Universidade Estadual de Londrina

LUANA RAINIERI MASSUCATO

ISOLAMENTO E CARACTERIZAÇÃO DE BACTÉRIAS SOLUBILIZADORAS DE FOSFATO E SEU EFEITO NAS CULTURAS DO MILHO E SOJA

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Tese de Doutorado apresentada ao Programa de Pós Graduação em Agronomia da Universidade Estadual de Londrina, como requisito parcial à obtenção do título de Doutor em Agronomia.

Orientador: Prof. Dr. Leandro Simões Azeredo Gonçalves

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A minha amada avó Ivanilde, minha intercessora
em vida terrena e espiritual.

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RESUMO

O fósforo é um macronutriente essencial para o desenvolvimento das plantas, no entanto, sua fixação no solo dificulta a absorção deste nutriente e, portanto, se torna necessário que uma grande quantidade de fertilizante fosfatado seja aplicada para que as culturas atinjam sua produtividade máxima. O uso de fertilizantes químicos é de alto custo, causa diversos prejuízos ao meio ambiente e para a atividade biológica do solo. Os microorganismos presentes no solo são fundamentais para manter o equilíbrio do meio, além melhorar a disponibilidade de nutrientes, auxiliar na defesa da planta ao aumentar o crescimento e desenvolvimento vegetal. Dentre as bactérias promotoras do crescimento vegetal, os gêneros *Bacillus* spp. e *Lysinibacillus* spp. tem mostrado capacidade promoção de crescimento e solubilização de fosfato. Diante disso, o objetivo deste trabalho foi identificar microorganismos que auxiliem na solubilização do P e na melhoria da atividade biológica do solo, visando encontrar bactérias que possibilitem a formulação de inoculantes microbianos para as culturas do milho e soja. Com base nessas características, 24 linhagens foram selecionadas para serem posteriormente avaliadas em condições de laboratório, casa de vegetação e campo. Dentre as cepas selecionadas, quatro (I04, I12, I13 e I17) apresentaram alto potencial para aumentar o crescimento radicular do milho e o teor de P da parte aérea. As cepas I13 (ag87) e I17 (ag94) foram identificadas por sequenciamento genômico como *Bacillus megaterium* e *Lysinibacillus* sp., respectivamente. Essas cepas apresentaram maiores incrementos de produtividade em relação ao tratamento controle com 30% P. Além disso, a combinação de ag87 e ag94 resultou em ganhos de produtividade ainda maiores, indicando um efeito sinérgico. As cepas previamente estudadas e consolidadas no milho, foram incorporadas às sementes de soja, e semeadas a campo, em Londrina, Maringá, Guarapuava e Entre Rios. A produtividade e a eficiência do uso de fósforo (EUP) foram avaliadas. As cepas ag87 e ag94 mostraram-se uma opção viável para inoculação de sementes de soja, aumentando a produtividade de grãos e a eficiência do uso do fósforo em adubações com baixa dose de P.

Palavras-chave: Bactérias promotoras do crescimento vegetal; *Bacillus* sp.; *Lysinibacillus* sp.; solubilização de fosfato; *Zea mays L.; Glycine max (*L.) Merril.

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ABSTRACT

Phosphorus is an essential macronutrient for plant development, however, its fixation in the soil makes it difficult to absorb this nutrient and, therefore, it is necessary that a large amount of phosphate fertilizer be applied so that crops reach their maximum productivity. The use of chemical fertilizers is expensive, causes several damages to the environment and to the biological activity of the soil. The microorganisms present in the soil are essential to maintain the balance of the environment, in addition to improving the availability of nutrients, helping to defend the plant by increasing plant growth and development. Among the plant growth-promoting bacteria, the genera Bacillus spp. and Lysinibacillus spp. has shown ability to promote growth and solubilization of phosphate. Therefore, the aim of this work was to identify microorganisms that help in the solubilization of P and in the improvement of the biological activity of the soil, aiming to find bacteria that allow the formulation of microbial inoculants for corn and soybean crops. Based on these characteristics, 24 lines were selected to be further evaluated under laboratory, greenhouse and field conditions. Among the selected strains, four (I04, I12, I13 and I17) showed high potential to increase corn root growth and shoot P content. Strains I13 (ag87) and I17 (ag94) were identified by genomic sequencing as Bacillus megaterium and Lysinibacillus sp., respectively. These strains showed greater increases in productivity compared to the control treatment with 30% P. In addition, the combination of ag87 and ag94 resulted in even greater productivity gains, indicating a synergistic effect. The strains previously studied and consolidated in corn were incorporated into soybean seeds and sown in the field in Londrina, Maringá, Guarapuava and Entre Rios. Productivity and phosphorus use efficiency (PEU) were evaluated. The strains ag87 and ag94 proved to be a viable option for inoculation of soybean seeds, increasing grain yield and the efficiency of phosphorus use in low-dose P fertilization.

Keywords: Plant growth promoting bacteria; *Bacillus* sp.; *Lysinibacillus* sp.; phosphate solubilization; *Zea mays L.; Glycine max (*L.) Merril.

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1 INTRODUÇÃO

A demanda por alimento tem aumentado cada vez mais com o passar dos anos, exigindo que as culturas sejam cada vez mais produtivas. A produtividade das culturas está, entre outros fatores, ligada à disponibilidade de nutrientes para seu pleno desenvolvimento. Dentre os nutrientes exigidos pelas plantas, o fósforo desempenha papel fundamental na morfologia e fisiologia vegetal. No entando, é o mineral com menor disponibilidade, o que está relacionada à sua baixa mobilidade no solo.

Devido à dificuldade em tornar o fósforo disponível para a absorção pela planta, os fertilizantes fosfatados são utilizados em grandes quantidades, onerando o custo de produção. Além disso, o excesso de fertilizantes fosfatados químicos tende a transformar o solo em alcalino e salino; a alcalinidade e a salinidade aumentam a precipitação dos fosfatos solúveis disponíveis, mudando suas propriedades físicas e químicas do solo. A utilização exacerbada deste fertilizante químico traz danos irreparáveis ao meio ambiente, como a eutrofização das águas, prejudicando sua biodiversidade e qualidade. Portanto, a procura por métodos que auxiliem a disponibilização de fósforo para as plantas sem que haja danos ambientais, econômicos e produtivos, é fundamental para a manutenção da capacidade produtiva das culturas.

Bactérias promotoras de crescimento vegetal (BPCVs) tem se mostrado importantes aliados na solubilização de fosfato insolúvel nas áreas de cultivo. Estudos mostram que BPCVs têm a habilidade de induzir o crescimento radicular, aumentando a área de absorção de nutrientes, além de converter fosfato mineral em fosfato solúvel, produzindo ácidos orgânicos, ácido málico, ácido acético, ácido oxálico, ácido cítrico e ácido guconico (VESSEY et al, 2004; TAHIR et al., 2013. SOLANGI et al., 2016) e enzimas fitases que permitem uma melhor ciclagem do fosfato orgânico, aumentando a degradação da matéria orgânica do solo. Bactérias do gênero *Bacillus spp.* se mostram vantajosas para a utilização como bioinoculantes pois, além de trazerem vantagens morfológias e fisiológicas que possibilitam maior desempenho vegetal, esse gênero produz estruturas de sobrevivência, como endósporos que possibilitam que a bactéria resista no ambiente mesmo em condições desfavoráveis, tornando-as mais tolerantes à possíveis estresses, como altas temperaturas, salinização e alcalinização do solo.

A utilização de inoculantes tem sido cada vez mais explorada devido suas inúmeras vantagens ambientais, econômicas e microbiológicas. Espécies como *Bacillus subitilis* e *B. megathelium* são alvo de estudos sobre sua contribuição na eficiência de uso do fosfato insolúvel presente nas áreas de cultivo (Bonten et al, 2014; Oliveira et al, 2008; Prabhu, Borkar e Garg, 2018; Mohamed, Farag e Youssef, 2018). Outros gêneros têm sido estudados quanto ao seu potencial de solubilização de fosfato e promoção de crescimento com o objetivo de produção de bioinoculantes para os principais cultivos, entretanto, são escassos os inoculantes adaptados para regiões tropicais. O objetivo do presente trabalho foi identificar cepas bacterianas com capacidade de promoção do crescimento vegetal e eficiência na solubilização do fosfato, buscando o desenvolvimento de bionoculantes para as culturas do milho e soja.

2 REVISÃO DE LITERATURA

2.1 CULTURA DO MILHO

A cultura do milho (*Zea mays* L.) se destaca há décadas no cenário agrícola brasileiro. Sua importância está relacionada a versatilidade de uso na alimentação humana, na indústria, como na produção de etanol, ou na alimentação animal, na forma de grão, forrageira ou transformado em ração (DUARTE et al., 2018). Da produção total de milho no Brasil estimada de 87,1 milhões de toneladas na safra 2020/2021, aproximadamente 70% são representadas pelo milho segunda safra (CONAB, 2022). Esses dados refletem a importância da implementação da safrinha, que, em conjunto com o incremento das exportações ao longo da última década, foram fundamentais para alavancar a produção brasileira (KIST et al., 2018). Os Estados Unidos é o maior produtor do grão, seguido pela China, e pelo Brasil ocupante da terceira posição no ranking, sendo que as maiores produções nacionais se concentram nos estados do Mato Grosso e Paraná (CONAB, 2022).

O gênero *Zea* é um grupo de gramíneas anuais e perenes nativas do México e América Central e inclui os teosintes, entre eles as espécies *Zea parviglumis* e *Z. luxurians,* consideradas atualmente como as ancestrais silvestre do milho domesticado (ALVES, 2019). Evidências indicam que o centro de origem e a domesticação do milho ocorreu na América Central há aproximadamente 9000 anos (DOEBLEY, 2004). O fluxo gênico foi fundamental para seu processo de evolução, sendo a seleção artificial realizada pelo homem responsável pela escolha de que mantivessem os grãos na espiga, descartando os que se desprendiam facilmente (ALVES, 2017). O milho sofreu diversas modificações com a finalidade de selecionar e multiplicar características que atendessem à demanda populacional, além de visar o desenvolvimento de cultivares com maior quantidade de proteína, resistência a pragas ou adaptados a regiões não produtoras do grão (KISTLER et al., 2018). A alta variabilidade genética da espécie permite que o cultivo ocorra em variados ambientes, podendo se mostrar adaptado à diversos tipos de estresses bióticos e abióticos (ALVES, 2017).

O ciclo do milho é dividido em estádios fenológicos vegetativos (V) e reprodutivos (R). No estádio V12, a disponibilidade de nutrientes é decisiva para a confirmação da produção e do rendimento da cultura, pois se definem o tamanho da espiga e número de grãos por fileira. O aparecimento do pendão ocorre no estádio VT, que marca a transição para o estádio reprodutivo do milho (KIANI et al., 2017).

Os micronutrientes e macronutrientes são determinantes no desempenho da cultura, ainda que o milho seja eficiente na absorção de tais elementos, devido seu sistema radicular bem desenvolvido, o acúmulo de nutrientes ainda é um fator limitante para a manutenção da nutrição das plantas (GRIGOLLI et al., 2016). Dentre os macronutrientes, o fósforo se destaca, já que a maior parte das áreas agrícolas do Brasil é deficiente deste nutriente devido a sua fixação no solo, principalmente em áreas com alto teor de argila (FIERER, 2017).

2.2 CULTURA DA SOJA

A soja (*Glycine max* (L.) Merrill) é uma planta originária da Ásia, cultivada em praticamente todo o mundo e considerada uma das culturas com maior importância econômica no território brasileiro. O Brasil, seguido dos Estados Unidos, são os maiores produtores à nível mundial, com produção de 125,552 (CONAB, 2022) e 123,31 milhões de toneladas (USDA, 2022), respectivamente.

A soja cultivada é uma planta herbácea, que pertence à família Fabaceae, subfamília Faboideae, gênero Glycine L., espécie *max,* com ciclo de desenvolvimento com duração de 90 a 150 dias (SEDIYAMA et al., 2009). O sistema radicular é classificado como pivotante e apresenta quantidade considerável de raízes secundárias (ARVIDSSON; HAKANSSON, 2014). O caule é ramificado e pubescente e suas folhas se apresentam em três tipos diferentes conforme o desenvolvimento da cultura: cotiledonares, unifolioladas e trifolioladas (ALVES, 2018). As folhas unifolioladas possuem apenas um folíolo que se insere do lado oposto do primeiro nó, já as trifolioladas são compostas por três folíolos inseridos nos nós da haste principal, localizado acima das unifolioladas, com disposição alternada e ordenadas em duas fileiras (ZANON et al., 2018).

Por ser uma planta autógama suas flores são hermafroditas e desenvolvem em rácemos terminais ou axilares, com coloração branca, púrpura ou roxa, e originam frutos do tipo legume, que são pubescentes, deiscentes, alocam de 1 a 5 sementes e sua cor pode variar entre amarelo-palha, cinza-claro ou cinza-escuro (ZANON et al., 2018). Os cultivares de soja são classificados quanto ao seu hábito de crescimento, os quais podem ser do tipo determinado, que é caracterizado pela interrupção do crescimento vegetativo a partir do florescimento; ou indeterminado, no qual esse crescimento não cessa com o início do florescimento (LOPES; LIMA, 2015). Esse último possibilita a variação da época de semeadura, assim a mesma pode ser antecipada ou retardada após o período recomendado nas regiões do Sul do Brasil (ZANON et al., 2016).

A escala fenológica caracteriza as plantas de soja de acordo com seu estádio de desenvolvimento, o que auxilia a comunicação entre o consultor técnico e o produtor rural nas recomendações de manejo dos tratos culturais, pois permite uma padronização na identificação das etapas da cultura. A descrição dessas etapas fundamenta-se na escala proposta por Fehr e Caviness (1977), que é a mais utilizada, onde há uma classificação em dois estádios, denominados de vegetativo (V) e reprodutivo (R), que são subdivididos numericamente a fim de especificar detalhadamente tais fases, no entanto, as duas primeiras são categorizadas como VE e VC, indicando o período de emergência e cotilédones, respectivamente.

2.3 ESTRESSE ABIÓTICO

O estresse é uma condição causada pelo ambiente que limita o crescimento e desenvolvimento das plantas, resultando em redução da produtividade. As consequências do estresse para o crescimento da planta dependem da severidade (intensidade) do fator causador do estresse, bem como da duração da perturbação, do número de exposições da planta ao fator de estresse durante seu ciclo de cultivo, do estádio de desenvolvimento da planta e do genótipo selecionado (GRATÃO et al., 2015; LIANG et al., 2018).

Os estresses podem ser de ordem biótica ou abiótica. Os fatores bióticos englobam ataques de pragas e doenças, como fungos, bactérias e vírus. Entretanto, os fatores abióticos são os que afetam a produtividade (HOGARTH, 2015). Isso ocorre porque a cultura está exposta à combinação de vários fatores abióticos, como a deficiência ou excesso hídrico, altas temperaturas, altos níveis de radiação solar, baixas temperaturas, deficiência nutricional ou até mesmo exposição à metais pesados (WANG et al., 2017).

As plantas desenvolvem diversas estratégias de defesa em situações de estresse. A primeira estratégia adotada é o aumento da taxa de respiração, que tem como finalidade manter os processos de manutenção fisiológica e reparo de danos celulares. Entretanto esta etapa resulta em um alto custo energético. A segunda estratégia é a tolerância ao estresse; neste caso, a planta possui maior plasticidade, convivendo com os fatores de estresse com menor gasto energético, promovendo recuperação mais eficiente após a perturbação. A terceira estratégia é consiste em evitar que ocorra o estresse, por exemplo, investindo previamente na formação de um sistema de raízes profundo e ramificado, que será mais eficiente na absorção de água e nutrientes (GRATÃO et al., 2015; WANG et al., 2017). Dependendo do material genético, estas três estratégias assumem diferentes papéis durante o ciclo da cultura, em função da variabilidade do ambiente de produção. De forma geral, podemos entender que a variação da produção de uma cultura depende do genótipo do material, que estabelece o nível de potencial máximo de produção; do ambiente de produção, que impõe limites ao desenvolvimento da expressão do potencial da cultura; e das respostas fisiológicas das plantas aos desafios impostos pelo ambiente (ALVES et al., 2018).

A compreensão de como os fatores ambientais podem induzir as plantas ao estresse é fundamental para o estabelecimento de manejo adequado da cultura e para a escolha do cultivar mais apropriado à região de produção. O que pode ser uma tarefa complexa, visto que normalmente as plantas estão expostas a diversos fatores de estresse, envolvendo a necessidade de técnicas adequadas para o monitoramento constante do estado fisiológico das culturas ao longo de seu ciclo (ASHRAF et al., 2015; KHALID et al., 2017; RADHAKRISHNAN; BAEK, 2017).

Na cultura do milho, a combinação dos fatores de estresse se dá de maneira diferente, em cada época de cultivo. Na primeira safra, a combinação das altas temperaturas do ar, alta irradiação e, em algumas situações, a baixa disponibilidade de água são os principais fatores causadores de estresse. Já no cultivo de segunda safra, a combinação das baixas temperaturas do ar, baixa disponibilidade de água, baixa umidade relativa do ar e os baixos níveis de irradiação reduz o desenvolvimento e a produtividade da cultura. Em termos globais, a falta de água é responsável pelas maiores perdas de produtividade da cultura (TARDIEU et al., 2018).

O impacto do déficit hídrico nas plantas gera um sinal para as folhas via ácido abscísico (ABA) pela corrente transpiratória, induzindo ao fechamento dos estômatos, de modo a reduzir a perda de água através da transpiração. Entretanto, esse mecanismo gera um *trade off* no organismo vegetal, pois enquanto o fechamento estomático auxilia na redução de perda de água, também ocasiona uma menor assimilação de CO_2 atmosférico e, com isso, as taxas fotossintéticas são reduzidas (NOURI et al., 2017). A falta de água também afeta processos bioquímicos relacionados a redução da taxa fotossintética, que ocorrem pela degradação ou inativação das enzimas responsáveis pela fixação de CO_2, como a PEPcase (fosfoenolpiruvato carboxilase, específica de plantas de metabolismo tipo C4, como o milho) e a Rubisco (ribulose bifosfato carboxilase/oxigenase) (PELEGRINI et al., 2013). Em situações severas de déficit hídrico combinado com altos níveis de irradiação solar, a eficiência fotoquímica pode ser afetada pela fotoinibição do fotossistema II, reduzindo a eficiência do transporte de elétrons, em virtude do excesso de elétrons e da degradação da membrana dos cloroplastos (ZANDALINAS et al., 2017).

O efeito da temperatura sobre o desenvolvimento das culturas varia em função da época de cultivo e, também da altitude da região cultivada. As temperaturas altas causam o aumento da respiração noturna; neste caso, ocorre o aumento da respiração de manutenção das plantas e parte dos fotoassimilados (que seriam utilizados na respiração para suprir a energia para o crescimento) são redirecionados para o reparo e na manutenção do metabolismo da planta, de modo a evitar que entre em estresse severo (DUTRA et al., 2017). Em regiões de alta altitude o risco de as plantas entrarem em estresse térmico pelas baixas temperaturas é maior. A baixa temperatura do ar afeta diretamente o metabolismo fotossintético das plantas, reduzindo a eficiência das enzimas PEPcase e Rubisco, responsáveis pela fixação de carbono na planta (KAUSHAL; WANI, 2016).

Os danos no aparato fotossintético das plantas resultam na formação de espécies reativas de oxigênio (EROs), que são produzidas pelo acúmulo de elétrons gerando oxigênio mais reativo que o oxigênio molecular, e causam danos à integridade e funcionalidade celular por meio da peroxidação de lipídios, oxidação de proteínas, DNA e outras moléculas orgânicas vitais ao funcionamento das plantas (TAIZ et al., 2017). No entanto, a planta possui um conjunto de enzimas antioxidantes que minimizam ou até anulam os efeitos causados pelas EROs. Dentre as enzimas mais estudadas, podem-se citar o superóxido dismutase (SOD), a catalase (CAT), o ascorbato peroxidase (APX), o dehidroascorbato redutase (DHAR), o monodehidroascorbato redutase (MDHAR), a glutationa redutase (GR) e a glutationa peroxidase (GPx). Esse conjunto de enzimas, aliado a outros agentes antioxidantes – como a prolina e o ácido salicílico –, auxilia as plantas a sobreviverem em condições de estresse, por longos períodos (TAIZ et al., 2017).

2.3.1 Estresse Nutricional

Tratando-se dos aspectos fisiológicos, a cultura do milho desempenha uma melhor resposta em solos bem estruturados, com favorável circulação hídrica e do ar, possibilitando uma melhor captação água e nutrientes. Na soja, o início da floração é considerado o período de maior velocidade de absorção e de maior exigência nutricional. O nitrogênio (N) é o elemento mais influente na produtividade do milho (DEBRUIN et al, 2017) e da soja (DOMINGOS; LIMA; BRACCINI, 2015), pois está relacionado a processos essenciais do metabolismo primário, como síntese de proteína, absorção iônica, fotossíntese entre outros, atuando assim, nas moléculas de aminoácidos e proteínas (OKUMURA et al., 2011). As fontes de N conhecidas são através da decomposição de matéria orgânica no solo; pela fixação não biológica através de processos naturais como descargas elétricas, porém em menor expressividade; com a aplicação de fertilizantes nitrogenados, que oneram a produção e causam danos ambientais; ou pela fixação biológica do N_2 atmosférico. A fixação biológica é consolidada na cultura da soja com o uso de bactérias diazotróficas do gênero *Bradyrhizobium* (DOMINGOS; LIMA; BRACCINI, 2015), sendo responsável por garantir a demanda de N pela cultura, mesmo em altos níveis de produtividade (TOFFOLO et al., 2022).

O potássio é um dos macronutrientes essenciais para o crescimento das plantas, formação de frutos, além da resistência a doenças fúngicas (YAN et al., 2017). Sintomas de deficiência de potássio (K) são notados em folhas mais velhas, que manifestam clorose nas pontas e margem das folhas, com posterior secamento, necrose (queima) e dilaceração de tecido. Os níveis adequados de potássio atuam na regulação estomática, estão envolvidos na ativação de inúmeras enzimas, melhoram o crescimento radicular e, por consequência de todas essas características, aumentam o rendimento da cultura. Além disso, o potássio é importante na fotossíntese, formação de frutos, resistência ao frio e a doenças fúngicas (BASAK et al., 2017).

Apesar da importância dos macronutrientes nitrogênio e potássio, o maior desafio na maior parte das áreas agrícolas do Brasil é o manejo da adubação de fósforo (PANTANO et al., 2016). Em parte, os problemas da adubação fosfatadas estão relacionados com a interação deste mineral com o solo, pois sua tendência é de se adsorver aos sólidos do solo (FERREIRA, 2015).

2.4 EFICIÊNCIA DO USO DO FÓSFORO

O fósforo é um macronutriente de grande importância para diversas atividades fisiológidas como fotossíntese, divisão celular e outros (MOHAMED; FARAG; YOUSSEF, 2018). No solo o fósforo pode ser encontrado de três formas distintas, como fosfato solúvel, fosfato inorgânico insolúvel ou fosfato orgânico solúvel. Sua forma orgânica pode ser obtida através da decomposição de matéria vegetal, animal e microbiana (PRABHU; BORKAR; GARG, 2018).

O P que se encontra no solo é absorvido pelas plantas em suas formas aniônicas ($H_2PO_4{}^-$ e $HPO_4{}^{2-}$), é parcialmente solúvel e apresenta forte fixação no solo. A disponibilidade desse nutriente é baixa em solos fortemente ácidos ou alcalinos, o que ocorre devido à formação de complexos de fosfato com Al e Fe em solos ácidos e complexos de Ca em solos alcalinos (MAHDI et al., 2011). O uso intensivo de adubos químicos ou a utilização repitida de cobertura morta torna o solo alcalino e salino. A alcalinidade e a salinidade aumentam a precipitação dos fosfatos solúveis disponíveis além da toxicidade dos íons de sal e as mudanças nas propriedades físicas e químicas do solo (YAMAGUCHI; BLUMWALD, 2005; SHAHBAZ; ASHRAF, 2013). Estudos estimam que cerca de 80% do P está ligado às partículas do solo e não disponível às plantas, o que reduz a eficácia da adubação fosfatada (RICHARDSON et al. 2009;), consequentemente, o fosfato é aplicado em grandes quantidades, excedendo a demanda da planta (PEREIRA; CASTRO, 2014). No entanto, a rocha fosfática é um recurso não renovável e limitado.

Atualmente o fósforo é obtido principalmente de fosfato de rocha extraído e geralmente é combinado em fertilizantes minerais com ácido sulfúrico, nitrogênio e potássio (CORDELL; DRANGERT; WHITE, 2008). Além disso, o uso desse fertilizante químico causa diversos impactos ambientais. Para cada tonelada de fosfato processado da rocha fosfática, são geradas cinco toneladas de fosfogesso, um subproduto tóxico da mineração de rocha fosfática, que contém em sua composição matais pesados, além das emissões signiticativas de carbono para sua produção (CORDELL; DRANGERT; WHITE, 2008). Outra preocupação é referente ao vazamento deste produto em rios, mares e lenções freáticos, onde causa a eutrofização do ambiente aquático, pois estimula a proliferação de algas, que formam uma camada na superfície da água, impedindo a entrada de luz e com isso prejudica o crescimento e desenvolvimento de plantas aquáticas fundamentais para a oxigenação da água, afetando assim toda a biodiversidade ali presente (SHARPLEY et al., 2005).

Diante disso, são exploradas alternativas que substituam ou diminuam o uso do fosfato inorgânico nas áreas de cultivo. Tem sido feitas pesquisas que demonstram que exopolissacarídeos microbianos podem desempenhar a solubilização de fosfato (IONESCU; BELKIN, 2009). Bactérias presentes no solo podem promover a produção de sideróforos que podem ter função de quelar o ferro de fosfatos de ferro insolúveis (VASSILEV; VASSILEVA; NIKLAEVAL, 2006; CABALLERO-MELLADO et al., 2007; HAMDALI et al., 2008).

2.5 BACTÉRIAS PROMOTORAS DE CRESCIMENTO

A rizosfera compreende um ecossistema composto por populações microbianas numerosas e diversificadas em estado de equilíbrio dinâmico, que interferem na qualidade biológica, física e química do solo (AHEMAD; KIBRET, 2013). Algumas bacterias secretam ezinmas extracelulares, como a catalase, hidrolase, triptofanase, urease e protease, que metabolizam substâncias para suprir suas necessidades metabólicas (SONG et al., 2021). As bactérias promotoras de crescimento vegetal (PGPR – Plant Growth-promoting Rhizobacteria) residem na rizosfera das plantas e melhoram o desempenho das culturas por meio da produção de compostos com efeito antibiótico, fixação de N_2, solubilização de P, mineralização de matéria orgânica, produção de fitohormonios, (KAUR; DEVI; VIJAS, 2018; GOSWAMI et al., 2016; KUMAR; MAURYA; RAGHUWANSHI, 2014), e produção de ácidos orgânicos, incluindo ácido acético, ácido lático e ácido pirúvico (SONG et al., 2021).

Várias espécies bacterianas podem solubilizar o P aderido ao solo, tornando-se disponível para as plantas e assim melhorando seu desenvolvimento e crescimento (PEREIRA; CASTRO, 2014). As bactérias que solubilizam fosfato (PSB – Phosphate sulubilizing bacteria) se utilizam de diversos mecanismos, como secreção de compostos minerais solventes, mineralização bioquímica de fosfato mediada por enzimas e assimilação de fosfato pela liberação de fósforo de substratos mais complexos (GURBANOV et al., 2021). A atividade da fosfatase é considerada uma das mais importantes enzimas envolvidas no processo de solubilização (RICHARDSON, 2005). A fosfatase é liberada no compartimento extracelular e catalisa a hidróslise do fosfato orgânico, transformando-o de composto de baixo peso molecular, o que leva à liberação de íons fosfato (MATOS et al., 2017). A produção de baixas concentrações de etileno também podem estimular o crescimento radicular, o que pode ser obtido pela PSB com atividade de ACC-desaminase, que converte etileno em amônia e ácido alfa-cetolbutírico (PEREIRA; CASTRO, 2014).

2.5.1 Gênero *Bacillus spp.*

O gênero *Bacillus* consiste em bactérias Gram-positivas, em forma de bastonete, que se movem por flagelos peritricosos e são aeróbias ou anaeróbias facultativas (CAWOY et al., 2011). Produzem endósporos que garantem a elas uma maior resistência à diversos ambientes (FERREIRA, 2015). Esse gênero é conhecido pelo controle de doenças por meio de síntese de metabóitos secundários, também se destacam pela formação de esporos que possibilitam alta viabilidade em comparação com as células vegetativas (BORIS, 2015).

O gênero *Bacillus* tem grande importância agrícola, sua interação simbiótica proporciona proteção às plantas de diversas maneiras, como produção de enzima, antibióticos e compostos orgânicos voláteis (PATEL; MINOCHEHERHOMJI, 2018), aumento da fixação de nitrogênio, solubilização de nutrientes, síntese de fitormônios e melhoria das condições do solo (KLOEPPER, 1999). Os metabólitos produzidos por essas bactérias também podem afetar a microflora na rizosfera, fornecendo um ambiente antagônico aos patógenos, ou podem desencadear respostas de defesa do hospedeiro (VELUSAMY; GNANAMANICKAM, 2008).

A produção de metabólitos secundários tem grande importância nos mecanismos que auxiliam o desenvolvimento vegetal. Belbahri et al. (2017) identificou os seguintes metabólitos presentes no gênero: surfactinas, mersacidina, difficidina, bacilisina, fengicina, macrolactina, bacuillaene e bacilibactina. Esima-se que 795 metabólitos secundários são produzidos pelas diferentes espécies de *Bacillus* (AWAD et al., 2012).

2.5.2 Gênero *Lysinibacillus* spp.

O gênero foi classificado em 2007 em trabalho desenvolvido por Ahmed et al. (2007), descrevendo essas bactérias como células móveis, com formato de bastonetes, que produzem endósporos elipsoidais ou esféricos. As *Lysinibacillus* spp. são bactérias Gram-positivas, tendo em sua camada de peptideoglicano da parede celular lisina e ácido aspártico, como aminoácidos diagnósticos, representando o peptideglicano como A4a (Lys-Asp). Suas colônias são caracterizadas por coloração amarelada, circulares, com margens irregulares e opacas (ZHU et al., 2014).

Os trabalhos envolvendo espécies do gênero indicam a capacidade de produção de uma ampla gama de bacteriocinas e antimicrobianas contra patógenos bacterianos e fúngicos. Além disso cepas de *Lysinibacillus sphaericus* foram identificadas como inseticida biológico que atua em larvas do complexo *Culexpipens* e *Anopheis* spp. (SILVA-FILHA et al., 2008). Algumas espécies do gênero atuam na remoção de Cr pela imobilização da magnetita (ZHU et al., 2021), na biodegradação de polietileno e polipropileno (JEON et al., 2021).

Recentemente, a capacidade de promoção de crescimento vegetal tem sido encontrada em espécies do gênero *Lysinibacillus*. Lelapalli et al. (2021) encontraram resultados que demonstram a capacidade promoção de crescimento vegetal e de solubilização de fósforo orgânico por cepas de *Lysinibacillus pakistanensis* isoladas de plantas de arroz. *Lysinibacillus varians* foram identificados como promotores de crescimento, promoção de IAA (Indol-acetic acid), fixação de nitrogênio, solubilização de P e redução da contaminação por cádmio (PAL; SENGUPTA, 2019). Em cultivo de pimenta, cepas de *L. xylanilyticus* mostraram capacidade de promoção de crescimento e biocontrole de doenças fúngicas de solo (PHAZNA et al., 2022).

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CAPÍTULO I

Eficiência da combinação de cepas de Ag 87 (*Bacillus megaterium*) e Ag 94 (*Lysinibacillus* sp.) como solubilizadores de fosfato e promotores do crescimento vegetal em milho

Efficiency of the combination of Ag 87 (*Bacillus megaterium*) and Ag 94 (*Lysinibacillus* sp.) strains as a phosphate solubilizer and growth promoter in maize

Efficiency of Combining Strains Ag87 (Bacillus megaterium) and Ag94 (Lysinibacillus sp.) as Phosphate Solubilizers and Growth Promoters in Maize

Abstract: Increasing phosphorus (P) use efficiency in agricultural systems is urgent and essential to significantly reduce the global demand for this nutrient. Applying phosphate-solubilizing and plant growth-promoting bacteria in the rhizosphere represents a strategy worthy of attention. In this context, the present work aimed to select and validate bacterial strains capable of solubilizing phosphorous and promoting maize growth, aiming to develop a microbial inoculant to be used in Brazilian agriculture. Bacterial strains from the maize rhizosphere were evaluated based on their ability to solubilize phosphate and produce indole acetic acid. Based on these characteristics, 24 strains were selected to be further evaluated under laboratory, greenhouse, and field conditions. Among the selected strains, four (I04, I12, I13, and I17) showed a high potential to increase maize root growth and shoot P content. Strains I13 (Ag87) and I17 (Ag94) were identified by genomic sequencing as *Bacillus megaterium* and *Lysinibacillus* sp., respectively. These strains presented superior yield increments relative to the control treatment with 30% P. In addition, combining Ag87 and Ag94 resulted in even higher yield gains, indicating a synergistic effect that could be harnessed in a commercial inoculant for Brazilian agriculture.

Keywords: *Zea mays* L.; microbial inoculants; phosphorus solubilization; phosphorus use efficiency

1. Introduction

Phosphorus (P) is an integral component of several important compounds in plant cells and is essential for plant growth and development (TAIZ; ZEIGER IAN MAX MØLLER; MURPHY, 2017). However, a large proportion of the P present in soils is not available to be absorbed by plants. In calcareous soils, Ca^{2+} increases P precipitation, while in acidic soils, Al^- and Fe^- oxides are responsible for P immobilization (BILLAH et al., 2019). In addition, the low mobility of this nutrient in the soil solution makes the mechanisms related to its acquisition ineffective, as they depend on the direct interception by roots.

An alternative to minimize P deficiency in acidic soils is to apply correctives and phosphate fertilizers, adapting the soil to the plant. Phosphate fertilizer, derived mainly from phosphate rocks, is widely used in agriculture and has contributed significantly to food production, a pillar of food security (BROWNLIE et al., 2021). However, only 15% of the P applied is absorbed by plants, and the addition of this inorganic fertilizer in excess can cause environmental problems, leading to the accumulation of heavy metals in soil, contamination of groundwater, and eutrophication of water sources (BAVEYE, 2015). Furthermore, a significant proportion of phosphate rock reserves for mineral extraction are concentrated in a relatively small area, mainly in Morocco and Western Sahara, making many countries dependent on imports to supply their P demands (CORDELL; DRANGERT; WHITE, 2009). For instance, in Brazil, approximately 4.5 million tons of inorganic fertilizers are imported per year (CONAB, 2022).

Increasing P utilization efficiency in agricultural systems is fundamental to substantially reducing its demand. An important strategy is using microbial inoculants with P solubilization activity in the soil (ALORI; GLICK; BABALOLA, 2017; BARGAZ et al., 2021; FATIMA et al., [s.d.]). These microorganisms use several solubilization and mineralization mechanisms that convert inorganic and organic P, respectively, into a bioavailable form, facilitating absorption by plant roots. Moreover, some of these microorganisms also demonstrate potential as plant growth promoters and biocontrol agents against plant pathogens (ALORI; GLICK; BABALOLA, 2017; BILLAH et al., 2019).

Many microorganisms, including bacteria, fungi, actinomycetes, and algae, exhibit the ability to solubilize and mineralize P. Among bacteria, strains of the genera *Pseudomonas*, *Mycobacterium*, *Micrococcus*, *Bacillus*, *Flavobacterium*, *Rhizobium*, *Mesorhizobium*, and *Sinorhizobium* have been reported to solubilize P (ALORI; GLICK; BABALOLA, 2017; KALAYU, 2019; SHARMA et al., 2013). These bacteria can make P available to plants through several mechanisms, some more related to enzymatic processes (phytases and/or phosphatases), while others involve cellular physiology, with the extrusion of H^+ ions and release of organic acids from microbial metabolisms (ALORI; BABALOLA, 2018).

Phosphate-solubilizing bacteria (PSB) have great potential in regions with phosphorus deficiency, such as the Brazilian Cerrado. Under natural conditions, Cerrado soil is characterized by low pH and low nutrient fertility, especially P. In addition, the soil of these regions has a high capacity for P fixation, mainly due to the high contents of iron and aluminon oxide. Despite these natural restrictions, this region was responsible for 78% of Brazilian maize production in the 2020/2021 harvest (CONAB, 2021). In this context, this study aimed to select and validate bacterial strains that solubilize phosphorus and promote maize growth, aiming to develop a microbial inoculant for Brazilian agriculture.

2. Materials and Methods

2.1. Screening of Bacterial Isolates—Phosphate Solubilization and Indole Acetic Acid Production

Ninety-two bacterial strains from the maize rhizosphere were evaluated for their ability to solubilize phosphate and produce indole acetic acid (IAA). These strains were isolated from maize rhizospheric soil collected in the municipality of Italva, Rio de Janeiro, Brazil. For P solubilization, 100 µL of each strain was plated in the National Botanical Research Institute’s phosphate growth medium (NBRIP) (NAUTIYAL, [s.d.]), containing inorganic iron phosphate ($FePO_4.2H_2O$). The inoculated plates were incubated at 25 °C for 10 days, and microorganism growth was monitored daily. The isolates were selected by growth and/or formation of a halo, indicating the solubilization of the compound in the culture medium. In the control treatment, the plates were inoculated with sterile saline solution (0.85%).

IAA production was determined according to the Salkowski colorimetric assay, as described by Sawar et al.(SARWAR; KREMER, 1995). The cell-free culture supernatants obtained in DYGS liquid medium supplemented with tryptophan (100 µg mL^{-1}) were incubated for 48 h at 28 °C and 180 rpm in an orbital shaker (Tecnal—TE 422, Piracicaba, São Paulo, Brazil). The concentration of IAA produced by each strain was estimated by measuring its optical density at 530 nm and using an IAA standard curve. Based on the results of P solubilization and IAA production, 26 bacterial strains were selected for growth promotion assays in maize under greenhouse conditions.

2.2. Preparation of Bacterial Isolates

Strains stored in cryovials containing liquid Tryptic Soy Broth (TSB) and glycerol in a 2:1 ratio at −80 °C were activated in Petri dishes containing LBA (Luria Bertani Agar, Neogen Corporation, United States) culture medium at 28 °C for 24 h. A pre-inoculum of each strain was prepared from pure colonies suspended in saline solution (0.85% sodium chloride), with turbidity adjusted to 0.5 in the McFarland nephelometric standard (1.5×10^8 CFU/mL). Thirty uL of these bacterial suspensions were transferred to 125 mL Erlenmeyer flasks containing 30 mL of Ag/02 culture medium (g L^{-1}: glucose 15.0, sucrose 10.0, yeast extract 10.0, micronized soy protein 10.0, KH_2PO_4 1.5, $MgSO_4$ 0.5, $MnSO_4$ 0.5, $CaCl_2$ 1.5, and pH 8.0) and incubated at 30 °C for 18–20 h at 200 rpm in an orbital shaker (Tecnal—TE 422, Piracicaba, São Paulo, Brazil) for inoculum production. For fermentation, 1000 mL Erlenmeyer flasks containing 400 mL of Ag/02 were inoculated with a 4 mL aliquot of the inoculum and incubated at 30 °C for 72 h at 200 rpm. After fermentation, the production concentration was adjusted to 2.0×10^9 CFU/mL.

2.3. Laboratory and Greenhouse Tests

Maize seeds of the cultivar P30F53 (Pioneer®, Tokyo, Japan) were inoculated with bacterial strains at a 2×10^9 CFU/mL concentration, constituting 26 treatments. The following treatments were used as controls: no inoculation, Biomaphos (*Bacillus subtilis* strain CNPMS B2084 and *Bacillus megaterium* strain CNPMS B119), and Nodugram (*Azospirillum brasilense* strain Abv5). The seeds were treated at a dose of 100 mL/60,000 seeds.

After inoculation, 15 seeds of each treatment were placed on germination paper with sterilized distilled water in a growth chamber (25 ± 2 °C and 70% relative humidity). For this experiment, four repetitions were used. Ten days after sowing, five seedlings were selected for the evaluation of average root diameter (ARD), root surface area (RSA), and root length (RL) by scanning them in a scanner with a resolution of 300 dpi. First the images were treated and analyzed by GiaRoots software (GALKOVSKYI et al., 2012). Then, the shoot and root systems of the seedlings were kept in an air forced oven at 60 °C for 72 h. Then, shoot and root dry mass (SDM and RDM, respectively, in g) were determined. The experiment was conducted in a completely randomized design.

For the greenhouse tests, two experiments were performed, one using sand as the substrate and the other using sand and soil (3:1). Twenty-eight treatments were evaluated, comprising 26 bacterial strains and 4 controls (Biomaphos, Nodugram, insoluble P, and no P). Seeds of the hybrid P30F53 (Pioneer®) were inoculated and sown in 970 mL pots containing sterilized sand or sand with soil collected in a cultivation area at School Farm, Universidade Estadual de Londrina (UEL). Two seeds were sown per pot, and, ten days after sowing (DAS), thinning was performed, keeping only one plant per pot. The experiments were carried out in a completely randomized design with six repetitions.

The treatments were irrigated with Hoagland's nutrient solution (HOAGLAND; ARMON, 1950), modified by Magnavaca et al. (MAGNAVACA; GARDNER; CLARK, 1987), A volume of 100 mL of the solution was applied every three days. The plants were removed 28 DAS and subjected to evaluation of the stem diameter. Subsequently, shoots and roots were separated and air-dried in an oven with forced ventilation for 72 h to measure SDM and RDM. After drying, shoots were ground and evaluated for P content. To determine the P content in the grains and shoots, the samples were dried in an oven at 70 °C for 72 h and ground with Willey MA340-type knives (Piracicaba, São Paulo, Brazil). Then, 0.1 g aliquots were digested in nitroperchloric solution (HNO_3:$HClO_4$) according to Malavolta et al. (MALAVOLTA; VITTI; OLIVEIRA, 1997). The P content was determined by the molybdenum blue spectrophotometric method (PRADHAN; POKHREL, 2013) and the readings were performed in an Agilent 8453 spectrophotometer (Agilent Technologies, Santa Clara, CA, USA) at a wavelength of 660 nm.

2.4. Genomic Sequencing of the Strains Ag87 and Ag94

Based on the results obtained in the laboratory and greenhouse, two strains were selected for field trials, which were cataloged in the AgBio microorganism bank and named Ag87 and Ag94. For complete genome sequencing of these strains and species identification, they were cultured in LB medium at 150 rpm at 28 °C for 48 h. DNA extraction was performed using a PureLinkTM Microbiome DNA Purification kit (Invitrogen, Thermo-Fisher Scientific, Waltham, MA, USA). DNA integrity was verified using a 1% agarose gel, and the DNA was quantified by spectrophotometry in a NanoDrop 2000/2000 c (ThermoFisher Scientific, Wilmington, DE, USA). The sequencing was performed on the Illumina MiSeq platform of the company SuperBAC, Mandaguari, Paraná, Brazil.

The quality of the readings and the cut-off parameters were considered and chosen using FastQC(ANDREWS, 2010). Using the Trimmomatic program (BOLGER; LOHSE; USADEL, 2014) the raw readings were filtered based on the parameters defined by FastQC. After the filters, the quality of the readings was analyzed again to check if the chosen parameters were adequate. A series of *de novo* assemblies were performed in two software programs (SPA-des and IDBA hybrid) (PENG et al., 2010) testing different assembly parameters and comparing them with each other in the QUAST program (GUREVICH et al., 2013). According to the reference genome provided in QUAST, key metrics such as total alignment size, number of contigs, largest contig, N50 values, and gene numbers were used to choose the best assembly. Using the CONTGuator web server, best-assembled contigs were aligned with the genomes of *Bacillus megaterium* DSM319 and *Lysinibacillus agricola* FJAT-51161 for strains Ag87 and Ag94, respectively, to generate the scaffolds. Gaps were filled manually, mapping reads using Bowtie2 and filling gaps using CLC Genomics Workbench 12 GUI (LANGMEAD; SALZBERG, 2012). The genome start point was determined by comparison with a reference strain genome, assuming the dnaA gene as the first gene.

The genomes of strains Ag87 and Ag94 were represented circularly and compared with other reference genomes using BRIG software (BLAST Ring Image Generator) (ALIKHAN et al., 2011). Genomic comparisons were performed with the program Gegenees (ÅGREN et al., 2012), SplitsTree (HUSON, 1998) was used to represent phylogenetic relationships in a tree. For species determination, ANI (average nucleotide identity) and dDDH (digital DNA–DNA hybridization) were performed among other *Bacillus* spp. and *Lysinibacillus* sp. using OrthoANI (LEE et al., 2016).

2.5. Field Trials—Harvest (2020/2021)

For the tests under field conditions, seeds of the maize cultivar P3340VYHR (Corteva®, Indianapolis, IN, USA) were used. The seeds were treated with biological products (Ag87, Ag94, Ag87 + Ag94, and the commercial product Biomaphos) in plastic bags using a dose of 100 mL/60,000 seeds.

The experiments were carried out during the 2020/2021 summer (first) harvest in the municipalities of Londrina, Maringá, Guarapuava, and Entre Rios do Oeste, state of Paraná, Brazil. The physical-chemical analyses of the soils and other characteristics related to the evaluation sites are presented in Table S1.

The experimental design adopted was complete randomized blocks with four repetitions. The plots consisted of eight rows of 6 m in length with a spacing of 0.45 m between rows and five plants per meter. Before setting up the experiment, the areas were fertilized with 25 kg P_2O_5 ha^{-1}, 60 kg K^2O ha^{-1}, and 21 kg N ha^{-1}. The amount of P_2O_5 applied in the experiments was 30% of the standard phosphate fertilization recommended for maize. The topdressing fertilization in the crop was carried out with 120 kg N ha^{-1} applied at the V6 development stage.

Three representative plants from each plot of the experiments were collected at the physiological maturation stage. The determination of P content in the grains and shoots was carried out as described in Section 2.3. Grain yield (GY, in kg ha^{-1} and 13% moisture) was obtained after manual harvesting and mechanical threshing of the plants in the six central rows of each plot. The components of P use efficiency (PUE) were determined according to Moll et al. (MOLL; KAMPRATH; JACKSON, 1982). P uptake efficiency (PUpE, in g of absorbed P per g of applied P) was calculated by the ratio between total plant P and P available to the plant. P utilization efficiency (PUtE, in g of grains produced per g of total plant P) was determined by the ratio between grain dry biomass and the amount of total plant P, while PUE (in g of grains produced per g of applied P) was calculated by the product of PUpE and PUtE.

2.6. Field Trials—Second Harvest (2021)

The experiments were carried out during the second harvest in the municipalities of Londrina and Maringá, state of Paraná, and in the municipalities of Itiquira, Sorriso, and Sapezal, state of Mato Grosso. The physical-chemical analyses of the soils and other characteristics related to the evaluation sites are presented in Table S2. The experimental design adopted was complete randomized blocks with four repetitions. The plots consisted of eight 6 m long rows with 0.45 m spacing between rows and five plants per meter for the Londrina and Maringá experiments, while for the Mato Grosso experiments, three plants per meter were used. In Londrina and Maringá, the areas were fertilized at sowing with 25 kg P_2O_5 ha^{-1}, 60 kg K_2O ha^{-1}, and 21 kg N ha^{-1}. Then, topdressing fertilization of 120 kg N^{-1} ha was applied at the V6 development stage. In Mato Grosso, the areas were fertilized with 51 kg K_2O ha^{-1} at the V1 stage and 100 kg N ha^{-1} at V4. Concerning phosphorus, 13 kg P_2O_5 ha^{-1} was applied before sowing in the 30% P control and biological treatments. For the 50 and 100% P controls, 22 and 45 kg P_2O5 ha^{-1} was applied, respectively. Grain yield (GY, in kg ha^{-1} and 13% moisture) was obtained after manual harvesting and mechanical threshing of the plants in the six central rows of each plot.

2.7. Data Analysis

The agronomic data were subjected to analysis of variance, Scott–Knott mean cluster analysis (laboratory and greenhouse assays), and Tukey's test (field assays). In addition, the greenhouse data were subjected to correlation and multivariate analysis using principal component analysis (PCA) and UPGMA hierarchical clustering based on standardized Euclidean distance. These analyses were performed by the R program using the packages "AgroR" (SHIMIZU; MARUBAYASHI; GONCALVES, 2021), "factoMiner" (LÊ; JOSSE; HUSSON, 2008), "pheatmap" (KOLDE, 2018) and "ggplot2" (WICKHAM, 2016).

3. Results

3.1 Screening of Bacterial Strains

Using NBRIP medium for cultivation, 42 strains were capable of solubilizing phosphate and producing IAA with values ranging from 0.15 to 18.30 µg mL^{-1}. Based on the traits of phosphate solubilization and IAA production, 24 strains were selected for laboratory and greenhouse experiments.

In the germination paper experiment, a significant effect of treatments was observed for all of the analyzed traits. The coefficient of variation ranged from 8.12 (RL) to 14.15 (SDM). For ARD, 17 bacterial strains obtained higher values than the control treatment, ranging from 0.0812 to 0.1054 mm (Table S3). For RSA, 13 strains obtained better results than the control, with emphasis on strains I04 and I10, while for RL, 11 strains were superior to the control, especially strains I04, I10, and I11. For DRM, the strains that stood out were I10, I13, and I25, with 65% more dry root mass, on average, than the control. Moreover, a total of 18 bacterial strains obtained higher values for this trait than the control. For SDM, the strains that stood out were I09, I20, and I26, with an average increase of 19% compared to the control.

For the greenhouse experiment, a significant effect was observed for most traits, except for SDM (experiment sand:soil, Exp_SS). The coefficient of variation ranged from 6.15 (RDM—experiment soil, Exp_S) to 12.15 (SDM—Exp_SS).

For the experiment in the sand, strains I04, I05, I13, and I17 obtained the highest values for RDM, with increments of 58, 51, 67, and 53%, respectively, in relation to the control (Pinsol) (Table S4). These strains also obtained higher values of RDM in the sand:soil experiment, with increments of 33, 25, 30, and 32%, respectively, in relation to the control (Pinsol). For this experiment, nine strains obtained values higher than the control. For SDM, no difference was observed between the treatments and the control (Pinsol). For shoot P content, no differences were observed for treatments compared to the control (Pinsol) in the sand experiment. By contrast, in the sand:soil experiment, the Biomaphos, I04, I10, I13, I17, and I22 treatments led to 26, 15, 21, 16, 25, and 11% more shoot P content than Pinsol.

According to the principal component analysis, the first two components explained 58.8% of the total variation (PCA1 and PCA 2 with 35.7 and 23.1%, respectively) (Figure 1a). The traits related to the root system (RL, RDM, ARD, and RSA) were correlated with each other (Figure 1a,b). However, they showed a negative correlation with SDM ($r = -0.45$, -0.78, -0.39, and -0.58, respectively). The traits evaluated in the germination paper did not correlate with the traits evaluated in the greenhouse. Regarding the greenhouse experiments, the RDM was correlated in both experiments ($r = 0.43$). RDM_Exp_SS also showed a positive correlation with SPC_Exp_S, SDM_Exp_S, and SDM_Exp_SS ($r = 0.41$, 0.52, 0.41, respectively). In turn, RDM_Exp_S obtained a positive correlation with SPC_Exp_S, SPC_Exp_SS and SDM_Exp_SS ($r = 0.41$, 0.48, and 0.43, respectively).

Based on the PCA and hierarchical clustering UPGMA, a wide distribution of treatments for agronomic traits and P content was observed (Figure 1a,c). The inoculation of strains I12, I13, and I04 favored greater increments for most of the traits evaluated in maize in all experiments (germination paper, greenhouse_sand, and greenhouse_sand:soil). Strains I17, I05, I03, I16, I19, I21, I24, I01, I02, I09, and I26 led to better results for most traits in the greenhouse experiments. However, they showed the lowest values in the experiment on germination paper. Among these strains, I17 stood out, obtaining high SDM_Exp_SS, SPC_Exp_SS, and RDM_Exp_S values. Strains I20, I14, I23, I07, I22, I11, I18, I10, I06, I25, I08, and I15 provided greater increases in maize in the germination paper experiment. Based on these results, strains I13 and I17 were selected for further experiments and were called Ag87 and Ag94.

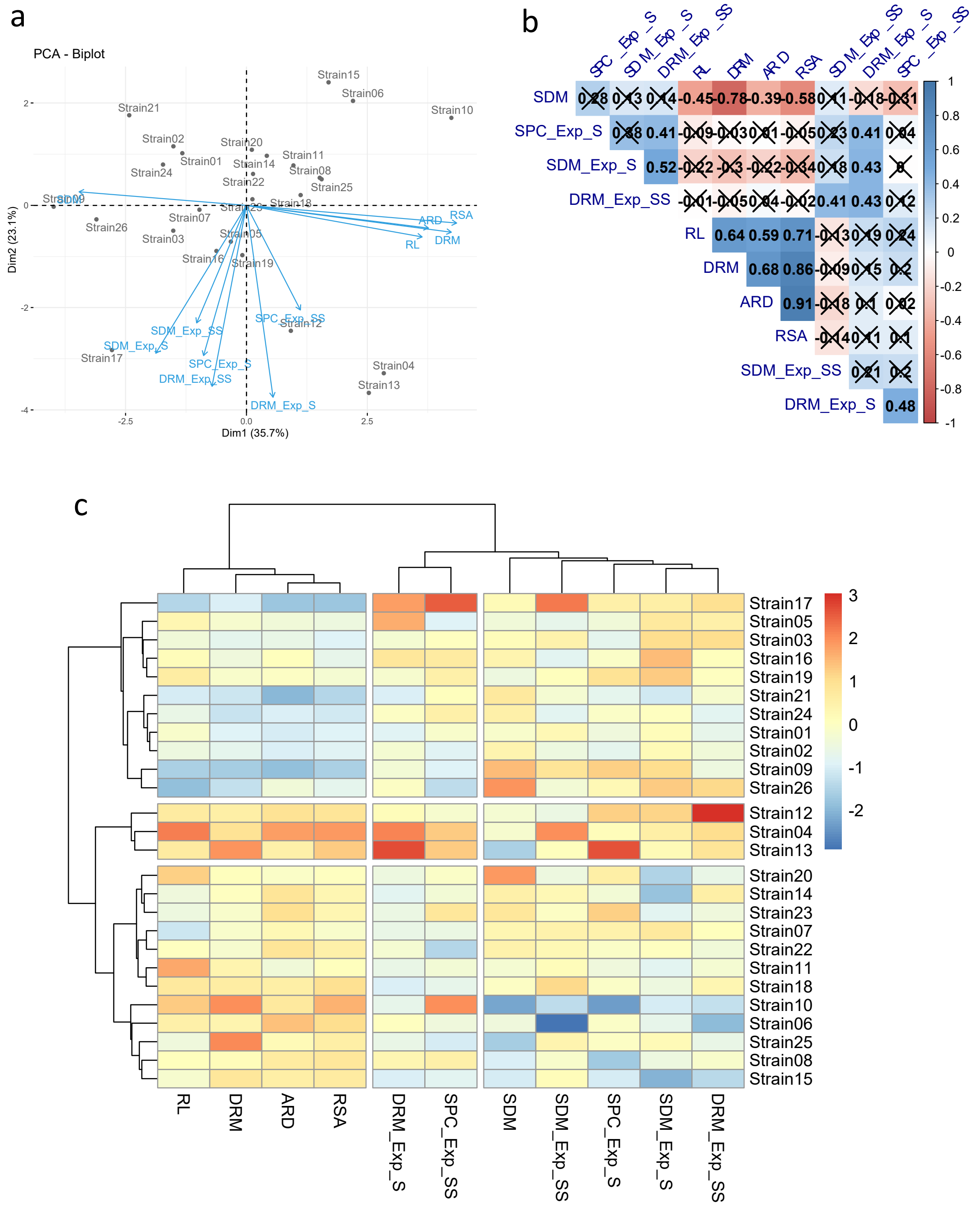

Figure 1. Multivariate analysis and correlation among traits evaluated in maize experiments (germination paper and greenhouse) with seeds inoculated with different phosphorus-solubilizing bacteria. (**a**) Principal component analysis; (**b**) Pearson correlation; and (**c**) UPGMA hierarchical grouping. (ARD: average root diameter, RSA: root surface area, RL: root length, RDM: root dry mass, SDM: shoot dry mass, and SPC: shoot phosphorus content. Exp_S: greenhouse experiment—sand substrate, Exp_SS: greenhouse experiment—sand:soil substrate (3:1)).

3.2. Genomic Analysis of Strains Ag87 and Ag94

The CLC Genomics Workbench 11 and IDBA Hybrid genome assembly strategies demonstrated the best results for assembly. First, a BLASTn search was performed using the largest contig to find a reference genome to be used in CONTIGuator. The strains CP001982 from *Bacillus megaterium* strain DSM319 and CP067341 from *Lysinibacillus agricola* strain FJAT-51161 were selected to align the contigs and generate the pseudoconting (scaffold) using CONTIGuator. The scaffolds contained 27 and 20 gaps, respectively, which were aligned against the raw sequencing reads using Bowtie2 and then evaluated and cured in CLC Genomics Workbench 11. In the genome of Ag87, two plasmids of 67,247 and 48,132 bp were found. The genomes of strains Ag87 and Ag94 showed read alignment rates of 93.44 and 90.53%, respectively, with sizes of 6,184,617 and 4,623,298 bp, respectively. Genome annotation via RAST verified a GC content of 38.1% for strain Ag87, with 6477 CDSs. From this total, 15 CDSs were related to rRNA and 119 to tRNA sequences (Figure S1). For strain Ag91, a GC content of 36.9% and 4709 CDSs were found, with 16 of those related to rRNA and 61 to tRNA sequences (Figure S1).

Comparing the strain Ag87 (GenBank accession CP098610) with the main species of the genus *Priestia*, it was observed that the average nucleotide identity (ANI) and digital DNA–DNA hybridization (dDDH) were higher with the groups of isolates of the *Priestia megaterium* species, ranging from 96.84 to 97.16% for ANI. For the strain Ag94 (GenBank accession CP096780), a greater genetic similarity was observed with the species of the genus *Lysinibacillus*. However, it was impossible to determine the species since the ANI values were <95% compared to the genomes of isolates with a defined species. The genome of the strain *Lysinibacillus* sp. SDF0037 obtained a value of 96.10% for ANI, suggesting that both belong to the same species. Nevertheless, deeper analyses are necessary to define a new species within the genus *Lysinibacillus*. In the comparison performed with orthoANI/GGDC, it was observed that strains Ag87 and Ag94 are located within the cluster containing most species of *Priestia megaterium* (Figure 2) and *Lysinibacillus* sp. (Figure 3), respectively. The circular genomes of the two strains are represented in Figures S2 and S3.

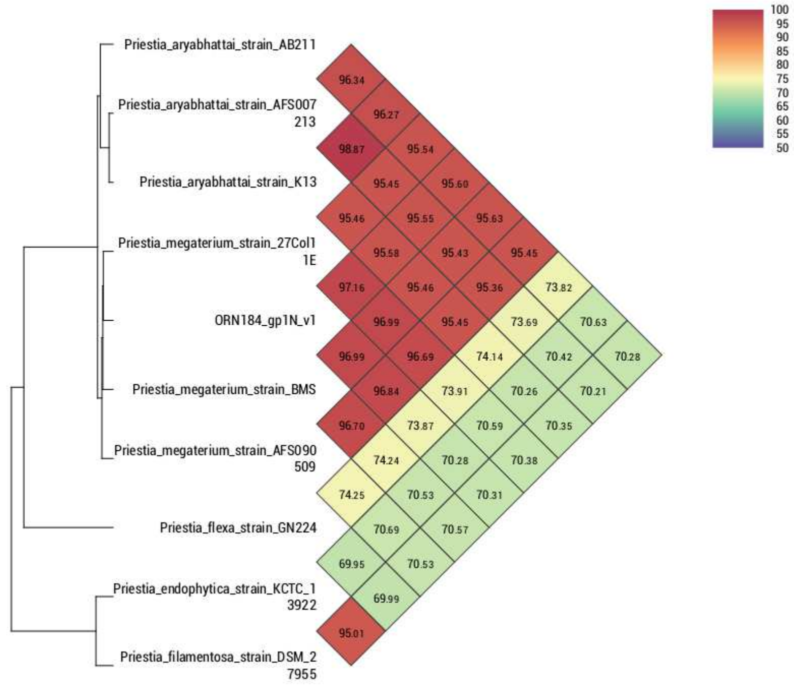

Figure 2. Heatmap generated with OAT software indicating the OrthoANI values of *Bacillus megaterium* strain Ag87 and the closely related *Bacillus* species.

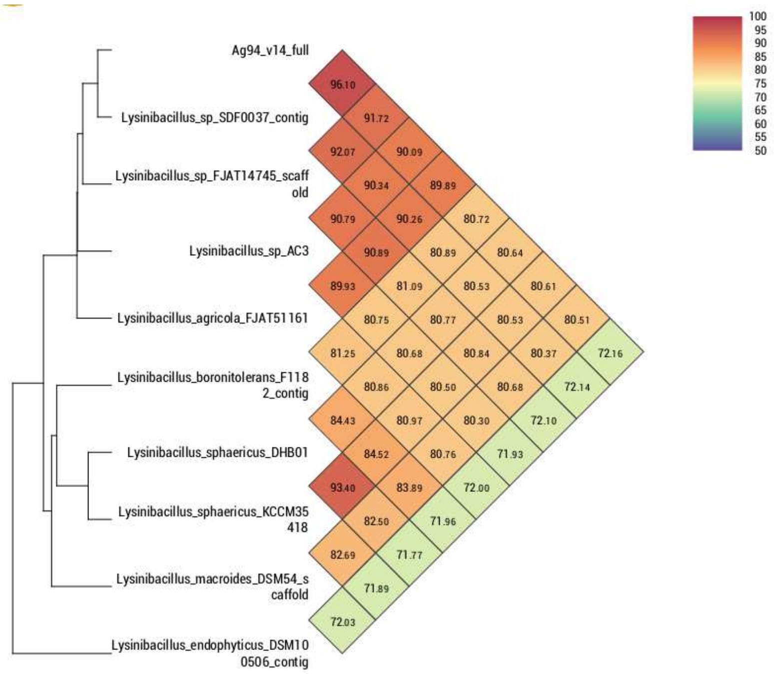

Figure 3. Heatmap generated with OAT software indicating the OrthoANI values of *Lysinibacillus* sp. strain Ag94 and closely related *Lysinibacillus* sp. species.

3.3. Field Data—Harvest 2020/2021

Based on the analysis of variance, a significant effect was observed for all sources of variation (environment, treatments, and environment × treatments) for grain yield (Table 1). The experimental coefficient of variation was 9.9, and all the assumptions of the analysis of variance were met (normality of errors, homogeneity of variance, and independence of errors). Among the environments, Londrina obtained the highest average yield, followed by Maringá, Entre Rios, and Guarapuava.

Table 1. Analysis of variance for grain yield in maize experiments conducted in the first and second harvests (2020/2021 and 2021, respectively) with seeds inoculated with different phosphorus-solubilizing bacteria.

Source of Variation	**First Harvest 2020/2021 Paraná**		**Second Harvest 2021 Paraná**		**Second Harvest 2021 Mato Grosso**	
	DF	**MS (Yield)**	**DF**	**MS (Yield)**	**DF**	**MS (Yield)**
Repetitions/E	12	1,077,861	6	471,722	9	304,887
Environment (E)	3	65,585,288 **	1	85,089,692 **	2	8,320,357 **
Treatments (T)	6	3,104,344 **	4	1,443,243 *	4	2,022,543 **
E × T	18	837,210 *	4	1,970,383 **	8	563,600 **
Error	81	455,739	24	394,322	36	156,392
CV(%)		9.90		12.62		7.78
Mean Londrina		8558.09		3516.60		-
Mean Maringá		7004.05		6434.40		-
Mean Guarapuava		4828.13		-		-
Mean Entre Rios		6888.12		-		-
Mean Itiquira		-		-		6321.41
Mean Sorriso		-		-		6653.19
Mean Sapezal		-		-		7553.43
Shapiro–Wilk		0.98 ^{ns}		0.97 ^{ns}		0.98 ^{ns}
Bartlett		31.10 ^{ns}		22.60 ^{ns}		14.55 ^{ns}
Durbin–Watson		2.24 ^{ns}		2.63 ^{ns}		2.51 ^{ns}

ns, **, and * indicate non-significance and significance at levels of 1 and 5% probability by the F test, respectively.

In Londrina, the highest yield was observed for 100% P (9228 kg ha^{-1}), followed by the Ag87 (9088 kg ha^{-1}), 50% P (8791 kg ha^{-1}), Ag87 + Ag94 (8653 kg ha^{-1}), and Ag94 (8339 kg ha^{-1}) treatments (Figure 4). Compared with the 30% P control, the strains Ag87, Ag94, and Ag87 + Ag94 obtained yield increases of 16.6, 7.07, and 11.1%, respectively. For Maringá, the highest yields were observed for 100% P, Ag87 + Ag94, and Ag94, with 7632, 7587, and 7220 kg ha^{-1}, respectively. These two biological treatments (Ag 94 and Ag87 + Ag94) showed yield increases of 7.3 and 12.8%, respectively, compared to the 30% P control.

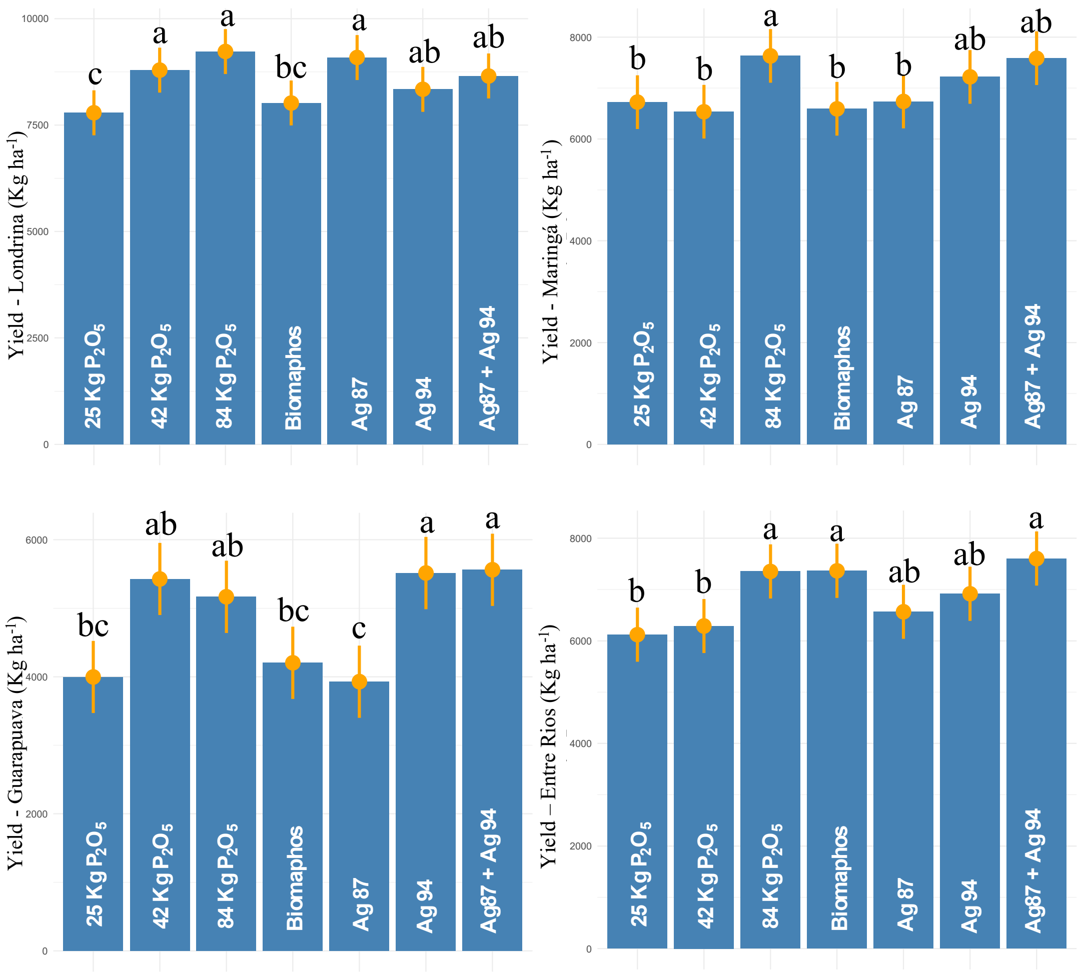

Figure 4. Tukey comparison ($p < 0.05$) for grain yield in maize experiments (first harvest—2020/2021) with seeds inoculated with different phosphorus-solubilizing bacteria at four sites in Paraná state—Brazil.

In Guarapuava, higher yield values were also observed for the 50% P, 100% P, Ag94, and Ag87 + Ag94 treatments, with 5427, 5167, 5514, and 5561 kg ha^{-1}, respectively. The strain Ag94 and the Ag87 + Ag94 combination obtained an average increase of 38% in productivity compared to the control (30% of P). For Entre Rios, the highest yields were observed for the Ag87 + Ag94, Biomaphos, 100% P, Ag94, and Ag87 treatments, with 7604, 7366, 6918, and 6566 kg ha^{-1}, respectively. These treatments obtained 24, 20, 20, 13, and 7% productivity increases, respectively. Based on these experiments, strain Ag94 and its combination with Ag87 obtained average yield increases of 16.3 and 21.45%, respectively, at the four sites compared to the 30% P control. Furthermore, these strains did not differ statistically from the 100% P control.

For the phosphorus use efficiency indices (PUpE_g, PUtE_g, and PUE_g), significant effects were observed for all sources of variation (environments, treatments, and environments × treatments). The coefficients of variation were 12.8, 14.1, and 11.7, respectively. For PUpE_g, the Ag94 and Ag87 + Ag94 treatments obtained the highest values for Londrina and Maringá, while for Guarapuava, the highest values came from Biomaphos and Ag87 (Table 2). For Entre Rios, the highest values were observed for the Biomaphos, Ag87, Ag94, Ag87 + Ag94, and 30% P treatments.

Differences between treatments for PUtE_g were observed only in Londrina and Entre Rios. However, no differences were observed in the application of biological products compared to the 30% P control, indicating the non-influence of biological products in relation to the phosphorus utilization efficiency. For PUE_g, only in Guarapuava was a difference between biological products observed in relation to the 30% P control, with higher values obtained for Biomaphos, Ag87, and Ag87 + Ag94.

Table 2. Tukey comparison ($p < 0.05$) for phosphorus uptake efficiency (PUpE_g), phosphorus utilization efficiency (PUtE_g), and phosphorus use efficiency (PUE_g) in maize experiments (first harvest—2020/2021) with seeds inoculated with different phosphorus-solubilizing bacteria at four sites in Paraná state—Brazil.

Treatments	**Londrina** 1/			**Treatments**	**Maringá**		
	PUpE_g	**PUtE_g**	**PUE_g**		**PUpE_g**	**PUtE_g**	**PUE_g**
25 kg P_2O_5	1.31 **b**	395 **ab**	516 **a**	25 kg P_2O_5	0.72 **b**	757 **a**	545 **ab**
42 kg P_2O_5	0.78 **c**	494 **a**	385 **b**	42 kg P_2O_5	0.47 **c**	689 **a**	323 **b**
84 kg P_2O_5	0.45 **d**	382 **ab**	172 **c**	84 kg P_2O_5	0.23 **d**	698 **a**	160 **c**
Biomaphos	0.92 **b**	489 **a**	451 **ab**	Biomaphos	0.71 **b**	808 **a**	573 **ab**

Ag 87	1.27 **b**	475 **a**	603 **a**	Ag 87	0.76 **b**	552 **a**	420 **b**
Ag 94	1.38 **a**	412 **ab**	568 **a**	Ag 94	0.90 **a**	593 **a**	533 **ab**
Ag 87 + 94	1.58 **a**	347 **b**	548 **a**	Ag 87 + 94	0.80 **ab**	790 **a**	632 **a**

Treatments	Guarapuava			Treatments	Entre Rios		
	PUpE_g	PUtE_g	PUE_g		PUpE_g	PUtE_g	PUE_g
25 kg P_2O_5	0.42 **c**	1836 **a**	771 **b**	25 kg P_2O_5	0.50 **ab**	1908 **a**	954 **a**
42 kg P_2O_5	0.44 **c**	1726 **a**	759 **b**	42 kg P_2O_5	0.34 **b**	1328 **b**	451 **c**
84 kg P_2O_5	0.15 **d**	1242 **a**	186 **c**	84 kg P_2O_5	0.19 **c**	1604 **a**	304 **c**
Biomaphos	0.79 **a**	1334 **a**	1053 **a**	Biomaphos	0.73 **a**	1372 **b**	1001 **a**
Ag 87	0.78 **a**	1638 **a**	1278 **a**	Ag 87	0.59 **ab**	1513 **b**	892 **b**
Ag 94	0.35 **c**	2012 **a**	704 **b**	Ag 94	0.52 **ab**	1964 **a**	1021 **a**
Ag 87 + 94	0.61 **b**	1763 **a**	1075 **a**	Ag 87 + 94	0.63 **ab**	1985 **a**	1250 **a**

[1/] Means followed by the same letter in the column do not differ statistically at 5% probability by the tukey test.

3.4. Field Data—Second Harvest 2021

For the experiments installed in Paraná (Londrina and Maringá), a significant effect was observed for all sources of variation (treatments, environments, and treatments × environments) for grain yield (Table 2). The experimental coefficient of variation was 12.6, and all the assumptions of the analysis of variance were met. The average yield was 3516.6 kg ha^{-1} in Londrina and 6434.40 kg ha^{-1} in Maringá. Regarding the treatments, Biomaphos, 100% P, and Ag87 + Ag94 resulted in the highest yields (4419, 3722, and 3736 kg ha^{-1}, respectively) in Londrina. By contrast, in Maringá, the highest yields were obtained by applying Ag87 + Ag94, 50% P, and 100% P (7398, 6611, and 6421 kg ha^{-1}, respectively) (Figure 5).

For the experiments conducted in Mato Grosso (Sorriso, Itiquira, and Sapezal), a significant effect was also observed for all sources of variation for grain yield (Table 2). The average productivity obtained by the environments was 6653.19, 6321.4, and 7553.43 kg ha^{-1}, respectively (Figure 6). For Sorriso, the highest yield was obtained for 100% P (7500 kg ha^{-1}), not differing statistically from Biomaphos, Ag87 + Ag94, and 50% P (6716.1, 6494.6, and 6432.2 kg ha^{-1}, respectively). Biomaphos and Ag87 + Ag94 led to productivity increases of 9 and 6% in relation to the 30% P control, respectively. In Itiquira, no differences were observed between treatments, while for

Sapezal, the highest productivity values were observed for the 100% P and Ag87 + Ag94 treatments (8377.5 and 7869.6 kg ha^{-1}, respectively). The application of Ag87 + Ag94 resulted in an average yield increase of 13% in relation to the 30% P control.

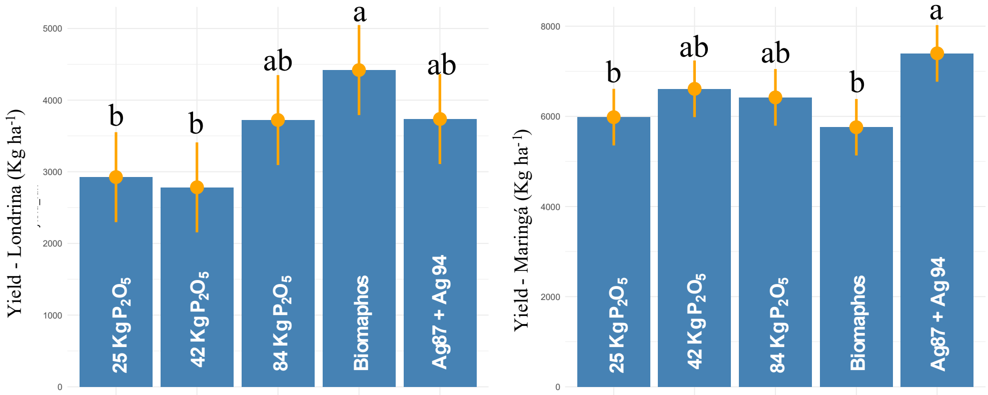

Figure 5. Tukey comparison ($p < 0.05$) for grain yield in maize experiments (second crop—2021) with seeds inoculated with different phosphorus-solubilizing bacteria at two sites in Paraná state—Brazil.

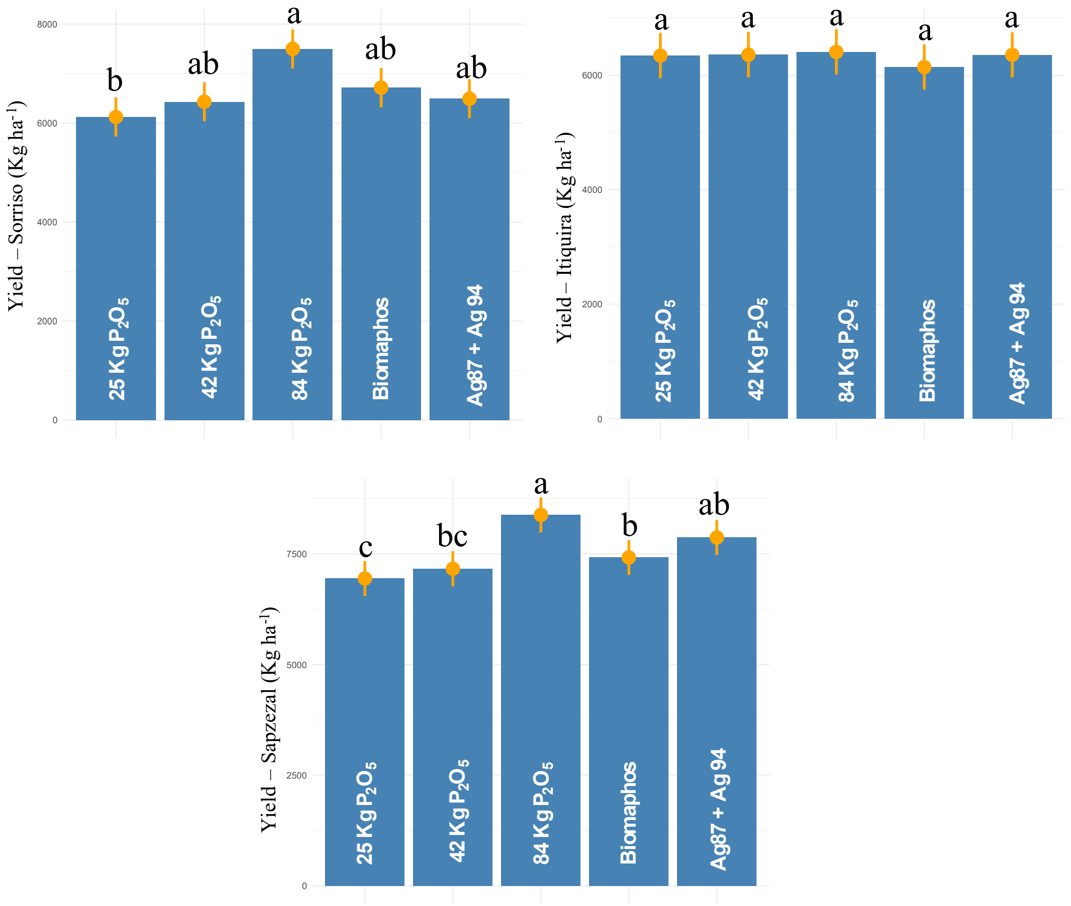

Figure 6. Comparison of Tukey ($p<0.05$) for grain yield in maize experiments (second crop – 2021) with seeds inoculated with different phosphorus-solubilizing bacteria at three sites in Mato Grosso state – Brazil.

4. Discussion

Phosphorus-solubilizing and plant growth-promoting rhizobacteria (PSB-PGPR) have gained prominence in world agriculture due to their beneficial effects on P use efficiency and improvement in radicular P acquisition (BARGAZ et al., 2021; RAWAT et al., 2021). To explore the biodiversity of microorganisms already adapted to the edaphoclimatic conditions in Brazil, we selected bacteria from the maize rhizosphere. Then, we performed various tests to choose bacterial strains with the potential for application as biological products capable of increasing P uptake efficiency and promoting plant growth.

For this purpose, rhizobacteria with the ability to solubilize phosphate and produce high amounts of IAA were selected. IAA plays an important role in root development, mainly in root hair and lateral root formation, improving nutrient absorption (KUDOYAROVA et al., 2017; ZEFFA et al., 2019). In this context, this combination, in addition to providing phosphate to plants, also stimulates the development of the organ, favoring greater capture of phosphorus. Kudoyarova et al. (KUDOYAROVA et al., 2017) verified that the *Paenibacillus illinoisensis* IB 1087 and *Pseudomonas extremeustralis* IB-Ki-13-1A strains, selected based on IAA production and phosphate solubilization, contributed efficiently to wheat root system development, favoring greater accumulation of biomass and phosphorus. Etesami et al. (ETESAMI; ALIKHANI; HOSSEINI, 2015)found that the trait IAA plays an important role in selecting plant growth-promoting bacteria in rice. In the present study, 70% of the evaluated strains increased root development compared to the control in the germination paper experiment.

In the greenhouse experiments, the efficiency of bacterial strains on maize root development was lower than that in the tests conducted on germination paper. The inoculation efficiency of plant growth-promoting bacteria can vary according to the plant genotype, plant development stage, bacterial strain, and environmental conditions, which impact colonization and interaction with the plant (ZEFFA et al., 2019). This fact is corroborated when analyzing the greenhouse experiments, in which there was greater effectiveness of bacterial strains evaluated in sand:soil conditions. The lower efficiency of PSB-PGPR inoculation in the sand experiment may be related to the nutritional conditions the plant was subjected to, disfavoring an effective interaction with maize. On the other hand, in the sand:soil experiment, soil nutrients may have favored this interaction, reflecting a greater promotion of the root system and higher shoot P content. Furthermore, similar to plant–endophyte interactions, the “balanced antagonism” hypothesis can be applied to plant and PGPR interactions (FESEL; ZUCCARO, 2016; SCHULZ; BOYLE, 2005), where phenotypic plasticity in host plants can range from mutualism to antagonism, depending on the plant genotype, environmental conditions, and bacterial isolate.

Based on the studies carried out on germination paper and in a greenhouse (sand and sand:soil), four bacterial strains (I04, I12, I13, and I17) demonstrated a high potential for maize root growth and shoot P content, indicating promising PSB-PGPR. Based on this information, strains I13 (Ag87) and I17 (Ag94) were selected for genomic studies and evaluation of their potential under field conditions. The strain Ag87 was identified as *Bacillus megaterium*. This species is commonly found in soils and is a member of the microbiome of several host plants, acting mainly as PGPR (HUANG et al., 2019; KUMAR; KUMAR; PATEL, 2018; SHARMA et al., 2013). In addition, strains of this species produce a wide range of bioactive compounds that promote plant growth and nutrient solubilization, mainly P and potassium (DUARTE et al., 2022; NASCIMENTO et al., 2020). Zhao et al. (ZHAO et al., 2021) found that the application of *B. megaterium* increased cucumber yield and improved soil phosphorus and potassium bioavailability.

The strain Ag94 was identified as *Lysinibacillus* sp. However, it was impossible to determine the species due to the ANI values (lower < 95%) compared to the genomes of isolates with a defined species. In this context, this strain may be a new species; therefore, further research is needed for this validation. *Lysinibacillus* species, previously described as members of the genus *Bacillus* (AHMED et al., 2007), have 37 described species (http://www.bacterio.net/lysinibacillus.html, accessed on 11 April 2022). Most species of this genus are isolated from soil environments, and several works have demonstrated their potential as PSB-PGPR (AGUIRRE-MONROY; SANTANA-MARTÍNEZ; DUSSÁN, 2019; LELAPALLI et al., 2021; SHABANAMOL et al., 2018). Evaluating several strains from the rice rhizosphere, Lelapalli et al. (LELAPALLI et al., 2021) found that *Lysinibacillus pakistanensis* PCPSMR15 has a high capacity for phosphate solubilization and growth promotion in beans, indicating that it is an important strain in the development of a commercial inoculant.

Under field conditions (2020/2021 harvest), the strains Ag87 and Ag94 obtained higher average yield increases than the 30% P control. In turn, when analyzed in combination, the average increase was even higher, indicating a positive effect when combining these strains. This fact is also corroborated for PUpE_g since the combined action of the strains increased P uptake efficiency compared to the control. In addition, the non-differentiation of inoculation (Ag87 + Ag94) with 100% P control indicates the possibility of reducing the P applied in maize. This reduction has great relevance for Brazilian agriculture due to the country's high dependence on imported fertilizers and the increase in the costs of phosphate fertilizers in recent years (WITHERS et al., 2018).

The results of increased grain yield in maize when inoculated with the strains Ag87 + Ag94 were also verified in the experiments carried out in the second harvest under different weather conditions. Therefore, this is the first study that demonstrates the efficiency of the combined action of *Bacillus megaterium* and *Lysinibacillus* in maize, aiming at increasing yield and phosphorous uptake efficiency, indicating the potential of these strains in developing commercial inoculants for Brazilian agriculture.

5. Conclusions

The combined selection of strains capable of phosphate solubilization and indole-acetic acid production allowed obtaining bacteria with the potential to promote maize growth. The strains I13 (Ag87) and I17 (Ag94) selected as PSB-PGPR were identified as *Bacillus megaterium* and *Lysinibacillus* sp., respectively. The combined inoculation of these strains increased maize grain yield and phosphorous use efficiency, indicating the potential of these strains to be used as commercial inoculants for Brazilian agriculture. Further studies are needed to evaluate the effect of these strains on other crops of agricultural importance. In addition, the optimization of the bioprocess for industrial production of these strains is needed.

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CAPÍTULO II

Avaliação das cepas Ag 87 (*Bacillus megaterium*) e Ag 94 (*Lysinibacillus* sp.) para rendimento de grão e eficiência do uso de fósforo no cultivo da soja

Evaluation of Ag87 (*Bacillus megaterium*) e Ag94 (*Lysinibacillus* sp.) strains for grain yield and efficiency in the use of phosphorus for soybean crop

Evaluation of Ag87 (*Bacillus megaterium*) e Ag94 (*Lysinibacillus* sp.) strains for grain yield and efficiency in the use of phosphorus for soybean crop

ABSTRACT

Phosphorus (P) is an essential nutrient for plants development, required in massive quantities in soybean crop. However, the low availability in Brazilian make looking for sustainable alternatives to increase it absorption by the plant. Plant growth-promoting bacteria (PGPB) has stood out by convert insoluble forms of phosphorus in soil to an accessible form. The aim of this work was to evaluate the performance of Ag87 (*Bacillus megaterium*) e Ag94 (*Lysinibacillus* sp.) as PGPB and their potential as phosphorus solubilizing, in soybean crop. Bacteria (belonging to the AgBio microorganisms bank) previously studied and consolidated in the maize, was incorporated into soybean seeds, and sown in the field, in Londrina, Maringá, Guarapuava and Entre Rios, in the state of Paraná – Brazil. Productivity and phosphorus use efficiency (PUE) were evaluated. Data obtained showed that Ag87 + Ag94 resulted in a productivity statistically equal to the control 100% treatment. The Ag87 strain showed the highest PUE in Guarapuava. Seed treatment with Ag94 resulted in higher PUE in Entre Rios, while the combination of Ag87 + Ag94 obtained higher PUE in Maringá. The strains showed superior results of PUE when compared to the control 25 Kg P_2O_5 in all locations, except Entre Rios. Strains Ag87 and Ag94 proved to be a viable option for inoculation of soybean seeds, increasing grain yield and phosphorus use efficiency in fertilizations with low dose of P recommended for the crop.

KEYWORDS: Bioinoculants, *Glycine max* (L.), microbial inoculation, microorganisms, phosphate solubilization, Plant Growth-Promoting Bacteria.

1. INTRODUCTION

Soybean (*Glycine max* (L.) Merril) is considered the main commodity of Brazilian market, showing exponential growth in the last few years, thus consolidating as the largest producer in the world (USDA, 2022). It is estimated that the average production for the 2021/2022 harvest will be around 124 million tons, with 62% of the production going to be exported (CONAB, 2022). However, abiotic factors have caused major concerns for crop yield.

Among the abiotic factors, nutritional stress is considered important because soils in tropical regions, in general, have high acidity, toxic levels of aluminum e low availability of nutrients, especially Phosphorus (P) (PAVINATO et al., 2020; ROY et al., 2016). Phosphorus is a element considered essential to the plants growth and development because it is involved in several essential mechanisms for their survival, such as the regulation of genes liked to photosynthesis, production of ATP (adenosine triphosphate) and phospholipids (LAWLOR; CORNIC, 2002), DNA (deoxyribonucleic acid) component and protein biosynthesis (CASTAGNO et al., 2021). This element can be found in the soil in its organics and inorganic form (FATIMA et al., 2021), and the main forms of phosphate uptake by plants are $H_2PO_4^-$ and HPO_4^{2-}.

Brazil imports more than 80% of the total fertilizers used in the country, with 23% of imports corresponding to Phosphate fertilizers, which influences in increase in production costs (CONAB, 2022b). Phosphate fertilizers are used in large quantities in order of meet the demand of large crops, because of the great part of applied Phosphate is fixed in the insoluble forms of iron and aluminum phosphate ($FePO_4$ e $AlPO_4$, respectively), or strongly adsorbed by colloids, becoming unavailable for the plant uptake (PENN; CAMBERATO, 2019).

Agricultural management strategies to improve the use of P in the soil by crops is essential in order to substantially reduce its demand. Among these strategies are the increase in soil pH by liming, crop rotation, double cultivation, cover crops between seasons, no-tillage and the use of modern fertilizers, in addition to the development of cultivars efficient in the use of P and inoculation of solubilizing phosphate microorganisms (SPM) (PAVINATO et al., 2020).

Microorganisms presents in the soil have been used due to their ability of solubilizing the Phosphorus in a soluble and uptake by plants. Wang et al. (2021) in studies with *Bacillus megaterium*, concluded that this bacterium induces auxin biosynthesis in *Arabidopsis* roots, stimulating the growth of the plant and lateral roots. Equivalent results were found by Song et al. (2022) with *Bacillus aryabhattai* isolated from the rhizosphere of soybean, further indicating that organic acids released by these microorganisms increased the P solubilization, besides promoting the germination of corn seeds and the seedling growth. Recent studies indicate that bacteria of the genus *Lysinibacillus* sp. has shown potential of promote the plant growth (AGUIRRE-MONROY; SANTANA-MARTÍNEZ; DUSSÁN, 2019b), osmotic resistance to oxidative stress (JHA et al., 2022), as well the capacity of P solubilization (Matos et al., 2017). Massucato et al. (2022), evaluating different strains of bacteria with the ability to solubilizing Phosphate and promoting plant growth in maize, found that the combined action of Ag87 (*B. megaterium*) e Ag94 (*Lysinibacillus* sp.) strains promoted productivity gains, indicating a synergic effect of these two strains. In view of the results obtained in related research, the present study aimed to evaluate the potential of Ag 87 and Ag94 strains in an isolated and/or combined manner in terms of productivity and P availability in soybean.

2. MATERIAL AND METHODS

Two bacteria strains were selected, identify as *Bacillus megaterium* and *Lysinibacillus* sp., cataloged in microorganisms bank of company AgBio and named as Ag87 e Ag94, respectively. Both strains were previously chosen from experiments in greenhouse and field in maize (MASSUCATO et al., 2022).

2.1. Preparation of bacterial isolates

Strains stored in cryovials containing liquid Tryptic Soy Broth (TSB) and glycerol in a 2:1 ratio at −80 ◦C were activated in Petri dishes containing LBA (Luria Bertani Agar, Neogen Corporation, United States) culture medium at 28 ◦C for 24 h. A pre-inoculum of each strain was prepared from pure colonies suspended in saline solution (0.85% sodium chloride), with turbidity adjusted to 0.5 in the McFarland nephelometric standard (1.5 × 108 CFU/mL). Thirty uL of these bacterial suspensions were transferred to 125 mL Erlenmeyer flasks containing 30 mL of Ag/02 culture medium (g L−1 : glucose 15.0, sucrose 10.0, yeast extract 10.0, micronized soy protein 10.0, KH2PO4 1.5, MgSO4 0.5, MnSO4 0.5, CaCl2 1.5, and pH 8.0) and incubated at 30 ◦C for 18–20 h at 200 rpm in an orbital shaker (Tecnal—TE 422, Piracicaba, São Paulo, Brazil) for inoculum production. For fermentation, 1000 mL Erlenmeyer flasks containing 400 mL of Ag/02 were inoculated with a 4 mL aliquot of the inoculum and incubated at 30 ◦C for 72 h at 200 rpm. After fermentation, the production concentration was adjusted to 2.0 × 109 CFU/mL

2.2. Field Trials

For the tests under field conditions, seeds of the soybean cultivar Credenz Result I2X (BASF®) were used. The seeds were treated with the biological products Ag87, Ag94, Ag87+Ag94, and the commercial product Biomaphos® (compost by the combination between *Bacillus subtilis* CNPMS B2084 – BRM034840 and *Bacillus megaterium* CNPMS B119 – BRM033112 strains) in plastic bags using a dose of 100 mL/50 kg seeds. As control treatments were used uninoculated seeds and three doses of P in the soil (25 KgP_2O_5, 42 KgP_2O_5 and 84 KgP_2O_5). The experiments were conducted during the 2020/2021 summer harvest in the municipalities of Londrina, Maringá, Guarapuava and Entre Rios do Oeste, state of Paraná, Brazil.

The experiment design adopted was complete randomized blocks with four repetitions. The plots were consisted of eight rows of 6 m in length with a spacing of 0.45 m between rows and five plants per meter. Before setting up the experiment, the areas were fertilized with 25 kg P_2O_5 ha^{-1}, 60 kg K_2O ha^{-1}, and 21 kg N ha^{-1}. The amount of P_2O_5 applied in the experiments was 30% of the standard phosphate fertilization recommended for soybean. Grain yield (GY, in kg ha^{-1} and 13% moisture) was obtained after manual harvesting and mechanical threshing of the plants in the six central rows of each plot. The components of P use efficiency (PUE) were determined by the ratio between grains produced and g of applied P, according (MOLL; KAMPRATH; JACKSON, 1982).

2.3. Data Analysis

The agronomic data were subjected to analysis of variance, after meeting the assumptions, the means were compared using the Tukey test ($p<0.05$). The analyzes and construction of the graphs were performed using the R program using the AgroR packages (SHIMIZU; MARUBAYASHI; GONCALVES, 2021).

3. RESULTS AND DISCUSSION

In the present study, *Bacillus megaterium* e *Lysinibacillus* sp. strains demonstrated significative effect of treatments and environment in the analysis of variance (Table 1). The coefficient of variation (CV) was considered low for both variables, with 13.47 and 21.66% for grain yield and PUE, respectively, showing a good experimental precision.

Among the environments, Guarapuava had the highest average for productivity (4079,41 kg ha^{-1}), followed by Entre Rios (3375.62 Kg ha^{-1}), Maringá (3074.65 kg ha^{-1}) and Londrina (3058.95 kg ha^{-1}). According to data gathered by CONAB (2022), the average yields observed in the four locations are consistent with the average yield values in Brazil in the 2020/2021 and 2021/2022 harvests, with 3528 kg ha^{-1} and 3526 kg ha^{-1}, respectively and the average of the state of Paraná for both seasons (CONAB, 2021; 2022), with 3535 kg ha^{-1} and 3678 kg ha^{-1}.

Table 1. Analysis of variance for grain yield and Phosphorus use efficiency in soybean experiments conducted in 2020/2021 harvest with seeds inoculated with different Phosphorus solubilizing bacteria.

Source of variation	**DF**	**Medium Square**	
		Grain Yield	**P use efficiency**
Block/environment	12	141508,7^{ns}	1790,45^{ns}
Treatments (T)	6	680907,6**	57238,6**
Environment (E)	3	6390671,4**	92850,06**
T x E	18	327153,4^{ns}	5829,55**
Error	72	209639,5	1126,27
CV(%)		13,47	21,66
Mean Londrina		3058,95	127,89
Mean Maringá		3074,05	153,56
Mean Entre Rios		3375,62	102,71
Mean Guarapuava		4079,41	235,48

ns, **, and * indicate non-significance and significance at levels of 1 and 5% probability by the F test, respectively.

Among the treatments, the 84 kg P_2O_4 control was the highest yield (3651.21 kg ha^{-1}) and it did not differ statistically from the Ag87 + Ag94 (3572.85 kg ha^{-1}), Ag94 (3540.44 kg ha^{-1}) treatments, P2O4 (3417.91 kg ha^{-1}) control and BiomaPhos® (3303.20 kg ha^{-1}) (Table 2). These data show the importance of using these bacteria to increase soybean grain yield, which can be caused by stimulating root growth by releasing components that stimulate the synthesis of phytohormones, or by the chelating capacity of substances exuded by bacteria that allow solubilization of the phosphate.

Table 2. Comparison of Tukey (p<0,05) avareges for grain yield and efficiency in the use of Phosphorous in soybean experiments conducted in the 2020/2021 harvest with seeds inoculated with different Phosphate solubilizing bacteria.

Treatments	Grain Yield		P use efficiency			
	Mean	Δ%	Londrina	Maringá	Entre Rios	Guarapuava

Ag87	3196,15 bc	3,18	156,77abc	183,71 ab	97,98 bc	357,89 a
Ag94	3540,44 ab	14,30	176,37 ab	182,21 ab	169,01 a	292,21 b
Ag87+Ag94	3572,65 a	15,34	134,11 bc	200,87 a	117,57 b	331,80 ab
Biomaphos	3303,20 abc	6,64	187,39 a	213,11 a	97,98 bc	224,22 c
25 KgP_2O_4	3097,47 c		129,83 c	163,50 b	128,60 ab	220,27 c
42 KgP_2O_4	3417,91 abc		76,05 d	80,47 c	67,60 cd	130,99 d
84 KgP_2O_4	3651,21 a		34,72 d	40,60 c	40,23 d	90,98 d

The yield data are similar to those found in previous studies in maize (MASSUCATO et al., 2022), which demonstrated that the seeds inoculation with Ag94 e Ag87 + Ag94 resulted in an average increase of 16.3 e 21.45% in maize yield in the four locations (Londrina, Maringá, Entre Rios and Guarapuava), when compared to the control of 30% do P recommended for maize crop , proving the capacity of this combination in both soybean and maize crops.

Bacillus megaterium strains are recognized in scientific community for their ability of promoting plant growth. Zhao et al. (2021), observed that the application of *Bacillus megaterium* strains had a positive impact on the functional properties of the soil microbial community, improving the availability of P e potassium (K). This species demonstrates the ability to control the expression of plant hormones such as auxin and thus induce the formation of lateral roots in *Arabidopsis* (Wang et al., 2021). Kang et al. (2014), in a study with mustard, found high rates of organic acids (malic acid and quinic acid) in treatments with the inoculation of *B. megaterium*, which act as chelating agents, increasing P absorption by plants, in addition to stimulating growth root and fresh biomass. An increase in photosynthetic pigments was also observed, improving photosynthesis rates and, consequently, stimulating plant growth (KANG et al., 2014). The use of *Bacillus megaterium* as a plant growth promoter and insoluble Phosphate solubilizer was confirmed by studies carried out by Hu et al. (2013) with oilseed rape, in zucchini by Zhao et al. (2021), and on wheat crop in Turkey by Turan et al. (2012).

Lysinibacillus sp. have been studied as the same aim, as proved by Jha et al. (2022) e Aguirre-Monroy et al. (2019) that strains of *L. fusiforme* e *L. sphaericus* produce siderophores, which are low molecular weight compounds that have the ability of remove iron, making insoluble P available and thus promoting plant growth, in addition to inducing plant hormone production and root growth in maize. Comparable results were related with the inoculation of *Lysinibacillus* sp. in soybean

(REIS et al., 2021), in which there was an increase in photosynthetic parameters of the plants, aiding in the synthesis of chlorophyll a and b. Jha et al., (2022) studding *L. fusiformis* and *L. sphaericus* strains in maize, stimulated the synthesis of auxin and gibberellic acid over time, thus stimulating the plant growth and, consequently, increasing the yield.

The combination of different strains proves to be advantageous, as observed with the commercial product BiomaPhos®, composed by the combination of strains of *Bacillus megaterium* and *Bacillus subtilis*. In Table 2 is possible to observe that the treatment inoculated with the commercial product did not differ statistically in the yield of the controls with 84 kg P_2O_4 e 42 kg P_2O_4. Equivalent results of grain yield by the action of the product were also observed in the study in maize (MASSUCATO et al., 2022) and in soybean (FIUZA et al., 2022).

The PUE is given by the ratio between dry biomass of grains and the amount of P applied. Treatments inoculated received 25 kg P_2O_4, which is equivalent to 30% of the amount of P recommended for the crop. Therefore, for a better understanding of the study, the inoculated treatments were compared with the control without inoculation fertilized with 25 kg P_2O_4, considering that the non-inoculated controls with the fertilization of 42 kg P_2O_4 and 84 kg P_2O_4, having the amount of P applied higher, result in a lower ratio. Thus, in Londrina, higher PUE was observed for the BiomaPhos® treatments, followed by Ag94 and Ag87 + Ag94 (Table 2). While for Maringá there was no statistical difference between treatments inoculated with bacteria, and all treatments were higher than the control 25 kg P_2O_4. In Guarapuava, the highest values of PUE were observed in treatments inoculated with Ag87 and with the combination of Ag87 + Ag94. Entre Rios was the only place where the control 25 kg P_2O_4 presented a result statistically equal to the Ag94 treatment and both were superior to the other treatments.

The highest levels of EUP in treatments inoculated with bacteria may be related to their ability to solubilize phosphate, in addition to the effects that promote plant growth. Plants inoculated with plant growth-promoting bacteria develop adaptation strategies for nutrient absorption, such as changes in the expression of iron transport, greater root development in order to explore greater soil area and activities of the rhizosphere to assist in the mobility of these nutrients (Jha et al., 2022).

In studies by Wang et al. (2021) it was also found that the induction of root growth happened even when the roots were no in contact with the bacterial strains, indicating that volatile metabolites may be involved in growth promotion. The presence of growth-promoting bacteria leads to the release of organic acids and metabolites that, in addition to promoting the synthesis of growth hormones, cause soil acidification, solubilizing the unavailable P (Aguirre-Monroy et al., 2019; Saeid et al., 2018). The pH is linked to availability of P to the plants in a way that the acidity of the soil influences to release of metallic ions, making the P insoluble in a absorbable form by the plants. PGPBs have the ability to reduce the pH of the medium through to release of organic acids synthesized through metabolic activities (SONG et al., 2022), such as the direct oxidation that occurs outside the cytoplasmic membranes of microorganisms (GURBANOV et al., 2021; SAEID; PROCHOWNIK; DOBROWOLSKA-IWANEK, 2018).

4. CONCLUSION

The results obtained by this study demonstrated that the positive performance of both *Bacillus megaterium* and *Lysinibacillus* sp. on soybean yield and Phosphorous use efficiency. The combination between both strains is efficient and show the potential of them in the development of commercial inoculant for soybean.

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ANEXOS

ANEXO A

Supplementary Table (S1) - Characterization of the soil used in the greenhouse experiment.

Characteristics$^{1/}$	Londrina – Greenhouse
Soil	Dystroferric Red Latosol
pH (H_20)	5.50
H+Al (cmolc dm^3)	3.24
K (cmolc dm^3)	0.83
Ca (cmolc dm^3)	5.81
Mg (cmolc dm^3)	2.50
Al (cmolc dm^3)	0.00
P (mg dm^3)	13.30
Organic matter (%)	9.51

ANEXO B

Supplementary Table (S2) - Characterization of environments used in maize and soybean experiments.

Characteristics[1/]	Londrina (2020/2021)	Maringá (2020/2021)	Guarapuava (2020/2021)	Londrina (2021/2021)	Londrina (2021/2022)	Guarapuava (2021/2022)
Geographical coordinates	23° 17’ S; 51° 10’ W	23° 11' S; 52° 03' W	25° 23' S; 51º 29' W	23° 17’ S; 51° 10’ W	23° 17’ S; 51° 10’ W	25° 23' S; 51º 29' W
Altitude (m)	550	555	1026	550	550	1026
Climate[2/]	Cfa	Cfa	Cfb	Cfa	Cfa	Cfb
Soil	Dystroferric Red Latosol	Dystroferric Red Latosol	Dystroferric Bruno Latosol	Dystroferric Red Latosol	Dystroferric Red Latosol	Dystroferric Bruno Latosol
pH ($CaCl_2$)	5,2	5,2	4,5	5,3	5,4	5,1
H+Al (cmolc dm^3)	3,3	3,2	5,5	3.5	3,2	3,9
K (cmolc dm^3)	0,8	0,5	0,3	1,2	0,9	0,9
Ca (cmolc dm^3)	5,4	2,9	2,1	5,8	5,4	2,7
Mg (cmolc dm^3)	1,6	1,3	0,6	1,2	1,5	1,2
Al (cmolc dm^3)	0,1	0,0	0,9	0,1	0,0	0,5
P (mg dm^3)	22,6	10,7	7,0	12,3	13,4	9,0
Organic Matter (%)	2,8	1,8	4,2	2,5	2,6	4,4

[1/] Physical-chemical analyses were performed using soil layer samples from 0 to 20 cm.

[2/] Köppen climate classification = Cfa, Humid subtropical climate; Cfb: Temperate oceanic climate.

ANEXO C

Supplementary Table (S3) - Analysis of variance for five agronomical traits evaluated in maize in greenhouse with seeds inoculated with different phosphate-solubilizing bacteria.

Source of Variation	DF	Mean Square[1/]				
		SD	PH	RDM	SDM	SPC
Treatments	14	6.72**	354.98**	1.34**	6.41**	1.95**
Error	75	2.16	82.08	0.32	1.63	0.26
Mean		11.90	74.2	1.85	6.58	4.43
CV (%)		12.37	12.21	30.49	19.39	11.63

[1] SD: stem diameter, PH: plant height, RDM: root dry mass, SDM: shoot dry mass, and SPC: shoot phosphorus content.

ns, ** e * indicates non-significance, significance at levels 1 and 5% of probability by the F test, respectively

ANEXO D

Supplementary Table (S4) - Mean clustering test (Scott-Knott) for stem diameter (SD), plant height (PH), root dry mass (RDM), shoot dry mass (SDM) and shoot phosphorus content (SPC) in maize seeds inoculated with phosphorus solubilizing bacteria in a greenhouse experiment.

Treatments	Traits				
	SD	PH	RDM	SDM	SPC
Control	12.18 **a**	72.83 **b**	1.85 **c**	6.16 **b**	3.99 **b**
Biomaphos	13.15 **a**	71.17 **b**	2.11 **b**	6.83 **a**	4.43 **b**
Strain01	11.27 **b**	70.83 **b**	1.65 **c**	6.25 **b**	3.79 **b**
Strain02	13.13 **a**	76.67 **a**	2.04 **b**	7.53 **a**	4.40 **b**
Strain03	13.67 **a**	84.67 **a**	2.68 **a**	8.78 **a**	5.39 **a**
Strain04	12.95 **a**	80.33 **a**	2.79 **a**	7.72 **a**	4.66 **a**
Strain05	11.62 **b**	67.33 **b**	1.25 **c**	6.03 **b**	5.06 **a**
Strain06	10.88 **b**	74.17 **b**	1.47 **c**	6.33 **b**	4.28 **b**
Strain07	9.60 **b**	58.33 **c**	1.14 **c**	4.74 **b**	5.12 **a**
Strain08	11.56 **b**	61.83 **c**	1.54 **c**	5.43 **b**	4.70 **a**
Strain09	11.37 **b**	71.50 **b**	1.98 **b**	6.67 **b**	4.40 **b**
Strain10	11.80 **b**	79.50 **a**	2.03 **b**	6.37 **b**	4.20 **b**
Strain11	12.15 **a**	80.50 **a**	2.10 **b**	7.29 **a**	3.85 **b**
Strain12	10.95 **b**	79.00 **a**	1.67 **c**	5.90 **b**	4.17 **b**
Strain13	12.24 **a**	84.33 **a**	1.83 **c**	7.34 **a**	4.78 **a**

[1/] Means followed by the same letter in the column do not differ statistically at 5% probability by the Scott-Knott test.

ANEXO E

Supplementary Table (S5) - Effects of phosphorus solubilizing bacteria on grain yield in maize in six experiments.

Treatments	Experiments[1/]					
	Env.1	Env.2	Env.3	Env.4	Env.5	Env.6
Control 25 Kg P_2O_5	8291 **b**	6475 **b**	4679 **b**	4486 **a**	8317 **b**	6778 **b**
Control 42 Kg P_2O_5	9108 **ab**	6555 **b**	5481 **ab**	5176 **a**	9978 **a**	7264 **ab**
Control 84 Kg P_2O_5	9477 **a**	7822 **a**	6088 **a**	5322 **a**	10856 **a**	7539 **a**
25 Kg P_2O_5 + Biomaphos	8608 **ab**	6842 **ab**	5407 **ab**	4953 **a**	10147 **a**	7294 **ab**
25 Kg P_2O_5 + Ag75	9210 **ab**	7369 **ab**	5829 **ab**	5043 **a**	11206 **a**	7306 **ab**

[1/] Env1.: Londrina (2020/2021), Env2.: Maringá (2020/2021), Env3.: Guarapuava (2020/2021), Env4.: Londrina (2021/2021), Env5.: Londrina (2021/2022) and Env6.: Guarapuava (2021/2022).

ANEXO F

Supplementary Table (S6) - Effects of phosphorus solubilizing bacteria on grain yield in soybean in five experiments.

Treatments	Experiments[1/]				
	Env.1	Env.2	Env.3	Env.4	Env.5
Control 25 Kg P_2O_5	2058 **b**	2052 **b**	3580 **b**	3195 **b**	2936 **b**
Control 42 Kg P_2O_5	2512 **ab**	2708 **ab**	3899 **ab**	3374 **ab**	3523 **ab**
Control 84 Kg P_2O_5	2945 **a**	2780 **a**	4313 **a**	4002 **a**	3449 **ab**
25 Kg P_2O_5 + Biomaphos	2240 **ab**	2344 **ab**	4494 **a**	3389 **ab**	3754 **a**
25 Kg P_2O_5 + Ag75	2843 **a**	2694 **ab**	4013 **ab**	3808 **ab**	3334 **ab**

[1/] Env1.: Londrina (2020/2021), Env2.: Maringá (2020/2021), Env3.: Guarapuava (2020/2021), Env4.: Londrina (2021/2022) and Env5.: Guarapuava (2021/2022).

ANEXO G

Supplementary Table (S7) - Biosynthetic Gene Clusters (BGCs) found within *Bacillus velezensis* Ag109 genome using the webserver antiSMASH 5.1.0.

Cluster	Type	From (pb)	To (pb)	Most similar known cluster		Similarity (%)
1	thiopeptide	281.447	311.183	-	-	-
2	NRPS	322.005	387.087	Surfactin	NRP:Lipopeptide	82
3	PKS-like	918.587	959.831	Butirosin A and B	Saccharide	7
4	Terpene	1045.457	1062.628			
5	TransAT-PKS	1392.865	1479.251	Macrolactin	Polyketide	100
6	TransAT-PKS, T3PKS, NRPS	1705.384	1805.935	Bacilaene	Polyketide + NRP	100
7	NRPS, TransAT-PKS, Betalactone	1874.238	2011.614	fengycin	NRP	100
8	Terpene	2034.651	2056.534	-	-	-
9	T3PKS	2140.205	2181.305	-	-	-
10	TransAT-PKS	2313.205	2406.997	Difficidin	Polyketide + NRP	100
11	NRPS, RiPP-like	3039.587	3091.378	Bacillibactin	NRP	100
12	Other	3619.927	3661.345	Bacilysin	Other	100

NRPS, non-ribosomal peptide synthetase; NRP, non-ribosomal peptide. PKS, polyketide synthetase. AT, acetyltransferase; T3PKS, type 3 Pks.

ANEXO H

Supplementary Figure (S1) - SEED classification of Ag75 genome. Pie chart depicting functional categories in Ag75 genome. The SEED annotated genome was compared to hundreds of genomes maintained within the SEED integration. RAST annotation (server possessed identified protein encoding genes (PEGs), RNA genes and repeat regions) was used to create the pie chart.