GUSTAVO ANDRÉS IGLESIAS BARRERA

PROMOTION OF MICROBIAL PHOSPHATE SOLUBILIZATION BY CLAY MINERALS

Thesis submitted to the Agricultural Microbiology Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Doctor Scientiae*.

Adviser: Maurício Dutra Costa

Co-advisers: Gilberto de Oliveira Mendes
Wendel Batista da Silveira

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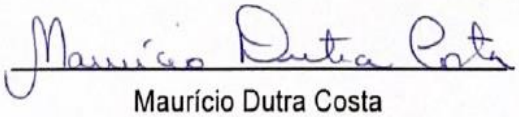

Maurício Dutra Costa

Adviser

To my family, colleagues, and friends

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“Gracias a la vida que me ha dado tanto. Me ha dado el sonido y el abecedario, con él, las palabras que pienso y declaro.”

Violeta Parra

ABSTRACT

IGLESIAS BARRERA, Gustavo Andrés, M.Sc., Universidade Federal de Viçosa, July, 2024. **PROMOTION OF MICROBIAL PHOSPHATE SOLUBILIZATION BY CLAY MINERALS**. Adviser: Maurício Dutra Costa. Co-advisers: Gilberto de Oliveira Mendes and Wendel Batista da Silveira.

A number of physical and chemical interactions between clays and microorganisms have been reported with strong influence on microbial physiology and ecology. However, clay influences on phosphate-solubilizing microorganisms (PSM) remains unknown. PSM are capable of converting low solubility phosphates into soluble orthophosphate that can be taken up by plants. PSM are commonly present in the soil, particularly in the rhizosphere where they maintain associative symbiosis with the host plant. This study aimed at investigating the influence of soil clays (kaolinite, gibbsite, goethite, and hematite) on phosphate solubilization by the fungus *Aspergillus niger*. In the first chapter, a literature review is presented addressing the potential use of PSM in agriculture. The interaction between clays and microorganisms is reported and the strategies to enhance microbial phosphate solubilization are described. In the second chapter, an investigation on the influence of kaolinite, gibbsite, goethite, and hematite, on organic acid production and phosphate solubilization by the fungal isolate *A. niger* FS1. The experiments were conducted in modified NBRIP medium containing 3 g L^{-1} of Araxá rock phosphate (RP) supplemented with clays at 250, 500, 1000, 1500, 2000, 2500, and 3000 g L^{-1}. The media were inoculated with 10^6 fungal conidia and incubated for 7 days at 28°C. All clays promoted increases in Araxá RP solubilization. The addition of gibbsite and kaolinite to the culture media led to the highest values of soluble P recorded (453.68 and 407.61 mg L^{-1}, respectively). Gibbsite increased oxalic acid production by the fungus 8.36 times that of the control treatment. Gibbsite and kaolinite also promoted higher yields of citric acid, with 15.69 and 8.93 mmol L^{-1}, respectively. In the third chapter, the hypothetical mechanisms of promotion of phosphate solubilization by clays were verified. Clays were added directly to the culture media as described above or placed in plastic capsules to prevent direct contact of the particles with the fungus. The effects of increasing concentrations of Fe-EDTA, $AlCl_3$, and silicic acid on Araxá RP solubilization and organic acid production were also evaluated. The encapsulation of clay particles did not decrease RP solubilization, indicating that the direct contact of the clays with the fungal mycelium is not required to induce RP solubilization. Significant increases in soluble P (3.89 times that of the control treatment) and oxalic acid were observed with increasing concentrations of $AlCl_3$. Fe-EDTA and silicic acid increased phosphate solubilization by only 29 and 11%, respectively, with no significant increases in oxalic and citric acid production. In the fourth chapter, we investigated the effect of kaolinite,

gibbsite, goethite, and hematite on the solubilization of Araxá, Argélia, Bayovar, Catalão, Marrocos, and Patos de Minas RPs and aluminum, calcium, and iron phosphate. For this, modified NBRIP medium was supplemented with 3 g L^{-1} of each P source above. The media were inoculated with 10^6 fungal conidia and incubated for 7 days at 28°C. Gibbsite was the most efficient clay at promoting the solubilization of all RPs tested, reaching 100% of solubilization for the majority. Kaolinite was particularly effective at the solubilization of iron phosphate, reaching 80.9% of solubilization. Only goethite was capable of enhancing aluminum phosphate solubilization (22.72%). In the fifth chapter, a brief review is presented discussing the question: Are there really microorganisms specialized in phosphate solubilization or is this process merely a side-effect of other microbial metabolic activities? All the findings presented here offer new possibilities for developing innovative strategies to enhance RP solubilization using soil clays. Our results shed light on the interactions between soil clays and fungal hyphae that may operate in the soil to increase P availability to plants. They also improve our understanding on P dynamics in the soil involving PSM and clay minerals.

Keywords: Soil clays, phosphate solubilization, organic acids, kaolinite, gibbsite, goethite, hematite, phosphate solubilizing microorganisms.

RESUMO

IGLESIAS BARRERA, Gustavo Andrés, M.Sc., Universidade Federal de Viçosa, Setembro, 2024. **PROMOÇÃO DA SOLUBILIZAÇÃO MICROBIANA DE FOSFATOS POR ARGILAS MINERAIS**. Orientador: Maurício Dutra Costa. Co-Orientadores: Gilberto de Oliveira Mendes e Wendel Batista da Silveira.

Uma série de interações físicas e químicas entre argilas e microrganismos têm sido relatadas, apresentando forte influência na fisiologia e ecologia microbiana. No entanto, as influências das argilas sobre os microrganismos solubilizadores de fosfato (MSF) permanecem desconhecidas. Os MSF são capazes de converter fosfatos de baixa solubilidade em ortofosfato solúvel, que pode ser absorvido pelas plantas. Esses microrganismos estão comumente presentes no solo, particularmente na rizosfera, onde mantêm uma simbiose associativa com a planta hospedeira. Este estudo teve como objetivo investigar a influência das argilas do solo (caulinita, gibbsita, goethita e hematita) na solubilização de fosfato pelo fungo *Aspergillus niger*. No primeiro capítulo, é apresentada uma revisão da literatura abordando o potencial uso dos MSF na agricultura. A interação entre argilas e microrganismos é discutida, e as estratégias para aumentar a solubilização microbiana de fosfato são descritas. No segundo capítulo, é realizada uma investigação sobre a influência da caulinita, gibbsita, goethita e hematita na produção de ácidos orgânicos e na solubilização de fosfato pelo isolado fúngico *A. niger* FS1. Os experimentos foram conduzidos em meio NBRIP modificado, contendo 3 g L^{-1} de fosfato natural (FR) de Araxá, suplementados com argilas nas concentrações de 250, 500, 1.000, 1.500, 2.000, 2.500 e 3.000 g L^{-1}. Os meios foram inoculados com 10^6 conídios fúngicos e incubados por 7 dias a 28°C. Todas as argilas promoveram aumentos na solubilização do FR de Araxá. A adição de gibbsita e caulinita ao meio de cultura proporcionou os maiores valores de P solúvel registrados (453,68 e 407,61 mg $L^{-1,}$ respectivamente). A gibbsita aumentou a produção de ácido oxálico pelo fungo em 8,36 vezes em relação ao tratamento controle. Além disso, a gibbsita e a caulinita também promoveram maiores rendimentos de ácido cítrico, com 15,69 e 8,93 mmol L^{-1}, respectivamente. No terceiro capítulo, foram verificados os mecanismos hipotéticos de promoção da solubilização de fosfato pelas argilas. As argilas foram adicionadas diretamente ao meio de cultura, conforme descrito acima, ou colocadas em cápsulas plásticas para evitar o contato direto das partículas com o fungo. Também foram avaliados os efeitos do aumento das concentrações de Fe-EDTA, $AlCl_3$ e ácido silícico na solubilização do FR de Araxá e na produção de ácidos orgânicos. O encapsulamento das partículas de argila não reduziu a solubilização do FR, indicando que o contato direto das argilas com o micélio fúngico não é necessário para induzir a solubilização do FR.

Observou-se aumento significativo no fósforo solúvel (3,89 vezes em relação ao tratamento controle) e na produção de ácido oxálico com o incremento das concentrações de $AlCl_3$. O Fe-EDTA e o ácido silícico promoveram aumento na solubilização do fosfato de apenas 29% e 11%, respectivamente, sem que houvesse aumentos significativos na produção de ácido oxálico e ácido cítrico. No quarto capítulo, investigamos o efeito da caulinita, gibbsita, goethita e hematita na solubilização dos FR Araxá, Argélia, Bayovar, Catalão, Marrocos e Patos de Minas, além de fosfatos sintéticos de alumínio, cálcio e ferro. Para isso, o meio NBRIP modificado foi suplementado com 3 g L^{-1} de cada fonte de fósforo mencionada. Os meios foram inoculados com 10^6 conídios fúngicos e incubados por 7 dias a 28°C. A gibbsita foi a argila mais eficiente em promover a solubilização de todos os FRs testados, alcançando 100% de solubilização na maioria dos casos. A caulinita demonstrou ser particularmente eficaz na solubilização do fosfato de ferro, atingindo 80,9% de solubilização. Apenas a goethita foi capaz de melhorar a solubilização do fosfato de alumínio (22,72%). No quinto capítulo, é apresentada uma breve revisão que discute a seguinte questão: existem realmente microrganismos especializados na solubilização de fosfato ou esse processo é apenas um efeito colateral de outras atividades metabólicas microbianas? Todas as descobertas aqui apresentadas oferecem novas possibilidades para o desenvolvimento de estratégias inovadoras que visem melhorar a solubilização dos FRs utilizando argilas do solo. Nossos resultados esclarecem as interações entre as argilas do solo e as hifas fúngicas, que podem atuar no solo para aumentar a disponibilidade de fósforo para as plantas. Além disso, eles aprimoram nossa compreensão sobre a dinâmica do fósforo no solo, envolvendo microorganismos solubilizadores de fósforo (PSM) e argilominerais.

Palavras-chave: Argilas, solubilização de fosfato, ácidos orgânicos, caulinita, gibbsita, goethita, hematita, microrganismos solubilizadores de fosfato.

SUMMARY

INTRODUCTION

In 2024, the world population exceeded 8 billion people, creating a significant demand for agricultural and forestry resources (Galanakis, 2024). To sustain agroforestry production, fertilizer applications are essential to fulfill the nutritional requirements of crops. In Brazil, the effectiveness of phosphate fertilizers in tropical soils is generally low due to phosphorus (P) fixation in the soil (Corrêa et al., 2011; Melo et al., 2015; Alovisi et al., 2020). This situation is further complicated by Brazil's reliance on imported phosphate fertilizers, leaving it vulnerable to international price fluctuations (Argenta et al., 2023). Recent years have seen high price volatility due to international conflicts in fertilizer-producing countries and post-pandemic effects (Hebebrand & Debucquet, 2023; Rice et al., 2023). Based on the current context of a growing global population, the United Nations has defined 17 objectives for sustainable development (United Nations, 2015). Goal number two has been defined as “zero hunger and sustainable agriculture”, emphasizing the need to increase food production worldwide while promoting sustainable agricultural practices. One of the challenges in agricultural production is the significant use of fertilizers to meet the crop´s requirements for nutrients. In Brazil, this challenge is exacerbated by the tendency of Brazilian soils to fix high amounts of P, reducing the effectiveness of phosphate fertilizers (Roy et al., 2016). Although Brazil possesses mineral P sources, these are for the most part underutilized in agriculture due to a perceived low agricultural suitability resulting primarily from their low reactivity and P content (Léon et al., 1986).

High quality P minerals are non-renewable resources on a human time scale. Early projections estimated P reserves to be around 16 billion tons, with potential depletion based on current consumption rates within 100 years (Cordell & White, 2011). However, the latest data, from 2024, indicate the existence of reserves containing approximately 74 billion tons (U.S. Geological Survey, 2024). Despite such a favorable scenario, the focus on developing biotechnological alternatives to sustainably meet P demand for agricultural crops remains crucial. One such alternative is the use of phosphate-solubilizing microorganisms (PSM) that can convert low solubility phosphates into soluble forms that can be taken up by plants (Rawat et al., 2020; Sarmah & Sarma, 2022; Liu et al., 2023). A diversity of microorganisms have been identified with this ability, including those that can solubilize low-reactivity RPs and reverse P fixation in the soil clay fraction (Mendes et al., 2013; Nascimento et al., 2021).

PSM are commonly bacteria and fungi present in the soil, particularly in the plant rhizosphere, as associative symbionts (Sarmah & Sarma, 2022). Among the fungi, the isolate *A. niger* FS1 has shown great potential for phosphate solubilization, but its

application in the manufacture of soluble P fertilizers still requires the improvement of the cultivation conditions that favor the production of organic acids responsible for the release of soluble P from poorly soluble mineral sources. Strategies to boost fungal phosphate solubilization can also be achieved through genetic techniques (Gonzalez et al., 2016).

Generally, PSM can be abundantly found in the rhizosphere soil where they thrive on carbon compounds exuded by the plant roots (Steinauer et al., 2016). The rhizosphere environment is complex and microbes living in this region are subjected to chemical, physical and biological conditions that differ radically from the laboratory culture medium. In the soil, microbial growth is also influenced by minerals, especially those that are present in the soil clay fraction. Due to their small particle size and large charge densities, clays can adsorb inorganic and organic ions, changing their availability to microbes (Cuadros, 2017). Clays can also protect bacteria from predation and change the patterns of hyphal growth in fungi (England et al., 1993; Cuadros, 2017). When added to agitated culture media, these particles are expected to cause different levels of mechanical injuries in fungal hyphae, leading to changes in fungal metabolism (Kotzybik et al., 2016). A common response to stress in fungi include the production of organic acids, such as oxalic acid, which has been shown to be one of the most effective organic acids to promote RP solubilization (Mendes et al., 2020).

Hence, the objective of this study was to investigate the effects of clay minerals (gibbsite, kaolinite, goethite, and hematite) on oxalic acid production and RP solubilization by *A. niger* FS1. For this, the fungal isolate was cultivated in media supplemented with Araxá RP and different clay concentrations. After cultivation, oxalic acid and soluble P in the supernatant were evaluated. The mechanisms involved in the promotion of phosphate solubilization by clays were also investigated. Finally, the ability of clays to promote the solubilization of different RPs and synthetic phosphates was tested. A minireview, critically addressing the concept of a “phosphate solubilizing microorganism”, was included.

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CHAPTER I

Literature review

LITERATURE REVIEW

Microbial phosphate solubilization: mechanisms and biotechnological strategies to address the fertilizer global crisis

Global fertilizer crisis

One of the major problems affecting agriculture worldwide is the rise in fertilizer prices (Snapp et al., 2023). Since 2020, cost with soil fertilization have increased by over 200 %. and phosphate fertilizers have experienced the highest price increments. The highest recorded prices were observed in 2023, surpassing those recorded because of the 2008 economic crisis (World Bank, 2024). The price increase intensified from 2020 onwards as a consequence of the global pandemic on the world's economy and significant increases in the cost of raw materials for the fertilizer industry, such as sulfuric acid, ammonia, and natural gas (Illinois et al., 2021).

China is the largest consumer of fertilizers in the world, accounting for approximately 25 % of the global consumption. Meanwhile, Brazil ranks as the fourth largest consumer of fertilizers (Guo et al., 2022). Unlike Brazil, China is a main fertilizer producer, contributing with 15% to global production (Good, 2022). As of June 2022, China has suspended the export of fertilizers to meet its significant domestic demand, thereby exacerbating issues in the global fertilizer market (Baffes & Koh, 2022). Brazil is heavily dependent on imported fertilizers, with the majority coming from Russia (22%) and Belarus (7%). These countries, however, experience considerable political instability, which is also reflected in the prices of fertilizers. Brazil imports over 87 % of phosphate fertilizers and reached record imports in January 2024 with 2.77 million tons. The primary suppliers are Morocco and Russia (World Bank, 2024).

Phosphorus fixation in Brazilian soils

There is a worldwide concern about the massive consumption of phosphate fertilizers in Brazil, particularly in soils with a high capacity for P fixation (Melo et al., 2015; Alovisi et al., 2020). The strong adsorption of orthophosphate to clay minerals in tropical soils leads to a low efficiency of P fertilizers, since most of the P applied becomes quickly unavailable to plants (Roy et al., 2016; Benicio, 2022). The concern on the use of phosphate fertilizers is increased by the fact that nonrenewable high-quality minerals are the preferred sources for phosphate fertilizer production (Cordell et al., 2009). P reservoirs in the world, however, have not been accurately measured, generating much debate on their life span (Reijnders, 2014).

The cycling dynamics of P applied to Brazilian soils as phosphate fertilizers has been extensively researched (Corrêa et al., 2011; Melo et al., 2015; Alovisi et al., 2020; Alves et al., 2022; Benício, 2022). Most of the country´s soils show a high P-fixing ability that can be as high as 15 kg ha^{-1} $year^{-1}$ (Roy et al., 2016). P fixation is determined by several factors, such as the degree of soil weathering and the content of iron and aluminum oxides, minerals with high P adsorption capacity (Novais et al., 2007; Lepsch, 2011). Other important features that decrease P availability in soils include the cation exchange capacity (CEC) and pH (Prado, 2003). Soils with a high CEC may indirectly lead to P precipitation due to their elevated contents of cations, such as iron, calcium, and aluminum (Lepsch, 2011). The low pH increases aluminum solubility. Aluminum can then react with P and form low-reactivity minerals (Scanlan et al., 2017). The electric charges on the surface of clay particles contribute to P adsorption. However, the intensity of P fixation will depend on the complex interaction between several chemical and physical soil factors (Fontes & Weed, 1996; Fan et al., 2021).

Phosphate solubilizing microorganism (PSM)

Phosphate-solubilizing microorganisms (PSM) are defined as those capable of transforming low reactivity P sources into readily available P forms usable by plants (Sharma et al., 2013; Soumare et al., 2020; Tariq & Ahmed, 2022; Li et al., 2023). There is a great diversity of PSM, *i.e.*, bacteria, actinobacteria, cyanobacteria, filamentous fungi, and yeasts (YandigerI et al., 2011; Mendes et al., 2013; Boubekri et al., 2021; Biswas et al., 2022). PSMs are primarily found in the rhizosphere, which is the soil region influenced by exudates from plant roots. This region is characterized by high microbial activity, as microorganisms use root exudates as a carbon source to generate energy to meet their metabolic requirements (Gupta & Sharma, 2020). Studies have shown that the release of malic acid by plants leads to an increase in PSM in the soil and enhanced microbial P solubilization (Barrera, 2020).

The fungal species *A. niger* is highly efficient at RP solubilization *in vitro* that can be attributed to its high production of organic acids (Yang et al., 2017). The genus *Aspergillus* is characterized by its ability to synthesize organic acids, such as citric and oxalic acid, which are important for the solubilization of RPs. *A. niger* has also been shown to have the ability to reverse P fixation processes in soils (Nascimento et al., 2021). This fungus has characteristics that make it suitable for use in research and industry, including rapid growth, adaptability to different culture media, and tolerance to adverse conditions, such as extremely acidic pH or high osmotic concentrations (Suliasih & Widawati, 2021).

Mechanisms of microbial phosphates solubilization

PSM are able to conduct P solubilization and mineralization processes. For that, they use several solubilization mechanisms, depending on how P is found in the soil. PSM have the ability to transform low-reactivity P into forms that are unavailable to plants (Maharana et al., 2021). The main mechanism by which microorganisms solubilizes inorganic P forms, as described by Prabhu et al. (2019), involves the production of organic acids. These compounds have the ability to chelate cations linked to P, thus making P available (Kumari et al., 2008; Kumari et al., 2008).

Organic acids can be produced by various microorganisms, such as filamentous fungi, yeasts, and bacteria (Mendes et al., 2013b; Hesham et al., 2020; Wang et al., 2020). These compounds are produced inside microbial cells as a result of central metabolic pathways (Yang et al., 2017). Several organic acids produced by PSM have been reported to have the ability to solubilize phosphates, such as citric, oxalic, malic, gluconic, aspartic, fumaric, acetic, lactic, and tartaric acids (Mendes et al., 2013; Alori et al., 2017; Zúñiga-Silgado et al., 2020; Kaur et al., 2021). In fungi, it has been reported that the most relevant organic acids for P solubilization are citric and oxalic acids, with the latter being particularly efficient (Mendes et al., 2020). For P-solubilizing bacteria, the significance of gluconic acid, citric, and oxalic acids has been reported (Stella & Halimi, 2015). Organic acids provoke decreases in medium pH and thus lead to P solubilization. Phosphate ions also can be released by replacing hydrogen ions (H^+) with cations (Prabhu et al., 2019).

Another phosphate solubilizing mechanism used by PSM involves direct H^+ extrusion, without the production of organic acids. H^+ is the result of metabolic processes carried out by PSM, such as the NH_4^+ assimilation, NH_4^+ and H_2S oxidation, or aerobic and anaerobic respiration (Krysenko & Wohlleben, 2022). The oxidation of NH_4^+ and H_2S lead to the production of H+ (Suliasih & Widawati, 2021). There are also microorganisms with the ability to synthesize inorganic acids, such as sulfuric, nitric, and carbonic acid. However, a lower efficiency at phosphate solubilization is observed when inorganic acids are used in comparison to oxalic acid (Mendes et al., 2020).

Siderophores are chelating substances with the ability to bind to metal cations (Cui et al., 2022). The production of siderophores is also an important mechanism for solubilizing insoluble phosphates, such as iron phosphate (Prado, 2003),

Currently, the production of exopolysaccharides (EPS) has also been reported as a mechanism for solubilizing P (Prabhu et al., 2019). The ability of EPS to bind to metals present in the soil has been observed. Studies show a positive relationship between the amount of EPS present in the soil and P solubilization (Yi et al., 2007).

The ability of PSM to solubilize phosphates varies among different taxonomic groups.

Fungi and actinobacteria stand out and are capable of solubilizing more than 100 mg L^{-1} of P from low reactivity RPs (Nascimento et al., 2021; Soumare et al., 2021). In general, for bacteria, rock phosphate solubilization levels are achieved, albeit lower but still significant compared to treatments without PSM inoculation (Oshoma et al., 2020; Afzal et al., 2022; Elhaissoufi et al., 2022). The solubilization capacity also depends on the P source, with low reactivity RPs being more difficult to solubilize. Additionally, P can be fixed in soils, making it less available for solubilization (Emami-Karvani & Chitsaz-Esfahani, 2021; Nascimento et al., 2021).

Factors involved in solubilization

One of the main factors affecting phosphate solubilization is the P source to be solubilized (Nahas, 1996; Amarasinghe et al., 2022). Research has focused mainly on the solubilization of calcium, aluminum, iron phosphates, and RPs (Xiao et al., 2008; Mendes et al., 2013; Soumare et al., 2021; Aliyat et al., 2022; Ríos-Ruiz et al., 2024). Tricalcium phosphates, in general, are the easiest to be solubilized (Liu et al., 2021; Sauka et al., 2021; Aliyat et al., 2022). Several studies reveal that PSM achieve greater solubilization with tricalcium phosphate as a P source (Posada et al., 2013; Spagnoletti et al., 2017; Flatian et al., 2021; Sauka et al., 2021). In general, calcium phosphates have greater stability as a function of increasing pH (Lindsay, 1979). Several authors report increases in tricalcium phosphate solubilization as a function of lower supernatant pH in experiments inoculated with PSM (Nahas, 1996; Sauka et al., 2021). Despite this characteristic, tricalcium phosphate solubilization under alkaline conditions has been reported (Prabhu et al., 2018). Under this condition, the presence of chelating compounds is also essential for the solubilization. A direct relationship between titratable acidity of the supernatants and solubilization has been observed (Nahas, 1996). For iron and aluminum phosphates, the relationship with pH is different. These P sources have greater stability at acidic pH (Lindsay, 1979). Even in experiments with PSM that can bring the pH to values below 3, no large amounts of Al and Fe phosphates can be solubilized (Wang et al., 2020). Interestingly, studies testing different microbial isolates in the solubilization of iron phosphate have shown greater solubilization by the isolates that led to a higher medium pH (Aliyat et al., 2022; Ríos-Ruiz et al., 2024). Organic acids have the ability to complex Fe and Al, but a larger amount of acid is required compared to calcium phosphate (Kumar et al., 2023). This is clearly observed in experiments where *A. niger* caused the highest titratable acidity with Fe phosphate as a P source, but the solubilization of this phosphate was lower than the solubilization of Ca phosphate, even though the titratable acidity in this treatment was lower (Mendes et al., 2013). An important factor for the solubilization of iron phosphate is the redox condition, where a reducing

environment favors the reduction of Fe^{3+} to Fe^{2+}, which forms soluble iron phosphates (Lindsay, 1979).

The solubilization of RPs is influenced by their geological formation (Léon et al., 1986). Phosphate rocks can be of igneous, sedimentary, or metamorphic origin, with the first two being the most commonly found in the world (Fayiga & Nwoke, 2016). RPs of igneous origin have lower reactivity (Léon et al., 1986). Due to a more organized crystalline structure and a greater occurrence of isomorphic substitutions where calcium ions can be replaced by other cations (Knubovets, 1993). RPs contain apatites [$Ca_{10}(PO_4)_6(F^-$ or OH^- or $Cl^-)$], with a high calcium phosphate content (Combes et al., 2016). Thus, in general, the solubilization of RPs by PSMs shown a positive correlation with a decrease in pH and the content of chelating substances (Nahas, 1996; Manzoor et al., 2016). Interestingly, for the solubilization of several inorganic phosphates, increases in solubilization have been observed when PSMs are under stress conditions (Nautiyal et al., 2000; Banerjee et al., 2010). This opens the possibility of studying forms of stress induction in microorganisms that may reflect in improving phosphate solubilization. Experiments with silica nanoparticles have shown the induction of oxidative stress in fungi and a response in metabolite synthesis (Kotzybik et al., 2016). This may be relevant for phosphate solubilization since a common response to several stress conditions in fungi is the production of organic acids (Goldberg et al., 2006). Thus, the evaluation of the effect of easily obtainable and economically viable particles can be an alternative for optimizing P solubilization. Particles that would meet these requirements could be soil clays, which are abundant in various environments.

Soil clays

Clays are mineral particles smaller than 2 um (Velde, 2013). These particles are formed through the weathering of primary or secondary minerals (Velde & Meunier, 2008). Clays represent 30 to 60% of the mass of clayey soils or between 5 and 15% of sandy soils (Lepsch, 2011). In Brazilian soils, the most commonly found clays are goethite, hematite, gibbsite, and kaolinite (Schaefer et al., 2008). Clays can be classified into two groups based on their composition: silicate clays and oxidic clays (Velde & Meunier, 2008; Lepsch, 2011). Silicate clays are those in which oxygen molecules are bonded to silica and aluminum. In the oxidic ones, the oxygen molecules are linked to iron and/or aluminum atoms.

Clay characteristics, such as their structure and composition, can have important effects on soil microorganisms (Amadou et al., 2022). The interactions between clays and microorganisms have been widely studied and classified into physical and chemical interactions (Burford et al., 2003; Cuadros, 2017; Fomina & Skorochod, 2020). There is extensive evidence in the literature of the ability of microorganisms to solubilize clays

(Friedrich et al., 1991; Kalinowski et al., 2000; Essington et al., 2005; Heckman et al., 2012). This allows for nutrient uptake from the elements that compose the clay particles or are adsorbed on them (Yang et al., 2023; Polák et al., 2018). Microorganisms dissolve clays through two main processes: acidolysis, when the microbe lowers the medium's pH, and complexolysis, through the synthesis of chelating substances (Fomina and Skorochod, 2020). In soil, obtaining iron from goethite and hematite is essential to sustain microbial growth and this is done through the production of siderophores (Kalinowski et al., 2000).

On the other hand, experiments have shown that the solubilization of gibbsite and kaolinite by fungi leads to increases in aluminum levels in the culture medium (Friedrich et al., 1991; Polák et al., 2018). This evidence is of great interest for P solubilization, as a common response of several organisms to protect themselves from aluminum stress is the increase in oxalic acid synthesis (Ahonen-Jonnarth et al., 2000; Jarosz-Wilkolazka & Gadd, 2003). In the case of kaolinite, its dissolution also results in the formation of silicic acid (Lindsay, 1979). This could also have an impact on the solubilization of metal-associated phosphates, due to the ability of this acid to complex metals (Exley et al., 2019). Clays also have the ability to adsorb pollutants and toxic elements (Alorabi et al., 2021; Mukhopadhyay et al., 2021). This could be particularly interesting in the solubilization of RPs since these rocks contain a wide variety of elements, including elements that are toxic to microorganisms such as fluorine (Mendes et al., 2013). However, there is currently no research that has tested the effect of clay particles on the microbial phosphate solubilization processes of phosphates.

On the other hand, clays present several physical interactions with microorganisms, which could directly or indirectly influence phosphate solubilization. One of the primary physical interactions involves the fungal ability to mechanically break down clay particles and aggregates. The hyphae can exert significant mechanical pressure, created by osmotic turgor of the fungal cells (Money, 2004; Fomina & Skorochod, 2020). Another interesting clay-fungus physical interaction is thigmotropism, where the growth of fungal hyphae responds to physical contact with particles (Bowen et al., 2007a). This mechanism is very relevant to guide fungal growth and allow fungi to explore the soil in search for nutrients (Bowen et al., 2007b; Fomina and Skorochod, 2020). Clays can also physically protect microorganisms from predators, increasing their survival in the soil (England et al., 1993). The presence of clays can also influence the formation of microbial structures such as biofilms, not only increasing their quantity but also enhancing their durability (Ma et al., 2017; Kim and Kwon, 2021). The effects of these particles, commonly found in the natural environment on P solubilization processes are still unknown. Therefore, it is important to analyze the impact of mineral clays on the production of organic acids and phosphate solubilization by PSMs.

Final considerations

The current context of great instability in the fertilizer markets has highlighted the urgent need for biotechnological alternatives that can address this situation in a sustainable manner, focusing on the preservation of natural resources. Microbial solubilization of phosphates emerges as a viable alternative to mitigate this dependence, but it is crucial to increase the efficiency of this process so that the use of phosphate-solubilizing microorganisms (PSM) may be viable, both in agriculture and in the fertilizer industry.

The utilization of particles linked to microorganisms, such as clays, seems to be a promising approach to enhance the ability of microbial solubilization. Nevertheless, it is crucial to carry out detailed studies that explore the influence of these particles on the phosphate solubilization processes and on the dynamics of the microorganisms engaged. Uncovering the fundamental mechanisms is essential to broaden the understanding of how these interactions can also take place at the ecosystem level, facilitating the establishment of effective interactions among microorganisms, clays, and plants.

Therefore, advancing our understanding on these processes can pave the way for the development of more effective and sustainable technologies that integrate biotechnological innovation with the conservation of natural resources. This, in turn, can contribute to a more resilient and productive agriculture.

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CHAPTER II

Clay minerals promote oxalic acid production and phosphate solubilization by *Aspergillus niger*

CLAY MINERALS PROMOTE OXALIC ACID PRODUCTION AND PHOSPHATE SOLUBILIZATION BY *Aspergillus niger*

ABSTRACT

Clays are common components of the soil, affecting the ecology and physiology of the soil microbiota. When added to agitated culture media, these particles are expected to cause different levels of mechanical injuries in fungal hyphae. A common response to stress in fungi include the production of organic acids, such as oxalic acid, which has been shown to be one of the most effective organic acids for RP solubilization. Thus, the objective of this study was to investigate the effects of soil clays on the biosynthesis of organic acids and the solubilization of Araxá rock phosphate (RP) by the fungus *A. niger* FS1. To achieve this, 50 mL of NBRIP medium was supplemented with gibbsite, hematite, kaolinite, and goethite at concentrations of 250, 500, 1000, 1500, 2000, 2500, and 3000 mg L^{-1}. The media were inoculated with 10^6 spores of *A. niger* FS1 and incubated for 7 days at 28°C. Medium supplementation with clays led to increases in organic acids and Araxá RP solubilization. The highest production of oxalic acid was observed in the treatment with gibbsite with a production of 44.33 mmol L^{-1}. Gibbsite and kaolinite also increased citric acid synthesis by the fungus. The highest release of soluble P was observed in the treatments with gibbsite and kaolinite, reaching 453.68 and 407.61 mg L^{-1} of soluble P, respectively. The production of oxalic acid increased by 736% compared to the control treatment without the addition of clay. The biomass of *A. niger* FS1 also increased with clay supplementation, with the highest accumulation observed in the treatments with gibbsite and kaolinite, at 24.4 g L^{-1} and 2.02 g L^{-1}, respectively. Supplementation with clays significantly reduced the sizes of fungal pellets. Gibbsite and kaolinite increased the efficiency of organic acid production and phosphate solubilization by *A. niger* FS1. This study provides insights into the potential use of clays for improving soluble P fertilizer production from low-reactivity RPs. To our knowledge this is the first report on the promotion of RP solubilization by soil clays.

KEYWORDS: Soil clays, organic acids, phosphate solubilizing microorganisms.

INTRODUCTION

Currently, one of the most limiting factors for agricultural and forestry production is the cost of fertilizers. Prices have sharply increased due to various international factors, including armed conflicts, increased demand from China to meet growing domestic production, and the indirect effects caused by the global pandemic on the fertilizer industry (Hebebrand & Debucquet, 2023; Rice et al., 2023). In Brazil, these effects have been evident, as over a third of the fertilizers imported by the country come from regions currently experiencing armed conflicts, such as Russia and Belarus. Russia contributes with 22% of the Brazilian imports, while Belarus contributes 6% (Homely, 2022; Argenta et al., 2023).

This negative scenario for world agriculture is even worse in Brazil where phosphate fertilizers are used in soils with a high phosphorus (P) fixation capacity (Melo et al., 2015; Alovisi et al., 2020; Pavinato et al., 2020). Leading to significant losses of the nutrient that cannot be used by crops. Thus, the development of technological processes that enable a more efficient utilization of P resources is fundamental.

An alternative to circumvent these problems is the use of phosphate-solubilizing microorganisms (PSM), which are capable of transforming less reactive phosphate forms into forms that are available to crops (Alori et al., 2017). Several microorganisms capable of carrying out this solubilization process have been described (Prabhu et al, 2019; Wan et al, 2020). These microorganisms have the ability to solubilize even brazilians RPs, which are considered to have low agricultural suitability due to their low reactivity and P content (Mendes et al., 2014).

However, increases in the efficiency of microbial phosphate solubilization processes are essential to reduce costs (Wan et al., 2020). An alternative to optimizing the process of phosphate solubilization is to study the effects of compounds, particles, and molecules commonly found in the native environments of these microorganisms. Most of the PSM are isolated from the rhizosphere, which is the soil region under the influence of plant roots. In the rhizosphere, plants exude various substances that interact with microorganisms (Gupta & Sharma, 2020). However, in this habitat, interactions with soil particles such as clays are also important for several microbial processes. (Cuadros, 2017; Gupta & Sharma, 2020). Mineral clays of soil can interact with microorganisms by physical and chemical mechanisms (Burford et al., 2003; Cuadros, 2017; Fomina & Skorochod, 2020). The effect of clays on the microbial acid production from organic compounds, which is relevant to microbial phosphate solubilization processes, is still unknown. The objective of this study was to investigate the effects of clay minerals (gibbsite, kaolinite, goethite, and hematite) on oxalic acid production and RP solubilization by *A. niger* FS1.

MATERIAL E METHODS

The experiments were conducted at the Laboratory of Microbial Ecology, Department of Microbiology, located at the Institute of Biotechnology Applied to Agriculture (BIOAGRO), Universidade Federal de Viçosa, Viçosa, MG. Kaolinite, hematite, goethite, and gibbsite clays (Sigma-Aldrich Brasil Ltda.) were used. The biological material used was the fungal isolate *A. niger* FS1, obtained from the collection of phosphate-solubilizing fungi at the Laboratory of Microbial Ecology. The fungus was maintained on potato dextrose agar (PDA) at 30°C and subcultured to fresh PDA every seven days. In all experiments, Araxá RP, a natural phosphate of igneous origin, was utilized. This phosphate has low reactivity and contains 13.97% phosphorus. The RP was sifted to standardize the particle size to 75 µm.

Effect of clays on the solubilization of Araxá natural phosphate and on the biosynthesis of organic acids by *A. niger*

125 mL Erlenmeyer flasks, containing 50 mL of modified NBRIP medium containing per liter: glucose, 10 g; Araxá natural phosphate, 3 g; $MgCl_2 \cdot 6H_2O$, 5 g; $MgSO_4 \cdot 7H_2O$, 0.25 g; KCl, 0.2 g; and $(NH_4)2SO_4$, 0.1 g (Nautiyal, 1999), supplemented with hematite, goethite, and gibbsite clays at concentrations of 250, 500, 1000, 1500, 2000, 2500, and 3000 mg L^{-1}. These flasks were then inoculated with 10^6 spores of *A. niger* FS1, which had been cultured on PDA medium for 7 days at 30°C. The spore suspension was prepared in a 0.1% Tween-80 solution. Each type of clay was tested individually. Control treatments without the clay adition, fungal inoculation, or Araxá phosphate were also included. The flasks were incubated in a horizontal shaker for 7 days at 30°C and 150 rpm. After incubation, the supernatants were filtered through JP42 filter paper (QuantyR). The pH was measured using a bench pH meter, while titratable acidity was determined through titration with NaOH. Soluble phosphorus was evaluated in the supernatant using the colorimetric technique (Braga and Defelipo, 1974), on a spectrophotometer Genesys 10S UV-VIS (Thermo Scientific). The percentage of P solubilization was calculated using the following equation: ((Soluble P measured in the supernatant) - (Soluble P measured in the control without clay and inoculation) * 100) / (Total P content of the P source).

Fungal biomass was determined by drying samples at 65°C till constant weight was achieved. Subsequently, to eliminate the weight of clay and RP residues, the dried samples were incinerated in a muffle furnace at 500°C for 5 hours. Finally, biomass was calculated by subtracting the weight of the mineral remains obtained after incineration from the dry weight measured at 60°C.

The quantification of organic acids and remnant glucose was conducted using high-

performance liquid chromatography (HPLC), as described by Van Hees et al. (1999), on a Shimadzu Prominence chromatograph equipped with a refractive index detector (RID), model RID-20A. For the analysis, an HPX 87H column (Aminex®) (300 mm x 7.8 mm) and a corresponding pre-column (Bio-Rad) were employed, maintained at a temperature of 45°C. A 5 mmol L^{-1} sulfuric acid solution was utilized as the mobile phase, delivered at a constant flow rate of 0.7 mL min^{-1}. Standard curves for organic acids were generated using oxalic and citric acid concentrations of 0, 2.5, 5, 10, 20, 40, 80, and 160 mmol L^{-1}. For glucose, the standard curve included concentrations of 0, 5.5, 11, 22, 44, and 88 mmol L^{-1}. Data processing were performed using Lab Solutions software, Shimadzu Corporation (2013).

Evaluation of the effect of clays on the size of fungal pellets

125 mL Erlenmeyer flasks, containing 50 mL of modified NBRIP medium (Nautiyal, 1999), supplemented with clays, hematite, kaolinite, goethite, and gibbsite at concentrations of 250, 500, 1000, 1500, 2000, 2500, and 3000 mg L^{-1}, were inoculated with 10^6 spores of *A. niger* FS1 grown in PDA medium for 7 days at 30°C. The spore suspension was prepared in a 0.1% Tween-80 solution. Each type of clay was tested individually. Control treatments without the clay addition and Araxá natural phosphate were also included. The flasks were incubated on a horizontal shaker for 7 days at 30°C and 150 rpm. After incubation, the supernatants were filtered using filter paper. The fungal pellets were then separated, and the diameter of 100 pellets per treatment was measured using the software "Meazure."

Determination of efficiency indices

With the data obtained previously, efficiency coefficients were calculated. These were: phosphate solubilization efficiency (BEPS), oxalic acid production efficiency (BEOA), citric acid production efficiency (BECA), phosphate solubilization efficiency per glucose consumed (GEPS), oxalic acid production efficiency per glucose consumed (GEOA), and citric acid production efficiency per glucose consumed (GECA). The coefficients were calculated using the following equation:

BEPS = [Available P (mg L^{-1}) / Biomass (g)]
BEOA = [Oxalic acid production (mmol) / Biomass (g)]
BECA = [Citric acid production (mmol) / Biomass (g)]
GEPS = [Available P (mg L^{-1}) / Consumed glucose (g)]
GEOA = [Oxalic acid production (mmol) / Consumed glucose (g)]

GECA = [Citric acid production (mmol) / Consumed glucose (g)]

Experimental design and statistical analyzes

The experiments were set up in a completely randomized design, following a factorial scheme corresponding to (4 x 7) x 3, where 4 represents the type of clay, 7 represents the clay dose, and 3 represents the number of repetitions. The data obtained underwent ANOVA, and the treatment means were compared using the Scott-Knott test at a 5% significance. The experiments of pellet size and solubilization in solid culture medium were set up in a completely randomized design with three replications. The data obtained were submitted to ANOVA and the treatment means were compared using the Tukey test at a 5% significance level.

RESULTS

Effect of clays on Araxá RP solubilization

Supplementation of the NBRIP medium with hematite, kaolinite, goethite, and gibbsite clays promoted increase in araxá RP solubilization in the presence of the fungus *A. niger* FS1, compared to the control without the clay addition of (Figure 1a). The control showed a solubilization of 108.58 mg L^{-1} Soluble P (Figure 1a). The highest solubilization was observed for the gibbsite treatment, with soluble P ranging from 216.78 to 453.68 mg L^{-1}, values recorded at a concentration of 1500 mg L^{-1} (Figure 1a). Followed by treatment with kaolinite, solubilization of soluble P ranged from 149.96 to 407.61 mg L^{-1}, observed at a concentration of 3000 mg L^{-1} (Figure 1a). Treatments with goethite and hematite showed lower levels of solubilization compared to those with gibbsite and kaolinite, but higher than the control without the addition of clay. The solubilization in the treatment with hematite varies from 159.35 to 245.18 mg L^{-1} of soluble phosphorus, with the highest solubilization value observed at a concentration of 2500 mg L^{-1} (Figure 1a). For Goethite, solubilization ranges from 153.27 to 192.95 mg L^{-1} of soluble P, reaching the highest level of solubilization at a concentration of 3000 mg L^{-1} (Figure 1a). Only in the treatment with gibbsite, a drop in solubilization was observed due to the increase in clay concentration to 1500 mg L^{-1} (Figure 1a). At a concentration of 1500 mg L^{-1}, the solubilization levels decreased in the following order: gibbsite > kaolinite > hematite > goethite > control. However, all treatments showed statistically significant improvement compared to the control without the clay addition, as determined by the Tukey test ($p < 0.05$) (Figure 1b). Treatments without inoculation of the fungus *A. niger* FS1 showed insignificant P solubilization values (Supplementary material).

As for the biomass of the fungus *A. niger* FS1, an increase was observed when

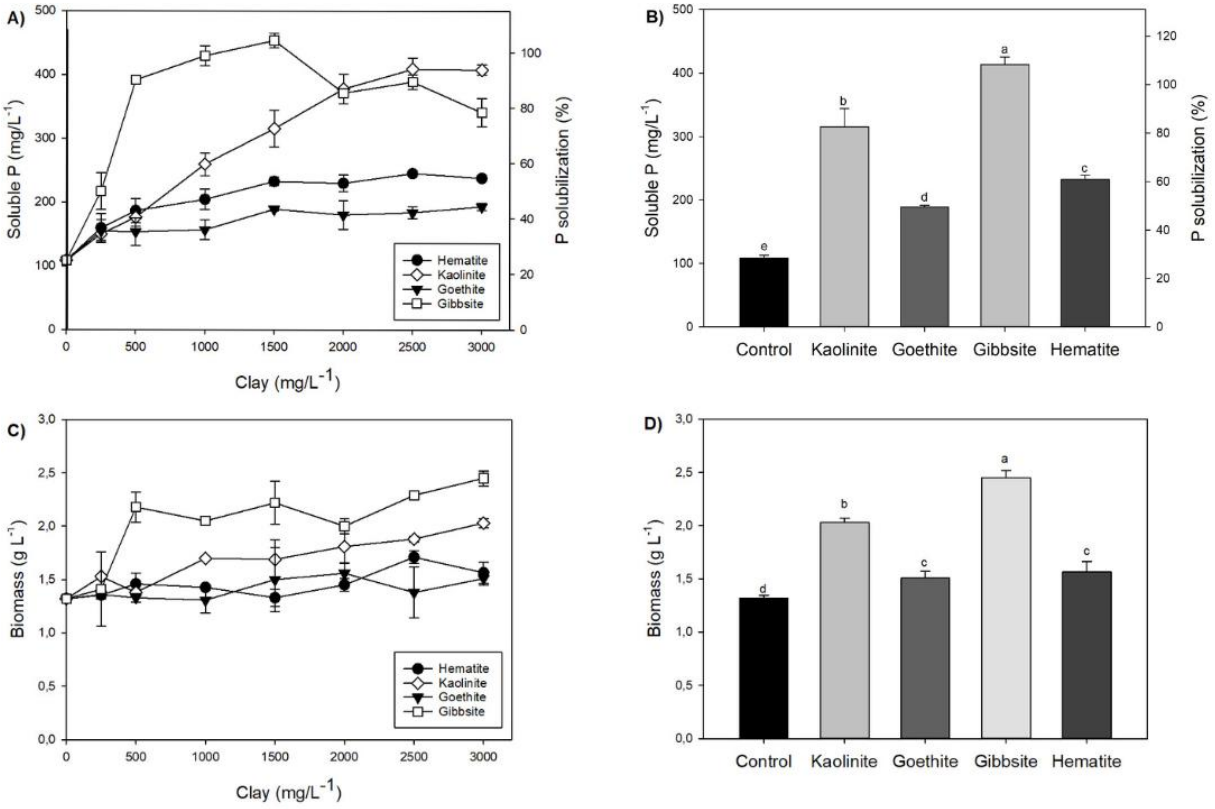

FIGURE 1. Solubilized P and biomass produced by *A. niger* FS1 on modified NBRIP medium supplemented with the clays hematite, kaolinite, goethite, and gibbsite at concentrations of 250, 500, 1000, 1500, 2000, 2500, and 3000 mg L^{-1}. **(A)** Soluble phosphorus measured in the supernatant. **(B)** Soluble phosphorus at the clay concentration of 1500 mg L^{-1}. **(C)** Biomass (g L^{-1}) produced in each clay concentration and **(D)** at the clay concentration of 3000 mg L^{-1}. Bars with the same lowercase letter do not differ significantly from each other according to Tukey's test ($p<0.05$).

NBRIP was supplemented with gibbsite, kaolinite, hematite, and goethite clays, compared to the control without the clay adition (Figure 1c). The highest biomass production was observed in the treatment supplemented with gibbsite, ranging from 1.4 to 2.45 g L^{-1}, as recorded for the treatment with a concentration of 3000 mg L^{-1} (Figure 1c). In the treatment with kaolinite, biomass values ranged from 1.53 to 2.02 g L^{-1}, with the highest value observed at a concentration of 3000 mg L^{-1} (Figure 1c). For the treatment with hematite, biomass accumulation ranged from 1.35 to 1.7 g L^{-1}. The highest value was registered at a concentration of 2500 mg L^{-1} (Figure 1c). The biomass accumulation in the treatment supplemented with goethite ranged from 1.36 to 1.55 g L^{-1}, with the highest value observed at a concentration of 2000 mg L^{-1} (Figure 1c). In general, the highest values of biomass accumulation were observed in the treatments with the highest clay concentrations.

Furthermore, no significant decreases in biomass were observed with an increase in clay concentration in any treatment (Figure 1c). In the treatments with a concentration of 3000 mg L^{-1} of each clay, significant differences were observed in the accumulation of biomass compared to the control without the addition of clay, according to the Tukey test ($p<0.05$) (Figure 1d). Biomass values at this clay concentration decreased in the following order: gibbsite > kaolinite > hematite > goethite > control (Figure 1d).

The pH and titratable acidity of the supernatant were also evaluated. As for the pH, a decrease was observed when the NBRIP medium was supplemented with gibbsite, kaolinite, hematite, and goethite clays (Figure 2a). The treatments supplemented with gibbsite exhibited the lowest pH values, ranging from 2.66 to 1.85. The latter value was observed in treatments with concentrations of 2000 and 3000 mg L^{-1} (Figure 2a). In treatments supplemented with kaolinite, pH values ranging from 2.88 to 2.37 were observed. The lowest value was observed in the treatment with a clay concentration of 3000 mg L^{-1} (Figure 2a). The treatments with the lowest variation in pH were those supplemented with hematite and gibbsite (Figure 2a). In the treatment supplemented with hematite, pH values ranged from 3 to 2.7. The lowest value was observed in the treatment at a concentration of 3000 mg L^{-1} (Figure 2a). At a concentration of 2000 mg L^{-1}, no significant differences were observed in the pH values between the control and the treatment with hematite, as determined by the Tukey test ($p<0.05$) (Figure 2b). No significant differences were observed between the hematite and goethite treatments. However, the latter treatment showed a significant difference from the control, as indicated by the Tukey test ($p<0.05$) (Figure 2b). The gibbsite and kaolinite treatments had a significant effect on the pH values compared to the control without the addition of clay. There were also significant differences between the two treatments, as determined by the Tukey test ($p<0.05$) (Figure 2b). At a concentration of 2000 mg L^{-1}, the pH values decreased in the following order: control > hematite > goethite > kaolinite > gibbsite (Figure 2d).

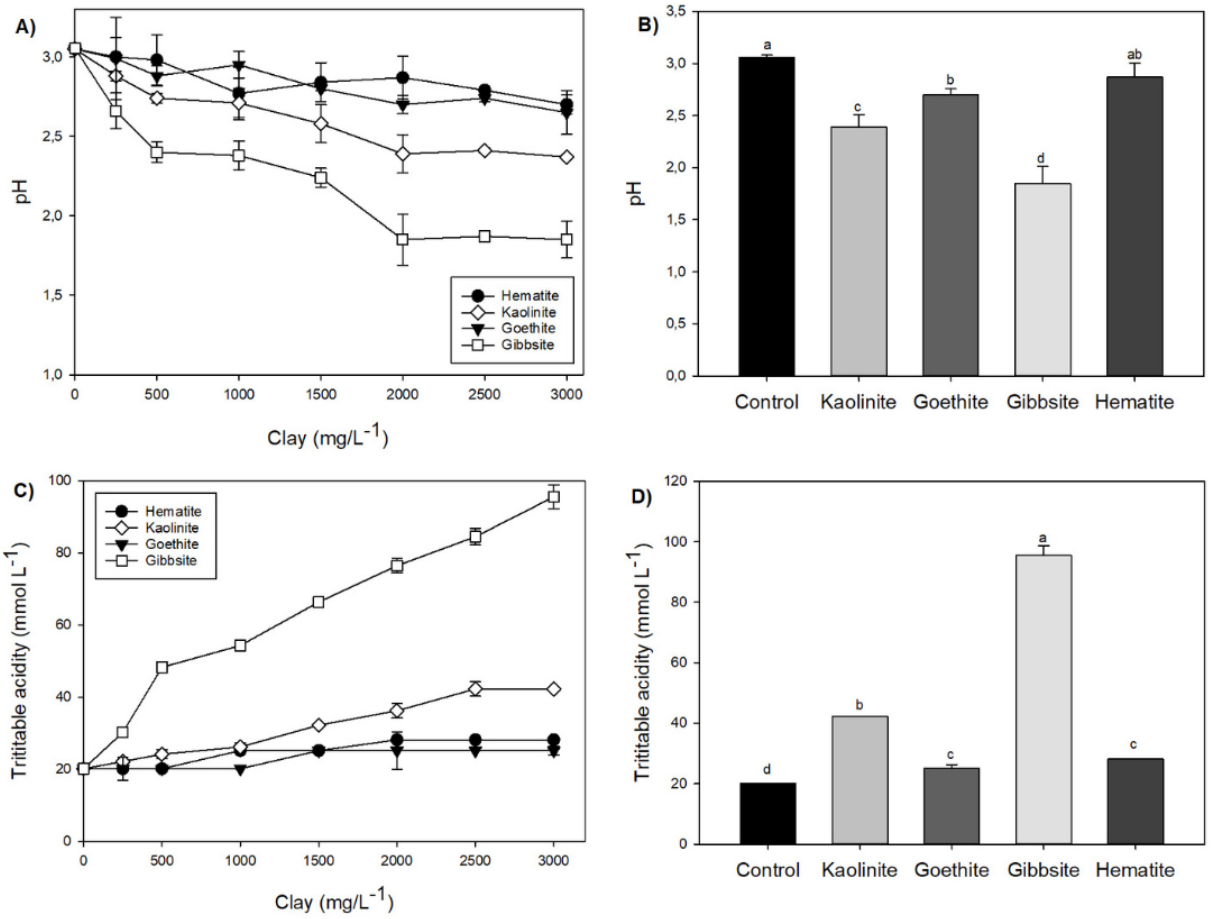

FIGURE 2. (A) pH measured in the supernatant of modified NBRIP medium, supplemented with clays (hematite, kaolinite, goethite, and gibbsite) at concentrations of 250, 500, 1000, 1500, 2000, 2500, and 3000 mg L^{-1}. **(B)** pH at a concentration of 2500 mg L^{-1} of clay. **(C)** Titratable acidity measured in the supernatant of modified NBRIP medium, supplemented with clays (hematite, kaolinite, goethite, and gibbsite) at concentrations of 250, 500, 1000, 1500, 2000, 2500, and 3000 mg L^{-1}. **(D)** Titratable acidity at a concentration of 3000 mg L^{-1} (d). Bars with the same lowercase letter do not differ significantly from each other according to Tukey's test ($p < 0.05$).

While the titratable acidity increased when the NBRIP medium was supplemented with gibbsite, kaolinite, hematite, and goethite clays compared to the control without the clay adition (Figure 2c). The highest titratable acidity was observed in the gibbsite treatments, ranging from 30.17 to 95.55 mmol L^{-1} (Figure 2c).

This last value was observed in the treatment at a concentration of 3000 mg L^{-1} (Figure 2c). In the treatment with kaolinite, the titratable acidity values ranged from 22.12 to 42.24 mg L^{-1}, with the highest value observed at the concentrations of 2500 and 3000 mg L^{-1} (Figure 2c). The titratable acidity increased from 20.09 to 28.1 mg L^{-1}, with the highest value observed at concentrations of 2000, 2500, and 3000 mg L^{-1} (Figure 2c). The lowest values of titratable acidity were observed in the treatments with goethite, ranging from 20.09 to 25.12 mmol/L. The highest value of 25.12 was observed at a concentration of 1500 mg L^{-1} (Figure 2c). At a concentration of 3000 mg L^{-1} of clay, all treatments were significantly superior compared to the control without the addition of clay, according to the Tukey's test ($p < 0.05$) (Figure 2d). Treatments with hematite and goethite did not differ significantly according to the Tukey's test ($p<0.05$) (Figure 2d). Titratable acidity values decreased in the following order: gibbsite > kaolinite > hematite > goethite > control (Figure 2d).

Supplementation of gibbsite, kaolinite, hematite, and goethite to the NBRIP had different effects on the production of organic acids and remanent glucose, depending on the type of clay evaluated (Figure 3). In the biosynthesis of oxalic acid, the highest values were observed in the treatment with gibbsite, with a variation from 7.08 to 44.33 mmol L^{-1} of oxalic acid. The highest value was observed in the treatment with a concentration of 3000 mg L^{-1} (Figure 3a). In the other treatments, no significant variations were observed. In the treatments with kaolinite, a variation of 4.2 to 7 mmol L^{-1} of oxalic acid was observed. The highest value of 7 mmol L^{-1} was found in the treatment with a concentration of 3000 mg L^{-1} of hematite (Figure 3a). In treatments with hematite, the variation in oxalic acid production ranged from 5.34 to 5.6 mmol L^{-1}. The highest value was observed in the treatment with a concentration of 1000 mg L^{-1} (Figure 3a). The smallest variation was observed in the treatments with goethite, which ranged from 5.3 to 5.5 mmol of citric acid, with the highest value observed in the treatment with a concentration of 2500 mg L^{-1} (Figure 3a). At a concentration of 3000 mg L^{-1}, the treatment with gibbsite supplement showed a significant increase in the production of oxalic acid, as observed in Tukey's test ($p<0.05$) (Figure 3b). The treatments with kaolinite, hematite, and gibbsite did not differ from the control without the clay adition in the production of oxalic acid, according to Tukey's test ($p < 0.05$) (Figure 3b). The production of oxalic acid at a concentration of 3000 mg L^{-1} of clay decreased in the following order: gibbsite > kaolinite > hematite > goethite > control (Figure 3b).

The highest biosynthesis of citric acid was observed in treatments with gibbsite, with a variation ranging from 5.51 to 15.69 mmol L^{-1}. The highest value of 15.69 mmol L^{-1} was

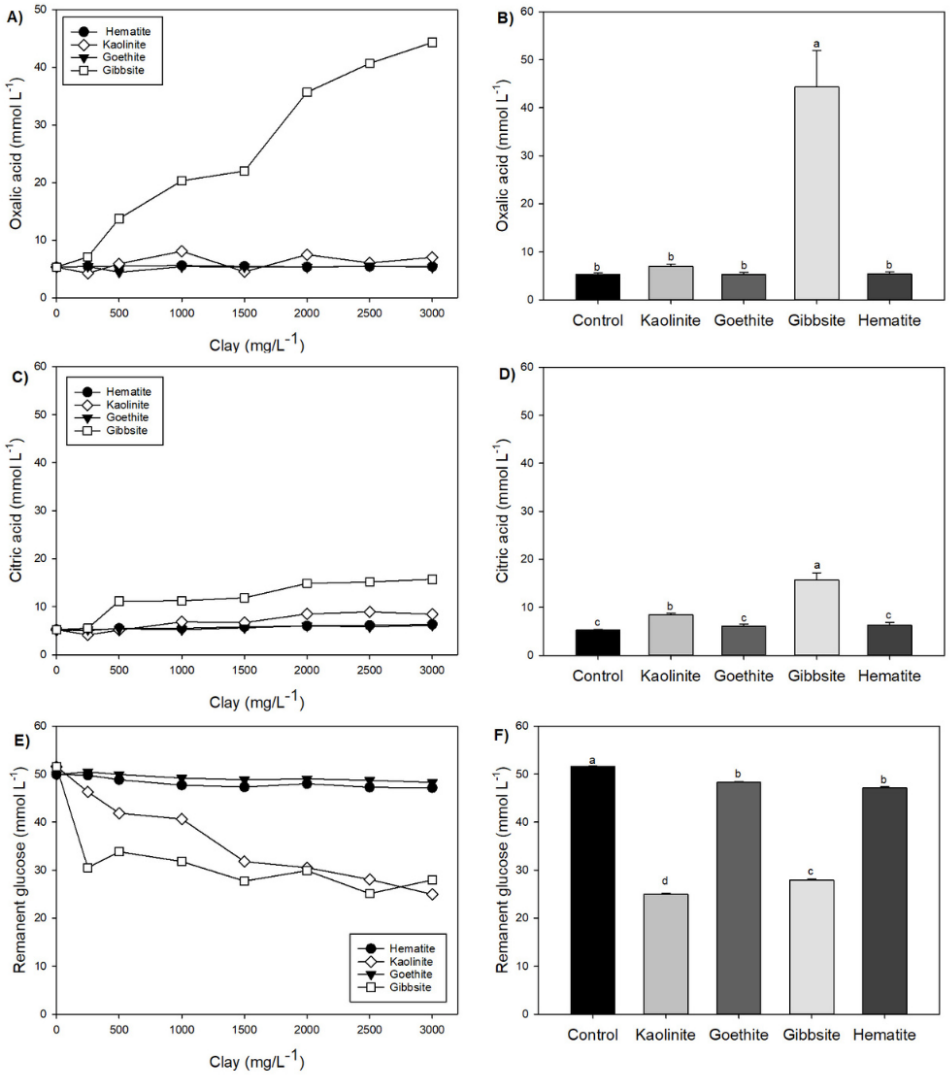

FIGURE 3. (A) Oxalic acid measured in the supernatant of modified NBRIP medium, supplemented with clays, hematite, kaolinite, goethite, and gibbsite at concentrations of 250, 500, 1000, 1500, 2000, 2500, and 3000 mg L^{-1}. **(B)** Oxalic acid at a concentration of 3000 mg L^{-1} of clay. **(C)** Citric acid measured in the supernatant of modified NBRIP medium, supplemented with clays, hematite, kaolinite, goethite, and gibbsite at concentrations of 250, 500, 1000, 1500, 2000, 2500, and 3000 mg L^{-1}. **(D)** Citric acid at a concentration of 3000 mg L^{-1} of clay. **(E)** Remanent glucose measured in the supernatant of modified NBRIP medium, supplemented with clays, hematite, kaolinite, goethite, and gibbsite at concentrations of 250, 500, 1000, 1500, 2000, 2500, and 3000 mg L^{-1}. **(F)** remanent glucose at a concentration of 3000 mg L^{-1} of clay. Bars with the same lowercase letter do not differ significantly from each other according to Tukey's test ($p<0.05$).

observed at a concentration of 3000 mg L^{-1} (Figure 3c). In treatments with kaolinite, a variation in citric acid biosynthesis was observed, ranging from 4.12 to 8.93 mmol L^{-1}. The highest value was observed at a concentration of 2500 mg L^{-1} (Figure 3c). For treatments with hematite, citric acid production was observed to vary between 5 and 6.3 mmol L^{-1}, with the highest value observed at a concentration of 3000 mg L^{-1} (Figure 3c). In treatments with goethite, a variation of 5.2 to 6.1 mmol L^{-1} of citric acid was observed. The highest citric acid production value was observed at a concentration of 3000 mg L^{-1} (Figure 3c). At a concentration of 3000 mg L^{-1}, there was a significant increase in citric acid biosynthesis in treatments with gibbsite and kaolinite compared to the control without the clay adition, as determined by Tukey's test ($p<0.05$) (Figure 3d). Treatments with hematite and goethite did not show significant differences in citric acid production at a concentration of 3000 mg L^{-1}, as determined by Tukey's test ($p<0.05$) (Figure 3d). The production of citric acid at a concentration of 3000 mg L^{-1} of clay decreased in the following order: gibbsite > kaolinite > hematite > goethite > control (Figure 3b).

A general decrease in remanent glucose was observed when the NBRIP medium was supplemented with gibbsite, kaolinite, hematite, and goethite (Figure 3e). In the treatment with goethite, a variation of 49.95 to 48.29 mmol L^{-1} was observed. The lowest level of remanent glucose was observed at a concentration of 3000 mg L^{-1} (Figure 3e). For the treatment with hematite, the remanent glucose variation ranged from 51.59 to 47.18 mmol L^{-1}, with the lowest value observed at a concentration of 3000 mg L^{-1} (Figure 3e). The greatest variation in remanent glucose was observed in treatments with gibbsite and kaolinite (Figure 3e). In the treatments with kaolinite, a variation of 51.59 to 24.97 mmol L^{-1} is observed, with the lowest value observed at a concentration of 3000 mg L^{-1} (Figure 3e).

Effect of clays on the size of fungal pellets

After incubating for 7 days at 28°C in NBRIP medium supplemented with gibbsite, kaolinite, hematite, and goethite clays separately, a decrease in the size of fungal pellets of *A. niger* FS1 was observed (Figure 4). At a concentration of 3000 mg L^{-1}, pellet diameters of 0.587 mm, 0.122 mm, 0.108 mm, and 0.1 mm were observed for the gibbsite, kaolinite, hematite, and goethite treatments, respectively (Figure 4a). At a concentration of 3000 mg L^{-1}, significant differences were observed between treatments with gibbsite, kaolinite, hematite, and goethite compared to the control without the clay adition, according to Tukey's test ($p<0.05$) (Figure 4a). The size of pellets at a concentration of 3000 mg L^{-1} of clay decreased in the following order: Control > gibbsite > goethite = hematite = kaolinite (Figure 4a). A decrease in the size of fungal pellets was observed depending on the amount of clay supplemented to the culture medium (Figure 4b).

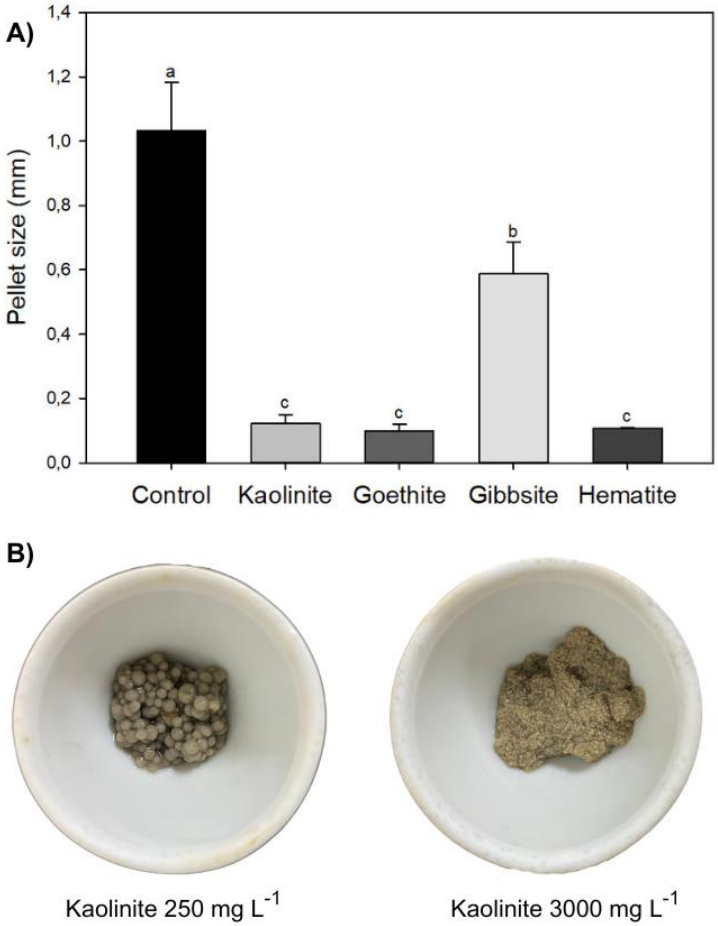

FIGURE 4. **(A)** Size of *A. niger* FS1 pellets incubated in modified NBRIP medium, supplemented with clays, hematite, kaolinite, goethite, and gibbsite at concentrations of 3000 mg L^{-1}. Bars with the same lowercase letter do not differ significantly from each other according to Tukey's test ($p < 0.05$). **(B)** *A. niger* FS1 pellets incubated in modified NBRIP medium, supplemented with kaolinite at concentrations of 250 and 3000 mg L^{-1}.

Efficiency indices

Clays supplementation modified phosphate solubilization efficiency (BEPS). An increase was observed for gibbsite treatments depending on the amount of clay supplemented up to 2500 mg L^{-1}, ranging from 3074.9 to 4102.7 mg soluble P/g biomass. After this concentration, a decrease was observed (Figure 5a).

For the kaolinite treatments, BEPS ranged from 1960.2 to 4348.45 mg of soluble P per gram of biomass, with the highest efficiency achieved at a concentration of 2500 mg L^{-1} (Figure 5a). In the treatments with hematite, a variation in BEPS of 2351.85 to 3498.2 mg of soluble P per gram of biomass was observed, with the highest concentration observed at 1500 mg L^{-1} (Figure 5a). For goethite, a BEPS variation of 2271.96 to 2661.5 mg of soluble P per gram of biomass was observed, with the highest values also found at a concentration of 2500 mg L^{-1} (Figure 5a).

As to the oxalic acid production efficiency (BEOA), a significant difference was observed between gibbsite and the other treatments (Figure 5b). In the gibbsite treatment, BEOA varied from 100.52 to 394.35 mmol L^{-1} of oxalic acid per gram of biomass produced (Figure 5b). The highest BEOA was observed at a concentration of 2000 mg L^{-1} (Figure 5b).

The highest citric acid production efficiency (BECA) were observed in the treatment with gibbsite (Figure 5c), ranging from 78.24 to 164.1 mmol L^{-1} of citric acid per gram of biomass produced. The highest BECA was observed at a concentration of 2000 mg L^{-1} (Figure 5c).

In the case of phosphate solubilization efficiency per glucose consumed (GEPS), a clear division into two groups of clays was observed (Figure 5d). The clays with the highest GEPS were goethite and hematite, showing no significant variations depending on the increase in clay concentration (Figure 5d). The highest GEPS in treatments supplemented with hematite was observed with the supplementation of 2000 mg L^{-1}, resulting in 170.22 mg of soluble P per gram of glucose consumed by the fungus (Figure 5d). For treatments supplemented with goethite, the highest GEPS was observed with 250 mg L^{-1}, resulting in 171.65 mg of soluble P for each gram of glucose consumed (Figure 5d). In treatments with gibbsite and kaolinite, lower GEPS values were observed compared to the other group of clays. For treatments with gibbsite, increases in GEPS were observed at the three lowest concentrations tested, ranging from 48.17 to 100.71 mg of soluble P for each gram of glucose consumed by the fungus (Figure 5d). In treatments with kaolinite, no significant changes in GEPS were observed depending on the amount of clay supplemented to the culture medium. The highest GEPS observed in kaolinite treatments was with 1000 mg L^{-1} of clay, resulting in 97.09 mg of soluble P for each gram of glucose consumed (Figure 5d).

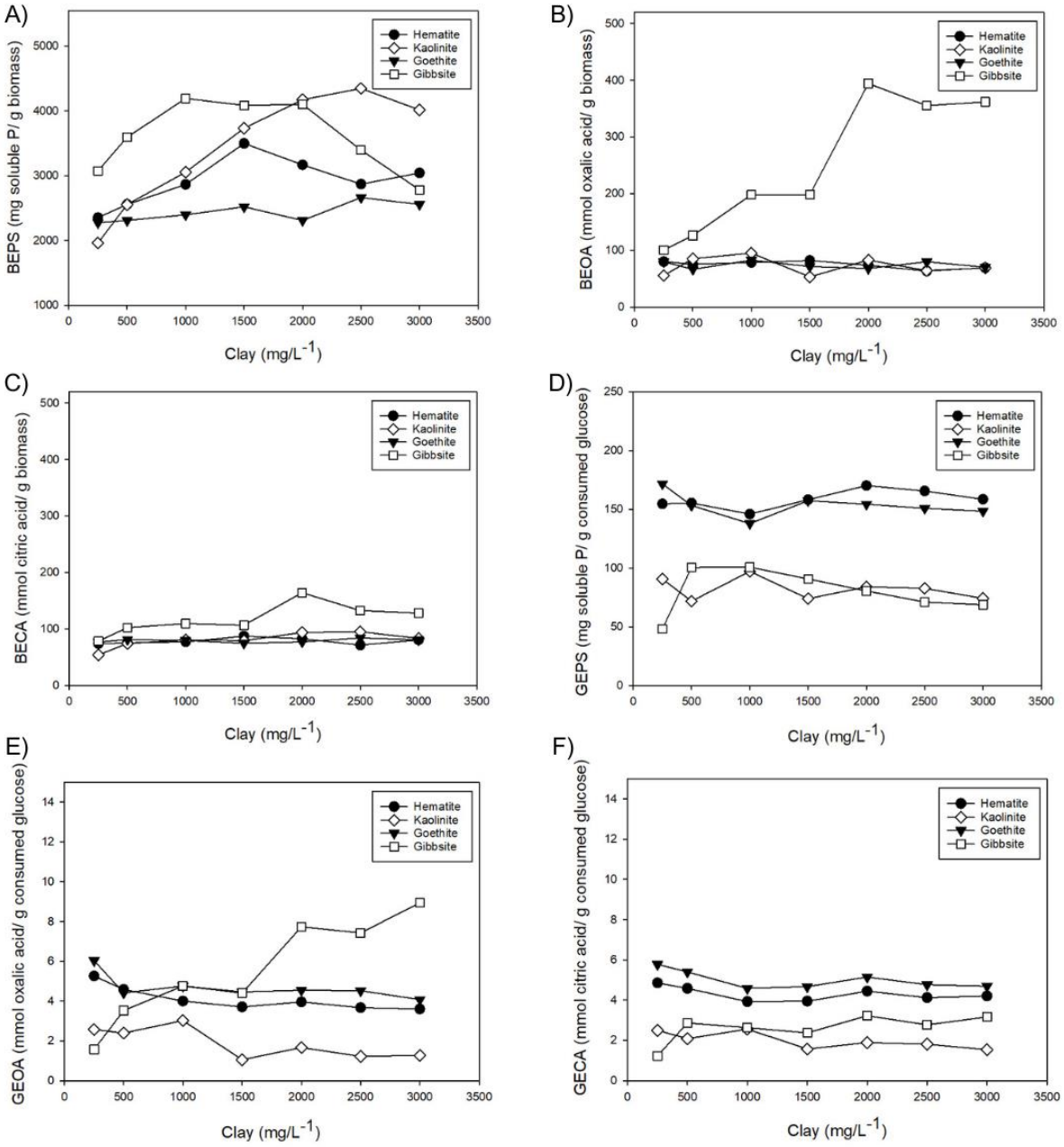

FIGURE 5. (A) Phosphate solubilization efficiency (BEPS), **(B)** Oxalic acid production efficiency (BEOA), **(C)** Citric acid production efficiency (BECA), **(D)** Phosphate solubilization efficiency per glucose consumed (GEPS), **(E)** Oxalic acid production efficiency per glucose consumed (GEOA), **(F)** Citric acid production efficiency per glucose consumed (GECA), measured in the supernatant of modified NBRIP medium, supplemented with clays (hematite, kaolinite, goethite, and gibbsite) at concentrations of 250, 500, 1000, 1500, 2000, 2500, and 3000 mg L^{-1}.

For the oxalic acid production efficiency per glucose consumed (GEOA), a significant increase was observed with gibbsite supplementation, ranging from 1.57 mmol L^{-1} of oxalic acid per gram of glucose absorbed at a concentration of 250 mg L^{-1} of gibbsite, up to 8.94 mmol L^{-1} of oxalic acid produced for each gram of glucose consumed at a concentration of 3000 mg L^{-1} gibbsite (Figure 5e). The second treatment where a greater GEOA was observed after the gibbsite treatments was with 250 mg L^{-1} of goethite, resulting in 6.03 mmol L^{-1} of oxalic acid per gram of glucose consumed. In this treatment, the GEOA decreased due to the increase in clay (Figure 5e). A similar trend was observed in hematite treatments, with the highest GEOA observed at the lowest clay concentration and decreasing with hematite supplementation. The highest GEOA in this treatment was 5.26 mmol L^{-1} of oxalic acid for each gram of glucose consumed (Figure 5e). The lowest GEOA was observed in treatments with kaolinite. Among these treatments, the highest GEOA was 3.02 mmol L^{-1} of oxalic acid for each gram of glucose consumed. In this treatment, no significant variations were observed depending on the increase in clay supplementation (Figure 5e).

Regarding the citric acid production efficiency per glucose consumed (GECA), two groups were observed. The treatments with goethite and hematite showed the highest GECA (Figure 5f). In both treatments, the highest GECA was observed with 250 mg L^{-1} of clay, yielding 5.77 and 4.85 mmol L^{-1} of citric acid per gram of glucose consumed, respectively (Figure 5f). The gibbsite and kaolinite treatments had lower GECA values, with the highest calculated values being 3.22 and 2.55 mmol, respectively (figure 5f).

DISCUSSION

Supplementing the culture medium with gibbsite, kaolinite, hematite, and goethite clays enhanced the Araxá RP solubilization (Figure 1). Even at the lowest concentration of 250 mg L^{-1}. Two responses to clay additions were observed. In the first response, the best solubilization results with gibbsite and kaolinite. In the best treatment, there was a 282% increase in solubilization for gibbsite and a 277% increase for kaolinite compared to the control without clay. Both recorded more than 100% solubilization of the supplied Araxá RP (Figure 1). Both clays also enhanced the efficiency of phosphorus solubilization by fungal biomass. (Figure 5a). The structure of these clays is α-$Al(OH)_3$ and $Al_2Si_2O_5(OH)_4$ respectively (Lindsay, 1979; Lepsch, 2011). Due to their chemical structure, these clays, despite being insoluble under normal conditions, are known for their ability to dissolve in an acidic medium (Lindsay, 1979; Lin et al., 2020). When a portion of these clays becomes solubilized, aluminum is released into the supernatant. This release of aluminum may indirectly contribute to the solubilization of phosphates by inducing oxidative stress and a protective response in the fungus *A. niger* FS1 through organic acid production (Hamel et al.,

1999). Several studies in both plants and microorganisms report that the response to stress caused by aluminum is an increase in the biosynthesis of organic acids (Hamel et al., 1999; Muhammad et al., 2019; Shi et al., 2020; Panchal et al., 2021; Kumar et al., 2023). This is supported by the analysis of organic acid production using HPLC, where a significant increase in oxalic acid production was observed (up to 736%, in the treatment with gibbsite at a concentration of 3000 mg L^{-1})(Figure 3b). Also, for citric acid production, an increase of 201% and 60% was observed for gibbsite and kaolinite, respectively, at a concentration of 3000 mg L^{-1} (Figure 3d). The efficiency of organic acid production by fungal biomass was also observed to increase in the gibbsite treatments. (Figure 5b-5c). The ability of these acids to solubilize P has been widely demonstrated (Kumari et al., 2008; Marra et al., 2019; Mendes et al., 2020; Kaur et al., 2021). The increase in the production of organic acids in the treatments with gibbsite and kaolinite is also confirmed by the rise in titratable acidity and the decrease in pH (Figure 2). Decrease in pH means an increase in the concentration of H^+ ions, which is also a mechanism for RP solubilization (Alori et al., 2017; Panchal et al., 2021).

Interestingly, it was observed that the solubilization of Araxá RP decreased in the treatment with gibbsite at concentrations above 1500 mg L^{-1}. However, the production of organic acids continued to increase with higher concentrations (Figure 3). This can be explained because, at these concentrations, the aluminum content is increasing, which can start to interact with the previously solubilized P, rendering it unavailable (Penn & Camberato, 2019). This does not happen at lower concentrations because aluminum has a stronger attraction to other elements commonly found in RPs, such as F, compared to P (Lindsay, 1979; Zhang et al., 2017). Thus, when the concentration of aluminum is low, it will initially interact with elements that have a greater chemical affinity. However, at very high concentrations, negative interactions with P begin to be observed (Penn & Camberato, 2019).

In the treatments with kaolinite, decreases in natural phosphate solubilization could not observed even at the highest concentrations. This can be explained by the presence of silicon (Si) in the structure of this clay (Lepsch, 2011). Once kaolinite is solubilized, silicic acid is formed (Lindsay, 1979). This compound can have a direct interaction in P solubilization, but it is also characterized by its affinity for aluminum, thus limiting its negative interaction with P (Exley et al., 2019). Furthermore, when comparing the solubility of gibbsite and kaolinite in an acidic medium, gibbsite exhibits higher solubility, resulting in a greater release of aluminum (Lindsay, 1979; Lin et al., 2020).

Hematite and goethite showed a smaller increases in Araxá RP solubilization. These clays include hematite and goethite. However, they still exhibited a significant increase compared to the control without the clay adition (Figure 1). RP solubilization was increased

by 125% and 77%, respectively (Figure 1). These clays have similarities in their structure; both belong to the group of oxide clays and contain iron in their composition. Hematite is represented by the chemical formula Fe_2O_3, while goethite is represented by FeO(OH) (Lepsch, 2011). Among the ferric oxide clays, in descending order according to their solubility in an acidic medium, are: maghemite > lepidocrocite > hematite > goethite (Lindsay, 1979). The increase in Araxá RP solubilization in the treatments with hematite and goethite cannot be explained by the production of organic acids, as there are no significant differences observed in the production of citric and oxalic acid compared to the control without the addition of clay (Figure 3). The increase in Araxá RP solubilization could be explained by the indirect effect of the increase in iron (Fe) content, which has a positive nutritional effect on microorganisms (Fourquez et al., 2014; Soares et al., 2022).This can be supported by the observed increase in significant biomass accumulation compared to the control without the clay adition (Figure 1d). Iron is essential for fungal growth as it is a component of several enzymes that play a crucial role in various metabolic processes, such as respiration. In respiration, iron is a component of the enzymes involved in the electron transport chain (Cornelis & Andrews, 2010; Misslinger et al., 2021). The increase in biomass can be related to the increase in phosphate solubilization because higher biomass can impact several solubilization mechanisms, including EPS production and direct H^+ extrusion (Prabhu et al., 2019). This last mechanism could be observed in the pH decrease observed in the treatments with kaolinite and goethite (Figure 2).

While the remanent glucose was analyzed in the supernatant, a decrease was observed in all treatments (Figure 3f). This is related to an increase in carbon consumption by the fungus (Hamad et al., 2015). This increase is explained by the accumulation of biomass in all treatments and the allocation of carbon towards the synthesis of organic acids, particularly in the treatments involving gibbsite and kaolinite (Yang et al., 2017). Interestingly, the fungus *A. niger* FS1 exhibited a low consumption of glucose even without clay supplementation. Only 7% of the glucose in the culture medium was consumed (Figure 4e). This may indicate that, under this condition, chemical elements present in natural Araxá RP, such as fluorine, can limit the growth of the fungus (Silva et al., 2014; Szostek, 2015; Geretharan et al., 2020). In clay treatments, these negative effects can be reduced due to the high levels of aluminum and iron that can chemically interact with fluorine, thus limiting its availability (Zhang et al., 2017).

It is necessary to study the physical effects of clay particles on the solubilization of phosphates. Due to the positive effects that clays could have on the solubilization of Araxá RP through physical interactions with the fungal mycelium (Cuadros, 2017; Fomina & Skorochod, 2020)..

In this experiment, certain physical interactions can be observed in the size of the

fungal pellets after incubation at 28°C for 7 days in the NBRIP medium supplemented with gibbsite, kaolinite, hematite, and goethite clays (Figure 4). The relationship between pellet size and the production of organic acids has been studied (Liao et al., 2007). Studies indicate that a larger pellet size with a thin layer of mycelium and an empty nucleus leads to a greater production of organic acids, mainly due to the increased supply of oxygen inside the pellet (Veiter et al., 2018). Interestingly, in this study, a decrease in pellet size was observed in treatments with clay concentration, which also corresponded to a higher production of organic acids (Figure 4c). The decrease in pellet size can be attributed to the higher concentrations of clay particles. These particles can act as a substrate for the fungal mycelium to adhere to the clay particles (Kelly et al., 2006; Kheirkhah et al. 2023). This can be confirmed since clays with lower solubility resulted in smaller pellets, with a decrease of around 90% in diameter compared to the control without the clay adition (Figure 4c). This explains why that in the treatment with gibbsite, a smaller decrease in the size of pellets was observed, around 50%, since this clay has greater solubility (Figure 4c).

Thus, gibbsite, a common clay found in Brazilian soils, exhibits significant potential to enhance the industrial production of economically important organic acids. Additionally, it promotes the solubilization of low-reactivity phosphates for agricultural applications. Further research is necessary to elucidate the specific processes and mechanisms that contribute to the increased solubilization of phosphates by soil clays..

CONCLUSIONS

The results obtained in this study demonstrated an increase in the solubilization of Araxá RP by the fungus *A. niger* FS1 when the clays gibbsite, kaolinite, goethite, and hematite were supplemented in the culture medium. This research highlights the significant potential of using gibbsite and kaolinite, which achieved nearly 100% solubilization of Araxá RP. The data suggest that the observed increases in solubilization may be related to enhanced synthesis of organic acids. The findings indicate variability in the performance of the clays based on their chemical composition. Possible physical interactions between the clays and the fungus were evidenced by the size of the fungal pellets observed after incubation. Further research is necessary to elucidate the mechanisms involved in promoting the solubilization of Araxá RP by these mineral clays. This study opens new avenues for developing biotechnological strategies aimed at implementing microbial solubilization of phosphates in critical sectors such as the fertilizer industry and agricultural and forestry production.

SUPPLEMENTARY MATERIAL

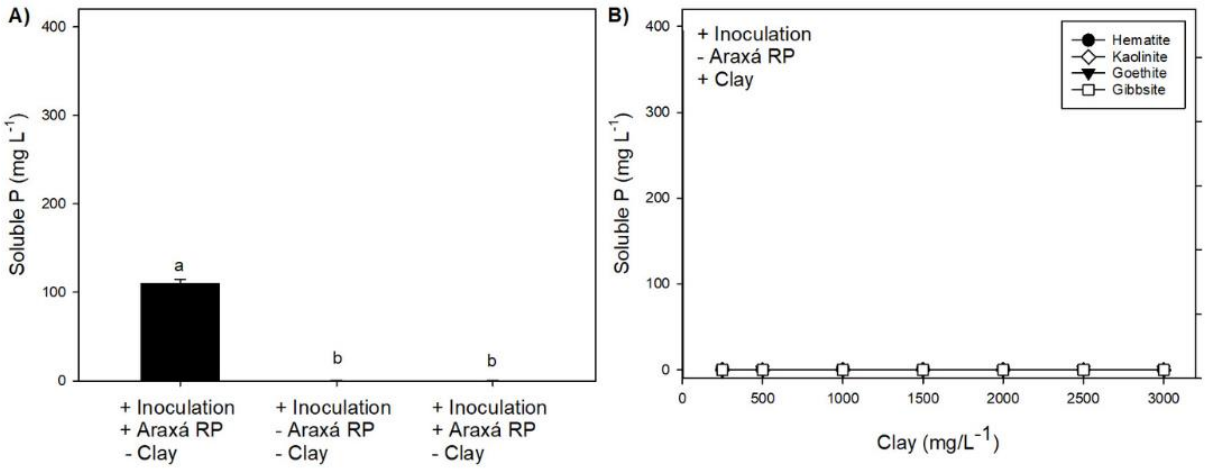

FIGURE 6. Experiment controls. **(A)** Soluble phosphorus (mg L^{-1}) of controls without clays supplementation. **(B)** Soluble phosphorus (mg L^{-1}) of controls supplemented with the clays hematite, kaolinite, goethite, and gibbsite at concentrations of 250, 500, 1000, 1500, 2000, 2500, and 3000 mg L^{-1}. Bars with the same lowercase letter do not differ significantly from each other according to Tukey's test ($p<0.05$).

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CHAPTER III

Aluminum release from kaolinite and gibbsite enhances phosphorus solubilization by *Aspergillus niger*

ALUMINUM RELEASE FROM KAOLINITE AND GIBBSITE ENHANCES PHOSPHORUS SOLUBILIZATION BY *Aspergillus niger.*

ABSTRACT

Clays determine several physical and chemical characteristics of the soil. They also play a fundamental role in water and nutrient retention. Clays also interact with microorganisms through physical and chemical processes, impacting several fundamental microbial processes that maintain the balance of biogeochemical cycles. Previous studies have shown the influence of clays on the microbial solubilization of phosphates, a process that can offer a sustainable alternative to meet the phosphorus needs of agricultural crops. However, the mechanisms through which clays increase phosphate solubilization remain unknown. Therefore, the objective of this study was to determine the mechanisms that enhance the solubilization of Araxá rock phosphate (RP) by the fungus *A. niger* FS1 when grown in the presence of the clays gibbsite, kaolinite, goethite, and hematite. To achieve this goal, the modified NBRIP medium was, supplemented with the clays gibbsite, kaolinite, goethite, and hematite both inside and outside capsules to analyze the importance of the physical contact between the fungal mycelium and the clays under investigation. Additionally, experiments were conducted with the supplementation of $AlCl_3$, silicic acid, and Fe-EDTA. to assess the impact of chemical elements present in clays on the solubilization of Araxá RP. The tested clays significantly increased the solubilization of Araxá RP. There were no significant differences between clay treatments inside or outside the capsules, suggesting that the increase in solubilization does not result from a physical fungus-clay interaction. Supplementation with $AlCl_3$ promoted significant increases in solubilization, surpassing 100% solubilization of Araxá RP, inducing mainly the synthesis of oxalic acid by *A. niger* FS1. A concentration of 0.6 mmol L^{-1} of silicic acid significantly increased phosphate solubilization. Concentrations of Fe-EDTA between 20 and 50 mg L^{-1} significantly increased phosphate solubilization without observed increases in the production of organic acids by the fungus *A. niger* FS1. The results indicate that the acidic environment produced by the fungus promotes the dissolution of clays. In the cases of gibbsite and kaolinite, this process leads to an increased concentration of Al, which triggers a protective response from the fungus. Consequently, this response enhances the synthesis of oxalic acid to decrease the availability of Al in the medium. Furthermore, the excess oxalic acid improves the solubilization of Araxá RP. Thus, this work allows for a better understanding of the mechanisms involved in increases in phosphate solubilization through the interaction of mineral clays with the fungus *A. niger* FS1. Understanding the mechanisms involved allows us to hypothesize how these interactions occur in the soil and highlights the potential for using phosphate-solubilizing microorganisms in conjunction with mineral clays in agriculture

to sustainably meet the phosphorus demand of crops.

KEYWORDS: Soil clays, phosphate solubilization, phosphate solubilizing microorganisms.

INTRODUCTION

Soil clays are defined as mineral particles present in the soil with a size of less than 0.002 mm resulting from the weathering of primary or secondary minerals (Lepsch, 2021). The proportion of clay in the soil is variable and this determines the soil texture. In sandy soils, the clay content varies from 5 to 15%, while in clayey soils, it ranges from 30 to 60% (Santos et al., 2018). There are several ways to classify clays, one of the most commonly used methods is based on the arrangement of elements that compose them. Clays are classified as silicate clays when oxygen atoms are linked to silicon and aluminum, and as oxidic clays when oxygen is linked only to iron or aluminum (Meunier, 2005; Lepsch, 2021). Silicate clays have an organized layered structure and are characterized by a high cation exchange capacity, a large surface area, and greater plasticity compared to oxidic clays (Velde & Meunier, 2008). Examples of silicate clays include kaolinite, montmorillonite, and illite. Oxide clays have lower plasticity and cation exchange capacity due to their less organized structure, lacking well-defined layers. Examples of oxide clays include gibbsite, hematite, and goethite (Meunier, 2005).

Clays play a fundamental role in soils, influencing processes and characteristics such as water retention, nutrient exchange, soil structuring, pH, and microbial activity (Meunier, 2005; Fan et al., 2021; Pessoa & Libardi, 2022). The relationship between microorganisms and clays is complex and diverse, interacting directly and indirectly in various microbial processes (Cuadros, 2017). Clays in the soil have various interactions with soil microorganisms, which can be primarily physical or chemical interactions (Cuadros, 2017; Li et al., 2019; Fomina & Skorochod, 2020). Among the physical interactions observed between microorganisms and clays, a notable aspect is their ability to adsorb microorganisms due to their electrical charge and high surface area (Hong et al., 2011; Huang et al., 2015). Another important clay-microorganism physical interaction is thigmotropism by filamentous fungi with clay particles, where physical contact directly influences their growth (Watts et al., 1998; Bowen et al., 2007; Fomina & Skorochod, 2020). Additionally, the formation of microbial structures using clay particles as biofilms promotes the adhesion of microorganisms to surfaces (Alimova et al., 2009; Ma et al., 2017; Kim & Kwon, 2021).

There are also chemical interactions between clays and microorganisms. The ability to transform clays, thus modifying their chemical composition, has been widely reported (Burford et al., 2003; Gadd, 2010; Hong et al., 2016; Wild et al., 2021; Zhang et al., 2021;

Jung et al., 2022). This ability of microorganisms allows them to access nutrients that are part of the clay composition or nutrients adsorbed on clays, which is essential to sustain microbial growth in oligotrophic environments such as the soil (Gadd, 2010).

Clays have a significant influence on extracellular enzymes synthesized by microorganisms, modifying their activity and stability in the environment (Olagoke et al., 2020; Pooni et al., 2021; Yandri et al., 2022).

It is clear how clays in the soil play a role in various microbial processes (Cuadros, 2017). One of the highly relevant processes that occur in the soil is the microbial solubilization of phosphates.

Previous experiments have demonstrated an increase in the solubilization of Araxá RP by the fungus *A. niger* FS1 when the culture medium is supplemented with clay minerals such as gibbsite, kaolinite, goethite, and hematite. These increases may result from physical interactions between clay particles and the fungal mycelium or from chemical interactions with compounds released during the dissolution of the clays, including aluminum, iron, and silicic acid. These interactions can impact the production of organic acids, thereby enhancing the solubilization of Araxá RP. This study aims to investigate the mechanisms that facilitate the increased phosphate solubilization by *A. niger* FS1 when grown in culture media supplemented with these clay minerals (gibbsite, kaolinite, goethite, and hematite).

MATERIAL E METHODS

The experiments were conducted at the Laboratory of Microbial Ecology, Department of Microbiology, located at the Institute of Biotechnology Applied to Agriculture (BIOAGRO), Universidade Federal de Viçosa, Viçosa, MG. Kaolinite, hematite, goethite, and gibbsite clays (Sigma-Aldrich Brasil Ltda.) were used. The biological material used was the fungal isolate A. niger FS1, obtained from the collection of phosphate-solubilizing fungi at the Laboratory of Microbial Ecology. The fungus was maintained on potato dextrose agar (PDA) at 30°C and subcultured to fresh PDA every seven days. In all experiments, Araxá RP, a natural phosphate of igneous origin, was utilized. This phosphate has low reactivity and contains 13.97% phosphorus. The RP was sifted to standardize the particle size to 75 µm.

Influence of physical contact between clays and the fungus *A. niger* FS1 on the solubilization of Araxá RP

Capsules were manufactured to contain the clays inside without interacting directly with the fungal mycelium. The capsules have a 0.22 µm filter at both ends to allow the flow of chemical elements in solution. 75 mg of each clay (Kaolinite, hematite, goethite, and gibbsite) were added inside the capsules. Treatment with the clays outside the capsules was

also conducted. The capsules were placed inside 25 mL Erlenmeyer flasks, containing 50 mL of modified NBRIP medium containing per liter: glucose, 10 g; Araxá natural phosphate, 3 g; $MgCl_2 \cdot 6H_2O$, 5 g; $MgSO_4 \cdot 7H_2O$, 0.25 g; KCl, 0.2 g; and $(NH_4)2SO_4$, 0.1 g (Nautiyal, 1999). These flasks were then inoculated with 10^6 spores of *A. niger* FS1, which had been cultured in PDA medium for 7 days at 30°C. The spore suspension was prepared in a 0.1% Tween-80 solution. Each type of clay was tested individually. Control treatments without the clay adition, fungal inoculation, or Araxá phosphate were also included. The flasks were incubated on a horizontal shaker for 7 days at 30°C and 150 rpm. After incubation, the supernatants were filtered through JP42 filter paper (QuantyR). The pH was measured using a bench pH meter, while titratable acidity was determined through titration with NaOH. Soluble phosphorus was evaluated in the supernatant using the colorimetric technique (Braga and Defelipo, 1974), on a spectrophotometer Genesys 10S UV-VIS (Thermo Scientific). The percentage of P solubilization was calculated using the following equation: ((Soluble P measured in the supernatant) - (Soluble P measured in the control without clay and inoculation) * 100) / (Total P content of the P source).

Fungal biomass was determined by drying samples at 65°C until a constant weight was achieved. Subsequently, to eliminate the weight of clay and RP residues, the dried samples were incinerated in a muffle furnace at 500°C for 5 hours. Finally, biomass was calculated by subtracting the weight of the mineral remains obtained after incineration from the dry weight measured at 60°C.

The quantification of organic acids and remnant glucose was conducted using high-performance liquid chromatography (HPLC), as described by Van Hees et al. (1999), on a Shimadzu Prominence chromatograph equipped with a refractive index detector (RID), model RID-20A. For the analysis, an HPX 87H column (Aminex®) (300 mm x 7.8 mm) and a corresponding pre-column (Bio-Rad) were employed, maintained at a temperature of 45°C. A 5 mmol L^{-1} sulfuric acid solution was utilized as the mobile phase, delivered at a constant flow rate of 0.7 mL min^{-1}. Standard curves for organic acids were generated using oxalic and citric acid concentrations of 0, 2.5, 5, 10, 20, 40, 80, and 160 mmol L^{-1}. For glucose, the standard curve included concentrations of 0, 5.5, 11, 22, 44, and 88 mmol L^{-1}. Data processing were performed using Lab Solutions software, Shimadzu Corporation (2013).

Effect of aluminum on the solubilization of Araxá RP by *A.* niger FS1

125 mL Erlenmeyer flasks, containing 50 mL of modified NBRIP medium, were supplemented with $AlCl_3$ at the concentrations of 0, 400, 800, 1600, 2400, 3200 and 4000 mg L^{-1}. These concentrations were calculated based on the theoretical yield of Al generated from gibbsite. These flasks were then inoculated with 10^6 spores of *A. niger* FS1, which had been

cultured in PDA medium for 7 days at 30°C. The spore suspension was prepared in a 0.1% Tween-80 solution. Control treatments without the addition of $AlCl_3$, fungal inoculation, or Araxá phosphate were also included. The flasks were incubated on a horizontal shaker for 7 days at 30°C and 150 rpm. After incubation, the supernatants were filtered through JP42 filter paper (QuantyR). The pH, titratable acidity, soluble P, fungal biomass and the production of organic acids was measured as described above.

Effect of silicic acid on the solubilization of Araxá RP by *A. niger* FS1

1125 mL Erlenmeyer flasks containing 50 mL of modified NBRIP medium were supplemented with silicic acid at concentrations of 0, 0.2, 0.4, 0.6, 0.8, 1.0, and 1.2 mmol L^{-1}. These concentrations were calculated based on the theoretical yield of silicic acid generated from kaolinite. The flasks were then inoculated with 10^6 spores of *A. niger* FS1, previously cultured in PDA medium for 7 days at 30°C. The spore suspension was prepared in a 0.1% Tween-80 solution. Control treatments without the addition of silicic acid or Araxá RP were also included. Treatments without fungal inoculation were included to analyze the direct effect of silicic acid on P solubilization. In the treatment without inoculation, the pH was adjusted to 2.0 to replicate the environment created by the fungus. The flasks were incubated on a horizontal shaker for 7 days at 30°C and 150 rpm. After incubation, the supernatants were filtered through JP42 filter paper (QuantyR). The pH, titratable acidity, soluble P, fungal biomass, and the production of organic acids were measured as described above.

Effect of iron on the solubilization of Araxá RP by *A.* niger FS1

125 mL Erlenmeyer flasks, containing 50 mL of modified NBRIP medium, were supplemented with Fe-EDTA. at the concentrations of 0, 1, 10, 20, 30, 40, 50 and 60 mg L^{-1}. These concentrations were calculated based on the theoretical yield of Al generated from goethite and hematite. These flasks were then inoculated with 10^6 spores of *A. niger* FS1, which had been cultured in PDA medium for 7 days at 30°C. The spore suspension was prepared in a 0.1% Tween-80 solution. Control treatments without the addition of Fe-EDTA., fungal inoculation, or Araxá phosphate were also included. The flasks were incubated on a horizontal shaker for 7 days at 30°C and 150 rpm. After incubation, the supernatants were filtered through JP42 filter paper (QuantyR). The pH, titratable acidity, soluble P, fungal biomass and the production of organic acids was measured as described above.

Determination of efficiency indices

With the data obtained previously, efficiency coefficients were calculated. These were: phosphate solubilization efficiency (BEPS), oxalic acid production efficiency (BEOA), citric acid production efficiency (BECA), phosphate solubilization efficiency per glucose consumed (GEPS), oxalic acid production efficiency per glucose consumed (GEOA), and citric acid production efficiency per glucose consumed (GECA). The coefficients were calculated using the following equation:

BEPS = [Available P (mg L^{-1}) / Biomass (g)]
BEOA = [Oxalic acid production (mmol) / Biomass (g)]
BECA = [Citric acid production (mmol) / Biomass (g)]
GEPS = [Available P (mg L^{-1}) / Consumed glucose (g)]
GEOA = [Oxalic acid production (mmol) / Consumed glucose (g)]
GECA = [Citric acid production (mmol) / Consumed glucose (g)]

Experimental design and statistical analyzes

The physical contact experiments were set up in a completely randomized design, following a factorial scheme of the type (4 x 2) x 3, where 4 represents the type of clay, 2 represents inside or outside the capsule, and 3 represents the number of repetitions.The data obtained were analyzed using ANOVA, and the treatment means were compared with the Tukey test at a 5% significance level, as well as the F-test when necessary. The experiment of silicic acid were set up in a completely randomized design, following a factorial scheme of the type (2 x 7) x 3, where 2 represents inoculated and not inoculated, 7 represents the number of concentrations tested and 3 represents the number of repetitions. The experiments of aluminum and iron were set up in a completely randomized design with 3 repetitions.

RESULTS

Influence of physical contact between clays and *A. niger* FS1 on the solubilization of Araxá RP

No significant differences were observed in the solubilization of Araxá rock among the treatments with and without direct contact of the fungal mycelium with all the supplemented clays, according to the Tukey test ($p < 0.05$) (Figure 1).

All tested clays significantly increased the solubilization of Araxá RP by the fungus *A. niger* FS1, compared to the control without clay addition where the soluble P in the

supernatant was 169.49 mg L^{-1}, resulting in a 40% solubilization respecting the total amount of phosphate used (Figure 1a). Clays increase the solubilization of Araxá natural phosphate in the following order: goethite = hematite < kaolinite < gibbsite (Figure 1a). The greatest solubilization of phosphate from Araxá rock was observed with the supplementation of gibbsite clay, with 406.53 mg L^{-1} of soluble P measured in the supernatant, corresponding to a solubilization of 97% of the total phosphate used (Figure 1a).

In treatments supplemented with kaolinite, an average of 264.11 mg L^{-1} of soluble P was measured in the supernatant, equivalent to a solubilization of 75% of the total phosphate used (Figure 1a). For treatments with hematite supplementation, 216.09 mg L^{-1} of soluble P was observed in the supernatant, equivalent to a solubilization of 51% of the total phosphate added. In treatments supplemented with goethite, an average of 203.43 mg L^{-1} of soluble P was observed in the supernatant, corresponding to 48% solubilization (Figure 1a).

The production of biomass by the fungus A. niger FS1 showed no statistical differences between treatments with and without physical contact of clays with the fungus mycelium, according to the Tukey test ($p < 0.05$) (Figure 1b). The treatments supplemented with gibbsite, kaolinite, and goethite presented biomasses of 2.34, 1.47, and 1.45 g L^{-1}, respectively, which were statistically superior to the control without clay addition, resulting in a biomass production of 1.1 g L^{-1} (Figure 1b). Treatments supplemented with goethite showed a biomass production of 1.3 g L^{-1} (Figure 1b). Therefore, biomass production by the fungus decreased in the following order: gibbsite > kaolinite = hematite = goethite (Figure 1b).

Regarding the pH measured in the supernatants, no significant differences were observed between treatments with and without physical contact of the *A. niger* FS1 fungus mycelium with each clay evaluated, according to the Tukey test ($p < 0.05$) (Figure 1c). The only treatment that presented a statistically different pH compared to the control was the treatment with the addition of gibbsite, with a measured pH of 1.82, while the control without the addition of clay had an average pH of 2.23 (Figure 1c). The pH values for the treatments with kaolinite, goethite, and hematite were 2.13, 2.12, and 2.20, respectively (Figure 1c). Thus, the pH measured in the supernatant of treatments with clay supplementation decreased in the following order: hematite = kaolinite = goethite > gibbsite (Figure 1c).

No significant differences were observed in the titratable acidity measured in the supernatant of the treatments with and without direct contact of the mycelium with the supplemented clays according to the Tukey test ($p < 0.05$) (Figure 1d). The clays gibbsite, kaolinite, and goethite showed significantly higher levels of titratable acidity compared to the control without clay addition (Figure 1d). The titratable acidity observed in the control treatment without clay addition was 24.47 mmol L^{-1}. The highest titratable acidity was observed in treatments with gibbsite, with an average of 60.68 mmol L^{-1} (Figure 1d). In

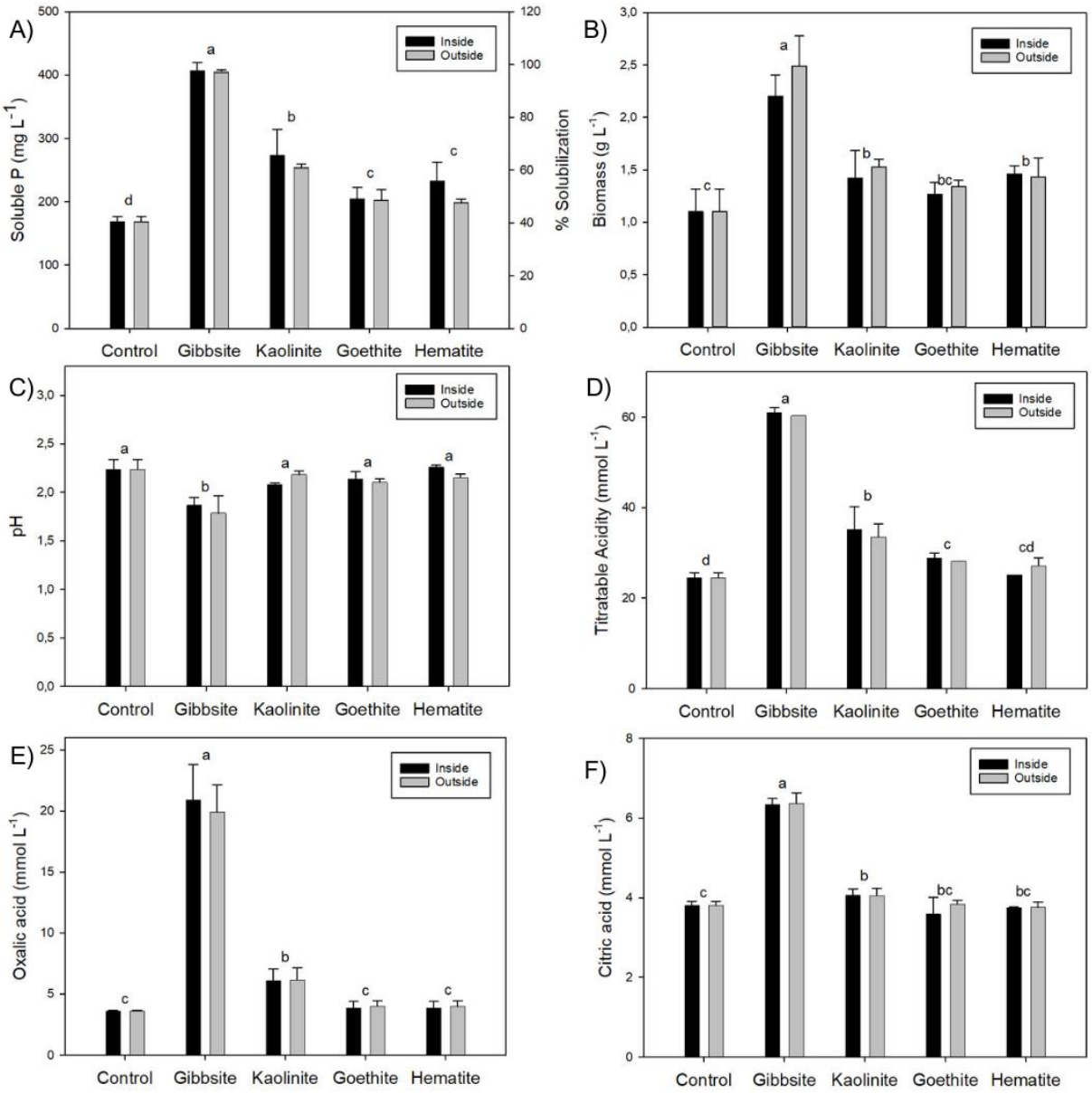

FIGURE 1. A) Soluble phosphorus (mg L^{-1}), **B)** Biomass (g L^{-1}), **C)** pH, **D)** Titratable acidity (mmol L^{-1}), **E)** Oxalic acid (mmol L^{-1}) and **F)** citric acid (mmol L^{-1}), measured in the supernatant of the modified NBRIP medium, supplemented with clays, hematite, kaolinite, goethite, and gibbsite. Black bars represent treatments with clays inside the capsules, while gray bars represent treatments with clays outside the capsules. Bars with the same lowercase letter do not differ significantly from each other according to Tukey's test ($p < 0.05$).

treatments with kaolinite, an average acidity of 34.36 mmol L^{-1} was observed (Figure 1d).
In treatments with goethite, the titratable acidity corresponded to 28.49 mmol L^{-1} (Figure 1d). The titratable acidity in treatments supplemented with hematite was not statistically different from the control without clay addition, according to the Tukey test ($p<0.05$), with an average titratable acidity of 26.15 mmol L^{-1} (Figure 1d). Thus, the titratable acidity measured in the supernatants of treatments with clay supplementation decreased in the following order: gibbsite > kaolinite > goethite = hematite (Figure 1d).

The results of high-performance liquid chromatography showed that there were no significant differences between treatments with and without contact of clays with the fungus regarding the production of organic acids and remanent glucose in the supernatant, according to the Tukey test ($p < 0.05$) (Figure 1e). The production of oxalic acid was statistically higher in the treatments with gibbsite and kaolinite, with a production of 20.39 and 6.25 mmol L^{-1}, respectively, compared to the control without clay addition, which presented a production of 3.6 mmol L^{-1} of oxalic acid (Figure 1e). The treatments with supplementation of goethite and hematite clays were not different from the control without clay addition, with an average production of 4.01 and 3.98 mmol of oxalic acid, respectively (Figure 1e).
Oxalic acid production decreased in the following order: gibbsite > kaolinite > goethite = hematite (Figure 1e).

Regarding the synthesis of citric acid by the fungus *A. niger* FS1, the production was statistically superior in the treatments supplemented with gibbsite and kaolinite, yielding 6.05 and 4.25 mmol L^{-1} respectively, compared to the control without clay addition, which produced 3.70 mmol L^{-1} (Figure 1f).

Treatments supplemented with goethite and hematite clays did not differ from the control without added clay, with an average production of 3.4 and 3.76 mmol of citric acid, respectively (Figure 1f). Citric acid production decreased in the following order: gibbsite > kaolinite = goethite = hematite (Figure 1f).

The glucose concentration present in the supernatant after incubation in all treatments was statistically equal to the control without clay addition, where a concentration of 30.6 mmol L^{-1} was measured. Treatments supplemented with gibbsite presented an average of 25.7 mmol L^{-1} glucose, statistically lower than the treatments with goethite and hematite, with 32.6 and 30.5 mmol L^{-1} respectively. The treatments with kaolinite presented a concentration of 28.5 mmol L^{-1} (Figure 2b). Thus, the concentration of glucose present in the supernatant decreased in the following order: goethite = hematite = kaolinite = gibbsite (Figure 2b).

Finally, the amount of clay remaining inside the capsules after incubation was measured, showing statistical differences in treatments with and without physical contact of

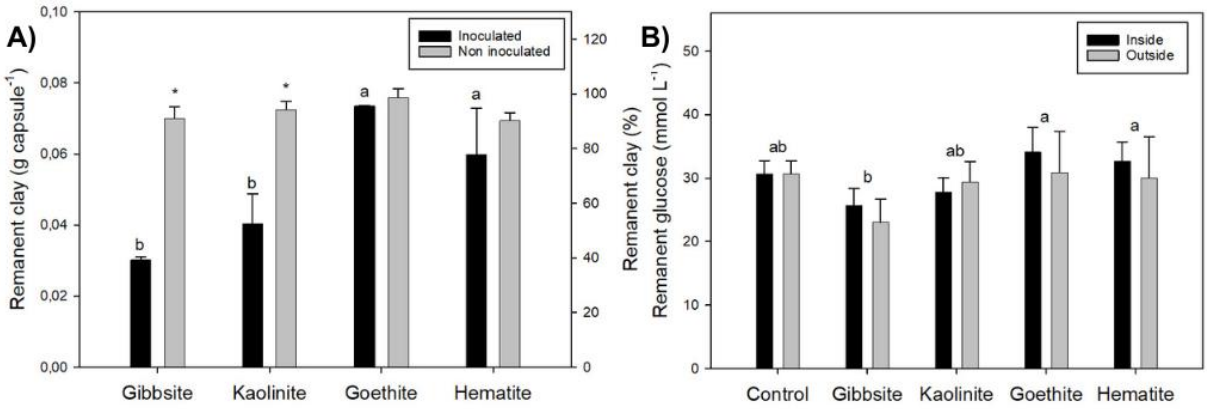

FIGURE 2. **A)** Remanent clay (g capsule^{-1}) measured inside the capsules after 7 days incubation. Black bars represent treatments inoculated with *A. niger* FS1, while gray bars represent treatments non inoculated. **B)** Remanent glucose (mmol L^{-1}) measured in the supernatant of the modified NBRIP medium, supplemented with clays, hematite, kaolinite, goethite, and gibbsite. Black bars represent treatments with clays inside the capsules, while gray bars represent treatments with clays outside the capsules. Bars with the same lowercase letter do not differ significantly from each other according to Tukey's test ($p < 0.05$). Bars with * symbols differ significantly between inoculated and not inoculated treatments according to F test ($p < 0.05$).

clays with the fungus *A. niger* FS1, in treatments supplemented with gibbsite and kaolinite, according to the F test ($p < 0.05$) (Figure 2a). Breaking down the interaction, no statistical differences were observed between treatments that were not inoculated with the fungus. However, at the level of inoculated treatments, goethite and hematite were statistically superior, with 0.058 and 0.071 g capsule^{-1} respectively, in comparison to treatments with gibbsite and kaolinite clays, which had 0.029 and 0.039 g capsule^{-1} respectively (Figure 2a). Thus, the amount of clay remaining inside the capsules at the level inoculated with the fungus *A. niger* FS1 decreased in the following order: Goethite = hematite > kaolinite = gibbsite (Figure 2a).

Effect of aluminum on the solubilization of araxá RP by *A. niger* FS1

An increase in the solubilization of Araxá RP was observed due to the increase in $AlCl_3$ concentration up to a concentration of (Figure 3a). Solubilization was higher than controls without the addition of AlCl3 from a concentration of 800 mg L^{-1}. At this concentration, an amount of 187.87 mg L^{-1} of soluble P was measured, corresponding to a solubilization of 44.2% of the added phosphate. In the control without clay addition, 101.41 mg L^{-1} of soluble P was found, representing 24.19% of the added phosphate (Figure 3a). The greatest solubilization was observed at a concentration of 3200 mg L^{-1}, where 345.5 mg L^{-1} was determined, equivalent to 75.99% solubilization of the added phosphate (Figure 3a).

The biomass of the fungus *A. niger* FS1 increased between concentrations of 800 to 3200 mg L^{-1} of $AlCl_3$; after these concentrations, a decrease in fungal biomass was observed (Figure 3b). Concentrations of 2400 to 4000 mg L^{-1} of $AlCl_3$ showed significantly higher biomass than the control without the addition of $AlCl_3$ (Figure 3b). The biomass of the control without the addition of $AlCl_3$ was 0.97 g L^{-1}, and the highest biomass observed was 1.6 g L^{-1} at a concentration of 3200 mg L^{-1} of $AlCl_3$ (Figure 3b).

It was observed that the pH decreases as a function of the increase in $AlCl_3$. The pH was significantly lower starting from a concentration of 400 mg L^{-1} of $AlCl_3$ compared to the control without the addition of $AlCl_3$ (Figure 3c). The lowest pH observed was 1.77 at a concentration of 3200 mg L^{-1} of $AlCl_3$ The pH measured in the control supernatant without the addition of $AlCl_3$ was 3.12 (Figure 3c).

Titratable acidity measured in the supernatant increased in relation to the increase in $AlCl_3$ concentration, being significantly higher than the control without the addition of $AlCl_3$ from a concentration of 1600 mg L^{-1} (Figure 3c). The highest titratable acidity measured in the supernatant was observed in the treatment with a concentration of 4800 mg L^{-1} of $AlCl_3$, with 144.16 mmol L^{-1}. In the control without the addition of $AlCl_3$, a titratable acidity of 26.82 mmol L^{-1} was determined (Figure 3c). In relation to the amount of glucose remaining in the

supernatant after incubation for 7 days, a decrease was observed due to the increase in $AlCl_3$ concentration. The glucose concentration was significantly lower starting from 1600 mg L^{-1} (Figure 3d). The lowest glucose concentration recorded was 6.94 g L-1, observed at an $AlCl_3$ concentration of 4800 mg L^{-1} (Figure 3d).

Regarding the production of organic acids, an increase in the production of oxalic acid was observed due to the increase in the concentration of $AlCl_3$ supplemented in the culture medium (Figure 3e). All concentrations of $AlCl_3$tested promoted a synthesis of oxalic acid statistically superior to the control without the addition of $AlCl_3$ (Figure 3e). The highest concentration of oxalic acid reached 36.27 mmol L^{-1}, produced at a concentration of 4800 mg L^{-1} of $AlCl_3$ (Figure 3e).

The production of citric acid increased as a result of the higher $AlCl_3$ concentration, with a significant increase observed at concentrations above 1600 mg L^{-1} according to the (Figure 3f). The highest concentration of citric acid was 6.48 mmol L^{-1}, observed at a concentration of 4800 mg L^{-1} of $AlCl_3$ (Figure 3f).

Effect of silicic acid on the solubilization of Araxá RP by *A. niger* FS1

Significant differences were observed between the inoculated and non-inoculated treatments at concentrations of 0, 0.8, 1, and 1.2 mmol L^{-1} of silicic acid. Araxá RP solubilization was superior in treatments inoculated with the fungus *A. niger* FS1 (Figure 4a).

Breaking down the interaction, within treatments not inoculated with the fungus, all concentrations of silicic acid tested were significantly higher than the control without the addition of silicic acid. The concentrations of 0.6 and 0.8 mmol L^{-1} of silicic acid were significantly higher than the other concentrations (Figure 4a). The highest solubilization was observed at a concentration of 0.6 mmol L^{-1} of silicic acid, resulting in an average of 140.35 mg L^{-1} of soluble P in the supernatant, equivalent to 33.48% solubilization of the total phosphate added (Figure 4a). In treatments inoculated with the fungus A. niger FS1, significant increases in solubilization were observed starting from a concentration of 0.6 mmol L^{-1} of silicic acid compared to the control without silicic acid addition (Figure 4a). The highest solubilization among the inoculated treatments was observed at a concentration of 1.2 mmol of silicic acid, resulting in 147.64 mg L^{-1} of soluble P in the supernatant, equivalent to 35.22% solubilization of the total RP from Araxá (Figure 4a).

No gains were observed in the biomass production of the fungus *A. niger* FS1, regardless of the concentration of silicic acid (Figure 4b).

In treatments inoculated with fungus, the pH of the control without the addition of silicic acid was 2.21. No significant differences were observed with increases in silicic acid concentration (Figure 4c).

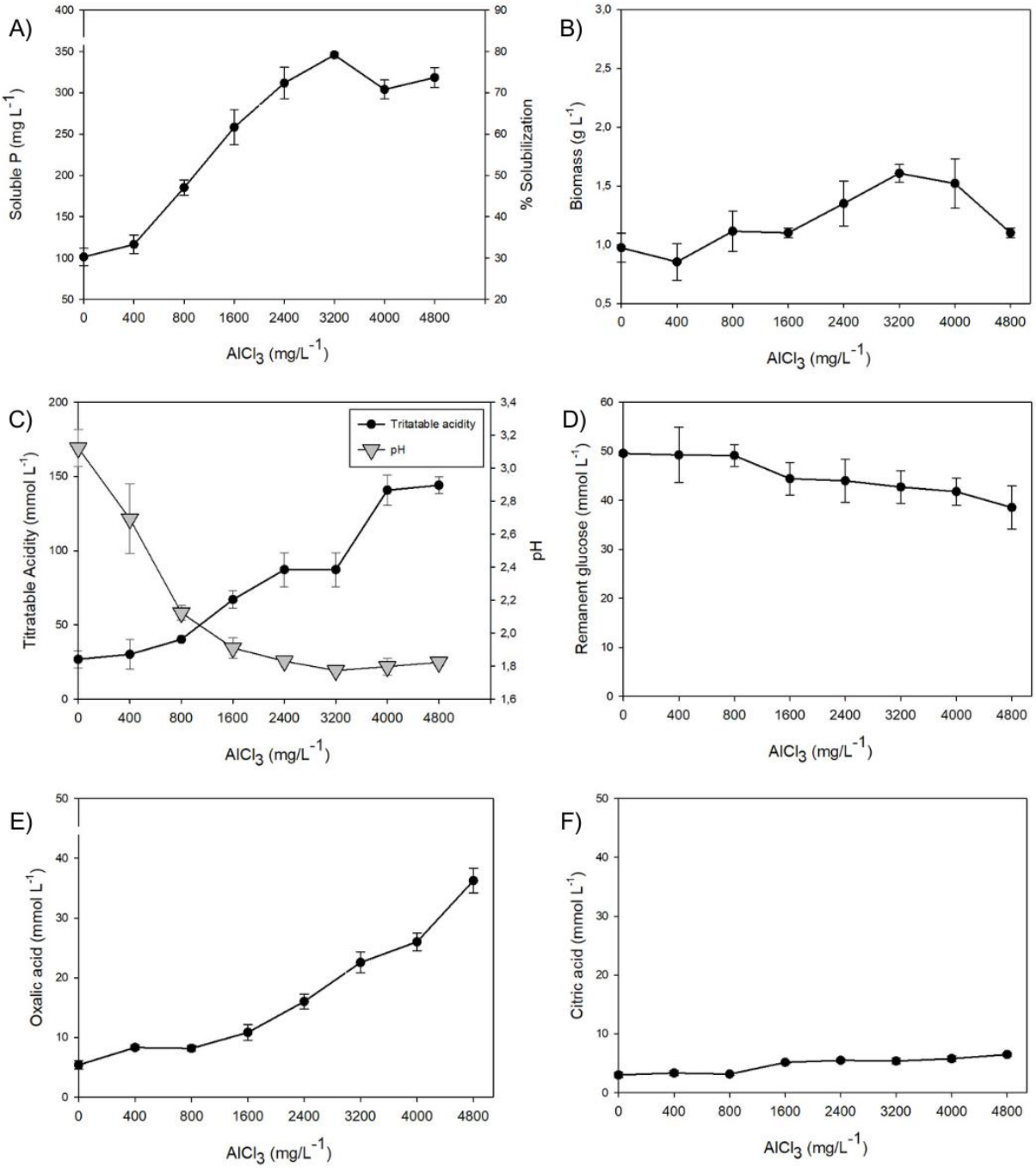

FIGURE 3. A) Soluble phosphorus (mg L^{-1}), **B)** Biomass (g L^{-1}), **C)** Titratable acidity (mmol L^{-1}) and pH, **D)** Remanent glucose (mg L^{-1}), **E)** Oxalic acid (mmol L^{-1}) and **F)** citric acid (mmol L^{-1}), measured in the supernatant of the modified NBRIP medium, supplemented 0, 400, 800, 1600, 2400, 3200 and 4000 mg L^{-1} of $AlCl_3$, inoculated with *A. niger* FS1, after 7 days incubation.

No significant differences were observed in the titratable acidity measured in the supernatant of the treatments inoculated with the fungus *A. niger* FS1 and with varying concentrations of silicic acid (Figure 4c).

There were no significant differences in the amount of glucose remaining in the supernatant after the 7-day incubation (Figure 4d).

The production of organic acids by the fungus *A. niger* FS1 was not altered by supplementation with silicic acid; no differences were observed in the production of oxalic and citric acid (Figure 4e-4f).

Effect of iron on the solubilization of Araxá RP by *A. niger* FS1

Fe-EDTA supplementation in the culture medium increased the solubilization of Araxá RP up to a concentration of 40 mg L^{-1}. After this concentration, a negative effect on solubilization was observed (Figure 5a). Concentrations of 20 to 50 mg L^{-1} of Fe-EDTA significantly increased the solubilization of Araxá RP.

Treatment with 60 mg L^{-1} of Fe-EDTA resulted in significantly lower solubilization compared to the control without added iron (Figure 5a). The highest solubilization was observed in the treatment with 40 mg L^{-1} of Fe-EDTA supplementation, reaching 160.7 mg L^{-1} of soluble P, which is equivalent to 37.6% solubilization of the added phosphate (Figure 5a).

Regarding biomass production by the fungus *A. niger* FS1, although the treatment supplemented with 40 mg L^{-1} of Fe-EDTA showed the highest average biomass production, there were no significant differences compared to the control without the addition of Fe-EDTA (Figure 5b).

A significant decrease in the pH of supernatants was observed in treatments with supplementation of 30 to 50 mg L^{-1} of Fe-EDTA compared to the control without the addition of Fe-EDTA (Figure 5c). The lowest pH recorded was 2.07 in the treatment with 40 mg L^{-1} Fe-EDTA. supplementation (figure 5c).

No significant differences were observed in the titratable acidity measured in the supernatant of treatments with all evaluated concentrations of Fe-EDTA (Figure 5c).

In relation to the amount of glucose remaining in the supernatant after incubation, a nonsignificant increase was observed in the highest Fe-EDTA. concentration (Figure 5d).

No effects of Fe-EDTA supplementation was observed on the production of organic acids. There were no increases in the synthesis of oxalic and citric acid with an increase in Fe-EDTA. concentration (Figure 5e-5f).

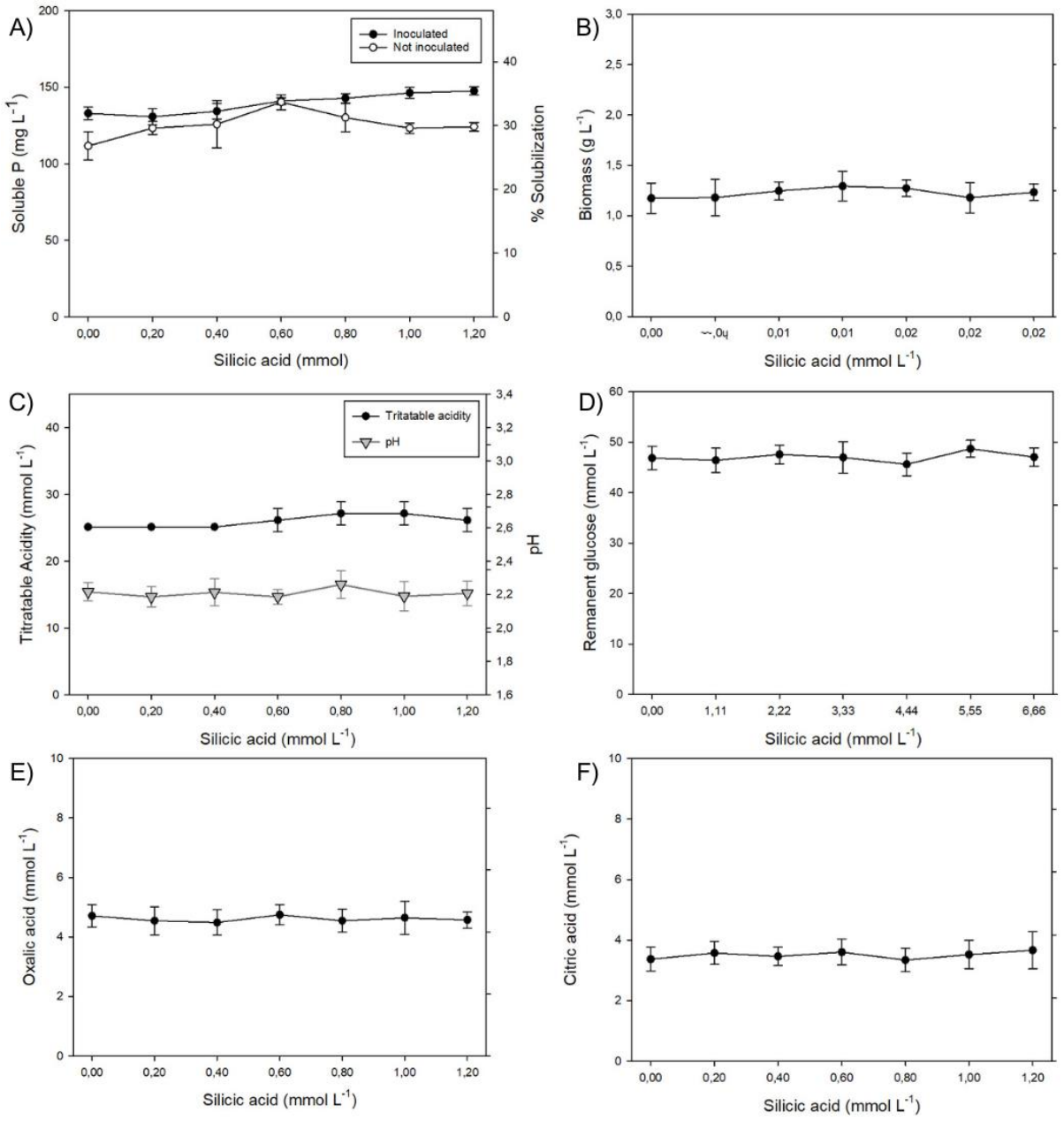

FIGURE 4. A) Soluble phosphorus (mg L^{-1}), **B)** Biomass (g L^{-1}), **C)** Titratable acidity (mmol L^{-1}) and pH, **D)** Remanent glucose (mg L^{-1}), **E)** Oxalic acid (mmol L^{-1}) and **F)** citric acid (mmol L^{-1}), measured in the supernatant of the modified NBRIP medium, supplemented 0, 0.2, 0.4, 0.6, 0.8, 1.0, and 1.2 mmol L^{-1} of silicic acid, inoculated with *A. niger* FS1, after 7 days incubation. White symbols represents treatment without inoculation with *A. niger* FS1.

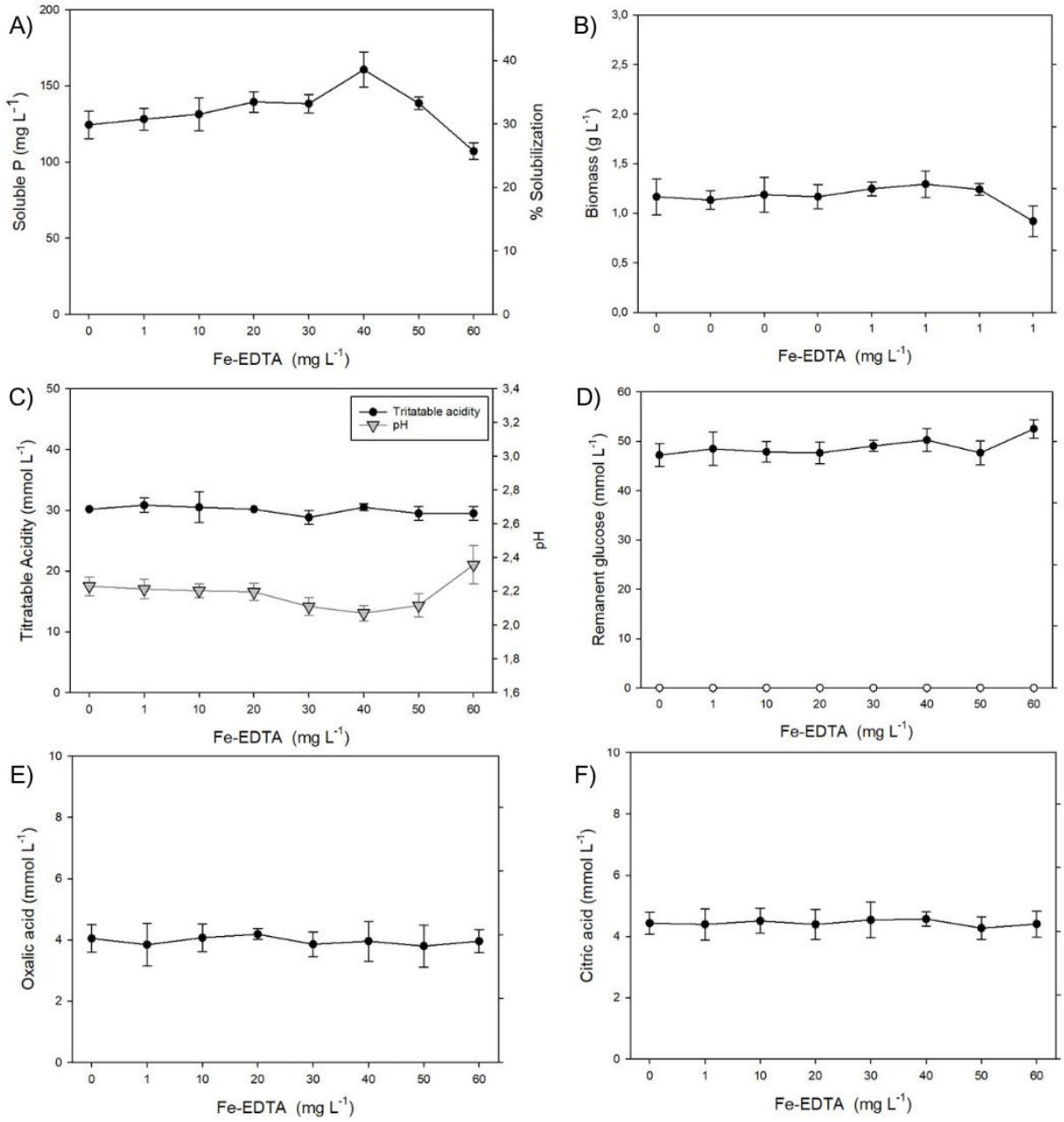

FIGURE 5. A) Soluble phosphorus (mg L^{-1}), **B)** Biomass (g L^{-1}), **C)** Titratable acidity (mmol L^{-1}) and pH, **D)** Remanent glucose (mg L^{-1}), **E)** Oxalic acid (mmol L^{-1}) and **F)** citric acid (mmol L^{-1}), measured in the supernatant of the modified NBRIP medium, supplemented 0, 1, 10, 20, 30, 40, 50 and 60 mg L^{-1} of Fe-EDTA, inoculated with *A. niger* FS1, after 7 days incubation.

Efficiency indices

Regarding the phosphate solubilization efficiency (BEPS), a significant increase was observed due to $AlCl_3$ supplementation (Figure 6a). The highest BEPS was observed at the highest concentration of $AlCl_3$ (4800 mg L^{-1}), corresponding to 5571.44 mg L^{-1} of soluble P for each gram of fungal biomass (Figure 6a). However, supplementation with silicic acid and Fe-EDTA did not alter the efficiency of biomass for P solubilization (Figure 6b-6c).

With respect to the oxalic acid production efficiency (BEOA), a significant impact of $AlCl_3$ supplementation was observed, increasing depending on the AlCl3 concentration (Figure 6d). The highest BEOA was observed at a concentration of 4800 mg L^{-1} of $AlCl_3$ with 659.61 mmol L^{-1} of oxalic acid for each gram of fungal biomass produced. No impacts on BEOA were observed due to silicic acid and Fe-EDTA supplementation (Figure 6e-6f).

The citric acid production efficiency (BECA) was impacted to a lesser extent by the $ALCl_3$ supplementation compared to BEOA. However, it was also significantly enhanced due to the increase in $AlCl_3$ concentration (Figure 6g). The highest BECA was calculated at a concentration of 4800 mg L^{-1} of $AlCl_3$ with 117.98 mmol L^{-1} of citric acid for each gram of fungal biomass (Figure 6g). The supplementation of silicic acid and Fe-EDTA did not affect the efficiency of biomass in the production of citric acid (Figure 6h-6i).

In relation to phosphate solubilization efficiency per glucose consumed (GEPS), increases were observed depending on the lower concentrations of $AlCl_3$ tested, up to a concentration of 800 mg L^{-1} with an efficiency of 161.38 mg L^{-1} of soluble P for each gram of glucose consumed by the fungus. From this concentration, a drop in efficiency was observed (Figure 7a). Concerning silicic acid supplementation, increases in GEPS were observed from the supplementation of 1 mmol L^{-1} of silicic acid, corresponding to 119.24 mg L^{-1} of soluble powder for each gram of glucose consumed (Figure 7b). Fe-EDTA supplementation also affected GEPS, generally showing increases in efficiency with the rise in Fe-EDTA concentration, with the highest BEPS calculated at a concentration of 60 mg L^{-1}, corresponding to 199.35 mg L^{-1} of P for each gram of glucose consumed (Figure 7c). However, a significant drop was calculated at the concentration of 50 mg L^{-1} Fe-EDTA.

Regarding the oxalic acid production efficiency per glucose consumed (GEOA) in general, a significant increase was observed due to $AlCl_3$ supplementation (Figure 7d). The greatest efficiency was observed at a concentration of 4800 mg L^{-1} of $AlCl_3$, corresponding to 11.86 mmol of oxalic acid for each gram of glucose consumed (Figure 7d). Supplementation with silicic acid did not cause significant changes in GEOA (Figure 7e).

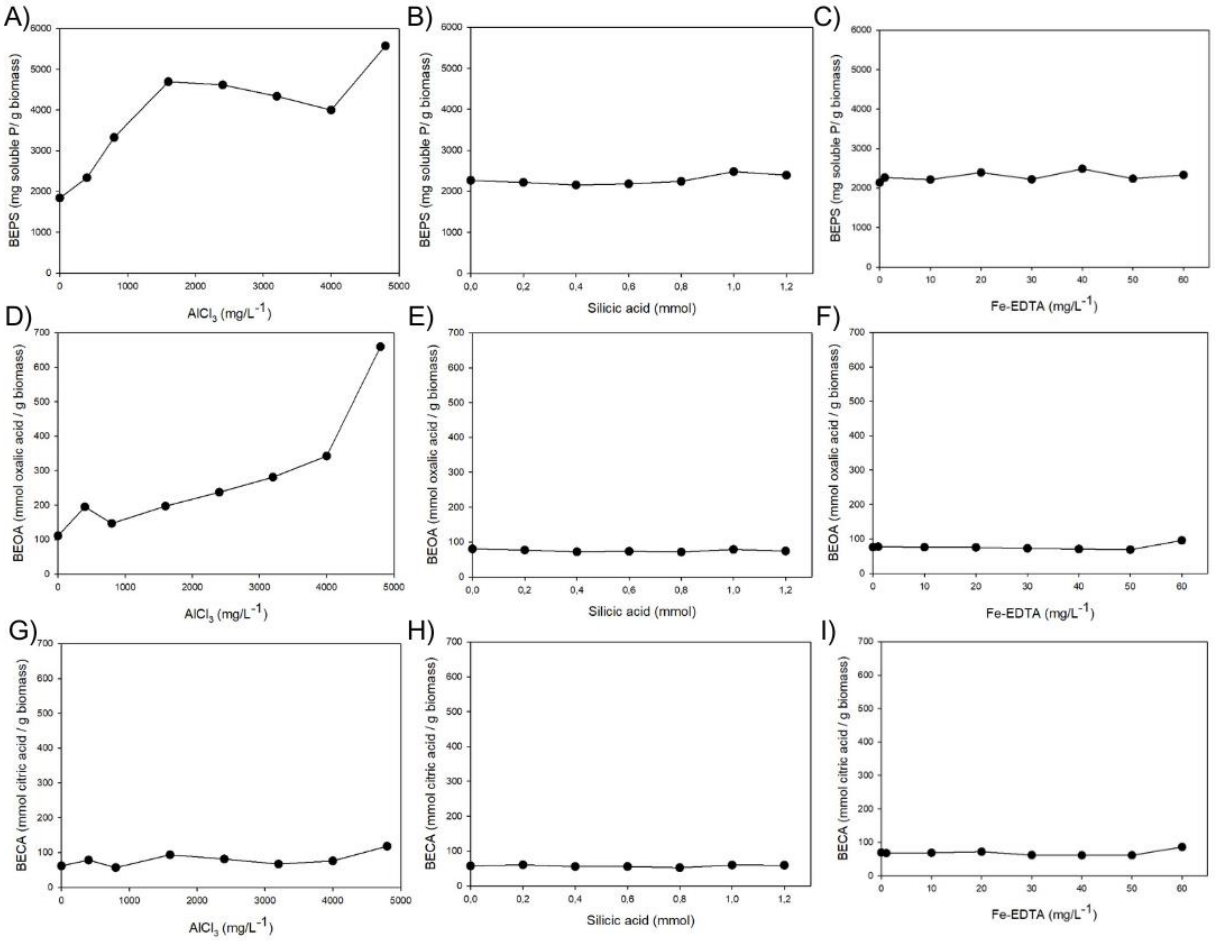

FIGURE 6. Phosphate solubilization efficiency (**BEPS)** (mg soluble P/g biomass) calculated using the data observed in treatments supplemented with **A)** 0, 400, 800, 1600, 2400, 3200 and 4000 mg L^{-1} of $AlCl_3$. **B)** 0, 0.2, 0.4, 0.6, 0.8, 1.0, and 1.2 mmol L^{-1} of silicic acid. **C)** 0, 1, 10, 20, 30, 40, 50 and 60 mg L^{-1} of Fe-EDTA. Oxalic acid production efficiency (**BEOA)** (mmol oxalic acid/g biomass) calculated using the data observed in treatments supplemented with **D)** 0, 400, 800, 1600, 2400, 3200 and 4000 mg L^{-1} of $AlCl_3$. **E)** 0, 0.2, 0.4, 0.6, 0.8, 1.0, and 1.2 mmol L^{-1} of silicic acid. **F)** 0, 1, 10, 20, 30, 40, 50 and 60 mg L^{-1} of Fe-EDTA. citric acid production efficiency (**BECA)** (mmol citric acid/g biomass) calculated using the data observed in treatments supplemented with **G)** 0, 400, 800, 1600, 2400, 3200 and 4000 mg L^{-1} of $AlCl_3$. **H)** 0, 0.2, 0.4, 0.6, 0.8, 1.0, and 1.2 mmol L^{-1} of silicic acid. **I)** 0, 1, 10, 20, 30, 40, 50 and 60 mg L^{-1} of Fe-EDTA.

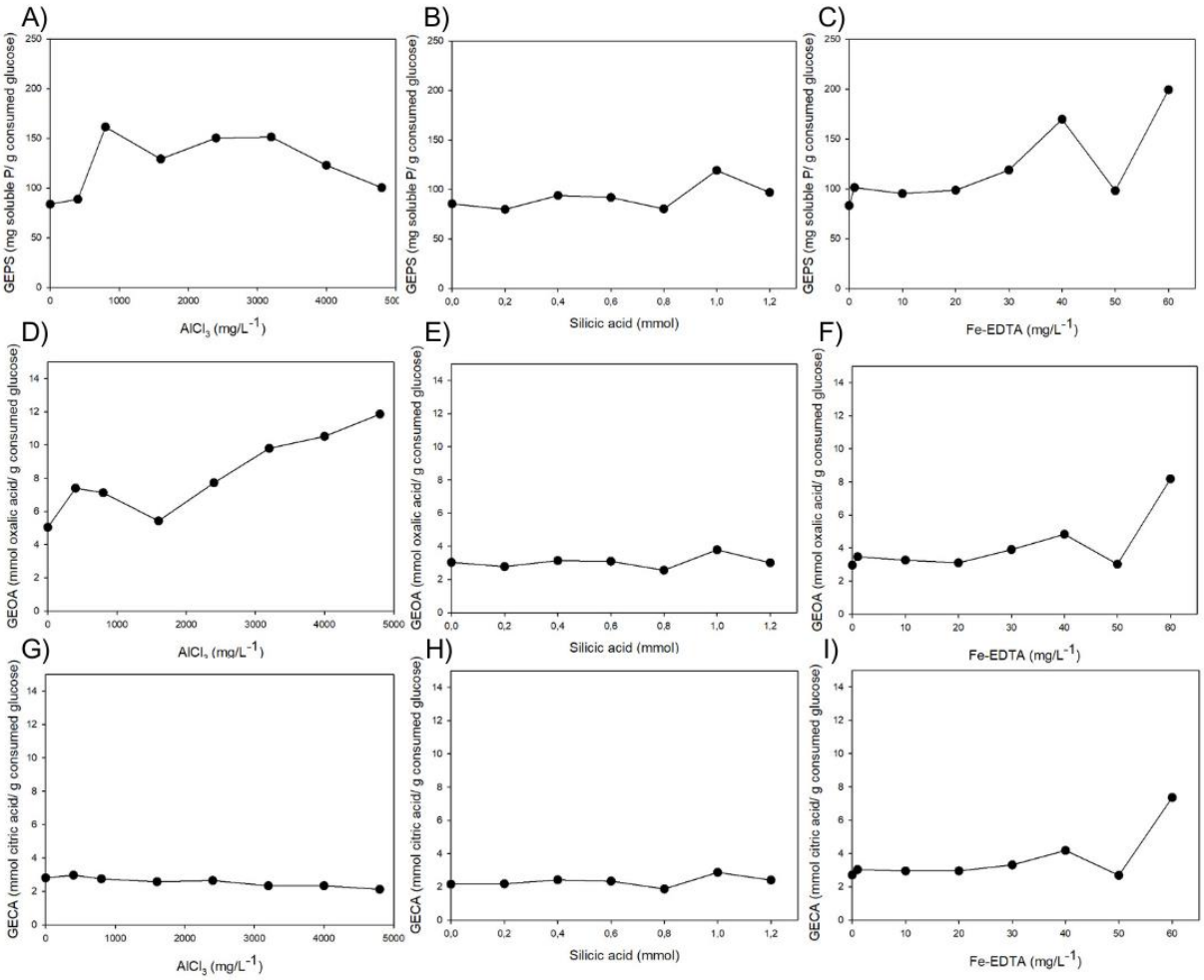

FIGURE 7. Phosphate solubilization efficiency per glucose consumed (**GEPS)** (mg soluble P/g consumed glucose) calculated using the data observed in treatments supplemented with **A)** 0, 400, 800, 1600, 2400, 3200 and 4000 mg L^{-1} of $AlCl_3$. **B)** 0, 0.2, 0.4, 0.6, 0.8, 1.0, and 1.2 mmol L^{-1} of silicic acid. **C)** 0, 1, 10, 20, 30, 40, 50 and 60 mg L^{-1} of Fe-EDTA. oxalic acid production efficiency per glucose consumed (**GEOA)** (mmol oxalic acid/g consumed glucose) calculated using the data observed in treatments supplemented with **D)** 0, 400, 800, 1600, 2400, 3200 and 4000 mg L^{-1} of $AlCl_3$. **E)** 0, 0.2, 0.4, 0.6, 0.8, 1.0, and 1.2 mmol L^{-1} of silicic acid. **F)** 0, 1, 10, 20, 30, 40, 50 and 60 mg L^{-1} of Fe-EDTA. Citric acid production efficiency per glucose consumed (**GECA)** (mmol citric acid/g consumed glucose) calculated using the data observed in treatments supplemented with **G)** 0, 400, 800, 1600, 2400, 3200 and 4000 mg L^{-1} of $AlCl_3$. **H)** 0, 0.2, 0.4, 0.6, 0.8, 1.0, and 1.2 mmol L^{-1} of silicic acid. **I)** 0, 1, 10, 20, 30, 40, 50 and 60 mg L^{-1} of Fe-EDTA.

Fe-EDTA supplementation resulted in increased efficiency at the highest concentration tested, corresponding to a GEOA of 8.18 mmol L^{-1} of oxalic acid for each gram of glucose consumed (Figure 7f).

Regarding the citric acid production efficiency per glucose consumed (GECA), no increases in efficiency were observed due to supplementation with $AlCl_3$ (Figure 10g) or with supplementation of silicic acid (Figure 7h). Increases in GECA were calculated concerning Fe-EDTA supplementation at the highest concentration tested (60 mg L^{-1}), corresponding to 7.36 mmol L^{-1} of citric acid for each gram of glucose consumed (Figure 7i).

DISCUSSION

All tested clays increased the solubilization of Araxá RP. The results indicate that this increase is not attributed to a physical interaction between the fungal mycelium and the clay particles, as there were no significant differences observed in treatments with different clays whether they were inside or outside the capsules (Figure 1). Several authors have reported that clays can chemically interact with microorganisms in addition to physical interactions (Burford et al., 2003; Fomina et al., 2006; Cuadros, 2017; Li et al., 2019; Fomina & Skorochod, 2020; Yang et al., 2023). Microorganisms can transform soil clays, releasing chemical elements from their composition or adsorbed on them (Heckman et al., 2012). Experiments have demonstrated the ability of fungi, such as *Aspergillus*, to extract aluminum from boehmite (Polák et al., 2018).

In relation to the chemical composition of clays, it is evident that those containing aluminum (Gibbsite and kaolinite) exhibited higher solubilization of P (Figure 1a). In treatments inoculated with the fungus, a portion of these clays dissolved, as evidenced by the quantity of clay remaining inside the capsules (Figure 2a).

The dissolution of gibbsite and boehmite by the fungi A. niger and *Aspergillus clavatus* has been reported, leading to an increase in the aluminum content in the culture medium (Polák et al., 2018). This is facilitated by the acidic pH resulting from the fungus's activity (figure 1c). These clays undergo transformation in an acidic environment, as illustrated by the equations outlined by Lindsay (1979):

$Al(OH)_3$ (Gibbsite) + $3H^+ \leftrightarrows Al^{3+}+3H_2O$

$Al_2Si_2O_5(OH)_4$ (Kaolinite) + $\leftrightarrows 2Al^{3+}+2H_4SiO_4+H_2O$

Thus, we can observe that in both clays, there was a release of Al3+. This could be attributed to the increase in P solubilization. A common fungal response to stress induced by $Al3^+$ is the release of organic acids, particularly oxalic acid, to mitigate stress (Ahonen-Jonnarth et al., 2000; Jarosz-Wilkolazka & Gadd, 2003; Peng et al., 2017). Interestingly, this stress response to aluminum is also reported in plants (Miyagi et al., 2012; Chauhan et al.,

2021; Dos Santos et al., 2022). This finding aligns with the data obtained, as treatments with gibbsite and kaolinite exhibited higher titratable acidity (Figure 1d) and increased synthesis of oxalic and citric acids (Figure 1e-1f).

The impact of aluminum on the synthesis of organic acids was demonstrated in the experiment involving $AlCl_3$ supplementation. It was observed that an increase in the concentration of this element led to elevated synthesis of oxalic and citric acids (Figure 3e-3f). Nevertheless, it is notable that the fungus primarily responds to aluminum by stimulating the synthesis of oxalic acid over other acids (Figure 3e). $AlCl_3$ supplementation also increased the efficiency of fungal biomass in producing oxalic acid (Figure 6d).

Interestingly, oxalic acid is reported to be one of the most efficient acids for the phosphate solubilization process (Mendes et al., 2020).

The synthesis of oxalic acid is a complex and highly regulated process involving several enzymes and regulatory proteins. However, these enzymes do not respond to phosphorus content (Yang et al., 2017). Respond mainly to the carbon source and pH of the medium (Arst & Peñalva, 2003; David et al., 2005; Poulsen et al., 2012).

Thus, we can observe that the fungus significantly increases the production of oxalic acid, not with the objective of solubilizing P, but rather for protection against the stress caused by aluminum. Unintentionally, the high concentration of oxalic acid produced significantly increases the solubilization of P.

Even though both clays contain aluminum in their composition, gibbsite was highlighted for its role in increasing P solubilization (Figures 1a). This may be possible because, according to Lindsay (1979), gibbsite has greater solubility, leading to a higher Al^{3+} content in the medium. Furthermore, the dissolution of kaolinite in acidic pH, in addition to generating Al^{3+}, produces silicic acid (Lindsay, 1979). Silicic acid is known to be essential in environments for maintaining balanced Al^{3+} concentrations (Exley et al., 2019). One of the most important functions of silicic acid in natural environments is the formation of aluminosilicates, thereby reducing the availability of aluminum for different organisms (Exley et al., 2019). Thus, this compound could be responsible for the lower stress caused by Al^{3+} to the fungus in its presence, resulting in a lower synthesis of organic acids as observed in gibbsite treatments (Figures 1e). Silicic acid could directly participate in the solubilization of P, as inorganic acids also play a role in the solubilization of phosphates (Prabhu et al., 2019). Silicic acid could also have positive effects on the growth of *A. niger*, by complexing toxic elements such as fluorine (Roberson & Barnes, 1978).

The results obtained from the silicic acid supplementation experiment suggest that silicic acid, in concentrations that could be produced by the solubilization of kaolinite, contributes to the solubilization of araxá RP (Figure 4a). However, the results show that the solubilization capacity of silicic acid is significantly lower compared to the effect observed

with increased oxalic acid content. This difference causes gibbsite to enhance a greater increase in P solubilization than kaolinite.

The goethite and hematite clays showed a significant increase in P solubilization, but lower than the gibbsite and kaolinite clays (Figure 1a). This could not be explained by the effect of aluminum since this element is not present in their composition. Furthermore, In these treatments, it is evident that the enhancement of phosphate solubilization is not correlated with an increase in organic acid synthesis (Figure 1e- 1f). Therefore, it is possible that the observed solubilization is the result of pH changes, even without statistically significant differences (Figure 1c). In general, the pH levels observed in treatments supplemented with goethite and hematite were slightly lower compared to the control (Figure 1c). pH is a logarithmic scale, meaning even small changes in the medium's pH can result in a significant difference in H^+ concentration. For example, a decrease in pH from 2.1 to 2.05 results in a 12% increase in the H^+ concentration (Covington et al. 1983) These slight differences in pH can lead to significant alterations in phosphate solubilization (Prabhu et al., 2019).

Hematite and goethite are characterized by greater stability at acidic pH, but partial solubilization at extreme pH caused by the fungus is possible (Lindsay, 1979). As a result of solubilization, an increase in Fe3+ content is observed, as shown in the following equation:

Fe_2O_3 (Hematite) + $3H^+ \leftrightarrows Fe^{3+} + \frac{3}{2}H_2O$

FeOOH (Goethite) + $3H^+ \leftrightarrows Fe^{3+} + 2H^2O$

The increase in Fe^{3+} availability can directly impact cellular processes such as respiration (Misslinger et al., 2021). Iron is a structural component of the enzyme complexes in the electron transport chain and is crucial for electron transfer during respiration (Joseph-Horne et al., 2001). During respiration, H^+ ions are expelled outside the cell, leading to a decrease in pH. This change may have a direct correlation with P solubilization (Prabhu et al., 2018).

This finding is consistent with the results of the experiment involving Fe-EDTA supplementation, where concentrations exceeding 30 mg L^{-1} led to a small but significant decrease in pH and an increase in P solubilization without a concurrent rise in the production of organic acids. Interestingly, increases in the efficiency of glucose consumption in the production of organic acids were observed in the treatments with the highest Fe-EDTA supplementation tested (Figure 7g-7i). However, this may be attributed to the low glucose consumption observed in these treatments (Figure 5d).

Concentrations above 50 mg L^{-1} caused a negative effect on the fungus, which was observed in biomass losses (Figure 5b). This may be caused by the antimicrobial activity of iron oxides (Seabra et al., 2017). In the capsule experiment, it was observed that the

remaining clay content for the treatments with goethite and hematite did not vary significantly (Figure 5a), indicating that there was no dissolution. However, considering that in the capsule experiments, an amount equivalent to 1500 mg L^{-1} of clay, a minimum dissolution of the clays could sustain a concentration equivalent to 30 mg L^{-1} of Fe-EDTA. The fact that these clays remain more stable in the culture medium can lead to a greater adsorption of toxic elements (Cuadros, 2017). In an acidic medium, these clays have a positive charge, improving their capacity to interact with anions like fluorine, which may also have a positive impact on fungal activity and on P solubilization.

The results of this study suggest that the enhanced solubilization of araxá RP by the fungus *A. niger* FS1, in the presence of mineral clay supplementation, is influenced by their chemical composition rather than any physical effects of the clay particles. The increase in P solubilization by gibbsite is attributed to the higher Al^{3+} content, leading to the synthesis of organic acids (Figure 8). Similarly, in the case of kaolinite, the rise in solubilization is linked to the Al3+ content, the induction of organic acid synthesis, and to a lesser extent, the impact of silicic acid (Figure 8). As for goethite and hematite clays, the improved solubilization is associated with an increase in cellular respiration, triggered by higher levels of Fe^{3+} (Figure 8). Goethite and hematite clays can also participate in the adsorption of toxic elements such as fluorine, resulting in positive effects on fungal activity.

The results obtained in this study provide a better understanding of how microbial phosphate solubilization processes can occur in the soil and how gibbsite can play a crucial role (Figure 9). Phosphate-solubilizing microorganisms are primarily found in the rhizosphere of plants, where they utilize plant rhizodeposition as a carbon source to support metabolic processes (Figure 9).

These microorganisms and plant roots engage in various metabolic processes that lead to the acidification of the environment. Under these conditions, gibbsite particles present in the soil can dissolve, thereby increasing the Al^{3+} content (Figure 9). In response to the increase in Al^{3+}, microorganisms and plants synthesize organic acids (Figure 9). Organic acids, such as oxalic acid, can interact with RPs in the soil, leading to the release of orthophosphate from the soil solution and the formation of calcium oxalate minerals (Figure 9).

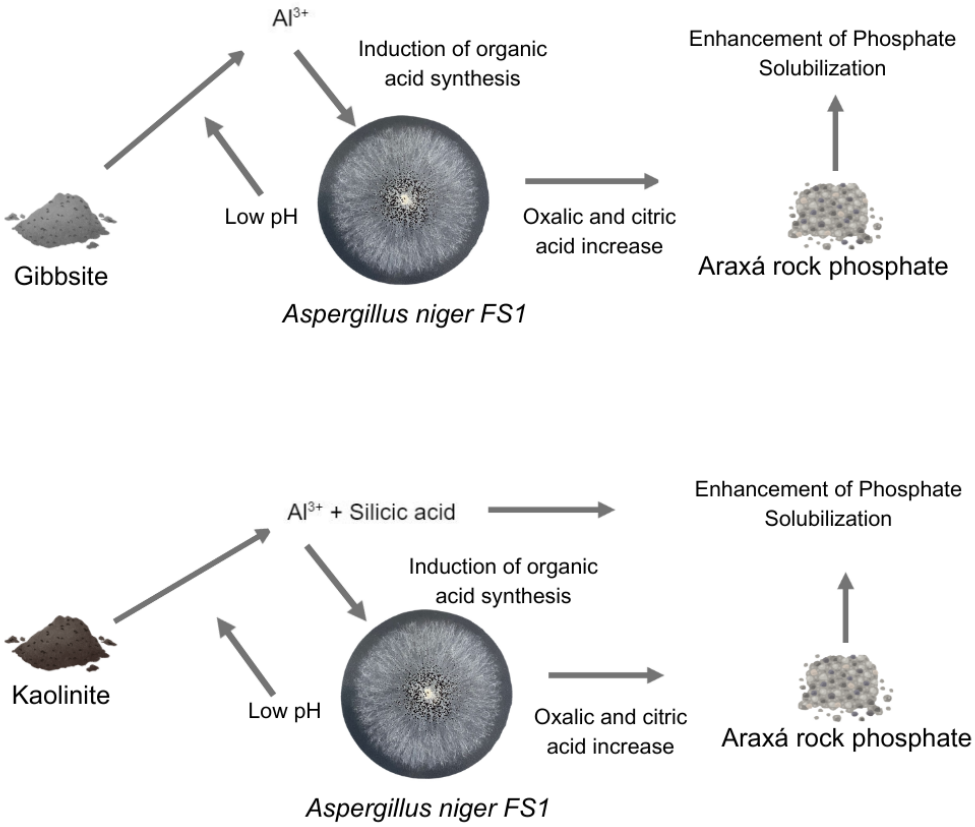

FIGURE 8. Main mechanisms involved in the increase in the solubilization of natural Áraxa RP by the fungus *A. niger* FS1 using clays. The dissolution of gibbsite in an acidic medium leads to high levels of Al^{3+}, causing a protective response to stress by the fungus, increasing the synthesis of organic acids, which inadvertently impacts the solubilization of P. Kaolinite also causes increases in the synthesis of organic acids by increasing Al^{3+} resulting from its dissolution, but this is less intense due to the presence of silicic acid, which also has a small participation in the solubilization of P.

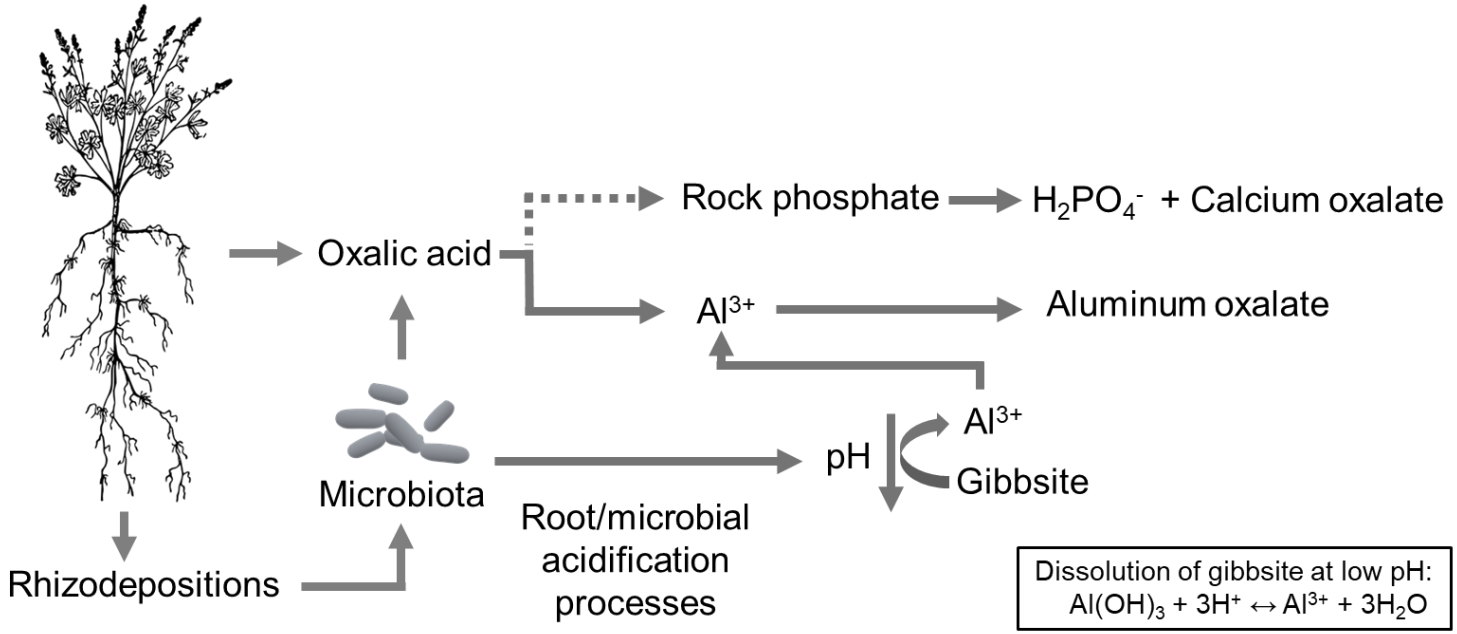

FIGURE 9. Hypothetical interaction between gibbsite and RP solubilization by microorganisms in the plant rhizosphere. Plant roots and rhizosphere microorganisms perform metabolic processes that lead to soil acidification, such as aerobic respiration, NH_4^+ uptake, nitrification, organic acids release etc. At low pH, gibbsite is unstable and releases Al^{3+} into the soil solution. To circumvent aluminum toxicity, plants and microorganisms produce oxalic acid to form nontoxic aluminum oxalate. Al^{3+} may also work as a signaling molecule to induce oxalic acid production. Oxalic acid production by microorganisms is dependent on the metabolism of rhizodepositions released by the plant. The excess of oxalic acid produced in response to aluminum ions can also react with RPs, leading to the release of orthophosphate into the soil solution and the formation of calcium oxalate minerals.

CONCLUSIONS

This study confirmed that different types of clay minerals (kaolinite, hematite, goethite, and gibbsite) significantly increase the solubilization of Araxá RP by the fungus *A. niger* FS1, with gibbsite being the most effective. The results indicate that this increase is not attributed to a physical interaction between the fungal mycelium and the clay particles, but rather to their chemical interaction. Experiments revealed that $AlCl_3$ supplementation notably enhances phosphate solubilization and organic acid production. Tests with silicic acid and iron demonstrate the positive influence of these elements on the phosphate solubilization processes. The primary mechanism responsible for significant increases in phosphate solubilization when gibbsite and kaolinite are supplemented is the rise in aluminum concentration. This leads to a protective response to aluminum stress, which, in turn, enhances the synthesis of organic acids, primarily oxalic acid. In the case of goethite and hematite clays, the mechanisms are less well-defined but could be primarily associated with the beneficial effects of iron on fungal metabolic processes such as respiration, as well as the clays' capacity to adsorb toxic elements.The ability of the fungus to solubilize phosphates in the presence of these minerals can provide a practical alternative to reduce the reliance on expensive chemical fertilizers and promote more environmentally friendly agricultural practices.

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SUPPLEMENTARY MATERIAL

Table 1. Soluble phosphorus (mg L^{-1}), biomass (g L^{-1}), titratable acidity (mmol L^{-1}), pH, oxalic acid (mmol L^{-1}), citric acid (mmol L^{-1}), remanent glucose (mg L^{-1}), measured in the supernatant of the modified NBRIP medium, supplemented 0, 400, 800, 1600, 2400, 3200 and 4000 mg L^{-1} of $AlCl_3$, inoculated with *A. niger* FS1, after 7 days incubation. Lines with the same lowercase letter do not differ significantly from each other according to the scott-knott test ($p<0.05$).

$AlCl_3$ (mg L-1)	Soluble P (mg L-1)	Biomass (mg L-1)	pH	Tritatable acidity (mmol)	Oxalic acid (mmol)	Citric acid (mmol)	Remanent glucose (g L-1)
0	101,41 e	0,049 b	3,12 a	26,82 c	5,39 g	3,01 c	8,93 a
400	116,52 e	0,043 b	2,69 b	30,17 c	8,32 f	3,34 c	8,87 a
800	185,27 d	0,056 b	2,12 c	40,23 c	8,18 f	3,16 c	8,85 a
1600	258,32 c	0,055 b	1,91 c	67,05 c	10,84 e	5,15 b	8,00 b
2400	311,83 b	0,068 a	1,83 c	87,17 b	16,02 d	5,49 b	7,93 b
3200	345,82 a	0,080 a	1,77 c	87,17 b	22,57 c	5,38 b	7,70 b
4000	304,02 b	0,076 a	1,80 c	140,81 a	26,03 b	5,77 b	7,53 c
4800	318,47 b	0,055 b	1,82 c	144,16 a	36,28 a	6,49 a	6,94 d

Columns with the same lowercase letter are not statistically different by the scott-knott test ($p < 0.05$). Nd= Treatments don't differ statistically

Table 2. Soluble phosphorus (mg L^{-1}), biomass (g L^{-1}), titratable acidity (mmol L^{-1}), pH, oxalic acid (mmol L^{-1}), citric acid (mmol L^{-1}), remanent glucose (mg L^{-1}), measured in the supernatant of the modified NBRIP medium, supplemented 0, 0.2, 0.4, 0.6, 0.8, 1.0, and 1.2 mmol L^{-1} of silicic acid, inoculated with *A. niger* FS1, after 7 days incubation. Lines with the same lowercase letter do not differ significantly from each other according to the scott-knott test ($p<0.05$).

Silicic acid (mmol)	Soluble P (mg L-1)	Biomass (mg L-1)	pH	Tritatable acidity (mmol)	Oxalic acid (mmol)	Citric acid (mmol)	Remanent glucose (g L-1)
0	133,01 b	58,67 nd	2,22 nd	25,15 nd	4,71 nd	3,37 nd	8,44 nd
0,2	130,84 b	59,00 nd	2,19 nd	25,15 nd	4,54 nd	3,58 nd	8,36 nd
0,4	134,36 b	62,33 nd	2,21 nd	25,15 nd	4,49 nd	3,46 nd	8,57 nd
0,6	141,14 a	64,67 nd	2,19 nd	26,15 nd	4,75 nd	3,60 nd	8,46 nd
0,8	142,90 a	63,67 nd	2,26 nd	27,16 nd	4,55 nd	3,34 nd	8,22 nd
1	146,42 a	59,00 nd	2,19 nd	27,16 nd	4,64 nd	3,52 nd	8,77 nd
1,2	147,64 a	61,67 nd	2,21 nd	26,15 nd	4,58 nd	3,66 nd	8,48 nd

Columns with the same lowercase letter are not statistically different by the scott-knott test ($p < 0.05$). Nd= Treatments don't differ statistically

Table 3. Soluble phosphorus (mg L^{-1}), biomass (g L^{-1}), titratable acidity (mmol L^{-1}), pH, oxalic acid (mmol L^{-1}), citric acid (mmol L^{-1}), remanent glucose (mg L^{-1}), measured in the supernatant of the modified NBRIP medium, supplemented 0, 1, 10, 20, 30, 40, 50 and 60 mg L^{-1} of Fe-EDTA, inoculated with *A. niger* FS1, after 7 days incubation. Lines with the same lowercase letter do not differ significantly from each other according to the scott-knott test ($p<0.05$).

Fe-EDTA (mg L-1)	Soluble P (mg L-1)	Biomass (mg L-1)	pH	Tritatable acidity (mmol)	Oxalic acid (mmol)	Citric acid (mmol)	Remanent glucose (g L-1)
0	124,51 c	58,33 nd	2,23 a	30,17 nd	4,43 nd	4,05 nd	8,50 nd
1	128,23 c	56,67 nd	2,21 a	30,84 nd	4,39 nd	3,84 nd	8,73 nd
10	131,37 c	59,33 nd	2,20 a	30,51 nd	4,51 nd	4,07 nd	8,62 nd
20	139,53 b	58,33 nd	2,20 a	30,17 nd	4,39 nd	4,18 nd	8,58 nd
30	138,38 b	62,33 nd	2,11 b	28,83 nd	4,54 nd	3,86 nd	8,84 nd
40	160,70 a	64,67 nd	2,07 b	30,51 nd	4,57 nd	3,96 nd	9,05 nd
50	138,67 b	62,00 nd	2,12 b	29,50 nd	4,27 nd	3,80 nd	8,59 nd
60	107,20 d	46,00 nd	2,26 a	29,50 nd	4,40 nd	3,96 nd	9,46 nd

Columns with the same lowercase letter are not statistically different by the scott-knott test ($p < 0.05$). Nd= Treatments don't differ statistically

CHAPTER IV

Gibbsite improves the solubilization of low-reactivity rock phosphates by *Aspergillus niger*

GIBBSITE IMPROVES THE SOLUBILIZATION OF LOW-REACTIVITY ROCK PHOSPHATES BY *Aspergillus niger*

ABSTRACT

The phosphate fertilizer industry utilizes high-quality natural rock phosphates (RPs), along with significant amounts of sulfuric acid, to solubilize RPs. After undergoing various industrial processes with high energy consumption, soluble phosphate fertilizers suitable for agricultural crops are produced. An alternative to these processes involves the use of phosphate-solubilizing microorganisms. Through various mechanisms, these microorganisms can solubilize RPs, including those with low reactivity that are typically considered to have low agricultural suitability. However, enhancing the capacity of these microorganisms is essential to make them a viable alternative to the current industry processes. Previous studies have shown that mineral clays can increase the solubilization of Araxá RP by the fungus *A. niger* FS1. The effects of these clays on other RPs and synthetic phosphates remain unknown. Therefore, this study aims to investigate the impact of gibbsite, kaolinite, goethite, and hematite clays on the solubilization of Araxá, Argelia, Bayovar, Catalão, Marrocos, and Patos de Minas natural RPs and Aluminium, tricalcium and iron synthetic phosphates by the fungus *A. niger* FS1. The experiment utilized the NBRIP cultivation medium supplemented with the different phosphates individually, as well as with the clays gibbsite, kaolinite, goethite, and hematite. Gibbsite exhibited significant potential in inducing the solubilization of natural RPs, achieving approximately 100% solubilization of all tested RPs. Kaolinite was particularly effective in the solubilization of iron phosphate, with a solubilization rate of 80.9%. Only goethite enhanced the solubilization of aluminum phosphate, with a solubilization rate of 22.72%. Therefore, the clay adition has shown great potential for enhancing the solubilization of phosphates by the fungus *A. niger* FS1.

KEYWORDS: Soil clays, phosphate solubilization, organic acids, kaolinite, gibbsite, goethite, hematite, phosphate solubilizing microorganisms.

INTRODUCTION

The global phosphate fertilizer industry depends on the mining of natural (RP) deposits (Withers et al., 2015). RP are minerals that contain phosphorus in the form of calcium phosphates known as apatites. These can be of different compositions, but the most common form found is fluorapatite (Van Kauwenbergh, 2010; Toama, 2017).

RPs are a non-renewable natural resource, but the size of the reservoirs of this resource is still controversial (Van Kauwenbergh, 2010; Cordell & White, 2011). Authors have estimated that this resource could be depleted in the current century (Vaccari, 2009; Cordell & Tema, 2010; Cordell & White, 2011). However, the most recent data indicate that RP reserves are around 74 billion tons (U.S. Geological Survey, Mineral Commodity, 2024), largely surpassing the most pessimistic projections that quantify reserves at around 16 million tons (Cordell & White, 2011). Nevertheless, there is a great need to use this resource more efficiently (Withers et al., 2015; Roy et al., 2016).

The phosphate fertilizer industry primarily utilizes high-quality RP, which contains a P_2O_5 content greater than 15% and exhibits high reactivity (Toama, 2017). Moreover, the industry consumes a significant amount of energy and inputs, such as inorganic acids, which pose a severe contamination threat to ecosystems (Nadarajan & Sukumaran, 2021). Biotechnological strategies enabling the utilization of lower-quality natural phosphates are crucial to enhance the availability of this resource.

A sustainable and efficient alternative is the use of phosphate-solubilizing microorganisms (PSM), which are defined as all microorganisms that have the ability to transform insoluble forms of phosphorus (P) unavailable to plants into soluble and available forms (Tariq & Ahmed, 2023; Li et al., 2023).

Experiments with PSM have demonstrated the potential to solubilize various sources of phosphorus, including RP (Mendes et al., 2013; Amarasinghe et al., 2022). However, it is essential to optimize biological solubilization processes to establish them as viable alternatives for the industrial production of fertilizers. Previous studies have shown that the solubilization of Araxá RP by the fungus A. niger FS1 increased when supplemented with clays such as gibbsite, kaolinite, goethite, and hematite. Gibbsite and kaolinite have been found to induce the synthesis of oxalic acid, to mitigate the accumulation of aluminum resulting from the dissolution of these clays. The oxalic acid released into the supernatant enhances the solubilization of Araxá RP. These mechanisms may also apply to other sources of P. However, the impact of clays on other rock phosphates remains unknown. Therefore, the aim of this research is to investigate the influence of supplementation with gibbsite, kaolinite, goethite, and hematite on the solubilization of various RPs and synthetic phosphates from different sources, each with unique compositions and phosphorus contents

MATERIAL E METHODS

The experiments were conducted at the Laboratory of Microbial Ecology, Department of Microbiology, located at the Institute of Biotechnology Applied to Agriculture (BIOAGRO), Universidade Federal de Viçosa, Viçosa, MG.. For the following experiments, 6 natural RPs were used: Araxá, Argelia, Bayovar, Catalão, Marrocos, and Patos de Minas, along with 3 synthetic phosphates: aluminum, calcium, and iron phosphate. The phosphorus contents of the phosphates are detailed in Table 1. Kaolinite, hematite, goethite, and gibbsite clays (Sigma-Aldrich Brasil Ltda.) were utilized. The biological material employed was the fungal isolate *A. niger* FS1, sourced from the collection of phosphate-solubilizing fungi at the Laboratory of Microbial Ecology. The fungus was cultured on potato dextrose agar (PDA) at 30°C and subcultured with fresh PDA every seven days.

Table 1. Physical and chemical characterization of rock and synthetic phosphates used.

Phosphate	Iron	Tricalcium	Aluminium	Patos de Minas	Argelia	Catalão	Marrocos	Bayovar	Araxá
P (%)	16.57	19.97	25.39	14.4	16.2	11.8	13.5	12.5	13.97
Particles size (mm)	< 0.050	< 0.050	< 0.050	< 0.075	< 0.075	< 0.075	< 0.075	< 0.075	< 0.075
Geological origin	-	-	-	Methamorphic	Sedimentary	Igneus	Sedimentary	Sedimentary	Igneus

Determination of the effect of clays on the solubilization of different phosphates by the fungus *A. niger* FS1

125 mL Erlenmeyer flasks, containing 50 mL of modified NBRIP medium containing per liter: glucose, 10 g; MgCl2·6H2O, 5 g; MgSO4·7H2O, 0.25 g; KCl, 0.2 g; and (NH4)2SO4, 0.1 g (Nautiyal, 1999), were supplemented with 3 g L^{-1} of each phosphate. The medium was also supplemented with 1500 mg L^{-1} of kaolinite, hematite, goethite, and gibbsite clays. These flasks were then inoculated with 10^6 spores of *A. niger* FS1, which had been cultured in PDA medium for 7 days at 30°C. The spore suspension was prepared in a 0.1% Tween-80 solution. Each type of clay was tested individually. Control treatments without the clay adition, fungal inoculation, or Araxá phosphate were also included. The flasks were incubated on a horizontal shaker for 7 days at 30°C and 150 rpm. After incubation, the supernatants were filtered through JP42 filter paper (QuantyR). A 10 mL aliquot was taken before filtration for the subsequent analysis of organic acids. The pH was measured using a bench pH meter, while titratable acidity was determined through titration with NaOH. Soluble phosphorus was evaluated in the supernatant using

the colorimetric technique (Braga and Defelipo, 1974), on a spectrophotometer Genesys 10S UV-VIS (Thermo Scientific). The percentage of P solubilization was calculated using the following equation: ((Soluble P measured in the supernatant) - (Soluble P measured in the control without clay and inoculation) * 100) / (Total P content of the P source).

Fungal biomass was determined by drying samples at 65°C until a constant weight was achieved. Subsequently, to eliminate the weight of clay and RP residues, the dried samples were incinerated in a muffle furnace at 500°C for 5 hours. Finally, biomass was calculated by subtracting the weight of the mineral remains obtained after incineration from the dry weight measured at 60°C.

The quantification of organic acids and remnant glucose was conducted using high-performance liquid chromatography (HPLC), as described by Van Hees et al. (1999), on a Shimadzu Prominence chromatograph equipped with a refractive index detector (RID), model RID-20A. For the analysis, an HPX 87H column (Aminex®) (300 mm x 7.8 mm) and a corresponding pre-column (Bio-Rad) were employed, maintained at a temperature of 45°C. A 5 mmol L^{-1} sulfuric acid solution was utilized as the mobile phase, delivered at a constant flow rate of 0.7 mL min^{-1}. Standard curves for organic acids were generated using oxalic and citric acid concentrations of 0, 2.5, 5, 10, 20, 40, 80, and 160 mmol L^{-1}. For glucose, the standard curve included concentrations of 0, 5.5, 11, 22, 44, and 88 mmol L^{-1}. Data processing were performed using Lab Solutions software, Shimadzu Corporation (2013).

Determination of efficiency indices

With the data obtained previously, efficiency coefficients were calculated. These were: phosphate solubilization efficiency (BEPS), oxalic acid production efficiency (BEOA), citric acid production efficiency (BECA), phosphate solubilization efficiency per glucose consumed (GEPS), oxalic acid production efficiency per glucose consumed (GEOA), and citric acid production efficiency per glucose consumed (GECA). The coefficients were calculated using the following equation:

BEPS = [Available P (mg L^{-1}) / Biomass (g)]

BEOA = [Oxalic acid production (mmol) / Biomass (g)]

BECA = [Citric acid production (mmol) / Biomass (g)]

GEPS = [Available P (mg L^{-1}) / Consumed glucose (g)]

GEOA = [Oxalic acid production (mmol) / Consumed glucose (g)]

GECA = [Citric acid production (mmol) / Consumed glucose (g)]

Experimental design and statistical analyzes

The experiments were set up in a completely randomized design, following a factorial scheme of the type (9 x 2) x 3, where 9 represents the type of phosphate, 2 represents the absence or presence of clay, and 3 represents the number of repetitions. Each clay was tested in independent experiments. The data obtained underwent ANOVA, and the treatment means were compared using the Tukey test at a 5% significance level, as well as the Scott-Knott test at a 5% significance when necessary.

RESULTS

Effect of clays on the solubilization of different phosphates by *A. niger F*S1

The impact of clays on the solubilization of phosphates by the fungus *A. niger* FS1 varied depending on the type of phosphate used (Figures 1). Generally, clays showed better performance in enhancing the solubilization of natural RPs (Figure 1). For iron phosphate, only kaolinite significantly increased solubilization compared to the control without clay addition, as indicated by the Tukey test ($p < 0.05$) (Figure 1a). In the kaolinite treatment, 400.2 mg L^{-1} of soluble P was detected in the supernatant, representing an 80.9% solubilization rate (Figure 1a). Therefore, the solubilization of iron phosphate decreased in the following sequence based on the clay type used: Kaolinite > hematite > gibbsite > goethite (Figure 1a).

No increases in calcium phosphate solubilization were observed in treatments with clay supplementation. The control without clay addition reached 100% solubilization (Figure 1b). Goethite significantly decreased the solubilization of calcium phosphate compared to the control without clay addition according to the Tukey test ($p < 0.05$) (Figure 1b). In this treatment, 555.29 mg L^{-1} was measured, equivalent to 92.68% solubilization of the added phosphate (Figure 1b). Thus, the solubilization of calcium phosphate decreased in the following order: Gibbsite = kaolinite = hematite > goethite (Figure 1b).

In treatments with aluminum phosphate, the lowest levels of solubilization were observed (Figure 1c). The only clay tested that significantly increased solubilization was goethite with 173.07 mg L^{-1} of soluble P, equivalent to a solubilization of 22.72% (Figure 1c). The gibbsite clay significantly decreased the solubilization of aluminum phosphate compared to the control without clay addition according to the Tukey test ($p < 0.05$) (Figure 1c). Thus, the solubilization of aluminum phosphate decreased in the following order: Goethite > kaolinite = hematite = gibbsite (Figure 1c).

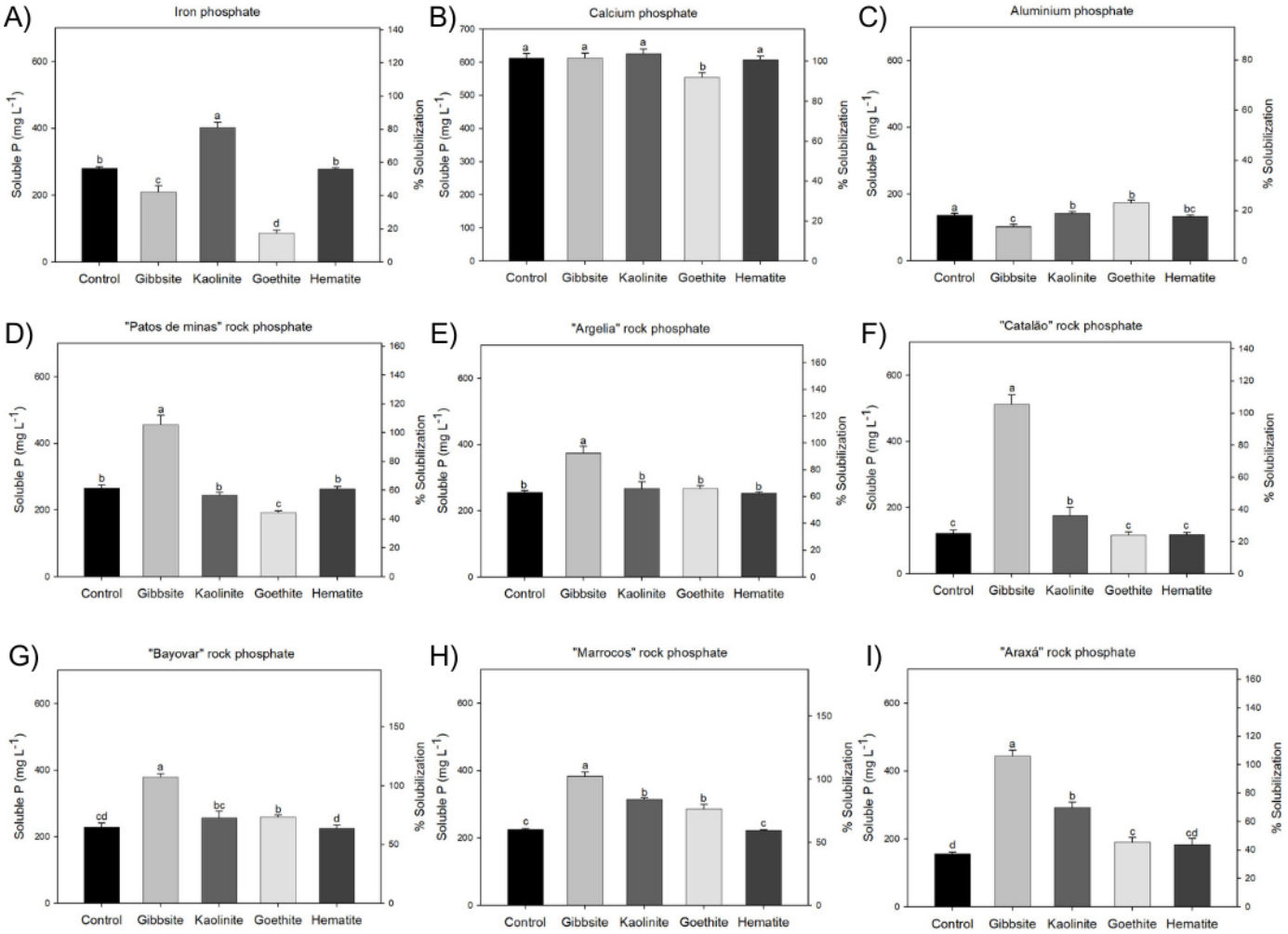

FIGURE 1. Soluble phosphorus (mg L^{-1}) measured in the supernatant of the modified NBRIP medium with **A)** Iron phosphate, **B)** Calcium phosphate, **C)** Aluminium phosphate, **D)** Patos de Minas RP, **E)** Argélia RP and **F)** Catalão RP, **G)** Marrocos RP, **H)** Bayovar RP and **I)** Araxá RP as P source, supplemented with clays, hematite, kaolinite, goethite, and gibbsite. Bars with the same lowercase letter do not differ significantly from each other according to Tukey's test ($p < 0.05$). Control corresponds to the treatment without clay supplementation.

Gibbsite significantly enhanced the solubilization of natural RPs, achieving 100% solubilization in all types of RPs tested with the exception of Argélia RP, where solubilization was 92% (Figures 1d-1i). For Patos de Minas RP, only gibbsite clay significantly increased solubilization compared to the control without clay addition, according to the Tukey test ($p<0.05$) (figure 1d). In this treatment, 456.07 mg L^{-1} of soluble P was measured, equivalent to 105.55% (Figure 1d). Thus, the solubilization of phosphate from Patos de Minas rock decreased in the following order: Gibbsite > hematite = kaolinite > goethite (Figure 1d).

In Argélia RP, a significant increase was observed in treatments supplemented with gibbsite, where 373.4 mg L^{-1} of soluble P was measured in the supernatant, corresponding to 92.22% solubilization of the total phosphate used (Figure 1e). The other phosphates tested did not show significant differences in phosphate solubilization compared to the control without clay addition according to the Tukey test ($p < 0.05$) (Figure 1e). Thus, the solubilization of Argélia RP decreased in the following order: Gibbsite > kaolinite = goethite = hematite (Figure 1e).

In treatments with Catalão RP, the most significant increase in solubilization occurred with clay supplementation. The highest increase was observed in treatments supplemented with gibbsite, where solubilization rose from 25.34% without clay addition to 105.56% with gibbsite supplementation (Figure 1f). Kaolinite also notably enhanced the solubilization of Catalão RP, resulting in 175.55 mg L^{-1} of soluble P measured in the supernatant, equivalent to 36.12% solubilization of the total phosphate added (Figure 1f). The order of decreasing solubilization was as follows: Gibbsite > kaolinite > hematite = goethite (Figure 1f).

For Bayovar RP, it was observed that gibbsite significantly increased phosphate solubilization compared to the control without clay addition according to the Tukey test ($p < 0.05$) (Figure 1g). In this treatment, 380.08 mg L^{-1} of soluble P was measured in the supernatant, equivalent to 107.36% solubilization of the total phosphate used (Figure 1g). The other clays tested showed no differences compared to the control, so the solubilization of Bayovar RP decreased in the following order: Gibbsite > goethite = kaolinite = hematite (Figure 1g).

Gibbsite, kaolinite, and goethite significantly increased the solubilization of Marrocos RP compared to the control group according to the Tukey test ($p < 0.05$) (Figure 1h), the supernatant of these treatments contained 382.87, 314.77, and 285.49 mg L^{-1} of soluble P, equivalent to 102.1%, 83.93%, and 73.41% solubilization of the total phosphate added, respectively (Figure 1h). Therefore, the solubilization of Marrocos RP decreased in the following order: Gibbsite > kaolinite = goethite > hematite (Figure 1h).

In the last RP tested, Araxá RP, gibbsite showed the greatest increase in

solubilization, with 444.22 mg L^{-1} of soluble P, equivalent to 105.99% solubilization of the total phosphate added (Figure 1i). Kaolinite and goethite also significantly increased solubilization compared to the control without clay addition according to the Tukey test ($p < 0.05$) (Figure 1i). In this treatment, 291.76 and 190.06 mg L^{-1} of soluble P were measured respectively, corresponding to 69.61% and 45.35% solubilization of total phosphate respectively (Figure 1i). The solubilization of phosphate from Araxá rock decreased in the following order: Gibbsite > kaolinite > goethite = hematite (Figure 1i).

Regarding the pH measured in the supernatant, there were no significant differences between the treatments supplemented with clay and the control without added clay for aluminum and iron phosphates, as indicated by the Tukey test ($p < 0.05$) (Table 2). However, for calcium phosphate, all clays resulted in a pH significantly lower than the control without clay addition (Table 2). Among natural RPs, only gibbsite exhibited a significantly lower pH than the control without added clay, with the lowest pH recorded at 1.95 in the treatment with Patos de Minas RP (Table 2).

The titratable acidity in treatments with iron phosphate was significantly higher with the addition of gibbsite compared to the control without the addition of clay, as indicated by the Tukey test ($p < 0.05$) (Table 2). The treatments with goethite and hematite showed a significantly lower titratable acidity than the control (Table 2). In treatments with calcium phosphate, only gibbsite led to a significant increase in titratable acidity (Table 2). For aluminum phosphate, both gibbsite and hematite outperformed the control without clay addition (Table 2).

In natural RPs, a significant impact on titratable acidity was observed in treatments supplemented with gibbsite. The greatest increase was noted in the treatment with Araxá RP, where the control without the addition of clay had an acidity of 26.82 mmol L^{-1}, while the treatment with gibbsite showed an acidity of 110.63 mmol L^{-1} (Table 2). In Catalao RP, treatments supplemented with gibbsite and kaolinite showed statistically higher titratable acidity compared to the control without clay addition, as indicated by the Tukey test ($p<0.05$) (Table 2). For the other natural RPs, only gibbsite significantly increased the titratable acidity (Table 2).

In general, the highest production of oxalic acid was observed in treatments with natural RPs (Table 3). For iron and aluminum phosphates, oxalic acid production was only significantly higher in treatments supplemented with gibbsite compared to the control without clay addition, as indicated by the Tukey test ($p < 0.05$) (Table 3). In treatments with calcium phosphate, oxalic acid contents were significantly higher in treatments with gibbsite and kaolinite (Table 3).

In all treatments with natural RPs, the oxalic acid content was significantly higher in treatments supplemented with gibbsite and kaolinite compared to the control without clay

Table 2. Titratable acidity (mmol L^{-1}) and pH measured in the supernatant of the modified NBRIP medium with different phosphates as P source, supplemented with clays, hematite, kaolinite, goethite, gibbsite, and control without clay supplementation. Lines with the same lowercase letter do not differ significantly from each other according to Tukey's test ($p<0.05$).

	Tritatable acidity (mmol)					pH				
Phosphate	Control	Gibbsite	Kaolinite	Goethite	Hematite	Control	Gibbsite	Kaolinite	Goethite	Hematite
Iron	50,29 Bb	55,32 Cb	77,11 Ba	25,15 Dd	31,85 Cc	2,29 Bnd	2,22 And	2,10nd	2,28 And	2,30 And
Tricalcium	92,20 Ab	102,26 Ba	92,20 Ab	97,23 Ab	98,90 Ab	2,39 Ba	1,98 Bb	1,97 Bb	2,07 Bb	2,04 Bb
Aluminium	30,17 Cc	45,26 Da	31,85 Dc	31,85 Cc	38,56 Bb	2,26 Bnd	2,34 And	2,37 And	2,38 And	2,39 And
Patos	28,50 Db	97,23 Ba	31,85 Db	28,50 Db	30,17 Cb	2,42 Ba	1,96 Bb	2,27 Aa	2,31 Aa	2,27 Aa
Argelia	31,85 Cc	103,93 Ba	39,56 Cb	39,56 Bb	38,56 Bb	2,36 Ba	2,06 Bb	2,24 Aa	2,33 Aa	2,29 Aa
Catalão	20,12 Ec	100,58 Ba	28,50 Db	21,79 Dc	25,15 Cb	2,41 Ba	2,17 Ab	2,23 Ab	2,45 Aa	2,41 Aa
Marrocos	36,88 Cc	105,61 Aa	43,58 Cb	36,21 Bb	43,58 Bc	2,39 Ba	2,00 Bb	2,22 Aa	2,34 Aa	2,32 Aa
Bayovar	35,20 Cb	102,20 Ba	31,85 Db	30,17 Db	33,53 Cb	2,48 Aa	2,10 Bb	2,30 Ab	2,30 Ab	2,24 Ab
Araxá	26,82 Db	110,64 Aa	31,85 Db	26,15 Db	26,82 Cb	2,31 Ba	1,99 Bb	2,09 Bb	2,28 Aa	2,31 Aa

Columns with same uppercase letter or lines with the same lowercase letter are not statistically different by the Scott-Knott test ($p < 0.05$). nd= Treatments don't differ

addition according to the Tukey test ($p < 0.05$) (Table 3). Treatments with gibbsite are superior to those supplemented with kaolinite (Table 3). The highest production of oxalic acid was 32.73 mmol in the treatment with Patos de Minas RP supplemented with gibbsite (Table 3).

With respect to the citric acid production, higher contents were measured in treatments with calcium phosphate, but there were no significant differences between treatments supplemented with clay and the control without added clay according to the Tukey test ($p<0.05$) (Table 3). The highest citric acid content was 10.54 mmol, observed in the treatment with calcium phosphate supplemented with kaolinite (Table 3). In treatments with iron, Patos de Minas, Algéria, Bayovar, and Araxá phosphates, treatments supplemented with gibbsite were significantly superior to the control without the clay adition (Table 3). The treatments supplemented with kaolinite, goethite, and hematite did not show significant differences compared to the control without clay addition according to the Tukey test ($p<0.05$) (Table 3).

In relation to remanent glucose, in iron and calcium phosphates, no significant differences were observed in the remanent glucose content after incubation compared to the control without clay addition, as indicated by the Tukey test ($p < 0.05$) (Table 4). In treatments with aluminum phosphate, only the treatment supplemented with gibbsite clay showed a significant decrease compared to the control without clay addition (Table 4).

In treatments with natural RPs supplemented with gibbsite, the lowest remanent glucose contents were observed (Table 4). The lowest remanent glucose value measured was 32.13 mmol L^{-1}, in the treatment with Marrocos RP supplemented with gibbsite clay (Figure 6). In all treatments with natural RPs, supplementation with gibbsite led to a significantly lower remanent glucose content compared to the control without clay addition according to the Tukey test ($p < 0.05$) (Table 4).

Regarding biomass production by the fungus *A. niger* FS1, significant increase was observed in the treatment inoculated with kaolinite, while in the treatment with iron phosphate and supplemented with goethite, lower biomass production was observed compared to the control without clay addition according to the Tukey test ($p < 0.05$) (Table 4). The treatments with calcium phosphate were the only ones where there were no significant differences in biomass production when supplemented with clay compared to the control. The treatment with calcium phosphate supplemented with gibbsite recorded the highest biomass value measured, at 2.58 g L^{-1} (Table 4). In all natural RPs, supplementation with gibbsite led to significant increases in biomass production. Only in Araxá RP, the treatment supplemented with kaolinite also resulted in gains in fungal biomass production compared to the control without clay addition, as indicated by the Tukey test ($p < 0.05$) (Table 4).

Table 3. Oxalic acid (mmol L^{-1}) and citric acid (mmol L^{-1}) measured in the supernatant of the modified NBRIP medium with different phosphates as P source, supplemented with clays, hematite, kaolinite, goethite, gibbsite, and control without clay supplementation. Lines with the same lowercase letter do not differ significantly from each other according to Tukey's test ($p<0.05$).

	Oxalic acid (mmol)					Citric acid (mmol)				
Phosphate	Control	Gibbsite	Kaolinite	Goethite	Hematite	Control	Gibbsite	Kaolinite	Goethite	Hematite
Iron	10,32 Ab	13,28 Ea	13,19 Ba	9,72 Bb	10,17 Bb	3,48 Cb	5,30 Ca	3,99 Bb	3,52 Cb	3,63 Cb
Tricalcium	15,24 And	18,85 Dnd	18,42 And	15,03 And	15,14 And	10,29 And	10,39 And	10,54 And	10,06 And	10,26 And
Aluminium	10,31 Aa	13,50 Ea	12,15Ba	9,90 Bb	10,18 Bb	3,69 Cnd	3,51 Dnd	4,07 Bnd	3,68 Cnd	3,68 Cnd
Patos	4,55 Bc	32,74 Aa	8,53 Cb	4,28 Cc	4,59 Cc	4,45 Bb	8,12 Ba	4,51 Bb	4,34 Ab	4,49 Bb
Argelia	5,34 Bb	25,65 Ba	6,70 Cb	5,12 Cb	5,42 Cb	4,52 Bb	5,96 Ca	4,69 Bb	4,56 Ab	4,56 Bb
Catalão	5,08 Bc	22,38 Ca	12,26 Bb	4,76 Cc	5,06 Cc	4,36 Bnd	4,82 Cnd	4,62 Bnd	4,28 And	4,36 Bnd
Marrocos	5,36 Bc	25,65 Ba	12,07 Bb	5,10 Cc	5,40 Cc	4,82 Bnd	5,59 Cnd	4,86 Bnd	4,68 And	4,82 Bnd
Bayovar	4,52 Bc	19,10 Da	5,89 Cb	4,13 Cc	4,74 Cc	3,59 Cb	7,72 Ba	3,88 Bb	3,57 Cb	3,63 Cb
Araxá	4,89 Bc	25,43 Ba	13,62 Bb	4,91 Cc	4,90 Cc	4,63 Bb	7,54 Ba	4,67 Bb	4,64 Ab	4,56 Bb

Columns with same uppercase letter or lines with the same lowercase letter are not statistically different by the Scott-Knott test ($p < 0.05$). nd= Treatments don't differ

Table 4. A) Biomass (g L^{-1}), **B)** Remanent glucose (g L^{-1}) measured in the supernatant of the modified NBRIP medium with different phosphates as P source, supplemented with clays, hematite, kaolinite, goethite, gibbsite, and control without clay supplementation. Lines with the same lowercase letter do not differ significantly from each other according to Tukey's test ($p<0.05$).

	Biomass (g L-1)					Remanent glucose (mmol L-1)				
Phosphate	Control	Gibbsite	Kaolinite	Goethite	Hematite	Control	Gibbsite	Kaolinite	Goethite	Hematite
Iron	1.52 Bb	1,30	2,28	0,98	1,58	41.96 Bnd	41.07 And	38.24 Bnd	42.29 Bnd	42.07 Bnd
Tricalcium	2.55 And	2,58	2,46	2,52	2,54	37.46 Cnd	34.96 Bnd	36.63 Bnd	38.13 Cnd	37.91 Cnd
Aluminium	1.08 Cb	1,00	1,42	1,37	1,30	51.56 Aa	44.96 Ab	46.62 Ab	50.78 Aa	50.78 Aa
Patos	1.54 Bb	2,41	1,63	1,58	1,62	51.62 Aa	34.91 Bc	46.07 Ab	50.78 Aa	50.89 Aa
Argelia	1.59 Bb	2,30	1,66	1,60	1,71	51.56 Aa	36.07 Bc	45.95 Ab	50.89 Aa	50.56 Aa
Catalão		2,41	1,42	1,22	1,31	48.73 Aa	38.52 Bb	45.51 Aa	49.56 Aa	48.45 Aa
Marrocos	1,67	2,40	1,68	1,64	1,60	43.46 Ba	32.19 Bb	43.07 Aa	43.46 Ba	42.96 Ba
Bayovar	1,61	2,48	1,65	1,64	1,64	46.68 Aa	33.85 Bb	44.34 Aa	46.51 Aa	46.18 Aa
Araxá	1,42	2,50	1,98	1,32	1,44	50.06 Aa	36.24 Bb	45.79 Aa	49.73 Aa	49.45 Aa

Columns with same uppercase letter or lines with the same lowercase letter are not statistically different by the Scott-Knott test ($p < 0.05$). nd= Treatments don't differ

With respect to the the phosphate solubilization efficiency (BEPS), supplementation with gibbsite led to significant increases in treatments with RP from Patos de Minas, Algeria, Catalão, Marrocos, Bayovar, and Araxá compared to the control without clay supplementation, according to the Tukey test ($p < 0.05$) (Table 5). Supplementation with gibbsite had a negative impact on BEPS in treatments with synthetic phosphates. However, the highest BEPS observed in treatments supplemented with gibbsite was in the treatment with tricalcium phosphate, yielding 4711.9 mg L^{-1} of soluble P per gram of biomass (Table 5). Supplementation with kaolinite significantly increased BEPS in treatments with tricalcium phosphate and in RPs Catalão, Marrocos, Bayovar, and Araxá, according to the Tukey test ($p < 0.05$). The highest efficiency was calculated in the treatment with tricalcium phosphate, producing with 5076.8 mg L^{-1} of soluble P per gram of biomass, was the highest efficiency observed in all treatments (Table 5). Supplementation with goethite significantly increased BEPS in treatments with RPs from Bayovar, and Araxá, according to the Tukey test ($p < 0.05$). The highest BEPS observed in treatments supplemented with goethite was observed in the treatment with phosphate, yielding 4395.5 mg of P per gram biomass. However, this efficiency was significantly lower than the control treatment without clay addition (Table 5). Hematite supplementation had a negative impact on the BEPS of most of the phosphates tested, but significant increases were observed in Marrocos and Araxá phosphates, phosphates according to the Tukey test ($p < 0.05$).

In relation to the oxalic acid production efficiency (BEOA), supplementation with gibbsite led to significant increases in all phosphates tested. The highest efficiency was observed in the treatment with aluminum phosphate, yielding 268.27 mmol of oxalic acid for each gram of biomass produced (Table 5). In treatments supplemented with kaolinite, significant increases were observed in the BEOA of all RPs and tricalcium phosphate, as indicated by the Tukey test ($p < 0.05$). The highest efficiency observed in treatments supplemented with kaolinite was in the treatment with Catalão RP, producing 172.3 mmol of oxalic acid for each gram of biomass (Table 5). Goethite only resulted in increases in BEOA in treatments with iron phosphate and Araxá RP, while no efficiency improvements were observed for hematite.

Regarding the citric acid production efficiency (BECA), supplementation with gibbsite significantly increased efficiency only in treatments with iron phosphate and in RPs Patos de Minas and Bayovar, according to the Tukey test ($p < 0.05$). The highest efficiency was observed in the treatment with iron phosphate, yielding 81.46 mmol L^{-1} of citric acid for each gram of biomass (Table 5). In treatments supplemented with goethite, increases in BECA were only observed with iron phosphate.

Table 5. Phosphate solubilization efficiency **(BEPS)** (mg soluble P/g biomass), Oxalic acid production efficiency **(BEOA)** (mmol oxalic acid/g biomass), Citric acid production efficiency **(BECA)** (mmol citric acid/g biomass) calculated using the data observed in treatments with different phosphates as P source, supplemented with clays, hematite, kaolinite, goethite, gibbsite, and control without clay supplementation.

	BEPS (mg soluble P/ g biomass)					BEOA (mmol oxalic acid / g biomass)					BECA (mmol citric acid / g biomass)				
Phosphate	Control	Gibbsite	Kaolinite	Goethite	Hematite	Control	Gibbsite	Kaolinite	Goethite	Hematite	Control	Gibbsite	Kaolinite	Goethite	Hematite
Iron	3675,9 Ba	3221,0 Ec	3518,0 Cb	1758,0 Id	3515,0 Bb	135,21 Bc	204,38 Ea	115,37 De	198,35 Ab	128,79 Bd	45,55 Ec	81,46 Aa	34,86 Ed	71,92 Bb	45,92c
Tricalcium	4804,8 Ab	4711,9 Ac	5076,8 Aa	4395,5 Ad	4774,8 Ab	119,40 Cb	146,10 Ha	149,33 Ba	118,99 Cb	118,90 Cb	80,60 And	80,55 And	85,49 And	79,60 And	80,54nd
Aluminium	2527,6 Ga	2031,8 Hb	1982,8 Ic	2520,5 Fa	2027,7 Gb	191,01 Ab	268,27 Ba	170,33 Ac	144,22 Be	155,76 Ad	68,39 Ca	69,81 Ba	57,00 Cb	53,53 Cb	56,37b
Patos	3453,2 Cb	3779,1 Ca	3002,8 Fd	2413,9 Ge	3245,4 Cc	59,07 Fc	271,32 Aa	104,43 Eb	53,93 Fc	56,61 Gc	57,82 Db	67,28 Ba	55,23 Cb	54,69 Cb	55,42b
Argelia	3213,5 Db	3247,8 Ea	3217,6 Db	3322,8 Cb	2952,3 Dc	67,07 Ec	223,04 Ca	80,71 Fb	63,69 Ec	63,28 Fc	56,73 Dnd	51,81 Dnd	56,55 Cnd	56,80 Cnd	53,23nd
Catalão	2177,6 Hc	4251,7 Ba	2472,6 Hb	1922,2 Hd	1805,4 He	90,10 Dc	185,48 Fa	172,73 Ab	78,11 Dd	77,05 Dd	77,39 Ba	39,93 Fc	65,12 Bb	70,17 Bb	66,47b
Marrocos	2696,3 Fe	3190,6 Fc	3747,3 Ba	3481,6 Bb	2778,3 Ed	64,05 Ec	213,78 Da	143,72 Bb	62,20 Ec	67,55 Ec	57,58 Da	46,61 Eb	57,89 Ca	57,05 Ca	60,30a
Bayovar	2835,8 Ec	3057,0 Gb	3099,3 Eb	3150,0 Da	2749,4 Ed	56,03 Fc	153,63 Ga	71,26 Gb	50,19 Fc	57,78 Gc	44,45 Eb	62,06 Ca	46,97 Db	43,39 Db	44,32b
Araxá	2195,5 He	3544,3 Da	2947,1 Gb	2865,3 Ec	2537,1 Fd	68,82 Ed	202,93 Ea	137,62 Cb	74,00 Dc	68,02 Ed	65,17 Ca	60,15 Ca	47,13 Db	69,88 Ba	63,29a

Columns with same uppercase letter or lines with the same lowercase letter are not statistically different by the Scott-Knott test ($p < 0.05$). nd= Treatments don't differ

For kaolinite and hematite, no significant increases were observed (Table 5). The phosphate solubilization efficiency per glucose consumed (GEPS) was also altered by clay supplementation (Table 6). Supplementation with gibbsite significantly enhanced the efficiency of GEPS only in the treatment with Catalão RP, as indicated by the Tukey test ($p < 0.05$). In this treatment, the efficiency was 167.81 mg L^{-1} of soluble P for each gram of fungal biomass (Table 6). Regarding supplementation with kaolinite, GEPS increased significantly compared to the control without the addition of clay in iron phosphates and in Marrocos and Araxá RPs according to the Tukey test ($p < 0.05$). The highest GEPS observed in kaolinite treatments was measured in Araxá phosphate with 167.06 mg of soluble P for each gram of fungal biomass (Table 6). On the other hand, supplementation with goethite increased GEPS in the greatest number of phosphates, being significantly higher compared to the control without the addition of clay in aluminum phosphates and Catalão, Marrocos, Bayovar, and Araxá RPs, according to the Tukey test ($p < 0.05$). The highest GEPS observed in these treatments was in aluminum phosphate with 203.92 mg L^{-1} of soluble P for each gram of biomass (Table 6). On the other hand, supplementation with hematite only promoted increases in the efficiency of treatment with tricalcium and Araxá RP with 101,72 e 167.94 mg of soluble P for each gram of biomass respectively (Table 6).

In relation to oxalic acid production efficiency per glucose consumed (GEOA), supplementation with gibbsite significantly increased efficiency compared to the control without the addition of clay in treatments with aluminum, tricalcium phosphate and in all RPs, except for Algeria RP, according to the Tukey test ($p < 0.05$). The highest GEOA in treatments supplemented with gibbsite was observed with Patos de Minas RP, with 8.83 mmol of oxalic acid for each gram of biomass produced (Table 6). In treatments supplemented with kaolinite, significant increases in GEOA were observed in treatments with tricalcium phosphate and in Calão, Marrocos, and Araxá RPs, according to the Tukey test ($p < 0.05$). The greatest efficiency of these treatments was observed in Araxá RP, with 7.80 mmol of oxalic acid per gram of fungal biomass (Table 6). In treatments supplemented with goethite and hematite clays, no significant increases were observed in the efficiency of glucose consumption for the production of oxalic acid (Table 6).

With respect to citric acid production efficiency per glucose consumed (GECA), increases were only observed in the treatment supplemented with gibbsite and iron phosphate, yielding 2.04 mmol of citric acid for each gram of fungal biomass (Table 6). Kaolinite, goethite, and hematite did not significantly enhance GECA, as indicated by the Tukey test ($p < 0.05$).

Table 6. Phosphate solubilization efficiency per glucose consumed (**GEPS)** (mg soluble P/g biomass), Oxalic acid production efficiency per glucose consumed (**GEOA)** (mmol oxalic acid/g biomass), Citric acid production efficiency per glucose consumed (**GECA)** (mmol citric acid/g biomass) calculated using the data observed in treatments with different phosphates as P source, supplemented with clays, hematite, kaolinite, goethite, gibbsite, and control without clay supplementation.

	GEPS (mg soluble P/ g consumed glucose)					GEOA (mmol oxalic acid/ g consumed glucose)					GECA (mmol citric acid/ g consumed glucose)				
Phosphate	Control	Gibbsite	Kaolinite	Goethite	Hematite	Control	Gibbsite	Kaolinite	Goethite	Hematite	Control	Gibbsite	Kaolinite	Goethite	Hematite
Iron	115,09 Gb	80,59 Fc	129,31 Ea	36,21 Id	114,80 Gb	4,23 Db	5,11 Da	4,24 Db	4,09 Db	4,21 Db	1,43 Fb	2,04 Aa	1,28 Cb	1,48 Fb	1,50 Eb
Tricalcium	188,88 Db	164,43 Ad	184,21 Ab	177,63 Ec	191,72 Ca	4,69 Db	5,10 Da	5,42 Ca	4,81 Db	4,77 Db	3,17 Dnd	2,81 And	3,10 And	3,22 Dnd	3,23 Cnd
Aluminium	192,57 Cb	53,69 Ge	88,18 Gd	203,92 Ca	155,62 Ec	14,55 Aa	7,09 Bc	7,58 Ac	11,67 Ab	11,95 Ab	5,21 Ba	1,84 Bd	2,54 Bc	4,33 Bb	4,33 Bb
Patos	377,18 Aa	122,92 Be	144,60 Dd	224,74 Bc	314,82 Ab	6,45 Cb	8,83 Aa	5,03 Cc	5,02 Cc	5,49 Cc	6,32 Aa	2,19 Ac	2,66 Bc	5,09 Ab	5,38 Ab
Argelia	361,52 Ba	106,62 Ce	154,89 Cd	321,56 Ab	284,61 Bc	7,55 Ba	7,32 Ba	3,89 Ec	6,16 Bb	6,10 Bb	6,38 Aa	1,70 Bd	2,72 Bc	5,50 Ab	5,13 Ab
Catalão	100,59 Hc	167,81 Aa	97,78 Fd	109,17 Hb	93,54 Hd	4,16 Db	7,32 Ba	6,83 Ba	4,44 Db	3,99 Eb	3,57 Da	1,58 Bc	2,58 Bb	3,99 Ca	3,44 Ca
Marrocos	104,01 Hc	91,07 Ee	140,29 Da	131,74 Gb	98,55 Hd	2,47 Ec	6,10 Ca	5,38 Cb	2,35 Ec	2,40 Fc	2,22 Ea	1,33 Bb	2,17 Ba	2,16 Ea	2,14 Da
Bayovar	143,71 Fb	97,40 De	127,39 Ed	159,75 Fa	134,51 Fc	2,84 Eb	4,89 Da	2,93 Fb	2,55 Eb	2,83 Fb	2,25 End	1,98 Bnd	1,93 Cnd	2,20 End	2,17 Dnd
Araxá	158,55 Ec	128,09 Bd	167,06 Bb	183,41 Da	167,94 Db	4,97 Db	7,33 Ba	7,80 Aa	4,74 Db	4,50 Db	4,71 Ca	2,17 Ab	2,67 Bb	4,47 Ba	4,19 Ba

Columns with same uppercase letter or lines with the same lowercase letter are not statistically different by the Scott-Knott test ($p < 0.05$). nd= Treatments don't differ statistically

DISCUSSION

Clay supplementation had varying effects on the solubilization of phosphates by the fungus *A. niger* FS1, depending on the phosphate source used. Generally, greater improvements were observed in the solubilization of natural RPs compared to synthetic phosphates (Figures 1). This can be attributed to the mechanisms through which clays enhance solubilization, primarily by increasing the synthesis of organic acids and acidifying the medium through enhanced H^+ extrusion (Chapter 3). Natural RPs contain a high concentration of apatites [Ca_{10} $(PO_4)_6$ (F^- or OH^- or CL^-)] which have a high calcium content, and are more responsive to these mechanisms (Nahas, 1996; Amarasinghe et al., 2022). Calcium phosphates are more readily solubilized due to their lower binding energy, higher solubility in acidic pH, and increased stability of the complexes formed (Lindsay, 1979; Amarasinghe et al., 2022). In treatments where calcium phosphate was used as the phosphate source, no enhancement in solubilization was observed with clay supplementation, as the control without clay addition achieved 100% solubilization (Figure 1).

Interestingly, in treatments with calcium phosphate, higher citric acid contents were observed in all treatments, including the control without the addition of clay (Table 3). This indicates that the profile of organic acids produced by the fungus is influenced by the phosphorus source. The synthesis of organic acids by the Aspergillus fungus is a complex and highly regulated process (Yang et al., 2017). Several enzymes and regulatory proteins have been identified, which respond mainly to the carbon source and pH of the medium (Arst & Peñalva, 2003; David et al., 2005; Poulsen et al., 2012).

Analyzing the specific effect of each clay on solubilization, it was observed that the clays do not have the same response in each RP used (Figure 1). This variation occurs because apatites in RPs exhibit several isomorphic substitutions where different cations can replace Ca^{+2}, thereby altering the solubilization characteristics and disrupting the complex chemical equilibrium that occurs after solubilization when the elements diffuse into the supernatant (Knubovets, 1993). Additionally, the RPs tested have diverse origins of formation, including igneous rocks (Araxá and Catalão), sedimentary rocks (Bayovar, Marrocos , and Algeria), or metamorphic rocks (Patos de Minas). Generally being the most reactive sedimentary rocks (Léon et al., 1986). However, gibbsite consistently showed strong results in all RPs, significantly enhancing phosphate solubilization. In treatments involving calcium phosphate and all RPs, solubilization exceeded and was close to 100% (Figures 1). In treatments with gibbsite, increases in the efficiency of biomass for the solubilization of phosphates were observed in all natural RPs (Table 5). This can be explained by the highest values of titratable acidity and content of oxalic and

citric acids observed in these treatments (Table 1-3). Furthermore, biomass was more efficient in producing oxalic acid in all phosphates tested when supplemented with gibbsite (Table 5). These increases are caused by the rise in aluminum content in the culture medium, leading to a protective response to stress primarily by increasing the production of organic acids (Chapter 3). The experiments highlighted the influence of aluminum, showing that the treatment with aluminum phosphate supplemented with gibbsite resulted in one of the highest efficiencies of oxalic acid production for each gram of fungal biomass produced (Table 5).

Surprisingly, low levels of oxalic acid were observed in the treatment with calcium phosphate, in comparison with the high titratable acidity of these treatments (Table 2-3). It is possible that the oxalic acid levels were underestimated due to the formation of calcium oxalate precipitates, due to the high calcium content in these treatments.

Gibbsite decreased the solubilization of iron aluminum phosphate, even though small but significant increases in the production of organic acids were observed (Table 3). This may be caused by the greater stability of these phosphates at acidic pH compared to calcium phosphate (Lindsay, 1979).

The excellent performance of gibbsite in enhancing the solubilization of various natural RPs indicates a high potential for utilizing this clay in biotechnological processes for fertilizer production or for directly employing the fungus *A. niger* FS1 in agricultural applications.

Kaolinite clay significantly increased the solubilization of iron phosphates from Catalão, Marrocos, and Araxá (Figure 1). The enhanced solubilization of natural phosphates may be attributed to the higher oxalic acid content in these treatments (Table 3). Additionally, silicic acid can form complexes with elements harmful to fungi, like fluorine (Roberson & Barnes, 1978). This phenomenon may explain the observed increase in fungal biomass production in the treatments with Catalão and Araxá RPs (Table 4).

In the treatment with iron phosphate, organic acids did not seem to explain the significant increase in solubilization (Figure 1). In this treatment, the increase could be explained by the silicic acid formed when dissolving kaolinite in acidic pH (Exley et al., 2019). Silicic acid in an aqueous medium containing iron can form stable complexes such as, $FeOSi(OH)_3^{2+}$, Fe_2Si_{1-2} ou Fe_3Si_{2-3} (Pokrovski et al., 2003).

The increase in iron phosphate solubilization with kaolinite supplementation has great potential for agriculture. Therefore, it is crucial to conduct further studies to gain a better understanding of the mechanisms involved.

In these experiments, a limited ability to increase the solubilization of phosphates with supplementation of goethite and hematite clays was observed. Goethite significantly

increased the solubilization of Bayovar and Araxá RPs (Figure 1). Hematite only promoted greater solubilization in the treatment with Araxá phosphate (Figure 1). It is clear that these increases in solubilization are not related to increases in the production of organic acids (Table 3) nor to increases in the efficiency of their synthesis (BEOA, BECA, GEOA, and GECA). Theoretically, these clays could increase phosphate solubilization by raising the iron content in the culture medium, which could lead to an increase in respiratory activity, due to the significant role of iron in the enzymes of the electron transport chain (Joseph-Horne et al., 2001; Grahl et al., 2012). On the other hand, these clays have lower solubility, allowing the stable clay particles to participate in the adsorption of elements toxic to the fungus, such as fluorine (Vinati et al., 2015). This can positively impact the growth of the fungus and the solubilization of phosphates. Further research is needed to enhance the understanding of how these clays increase the solubilization of natural RPs.

The results of these experiments highlight the significance of the chemical composition of various P sources. Gibbsite and kaolinite exhibit significant potential in enhancing the solubilization of low-reactivity phosphates, offering an intriguing biotechnological option to support the nutritional requirements of agricultural crops. Subsequent studies are needed to analyze the potential in situ application of these clays or their integration into phosphate fertilizer production processes.

CONCLUSIONS

The clay adition, particularly gibbsite and kaolinite, significantly increases the solubilization rates of different rock phosphates. Gibbsite and kaolinite also stimulates greater production of organic acids, which are crucial for the solubilization process. However, the impact of clays on synthetic phosphates varied, suggesting the importance of the composition of the P source. The results suggest the feasibility of using mineral clays in combination with phosphate-solubilizing microorganisms to enhance phosphate solubilization. Further research is required to evaluate the applicability of these strategies in agriculture or within the fertilizer industry.

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CHAPTER V

SHORT REVIEW:
Microbial phosphate solubilization: incidental or intentional?

SHORT REVIEW:

MICROBIAL PHOSPHATE SOLUBILIZATION: INCIDENTAL OR INTENTIONAL?

Gustavo Andrés Iglesias Barrera

Abstract

Phosphorus (P) is an essential nutrient for plants, and its use in agriculture is crucial to ensure crop productivity. However, the rising prices of phosphate fertilizers have led to an increasing use of biological inputs containing P-solubilizing microorganisms (MSP) in agriculture. These microorganisms convert insoluble forms of phosphorus into soluble forms, making them accessible to plants. Various groups of microorganisms, such as fungi, bacteria, and actinobacteria, possess this capability. Nevertheless, the majority of identified MSP are heterotrophic microorganisms that derive energy and nutrients from the decomposition of plant residues or through symbiosis with plants. Why would they produce metabolites targeted at mineral acquisition when, as heterotrophs, they can get inorganic nutrients from living or dead plant and animal tissues? We suggest that the ability to solubilize phosphate in these heterotrophic microorganisms is mostly incidental and results from primary metabolic processes related their lifestyles, phosphate solubilization being a bonus. Criteria to define microorganisms that solubilize P in a *stricto sensu* manner, namely those that carry out phosphate solubilization through regulated processes targeted specifically at P acquisition are proposed. Furthermore, techniques for prospecting *stricto sensu* solubilizers are presented aiming at guiding future research towards the development of more efficient and sustainable agricultural practices.

Introduction

Phosphorus (P) is an essential nutrient for plant growth and development, as it is fundamental for various physiological and metabolic processes of plants (Raghothama, 2015; Malhotra et al., 2018). P is a crucial component of vital molecules, such as adenosine triphosphate (ATP), the primary molecule for storing and transferring energy (Carstensen et al., 2018). Additionally, P is a key element in DNA and RNA nucleic acids and is essential for cellular replication and protein synthesis (Walsh, 2020). Moreover, P is a structural component of phospholipids, crucial for forming cell membranes, maintaining structural integrity of the cell, and facilitating substance transport (Suriyagoda et al., 2022). Notably, P is vital for the growth of plant roots, with a significant decrease in root system size, particularly fine roots, under P-deficient conditions (Malhotra et al., 2018; Liu, 2021). This

nutrient plays a major role in the development of flowers and fruits, thereby being essential for enhancing the productivity of agricultural crops (Dixon et al., 2020; Fathi & Afra, 2023).

Currently, the need to meet the nutrient requirements of agricultural crops in a sustainable manner has led to a significant increase in the use of biological inputs in agriculture (Doll et al., 2020). Microorganisms with the ability to solubilize low reactivity phosphates minerals into plant-usable orthophosphate are increasingly sought for agricultural applications (Timofeeva et al., 2022; Kaur et al., 2024). However, a question arises: Are there microorganisms specialized in performing these processes, or is this a result of other microbial metabolic activities? Differently from biological nitrogen fixation, microbial phosphate solubilization reported so far seems to be more a side effect of other important microbial activities, such as cell respiration, plant residue degradation, NH_4^+ oxidation etc, than a regulated activity targeted at P acquisition (Parks et al., 1990: Prabhu et al., 2018; Wang et al., 2020). If is there any, regulation of phosphate solubilization, especially by fungi, is still poorly understood. Here we explore the diversity, lifestyles, and solubilization mechanisms of microorganisms loosely identified as phosphate solubilizers and propose a few criteria for defining microorganisms specialized at phosphate solubilization (*stricto sensu* phosphate solubilizers). We aim at enhancing our understanding of what phosphate solubilization represents at the cellular and ecosystem level, improving the current concepts of what a phosphate-solubilizing microorganism really is, and refocusing research on novel phosphate solubilizers that may have been overlooked for years because of the lack of a precise definition of the actors and processes involved.

Phosphate fertilizers: limitations and challenges

Due to its significance, the application of phosphate fertilizers in agriculture to meet the demands of agricultural crops is essential, especially in soil with high P-fixing capacity, such as tropical soils (Corrêa et al., 2011; Melo et al., 2015; Zou et al., 2022). Currently, the cost of fertilizers has emerged as a limiting factor for agricultural production (Brownlie et al., 2023). Since 2020, there has been a significant global increase in fertilizer prices (Hebebrand & Debucquet, 2023). In Brazil, despite the presence of natural rock phosphate sources, they are deemed to have low agricultural suitability (Léon et al., 1986). Consequently, nearly 95% of phosphate fertilizers in Brazil are imported, making them highly susceptible to international price fluctuations and geopolitical instabilities (Russo & Figueira, 2023; Rice et al., 2023). Brazil imports both field-ready fertilizers and natural rock phosphates for further processing by the fertilizer industry (World Bank, 2024).

This situation, accompanied by the boom in the use of biologicals inputs in agricultural crops, has led to more and more discussion about P-solubilizing microorganisms (PSM) and

their potential to improve the low efficiencies of phosphate fertilizers, generally estimated to range from 10 to 20 % of the P applied to the soil (Baligar et al., 2001; Silva et al., 2023). These microorganisms are understood as those with the ability to convert poorly soluble forms of phosphorus into soluble forms that plants can utilize (Sarmah & Sarma, 2022).

Currently in Brazil, commercial products based on PSM that can be used directly in the field to enhance P availability to agricultural crops have been developed (Ministério da Agricultura e Pecuária, 2020). PSM can also be used in batch and continuous cultivation and in solid state fermentation to solubilize low reactivity rock phosphates, allowing the generated products to be subsequently applied in the field (Vassileva et al., 2009; Gaind, 2016; Wang et al., 2022). In the literature, various microorganisms with the ability to efficiently solubilize several low reactivity P sources *in vitro*, such as igneous and metamorphic rock phosphates, have been identified (Mendes et al., 2014; Xiao et al., 2020). It is noteworthy that an *Aspergillus niger* isolate has been shown to desorb P from a soil with high P-fixing capacity *in vitro*, suggesting that some PSM can access and make available non-labile P reservoirs in the soil (Nascimento et al., 2021).

Phosphate-solubilizing microorganisms: diversity and lifestyles.

Several groups of microorganisms have been described with the ability to solubilize phosphates, including filamentous fungi, yeasts, bacteria, actinobacteria, and cyanobacteria (Yandigeri et al., 2011; Mendes et al., 2014; Chen et al., 2022; Teles et al., 2024).

Most microbial species that have been identified as phosphate solubilizers are heterotrophic microorganisms that thrive on decomposing plant residues or form mutualistic and associative symbioses with plants (Prabhu et al., 2019). As heterotrophs, they have specialized in obtaining energy, carbon, and nutrients from organic substrates available as dead organic matter or metabolites supplied by their symbiotic hosts (Benner, 2010). It seems odd that such heterotrophs would have to express mechanisms to dissolve inorganic materials when their basic growth medium comprises a plethora of organic residues that contain nutrient elements in their composition. Interestingly, many fungal species known to solubilize phosphates are ecologically classified as r strategists (Atlas & Bartha, 1998) since they grow rapidly in nutrient-rich organic materials and produce a large number of small propagules to disperse to other locations as soon as their growth substrate becomes nutritionally depleted (Gadgil & Solbrig, 1972).

Differently from algae, cyanobacteria, lichens, and other pioneer photosynthetic bacteria that can grow on inhospitable rock surfaces and minerals, microbial heterotrophs do not have as their typical environment inorganic materials from which they are supposed to obtain mineral nutrients (Reisser, 2007; Jung & Büdel, 2021; Kaštovský et al., 2021).

Coherent with their life strategies, their ability to solubilize phosphate minerals seem to be more incidental than specifically targeted at the acquisition of P from surrounding primary and secondary minerals. If such is the case, these PSM will be henceforth designated *lato sensu* PSM.

Mechanisms for microbial phosphate solubilization

The main mechanisms that lead to phosphate solubilization include medium acidulation and cation chelation (Amarasinghe et al., 2022). The first one is accomplished through H^+ extrusion and organic acid release, and the second, involves the chelation of phosphate-accompanying cations by the released organic acids (Prabhu et al., 2019). Interestingly, H^+ extrusion and organic acid release are related to many biochemical cellular processes that are not primarily directed to orthophosphate release from phosphate minerals [Fuhrmann, 2021] (Figure 1). For example, H^+ extrusion takes place as a result of aerobic respiration, NH_4^+ uptake, and NH_4^+ and S^{2-} autotrophic oxidation, among others (Joseph-Horne et al., 2001; Vylkova, 2017). The picture is even more complex regarding organic acids which participate in the lysis of the plant cell wall, soil organic matter degradation, and tolerance to toxic chemical elements, such as aluminum and heavy metals (Gadd, 1999; Ahonen-jonnarth et al., 2000; Klugh & Cumming, 2007).

One of the most relevant organic acids reported for phosphate solubilization is oxalic acid (Mendes et al., 2020). This compound appears to have several functions besides promoting the release of orthophosphate from low solubility P sources (Gadd, 1999). Oxalic acid is efficient at the destabilization of lignocellulosic complexes, facilitating the degradation of plant residues (Mäkelä et al., 2002; Andlar et al., 2018). Microorganisms may have acquired the ability to use oxalic acid in this process along evolution. By doing so, whatever excess of oxalic acid produced could be further used to promote the release of additional amounts of nutrients from minerals to fulfill the needs of the decomposer microbial biomass. Thus, within an ecological perspective, inorganic nutrients made available through a surplus of oxalic acid could balance the low C:nutrient ratios of decomposing plant residues leading to larger yields of microbial biomass, that, in turn, would speed carbon and nutrient cycling (Figure 2). N inputs from biological nitrogen

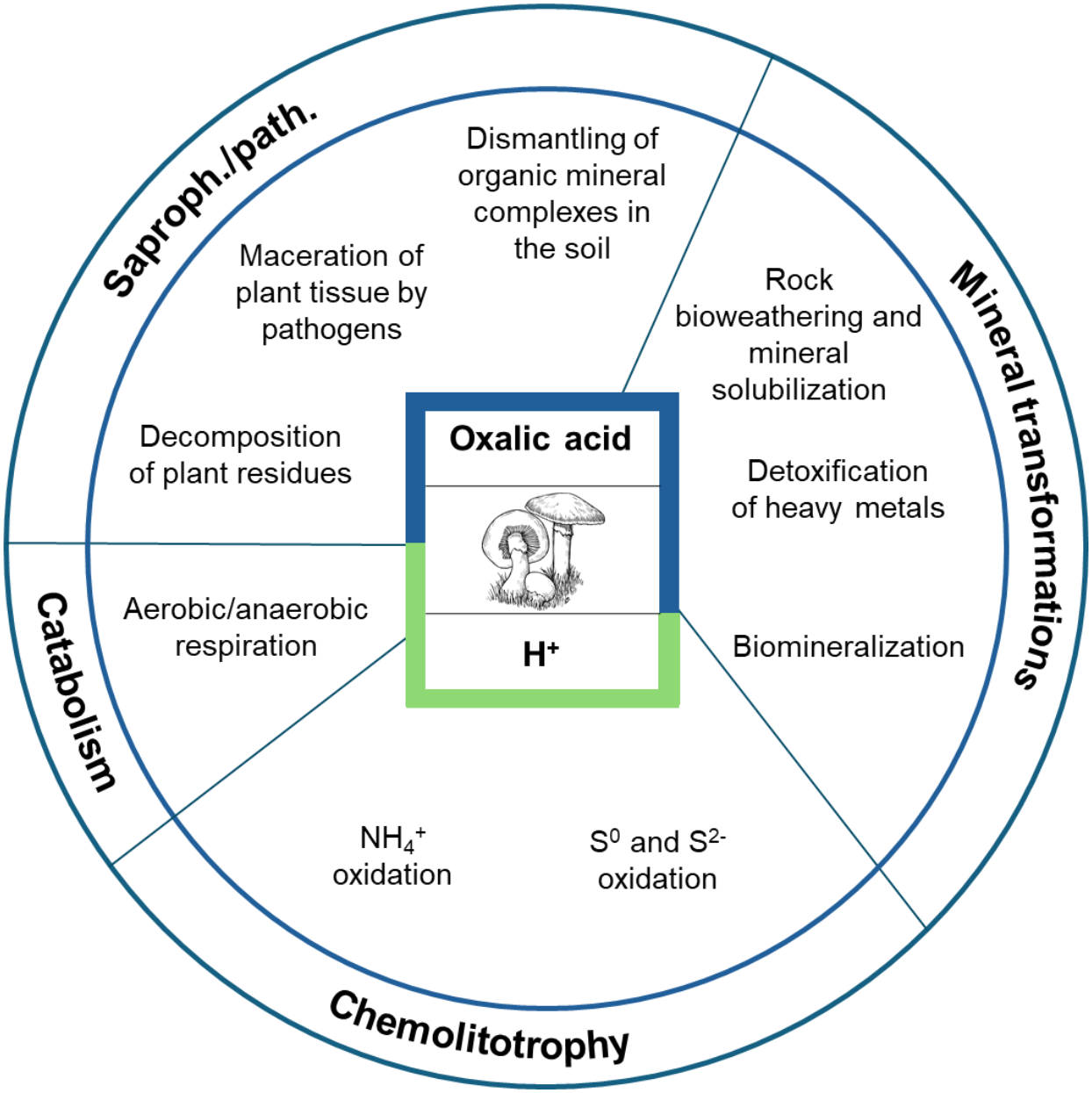

FIGURE 1. Microbial processes involving the production of oxalic acid and H^+ by microbial cells. Oxalic acid plays a role in the decomposition of lignocellulolytic material in plant residues. The compound is also involved in pathogenesis, provoking the maceration of infected tissues. In the soil, oxalic acid contributes to the destabilization of organic mineral complexes, leading to the release of organic matter into the soil solution and its subsequent oxidation by microorganisms. This organic acid can also promote the weathering of rocks and the solubilization of minerals. It can also detoxify chemical elements through chelation and form new oxalate minerals (biomineralization). H^+ is normally released by respiring cells. The chemolitotrophic oxidation of NH_4^+, S^0, and S^{2-}, leads to the release of H^+. Medium acidification by H^+ allows the solubilization of rock phosphates.

fixation carried out by prokaryotes would supply N, which is frequently the most limiting nutrient to plants and microbes (Figure 2). Oxalic acid is also crucial for medium acidification by a diversity of fungal species. Organic acids have been reported to maintain a low medium pH to favor enzymes with optimum activity in acidic media and to hinder the growth of other microbial groups (Palmieri et al., 2019). For fungi, considered to be efficient organic acids producer, several enzymes have been reported to participate in the synthesis of organic acids, but, interestingly, none of them seems to be regulated by the P content in the extracellular environment (Kobayashi et al., 2014; Yang et al., 2017).

In bacteria, genes related to phosphate solubilization have been identified. These genes are primarily involved in gluconic acid production. Clones lacking such genes are uncapable of phosphate solubilization diagnosed by the formation of solubilization halos in culture media containing low-solubility inorganic phosphates, pespecially tricalcium phosphate (Goldstein & Liu, 1987; Krishnaraj & Goldstein, 2001; Rodríguez et al., 2007).

Siderophores and exopolysaccharides are commonly reported as being supposedly linked to microbial phosphate solubilization (Prabhu et al., 2019). However, both metabolites also seem to be related to other functions, while phosphate solubilization would only be secondary. Siderophores are low molecular weight molecules with a high affinity for iron (Saha et al., 2015). Siderophores are synthesized by microorganisms to capture iron in environments where this nutrient is limiting. Phosphate solubilization by siderophores takes place when iron in iron phosphates is chelated by these compounds, thus leaving the phosphate ions available in solution (Cui et al., 2022). For exopolysaccharides, even though they have been related to P solubilization, their main function for microorganisms is adhesion to surfaces, protection from desiccation, toxic metals, and predation, and biofilm formation (Matz et al., 2008; Alavi & Hansen, 2013; Rana & Upadhyay, 2020). No explanatory hypothesis on the role of exopolysaccharides on phosphate solubilization has been proposed.

Thus, we suggest that most of the phosphate solubilizing activity brought about by heterotrophic microbes as well as via other mechanisms related to a chemolithotroph lifestyle is more incidental than represent a finely tuned activity targeted specifically at P acquisition. Phosphate solubilization by heterotrophic microorganisms seems to be a side effect primarily linked to their central roles in nature as residue decomposers. The question remains whether *stricto sensu* phosphate solubilizers, if they really exist, could actually be isolated and used in agriculture. Do they carry out distinct mechanisms from those that are currently known? What would be the criteria for defining a *stricto sensu* phosphate solubilizer microorganism?

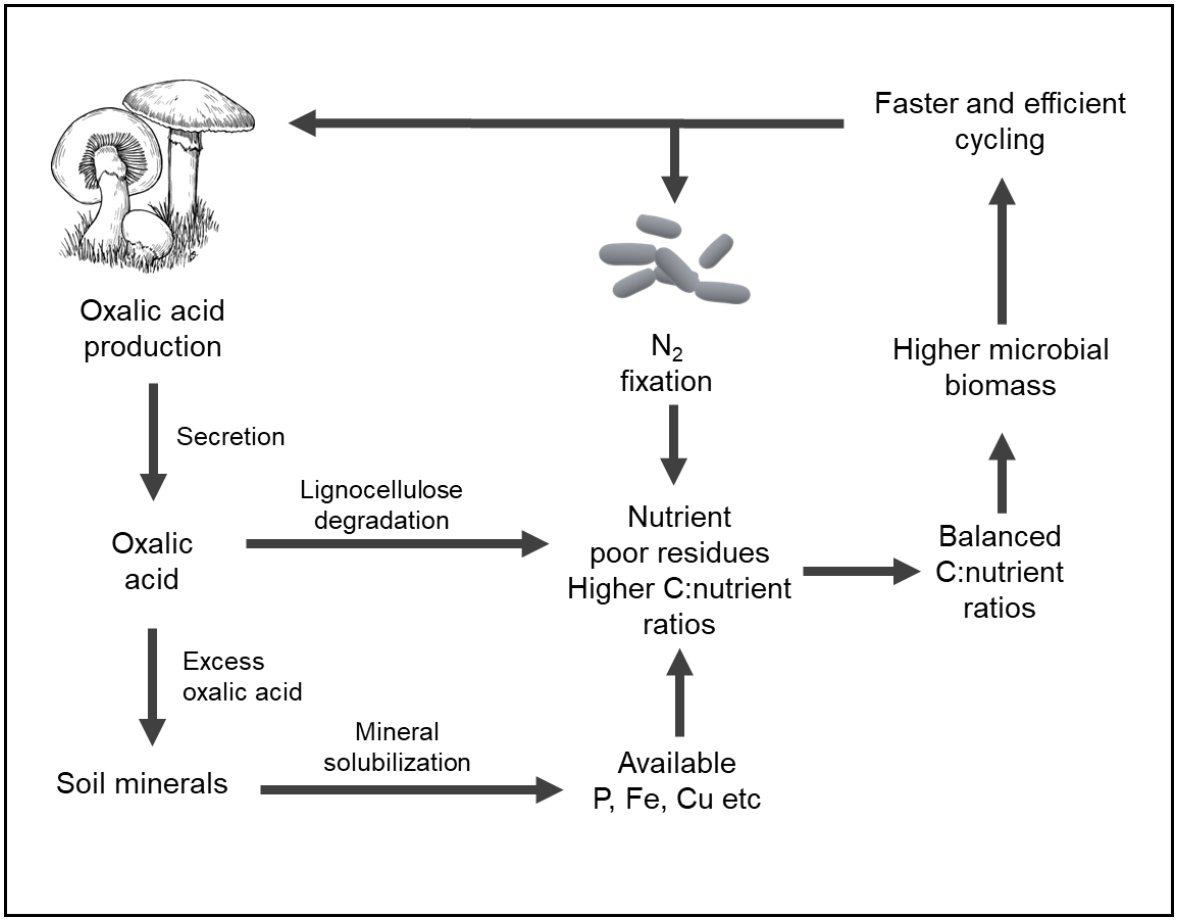

FIGURE 2. Ecosystem-level phosphate solubilization by saprotrophic fungi leads to a more balanced carbon:nutrient ratios of decomposing plant residues. Saprotrophic fungi produce oxalic acid firstly aiming at the degradation of lignocellulolytic residues. An excess of oxalic acid would be beneficial to saprotrophs since it would allow the solubilization of primary and secondary soil minerals that would balance carbon:nutrient ratios of the decomposing material. Limiting carbon:nitrogen ratios would be improved via nitrogen fixation performed by bacteria and archaea. Balanced carbon:nutrient ratios would allow higher microbial biomass yields that, in turn, would lead to a faster and more efficient cycling in the ecosystem. Fungi with higher oxalic acid production would be benefited and selected along evolution since an excess of this metabolite would allow fungal growth in a non-limiting availability of mineral nutrients. Because oxalic acid is so efficient at residue decomposition and mineral solubilization, fungi able to produce an excess of this metabolite would be favored during organic matter cycling in the environment.

Stricto sensu phosphate solubilizers

Here we propose that microorganisms that carry out phosphate solubilization aiming at P acquisition through strictly P-regulated mechanisms and not incidentally, because of other metabolic activity, should be called "*stricto sensu*" phosphate solubilizers. The existence of such microorganism is suggested by the fact P accumulation in plants inoculated with phosphate solubilizers does not always positively correlate with microbial P solubilization *in vitro*. In fact, there are many reports showing that some microbial isolates highly effective at P solubilization *in vitro* contribute little or nothing to P accumulation in the inoculated plants (Hameeda et al., 2008; Taurian et al., 2009; Oteino et al., 2015; Mei et al., 2021). The reverse has also been reported. The fact that the screening for phosphate solubilization focus on the ability of a given isolate to carry out the process *in vitro* and beyond the microbial needs for P, many isolates that could represent *stricto sensu* solubilizers are generally outright eliminated. Therefore, the current screening strategies would make it difficult the discovery of new and regulated mechanisms of phosphate solubilization induced by P and/or the host plant, putting s*tricto sensu* solubilizers always at the risk of passing totally unnoticed during *in vitro* screening. For a microorganism to be classified as a *stricto sensu* solubilizer, several criteria should be met (Table 1).

S*tricto sensu* P solubilization should take place in the plant rhizosphere to meet the plant´s requirement for P (Figure 3). Thus, *stricto sensu* solubilizers should establish associative symbiosis with the host. Low reactivity phosphate solubilization and P desorption can take place in the rhizosphere and released P can be either directly absorbed by the plant or immobilized in the rhizosphere microbial biomass. Further cycling of microbial biomass P would supply plants with adequate amounts of the element. In fact, microbial biomass P has recently been shown to be cycled quickly upon microbial death in the rhizosphere, being one of the major P sources for absorption by plants (Sokol, *et al.*, 2022). As associative symbionts, *stricto sensu* solubilizers should not form symbiosomes within plant cells or tissues. These microorganisms are likely to differ from *lato sensu* solubilizers that maintain loose interactions with the hosts, with low or no host specificity. It is expected that more intricate interactions may have coevolved between *stricto sensu* solubilizers and plants, indicating the coevolution of both partners and, possibly, but not necessarily, a higher degree of interdependence. Likewise, a *stricto sensu* solubilizers should be able to turn on phosphate solubilization mechanisms in response to plant signals released during P starvation (Table 1). This would enable them to interact bidirectionally with plants to maintain a balance in the availability of this nutrient, ensuring equilibrium of P levels in the rhizosphere

Table 1. Criteria for defining a *"stricto sensu"* phosphate solubilizing microorganism.

Criteria
1. P solubilization should be strictly regulated by P availability in the medium
2. P solubilization should be distinguishable from P mineralization by phosphatase activity
3. P solubilization should be specifically directed to phosphate acquisition by the microbe or plant symbiont
4. P solubilization should respond to plant rhizosphere signals
5. P solubilization should operate in association with the host based in a non-energy/P-wasting interaction, strictly regulated to meet the plant´s P demand.
6. P solubilization should be traced back to a specific protein or metabolic pathway clearly leading to an increased P availability in the medium
7. P solubilization should not provoke changes in root structure
8. P solubilization should be conducted by rhizosphere microbes as associative symbionts and not as saprophytes

soil. In fact, plants modify the quality and quantity of root exudates when grown under P deficiency, malic acid being one of the main compounds released by roots under such condition (Pantigoso et al., 2020; Tang et al., 2020). Interestingly, the addition of malic acid to the soil in experiments aiming at the determination of the soil P solubilization potential has revealed that it increases the numbers of phosphate solubilizers, the P content in the microbial biomass, and P in solution (Barrera, 2020).

The activity of *stricto sensu* solubilizers should also be clearly distinguishable from phosphatase activity, that is to say, increases in P availability in the soil should not result from phosphatase activity either singly or in combination with other mechanisms of mineral P solubilization. Phosphatases are primarily involved in the mineralization of organic P (Nannipieri et al., 2010). Microorganisms classified as *stricto sensu* P solubilizers should be specialized at solubilizing inorganic P through acidulation, complexation, or through other mechanisms still unknown to science. However, dissolved P should be clearly distinguishable from mineralized P through the use of improved P fractionation techniques sensible enough to track P transfer from distinct soil pools to the plant.

Microorganisms that solubilize P in a *stricto sensu* fashion should conduct the process in a clearly regulated way dependent on P availability. Low P levels in the soil would induce phosphate solubilization by such microorganisms, while excess P would hinder phosphate solubilization. This regulation is fundamental, as seen in other important microbial processes like biological nitrogen fixation, where the nitrogenase complex is strongly inhibited by combined nitrogen (Soumare et al., 2020). In such systems, no excess of the nutrient is produced beyond the needs of the symbiotic partners, avoiding wasting energy in the production of NH_4^+ that would not be used for the benefit of the symbiosis (Lodwig & Poole, 2003). This is also evident in free-living diazotrophs that perform biological nitrogen fixation only to sustain their requirements (Reed et al., 2011). In this context, increases in nitrogen availability are associated with a rise in rapidly cycled biomass in the soil rather than a direct release of fixed nitrogen directly in the soil (Smercina, 2019).

Another confusing factor that must be eliminated in the screening of *stricto sensu* solubilizers is that the microorganism should not induce changes in host root architecture (Table 1). Plant growth promoting microorganism stimulate plant growth and productivity through a series of direct and indirect mechanisms that operate singly or in a combined fashion (Glick, 2012). For example, many nitrogen fixers are also capable of solubilizing phosphate, producing AIA, ACC deaminase, and other factors involved in plant growth stimulation (Roesch et al., 2007; Higdon et al., 2020). Modifications in plant root structure

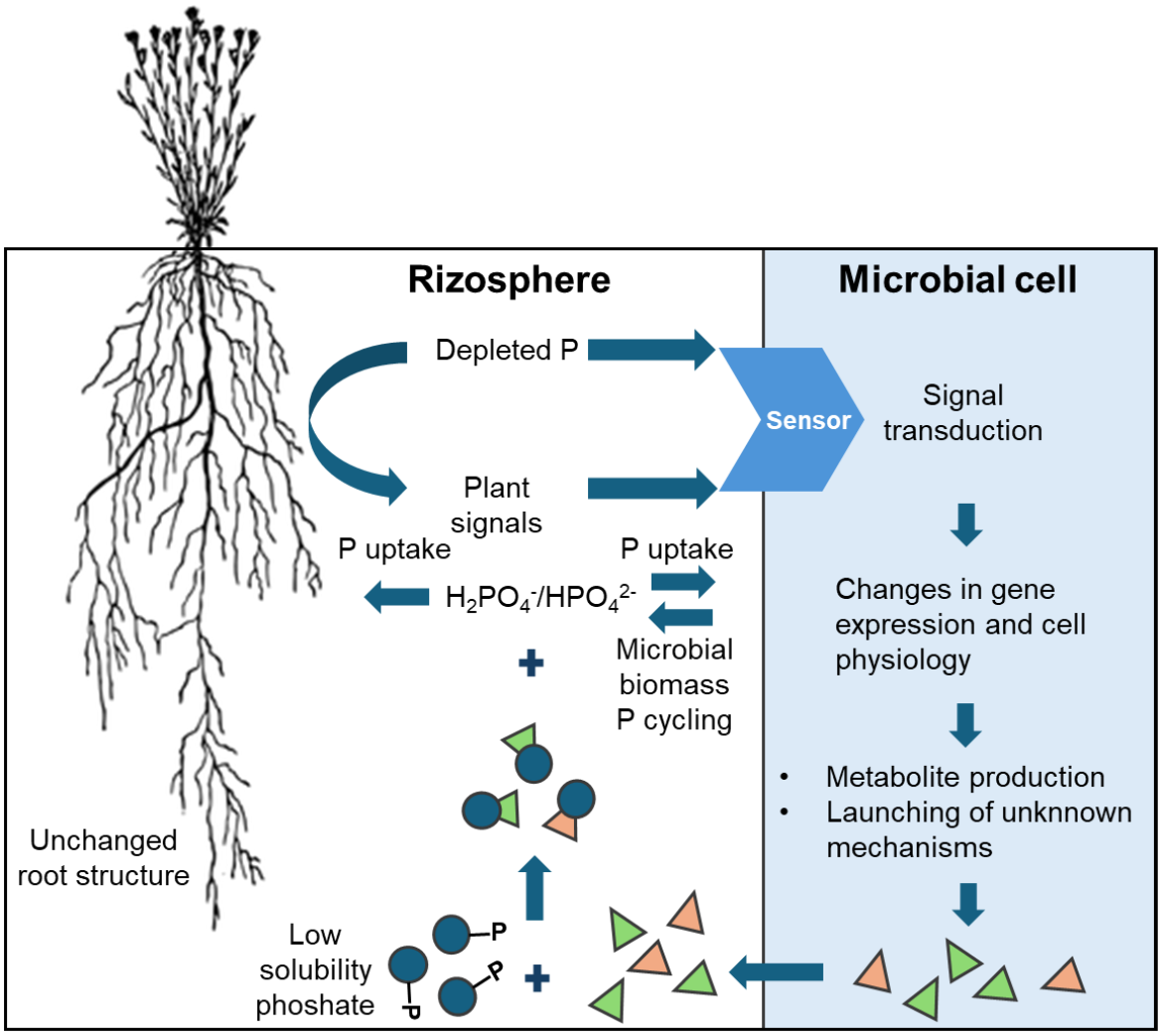

FIGURE 3. Hypothetical interaction between plants and *stricto sensu* phosphate solubilizing microorganisms in the rhizosphere. P depletion in the rhizosphere soil can be perceived by the plant roots and microbial cells. Under low phosphate availability, the plant exudes molecular signals that interact with microbial sensor proteins launching a signal transduction pathway that changes the expression of specific genes coding proteins involved in phosphate solubilization. The new pattern of gene expression changes cell physiology leading to the production of phosphate solubilizing metabolites and, or launching unknown mechanisms that operate in the dissolution of low reactivity phosphate minerals. Organic acids, such as oxalic acid, and other metabolites are synthesized and secreted to the soil where they will act upon the solubilization reaction leading to the release of orthophosphate into solution. *Stricto sensu* phosphate solubilizers promote increases in P availability that will lead to increased P uptake by the plant, independently of changes in root structure. It is noteworthy that phosphate release by *stricto sensu* microbes is strictly regulated to meet the host plant and the microbial demand, with no significant excess P being generated that could be used by other microbial populations or plant individuals. Cycling of microbial biomass in the rhizosphere could also provide significant amounts of orthophosphate previously solubilized and temporarily immobilized by *stricto sensu* solubilizers.

such as increased branching, smaller root diameters, and higher quantity of root hairs provoked by plant growth promoting microbes could increase water and nutrient accessibility leading to increases in plant P content without any microbial phosphate solubilization (Püschel et al., 2020). Thus, separating increases in P uptake from phosphate solubilization from those derived from a larger root is paramount. Screening *stricto sensu* solubilizers *in planta* should also avoid root colonization by arbuscular mycorrhizal fungi, since hyphal growth in the soil is one of the main mechanisms for increased water and nutrient uptake by mycorrhizal plants, especially P (Huang et al., 2020).

Prospecting s*tricto sensu* phosphate-solubilizing microorganisms

The current techniques and protocols used to isolate and study P-solubilizing microorganisms do not allow the identification of those considered to be *stricto sensu* solubilizers (Nautiyal, 1999; Prabhu et al., 2019). This is because we can only measure those that over-perform this process *in vitro* in a seemingly unregulated way. The most common techniques for isolating phosphate solubilizers involve the use of solid culture media with a low-solubility P source. In these media, P-solubilizing microorganisms are visually identified by the formation of solubilization halos around the colony (Nautiyal, 1999). One of the P sources commonly used is tricalcium phosphate that can be easily solubilized by simple media acidification (Tung, 1998). Solubilization experiments are also set up in liquid media for isolate selection and further optimization of P solubilization (Turan et al., 2006). A *stricto sensu* P solubilizer, as proposed here, may not be identifiable with the use of these techniques. Being *stricto sensu* solubilization a regulated strategy for P acquisition, large solubilization halos would be hardly formed and the process would be certainly turned off by the increasing P levels in liquid media to avoid unnecessary energy expenditures.

Screening of *stricto sensu* phosphate solubilizers must include microorganisms that do not necessarily express mechanisms of phosphate solubilization *in vitro,* but, notwithstanding that, when inoculated into plant roots, do increase plant P uptake. Many microbial isolates presenting this behavior are frequently reported in the literature (Qin et al., 2011; Battini et al., 2017; Matse et al., 2019). Therefore, screening cannot be limited to *in vitro* tests, but should necessarily involve *in planta* experiments. If not done so, *stricto sensu* solubilizers may be eliminated during screening for not performing as *lato sensu* solubilizers, for which the isolation and testing techniques were developed.

Though many microbial species have been identified as phosphate solubilizers, actual proof of which of the many mechanisms potentially involved is operational in the plant rhizosphere is still lacking. This is partially due to a lack of understanding of the genetic determinants involved in phosphate solubilization. Some advances have been obtained for

bacterial solubilizers for which some genes related to phosphate solubilization have been identified (Goldstein & Liu, 1987; Krishnaraj & Goldstein, 2001; Rodríguez et al., 2007). Evidences of partial or total loss of the solubilizing ability have been obtained through mutagenesis, but no *in situ* gene expression experiments have been done that could suggest the activation of phosphate solubilization in the rhizosphere. The number of studies focusing the sequencing of bacterial and fungal *lato sensu* solubilizers genomes is growing, but how genomic information could help detecting *stricto* sensu solubilizers is questionable since the genetics of phosphate solubilization in *stricto* and *lato sensu* populations is likely to involve substantially different systems. However, once the genetic determinants of *stricto sensu* solubilization are determined, mutagenesis techniques could be employed to determine the role of specific proteins and metabolic pathways involved as well as to improve microbial strains to provide adequate levels of P to plants.

Another strategy that could help identifying microorganisms, new metabolites, and new mechanism involved in *stricto sensu* solubilization is metabolomics. Experiments could be set up using media with and without P and analyzing the corresponding metabolome. From that, new metabolites could be detected whose regulation should be influenced by P availability. Further work would involve the determination of the potential cation chelating ability of the corresponding metabolites, their efficiency at rock phosphate solubilization and at P desorption, and the potential genes coding for proteins involved in their synthesis.

Finally, accurate and precise methods to determine changes in organic and inorganic P fractions in the soil and in the rhizosphere are essential. This would allow a panorama of the different P sources that are likely to be made available by *lato* and *stricto sensu* microorganisms through the mechanism they express. Additionally, data on P transfer from one reservoir to another would allow the distinction between which P form is being microbially solubilized and indicate mechanisms possibly involved in the process. Techniques involving accurate determinations of P reservoirs in the soil, the dynamics of the nutrient in the rizosphere, as well as the strength with which P is attached to soil surfaces can be evaluated through advanced techniques employing synchrotron light (Hurtarte et al., 2020).

Final considerations

Here we have attempted to conceptualize microbial phosphate solubilization within two specific strategies used by microorganisms. Hopefully, this review will stimulate a debate on what a phosphate solubilizing microorganisms really is, allowing refocusing research to novel unknown phosphate solubilizers and their mechanisms as well as providing a theorical framework to understand how *stricto sensu* solubilizers work on an ecosystem scale. A

deeper understanding of microbial phosphorus (P) solubilization and a more precise identification of the specific groups responsible for this process will enable research to be effectively targeted towards those microorganisms with a higher potential for interacting with plants, which is crucial for agricultural applications. This understanding would also facilitate a better grasp of the mechanisms underlying these processes in the environment.

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