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RAFAEL DE ALMEIDA LEITE

GENE PROSPECTION AND MECHANISMS OF PHOSPHATE SOLUBILIZATION IN SELECTED BACTERIAL AND FUNGAL STRAINS

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Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós- Graduação em Ciência do Solo, área de concentração em Biologia, Microbiologia e Processos Biológicos do Solo, para a obtenção do título de Doutor.

Profa. Dra. Fatima Maria de Souza Moreira
Orientadora

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**PROSPECÇÃO DE GENES E MECANISMOS DE SOLUBILIZAÇÃO DE
FOSFATOS DE BACTÉRIAS E FUNGOS SELECIONADOS**

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Solo, área de concentração em Biologia,
Microbiologia e Processos Biológicos do
Solo, para a obtenção do título de Doutor.

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RESUMO GERAL

O fósforo (P) é um dos elementos mais requeridos pelos organismos. Na agricultura, P é suprido através da adubação mineral fosfatada, em formas prontamente disponíveis para as plantas. No solo, a dinâmica do P é complexa e uma pequena fração do fertilizante pode estar disponível para as plantas e microrganismos, enquanto que grande parte do P pode ser fortemente retido na fração mineral do solo ou ser incorporado à biomassa microbiana. Microrganismos desempenham um papel importante na ciclagem de P e bactérias e fungos são os principais contribuintes para a ciclagem deste elemento. Neste sentido, há inúmeros relatos da solubilização de fosfato por bactérias e fungos e alguns dos mecanismos, apesar de conhecidos, ainda precisam ser melhor compreendidos para alcançar potencial biotecnológico. Os objetivos deste trabalho são avaliar o potencial e os mecanismos de estirpes de bactérias e fungos na solubilização de fosfatos de cálcio e ferro, avaliar o potencial das estirpes bacterianas em promover o crescimento de milho e identificar os genes relativos à produção de ácidos orgânicos nas estirpes bacterianas. Em um primeiro trabalho, foram realizados experimentos de solubilização *in vitro* de estirpes de bactérias de forma a comparar potenciais e mecanismos desses organismos. O sequenciamento de DNA genômico das estirpes foi realizado para identificar as espécies e buscar genes relacionados à produção de ácidos orgânicos. As bactérias foram inoculadas em plantas de milho e foi avaliado o potencial de promoção de crescimento para a planta. *Paraburkholderia fungorum* UFLA 04-21 e *Pseudomonas anuradhapurensis* UFPI B5-8A solubilizaram in vitro mais de 60% de fosfato e produziram grandes quantidades de ácidos cítrico/maleico e glucônico/tartárico, respectivamente. Todos os 11 ácidos orgânicos estudados foram identificados, apesar de a maioria das estirpes não apresentarem todos eles. A maior parte dos genes e enzimas relacionados à produção de ácidos orgânicos foi encontrada nos genomas. Plantas inoculadas com as estirpes UFPI B5-6, UFPI B5-8A e UFLA 03-10 acumularam mais biomassa do que as plantas fertilizadas com fosfato Bayóvar. As estirpes UFLA 03-10 e UFPI B5-8A aumentaram o acúmulo da maioria dos macronutrientes, incluindo P, e alguns micronutrientes. No segundo trabalho, estirpes de bactérias e fungos foram comparadas quanto aos potenciais e mecanismos de solubilização de fosfato de cálcio e de ferro. *Acinetobacter pittii* UFLA 03-09 e *Penicillium flavigenum* E24 foram os microrganismos que mais solubilizaram o fosfato de cálcio. O fosfato de ferro foi solubilizado em uma extensão consideravelmente menor devido às características do fosfato e os mais promissores foram *Brukholdeia cepacia* LMG 1222 e *Penicillium flavigenum* E24. Bactérias e fungos tiveram correlações significativas entre P solúvel e ácidos orgânicos quando cultivados em meio de fosfato de cálcio, mas apenas os fungos tiveram essa correlação significativa para fosfato de ferro. Todos os ácidos orgânicos de baixo peso molecular estudados foram identificados nas estirpes e a análise genômica identificou vários genes ligados à produção dos ácidos orgânicos. Os resultados contribuem para o entendimento dos processos de solubilização de fosfatos por microrganismos, especialmente considerando estirpes de bactérias e fungos com potencial de promoção de crescimento de plantas.

Palavras-chave: Fósforo. Microrganismos. Ácidos orgânicos. Sideróforos. Mecanismos. Promoção de crescimento de plantas.

GENERAL ABSTRACT

Phosphorus (P) is one of the most required elements by organisms. In agriculture, P is mainly supplied through mineral phosphate fertilization, in forms readily available to plants. In soil, P dynamics are complex and only a small fraction of the phosphate fertilizer may be available for plants and microorganisms, while a large part of P can be strongly retained in the mineral fraction of the soil or incorporated into microbial biomass. Microorganisms play an important role in the P cycling and bacteria and fungi are the main contributors to the cycling of this element between the organic and mineral fractions. In this sense, there are numerous reports of phosphate solubilization by bacteria and fungi and the some mechanisms, despite already known, still need to be better understood to reach biotechnological potential. The objectives of this work were to evaluate the potential and mechanisms of phosphate-solubilizing bacterial and fungal strains in the solubilization of calcium and iron phosphates, evaluate the potential of bacterial strains to promote maize growth and identify genes related to organic acid production in the bacterial strains. In the first work, *in vitro* solubilization experiments with bacterial strains were carried out in order to compare the potentials and mechanisms of these organisms. Then, genomic DNA was sequenced to identify the species and search for genes related to the production of organic acids. The strains were inoculated into maize plants and the plant's growth promotion potential was evaluated as a function of the strains' solubilization capacity. *Paraburkholderia fungorum* UFLA 04-21 and *Pseudomonas anuradhapurensis* UFPI B5-8A solubilized more than 60% of phosphate *in vitro* and produced large amounts of citric/maleic and gluconic/tartaric acids, respectively. All 11 organic acids studied were identified, although most strains did not produced all of them. Most of the genes and enzymes related to the production of organic acids were found in the genomes. Plants inoculated with UFPI B5-6, UFPI B5-8A, and UFLA 03-10 strains accumulated more biomass than plants fertilized with Bayóvar phosphate alone. Strains UFLA 03-10 and UFPI B5-8A increased the accumulation of most macronutrients, including P, and some micronutrients. In the second work, bacterial and fungal strains were compared regarding the potentials and mechanisms of calcium and iron phosphate solubilization. *Acinetobacter pittii* UFLA 03-09 and *Penicillium flavigenum* E24 were the microorganisms that solubilized most of the calcium phosphate. Iron phosphate was solubilized to a considerably lesser extent due to phosphate characteristics and the most promising microorganisms were *Brukholdeia cepacia* LMG 1222 and *Penicillium flavigenum* E24. Bacteria and fungi had significant correlations between soluble P and organic acids when grown in calcium phosphate medium, but only fungi had a significant correlation for iron phosphate. All the low molecular weight organic acids studied were identified in the strains and the genomic analysis identified several genes linked to the production of organic acids. The results contribute to the understanding of phosphate solubilization processes by microorganisms, especially considering bacterial and fungal strains with high potential for plant growth promotion.

Keywords: Phosphorus. Microorganisms. Organic acids. Siderophores. Mechanisms. Plant growth promotion

SUMÁRIO

PRIMEIRA PARTE – INTRODUÇÃO E REVISÃO BIBLIOGRÁFICA

1 INTRODUÇÃO

O fósforo (P) é um dos grandes limitadores para o crescimento de plantas por ser um elemento indispensável para processos metabólicos fisiológicos e bioquímicos ao ponto que a disponibilidade ou a falta do elemento controla, o crescimento e produtividade das plantas, especialmente em agrossistemas. A maior fonte de P utilizada na agricultura vem de rochas fosfatadas, que constituem um recurso finito e não renovável (SCHOLZ et al., 2013), e que, em função da sua natureza e interação com o solo, requer uma melhor eficiência de uso para reduzir impactos ambientais e criar a sustentabilidade das fontes naturais (CARPENTER; BENNETT, 2011; SIMPSON et al., 2011).

Devido à baixa solubilidade do fosfato de rocha, ele precisa passar por um processo de acidificação resultando em fosfato solúvel em água altamente concentrado, facilmente disponível para absorção pelas plantas após a aplicação. Este processo garante a disponibilidade de fosfato para as plantas, mas envolve altos custos e o P no solo torna-se altamente propenso à lixiviação e retenção do solo (CHIEN et al., 2011). As reservas mundiais de fosfato de rocha estão se esgotando e a qualidade do fosfato remanescente está diminuindo (SCHOLZ et al., 2013).

Entretanto, o P retido no solo não está completamente perdido, assim como o fosfato de rocha não é de todo inviável para uso na agricultura. O processo de solubilização de fosfatos é capaz de acessar essas fontes de P pouco biodisponíveis. Este processo é realizado por diversos microrganismos e até mesmo por plantas superiores. Entretanto, apesar de alguns mecanismos por trás da solubilização de fosfastos serem conhecidos e consolidados, um panorama completo deste processo biológio ainda não é definido, bem como a interação dos possíveis mecanismos durante o processo de solubilização. O principal mecanismo reportado é a produção de ácidos orgânicos de baixo peso molecular, que, através de algumas reações com os cátions ligantes dos fosfatos ou por interação com o solo, favorecem o processo de solubilização (KIM et al., 1997; CHEN et al., 2006). Estes ácidos orgânicos são produzidos pela ativação de genes em diversas rotas metabólicas, e têm sido identificados em alguns trabalhos, mas ainda em quantidade pouco representativa.

Neste trabalho, algumas hipóteses são levantadas. Em um primeiro momento, a hipótese é se bactérias com potencial de solubilização de fosfato *in vitro* são capazes de promover a solubilização via o mecanismo de liberação de ácidos orgânicos de baixo peso

molecular; e que estas bactérias são capazes de promover a solubilização de um fosfato de rocha pouco reativo em solo, e, por consequência, promover o crescimento de plantas de milho. Em um segundo momento, foram comparados diversos isolados de fungos e estirpes de bactérias e, baseado em diversas fontes da literatura, levantou-se a hipótese que os fungos teriam potencial de solubilização maior que as bactérias. Ao mesmo tempo, também levantou-se a hipótese de os mecanismos de solubilização de fosfato de cálcio e ferro estarem ligados a liberação de ácidos orgânicos e sideróforos.

O objetivo geral deste trabalho é avaliar o potencial de estirpes de bactérias e fungos solubilizadores de fosfato na solubilização de fosfato de cálcio e ferro, identificando possíveis mecanismos relacionados à solubilização; e identificar mecanismos de solubilização e os genes relacionados à produção de ácidos orgânicos, além da promoção de crescimento de plantas em estirpes de bactérias.

2 REVISÃO BIBLIOGRÁFICA

2.1 Dinâmica do fósforo no solo

As plantas absorvem P na forma de ânions da solução do solo, majoritariamente como ortofosfato. Em pH relativamente ácido, o ortofosfato encontra-se na forma de $H_2PO_4^-$ ($pK_{a1} = 2.14$); enquanto o pH aumenta, são formados íons HPO_4^{-2} ($pK_{a2} = 7.20$), que podem se transformar em PO_4^{-3} ($pK_{a3} = 12.37$) em solo alcalino (POWELL et al., 2005; YADAV; VERMA, 2012). Da fração orgânica do P no solo, a maioria dos compostos foram identificados como ésteres de ácido ortofosfórico nas formas de fosfato inositol, fosfolipídeos e ácidos nucleicos (YADAV; VERMA, 2012; BÜNEMANN, 2015), e podem constituir, em média, de 30-50% do P nos solos, mas com alta variação (5 a 90%) (SHARPLEY, 1985). A fração inorgânica pode ser dividida em dois grupos de maior ocorrência, conforme os cátions ligantes, formando diferentes minerais: fosfato de cálcio, principalmente na forma de apatita, um fosfato praticamente insolúvel; e os fosfatos de ferro e alumínio, alguns de baixa solubilidade, que pouco contribuem para a nutrição de plantas (YADAV; VERMA, 2012). Enquanto P associado com óxidos/hidróxidos de Fe (Fe-P) são sensíveis a condições redutoras, P precipitado com Al (Al-P) e Ca (Ca-P) são sensíveis a mudanças de pH, e as formas orgânicas são mais biodisponíveis (ZHU; LI; WHELAN, 2018).

Apesar de muitos solos apresentarem um considerável estoque de P, apenas uma pequena fração (< 1 %) do P total inorgânico e orgânico encontra-se dissolvida, e, portanto, disponível para as plantas (BÜNEMANN, 2015), enquanto a maior parte é removida da solução por mecanismos de sorção, precipitação e imobilização microbiana, ou é perdida por escoamento superficial ou fluxo subsuperficial. A soma dessas características mostra que o P é um elemento que apresenta grande potencial de limitar o desenvolvimento de sistemas naturais e cultivados pelo fato de ser removido de formas disponíveis para as plantas (HEATHWAITE; DILS, 2000; ROBERTS; JOHNSTON, 2015). Essa característica tem implicação direta na adubação de fertilizantes fosfatados, pois mais de 80% do P aplicado na forma de fertilizante mineral pode se tornar indisponível para absorção pelas plantas rapidamente após aplicação por processos de sorção, precipitação e imobilização microbiana (ZHU; LI; WHELAN, 2018).

Por outro lado, a alta capacidade de fixação do P nos solos e a baixa eficiência na fertilização e absorção pelas plantas acumulam o P no solo em formas pouco disponíveis

(WITHERS; EDWARDS; FOY, 2001). Assim, para satisfazer a necessidade de produção nos sistemas agricultáveis, o P deve ser constantemente reabastecido de maneira a sempre fornecer formas assimiláveis para as plantas. Essa acumulação é conhecida como legado de P (*legacy* P) e pode ser calculada como a diferença entre as entradas de P no sistema, a partir de efeitos de fertilização mineral e orgânica, intemperismo de rochas e deposição atmosférica; e as saídas de P do sistema, por perdas através de escoamento superficial, lixiviação, retirada pelas plantas, etc. (BOUWMAN; BEUSEN; BILLEN, 2009; SATTARI et al., 2012).

Este reservatório ou legado de P pode contribuir para o P dissolvido na solução do solo que pode ser absorvido pelas plantas, porém ao longo de muitos anos. Enquanto a quantidade de P prontamente disponível está abaixo do nível crítico, a taxa de liberação de P do legado de P pode não ser suficiente para rapidamente suprir as necessidades das plantas e manter níveis elevados de produtividade (BOUWMAN; BEUSEN; BILLEN, 2009). As frações de P entre as fases mineral, orgânica, dissolvida e aquela imobilizada na biomassa microbiana podem resultar em baixo equilíbrio, o que é refletido como deficiência do nutriente nas plantas e baixa produtividade. Entretanto, isso pode ser ajustado com os processos e reações que ocorrem no solo, via solubilização e precipitação (equilíbrio mineral), sorção e dessorção (interações entre o P e a superfície dos minerais), e mineralização e imobilização (transformações biológicas entre as fases orgânica e inorgânica do P) (ZHU; LI; WHELAN, 2018).

2.2 Microrganismos solubilizadores de fosfato

Microrganismos são um componente integrante do ciclo do P no solo e desempenham papel na transferência de P entre diferentes compartimentos. Grande parte da ciclagem de P, orgânico e inorgânico, pode ser atribuído a bactérias e fungos (ZHU; LI; WHELAN, 2018). Dessa forma, há um interesse na manipulação desses microrganismos solubilizadores de fosfato do solo para melhorar a nutrição fosfatada das plantas, aumentando a eficiência de uso do P nos sistemas agricultáveis (RICHARDSON, 2011). Microrganismos solubilizadores são aqueles que dissolvem o P inorgânico (mineral) insolúvel e mineralizam o P orgânico insolúvel com o consequente aumento da biodisponibilidade do P do solo para as plantas (SHARMA et al., 2013). A ideia de que microrganismos podem ser utilizados na nutrição fosfatada de plantas não é recente. O papel dos microrganismos de rizosfera na solubilização de fosfato data de 1903 (KHAN;

ZAIDI; WANI, 2007), mas até então, os trabalhos consideravam toda a população microbiana do solo, e os mecanimos da solubilização não eram claros, apesar de se ter uma ideia que estavam ligados à acidificação. Um dos primeiros trabalhos com culturas puras já mostrava que bactérias do solo podem contribuir para a nutrição fosfatada das plantas através da solubilização de fosfato de cálcio (GERRETSEN, 1948). No mesmo ano, Pikovskaya (1948) sugeriu um meio de cultura (PVK) para o isolamento e triagem de microrganismos solubilizadores de fosfato. Atualmente, os dois meios mais utilizados em isolamento e testes de solubilzação são o PVK e o mais recente National Botanical Research Institute Phosphate Medium (NBRIP) proposto por Nautyial (1999) como mais eficiente que o meio PVK.

Em se tratando de microrganismos solubilizadores de fosfato, é mais comum a ocorrência de estirpes com capacidade de solubilização de Ca-P que formas de Fe-P e Al-P, tanto em meio líquido quanto em meio sólido. Os microrganismos solubilizadores estão distribuídos em diversos filos: Actinobacteria, Ascomycota, Bacteroidetes, Basidiomycota, Euryarchaeota, Firmicutes, Mucoromycota e Proteobactérias. Em uma revisão sistemática, Kour et al. (2021) identificaram as seguintes proporções entre os grupos de microrganismos solubilizadores de P: o filo dominante entre as bactérias é Proteobacteria (38 %), seguido por Firmicutes (22 %); 9 % pertencem a Euryarchaeota; 6 % pertencem a Actinobacteria; e 1 % pertence a Bacteroidetes. Entre os fungos, os filos menos representados são Mucoromycota (1 %) e Basidiomycota (3 %), enquanto 20 % pertencem a Ascomycota. Os gêneros bacterianos mais reportados em trabalhos de solubilização de fosfato são *Bacillus*, *Pseudomonas* e *Acinetobacter*. Por outro lado, trabalhos com fungos também são mais comuns com formas de Ca-P e os gêneros mais recorrentes são *Aspergillus* e *Penicillium* (KOUR et al. 2021).

Vários inoculantes têm sido desenvolvidos para aumentar a biodisponibilidade de P no solo com estirpes de diversos gêneros, como *Pseudomonas* spp., *Bacillus* spp., *Rhizobium* spp., *Klebsiella* spp., *Penicillium* spp., *Aspergillus* spp., *Rhizopus* spp., e *Streptomyces* spp (MAHAJAN; GUPTA, 2009). Os benefícios incluem o baixo custo, não têm problemas ambientais conhecidos e podem ser usados para complementar fertilizantes químicos. Estes inoculantes podem ser associados com fungos micorrízicos arbusculares para aumentar ainda mais a eficiência no uso do P. Liu et al. (2016) estimaram uma redução de aproximadamente 80% do fertilizante fosfatado com rendimentos semelhantes na produtividade de milho após a inoculação com fungos

micorrízicos arbusculares. A associação de microrganismos solubilizadores associado a fungos micorrízicos pode ser uma alternativa viável para reduzir a adsorção permanente de P em solos tropicais e promover o crescimento e produtividade de plantas.

Entretanto, a distinção entre os papéis dos processos microbianos em comparação com os efeitos dos mecanismos vegetais na mobilização de P em solos ainda é pouco entendido. É conhecido que as raízes das plantas efetivamente aumentam a aquisição de P através do crescimento e arquitetura radicular modificados e produção de metabólitos que afetam diretamente a disponibilidade de P (RAGHOTHAMA, 1999). Processos como acidificação da rizosfera, exsudação de ânions orgânicos e secreção de fosfatases das raízes das plantas ocorrem em resposta à deficiência de P, e são mecanismos estabelecidos, pelos quais as plantas adquirem P (HINSINGER et al., 2005). Apesar da complexidade na separação dos potenciais de solubilização de microrganismos e plantas, a importância da biomassa microbiana para a ciclagem de P no solo e o potencial desse P para contribuir para a nutrição de plantas é inegável (RICHARDSON et al., 2011).

2.3 Mecanismos e genes de solubilização de fosfatos por microrganismos

De maneira geral, é amplamente aceito que um dos mecanismos de solubilização de fosfato é a liberação de ácidos orgânicos de baixo peso molecular, e diversos trabalhos mostram a liberação de diferentes tipos de ácidos orgânicos frente a formas de fosfato insolúvel (KIM; MCDONALD; JORDAN, 1997; CHEN et al., 2006; MARRA et al., 2012, 2015). Além da dissociação do ácido, liberação de H^+, e consequente acidificação do meio, que pode ser um outro mecanismo de solubilização de P (BRAZ; NAHAS, 2012), os grupos carboxil das moléculas dos ácidos orgânicos podem se ligar aos cátions ligantes dos fosfatos precitados, aumentando a disponibilidade de P para plantas (BOLAN et al., 1994; KPOMBLEKOU; TABATABAI, 1994; STRÖM et al., 2001), ou mesmo se ligar aos sítios de sorção nos minerais do solo, impedindo a adsorção permanente do P (ALI; DZOMBAK, 1996; GEELHOED; VAN RIEMSDIJK; FINDENEGG, 1999; STRÖM et al., 2001). A redução do pH também pode estar relacionada à solubilização do P. Chen et al. (2006) encontraram, *in vitro,* uma significativa correlação negativa entre pH e P solúvel (R = -0,80; p < 0,01), com a máxima solubilização (421,8 mg L^{-1}) para a máxima redução de pH (4,9), ao passo que as menores concentrações de P solúvel (31,5 mg L^{-1}) foram associadas à pouca redução de pH (de 6,8-7,0 para 6,0).

Entretanto, nem sempre é possível encontrar uma relação direta entre a solubilização e acidificação, indicando que outros mecanismos podem ter participação no processo, como, por exemplo, a produção de sideróforos (CUI et al., 2022). Em meio de cultura sólido, Marra et al. (2011) não encontraram halo de solubilização em meio enriquecido com $FePO_4.2H_2O$, enquanto observaram solubilização para $CaHPO_4$ e $Al(H_2PO_4)_3$. Em meio líquido, os autores verificaram aumento no P solúvel para as três fontes utilizadas, com algumas estirpes com atividade de solubilização para apenas uma ou para mais fontes, e com a ressalva de que em alguns casos, houve redução de pH associada à uma perda de P solúvel do meio, tendo isso sido atribuído à precipitação em função da acidez e à imobilização do P na biomassa especialmente para $CaHPO_4$ e $FePO_4.2H_2O$. Marra et al. (2012) também não encontraram solubilização em meio sólido de $FePO_4.2H2O$ para nenhuma das estirpes avaliadas, isoladas de áreas de floresta tropical, enquanto oberservaram halos de solubilização para $CaHPO_4$ em 36 das 82 estirpes avaliadas, e para $Al(H_2PO_4)_3$ em 16 das estirpes. Em meio líquido, entretanto, os autores verificaram que houve solubilização em 42 estirpes para $CaHPO_4$, 28 estirpes para $FePO_4.2H_2O$ e 13 estirpes para $Al(H_2PO_4)_3$, sendo que algumas estirpes que não apresentaram atividade solubilizadora em meio sólido foram consideradas eficientes na solubilização de fosfato em meio líquido.

No geral, parece haver uma maior ocorrência de trabalhos que testam a solubilização de P em condições de Ca-P, e, mesmo nos trabalhos que testam outras fontes pouco solúveis de P, o Ca-P parece ser solubilizado por um número maior de estirpes testadas. Todavia, considerando os solos tropicais, e, especialmente os do Brasil, grande parte destes são oxídicos e apresentam elevados teores de (hidr)óxidos de Fe e Al, que precipitam e indisponibilizam o P solúvel disponível para as plantas. Assim sendo, há uma necessidade de avaliar o potencial de solubilização de estirpes de bactérias para essas fontes, sob a premissa de utilizar o legado de P. A premissa é que a inoculação de microrganismos solubilizadores de P possam contribuir para a nutrição fosfatada das plantas ao acessar parte do P pouco disponível dos solos, dessa forma, contribuindo para a redução da necessidade da adubação fosfatada e assegurando maior sustentabilidade no uso dos recursos naturais.

O processo de solubilização de fosfatos ainda carece de informações mais aprofundadas quanto à identificação e expressão do genes relacionados. Pesquisas têm identificado alguns genes relacionados à produção de ácidos orgânicos há algum tempo,

mas, em geral, essa identificação genômica tem foco no ácido glucônico. Goldstein e Liu (1987) identificaram a codificação do gene relacionado à PQQ sintetase em *Erwinia herbicola* e a expressão desse gene com a produção de ácido glucônico quando transferido para *Escherichia coli*. Até hoje, o ácido glucônico é o mais estudado e relacionado ao processo de solubilização. Uma das enzimas do ciclo glicolítico que participa na síntese deste ácido em bactérias é a glicose desidrogenase (codificada pelo *gcd*) na via de oxidação direta de glicose, na qual a pirroloquinolina quinona – PQQ – (gene *pqq*) atua como cofator redox (SULEMAN et al., 2018). Outra enzima relacionada ao ácido glucônico é a glicose 1-desidrogenase (codificada pelo gene *gdh*), mas que não requer o cofator PQQ (SANTOS-TORRES et al., 2021). Em termos de capacidade de solubilização por cada ácido orgânico, é possível que outros ácidos tenham maior capacidade química de solubilização. Por exemplo, os ácidos cítrico e oxálico possuem propriedades quelantes que permitem a formação de complexos mais estáveis com o Ca, enquanto o ácido glucônico, que apresenta apenas um grupo carboxílico, é proposto a solubilizar fosfato pela liberação de H^+ (FOX et al., 1990; KPOMBLEKOU E TABATABAI, 1994, GOLDSTEIN, 1995). Entretanto, a quantidade de pesquisas envolvendo ácidos orgânicos, como cítrico, fórmico, málico, e oxálico, é escassa, e a genômica envolvendo esses ácidos é mais rara ainda, e poucos trabalhos oferecem uma gama maior de análise genômica para a produção desses ácidos (SANTOS-TORRES et al., 2021; SILVA et al., 2021).

No entanto, ácidos orgânicos e acidificação podem não ser os únicos mecanismos que promovem a solubilização de fosfato. Enquanto experimentos com fosfato de cálcio e fosfato de rocha são mais comuns e podem ser altamente correlacionados com acidificação e com a quantidade de ácidos orgânicos (CHEN et al., 2006; MARRA et al., 2015), estudos sobre solubilização de fosfato de ferro são mais escassos. Os fosfatos de ferro são menos propensos a se dissociar na faixa de pH de 2 a 8, que é a faixa onde a maioria dos microrganismos habita, resultando em baixos resultados ao solubilizar fosfatos ligados ao ferro (JIN et al., 2006; LI et al., 2015). Apesar de sua importância na solubilização e quelação, os ácidos orgânicos podem não ser tão eficazes na solubilização do fosfato de ferro, como são eficazes na solubilização do fosfato de cálcio (MARSCHNER et al. 2011). Nesse contexto, os sideróforos, um metabólito com forte atividade quelante sobre o Fe, podem ter um papel importante na liberação de P ligado ao ferro (CUI et al., 2022). Normalmente, plantas e microrganismos liberam sideróforos para

eliminar o ferro em condições de baixa biodisponibilidade (MARSCHNER et al., 2011). Como os ácidos orgânicos têm um efeito limitado sobre os fosfatos de ferro, a produção de sideróforos pode ajudar a explicar os mecanismos de solubilização do fosfato em solos de condições ácidas.

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SEGUNDA PARTE - ARTIGOS

ARTIGO 1 – Genomic annotation of genes related to the production of organic acids in phosphate-solubilizing bacteria that promote growth and nutrition in maize plants

(Artigo formatado conforme as regras do periódico Journal of Cleaner Production)

Genomic annotation of genes related to the production of organic acids in phosphate-solubilizing bacteria that promote growth and nutrition in maize plants

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Abstract

Bacteria can provide P to plants by solubilizing insoluble rock phosphates. Although P solubilization mechanisms are not completely understood, the secretion of low-molecular organic acids and acidification are the most accepted. Besides, genes involving the production of these organic acids may already be identified within genomes of phosphate solubilizing microorganisms. The objective of this study was to verify the Ca phosphate solubilization, production of low-molecular organic acids, genes related to the synthesis of these organic acids, and the growth and nutrient accumulation of maize plants inoculated with phosphate solubilizing bacteria and fertilized with Bayóvar rock phosphate. Ca phosphate solubilization and organic acids production were evaluated *in vitro* for five bacterial strains. Genomic DNA was extracted for strain identification and annotation of genes related to the organic acids. A greenhouse experiment was performed with the strains plus 150 mg dm^{-3} P$_2$O$_5$ as Bayóvar rock phosphate each to assess phosphate solubilization contribution to *Zea mays*. *Paraburkholderia fungorum* UFLA 04-21 and *Pseudomonas anuradhapurensis* UFPI B5-8A solubilized *in vitro* over 60% of phosphate and produced high amounts of citric/maleic and gluconic acids, respectively. All 11 organic acids studied were identified, despite most strains not showing all of them and enzymes related to the organic acids production were found in bacterial genomes. We also identified most genes related to some of the organic acids Plants inoculated with strains UFPI B5-6, UFPI B5-8A, and UFLA 03-10 accumulated more biomass than the plants fertilized with BRP only. Strains UFLA 03-10 and UFPI B5-8A increased the accumulation of most macronutrients, including P, and some micronutrients. The results show that is feasible to use phosphate solubilizing bacteria to increase maize growth and nutrient accumulation based on Bayóvar rock phosphate fertilization.

Key Words: genome, phosphate solubilization, plant growth promotion, *Zea mays* L.

1 Introduction

Phosphorus (P) is a major limiting nutrient required for all living organisms to complete their cycle. High demands of this nutrient in agriculture are required to ensure crop growth and productivity. In soil, plants absorb P from the soil solution in the form of orthophosphate, but in most tropical soils, P natural availability is low and most of the P added as mineral fertilizer quickly becomes unavailable. Reactions of adsorption with soil Fe- and Al- hydr(oxides) and precipitation with soil cations, mainly with Al^{3+} and Fe^{3+} in acidic soils or Ca^{2+} in calcareous soils, are fairly common and reduce the effectivity of the fertilization (Smyth and Sanchez, 1982; Abdala et al., 2015, Bunemann, 2015). Therefore, P needs to be constantly replenished to ensure satisfactory crop yield (Roberts and Johnston, 2015).

Mineral P fertilizer used in agriculture is obtained from natural rock phosphate. However, due to the low solubility of the rock phosphate, it needs to go through an acidification process resulting in highly concentrated water-soluble phosphate, easily available for plant uptake after application. This process ensures the availability of phosphate to plants, but high costs are involved and the P in the soil becomes highly prone to leaching and soil retention (Chien et al., 2011). Worldwide reserves of rock phosphate are being depleted and the quality of the remaining rock phosphate is reducing (Scholz et al., 2013). Furthermore, there are different types of rock phosphates concerning reactivity and the most reactive forms are from sedimentary rocks, such as Bayóvar rock phosphate (BRP) (Léon et al., 1986). Fertilization with rock phosphates, however, may not be enough for early plant requirements due to its low solubility. A viable way to supply P to crops and overcome the high losses from the soluble phosphates is to inoculate plants with microorganisms capable of phosphate solubilization (Zhang et al., 2020).

Microorganisms play an important role in nutrient cycling. Some microorganisms, mainly bacteria and fungi, are able to solubilize insoluble forms of phosphate and release soluble P. The main mechanism attributed to phosphate solubilization by microorganisms is the production of several types of low-molecular-weight organic acids (Kim et al., 1997; Chen et al., 2006; Marra et al., 2012; Leite et al., 2020). The rationale is that the carboxyl groups from the organic acids bond with the cations responsible for the phosphate precipitation, increasing the availability of P (Bolan et al., 1994, Ström et al., 2001) or bond with the soil sorption sites, preventing P permanent adsorption (Geelhoed el al., 1999, Ström et al., 2001). Organic acids such as gluconic, oxalic, citric, lactic, and acetic are the most commonly found by chromatography during *in vitro* phosphate solubilization (Santos-Torres et al., 2021). However, a genomic perspective involving the production of organic acids, such as the identification of the genes and the related enzymes, is not yet a common feature within phosphate solubilization research. Studies on the genes related to the gluconic acid, such as *gcd*, *pqq*, and *gdh* are the most common, but studies on genes of other organic acids are scarce (Santos-Torres et al., 2021).

Besides, other mechanisms also might be involved in P solubilization, such as acidification (Chen et al., 2006, Braz and Nahas, 2012), siderophore production (Hamdali et al., 2008), and exopolysaccharide production (Yi et al., 2008). Although there are still gaps in understanding the whole picture behind P solubilization, the inoculation of P solubilizing bacteria with insoluble P has already been shown to improve plant growth and nutrition (Estrada et al., 2013; Pereira and Castro, 2014; Costa et al., 2015; Leite et al., 2020).

In Brazil, maize (*Zea mays*) is one of the most important crops, with the current production surpassing 18 million tons over 4 million hectares grown yearly (Conab, 2020). A large amount of the crop is planted in highly weathered soils with high content

of Fe- and Al- hydr(oxides), i.e. soils with high P fixing capacity (Hinsinger, 2001). In these conditions, P fertilization is constantly required in large amounts to ensure enough soluble P for plant growth and high productivity (Fageria et al., 2015). The use of rock phosphate combined with phosphate solubilizing bacteria can represent an alternative method for plant fertilization to improve maize nutrition and reduce production costs. Therefore, the objective of this study was to verify the Ca phosphate solubilization, the production of low-molecular organic acids, the genes related to the synthesis of the organic acids, and the growth and nutrient accumulation of maize plants inoculated with phosphate solubilizing bacteria and fertilized with Bayóvar rock phosphate. In addition, we identified the bacterial strains at species level by average nucleotide identity.

2 Material and Methods

2.1 Strains characterization and cultivation

In the present study, the five bacterial strains used were provided by the Laboratory of Soil Microbiology of Universidade Federal de Lavras. (UFLA), Brazil. Strains UFLA 04-21 and UFLA 04-155 (both *Paraburkholderia* sp.) were isolated from nodules of *Macroptilium atropurpureum* collected from soil under agriculture and secondary forest in the initial stage of regeneration (4°21′ and 4°26′ S, and 69°36′ and 70°1′ W) on the Amazonian region (Lima et al., 2009). Strain UFLA 03-10 (*Paenibacillus* sp.) was isolated from nodules of *Vigna unguiculata* on soil of a tropical semideciduous forest (18°35′52.7″ S and 46°29′10.8″ W) in state of Minas Gerais, Brazil (Marra et al., 2012). Strains UFPI B5-6 and UFPI B5-8A (*Enterobacter* sp. and *Pseudomonas* sp, respectively) were isolated from nodules of *Vigna unguiculata* grown on floodplain soil under native forest (09°04′28″ S and 44°21′31″ W) in the southeast state of Piauí, Brazil

(Costa et al., 2016). Some of these strains presented favorable results in promoting rice (Costa et al., 2015) and maize (Leite et al., 2020) growth by phosphate solubilization in previous experiments. The strains were cultivated separately in 79 medium (Fred and Waksman, 1928) under 110 rpm at 28 ºC, for 48 h (approximately 10^8 CFU mL^{-1}).

2.2 Phosphate solubilization and organic acids quantification *in vitro*

An *in vitro* assay to test the ability of the strains to solubilize phosphate was performed in a completely randomized design with three replicates. The treatments consisted of the abovementioned bacterial strains plus a control without inoculation. The medium used was the NBRIP (Nautiyal, 1999), composed of: 10 g of glucose, 5 g of $Ca_3(PO_4)_2$, 5 g of $MgCl_2·6 H_2O$, 0.25 g of $MgSO_4·7 H_2O$, 0.2 g of KCl, and 0.1 g of $(NH_4)_2SO_4$, in which pH was adjusted to 6.8. Each flask received 1 mL of the inoculum prepared as mentioned (approximately 10^8 CFU mL^{-1}), according to the respective treatment. Incubation in the phosphate medium was carried out for seven days at 28 °C and 110 rpm. After incubation, the growth medium was centrifuged at 3000 rpm for 15 minutes and the extract was filtered in a 0.45 µm cellulosic membrane. Determination of soluble P was done by inductively coupled plasma optical emission spectroscopy (ICP-OES) and pH by a standard laboratory pH meter.

The identification and quantification of the organic acids were done via high-performance liquid chromatography. The Supelcogel C-610H 9 µm 30 cm × 7.8 mm chromatographic column was used to retain the acids in the samples and the following certified analytical acids used as standards: acetic, citric, formic, fumaric, gluconic, lactic, maleic, malic, malonic, oxalic, and tartaric. The mobile phase used was a 0.1 % H_3PO_4 running at a 0.5 mL min^{-1} flow rate for 45 minutes at 210 nm wavelength. The analytical procedure followed the instructions of the column manufacturer. The average retention

times identified on three replicates for three consecutive days of the standard curve analysis were: oxalic at 9.15 min, citric/maleic at 11.50 min, gluconic/tartaric at 12.30 min, malic at 13.56 min, malonic at 13.94 min, lactic at 17.04 min, formic at 18.51 min, acetic at 20.12 min, and fumaric at 21.13 min. The quantification of the acids was done using calibration curves built with the analytical standards.

2.3 Genome sequencing, assembling and quality assessment

Bacterial genomic DNA was extracted with the Promega Wizard® DNA Extraction Kit according to the instructions of the manufacturer. The DNA libraries were prepared with the NexteraXT kit (Illumina, CA, USA) according to the instructions of the manufacturer. The final amount of the library was measured with KAPA SYBR® FAST qPCR with the system QuantStudio® 5 (Applied Biosystems, CA, USA). The libraries were assessed with the TapeStation HSD1000 ScreenTape (Agilent Technologies, CA, USA). Illumina® 8-nt dual indices were used. Based on the quality measurements, equimolar mixtures of the libraries were used and the reading of the fragments was done in two directions (2×150 bases) in the Illumina® HiSeq X platform (Illumina, CA, EUA). Genome assembly was done with the algorithm SPADes 3.13.0 (Bankevich et al. 2012). Quality and completeness were checked with CheckM 1.0.18 (Parks et al. 2015). Both algorithms were used within the KBase platform (https://www.kbase.us/).

2.4 Genome comparison and species identification

Average nucleotide identity (ANI) technique was applied for genome comparison and species identification at the species level. After confirmation of the genera by extracting and comparing the 16 S rRNA from the genome, we proceeded by comparing each strain with all type strains from the same genus available in the GenBank (National

Centre for Biotechnology Information (NCBI); http://www.ncbi.nlm.nih.gov/). The ANI was assessed in the platform http://enve-omics.ce.gatech.edu/enveomics/ by comparing each strain with each obtained type strain sequence.

2.5 Greenhouse pot experiment

A greenhouse experiment was carried out in a completely randomized design and four replicates. The treatments were as follows: a control with 150 mg dm^{-3} of P_2O_5 as Bayóvar rock phosphate (BRP) without inoculation; five treatments inoculated with the strains UFLA 04-21, UFLA 04-155, UFLA 03-10, UFPI B5-6, and UFPI B5-8A, plus 150 mg dm^{-3} of P_2O_5 as BRP each; four doses of soluble P (50, 150, 200, and 250 mg dm^{-3} of P_2O_5) as monoammonium phosphate (MAP) ($NH_4H_2PO_4$); and a control without P fertilization or bacterial inoculation.

The experiment was carried out in 4 dm^3 polypropylene pots filled with a red-yellow Latosol (Oxisol) with a clayey texture, low P content and collected from the 0-20 cm layer in Lavras, state of Minas Gerais. The soil was air dried, homogenized, and sifted through a 4 mm mesh. The following chemical and physical characteristics were identified: pH (H_2O) = 5.4; P (Mehlich I) = 0.84 mg dm^{-3}; K^+ = 24.0 mg dm^{-3}; Ca^{2+} = 1.7 cmol$_c$ dm^{-3}; Mg^{2+} = 0.1 cmol$_c$ dm^{-3}; Al^{3+} = 0.1 cmol$_c$ dm^{-3}; $H+Al^{3+}$ = 2.9 cmol$_c$ dm^{-3}; ECEC = 4.76 cmol$_c$ dm^{-3}; base saturation (V) = 39.11 %; aluminum saturation = 5.10 %; organic matter = 2.11 %; equilibrium P = 3.54 ml L^{-1}; Zn^{2+} = 1.59 mg dm^{-3}; Fe^{2+} = 29.78 mg dm^{-3}; Cu^{2+} = 3.15 mg dm^{-3}; Mn^{2+} = 4.98 mg dm^{-3}; B = 0.13 mg dm^{-3}; S = 28.07 mg dm^{-3}; sand = 17 %; silt = 10 %; clay = 73 %. In a previous study, the soil was estimated with 2.1 x 10^4 CFU g^{-1} of phosphate solubilizing microorganisms (Costa et al., 2015).

Based on the chemical characteristics, liming was performed to increase base saturation up to 50 % using $CaCO_3$ and $MgCO_3$ at a 4:1 (w/w) ratio. After liming, the soil

was incubated for 40 days and moisture was kept at 60% of the total pore volume. The fertilization was done using (mg dm^{-3}): 450 of N (as urea), 350 of K (as KCl and K_2SO_4), 40 of S (as K_2SO_4), 1.5 of Cu (as $CuSO_4.5H_2O$), 3.6 of Mn (as $MnCl_2.4H_2O$), 5.0 of Zn (as $ZnSO_4.7H_2O$), 0.8 of B (as H_3BO_3), and 0.15 of Mo (as $(NH_4)_6Mo_7O_{24}.4H_2O$). The N fertilization was calculated considering the amount provided by the urea and the different MAP doses. Both N and K fertilization were divided into four applications (sowing, 15, 30, and 45 days after sowing).

Maize seeds were surface sterilized using 98 % ethanol for 30 seconds, 2 % sodium hypochlorite for 2 minutes, and successive washes in sterile deionized water. Seeds were germinated for 72 h at 28 ℃ in sterilized petri dishes with wet cotton and filter paper. Each pot received 6 seeds and every seed was inoculated with 1.0 mL of each strain (approximately 10^8 CFU seed^{-1}). After eight days, plants were thinned to 2 plants per pot.

Plants were harvested 60 days after sowing. Shoot and root were airflow-dried at 60 ℃ and shoot dry matter (SDM), root dry matter (RDM), and total dry matter (TDM) were assessed considering the two plants per pot. The total amount of nutrients (N, P, K, Ca, Mg, S, Cu, Fe, Mn, Zn, and B) in the shoots were then analyzed according to Malavolta et al. (1997) and obtained by multiplying SDM (mg pot^{-1}) by the concentration of each element analyzed in both plants of each pot.

2.6 Statistical analysis

Shapiro-Wilk's ($p < 0.05$) and Bartlett's test ($p < 0.05$) were performed to assess normality and homoscedasticity of the residues, but the data did not follow those assumptions. Instead, we fitted the data into a generalized least square (gls) model and compared it to the linear model according to Akaike's information criteria. Groups of

variances were built according to variance of the residues prior to the normality assessment. Data were then subjected to Tukey's range test ($p < 0.05$) to compare the treatments. Two Pearson's correlations were drawn between soluble P and pH and between soluble P and total organic acids using the function ggscatter from the package ggpubr (Kassambara, 2020). All statistical analysis and figures were done in the R environment (R Core Team, 2022) and the R Studio platform (R Studio, 2022).

3 Results

3.1 Bacterial species identification

The analysis of the 16 S sRNA gene extracted from the genome confirmed the previous identification at genus level. All strains showed an ANI $\geq$ 95-96 %, which is the adequate grade to consider strains as the same species (Richter and Rosselló-Mora, 2009). Results are displayed in Table 1.

3.2 Phosphate solubilization and organic acids quantification *in vitro*

All strains solubilized more P than the natural dissociation found in the non-inoculated control ($p < 0.05$). The most prominent strain found was UFLA 04-21 of *Paraburkholderia fungorum*, able to solubilize 71 % of the total 1000 mg of insoluble P (Figure 1). This strain also considerably reduced the final pH of the medium, reaching 4.42 from the original 6.8. Another prominent strain was UFPI B5-8A of *Pseudomonas anuradhapurensis*, reaching 60 % of P solubilization and 4.43 pH. The least effective strain was UFLA 03-10 of *Paenibacillus peoriae*, with only 9 % of soluble P, associated with a 5.5 pH.

All 11 organic acids used as standards were identified (Table 2), despite most strains not showing all of them. Strain UFLA 03-10 was the only strain that produced all organic acids analyzed. The highest amount of total organic acids was found for strain UFPI B5-8A (2566 ± 306 mg L^{-1}), despite this strain not showing the highest phosphate solubilization. However, this strain showed a relatively high amount of gluconic/tartaric acid, in which 99 % of the total (2539 ± 303 mg L^{-1}) corresponded to these two acids and the reaming 1 % was the sum of the other nine analyzed acids. Interestingly, strain UFLA 04-21, a notable *in vitro* phosphate solubilizer, only accounted for 376.9 ± 144.9 mg L^{-1} of organic acids, having citric/maleic acids as 90 % of the total.

The Pearson's correlation between soluble P and the final pH in the solution (Figure 2) shows an expected negative correlation ($R = -0.8$, $p < 0.001$) indicating that acidification of the medium is positively correlated with the amount of phosphate solubilized. In addition, the correlation between soluble P and total organic acids indicated a weaker yet significant and positive ($R = 0.57$, $p = 0.013$) association between these two variables (Figure 3).

3.3 Annotation of genes related to the solubilization of phosphates

The genes and enzymes for the acetic, citric, gluconic, lactic, and malic acids were found in most strains but those related to the production of oxalic acid were not found in any of the strains (Figure 4). Four strains (except the *Paenibacillus peoriae* UFLA 03-10) showed genes related to glucose dehydrogenase, but only *Pseudomonas anuradhapurensis* UFPI B5-8A had the *pqq* gene indicating the production of the co-factor pyrroloquinoline quinone (PQQ), probably producing gluconic acid by the glucose dependent 1-dehydrogenase-pyrroloquinoline quinone pathway. Genes related to the acetic acid were found through the enzyme aldehyde dehydrogenase in all strains, except

the *Paraburkholderia fungorum* UFLA 04-155. The potential to produce lactic acid was confirmed with the enzyme L-lactate dehydrogenase in all strains. Additionally, *Enterobacter bugandensis* UFPI B5-6 also had the gene to produce the isomer D-lactate dehydrogenase. Citric and malic acids were found for all strains in all forms of searched genes/enzymes. The entries for the enzymes glyoxylate dehydrogenase and oxaloacetate hydrolase were searched for the pathways of oxalic acid biosynthesis but neither yielded results. Further details about the genes and the complete name and identification of the enzymes can be found in the supplementary material (Table S1).

3.4 Maize growth and nutrition using rock phosphate as P source

There was a significant effect of the treatments on the production of RDW, SDW, and TDW (Table 3) and on the macronutrient accumulation in the plants (Table 4). Plants fertilized with the highest doses of the soluble P as MAP (150, 200, and 250 mg P dm^{-3}) had the highest accumulation of RDW, SDW, and TDW. All inoculated plants, except those inoculated with UFLA 04-21, had higher RDW than the non-inoculated plants fertilized with BRP. The increase in RDW in the inoculated treatments ranged from 49 % (strain UFPI B5-6) to 417 % (UFPI B5-8A) in comparison with the BRP control.

Considering the production of SDW and TDW, plants inoculated with strains UFPI B5-6, UFPI B5-8A, and UFLA 03-10 accumulated more biomass than the plants fertilized with BRP only (Table 2). The SDW was 200 % higher for the strains UFLA 03-10 and UFPI B5-8A and ranged from 200 % (UFLA 03-10) to 257 % (UFPI B5-8A) in TDW. Strain UFPI B5-8A promoted a favorable plant accumulation of RDW and TDW comparable or better than the plants fertilized with soluble P at the dose of 50 mg P dm^{-3}.

Regarding the macronutrient accumulation in the plant shoot (Table 4), the highest accumulations were found in the treatments with the highest soluble P doses (150, 200, and 250 mg P dm^{-3}) and the lowest accumulations were found in the control without both fertilization and inoculation, as observed for biomass production. The N accumulation was higher in plants inoculated with strains UFLA 04-21, UFPI B5-8A, and UFLA 03-10 than on the other inoculations and the plants fertilized with BRP. As for P accumulation, strain UFLA 03-10 promoted accumulation of P in the shoots as much as the plants fertilized with soluble P (50 mg dm^{-3}). Strains UFPI B5-6 and UFPI B5-8A also promoted higher P accumulation than the BRP fertilized plants, ranging from 37% to 61% more P in the shoots.

Strains UFPI B5-6, UFPI B5-8A, and UFLA 03-10 promoted superior K and Mg accumulation in the shoots than the BRP fertilization and inoculation with the two *Paraburkholderia* strains UFLA 04-155 and UFLA 04-21. Noteworthy, plants inoculated with strain UFPI B5-8A accumulated as much K as plants fertilized with 50 mg dm^{-3} of soluble P, with 123% more K than the BRP fertilized plants. The highest accumulations of Ca in the inoculated treatments were also obtained with two of the strains that stood out in the accumulation of P, K and Mg (UFPI B5-8A and UFLA 03-10), in addition to the strain UFLA 04-21. The only strain that increased S accumulation in the plants was UFLA 03-10 in comparison with the BRP treatment. Regarding the micronutrients, UFPI B5-8A and UFLA 03-10 were the best inoculations for most micronutrients. Further details can be found in Supplementary table 2.

4 Discussion

4.1 Phosphate solubilization, organic acids quantification, and annotation of genes related to the solubilization of phosphates

In this work, all strains were able to promote *in vitro* phosphate solubilization. Strains of the species *Paraburkholderia fungorum* (UFLA 04-21) and *Pseudomonas anuradhapurensis* (UFPI B5-8A) were the most effective in solubilizing phosphate. These genera are often referred to as phosphate solubilizers, especially *Pseudomonas*, considered one of the most predominant genus among phosphate solubilizers, after *Bacillus* (Kour et al., 2021). The considerable ability to solubilize phosphate from these strains was able to turn up to 70 % of insoluble phosphate into soluble forms. Furthermore, this method only evaluated the soluble P released in the medium. It is likely that more P was solubilized and it was incorporated into the metabolism of the cells, in both organic and inorganic forms. The main mechanism attributed to phosphate solubilization is the release of low molecular-weight organic acids that both bind the cations responsible for the phosphate precipitation (in soil, mainly Ca, Fe, and Al) and block the adsorptions sites of phosphate in soil colloids (Geelhoed et al., 1999, Ström et al., 2001, Chen et al., 2006).

Interestingly, Pearson's correlation between the amount of organic acids and solubilized P did not adjust as well the correlation between soluble P and pH. The association of soluble P and the total amount of organic acids of strain UFLA 04-21 did not fit along with the other strains since it had the highest amount of soluble P associated with high acidification, but a low amount of total organic acids. Strain UFLA 04-21 produced the highest amount of citric/maleic acid and was able to reach the lowest pH, but with a relatively low amount of the other organic acids. This could partially explain the potential acidification caused by the exceedingly amount of these acids and the high

amount of soluble P. Citric acid has three carboxylic groups and one hydroxyl group while maleic acid has two carboxyl groups and two hydroxyl groups (Haynes et al., 2016). The number of exchange sites in these two acids (carboxyl and hydroxyl groups), associated with a low first acid dissociation constant (pKa = 1.94) of maleic acid could explain part of the large amount of P solubilized in the medium by both ion exchange with calcium and acidification of the medium by H^+ release (Ström et al., 2001). However, other uncounted mechanisms might be occurring simultaneously since this strain and others had relatively similar amounts of citric/maleic acids, but the soluble P was far considerably different.

The large amount of soluble P produced by *Pseudomonas anuradhapurensis* UFPI B5-8A, however, might have another explanation. In this case, the strain produced a substantial quantity of gluconic/tartaric acids. Gluconic acid has one carboxyl group and four hydroxyls while tartaric acid has two carboxyl groups and two hydroxyl groups (Haynes et al., 2014, Ramachandran et al., 2006). Gluconic acid is sometimes referred to as low-effective in solubilizing phosphate for its monocarboxylic trait and low stability constant with calcium (Jones et al., 1998; Mendes et al., 2014). In our experiment, however, it was effective in solubilizing P even with the lesser number of exchange sites that probably was compensated by the large amount of acids produced, leading to a relativity similar soluble P production and pH acidification.

Oxalic acid applied to rock phosphates in a simulation of microbial phosphate solubilization was found to be the organic acid that most solubilized rock phosphates (Mendes et al., 2020). The authors suggest an industrial production of microbial-based oxalic acid instead of the synthetic sulfuric acid to solubilize phosphate from rocks as in other bioleaching systems (Ghosh and Paul, 2016; Vakilchap et al., 2016). In this work, two sets of different acids (critic/maleic and gluconic/tartaric) could be related to the

solubilization process, while oxalic acid production was relatively low. Citric and oxalic acids have chelating properties that allow the formation of stable complexes with Ca^+, while gluconic acid is proposed to solubilize phosphate by releasing H^+ (Fox et al., 1990; Kpomblekou and Tabatabai; 1994, Goldstein, 1995). Even if the strains are not very effective *in vivo* systems (Table 3), as shown for strain UFLA 04-21 (Table 2), they were very effective in releasing P *in vitro* and could also be assets for microbial-produced organic acids of industrial importance, such as gluconic and citric acids. Different behavior in P solubilization found *in vivo* systems is probably due to a highest complexity of nutrition and environment variables in the rhizosphere as compared to the controlled characteristics of culture medium.

The genome comparison through the ANI showed that all strains belonged to already described bacterial species. Nevertheless, these strains, all isolated from Brazilian soils, are well adapted and promote the growth of diverse plants, as well as solubilize phosphates (Oliveira-Longatti et al., 2013; Costa et al., 2015; Leite et al., 2020). For most genomic traits regarding the production of the evaluated organic acids, these strains also presented the related genes and enzymes. In the cases of *Paenibacillus peoriae* UFLA03-10 and gluconic acid, *Paraburkholderia fungorum* UFLA 04-155 and acetic acid, and oxalic acid for all strains, the gene was not found within the genomic DNA, but the production of the organic acids was found via liquid chromatography. Conversely, *Pseudomonas anuradhapurensis* UFPI B5-8A did not produce acetic and citric acids and *Paraburkholderia fungorum* UFLA 04-155 did not produce lactic acid, despite having the genes that codify the enzymes.

The genomes of the *Enterobacter bugandensis* UFPI B5-6 and *Pseudomonas anuradhapurensis* UFPI B5-8A show that both strains have genes to produce gluconic acid via the glucose dependent 1-dehydrogenase-pyrroloquinoline quinone pathway, but

genes for the co-factor PQQ were only found in the *Pseudomonas*. The two other strains, *Paenibacillus peoriae* UFLA 03-10 and *Paraburkholderia fungorum* UFLA 04-155, had the genes to produce glucose 1-dehydrogenase, an alternative option to produce gluconic acid via the quinoprotein glucose dehydrogenase pathway (Santos-Torres et al., 2021). Despite genes encoding for the production of gluconic acid in the *Paenibacillus peoriae* UFLA 03-10 strain were not found, research indicates the presence of *gcd* genes in the genus (Eastman et al., 2014). It is possible that in this case, as well as other cases in which the genes were not found but the synthesis of the relative organic acid was identified, the annotation tool and the database used could not identify the gene sequence. Conversely, the low amount of gluconic/tartaric acid found for strain *Paenibacillus peoriae* UFLA 03-10 could only be derived from the tartaric acid, since we could not separate these two acids in the chromatography analysis, thus confirming the absence of the genomic apparatus for gluconic acid.

4.2 Maize growth and nutrition using rock phosphate as P source

The combination of phosphate solubilizing bacteria with low soluble rock phosphates is an economic alternative to improve plant growth and nutrition and reduce the need for soluble phosphate fertilizers that are costly, finite, more prone to losses, and enhance the costs of food production. We demonstrated the phosphate solubilizing activity of bacteria in enhancing plant growth and nutrition of maize plants using Bayóvar rock phosphate in soil as the sole source of P, which is a low-soluble natural source of P with a relatively lower cost than soluble MAP. Strains UFLA 03-10, UFPI B5-6, and UFPI B5-8A were able to promote plant growth and increase SDM and TDM in comparison with the plants that received only insoluble phosphate (Table 3). Furthermore, these strains were also able to promote N, P, K, Mg, Cu, and Zn

accumulation on the shoots (Table 4; Table S2).

These results indicate the potential of bacterial strains to promote maize growth cultivated in non-sterile Oxisol fertilized with rock phosphate. Strains UFLA 03-10, UFPI B5-6, and UFPI B5-8A increased the P accumulation in plants fertilized with BRP and, consequently, increased plant growth as well. Interestingly, *Paenibacillus peoriae* UFLA 03-10 was not considered a promising phosphate-solubilizer in the *in vitro* experiment, but increased plant biomass accumulation. Conversely, *Paraburkholderia fungorum* UFLA 04-21 showed outstanding phosphate solubilization in vitro, but low results in maize growth promotion. In a previous study with maize plants also cultivated in non-sterile Oxisol, strain UFPI B5-8A was able to promote plant growth but not P uptake of plants fertilized with biochar enriched with BRP, while strains UFLA 03-10 and UFLA 04-155 did not differ from the non-inoculated control (Leite et al., 2020).

Despite the differences from the non-inoculated controls, the high clay content of the soil may have hindered the development of the maize plants. In this case, the clayey nature of the soil contributed to a higher P fixation, and the solubilized P from the rock phosphate might have been rapidly adsorbed by the soil rather than uptaken by the roots, a common feature in weathered Brazilian soils (Smyth and Sanchez, 1982). The maize growth achieved in another work studying these strains was higher, but the differences can be mainly attributed to the lower clay content (230 g kg^{-1}), causing lower P fixation (Leite et al., 2020), and to the benefits of the biochar, such as nutrient release, porosity, water retention and others (Rafique et al., 2017).

In both cases (raw BRP and biochar enriched with BRP), there are some drawbacks regarding the rate of P release. Rock phosphates are low-soluble sources of P and therefore, it might not be enough for a readily available P fertilization. Biochar is used as a slow-release source of nutrients and also provides nutrients at a much lower rate

than soluble fertilizers (Zhao et al., 2016; Lustosa-Filho et al., 2017, 2019). Nevertheless, it is possible to enhance plant growth and nutrition based on low-soluble P sources using phosphate solubilizing bacteria as was shown here and elsewhere (Pereira and Castro, 2014; Costa et al., 2015, Leite et al., 2020) and this combination might represent an alternative long-term or slow-release phosphate fertilizer to associate with soluble and readily available P fertilizers. Alternatively, this long-term P release could be interesting for perennial plants that require nutrients at a lower rate than annual plants, such as trees planted to recover degraded areas. Even though the soil was estimated with a count of 2.1 x 10^4 CFU g^{-1} of phosphate solubilizing microorganisms (Costa et al. 2015), some inoculated bacterial strains in this study were more efficient than the native population.

Strains UFLA 04-155 and UFLA 04-21 (both *Paraburkholderia fungorum*) did not promote the growth of the maize plants despite the positive results for *in vitro* calcium phosphate solubilization. It is not always possible to reproduce the *in vitro* effects in soil experiments. The complex relationships in the soil and the interaction with the plant may stimulate or prevent certain traits of microorganisms. For example, strain UFLA 04-21 was able to promote growth and nutrient accumulation in rice plants while strain UFLA 04-155 did not, both fertilized with BRP (Costa et al., 2015). Furthermore, despite the two *Paraburkholderia* strains (UFLA 04-21 and UFLA 04-155) had been previously categorized as free-living N_2 fixing (Oliveira-Longatti et al., 2013), neither of them increased the N accumulation in the plants.

Other growth promotion traits related to the bacterial strains used might be involved in the growth and nutrition of maize plants, such as IAA production and free-living N fixation. This characteristic might explain the growth promotion induced by the *Paenibacillus* UFLA 03-10, since this strain did not exceed in solubilize phosphate. The bacterial genera used in this work are known for potential growth promoting traits in

cultivated plants. In maize plants, increases in shoot and root growth were achieved with phosphate solubilizing diverse strains of *Paraburkholderia* (Young et al., 2013, Zhao et al., 2014, Tagele et al., 2018), *Paenibacillus* (Li et al., 2017), *Pseudomonas* (Vyas and Gulati, 2009, Li et al., 2017) and *Enterobacter* (Naveed et al., 2014). Noteworthy, some of the strains mentioned above also presented other growth promotion traits, such as antifungal activity and hormone production.

Finding alternatives to the high use of soluble manufactured fertilizers is essential in the near future, especially in cases where most of the fertilizer becomes unavailable to plants due to soil fixation. Although we might not be able to replace all soluble phosphate fertilizer, alternative low-soluble sources may be a solution to efficiently provide P to the plants. Furthermore, bacteria that can solubilize BPR and provide P to plants as well as promote growth by other mechanisms, and can be the biofertilizer to associate with low-soluble rock phosphates for P fertilization (Costa et al., 2015, Rawat et al., 2020). This combination is a possible way to promote maize growth and increase plant yield.

5 Conclusions

We verified the solubilization of phosphate from bacterial strains of different genera. All strains were able to solubilize phosphate and secrete several types of low molecular weight organic acids. *Paraburkholderia fungorum* UFLA 04-21 and *Pseudomonas anuradhpurensis* UFPI B5-8A solubilized over 60 % of the total phosphate. Genes from acetic, citric, gluconic, lactic, and malic were identified in the genome of the strains. Significant correlations between soluble P and pH and soluble P and the amount of organic acids were found. The results show that is feasible to use selected phosphate solubilizing bacterial strains to increase maize growth and nutrient accumulation based on BRP fertilization. Inoculation of maize plants with strains UFPI B5-8A (*Pseudomonas*

anuradhapurensis) and UFLA 03-10 (*Paenibacillus peoriae*) increased plant growth by solubilization of calcium phosphate as Bayóvar rock phosphate. Strains UFLA 03-10 and UFPI B5-8A also stood out increasing the accumulation of macronutrients N, P, K, Ca, Mg, and micronutrients Cu, Fe, Mn, and Zn.

Author contribuitions

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Competing interests

No potential competing interest was reported by the authors.

Data availability statement

The data that support the findings of this study are available from the corresponding author, FMSM, upon reasonable request.

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Table 1: Identification and growth promoting traits of evaluated strains.

Strain	Previous identification	Current identification	ANI (%)	Access code of the assembly of the most similar species in the NCBI	IAA DYGS medium ($\mu g\ ml^{-1}$)* Tryptophan		Free-living N_2 fixation
					-	+	
UFLA 04-21	*Paraburkholderia* sp.[1]	*Paraburkholderia fiungorum*[1]	99	GCF_000961515.1	2.35 [4]	6.51 [4]	+ [4]
UFLA 04-155	*Paraburkholderia* sp.[1]	*Paraburkholderia fiungorum*[1]	99	GCF_000961515.1	6.29 [4]	4.53 [4]	+ [4]
UFLA 03-10	*Paenibacillus* sp.[2]	*Paenibacillus peoriae*[2]	96	GCF_000236805.1	ND	ND	ND
UFPI B5-6	*Enterobacter* sp.[3]	*Enterobacter bugandensis*[3]	99	GCF_900324475.1	2.87 [3]	26.54 [3]	- [3]
UFPI B5-8A	*Pseudomonas* sp.[3]	*Pseudomonas anuradhapurensis* [3]	97	GCF_014269225.2	5.40 [3]	9.71 [3]	- [3]

ND: Not determined; *Production of indole-3-acetic acid (IAA) in DYGS medium without (-) and with (+) tryptophan. [1]Ferreira et al., 2012; [2]Marra et al., 2012; [3]Costa et al., 2016; [4]Oliveira-Longatti et al., 2013.

Table 2: Production of low-molecular organic acids in NBRIP medium (mg L^{-1}) from the bacterial strains UFLA 03-10 (*Paenibacillus peoriae*); UFLA 04-21 (*Paraburkholderia fungorum*); UFLA 04-155 (*Paraburkholderia fungorum*); UFPI B5-6 (*Enterobacter bugandensis*); UFPI B5-8A (*Pseudomonas anuradhapurensis*).

	UFLA 03-10	UFLA 04-21	UFLA 04-155	UFPI B5-6	UFPI B5-8A	Control
Oxalic	0.83 ± 0.17	0.86 ± 0.06	4.07 ± 0.24	4.35 ± 0.41	1.45 ± 0.24	0.58 ± 0.02
Citric / Maleic	9.46 ± 1.29	340.1 ± 137.4	274.5 ± 55.9	259.1 ± 35.2	-	-
Gluconic / Tartaric	3.10 ± 1.18	2.71 ± 0.88	109.7 ± 6.2	103.4 ± 1.9	2539 ± 303	4.19 ± 0.77
Malic	38.8 ± 3.8	17.2 ± 2.6	101.7 ± 34.3	120.7 ± 22.8	7.77 ± 0.40	50.6 ± 8.6
Malonic	3.21 ± 1.88	-	4.52 ± 1.12	4.42 ± 0.27	17.0 ± 2.6	7.44 ± 1.85
Latic	1.12 ± 0.17	7.61 ± 1.80	-	3.92 ± 0.31	0.20 ± 0.02	-
Formic	0.24 ± 0.11	0.13 ± 0.02	-	-	0.32 ± 0.09	0.22 ± 0.05
Acetic	27.3 ± 5.7	6.95 ± 1.90	27.4 ± 2.4	22.9 ± 3.4	-	21.2 ± 1.5
Fumaric	2.62 ± 0.53	1.24 ± 0.21	6.05 ± 0.57	8.86 ± 0.40	-	-
Total	86.7 ± 14.1	376.9 ± 144.9	527.9 ± 100.8	527.8 ± 64.0	2566 ± 306	84.2 ± 12.8

Means ± standard deviation

Table 3: Root dry weight (RDW), shoot dry weight (SDW) and total dry weight (TDW) of maize plants (*Zea mays* L.) cultivated under different conditions of P fertilization and inoculation (or not) with phosphate solubilizing bacterial strains, 60 days after sowing in pots containing red-yellow Latosol (Oxisol). Control: not fertilized, non-inoculated; BRP: Bayóvar Rock Phosphate; MAP: Monoammonium phosphate; UFLA 04-155 (*Paraburkholderia fungorum*); UFLA 04-21 (*Paraburkholderia fungorum*); UFPI B5-6 (*Enterobacter bugandensis*); UFPI B5-8A (*Pseudomonas anuradhapurensis*); UFLA 03-10 (*Paenibacillus peoriae*). Pots were cultivated with two plants.

Treatments	RDW	SDW	TDW
	--------------- g pot^{-1} ---------------		
Control	0.24 g	0.55 f	0.79 h
150 mg dm^{-3} P (BRP)	1.32 f	2.84 e	4.17 g
UFLA 04-155 + 150 mg dm^{-3} P (BRP)	3.14 e	3.08 de	6.22 ef
UFLA 04-21 + 150 mg dm^{-3} P (BRP)	1.38 f	3.77 de	5.15 fg
UFPI B5-6 + 150 mg dm^{-3} P (BRP)	2.67 e	4.59 cd	7.26 de
UFPI B5-8A + 150 mg dm^{-3} P (BRP)	5.05 c	5.69 c	10.74 c
UFLA 03-10 + 150 mg dm^{-3} P (BRP)	2.98 e	5.68 c	8.66 d
50 mg dm^{-3} P (MAP)	4.07 d	7.79 b	11.86 c
150 mg dm^{-3} P (MAP)	7.74 b	17.76 a	25.50 b
200 mg dm^{-3} P (MAP)	10.73 a	17.08 a	27.81 a
250 mg dm^{-3} P (MAP)	10.82 a	21.65 a	32.47 ab

Means followed by the same letter do not differ according to Tukey's test (P < 0.05).

Table 4: Accumulation of macronutrients in the shoots of maize plants (*Zea mays* L.) cultivated under different conditions of P fertilization and inoculation (or not) with phosphate solubilizing bacterial strains, 60 days after sowing in pots containing red-yellow Latosol (Oxisol). Control: non-fertilized, non-inoculated; BRP: Bayóvar Rock Phosphate; MAP: Monoammonium phosphate; UFLA 04-155 (*Paraburkholderia fiungorum*); UFLA 04-21 (*Paraburkholderia fiungorum*); UFPI B5-6 (*Enterobacter bugandensis*); UFPI B5-8A (*Pseudomonas anuradhapurensis*); UFLA 03-10 (*Paenibacillus peoriae*). Pots were cultivated with two plants.

Treatments	N	P	K	Ca	Mg	S
	------------------------ (mg pot^{-1}) ------------------------					
Control	25.5 f	0.50 e	9.3 g	9.7 h	2.6 g	2.00 e
150 mg dm^{-3} P (BRP)	97.3 e	5.36 d	61.4 f	34.7 fg	12.1 f	7.29 d
UFLA 04-155 + 150 mg dm^{-3} P (BRP)	117.9 de	5.54 d	75.7 ef	31.5 g	15.5 ef	8.01 d
UFLA 04-21 + 150 mg dm^{-3} P (BRP)	148.0 cd	6.13 d	72.1 ef	46.3 de	14.6 f	9.34 cd
UFPI B5-6 + 150 mg dm^{-3} P (BRP)	120.1 de	9.04 c	89.7 de	40.3 ef	22.3 d	8.64 d
UFPI B5-8A + 150 mg dm^{-3} P (BRP)	155.2 c	8.66 c	137.0 b	53.0 d	20.9 d	9.39 cd
UFLA 03-10 + 150 mg dm^{-3} P (BRP)	170.8 c	11.40 b	101.3 cd	53.4 d	19.6 de	10.58 c
50 mg dm^{-3} P (MAP)	237.4 b	12.02 bc	153.1 bc	71.22 c	36.2 c	14.58 b
150 mg dm^{-3} P (MAP)	490.2 a	20.25 a	340.8 a	137.5 ab	68.0 b	32.80 a
200 mg dm^{-3} P (MAP)	411.7 a	21.75 a	300.9 a	126.3 b	70.8 abc	31.79 a
250 mg dm^{-3} P (MAP)	540.2 a	24.82 a	396.6 a	160.1 a	104.1 a	39.47 a

Means followed by the same letter do not differ according to Tukey's test (P < 0.05)

Figure 1: Phosphate solubilization and pH from the bacterial strains UFLA 03-10 (*Paenibacillus peoriae*); UFLA 04-21 (*Paraburkholderia fungorum*); UFLA 04-155 (*Paraburkholderia fungorum*); UFPI B5-6 (*Enterobacter bugandensis*); UFPI B5-8A (*Pseudomonas anuradhapurensis*). Means followed by the same letter do not differ according to Tukey's test (P < 0.05). Vertical bars indicate the standard error for each treatment.
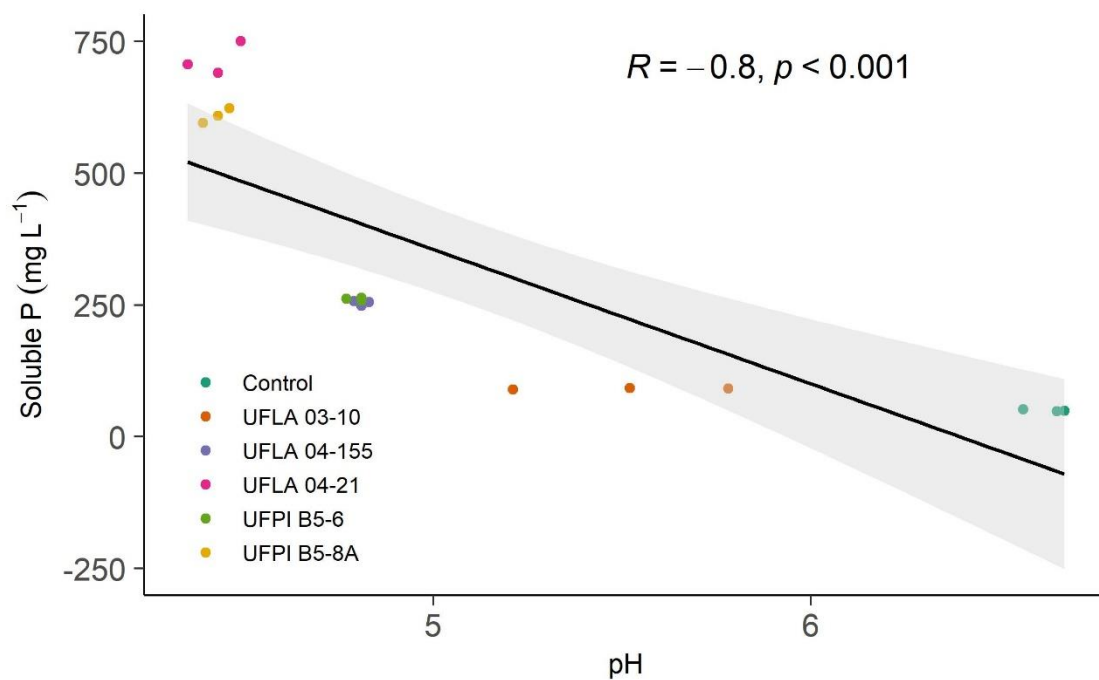
Figure 2: Pearson's correlation between soluble P and pH of an *in vitro* phosphate solubilization experiment with the bacterial strains UFLA 03-10 (*Paenibacillus peoriae*); UFLA 04-21 (*Paraburkholderia fungorum*); UFLA 04-155 (*Paraburkholderia fungorum*); UFPI B5-6 (*Enterobacter bugandensis*); UFPI B5-8A (*Pseudomonas anuradhapurensis*).
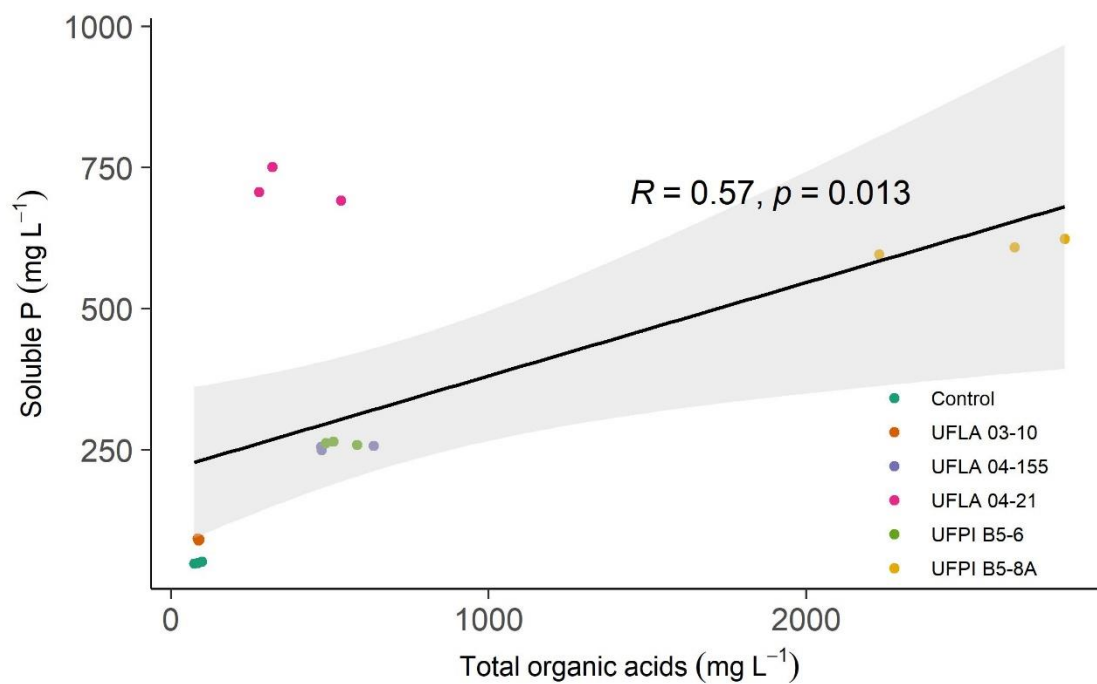
Figure 3: Pearson's correlation between soluble P and the total amount of organic acids of an *in vitro* phosphate solubilization experiment with the bacterial strains UFLA 03-10 (*Paenibacillus peoriae*); UFLA 04-21 (*Paraburkholderia fungorum*); UFLA 04-155 (*Paraburkholderia fungorum*); UFPI B5-6 (*Enterobacter bugandensis*); UFPI B5-8A (*Pseudomonas anuradhapurensis*).

Organic acid	UFLA 03-10	UFLA 04-155	UFLA 04-155	UFPI B5-6	UFPI B5-8A
			Genes		
Gluconic acid		*gdh*	*gdh*	*gcd* *gdh* A	*gcd* *gdh* A *pqq* B,C,D1,D2,E,F
Lactic acid	*ldh* 2	*lld* D	*lld* D	*lld* D *dld*	*lld* D
Acetic acid	*ald* HT	*ald* A		Putative aldehyde dehydrogenase	*ald* H
Citric acid	*pdh* A *pdh* B *cit* A2 *pdh* C	*pdh* A *pdh* B *glt* A	*pdh* B *glt* A	*ace* E *glt* A *ace* F	*ace* E *glt* A *ace* F
Oxalic acid					
Malic acid	*fum* C *mdh*	*fum* C *mdh*	*fum* C *mdh*	*fum* C *mdh*	*fum* C *mdh*

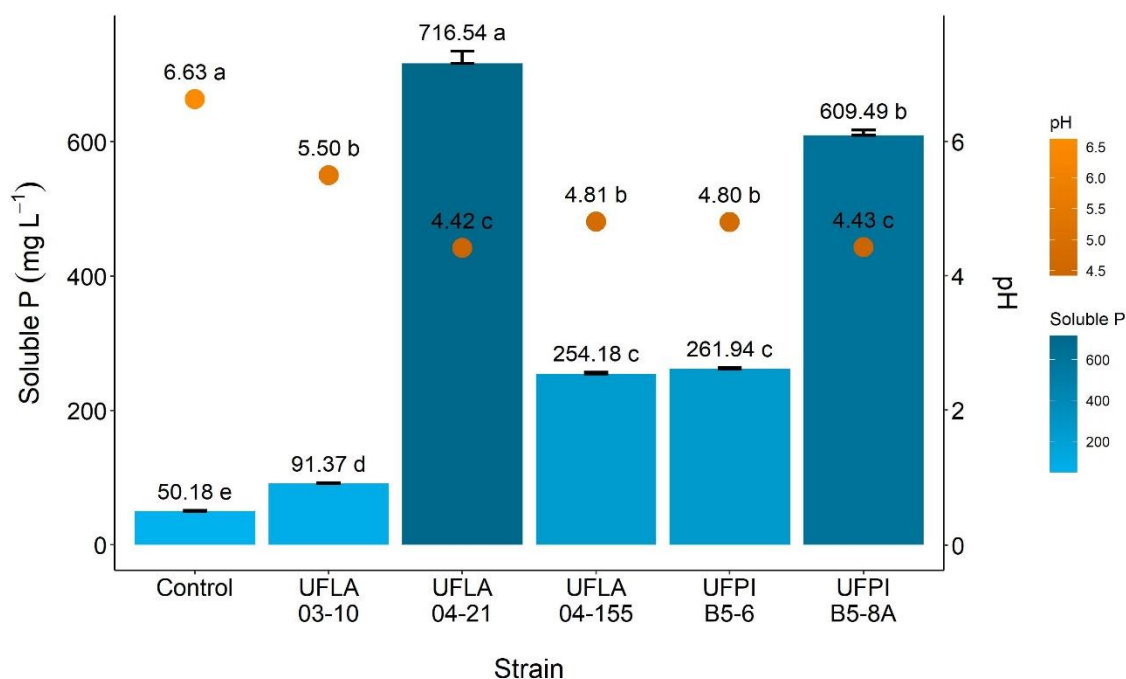**Figure 4**: Genes related to the main organic acids found in phosphate solubilizing strains UFLA 03-10 (*Paenibacillus peoriae*); UFLA 04-21 (*Paraburkholderia fungorum*); UFLA 04-155 (*Paraburkholderia fungorum*); UFPI B5-6 (*Enterobacter bugandensis*); UFPI B5-8A (*Pseudomonas anuradhapurensis*). Green box means presence of the gene, while red boxes mean absence of the gene. The name of the gene refers to the entry found in NCBI and KEGG databases.

Supplementary material

Table S1: Genes and enzymes related to the production of the organic acids acetic, citric, gluconic, lactic, malic, and oxalic in the phosphate solubilizing bacterial strains UFLA 04-155 (*Paraburkholderia fungorum*); UFLA 04-21 (*Paraburkholderia fungorum*); UFPI B5-6 (*Enterobacter bugandensis*); UFPI B5-8A (*Pseudomonas anuradhapurensis*); UFLA 03-10 (*Paenibacillus peoriae*)

Strain	Organic acid	Gene ID	Enzyme	Enzyme code	% Cover/ID
UFLA 03-10	Gluconic acid	-	-		
	Lactic acid	ldh2	L-lactate dehydrogenase 2	EC:1.1.1.27	96/50.55
	Acetic acid	aldHT	Aldehyde dehydrogenase, thermostable	EC:1.2.1.5	98/46.32
	Citric acid	pdhB	Pyruvate dehydrogenase E1 component subunit beta	EC:1.2.4.1	100/69.33
		pdhA	Pyruvate dehydrogenase E1 component subunit alpha	EC:1.2.4.1	98/58.86
		citA2	Citrate synthase 2	EC:2.3.3.16	100/63.34
		pdhC	Dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex	EC:2.3.1.12	93/55.34
	Oxalic acid	-	-		
	Malic acid	fumC	Fumarate hydratase class II	EC:4.2.1.2	98/78.86
		mdh	Malate dehydrogenase	EC:1.1.1.37	99/69.02
UFLA 04-21	Gluconic Acid	gdh	Glucose 1-dehydrogenase	EC:1.1.1.47	91/47.55
	Latic acid	lldD	L-lactate dehydrogenase	-	98/45.62
	Acetic acid	aldA	Putative aldehyde dehydrogenase AldA	EC:1.2.1.3	98/45.62
	Citric acid	pdhB	Dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex	EC:2.3.1.12	100/71.17
		pdhA	Pyruvate dehydrogenase E1 component	EC:1.2.4.1	100/64.52
		gltA	Citrate synthase	EC:2.3.3.16	99/70.23

	Oxalic acid	-	-		
	Malic acid	fumC	Fumarate hydratase class II	EC:4.2.1.2	98/77.29
		mdh	Malate dehydrogenase	EC:1.1.1.37	99/69.02
UFLA 04-155	Gluconic Acid	gdh	Glucose 1-dehydrogenase	EC:1.1.1.47	96/44.57
	Latic acid	lldD	L-lactate dehydrogenase	-	99/40.37
	Acetic acid	-	-		
	Citric acid	pdhB	Dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex	EC:2.3.1.12	100/71.17
		gltA	Citrate synthase	EC:2.3.3.16	73/72.01
	Oxalic acid	-	-		
	Malic acid	fumC	Fumarate hydratase class II	EC:4.2.1.2	98/77.29
		mdh	Malate dehydrogenase	EC:1.1.1.37	100/98.78
UFPI B5-6	Gluconic Acid	gcd	Quinoprotein glucose dehydrogenase	EC:1.1.5.2	100/90.83
		gdhA	Quinoprotein glucose dehydrogenase A	EC:1.1.99.35	96/61.86
	Latic acid	lldD	L-lactate dehydrogenase	EC:1.1.-.-	100/95.95
		dld	Quinone-dependent D-lactate dehydrogenase	EC:1.1.5.12	97/87.70
	Acetic acid	-	Putative aldehyde dehydrogenase	EC:1.2.1.3	100/44.49
	Citric acid	aceE	Pyruvate dehydrogenase E1 component	EC:1.2.4.1	100/93.57
		gltA	Citrate synthase	EC:2.3.3.16	100/96.25
		aceF	Dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex	EC:2.3.1.12	100/85.92
	Oxalic acid	-	-		
	Malic acid	fumC	Fumarate hydratase class II	EC:4.2.1.2	99/89.42

		mdh	Malate dehydrogenase	EC:1.1.1.37	100/92.88
UFPI B5-8A	Gluconic Acid	gdhA	Quinoprotein glucose dehydrogenase A	EC:1.1.99.35	96/48.15
		gcd	Quinoprotein glucose dehydrogenase	EC:1.1.5.2	95/47.24
		pqqD2	PqqA binding protein 2	-	100/74.44
		pqqC	Pyrroloquinoline-quinone synthase	EC:1.3.3.11	100/99.20
		pqqF	Coenzyme PQQ synthesis protein F	EC:3.4.24.-	99/75.16
		pqqE	PqqA peptide cyclase	EC:1.21.98.4	97/96.81
		pqqB	Coenzyme PQQ synthesis protein B	-	100/96.04
		pqqD1	PqqA binding protein 1	-	100/96.70
	Latic acid	lldD	L-lactate dehydrogenase	-	100/97.38
	Acetic acid	aldH	NADP/NAD-dependent aldehyde dehydrogenase PuuC	EC:1.2.1.5	98/58.86
	Citric acid	aceE	Pyruvate dehydrogenase E1 component	EC:1.2.4.1	100/89.34
		gltA	Citrate synthase	EC:2.3.3.16	99/89.25
		aceF	Dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex	EC:2.3.1.12	100/76.50
	Oxalic acid	-	-		
	Malic acid	fumC2	Fumarate hydratase class II 2	EC:4.2.1.2	100/78.60
		mdh	Malate dehydrogenase	EC:1.1.1.37	100/92.88

Table S2: Accumulation of micronutrients in shoots of maize plants (*Zea mays* L.) cultivated under different conditions of P fertilization and inoculation (or not) with phosphate solubilizing bacterial strains, 60 days after sowing in pots containing red-yellow Latosol (Oxisol). Control: not fertilized, non-inoculated; BRP: Bayóvar Rock Phosphate; MAP: Monoammonium phosphate; UFLA 04-155 (*Paraburkholderia fungorum*); UFLA 04-21 (*Paraburkholderia fungorum*); UFPI B5-6 (*Enterobacter bugandensis*); UFPI B5-8A (*Pseudomonas anuradhapurensis*); UFLA 03-10 (*Paenibacillus peoriae*)

Treatments	Cu	Fe	Mn	Zn	B
	------------------------ (μg pot^{-1}) ------------------------				
Control	7.97 f	65.74 f	53.08 g	27.44 h	29.38 e
150 mg P dm^{-3} (BRP)	23.68 e	749.76 e	217.09 f	234.24 f	139.17 d
UFLA 04-155 + 150 mg P dm^{-3} (BRP)	26.67 e	1431.77 d	292.94 e	151.48 g	149.52 d
UFLA 04-21 + 150 mg P dm^{-3} (BRP)	34.29 d	1019.96 e	330.01 e	361.03 e	157.30 d
UFPI B5-6 + 150 mg P dm^{-3} (BRP)	49.87 c	810.61 e	349.14 e	223.05 f	155.32 d
UFPI B5-8A + 150 mg P dm^{-3} (BRP)	44.12 c	1219.34 d	459.34 d	261.33 f	163.62 d
UFLA 03-10 + 150 mg P dm^{-3} (BRP)	41.05 d	824.58 e	529.89 d	459.73 d	248.71 b
50 mg P dm^{-3} (MAP)	50.43 c	1267.90 d	648.67 c	891.84 a	192.64 c
150 mg P dm^{-3} (MAP)	90.26 b	2180.70 c	1264.27 a	756.70 b	180.80 c
200 mg P dm^{-3} (MAP)	90.30 b	3217.33 b	968.29 b	584.29 c	272.24 b
250 mg P dm^{-3} (MAP)	167.44 a	3584.47 a	1240.54 a	469.84 d	426.58 a
CV (%)	10.71	10.05	11.60	12.41	10.23

Means followed by the same letter do not differ according to Tukey's test (P< 0.05).

There was variability among the treatments for micronutrients accumulated on the shoots and the highest accumulations were found in the plants fertilized with soluble P (Table S1). All inoculated plants except treatment UFLA 04-155 accumulated more Mn and Cu than the BRP fertilized plants. Strains UFPI B5-6 and UFPI B5-8A stood out accumulating Cu as much as the plants fertilized with 50 mg dm^{-3} of soluble P.

Regarding Fe accumulation, only strains UFLA 04-155 and UFPI B5-8A had more Fe content than the BRP fertilized plants and in the same amount that were found on the 50 mg dm^{-3} treatment. For Zn and B accumulation, plants inoculated with strains UFLA 04-21 and UFLA 03-10 (Zn) and strain UFLA 03-10 (B) were better than plants BRP fertilized. Among the treatments with the soluble P doses, the accumulation of Cu, Fe, and B were higher on the dose of 250 mg dm^{-3}. For Mn, the highest accumulation was at the doses of 150 and 250 mg dm^{-3}. For Zn, however, there was a significant decrease in accumulation according with the soluble P dose and the highest amount was found at the dose of 50 mg dm^{-3}.

SEGUNDA PARTE - ARTIGOS

ARTIGO II – Mechanisms and genes involved in phosphate solubilization by bacterial and fungal strains

(Artigo formatado conforme as regras do periódico World Journal of Microbiology and Biotechnology)

Mechanisms and genes involved in phosphate solubilization by bacterial and fungal strains

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Abstract

Microorganisms play an important role in P cycling. Bacteria and fungi can solubilize phosphates through several mechanisms and the most studied is the secretion of low molecular weight organic acids. However, other mechanisms may also participate in the process, such as the production of siderophores. The objective of this study was to screen through 7 bacterial and 30 fungal strains for potential calcium and iron phosphate solubilization and study mechanisms of solubilization of these two phosphate sources, including organic acids and siderophore production. Furthermore, we sequenced the genomic DNA from the bacterial strains to identify species and annotate genes related to the production of organic acids. Phosphate solubilization, organic acids, and siderophore production were evaluated *in vitro*. Several bacteria and fungi were able to solubilize phosphates, secrete diverse organic acids, and produce siderophores. *Acinetobacter pittii* UFLA 03-09 and *Penicillium flavigenum* E24 were the microorganisms that most solubilized calcium phosphate. Iron phosphate was solubilized to a considerably less extent due to the characteristics of the phosphate and the most promising microorganism were *Brukholdeia cepacia* LMG 1222 and *Penicillium flavigenum* E24. Bacteria and fungi had significant correlations between soluble P and organic acids when grown in calcium phosphate medium, but only the fungi had this significant correlation for iron phosphate. All studied low molecular weight organic acids were identified in the strains and the genomic analysis identified several genes linked to the production of the organic acids. We identified mechanisms of phosphate solubilization in bacteria and fungi with considerable growth-promotion potential and the next step is to verify this effect on mixed inoculation.

Introduction

Phosphorus (P) is a major nutrient required by all organisms. In agriculture, P is supplied mainly through fertilizers produced from phosphate rocks, a finite and non-renewable resource (Scholz et al., 2013). Although may exist a large amount of total P in the soil, only a small fraction is readily available for plant uptake (Sohrt et al., 2017). Most of the inorganic P is retained in the soil clay fraction or precipitated with cations, such as calcium in alkaline soils, and iron and aluminum in acidic soils (Gustafsson et al., 2012; Bünemann, 2015). In soil, P can be adsorbed by particles, such as iron and aluminum oxides and kaolinite (Fontes and Weed, 1996). The part of the P pool that is not soluble or retained in the soil is immobilized in the organic fraction, either in the composition of organisms or the organic matter. As a result, a low available P remains in the soil solution and P fertilization has to be done in large amounts due to a low efficiency from the interaction of the element and soil components (Fageria et al., 2016).

There are naturally occurring mechanisms that cycle P through the different pools where it is retained. These mechanisms are primarily governed by reactions of sorption, desorption, precipitation, solubilization, immobilization, and mineralization (Zhu et al., 2018). Microorganisms play an important role in P cycling as they drive or influence these reactions and bacteria and fungi are considered the main contributors to P cycling through organic and inorganic pools (Tian et al., 2021). There are several genera of bacteria related to P solubilization and the most predominant are *Bacillus*, *Pseudomonas*, and *Acinetobacter* (Marra et al., 2012; Kour et al., 2021). As for filamentous fungi, the two main genera related to P solubilization are *Aspergillus* and *Penicillium* (Khan et al., 2010; Kour et al., 2021), that usually are the ones detected more frequently by culture dependent techniques due to their high sporulation. However, soil particle washing technique allow

many other genera to be isolated and most of them proved able to solubilize Ca phosphates (Gudiño-Gomezjurado et al., 2015; 2022).

The main mechanisms attributed to phosphate solubilization by microorganisms are the production of organic acids (OA) that act both as organic ligands for cations and as a source of H^+ for acidification (Ström et al., 2001; Chen et al., 2006). Several types of OA have been identified during phosphate solubilization processes. The most common are gluconic, oxalic, citric, acetic, lactic, and succinic acids but other organic acids can also be found (Santos-Torres et al., 2021, Leite et al., 2020). In solution, these organic acids dissociate and the free negative charges on the carboxyl and hydroxyl groups bind cations that potentially would precipitate with the orthophosphate ions, thus increasing the level of available orthophosphates (Yadav and Verma, 2012). In addition, this dissociation also releases H^+, which reduces the solution pH and helps in solubilization, especially for calcium phosphates (Chen et al., 2006). In soil, another possibility is when the carboxyl groups react with soil particles and occupy sorption sites where otherwise the phosphates would be permanently adsorbed (Geelhoed et al., 1999, Ström et al., 2001). Nonetheless, research on the genomics of these mechanisms is scarce. Genes such as *gcd* and *pqq* responsible for the production of gluconic acid are the most researched ones, but genes for other organic acids and other possible mechanisms are poorly studied.

However, organic acids and acidification may not be the only mechanisms that promote phosphate solubilization. While experiments using calcium phosphate and rock phosphates are common and can be highly correlated with acidification and with the amount of organic acids (Chen et al., 2006; Marra et al., 2015), studies on iron phosphate solubilization are scarce. Iron phosphates are less prone to dissociate in acidic environments in the pH range from 2 to 8, which is the range where most microorganisms inhabit, thus rendering low results when solubilizing phosphates bound to iron (Jin et al.

2006; Li et al. 2015). Despite their importance in solubilization and chelation, organic acids may not be as effective in solubilizing iron phosphate, as they are effective in solubilizing calcium phosphate (Marschner et al. 2011). In this context, siderophores, a metabolite with a strong chelating activity over Fe, may have an important role in releasing P bound to iron (Cui et al. 2022). Usually, plants and microorganisms release siderophores to scavenge iron under conditions of low bioavailability (Marschner et al. 2011). Since organic acids have a limited effect on iron phosphates, siderophore production may help explain phosphate solubilization mechanisms in soils of acidic conditions.

We hypothesized that fungal isolates would be more efficient in solubilizing phosphates, as it is commonly reported in the literature. Furthermore, we hypothesized that phosphate solubilization would be associated with the secretion of organic acids or siderophores. Therefore, the objective of this study was to screen 7 bacterial and 30 fungal strains for potential calcium and iron phosphate solubilization and study mechanisms of solubilization of these two phosphate sources, including organic acids and siderophore production. Furthermore, we sequenced the genomic DNA from the bacterial strains to identify genes related to the production of organic acids. Additionaly, we identified the bacterial strains at species level by average nucleotide identity.

Material and Methods

Strains selection and cultivation

Thirty fungal isolates from different environments, genera and species, all belonging to the Coleção Micológica de Lavras, were chosen to compose the fungi group in this work. The identification and origin of the different fungi are presented on Table 1.

All isolates were previously identified according to the 18S rDNA. The fungal isolates were activated in potato-dextrose agar medium at 25 °C for 14 days prior to inoculation in the phosphate medium.

The following bacterial strains were chosen due to their ability to solubilize calcium and iron phosphates both in solid and liquid medium: *Acinetobacter* sp. UFLA 03-09; *Brevibacillus* sp. UFLA 03-104; *Paenibacillus* sp. UFLA 03-106, *Paenibacillus* sp. UFLA 03-116; *Paraburkholderia* sp. UFLA 04-155; *Rhizobium tropici* CIAT 899; *Burkholderia cepacia* LMG 1222 (Marra et al. 2012; Silva et al., 2012; Oliveira-Longatti et al. 2015). The strains were cultivated in 79 liquid medium (10 g mannitol; 1 mL K_2HPO_4 10%; 4 mL KH_2PO_4 10%; 2 mL $MgSO_4.H_2O$ 10%; 1 mL NaCl 10%; 0.4 g yeast extract; 1000 mL distilled water; pH 6.8-7.0) (Fred and Waksman 1928) for 3 days at 28 °C before inoculation in the phosphate medium.

Bacterial genomic identification

The bacterial strain used in this work had been previously identified according to the 16 S rRNA. We sequenced the genomic DNA from the strains UFLA 03-10, UFLA 03-104, UFLA 03-106, UFLA 03-116, and UFLA 04-155 and here we present the strains identification at species level. The genomic DNA was extracted with the Promega Wizard® DNA Extraction Kit following the instructions of the manufacturer. The DNA libraries were prepared with the NexteraXT kit (Illumina, CA, USA) according to the instructions of the manufacturer. The final amount of the library was measured with KAPA SYBR® FAST qPCR with the system QuantStudio® 5 (Applied Biosystems, CA, USA). The libraries were assessed with the TapeStation HSD1000 ScreenTape (Agilent Technologies, CA, USA). Illumina® 8-nt dual indices were used. Based on the quality measurements, equimolar mixtures of the libraries were used and the reading of the

fragments was done in two directions (2×150 bases) in the Illumina® HiSeq X platform (Illumina, CA, EUA). Genome assembly was done with the algorithm SPADes 3.13.0 (Bankevich et al. 2012). Quality and completeness were checked with CheckM 1.0.18 (Parks et al. 2015). Both algorithms were used within the KBase platform (https://www.kbase.us/).

The average nucleotide identity (ANI) technique (Konstantinidis and Tiedje, 2005) was applied for genome comparison and species identification at the species level. After confirmation of the genera by extracting and comparing the 16 S rRNA from the genome, we proceeded by comparing each strain with all type strains from the same genus available in the GenBank (National Centre for Biotechnology Information (NCBI); http://www.ncbi.nlm.nih.gov/). The ANI was assessed in the platform http://enve-omics.ce.gatech.edu/enveomics/ by comparing each strain with each obtained type strain sequence. Results are displayed in Table 2.

In vitro phosphate solubilization

An *in vitro* screening test to evaluate calcium and iron phosphate solubilization was carried out with all 30 fungal strains and seven bacterial strains compared to a control without inoculation for each source of phosphate. Though the bacterial strains had already been tested, the medium and/or the phosphate source were different and we performed the experiment to compare with the fungi in the same medium with the same mineral phosphate source. The insoluble phosphate enriched medium used was the PVK medium (Pikovskaya 1948) composed of (L^{-1}): 10 g of glucose, 0.5 g of $(NH_4)_2SO_4$, 0.2 g of NaCl, 0.1 g of $MgSO_4.7H_2O$, 0.2 g KCl, 0.5 g of yeast extract, 0.002 g of $MnSO_4.H_2O$, and 0.002 g of $FeSO_4.7H_2O$. The sources of phosphate used were $Ca_3(PO_4)_2$ and $FePO_4$ at 1000 mg L^{-1} of P. The experiment had a completely randomized design with three

replicates. Despite the PVK medium containing a small amount of yeast extract, and therefore an uncounted amount of P, we chose to use this medium since it is a traditional medium for phosphate solubilization experiments with fungi, and it could contain some growth factors for the fungi, other than a completely defined medium. In addition, the yeast extract could provide an initial amount of P to boost the solubilization, especially considering iron phosphate. Furthermore, any amount of soluble P in the medium would be shown in the uninoculated control treatments.

The inoculation of the fungi in the PVK liquid medium was done by cutting a 5 mm disk of the mycelium and placing directly in the medium. The bacterial strains were inoculated by applying 1.0 mL of the inoculant in the medium. Erlenmeyer flasks containing 50 mL of enriched PVK medium and the respective microbial inoculum were grown for 7 days at 28 °C and 110 rpm. After incubation, the medium was centrifuged at 3000 rpm for 15 min and filtered through a 0.45 µm cellulosic membrane. The supernatant was used to determine soluble P, pH, and organic acids. Soluble P in the extract was quantified by inductively coupled plasma optical emission spectroscopy (ICP-OES).

Organic acids identification and quantification

From the previous experiment, we selected five bacterial strains and five fungal isolates to investigate possible mechanisms of phosphate solubilization. The organic acids identification and quantification were done in a high-performance liquid chromatographer (HPLC). Samples were injected in a Supelcogel C-610H 9 µm 30 cm × 7.8 mm column to retain the organic acids at different times. Certified samples of acetic, citric, formic, fumaric, gluconic, lactic, maleic, malic, malonic, oxalic, and tartaric acids were used as analytical standards. The analytical procedure was performed according to the manufacturer instructions for organic acid analysis. The mobile phase was a 0.1 %

H_3PO_4 running at a 0.5 mL min^{-1} flow rate for 45 minutes at 210 nm wavelength. The quantification of organic acids was done by calibration curves built with the analytical standards. The identified acids and their respective retention times were as follows: oxalic at 9.15 min, citric/maleic at 11.50 min, gluconic/tartaric at 12.30 min, malic at 13.56 min, malonic at 13.94 min, lactic at 17.04 min, formic at 18.51 min, acetic at 20.12 min, and fumaric at 21.13 min. Three replicates were used.

Genomic annotation of genes related to the solubilization of phosphates

The genes that codify the enzymes that participate in the synthesis of the acetic, citric, gluconic, lactic, malic, and oxalic were annotated. The genes and enzymes identifications were based on the following publications: Kobayashi et al. (2014), Shariati et al. (2017), Dai et al. (2018), and Santos-Torres et al. (2021). The genome assembly of *Burkholderia cepacia* LMG 1222 was obtained from the NCBI deposited under the registration ASM354646v1. Genome annotation was performed with the Rapid Annotation using Subsystem Technology (RAST) program within the platform KBase (https://www.kbase.us/). The annotation process consisted of the search for the complete name of the enzyme or the name of the gene in the RAST tool. When possible matches were found, the protein sequence of the gene was compared with the database from the Basic Local Alignment tool – Blastp suite – from the NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi). This procedure had the objective to identify the value of similarity between our sequences and those from the database. After selecting the best matches according to the completeness and identity of the sequences, the gene/enzyme was confirmed using the Uniprot (https://www.uniprot.org/) entry available in the deposited sequence information. Enzymes were verified in the databases KEGG: Kyoto Encyclopedia of Genes and Genomes (https://www.genome.jp/kegg/) and

BRENDA (https://www.brenda-enzymes.org). After this procedure, we considered the presence (or not) of the gene/enzyme in the genome of the strains.

Siderophore analysis

The same selected strains and isolates from the initial experiment were also investigated for the production of siderophores. The rationale is that the siderophore produced by the microorganisms forms complexes with the Fe bound to the phosphate, thus leaving soluble P in the solution. Siderophore qualitative assay was done according to the chromo azurol sulphonate (CAS) method (Schwyn and Neilands, 1987). Briefly, a 1:10 mix of CAS reagent was mixed with LB agar medium, respectively. The cultivation of microorganisms was done as described previously. Inoculation was performed with 20 µL of each bacterial strain while fungi were inoculated with a 5 mm disk of the mycelium on the solid CAS plates. Positive results were considered when an orange halo appeared around the colonies (Blanco and Castro, 2021).

Data analyses

Data were assessed for normality and homoscedasticity according to Shapiro-Wilk's test and Bartlett's test, respectively ($P < 0.05$). Since data did not follow those assumptions, we fitted them into a generalized least square model and compared it to the linear model according to Akaike's information criteria. Groups of variances were built according to variance of the residues prior to the normality assessment. Means were compared by contrast analysis with the control without inoculation and with the inoculation with *Burkholderia cepacia* LMG 1222 ($P < 0.05$), which was the positive control for its performance in previous works (Marra et al., 2012). Pearson's correlations were drawn between soluble P and pH and between soluble P and total organic acids

using the function the package ggpubr (Kassambara, 2020). All statistical analysis and figures were done in the R environment (R Core Team, 2022) and the R Studio platform (R Studio, 2022).

Results

In vitro phosphate solubilization

Significant amounts of soluble phosphate were found for both calcium and iron phosphates (Figures 1 and 2). We compared the treatments with the control without inoculation and with the *Burkholderia cepacia* LMG 1222, which was our positive control. All fungal and bacterial strains were able to grow on the liquid medium containing only insoluble phosphate as a P source. This was attested by visualizing the presence of mycelia or by the turbidity of the medium in comparison with the control without inoculation. Conversely, the controls without inoculation did not show signs of microbial growth, suggesting that there was no contamination.

The most prominent strain evaluated in calcium phosphate was *Acinetobacter pittii* UFLA 03-09, able to solubilize 68 % of the total insoluble P initially added to the medium (1000 mg L^{-1} of P). The *Penicillium flavigenum* E24 was the most efficient fungal isolate and the second most efficient microbial tested and solubilized 40 % of the total calcium phosphate. Other fungal isolates also presented efficient solubilizing ability, such as two *Paraconyothirium* isolates F3697 and F3698 (290 and 201 mg L^{-1}, respectively), and *Penicillium flavigenum* 3.1A (203 mg L^{-1}). Among the other bacterial species, calcium phosphate solubilization ability was similar, except for *Paraburkholderia fungorum* strain UFLA 04-155, which left only a small amount of residual soluble P in the medium (11.62 mg L^{-1}), lower even than the amount hydrolyzed

in the control treatment (51 mg L^{-1}). Amongst the fungi, only *Induratia coffeana* CML 4010 showed lower soluble P than the control (41.8 mg L^{-1}).

Almost all inoculated microbes had equal or lower pH in comparison with the control (Figure 1). *Induratia coffeana* isolate CML 4010 showed a slightly more alkaline pH than the control. Amongst the fungi, the most acidic pHs appear to the linked with the higher soluble P, as expected. *Acinetobacter pittii* UFLA 03-09 considerably reduced the pH in association with a high soluble P as compared to the other bacterial strains. *Paraburkholderia fungorum* UFLA 04-155 showed a high pH associated with low soluble P in the medium.

Iron phosphate solubilization showed a different pattern from that observed for calcium phosphate, and other strains were more prominent. The highest amount of soluble P derived from iron phosphate solubilization was achieved by *Burkholderia cepacia* LMG 1222, accounting for 36 mg L^{-1} of soluble P. *Penicillium flavigenum* E24 was the most efficient fungus, followed by *Fusarium* sp. FPW (24 mg L^{-1}), *Paraconiothyrium cyclothyrioides* strains CML 3696 (20.5 mg L^{-1}), and the bacterial strain *Rhizobium tropici* CIAT 899 (23.5 mg L^{-1}). Interestingly, all *Induratia coffeana* isolates showed lower soluble P than the control. Other bacterial strains also showed no signs of solubilization, despite their growth.

The majority of the fungi tested considerably reduced pH in the presence of iron phosphate, although this acidification was not always linked to higher phosphate solubilization. For example, the most efficient fungus *Penicillium flavigenum* E24 reached the same pH as *Aspergillus keveii* CF292, despite the last one showing even less soluble P than the control. All bacterial strains also reduced pH, some in greater extent, but also not always linked to higher soluble P in the medium.

Bacterial calcium solubilization showed a high correlated (R = -0.95) and significant relationship ($p < 0.001$) between soluble P in the medium and the final pH as drawn by Pearson's correlation (Figure 2). Even though one strain was far superior in releasing P and acidifying the medium, altogether all strains showed a direct and negative relationship between these two variables, as expected. Fungal isolates also showed the same direct, negative ($R = -0.89$), and significant ($p < 0.001$) relationship between soluble P and pH for calcium phosphate.

Pearson's correlation between soluble P derived from iron phosphate and pH for bacterial strains ruled out any direct relationship as it was not significant (p = 0.315). Amongst the fungi cultivated in iron phosphate, a significant relationship was found (p < 0.001), with a coefficient of correlation R = -0.51 indicating an increase in soluble P as the pH decreases.

Organic acids quantification

All bacterial strains produced at least three different organic acids from the 11 acids that were used as standard. Citric/maleic and acetic acids were found only in two and three combinations of strains and sources of P, respectively. The highest OA production was achieved by *Acinetobacter pittii* UFLA 03-09 with an overwhelming 2546±104 mg mL^{-1} growing on calcium phosphate. This amount corresponds to 99 % of the total amount of OA produced by the strain. However, in the medium enriched with iron phosphate, this strain did not produce expressive amounts of OA. The strain UFLA 04-155 of *Paraburkholderia fungorum* had one of the lowest solubilization on both phosphate sources. However, when cultivated with iron phosphate, it produced a high amount of OA, as well as the highest amounts of oxalic, citric/maleic, gluconic/tartaric and malonic acids. Interestingly, *Burkholderia cepacia* LMG 1222, which achieved the

highest iron phosphate solubilization, produced the lowest amount of OA and only three diverse acids. Among the five strains, UFLA 03-104 (*Brevibacillus centrosporus*) and UFLA 04-155 (*Paraburkholderia fungorum*) produced more OA when the medium was supplemented with iron phosphate.

Regarding the fungi, all isolates produced at least one type of OA. Citric/maleic acids were produced only by *Penicillium flavigenum* E24. This isolate also produced the highest amount of OA and gluconic/tartaric acid, on both phosphate sources. Interestingly, *Induratia coffeana* CML 4010 produced several types of organic acids on both sources, despite its inability to solubilize phosphates.

Pearson's correlation between soluble P and the amount of OA showed a positive and significant correlation for both bacteria and fungi growing on calcium phosphate ($R = 0.97$, $p < 0.001$ and $R = 0.73$, $p = 0.002$, respectively). Under iron phosphate, however, only the fungi correlation was significant ($p < 0.001$), even though it was inversely proportional ($R = -0.9$). The correlation for bacteria on iron phosphate was not significant.

Bacterial species identification and genomic annotation of genes related to the solubilization of phosphates

All bacterial strains were identified as previously described species according to the ANI test (ANI $\geq$ 95 %). Results are displayed in Table 2.

After the screening in the first experiment, we selected the bacterial strains to investigate the genes related to the metabolism of acetic, citric, gluconic, lactic, and malic that were found in at least two of the strains (Figure 5). Conversely, the genes involved in the synthesis of oxalic acid were not found in any of the strains. The *Acinetobacter pittii* UFLA 03-09 had the highest diversity of genes related to all investigated organic acids (except oxalic acid). We did not find the genes to produce gluconic and lactic acids

in *Brevibacillus centrosporus* UFLA 03-104 and *Paenibacillus chondroitinus* UFLA 03-116 and the gene to produce acetic acid in *Paraburkholdeia fungorum*. Although the *Burkholderia cepacia* LMG 1222 has the *pqq* genes that codify the co-factor pyrroloquinoline quinone (PQQ), part of the glucose dependent 1-dehydrogenase-pyrroloquinoline quinone pathway, we did not find the gene related to the associated glucose dehydrogenase. Additionally, *B. cepacia* LMG 1222 also has the gene to produce two isomers of the lactate dehydrogenase enzyme (*ldh*A and *lld*D).

Siderophore assay

Three bacterial strains showed an orange halo in the blue CAS medium: *Acinetobacter pittii* UFLA 03-09, *Paraburkholderia fungorum* UFLA 04-155, and *Burkholderia cepacia* LMG 1222. The *Acinetobacter* strain even turned all medium in the plate into orange. Strain UFLA 03-104 of *Brevicaillus centrosporus* did not show halo and *Paenibacillus chondroitinus* UFLA 03-116 did not grow in the CAS + King B medium. Regarding the fungi, all isolates showed an orange halo, except the isolate CML 4010 of *Induratia coffeana*.

Discussion

Learning how to improve P efficiency in the near future will be of paramount importance for sustainable agriculture and food security. Despite the numerous interactions and reactions that the element performs in the soil, a considerable part of the total P is not lost from the system but remains inaccessible. Microorganisms represent a viable and relatively easy way to access the retained P as well as to get the element cycling through the different biotic and mineral compartments in the soil (Zhu et al. 2018). The potential for phosphate solubilization of several phyla of microorganisms is being studied

for decades, but it remains unclear, especially considering the myriad of soil characteristics and their own interaction with other microorganisms and plants (Kour et al. 2021). Here, we sought to compare strains of bacteria with recognized phosphate solubilization ability with several species of fungi, isolated from different systems, and to clarify mechanisms used from by microorganisms groups to solubilize phosphate.

In our experiment, the most promising microorganism for solubilization of calcium phosphate was the strain UFLA 03-09 of *Acinetobacter pittii*, which accounted for 685 mg L^{-1} of soluble P, representing the capacity to solubilize 68.5% of the $Ca_3(PO_4)_2$ added. The genus is well known for its potential to solubilize phosphates. In previous works, UFLA 03-09 ranged from 30 to 70 % of solubilization in NBRIP medium using 100 mg L^{-1} of insoluble P (Marra et al. 2015; 2019). This strain was also considered for its potential for solubilization of a biochar-based rock phosphate fertilizer (Leite et al. 2020). Other authors also have been studying species within this genus as phosphate solubilizers with outstanding results (Gulati et al. 2010; Xie et al. 2021). This strain also produced a great amount of gluconic acid, which is commonly correlated with soluble P in this genus (Ogut et al. 2010). Gluconic acid production in *Acinetobacter* appears to be an important mechanism of phosphate solubilization. An *Acinetobacter gcd* gene responsible for encoding the glucose dehydrogenase enzyme, when transferred to a *Pseudomonas*, was responsible to increase and maintain phosphate solubilization for a longer period than the wild strain (Xie et al. 2021). However, it is not clear if that excess of gluconic acid is produced as a response to P starvation or if it is produced as a normal carbon metabolism pathway, since some *Acinetobacter* can use the glucose via the Entner–Doudoroff pathway, which already requires gluconic acid (Ogut et al. 2010; Rozova et al. 2021; Ma et al. 2022). This uncertainty about the phosphate solubilization

process extends to other organic acids and research about the activation of mechanisms of solubilization still has to be done.

Interestingly, *Paraburkholderia fungorum* UFLA 04-155 considerably reduced the available P present in the PVK medium as compared to the treatment without inoculation. It is possible that this fast-growing strain initially absorbed all available P, but since it does not have a strong ability to solubilize $Ca_3(PO_4)_2$ it likely only survived with the lowest amount of P available and cycled the element after cell death. This strain was previously tested for solubilization a rock phosphate after inoculation on rice plants and did not promote plant growth (Costa et al. 2015), but it was able to solubilize P in the presence of $CaHPO_4$ with efficient results (Oliveira-Longatti et al. 2013) and with a biochar enriched with rock phosphate (Leite et al. 2020). In the presence of iron phosphate, this strain did not release soluble P different from the control. However, it produced a large amount of total OA, especially oxalic, citric/maleic, gluconic/tartaric, and malic. Since the natural dissociation of iron phosphate P is low (as shown by the low soluble P in the control), the low amount of P for the bacterial growth could have induced the activation of genes and the production of more OA as a response to P starvation (Gyaneshwar et al. 1999). Despite not releasing soluble P, the genus *Paraburkholderia* is known to have species with phosphate solubilizing abilities (Vio et al. 2020).

Amongst the fungi, *Penicillium flavigenum* E24 showed an interesting potential for calcium phosphate solubilization, releasing 40 % of the P added as $Ca_3(PO_4)_2$. The isolate E24 also showed a large amount of gluconic acid on both P sources, along with higher production of lactic and malonic acids than the other microorganisms. The high production of gluconic acid explains the solubilization process since this acid mainly solubilizes phosphate via the release of H^+ (Goldstein, 1995). The genus is known for its ability to produce gluconic acid (Crognale et al. 2008). The *P. flavigenum* 3.1A, isolated

at the same place as E24, also showed a promising but less efficient calcium phosphate solubilization (20 % of the total P). Within the same genus, however, not all species have the potential to solubilize phosphate. In our case, P. *chermesinum* 102 did not show the potential to mobilize calcium from $Ca_3(PO_4)_2$. *Penicillium* and *Aspergillus* are considered the two most important genera of fungi related to phosphate solubilization (Khan et al. 2010, Kour et al. 2021). Here, the *Aspergillus* isolates were not as efficient as the *Penicillium* isolates, and the three representatives of the genus were not considered potential solubilizers, even though they all had the ability to grow utilizing $Ca_3(PO_4)_2$ as the major source of P. Although *Aspergillus* has species with industrial importance in producing gluconic (Khattab and Bazaraa, 2005) and oxalic acids (Li et al. 2015; Mendes et al. 2014), *Aspergillus sydowii* GMA3 produced low amounts of OA, especially these acids. It is common to see differences in the potential for solubilization of phosphates within the same genus and even in the same species (Mendes et al. 2014, Adhikari and Pandey 2019). Interestingly, the hypothesis that fungal strains are more efficient in phosphate solubilization, as it is commonly reported in the literature, was not confirmed, especially for P-Ca. For P-Fe also most fungi showed lower solubilization than bacteria (Figure 2).

We report an interesting potential for solubilization for the genus *Paraconiothyrium*, able to reach 29 % of soluble P. The two best isolates of *P. cyclothyrioides*, CML 3697 and CML 3698, were both isolated from roots of *Urochloa*, but in different regions of Brazil (Gama et al. 2020), and also presented antifungal activity (Gama et al. 2020, Alves et al. 2021) and water stress tolerance induction on forage grass species (Azevedo et al. 2021). This genus was described in 2004 to accommodate other species (Verkley et al. 2004), but, at present, no research relating it to the solubilization of phosphates was found. Despite their potential to solubilize P, especially from calcium

phosphate, the amount of the organic acids was relatively low. It is possible that the phosphate solubilization mechanism of this species is more related to the acidification of the environment since the pH was low. Reyes et al. (1999) reported an H^+ pump in *Penicillium rugulosum*, which could explain the acidification of the medium and the consequent soluble P availability. Altogether, characteristics of growth promotion of these isolates of *Paraconiothyrium* and the ability to colonize grasses indicate a possible inoculant for plant growth.

Regarding iron phosphate, it is noticeable that the ability of all bacteria and fungi to solubilize $FePO_4$ is considerably lower compared to $Ca_3(PO_4)_2$ (Jin et al. 2006). The probable explanation is due to the stronger energy between Fe and P shared by the triple bond. Furthermore, P bound to iron has a lower dissolution rate and a relatively higher p*Ksp* than calcium phosphate (Tian et al. 2021). The highest soluble P achieved was 36 mg L^{-1} by *Burkholderia cepacia* LMG 1222, ahead of the 30 mg L^{-1} solubilized by *Penicillium flavigenum* isolate E24. The majority of the fungi even reduced the amount of soluble P in the medium, probably as a response to nutrient starvation. In acidic soils, free P not only forms secondary oxide minerals but also precipitates with Fe and Al (Osorio and Habte, 2013; Yuan et al. 2017), thus rendering the phosphate solubilization activity more difficult than *in vitro* systems. In addition, particle adsorption and the soil buffering capacity from acidic soils may limit the activity of the organic acids (Marschner et al. 2011) and other mechanisms, such as siderophores, may enhance P liberation.

Pearson's correlation also supported the established hypothesis that calcium phosphate solubilization is directly linked to acidification and the release of acids. Furthermore, the amount of organic acids was positively correlated with soluble P suggesting a role of these set of molecules in phosphate solubilization for both bacteria and fungi, as expected. However, both pH and OA did not have significant correlations

when the bacterial strains grew on the iron phosphate medium, thus confirming the difficulty in solubilizing iron phosphate and the participation of another mechanism.

The ANI technique showed that all bacterial strains used in this work belong to already described species. These strains were tested for diverse growth-promoting characteristics in previous works (Marra et al. 2012, Oliveira-Longatti et al. 2014, Leite et al. 2020). We now present genomic traits related to the synthesis of organic acids and their relationship with the solubilization of calcium and iron phosphates. For most genes related to the production of the evaluated organic acids, these strains also presented the genes and enzymes. Although strains ULFA 03-104 and UFLA 03-116 were identified as possible producers of gluconic lactic acids, we did not find the genes related to these acids. In the case of gluconic acid, since the chromatographer could not separate gluconic and maleic acids, we cannot rule out the amount of organic acid in the referred retention time being only due to one or the other acid. Furthermore, the lack of gene identification may be due to a limitation in the database for the genera of these strains (*Brevibacillus* and *Paenibacillus*, respectively). Conversely, citric acid genes were found in all strains, but production was found only in the *Brevibacillus centrosporus* UFLA 03-104 and *Paraburkholderia fungorum* UFLA 04-155. Interestingly, *Acinetobacter pittii* UFLA 03-09 has the genes to produce gluconic acid via the glucose dependent 1-dehydrogenase-pyrroloquinoline quinone pathway and via the quinoprotein glucose dehydrogenase pathway (Santos-Torres et al., 2021). This characteristic provides insights into the high production of gluconic acid through different pathways but transcriptomic analyses would be interesting to complete relate the activation of those genes and the phenotype.

Different media and components of the media, such as carbon source and the type of phosphate, can interfere in the solubilization process (Nautiyal, 1999, Mendes et al. 2014, Marra et al. 2019). Thus, the solubilization mechanism activated in a determined

set of conditions might change with the consequent synthesis of different types of organic acid. This suggests a complex genetic regulation system with the activation or inactivation of different metabolic pathways in the presence of a certain environmental condition (Rodríguez et al. 2006). Although genetic regulation in recombinant mutants and the over-expression of transferred genes have been studied for decades (Goldstein and Lui et al. 1995), the expression of genes related to the synthesis of organic acids under different types of phosphate is poorly studied. This is of uttermost importance to fully understand the mechanism of phosphate solubilization that occurs naturally in the soil and, in future perspective, access to the extensive P deposit in agricultural soils.

The other investigated mechanism was the production of siderophores. Siderophores are a group of secondary metabolites that has a high affinity for Fe and are produced by secreted by microorganisms under conditions of low Fe availability (Marschner et al. 2011, Gu et al. 2020). The rationale is similar to the effect of the organic acids. The siderophore can form strong chelates with Fe, thus releasing the orthophosphate molecule. In this work, we did not verify the secretion of siderophores in bacteria *Paenibacillus chondroitinus* UFLA 03-116 and in the fungus *Induratia coffeana* CML 4010. From these microorganisms, we did not find reports of siderophore production only for the *Induratia* genus. These are important molecules with diverse effects with positive consequences even for plant growth promotion and biocontrol (Zeng et al. 2018, Gao et al. 2022). Current research indicates the stimulation of siderophore biosynthesis and solubilization in the presence of $FePO_4$ (Cui et al. 2022). Despite the importance of this group of molecules, little is understood about their participation in the soil-organisms' relationships. A next important step would be to quantify and identify the types of siderophores that these microorganisms produce.

Conclusions

Most bacteria and fungi were able to solubilize phosphate through the secretion of low-molecular organic acids and/or siderophores. A bacterial strain UFLA 03-09 (*Acinetobacter pittii*) had an outstanding phosphate solubilization ability, thus contradicting literature reports. Bacteria and fungi had significant correlations between soluble P and organic acids when grown in a medium with calcium phosphate, but only the fungi had this significant correlation for iron phosphate. Bacterial strains had the genes to synthesize most of the studied organic acids. In some specific cases, solubilization might be linked to other mechanisms, such as acidification, and studies to unravel other possible mechanisms are necessary. The next step would be to verify these effects on mixed inoculations to further apply a possible mixed inoculant in soil and plants.

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Table 1: Identification and origin of the fungal isolates

CML code	Previous code	Species	Origin (municipality, state, Brazil)	Host / Origin
CML 4009	-	*Induratia coffeana*	Viçosa, MG	Organic coffee
CML 4010	-	*Induratia coffeana*	Viçosa, MG	Organic coffee
CML 4011	-	*Induratia coffeana*	Viçosa, MG	Organic coffee
CML 4012	-	*Induratia coffeana*	Viçosa, MG	Organic coffee
CML 4013	-	*Induratia* sp.	Viçosa, MG	Organic coffee
CML 4014	-	*Induratia coffeana*	Viçosa, MG	Organic coffee
CML 4015	-	*Induratia* sp.	Viçosa, MG	Organic coffee
CML 4016	-	*Induratia coffeana*	Viçosa, MG	Organic coffee
CML 4017	-	*Induratia coffeana*	Viçosa, MG	Organic coffee
CML 4018		*Induratia coffeana*	Viçosa, MG	Organic coffee
CML 4019	-	*Induratia coffeana*	Viçosa, MG	Organic coffee
CML 4020	-	*Induratia coffeana*	Viçosa, MG	Organic coffee
CML3699		*Paraconiothyrium estuarinum*	Campo Grande, MS, Pasture field	*Brachiaria humidicola*
CML3695	-	*Paraconiothyrium estuarinum*	Juiz de Fora, MG, Experimental plots	*Panicum maximum*
CML3698	-	*Paraconiothyrium cyclothyrioides*	Campo Grande, MS, Brazil, Pasture field	*Brachiaria humidicola*
CML3697	-	*Paraconiothyrium cyclothyrioides*	Juiz de Fora, MG, Experimental plots	*Brachiaria ruziziensis*
CML3696	-	*Paraconiothyrium cyclothyrioides*	Juiz de Fora, MG, Experimental plots	*Panicum maximum*
CML3767	A2C47	*Periconia ignaria*	Aiuruoca, MG	*Eremanthus erythropappus*
CML3766	A2C54	*Arcopilus* sp.	Aiuruoca, MG	*Eremanthus erythropappus*
CML3768	A2C32	*Epicoccum nigrum*	Aiuruoca, MG	*Eremanthus erythropappus*
CML3769	A2S61	*Epicoccum nigrum*	Aiuruoca, MG	*Eremanthus erythropappus*

CML2971	185A	*Epicoccum nigrum*	Iuiú, BA	*Honourado* grotto (soil)
CML2965	E24	*Penicillium flavigenum*	Vazante, MG	*Lapa Nova* grotto (soil)
CML3827	3.1A	*Penicillium flavigenum*	Vazante, MG	*Lapa Nova* grotto (soil)
CML2964	E25	*Aspergillus aureolatus*	Vazante, MG	*Lapa Nova* grotto (soil)
CML2970	ONI5	*Lecanicillium aphanocladii*	Pains, MG	*Cavalinho* grotto (air)
CML2968	CF292	*Aspergillus keveii*	Coronel José Dias, PI	*Cora de Frade* grotto (air)
CML2966	102	*Penicillium chermesinum*	Lavras, MG	Lab contaminant (air)
CML2969	FPW	*Fusarium* sp.	Lavras, MG	Lab contaminant (air)
CML2967	GMA3	*Aspergillus sydowii*	Delmiro Gouveia, AL	*Morcegos* grotto

Table 2: Identification and origin of the bacterial strains

Strain	Species	ANI (%)	Access code of the assembly of the most similar species in the NCBI	Origin (municipality, state, Brazil)
UFLA 03-09	*Acinetobacter pittii*	97	GCF_000369045.1	Patos de Minas, MG
UFLA 03-104	*Brevibacillus centrosporus*	95	GCF_003710815.1	Patos de Minas, MG
UFLA 03-106	*Paenibacillus peoriae*	96	GCF_000236805.1	Patos de Minas, MG
UFLA 03-116	*Paenibacillus chondroitinus*	95	GCF_004000765.1	Patos de Minas, MG
UFLA 04-155	*Paraburkholderia fungorum*	99	GCF_000961515.1	Secondary forest, AM

Table 3: Organic acids profile of phosphate solubilizing bacteria on PVK medium containing $Ca_3(PO_4)_2$ and $FePO_4$. UFLA 03-09 = *Acinetobacter pittii*; UFLA 03-104 = *Brevibacillus centrosporum*; UFLA 03-116 = *Paenibacillus chondroitinus*; UFLA 04-155 = *Paraburkholderia fungorum*; LMG 1222 = *Brukholderia cepacia*

	UFLA 03-09		UFLA 03-104		UFLA 03-116		UFLA 04-155		LMG 1222	
	Ca	Fe	Ca	Fe	Ca	Fe	Ca	Fe	Ca	Fe
Oxalic	1.20±0.03	0.53±0.03	1.72±0.24	0.51±0.03	1.87±0.11	0.57±0.03	-	16.30±2.59	2.80±0.09	1.23±0.13
Citric / Maleic	-	-	-	-	1.72±0.18	-	-	48.84±4.37	-	-
Gluconic / Tartaric	2546±104	5.86±0.61	-	4.39±0.14	2.58±0.24	4.15±0.37	5.64±1.66	229.7±34.6	4.75±1.41	-
Malic	-	13.97±2.30	11.08±4.08	16.73±1.31	31.95±1.80	16.43±0.72	28.33±1.08	-	30.44±6.44	-
Malonic	-	21.58±4.08	-	25.36±1.34	-	24.83±1.40	-	56.32±5.86	-	-
Latic	3.43±0.34	2.82±0.44	2.45±1.19	2.94±0.14	1.07±0.21	3.07±0.23	0.44±0.03	2.39±0.11	0.23±0.07	0.77±0.33
Formic	0.17±0.01	0.17±0.02	-	0.18±0.0	-	0.24±0.01	-	0.88±0.19	0.54±0.07	0.61±0.16
Acetic	8.58±2.18	-	-	-	16.13±4.22	-	-	5.53±0.16	-	-
Fumaric	-	0.22±0.00	1.16±0.4	0.22±0.0	1.02±0.23	0.23±0.00	-	0.41±0.02	-	-
Total	2560±107	45.16±7.48	16.41±5.91	50.33±2.96	56.34±6.99	49.52±2.76	34.41±2.77	360.37±47.9	38.76±8.08	2.61±0.62

Means ± standard deviation

Table 4: Organic acids profile of phosphate solubilizing fungi on PVK medium containing $Ca_3(PO_4)_2$ and $FePO_4$. CML 3697 and CML 3698 = *Paraconiothyrium cyclothyrioides*; E24 = *Penicillium flavigenum*; GMA3 = *Aspergillus sydowii*, CML 4010 = *Induratia coffeana*

	CML 3697		CML 3698		E24		GMA3		CML 4010	
	Ca	Fe	Ca	Fe	Ca	Fe	Ca	Fe	Ca	Fe
Oxalic	1.35±0.62	0.49±0.01	0.57±0.11	0.48±0.00	0.64±0.09	0.56±0.11	0.72±0.08	-	0.66±0.07	0.49±0.01
Citric / Maleic	-	-	-	-	-	0.28±0.09	-	-	-	-
Gluconic / Tartaric	5.73±1.19	6.44±0.29	-	-	1434±48	405.8±115.8	-	2.50±0.04	4.55±0.70	6.72±0.29
Malic	22.30±7.36	21.13±5.92	64.74±4.72	19.11±9.48	37.83±8.91	6.09±2.26	7.58±3.42	20.73±3.03	30.14±8.47	24.23±0.40
Malonic	-	-	1.96±0.12	-	47.65±4.65	93.1±20.5	-	-	-	-
Latic	0.53±0.26	1.66±0.02	0.49±0.31	1.18±0.20	72.5±12.6	68.8±10.1	0.48±0.17	3.23±1.06	8.07±6.03	15.45±1.44
Formic	-	0.13±0.01	0.24±0.09	0.12±0.01	-	-	-	0.17±0.01	0.11±0.07	0.12±0.01
Acetic	14.78±1.59	1.62±0.55	40.96±12.47	1.87±0.87	-	-	8.26±4.75	3.79±1.89	46.23±14.11	32.88±5.19
Fumaric	0.26±0.01	0.30±0.04	-	0.42±0.34	6.32±0.59	7.83±1.42	0.21±0.00	0.21±0.01	0.31±0.04	0.32±0.00
Total	44.9±11.1	31.8±6.8	108.9±17.8	23.2±10.9	1599±75	582±150	17.2±8.4	30.6±6.1	90.1±29.5	80.2±7.3

Means ± standard deviation

Figure 1: Calcium phosphate solubilization and pH by bacterial strains and fungal isolates on NBRIP medium. Vertical bars indicates the standard error from each treatment. Means were compared by contrast analysis (P < 0.05) with the control without inoculation (*) and with the treatment inoculated with the *Burkholderia cepacia* LMG 1222 (#).
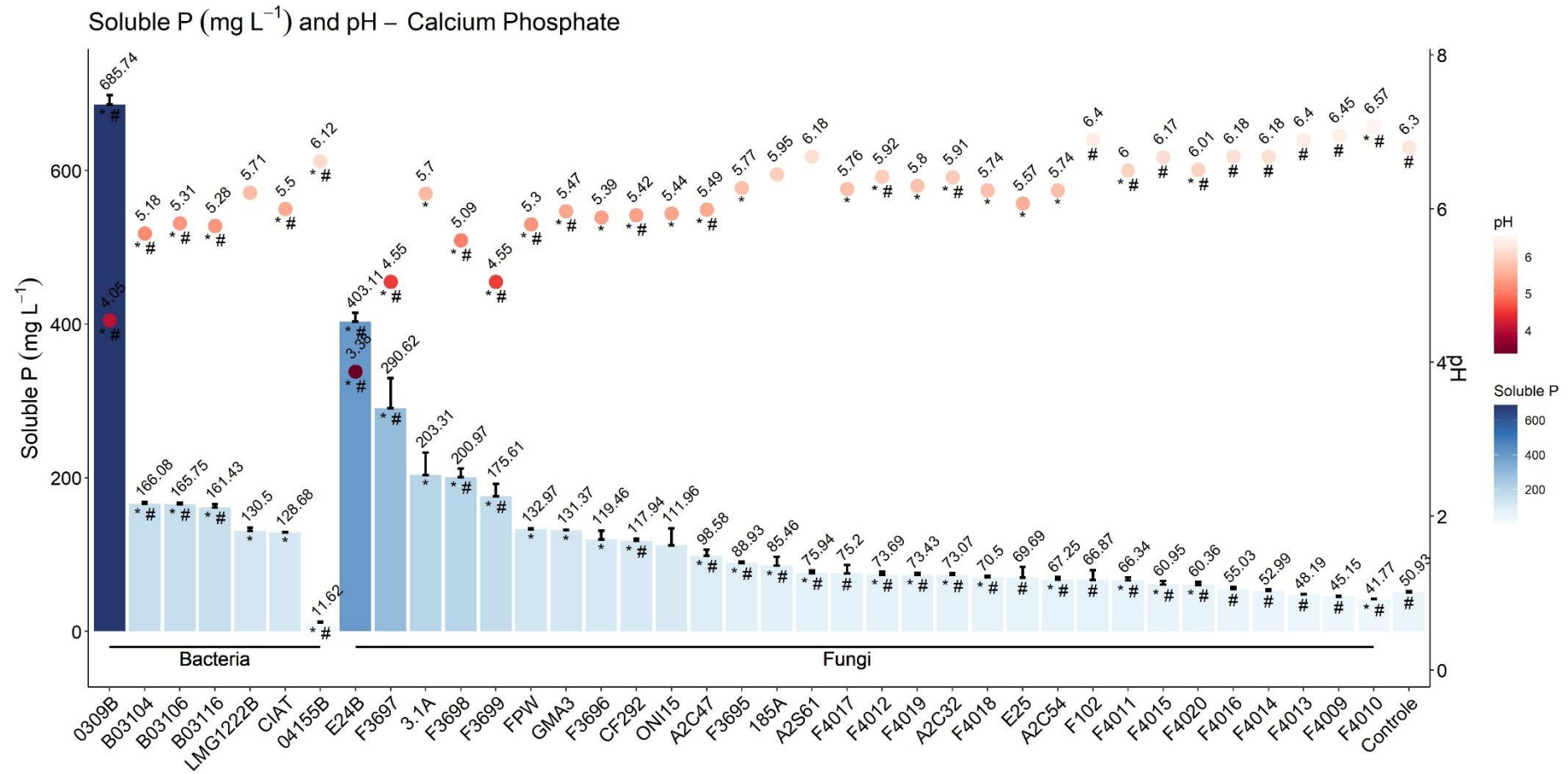
Figure 2: Iron phosphate solubilization and pH by bacterial strains and fungal isolates on NBRIP medium. Vertical bars indicates the standard error from each treatment. Means were compared by contrast analysis (P < 0.05) with the control without inoculation (*) and with the treatment inoculated with the *Burkholderia cepacia* LMG 1222 (#).
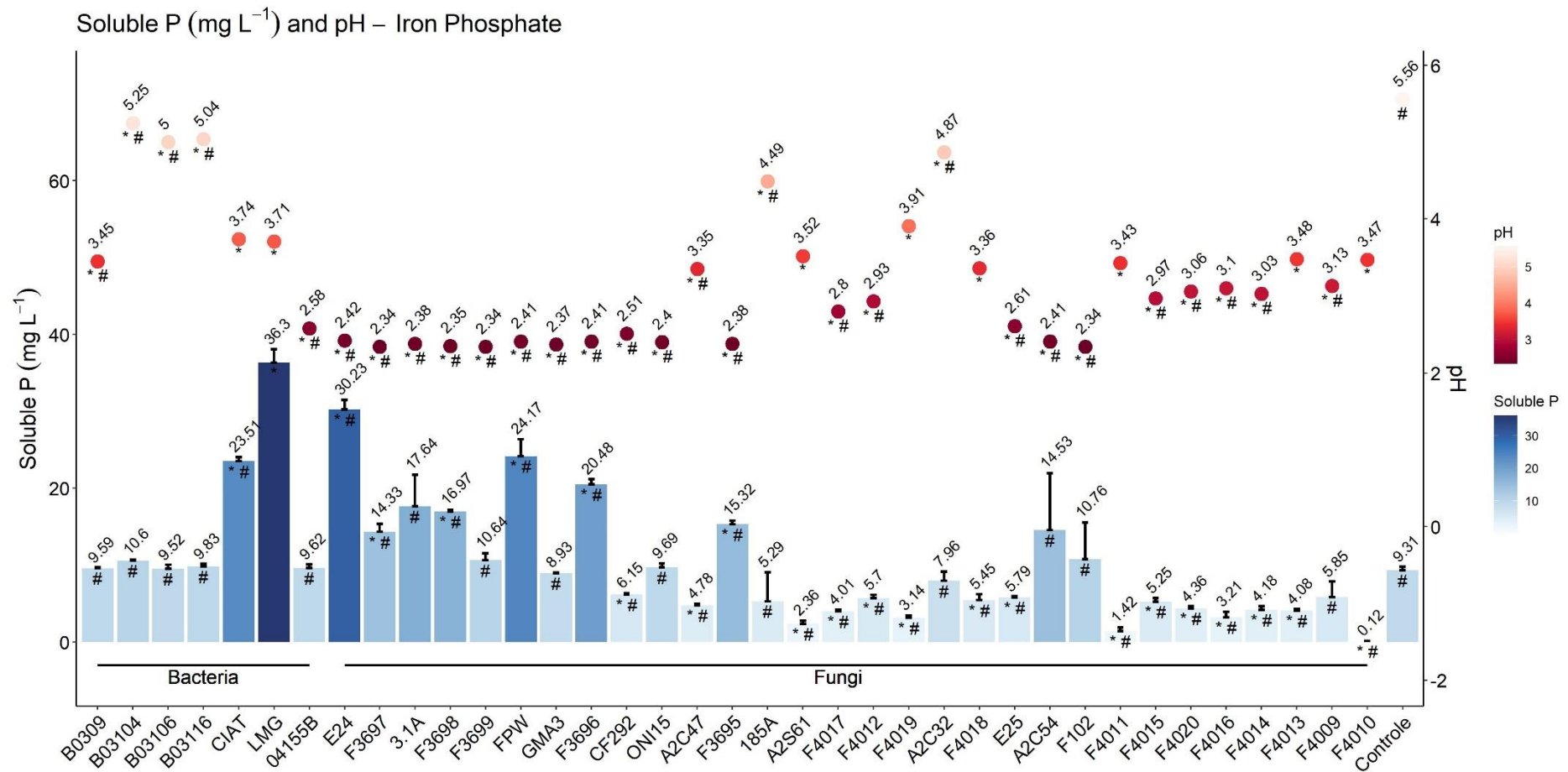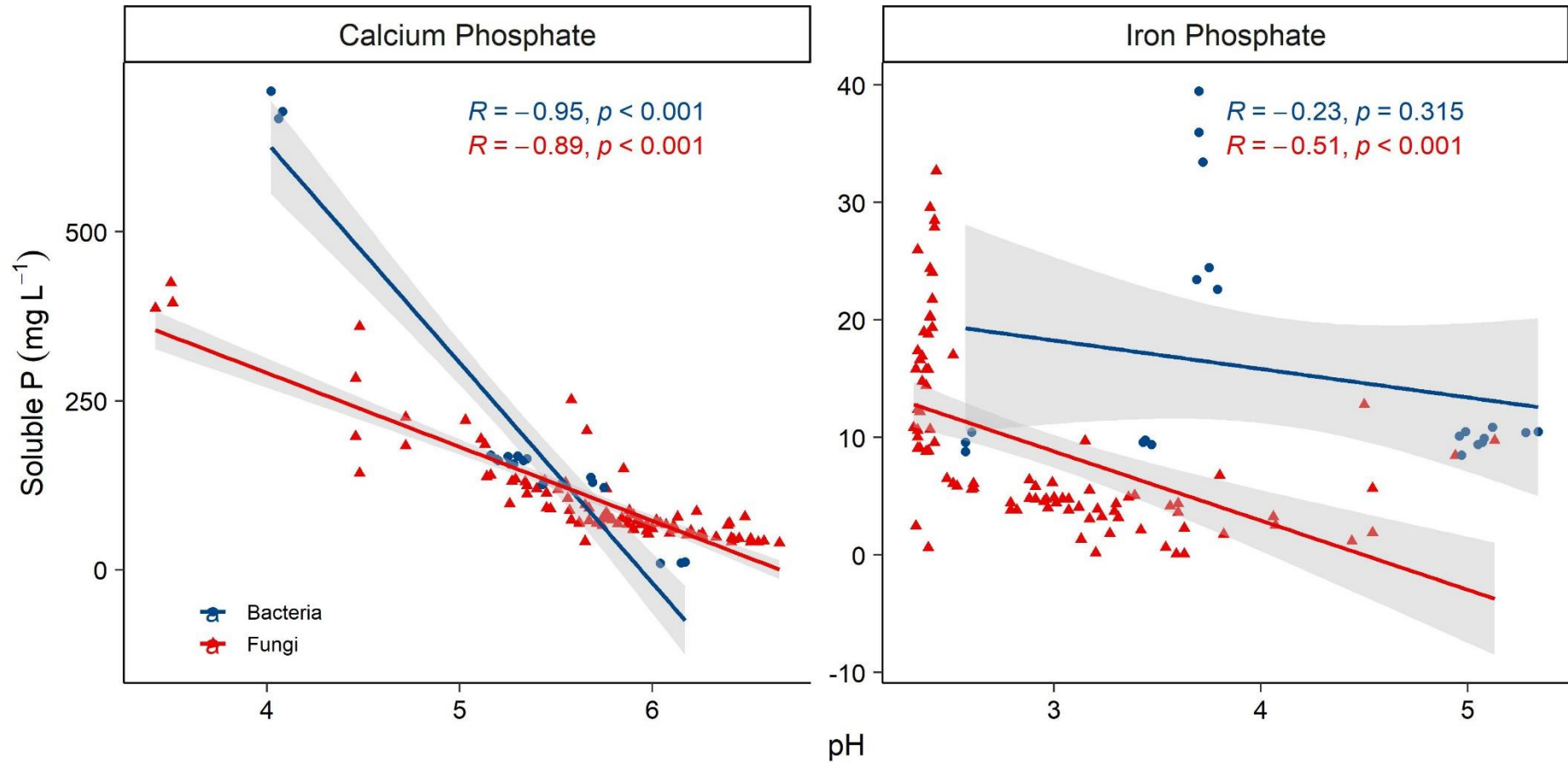
Figure 3: Pearson's correlation between soluble P and the pH of an *in vitro* phosphate solubilization experiment with the bacterial strains and fungal isolates.
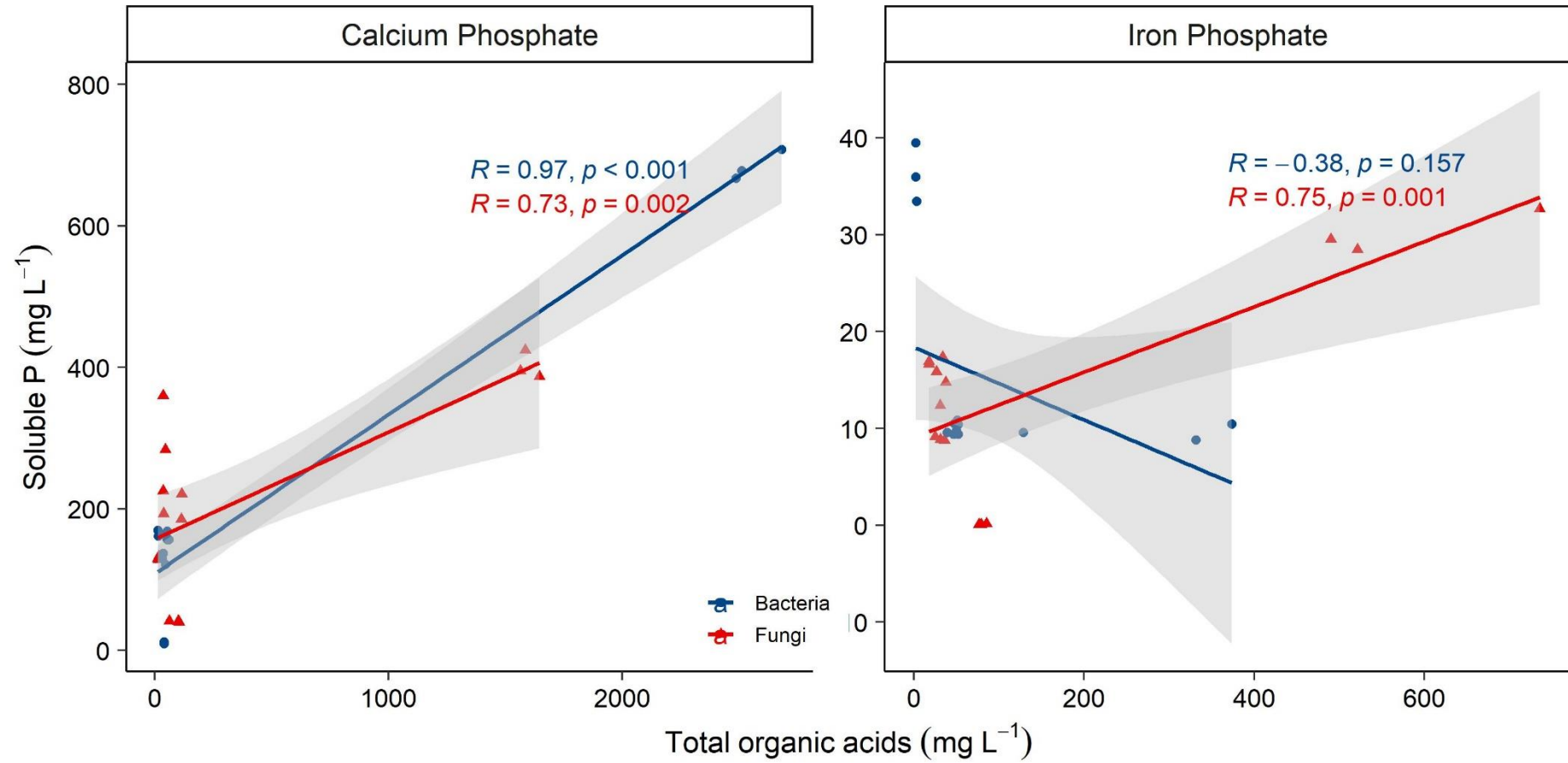
Figure 4: Pearson's correlation between soluble P and the total amount of organic acids of an *in vitro* phosphate solubilization experiment with the bacterial strains and fungal isolates.

Organic acid	UFLA 03-09	UFLA 03-104	UFLA 03-116	UFLA 04-155	LMG 1222
			Genes		
Gluconic acid	*gcd* *gdh* A *pqq* B,C,D,E			*gdh*	*pqq* B,C,D,E
Lactic acid	*lld* D			*lld* D	*ldh* A *lldD*
Acetic acid	*alkH*	*aldHT*	*ald* A		*ald* A
Citric acid	*ace* E *glt* A *ace* F	*pdh* A *pdh* B *glt* A *pdh* C	*pdh* A *pdh* B *glt* A *pdh* C	*pdh* B *glt* A	*pdh* A *pdh* B *glt* A
Oxalic acid					
Malic acid	*fum* C *mdh*	*fum* C *mdh*	*fum* C *mdh*	*fum* C *mdh*	*fum* C *mdh*

Figure 5: Genes related to the main organic acids found in phosphate solubilizing strains UFLA 03-09 (*Acinetobacter pittii*); UFLA 03-104 (*Brevibacillus centrosporum*); UFLA 03-116 (*Paenibacillus chondroitinus*); UFLA 04-155 (*Paraburkholderia fungorum*); LMG 1222 (*Brukholderia fungorum*). Light blue box means presence of the gene, while yellow boxes mean absence of the gene. The name of the gene refers to the entry found in NCBI and KEGG databases.

Supplementary material

Table S1: Genes and enzymes related to the production of the organic acids acetic, citric, gluconic, lactic, malic, and oxalic in the phosphate solubilizing bacterial strains UFLA 03-09 = *Acinetobacter pittii*; UFLA 03-104 = *Brevibacillus centrosporum*; UFLA 03-116 = *Paenibacillus chondroitinus*; UFLA 04-155 = *Paraburkholderia fungorum*; LMG 1222 = *Brukholderia cepacia*

Strain	Organic acid	Gene ID	Enzyme	Enzyme code	% Cover/ID	Strain
UFLA 03-09	Gluconic Acid	gdhA	Quinoprotein glucose dehydrogenase A	EC:1.1.99.35	100	99.88
		gcd	Quinoprotein glucose dehydrogenase	EC:1.1.5.2	99	60.05
		pqqC	Pyrroloquinoline-quinone synthase	EC:1.3.3.11	100	99.6
		pqqE	PqqA peptide cyclase	EC:1.21.98.4	100	99.22
		pqqD	PqqA binding protein	-	100	97.87
		pqqB	Coenzyme PQQ synthesis protein B	-	100	98.02
	Latic acid	lldD	L-lactate dehydrogenase	EC 1.1.2.3	99	98.95
	Acetic acid	alkH	Aldehyde dehydrogenase	EC:1.2.1.3	91	39.78
	Citric acid	aceE	Pyruvate dehydrogenase E1 component	EC:1.2.4.1	96	60.66
		gltA	Citrate synthase	EC:2.3.3.16	100	97.41
		aceF	Dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex	EC:2.3.1.12	91	54.72
	Oxalic acid	x				
	Malic acid	fumC	Fumarate hydratase class II	EC:4.2.1.2	99	75.05
		mdh	Malate dehydrogenase	EC:1.1.1.37	100	99.09
UFLA 03-104	Gluconic Acid	x				
	Latic acid	x				
	Acetic acid	aldHT	Aldehyde dehydrogenase, thermostable	EC:1.2.1.5	99	48.15

	Citric acid	pdhB	Pyruvate dehydrogenase E1 component subunit beta	EC:1.2.4.1	98	49.21
		pdhA	Pyruvate dehydrogenase E1 component subunit alpha	EC:1.2.4.1	95	65.61
		citZ	Citrate synthase 2	EC:2.3.3.16	100	66.58
		pdhC	Dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex	EC:2.3.1.12	99	53.21
	Oxalic acid	x				
	Malic acid	fumC	Fumarate hydratase class II	EC:4.2.1.2	100	79.00
		mdh	Malate dehydrogenase	EC:1.1.1.37	100	77.24
UFLA 03-116	Gluconic Acid	x				
	Latic acid	x				
	Acetic acid	aldA	Aldehyde dehydrogenase	EC:1.2.1.3	99	80.16
	Citric acid	pdhB	Pyruvate dehydrogenase E1 component subunit beta	EC:1.2.4.1	96	60.66
		pdhA	Pyruvate dehydrogenase E1 component subunit alpha	EC:1.2.4.1	99	61.17
		gltA	Citrate synthase 2	EC:2.3.3.16	100	64.96
	Oxalic acid	pdhC	Dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex	EC:2.3.1.12	99	52.28
	Malic acid	x				
		fumC	Fumarate hydratase class II	EC:4.2.1.2	99	70.65
		mdh	Malate dehydrogenase	EC:1.1.1.37	99	72.03
LMG 1222	Gluconic Acid	x				

		pqqC	Pyrroloquinoline-quinone synthase	EC:1.3.3.11	100	99.6
		pqqE	PqqA peptide cyclase	EC:1.21.98.4	97	59.57
		pqqD	PqqA binding protein	-	86	50.62
		pqqB	Coenzyme PQQ synthesis protein B	-	98	61.51
		x	x	x	x	x
	Latic acid	ldhA	D-lactate dehydrogenase	-	96	49.69
		lldD	L-lactate dehydrogenase	EC:1.1.-.-	97	40.06
	Acetic acid	aldA	Aldehyde dehydrogenase	EC:1.2.1.3	96	73.17
	Citric acid	pdhA	Pyruvate dehydrogenase E1 component	EC:1.2.4.1	100	78.29
		gltA	Citrate synthase	EC:2.3.3.16	99	70.70
		pdhB	Dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex	EC:2.3.1.12	100	70.34
	Oxalic acid					
	Malic acid	fumC	Fumarate hydratase class II	EC:4.2.1.2	98	76.03
		mdh	Malate dehydrogenase	EC:1.1.1.37	100	99.70
UFLA 04-155	Gluconic Acid	gdh	Glucose 1-dehydrogenase	EC:1.1.1.47	96	44.57
	Latic acid	lldD	L-lactate dehydrogenase	-	99	40.37
	Acetic acid	x	x			
	Citric acid	pdhB	Dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex	EC:2.3.1.12	100	71.17
		gltA	Citrate synthase	EC:2.3.3.16	73	72.01
	Oxalic acid	x	x			
	Malic acid	fumC	Fumarate hydratase class II	EC:4.2.1.2	98	77.29
		mdh	Malate dehydrogenase	EC:1.1.1.37	100	98.78

CONSIDERAÇÕES FINAIS

O fósforo é um importante nutriente para que todos os organismos vivos completem seu ciclo. Encontrar alternativas ao alto uso de fertilizantes industrializados solúveis será essencial em um futuro próximo, principalmente considerando os fertilizantes fosfatados, em que a maior parte do fertilizante rapidamente pode ser tornar indisponível para as plantas em solos mais intemperizados, como em boa parte dos solos brasileiros. Embora substituir completamente fertilizantes fosfatados solúveis seja uma tarefa complexa, fontes alternativas pouco solúveis podem ser uma solução para fornecer P eficientemente às plantas. Neste sentido, a associação com microrganismos solubilizadores de fosfato seria imprescindível para potencializar o fornecimento do P para as plantas. Entretanto, apesar de alguns mecanismos de solubilização de fosfatos já serem consolidados, há, ainda, lacunas no entendimento da ativação e desativação desses mecanismos e a interação entre eles e outros processos biológicos dos microrganismos do solo. Neste trabalho, o objetivo geral foi de avaliar a solubilização de fosfatos por microrganismos, juntamente com a identificação dos mecanismos de solubilização. Verificamos que as bactérias com potencial de solubilização de fosfato *in vitro* foram capazes de promover a solubilização via o mecanismo de liberação de ácidos orgânicos de baixo peso molecular. Além disso, foram capazes de promover o crescimento e nutrição de plantas de milho usando fosfato de rocha como fonte de P. Em seguida, dentro das condições do trabalho, uma estirpe de bactéria foi mais eficiente que diversos isolados de fungos, contrariando os registros de literatura. Além disso, a solubilização de fosfato de cálcio e ferro de bactérias e fungos estavam ligados a liberação de ácidos orgânicos e sideróforos. A análise genômica das estirpes bacterianas identificou as espécies e identificou diversos genes que codificam as enzimas relacionadas à síntese dos ácidos orgânicos. Estes resultados contribuem para avançar no conhecimento do processo de solubilização de fosfatos por microrganismos do solo.