em diferentes condições e culturas, além de estudos complementares de caracterização funcional e estabilidade, são essenciais para a confirmação dos benefícios potenciais indicados pelas análises *in vitro* e em condições controladas de casa de vegetação, permitindo o desenvolvimento de bioinsumos eficazes, seguros e adaptados às condições reais de cultivo.