JAQUELINE MARIA DO NASCIMENTO

CINÉTICA DE SOLUBILIZAÇÃO DE FOSFATOS DE ROCHA E PROMOÇÃO DO CRESCIMENTO DE *Eucalyptus grandis* POR *Aspergillus niger*

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Microbiologia Agrícola para obtenção do título *Doctor Scientiae*.

Orientador: Maurício Dutra Costa

Coorientadores: Gilberto de Oliveira Mendes
Leonardus Vergütz

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Assentimento:

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Aos meus pais e meu irmão.

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2. Seja sempre uma pessoa melhor. A pessoa que Deus quer que você seja.

“Não permita que alguém saia da sua presença sem se sentir melhor ou mais feliz”.

(Madre Tereza)

RESUMO

NASCIMENTO, Jaqueline Maria, D.Sc., Universidade Federal de Viçosa, novembro de 2022. **Cinética de solubilização de fosfatos de rocha e promoção do crescimento de *Eucalyptus grandis* por *Aspergillus niger*.** Orientador: Maurício Dutra Costa. Coorientadores: Gilberto de Oliveira Mendes e Leonardus Vergütz.

O fósforo (P) adicionado ao solo está sujeito a reações que o tornam indisponível para absorção radicular e nutrição das plantas. Os ácidos orgânicos apresentam a capacidade de solubilizar P de rochas fosfáticas (RF) e ainda podem atuar na disponibilização de P fixado ao solo. Pouco se conhece sobre a cinética de solubilização de P em RF por ação de ácidos orgânicos, as transformações desse mineral durante o processo de solubilização e capacidade de microrganismos solubilizadores em disponibilizar o P adsorvido ao solo e promover crescimento vegetal. Os objetivos com esse trabalho foram estudar a cinética de solubilização de RF e avaliar alterações morfológicas, químicas e mineralógicas após contato com ácidos orgânicos além de, avaliar a liberação de P e a contribuição para a nutrição e crescimento de plantas de eucalipto por *Aspergillus niger* FS1. A cinética de solubilização de RF foi realizada utilizando a técnica de stirred flow, com soluções dos ácidos orgânicos. As amostras foram coletadas por 160 mimutos e o P foi quantificado. Ácido oxálico 10 mmol L^{-1} foi o tratamento que apresentou maior taxa máxima de solubilização para os RF. A eficiência de solubilização aumentou quando o ácido oxálico foi combinado com ácido cítrico. Para estudos sobre a morfologia, composição química e mineralogia dos RF após o contato com ácidos orgânicos foi colocado separadamente 0,3 g de cada RF em frascos Erlenmayer 250 ml e misturados com 100 ml de soluções de ácidos orgânicos a 10 mmol L^{-1}. Os frascos foram incubados a 28 °C, 150 rpm por 48 horas. O material residual foi submetido à MEV com EDS acoplado e à DRX. O tratamento que mais promoveu alterações, morfológicas, químicas e mineralógicas foram aqueles que tinham ácido oxálico em sua composição. O elemento que mais sofreu diminuição nas amostras foi o P. As análises de DRX confirmaram a identidade dos minerais de oxalato de cálcio formados, sendo, whewelita, wedelita e caoxita. A whewelita foi a forma encontrada em todos os RF reativos e a wedelita e caoxita estavam presente nos RF de baixa reatividade. Para avaliar a liberação de P em solo altamente intemperizados e a contribuição para a nutrição e crescimento de plantas de eucalipto foi feita adsorção de P ao solo com quantidades que variaram de 5 a 25 % da CMAP e incubou-se por 40 dias. Tratamentos com $Ca_2(H_2PO_4)_2$ e RF de Araxá também foram avaliados. *Aspergillus niger* FS1 aumentou a massa seca de raiz das plantas de eucalipto em todos os tratamentos, além de auxiliar no

acúmulo de macronutrientes na planta. O acúmulo de P aumentou 234 % na parte aérea e 650 % na raiz. Os microrganismos solubilizadores de P e seus metabólitos são importantes para o processo de solubilização de P de RF e para promoção de crescimento vegetal. Destacando grande potencial de aplicação biotecnológica desses microrganismos e de seus metabólitos. Nossos dados contribuem para a construção de alternativas à adubação fosfatada convencional para culturas agrícolas e florestais buscando diversificar formas de manejo de P.

Palavras-chave: Ácidos orgânicos. Fósforo. Microrganismos.

ABSTRACT

NASCIMENTO, Jaqueline Maria, D.Sc., Universidade Federal de Viçosa, November, 2022. **Rock phosphate solubilization kinetics and growth promotion of *Eucalyptus grandis* by *Aspergillus niger*.** Adviser: Maurício Dutra Costa. Co-advisers: Gilberto de Oliveira Mendes and Leonardus Vergütz.

Phosphorus (P) added to the soil is subject to reactions that make it unavailable for root absorption and plant nutrition. Organic acids can solubilize P from rock phosphates (RP) and can also act in the availability of P fixed to the soil. Little is known about the solubilization kinetics of P in RP by the action of organic acids, the transformations of this mineral during the solubilization process and the ability of solubilizing microorganisms to make the adsorbed P available to the soil and promote plant growth. The objectives of this work were to study the kinetics of RP solubilization and to evaluate morphological, chemical, and mineralogical alterations after contact with organic acids, in addition to evaluating the release of P and the contribution to the nutrition and growth of eucalyptus plants by *Aspergillus niger* FS1. The RP solubilization kinetics was performed using the stirred flow technique, with organic acid solutions. Samples were collected for 160 minutes, and P was quantified. Oxalic acid 10 mmol L^{-1} was the treatment that showed the highest maximum solubilization rate for RP. The solubilization efficiency increased when oxalic acid was combined with citric acid. For studies on the morphology, chemical composition, and mineralogy of the RP after contact with organic acids, 0.3 g each RP was placed separately in 250 ml Erlenmayer flasks and mixed with 100 ml of organic acid solutions at 10 mmol L^{-1}. Flasks were incubated at 28°C, 150 rpm for 48 hours. The residual material was submitted to SEM with EDS coupled and XRD. The treatment that most promoted morphological, chemical, and mineralogical alterations were those that had oxalic acid in their composition. The element that suffered the most decrease in the samples was P. XRD analyzes confirmed the identity of the calcium oxalate minerals formed, namely, whewelite, wedelite and kaoxite. Whewelite was the form found in all reactive RPs and wedelite and kaoxite were present in low reactivity RPs. To evaluate the release of P in highly weathered soil and the contribution to the nutrition and growth of eucalyptus plants, P was adsorbed to the soil with amounts ranging from 5 to 25 % of MPAC and incubated for 40 days. Treatments with $Ca_2(H_2PO_4)_2$ and RP from Araxá were also evaluated. *Aspergillus niger* FS1 increased the root dry mass of eucalyptus plants in all treatments, in addition to helping the accumulation of macronutrients in the plant. P accumulation increased by 234 % in shoots and 650 % in roots. P solubilizing

microorganisms and their metabolites are important for the P solubilization process of RP and for promoting plant growth. Highlighting the great potential for biotechnological application of these microorganisms and their metabolites. Our data contribute to the construction of alternatives to conventional phosphate fertilization for agricultural and forestry crops, seeking to diversify forms of P management.

Keywords: Organic acids. Phosphorus. Microorganisms.

SUMÁRIO

INTRODUÇÃO GERAL

Em 2018, o Brasil importou cerca de 5,6 milhões de toneladas de fertilizantes fosfatados solúveis empregados na agricultura brasileira. A utilização de fontes de fósforo (P) nacionais é limitada na agricultura pelo fato de o Brasil possuir, em sua maioria, rochas fosfáticas (RFs) de baixa reatividade. Apesar da baixa reatividade dos fosfatos nacionais, há interesse crescente em se desenvolver tecnologias baseadas no uso de microrganismos solubilizadores de fosfato que permitam o melhor aproveitamento dessas fontes na agricultura.

Alguns microrganismos podem solubilizar RFs, melhorando o aproveitamento desse material pelas plantas. Esses microrganismos utilizam mecanismos metabólicos de acidificação do meio pela liberação de prótons e ácidos orgânicos e a complexação de íons acompanhantes para a solubilização das RFs. O processo de solubilização depende também do tipo de fosfato, sendo os sedimentares mais solúveis que os ígneos e os metamórficos. As apatitas, em geral, são mais solúveis em meios ácidos. Além disso, os ácidos orgânicos, tais como os ácidos oxálico e cítrico, também aumentam a solubilização desse material por meio da formação de complexos com o cálcio. Assim, o uso de ácidos orgânicos, bem como dos próprios microrganismos que os produzem, tem sido o foco de pesquisas na área de biotecnologia visando tecnologias que permitam maior aproveitamento desse nutriente. No entanto, a cinética de solubilização de apatitas por microrganismos ou metabólitos microbianos permanece desconhecida em sistemas de solubilização contínuos. Esses sistemas permitiriam conhecer o processo de solubilização e ainda estudar as partículas residuais dos fosfatos depois do contato com os ácidos orgânicos.

O P por estar sujeito aos processos de adsorção e fixação na fase sólida do solo, torna-se rapidamente indisponível às plantas, principalmente em solos mais eletropositivos, ricos em óxidróxidos de Fe e Al. Após a fertilização com P, o elemento liga-se à fase sólida do solo, por meio de ligações fracas ou de interações eletrostáticas, permanecendo em equilíbrio com a solução, nessa fase o P é chamado de P-lábil. Com o passar do tempo, essas ligações vão se fortalecendo e o P se prende à superfície das argilas por meio de ligações mono e bidentadas. Assim, o P torna-se indisponível, passando a compor fase sólida do solo, formando o P-não lábil. Nesse ponto, grande quantidade de energia torna-se necessária para a quebra da ligação estabelecida e que o elemento volte ao equilíbrio com a solução do solo e esteja disponível para a absorção pelas raízes.

Os microrganismos solubilizadores de fosfato e metabólitos podem influenciar os fenômenos de adsorção e dessorção de P no solo. Assim, os mecanismos utilizados pelos microrganismos do solo para solubilizar RFs podem também atuar liberando o P adsorvido ao solo. Cerca de 20 a 30 % do P fixado ao solo pode ser devolvido a solução em avaliações *in vitro.* O que indica que essa poderia ser a saída para reaver os grandes *pools* de P fixado em solos agricultáveis que tem como uma das suas características serem altamente intemperizados, como os solos tropicais.

Diante do exposto, os objetivos desse trabalho foram estudar a cinética de solubilização de fosfato de rocha por ácidos orgânicos e avaliar a absorção de P fixado em solo altamente intemperizado por plantas de *Eucalyptus grandis* inoculadas com *Aspergillus niger* FS1.

Capítulo I

MICRORGANISMOS SOLUBILIZADORES DE FOSFATO: VIA ALTERNATIVA PARA A PROBLEMÁTICA DO FÓSFORO EM SOLOS TROPICAIS: REVISÃO

MICRORGANISMOS SOLUBILIZADORES DE FOSFATO: VIA ALTERNATIVA PARA A PROBLEMÁTICA DO FÓSFORO EM SOLOS TROPICAIS: REVISÃO

Cenário atual do consumo de fertilizantes

No mundo foram utilizados cerca de 46 milhões de toneladas de P_2O_5 em 2020. A perspectiva é que em 2022 sejam utilizados 50 milhões de toneladas desse fertilizante em todo o mundo (FAO, 2020). O Brasil utilizou cerca de 5,2 milhões de toneladas de fertilizantes fosfatados para atender a demanda agrícola em 2020. Sendo metade desse montante, cerca de 3,2 milhoes de toneladas, proveniente de importação. O consumo anual de P na agricultura tende a aumentar 2,2 % ao ano (Bouwman et al., 2013). Observada a grandeza desses números é nítida a importância que os fertilizantes fosfatados têm na produção agrícola.

Atualmente, quantidades muito elevadas de fertilizantes fosfatados são requeridas para manter a agricultura, com maior destaque para a agricultura intensiva que tem sido praticada nos trópicos, em especial no Brasil (Menezes-Blackburn, 2016). Os depósitos de RFs de origem sedimentar são a principal fonte de P atualmente. Países como China, Marrocos, África do Sul e Estados Unidos detém cerca de 83 % das reservas mundiais de RFs sedimentares de alta reatividade (Vaccari, 2009) sendo 74 % só em Marrocos (Menezes-Blackburn, 2018). No Brasil existem depósitos de RFs de origem ígnea, que normalmente possuem teores de P mais baixos em torno de 4 a 16 %.

Os maiores produtores de RFs são China, produzindo entre 80 e 140 milhões de toneladas/ano, Marrocos com produção de 36 milhões de toneladas/ano, EUA com cerca de 23 milhões de toneladas/ano. Esses países produzem aproximadamente de 73 % de toda RF utilizada para produção mundial de fertilizantes fosfatados. O Brasil junto outros países como África do Sul e Austrália produz até 10 milhões de toneladas/ano.

No Brasil as reservas de RFs chegam a 2,9 bilhões de toneladas, o que representa 317 milhões de tonelads de P_2O_5, considerando teor de 10 % (Menezes-Blackburn, 2016). Essa dependência de recursos naturais não renováveis pode se tornar problemática por se tratar de fonte finita que vem sendo esgotada rapidamente (Cordell et al., 2009; Mueller et al., 2012; Menezes-Blackburn, 2016). Portanto, levando em conta que as fontes de P são recursos finitos e o uso tem sido constante alguns autores destacam possível crise global de P em um futuro próximo (Abelson, 1999; Vaccari, 2009; Jones et al., 2015).

As reservas de rochas fosfáticas que possuem melhor qualidade, ou seja, maior teor de P, menor quantidade de impurezas e melhor reatividade no solo são concentradas em poucos

países e, portanto, questões geopolíticas exercerão cada vez mais influência na produção e distribuição de fonte de P (Granada et al., 2018). Além disso, à medida que esses recursos vão sendo utilizados os custos de extração tendem a aumentar, uma vez que, as reservas de melhor qualidade se esgotam primeiro. Assim, os fertilizantes fosfatados se tornam recurso mais limitado e consequentemente mais caro, tendo grandes impactos na produção agrícola (Abelson 1999; Cordell et al., 2009).

Rochas fosfáticas

As rochas fosfáticas (RFs) ou fosfatos naturais apresentam ampla variação na composição, nos tipos de minérios presentes, na textura e na origem geológica, mas, geralmente, apresentam pelo menos uma característica em comum: são constituídos por minerais do grupo das apatitas (Kaminski e Peruzzo, 1997). Os minerais apatíticos formaram-se em diferentes regimes geológicos: ígneos, metamórficos ou sedimentares.

Os depósitos de RFs de origem ígnea ou magmática são frequentemente pobres em sílica, possuem textura fina e contêm rochas do tipo carbonatitos e ultrabásicas. A fluorapatita é o principal mineral fosfático constituinte dos fosfatos Jacupiranga e Catalão, no Brasil, e Tennessee, nos EUA. Esses fosfatos são considerados fosfatos duros e de baixa reatividade (Kaminski e Peruzzo, 1997).

As RFs de origem sedimentar possuem história geológica complexa e variada, podendo ser detríticos, precipitados químicos ou conter quantidades significativas de apatita fóssil (orgânica). Os minerais predominantes são apatitas com alto grau de substituições isomórficas por carbonato. São encontrados em áreas desérticas ou de clima seco. São muitas vezes identificados como francolitas ou fosforitas. São oferecidos no mercado de fertilizantes como RFs reativas, com utilização diretamente na agricultura. Nesse grupo citam-se as RFs da Carolina do Norte nos EUA, de Gafsa na Tunísia, de Sechura no Peru e de Arad em Israel (Leon et al., 1986).

As RFs de origem metamórfica representam categoria intermediária entre as rochas sedimentares e ígneas, mas são rochas duras e apresentam outros minerais misturados (McClellan e Kauwenberg, 1990). São também identificadas como rochas metassedimentares, como por exemplo, os fosfatos naturais de Patos de Minas, no Brasil. As RFs podem ser quimicamente solubilizados com ácidos inorgânicos, principalmente a partir da reação com ácido sulfúrico. Este ácido é, portanto, insumo intermediário básico para a produção

fertilizantes fosfatados solúveis. Atualmente, oito países concentram cerca de 45 % da produção global de ácido sulfúrico (Bruijne et al., 2009).

O ácido sulfúrico é produzido utilizando o enxofre com principal matéria prima. A produção mundial de enxofre em 2020 foi de aproximadamente de 78 milhões de toneladas. Os principais produtores são a China, EUA, Canadá e Iraque. Cerca de 70 % da produção de enxofre é a partir do processamento de combustíveis fósseis e, 20 % aproximadamente é proveniente da recuperação de sulfetos e minerais sufetados (Bruijne et al., 2009). Em 2019, os principais fornecedores de enxofre para o Brasil foram Rússia, EUA e Cazaquistão e so fornecedores de ácido sulfúrico foram a Espanha, Bélgica e o México. Espera-se que a produção mundial cresça e a estimativa é que em 2026 sejam produzidos 311 milhões de toneladas. Em 2019, o Brasil importou cerca de 1,5 milhões de toneladas de enxofre para atender ao mercado nacional. Nesse mesmo ano foram produzidos cerca de 3,3 milhões de toneladas de ácido sulfúrico para indústria de fertilizantes fosfatados (Bruijne et al., 2009).

Um dos problemas que pode ser observado é que os maiores produtores desses insumos também são os maiores consumidores, e sua produção é quase toda empregada no mercado interno. Os países como o Brasil que dependem desses insumos ficam a mercê de variação de preço devido a variação na oferta desses insumos no mercado. Associada a utilização do ácido sulfúrico no beneficiamento das RFs existe a grande produção de fosfogesso, como subproduto da fabricação de ácido fosfórico. A produção desse subproduto é também ponto chave na indústria de fertilizantes fosfatados. Estudos vem sendo feitos para o melhor aproveitamento desse subproduto, entretanto, ainda é um recurso que possui pequena escala de aproveitamento (Brasil et al., 2020).

O desenvolvimento de alternativas à utilização de ácido sulfúrico pode trazer benefícios ao mercado para que ele se torne mais sustentável e menos dependente de recursos externos, ficando menos exposto a grandes variações de preço e disponibilidade do produto (Xiao et al., 2008; Sharma et al., 2013).

A importância do Fósforo

O fósforo (P) faz parte de um conjunto de 17 elementos essenciais para o crescimento vegetal. É o segundo nutriente mais importante para o desenvolvimento das plantas, sendo o primeiro o nitrogênio (Raghothama, 1999; Hou et al., 2020). O P é o elemento que mais limita o crescimento das plantas. A limitação de P é mais marcante em regiões tropicais devido a presença de solos altamente intemperizados (Hou et al., 2020). No entanto, também ocorre

frequentemente em outras regiões, ocorrendo de forma generalizada na produção de plantas acima do solo em ecossistemas terrestres. A magnitude da limitação de P está relaciona a condições de clima, propriedades do ecossistema e regimes de fertilização (Hou et al., 2020).

O P está atrelado a moléculas vitais que fazem parte do metabolismo celular, como ácidos nucleicos, coenzimas, fosfolipídeos e fosfoproteínas, processos de produção de energia (ATP), fotossíntese e respiração, reações de oxi-redução, além de, ativação/inativação de enzimas (Hinsinger, 2001; Kapri e Tewari 2010). Embora seja nutriente tão importante, o P é requerido em concentrações baixas, da ordem de 0,1 a 0,5 % da massa seca produzida (Sultenfuss e Doyle, 1999; Kapri e Tewari, 2010). E, apesar disso, a deficiência de P nos solos é uma das principais limitações na produção agrícola (Condron e Newman, 2011). Estimativas indicam que 5,7 bilhões de hectares no mundo sejam deficientes em P (Mouazen e Kuang, 2016). É encontrado nas formas orgânicas (Po) e inorgânica (Pi). Uma vez na solução do solo se transforma em $H_2PO_4^-$ e HPO_4^{-2} e é pouco móvel no solo. Grande parte do P (95-99 %) presente no solo não é solúvel em água e, portanto, não fica disponível para absorção pelas raízes (Vance et al., 2003; Pradhan e Sukla, 2005).

O transporte de P no solo se dá por meio da difusão do elemento pela solução do solo, concomitantemente ocorre reabastecimento da solução a partir do P-lábil que está adsorvido à fase sólida do solo (Nye, 1997; Novais e Smith, 1999). A planta absorve esse nutriente na forma de ortofosfato divalente ($H_2PO_4^-$) e monovalente (HPO_4^{2-}), sendo a última preferencialmente absorvida pelas raízes (Raghothama, 1999; Novais et al., 2007). Nessas formas, as concentrações que permanecem na solução do solo são muito baixas, da ordem de 0,1 a 10 μM (Hinsinger, 2001).

A planta pode adquirir o P por meio de processos simples de absorção através das raízes, pela exsudação de enzimas, a exemplo das fosfatases, e pela exsudação de compostos orgânicos. Esses compostos alteram as reações químicas que ocorrem na rizosfera, moldando a população de microrganismos a ela associada que auxiliam a planta na aquisição de nutrientes (Marschner et al., 1986; Vance et al., 2003; Devau et al., 2011). Dentre os macronutrientes essenciais às plantas, o P é aquele que tem a biodisponibilidade mais limitada devido a sua baixa mobilidade e alta afinidade com óxidos de Fe e Al, característico em solos tropicais (Vance et al., 2003).

A dinâmica do Fósforo no solo

A disponibilidade de P na solução do solo é influenciada por fatores como pH, força iônica, conteúdo de matéria orgânica, presença de cátions como Fe, Ca e Al, além de presença

de ânions que possam ocupar preferencialmente o sítio de ligação do P nos colóides do solo (Achat et al., 2016). As concentrações de P no solo são controladas por reações que envolvem processos de adsorção e dessorção de P nas superfícies de minerais de argila do solo, pela solubilização de minerais fosfatados e pela degradação de compostos orgânicos que possam conter P em suas estruturas (Matar et al., 1992; Comerford, 1998; Hinsinger, 2001).

A forma de adsorção do P nas argilas é variada. Dependendo do tipo de argila essa ligação pode ser mais forte ou mais fraca empregando maior ou menor energia no processo. Inicialmente as ligações que são formadas no processo de adsorção são fracas de origem eletrostática entre o P e superfície dos colóides. Ao longo do tempo vão se tornando cada vez mais fortes, dando início ao processo de fixação (Novais e Smyth, 1999). As taxas de dessorção são mais baixas do que as taxas de adsorção, conferindo o ao comportamento do P na forma de histeresis descrito por Okajima et al. (1983). Nesse comportamento de histeresis o processo de adsorção não é totalmente revertido, ou seja, parte do fosfato adsorvido às argilas não pode ser devolvido ao solo, na sua forma disponível, permanecendo fixado ao solo (Novais et al., 2007).

A fixação de P nos solos é um processo no qual o P adsorvido não mantém mais equilíbrio com o P da solução, passando da sua forma lábil para sua forma não-lábil. Assim, ao ser fixado ao solo o P passa a ficar indisponível para absorção das raízes (Vance et al., 2003). O P adiconado ao solo reage com as superficies das argilas, adsorvendo-se, promovendo o deslocamento de ânions com menor afinidade da fase sólida do solo (Li e Stanforth, 2000; Syers et al., 2009). Esse processo é maior e mais significativo em solos que são altamente intemperizados, que possuem altos níveis de oxi-hidróxidos de Fe e Al e elevado número de cargas positivas onde a fixação a curto prazo, horas a dias, é altamente relevante (Matar et al., 1992; Comerford, 1998; Hinsinger et al., 2011).

O P no solo ainda pode sofrer outras reações que também o tornam indisponíveis, a exemplo da precipitação com outros elementos presentes como o Ca^{2+} e Mg^{2+}, em pH elevado, formando minerais secundários e a imobilização em compostos orgânicos de P, formando o Po (Hinsinger et al., 2011).

De um modo geral, a fixação de P nos solos faz com que os produtores apliquem fertilizantes em suas lavouras duas ou mais vezes além do requerimento das culturas (Syers et al., 2009; Roy et al., 2016). A saturação do sistema solo com P a partir de fertilizantes fosfatados tem sido estratégia agrícola aplicadas em diferentes solos para contornar a alta capacidade de fixação de P com o intuito manter concentrações ideais desse elemento na solução de solo para atender a exigência nutricional das plantas (Fox e Kamprath, 1970). No

entanto, apesar de ser prática eficiente torna a produção mais onerosa devido ao baixo aproveitamento dos nutrientes, já que pequena fração do que é disponibilizado é de fato absorvido pelas culturas (Batten, 1992).

Estima-se que todos os anos as entradas de P nos solos agricultáveis sejam da ordem de 2 a 8 milhões de toneladas e que desse montante, metade, cerca de 1 a 4 milhões, permaneça no solo como P residual (Roy et al., 2016). As projeções preveem ainda que 4 a 14 milhões de toneladas entrarão no sistema agrícola até 2050 e que desse total 2 a 7 milhões permanecerão fixados nos solos (Roy et al., 2016). De acordo com alguns modelos, se houvesse a utilização desse pool de P residual fixado no solo seria possível reduzir a demanda global de fertilizantes fosfatados em até 50 % até 2050 (Sattari et al., 2012).

O acesso a essa reserva de P nos solos poderia ser alcançada com a aplicação de tecnologias apropriadas para a mobilização desse elemento do solo para suas formas disponíveis (Menezes-Blackburn, 2018). Alguns autores relatam modelos estudados que indicam que o P fixado nos solos possa atender as demandas de P nos campos agrícolas por 9 a 22 anos, variando de acordo com sua disponibilidade (Sattari et al., 2012; Rowe et al., 2016). Esses dados indicam que possível produção baseada no uso do P fixado, com poucas entradas de fertilizantes fosfatados tem se tornado cada vez mais preocupação global, que trazem benefícios econômicos, agronômicos e ambientais (Sattari et al., 2012; George et al., 2016).

Em solos com fertilidade construída, onde são feitas adubações constantes com nutrientes principalmente P, as análises de solos dessas áreas mostram que existe quantidade de P remanescente altíssima e que ainda assim os produtores decidem por manter adubação considerável com P para não colocar em risco o rendimento da lavoura. O grau de saturação do solo com P e as taxas de fertilização são inversamente proporcionais a capacidade de retenção de P no solo, ou seja, ao longo do tempo solos intensamente adubados tem quantidade de P tão elevada que a capacidade de retenção de P do solo torna-se insignificante (Hooda et al., 2001).

Portanto, apoiando-se em pesquisas e abordagens biotecnológicas adequadas e viáveis é possível que pelo menos parte desse P seja novamente disponibilizado às culturas, desde que as estratégias de fertilização de P sejam revistas, no sentido de diminuir as entradas de P no sistema solo (Stutter et al. 2012; Roy et al., 2016; Menezes-Blackburn, 2018). Assim, o manejo de P fixado nos solos deve considerar a capacidade de reposição de P em solução em condições ambientais específicas, levando em conta a quantidade e o tempo que o P fixado pode sustentar a produtividade das culturas, fazendo sempre o monitoramento para

determinar o momento que se deve adicionar fertilizantes para que não haja perdas no rendimento das culturas.

Como os microrganismos e seus exsudatos atuam na disponibilização de P no solo?

O uso de microrganismos solubilizadores de fosfato (MSF) vem emergindo como alternativa biotecnológica para o manejo da fertilização fosfatada, um mecanismo de baixo custo e que aumenta a eficiência agronômica das rochas fosfáticas (RFs) (Sauer et al., 2008). Alguns estudos demonstraram que o produto obtido do tratamento de RFs com MSF (Vassilev et al., 1996), ou mesmo a aplicação direta desses microrganismos no solo (Jain et al., 2010), aumentam o crescimento e o acúmulo de P pelas plantas. A solubilização de P por MSF resulta da produção de ácidos orgânicos, que são capazes de formar complexos estáveis com cátions que normalmente se ligam ao P em formas pouco solúveis. A acidez gerada desestabiliza os minerais fosfatados, auxiliando na liberação de P (Fox et al., 1990). O P adsorvido em minerais pode ser liberado por ácidos orgânicos de baixo peso molecular, tais como ácido cítrico, málico, glucônico e oxálico, produzidos por microrganismos do solo (Plassard et al., 2011; Mendes et al., 2014a). Além disso, o P fixado às argilas do solo também pode ser liberado por *A. niger* FS1 e seus metabólitos (Nascimento et al., 2021). Para a maioria dos MSF, a produção de ácidos orgânicos é o principal mecanismo de solubilização (Richardson 2001; Richardson e Simpson 2011).

Os ácidos orgânicos contribuem para a redução do pH à medida que se dissociam num equilíbrio dependente do seu pKa nos seus respectivos ânions e prótons. Os íons H^+ favorecem a solubilização da RF deslocando o equilíbrio da dissolução da solução (Arcand et al., 2006). Dessa forma, a solubilização de minerais por ácidos orgânicos pode ocorrer por meio de acidólise, pelos prótons liberados de grupos carboxila, e das reações de complexação/quelação dos metais presentes na estrutura do mineral (Matsuya e Matsuya, 1994; Fomina et al., 2005). A capacidade de complexação depende das características químicas do ácido orgânico, a exemplo do número e da posição de grupos carboxila, da constante de estabilidade do complexo orgânico-metal, da concentração do ácido, do tipo de metal em solução e do pH do meio (Bolan et al., 1994; Kpomblekou-A e Tabatabai, 1994).

Nos microrganismos, semelhantemente às plantas, a excreção microbiana de H^+ ocorre em resposta à assimilação de cátions relacionados principalmente a fonte de N, onde o H^+ excretado é trocado pelo NH_4^+ (Banik e Dey, 1982). De forma geral, mas RF é solubilizado

quando fontes amoniacais de nitrogênio são utilizadas em relação as fontes nítricas (Ahuja et al., 2007; Chuang et al., 2007). Fontes nítricas proporcionam maiores taxas de crescimento sem, contudo, proporcionar boas taxas de solubilização (Narsian e Patel, 2000). A fonte de P também pode influenciar comportamento dos isolados sob diferentes fontes de nitrogênio.

Espécies do gênero *Aspergillus* e *Penicillium* estão entre as mais eficientes na solubilização de fosfato. Entre as bactérias solubilizadoras destacam-se as espécies do gênero *Pseudomonas*, *Bacillus* e *Rhizobium* (Silva Filho et al., 2002). Diversos microrganismos do solo solubilizam diferentes formas de fosfatos inorgânicos. *Penicillium radicum*, por exemplo, é capaz de solubilizar $Ca_3(PO_4)_2$, $CaHPO_4$, $FePO_4.4H_2O$ e $AlPO_4$ (Whitelaw et al., 1999). Fatores relacionados ao estado nutricional microbiano, como as diferentes fontes de fósforo, nitrogênio e carbono podem alterar a capacidade de solubilização de fosfato pelos microrganismos. Alterações nas fontes de fosfatos insolúveis proporcionam distintos padrões de crescimento e produção de ácidos orgânicos (Reyes et al., 1999; Reyes et al., 2002) e, em consequência, diferentes níveis de solubilização.

Além da concentração, o tamanho da partícula da RF também tem efeito sobre a quantidade de P solubilizado. Quanto maiores as partículas, menores são as quantidades de P liberadas da RF (Xiao et al., 2008). Durante o processo de solubilização, os MSF ficam expostos a elementos químicos liberados a partir da RF, como o flúor, que pode provocar forte diminuição no processo de solubilização (Mendes et al., 2013). Estratégia utilizada para remoção de F^- é sua adsorção em vários tipos de materiais complexos como, por exemplo, o biochar ou biocarvão (Mendes et al., 2014b; Vassilev et al., 2013a; Vassilev et al., 2013b). Testes com mutantes de *A. niger* tolerantes ao fluoreto, com potencial de solubilização de fosfato vêm sendo realizados no intuito de se obter linhagens superiores às selvagens (Silva et al., 2014). Duas linhagens mutantes de *Aspergillus tubingensis* com potencial solubilizador maior que da linhagem selvagem exibiram máxima solubilização tanto em meio de cultura com RF quanto com fosfato tricálcico (Achal et al., 2007).

Já na rizosfera os microrganismos desempenham um papel fundamental na ciclagem biogeoquímica das formas de Po e Pi (Richardson 1994; Whitelaw 2000; Jakobsen et al. 2005b; Harvey et al. 2009; Richardson et al. 2009a; Khan et al. 2010; Richardson e Simpson 2011). Do total de bactérias e fungos que são cultiváveis, porcentagem significativa tem sido relatada como solubilizadores de formas inorgânicas de P (Kucey et al. 1989; Bowen e Rovira 1999; Mendes et al., 2014a).

Os microrganismos promovem aumento no volume das raízes e alteram a geometria da rizosfera, possibilitando maior exploração do solo pelas raízes, o que é muito eficiente visto a

baixa mobilidade de P no solo (Richardson et al., 2009). De forma geral, na rizosfera os microrganismos tornam o Pi disponível através da mobilização de formas orgânicas de P a partir da produção de enzimas, a exemplo, das fitases e das fosfatases, da excreção de prótons e redução o pH do meio e da produção de ácidos orgânicos de baixo peso molecular (Comerford, 1998; Hinsinger, 2001; Jorquera et al., 2008; Hinsinger et al., 2011).

A capacidade que os ácidos orgânicos possuem em diminuir a fixação do P está relacionada à capacidade de complexação de metais no solo a exemplo do Fe e o Al, impedido que se combinem ao fosfato (Pavinato e Rosolem 2008; Nziguheba e Smolders 2008; Cessa et al., 2010). Além disso, ânions orgânicos tem potencial de adsorção em solos de carga variável, gerando cargas negativas na superfície das argilas, reduzindo ainda mais a adsorção de P (Bolan et al., 1994; Sanyal e Dedatta, 1991; Hinsinger, 2001). A competição dos ácidos orgânicos com o fosfato depende de fatores como, concentração, forma da molécula e persistência no solo e sua adsorção aos colóides do solo envolve grande quantidade de energia (Cessa et al., 2010; Taddese, 2019).

As concentrações de ácidos orgânicos nas raízes variam de 0 a 0,1 mM. Na rizosfera de algumas plantas essa concentração é estimada em até 50 mM (Jones et al., 1996). A concentração crítica de ácidos orgânicos para que ocorra a liberação de P varia entre 8,5 a 33 mM (Wouterlood et al., 2004). A mobilização de P por ação dos ácidos orgânicos varia ainda de acordo com o tipo de solo (Jones e Darrah, 1994; Jones et al., 2003). Portanto, em solos altamente intemperizados que apresentam alta capacidade de adsorção, presença de óxidos de Fe e Al, a adição de 100 μM Kg^{-1} de ácido cítrico promoveu aumento na disponibilidade de P na solução do solo, enquanto que essa mesma dose em solos com predominância de argilas 2:1 o efeito foi contrário resultando em diminuição da disponilidade de P (Duputel et al., 2013).

Dentre os exsudados que mais disponibilizam P na solução de solo encontram-se os ácidos cítrico e oxálico (Guppy et al., 2005; Nascimento et al., 2021). Ácidos húmicos e fúlvicos também exercem tal função e conseguem disponibilizar esse elemento (Guppy et al., 2005). Já é conhecido o efeito positivo do ácido cítrico no crescimento de plantas pelo aumento da disponibilidade de P através da solubilização de Al-P, Fe-P e Ca-P (Guan et al., 2005; Wei et al., 2010; Santos et al., 2011). Parte do P no solo também pode estar ligada ao Ca^{2+}, em solos que estão em condições de neutralidade, ou seja, com pH próximo a 7,0. No entanto, o composto químico formado é mais susceptível a solubilização pelos microrganismos rizosféricos que tem a capacidade de produzir acidez no meio em que se encontram (Souchie et al., 2007).

Produção de ácidos orgânicos por microrganismos solubilizadores de fosfato

A produção de ácidos orgânicos é considerada o principal mecanismo de solubilização de fosfatos inorgânicos por microrganismos (Ryan et al., 2001; Arcand et al., 2006). Fungos filamentosos apresentam capacidade natural de converter produtos de degradação da biomassa, como glicose, em vários ácidos orgânicos, como os ácidos oxálico, cítrico, glucônico, málico (Liaud et al., 2014). A produção microbiana de ácidos orgânicos é abordagem promissora para obtenção de produtos químicos por meio de fontes renováveis de carbono e substratos de baixo custo (Sauer et al., 2008), tais como resíduos da agricultura, das indústrias de alimentos, biocombustíveis e de processos biotecnológicos (Strasser et al., 1994; Musial et al., 2011; Betiku et al., 2016).

O fungo *A. niger* é conhecido por sua elevada capacidade de produzir ácido cítrico, embora produza grandes quantidades de outros ácidos orgânicos, dentre eles, o ácido oxálico. Em determinadas condições de cultivo, *A. niger* pode expressar diferentes genes que desencadeiam a produção de diferentes quantidades e tipos de ácidos orgânicos (Strasser et al., 1994). A depender dos elementos químicos que são liberados durante o processo de solubilização, diferentes quantidades e tipos de ácidos orgânicos serão produzidos (Scervino et al., 2010), influenciando, consequentemente, a solubilização de P. Ácido glucônico foi produzido em maior quantidade por *A. niger* quando $Ca_3(PO_4)_2$ foi utilizado como fonte de P, e quando se utilizou $FePO_4$ e $AlPO_4$ houve maior produção de ácido oxálico (Chuang et al., 2007).

A eficiência de solubilização, no entanto, depende do tipo de ácido orgânico produzido e de sua concentração no meio. A relação entre prótons adicionados e P liberado para ácidos orgânicos é superior à de ácidos inorgânicos, indicando que outros mecanismos além da acidificação estão envolvidos na solubilização de RF, como a quelação ou complexação de metais associados ao fosfato (Mendes et al., 2020). O ácido oxálico vem se mostrando mais eficiente na solubilização de P a partir de RFs. O ácido oxálico, a concentração de 1 mmol L^{-1} é mais efetivo do que H_2SO_4 em liberar P de duas amostras de RFs, Kodjari e North Florida (Kpomblekou-A e Tabatabai, 1994). O aumento da concentração dos ácidos orgânicos de 1 para 10 mmol L^{-1} ocasionou aumento de quase 10 vezes na liberação de fósforo a partir dos RFs (Kpomblekou-A e Tabatabai, 1994).

Produção de inoculantes

O uso de inoculantes microbianos com interesse específico na capacidade de disponibilizar P para as culturas foi proposto como um dos componentes de um sistema integrado de manejo de nutrientes (Kucey et al. 1989; Bowen e Rovira 1999; Adesemoye e Kloepper 2009; Harvey et al. 2009; Jakobsen et al., 2005b; Khan et al. 2010). No entanto, a forma como foram selecionados apresentam desvantagem competitiva em relação aos microrganismos nativos do solo. Atualmente a falta de estudos e investimento tecnológico fazem com que essa prática não seja amplamente aplicada para utilização de P fixado do solo. Outras questões como impacto limitado no crescimento das plantas, especificidade e colonização ineficiente e baixa sobrevivência de inóculo ainda são impecílios (Jakobsen et al. 2005b; Menezes-Blackburn, 2018).

As pesquisas sobre inoculantes microbianos na rizosfera se concentraram principalmente em microrganismos de vida livre que estabelecem associações não especificas e benéficas com várias plantas. Além disso, esses microrganismos também devem ser fáceis de serem produzidos em grande quantidade com potencial de resistência na rizosfera, a exemplo dos esporuladores (Bowen e Rovira 1999; Harvey et al. 2009; Khan et al. 2010).

Exitem no mercado a presença de alguns produtos solubilizadores de fosfato a base de bactérias. Apesar de serem eficientes na solubilização de P no solo esses microrganismos são mais efetivos na solubilização de formas de P ligadas a Ca. Não se conhece a capacidade de acessar formas mais complexas como P-Al, P-Fe e P ligado as argilas do solo, o que seria fundamental para solos de regiões tropicais.

As associações simbióticas com fungos ectomicorrízicos é conhecida como um sucesso na nutrição de P em espécies agro-florestais, particularmente em solos com baixo teor de P (Richardson e Simpson, 2011). Fungos micorrízicos arbusculares também são conhecidos por esse importante papel, porém as dificuldades relacionadas a cultivo em meios artificiais e falta de estabelecimento de associações específicas limitou seu desenvolvimento como inoculante para a rizosfera (Smith e Read 2008; Jakobsen et al. 2005b).

Por outro lado, o uso de microrganismos que não são associados a rizosfera, a exemplo de *A. niger* FS1, foi destaque na disponibilização de P na solução a partir do próprio solo, indicando sua capacidade de reverter em partes o processo de fixação de P no solo (Nascimento et al., 2021). Esse experimento foi realizado *in vitro* e, portanto, é possível observar essa quantidade de P sendo liberada em condição ótima para o microrganismo, em que ele pode expressar seu potencial. *Aspergillus niger* FS1 mostrou ser eficiente na solubilização de formas de P ligadas a Fe, Al e Ca, liberando 71, 36 e 100 % de P nas formas de $AlPO_4$, $FePO_4$ e $Ca_3(PO_4)_2$ (Mendes et al., 2014). Finalmente, *Aspergillus niger* FS1

também tem se mostrado eficiente em solubilizar formas de RFs de baixa reatividade como RFs de Araxá e Catalão (Mendes et al., 2014)

O potencial de microrganismos indígenas do solo também apresenta grande capacidade de liberar P do solo principalmente e partir de excreção de ácidos orgânicos, açucares e aminoácidos. Com destaque para a combinação desses compostos leva a disponibilidade de P maior do que aquela necessária para sua demanda metabólica (Barrera, 2020).

A liberação de P por microrganismos ou por seus metabólitos não causa alteração na matriz do solo, ou seja, apesar de acreditar-se que o P liberado nesses experimentos é de origem inorgânica que já passou pelo processo adsorção e agora encontra-se fixado, nenhuma alteração na estrutura do solo, principalmente com relação as argilas, não foi observada (Nascimento et al., 2021). A adição de microrganismos ao solo permite a exploração eficiente das alterações do P no solo, acelera a ciclagem entre as formas orgânicas e inorgânicas e aumenta a disponibilidade de P para as plantas.

Perspectivas

A agricultura tropical vem sendo criticada internacionalmente em face da aplicação de P em solos com alto poder de fixação do elemento. Essa prática é considerada um desperdício de recurso natural não renovável (Roy et al., 2016).

A otimização do processo microbiano de solubilização de RFs de menor qualidade, como os presentes no Brasil, permitirá a produção de fertilizantes fosfatos solúveis diminuindo a necessidade de importação de RFs e diminuindo a dependência do ácido sulfúrico, que implica na redução de custos e do impacto ambiental. Essa mudança é particularmente importante, considerando que o Brasil importa cerca de 80 % de todo fertilizante empregado na agricultura, sendo forma de diminuir esse montante construindo alternativas que busquem a exploração da riqueza nacional.

O uso de recursos microbianos do solo em relação ao acesso ao *pool* de P fixado apresenta grande potencial, principalmente em regiões tropicais e deve ser explorado minuciosamente. Os dados evidenciam que eles podem atuar na disponibilização de P fixado e isso pode indicar redução no uso de fertilizantes tornando o processo produtivo mais rentável e sustentável, apresentando-se como excelente ferramenta biotecnológica.

São necessárias mais pesquisas para desvendar o verdadeiro potencial dos microrganismos em casa de vegetação e em campo, contudo, o estudo de microrganismos

capazes de reverter o processo de fixação *in vitro*, é o primeiro passo que traz informações importantes e necessária para a continuidade das pesquisas.

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Capítulo II

ROCK PHOSPHATE SOLUBILIZATION BY ORGANIC ACIDS: I - KINETICS OF SOLUBILIZATION

ROCK PHOSPHATE SOLUBILIZATION BY ORGANIC ACIDS: I - KINETICS OF SOLUBILIZATION

ABSTRACT

The use of phosphate solubilizing fungi has emerged as a biotechnological alternative for the transformation of low-reactivity natural rock phosphates (RPs) into soluble phosphate fertilizers. Oxalic acid, produced by *Aspergillus* and *Penicillium* species, is one of the most efficient metabolites in the solubilization of these rocks. This acid can be produced from different carbon sources, mainly residues from agro-industrial processes, which can make the RP solubilization process cheaper and less harmful to the environment. Despite the considerable literature on RP microbial solubilization in a batch system, little is known about the solubilization kinetics of this material in a continuous system, which allows product removal and determination of the kinetics governed by selected microbial metabolites. Knowledge about the process is important to propose new strategies to optimize the RP microbial solubilization process. Thus, the work aims to study the kinetics of RP solubilization in a continuous flow system with organic acid solutions. The kinetics of solubilization of Argélia, Bayóvar, Catalão, Crandalita, Gafsa and Patos de Minas RPs was carried out using the stirred-flow technique, with solutions of oxalic, citric acids at the final concentrations of 10 mmol L^{-1} separately and their combination at the final concentration of 10 mmol L^{-1} (5 mmol L^{-1} for each component). Additional treatments were water pH 2 and water pH 7. The samples were collected for 160 minutes in a continuous flow of 1 mL min^{-1} and the P analyzed by colorimetry. Oxalic acid at 10 mmol L^{-1} was the most efficient compound in Catalão and Patos de Minas RPs solubilization. At the end of the experiment, 31.74 and 33.54 % of the P contained in the RPs had been solubilized, respectively. The efficiency of oxalic acid was increased when combined with citric acid at 10 mmol L^{-1}. The phosphate solubilization process showed percentages of P solubilization of 44.2 and 44.7 %. Citric acid at 10 mmol L^{-1} was the most efficient compound in Argélia, Bayóvar, and Gafsa RPs solubilization. At the end of the experiment, 43.91, 49.78 and 37.85 % of the P contained in the RPs had been solubilized, respectively. The efficiency of citric acid wasn't increased when combined with oxalic acid. There was no difference between the treatments tested for the solubilization of Crandalite RP. The average solubilization obtained for this phosphate was 4.5 %. Oxalic acid is the most efficient organic acid tested in the solubilization of P from Argélia, Bayovar, Catalão, Gafsa and Patos de Minas RP in stirred flow system.

Improvements in solubilization efficiency can be obtained by combining this compound with citric acid for low-reactivity RPs.

Keywords: Oxalic acid. Citric acid. Phosphorus. Complexation. Acidolysis.

INTRODUCTION

The use of soluble phosphate is costly to agricultural production, since most, abour 50 %, of all phosphate fertilizers used in Brazil are imported (FAO, 2020). The commercial exploitation of Brazilian rocks phosphates (RPs) is still low and, to meet its agricultural demand for fósforo (P), the country has become the fourth largest importer of RP in the world (Globalfert - globalfert.com.br). Many RP deposits show poor quality, with low-reactivity rocks (Novais et al., 1999; Vassilev et al., 2001).

Due to P adsorption and fixation in Brazilian soils, the use of soluble phosphate fertilizers is high, and it becomes necessary to increase P doses applied to the soil to compensate for P losses due to adsorption to clays and reactions with cations, such as Al^{3+}, Ca^{2+} and Fe^{3+} (Novais et al., 2007; Sattari et al., 2012). This practice has placed Brazil under criticism over the indiscriminate use of finite phosphate sources that could result in a shortage of this nutrient soon (Cordell et al., 2009; Roy et al., 2016)

RPs present different natural reactivities depending on their origin as igneous, sedimentary, or metamorphic (Correa et al., 2005). RPs can be chemically solubilized with inorganic acids, mainly from the reaction with sulfuric acid. It promotes the destabilization of apatite structure, leading to increased available P content (Goldstein et al., 1993; Novais et al., 2007). This acid is, therefore, a basic intermediate for phosphate fertilizers. Currently, eight countries concentrate around 45 % of the global production of sulfuric acid (Bruijne et al., 2009). Reserves of high-quality RPs that are cost-effective with this technology are being depleted (Cordel and White, 2011, Vaccari and Strigul, 2011). Biotechnological alternatives that can be used in the P solubilization process that are more sustainable and allow less dependence on this input. (Xiao et al., 2008; Sharma et al., 2013).

Many microorganisms can solubilize RPs by the excretion of organic acids, for exemple, oxalic and citric acids, and the acidification of the surrounding medium (Leitão et al., 2010; Maheswar and Sathiyavani, 2012; Mendes et al., 2014). Organic acids released by root and microbial cells chelate cations in RPs and in the soil, causing equilibrium to be

displaced towards products, with consequent P release (Kpomblekou-A and Tabatabai, 1994; Nautiyal et al.,2000).

Despite the considerable amount of literature available on microbial RP solubilization in batch systems (Moreira and Siqueira, 2006; Mendes et al., 2013; Vassilev et al., 2013; Vassilev et al., 2013a) little is known about the kinetics of RP solubilization in continuous systems that allow product removal and the determination of solubilization kinetics governed by selected microbial metabolites.

The continuous stirred flow system consists of a chamber with inlet and outlet holes. A small amount of material is placed inside the chamber and retained by a membrane filter immediately below the exit hole. A peristaltic pump is used to keep the flow rate of the solution constant. A fraction collector is used to sample the effluent (Sparks et al., 1998; Strawn and Sparks, 2000). This technique relates attributes of the batch and flow system. Due to mixing, the mass transfer phenomenon is practically negligible. When the system is well shaken, the concentration in the chamber and in the collected samples are the same (Sparks et al., 1998). This method allows quick measurements to be made throughout the solubilization process. Facilitating the understanding of the kinetics of P release from the RFs by each extracting solution used.

Oxalic and citric acids are reputed to be efficient at RP solubilization (Kpomblekou-A and Tabatabai, 1994; Mendes et al., 2013; Mendes et al 2020), but the changes in RP mineralogy, morphology, and chemical composition along the process of solubilization remains unknown. Knowledge on changes in RP mineralogy, morphology, and chemical composition during microbial solubilization is important for the proposal of new strategies for the optimization of microbial RP solubilization. The objectives of this work were to study the solubilization kinetics of RP in a continuous stirred flow system with solutions of organic acids.

MATERIALS AND METHODS

Samples of different natural RPs was used in the experiments. The P percentage is described below (Table 1) (Duarte, 2017).

Table 1- P percentage of rock phosphates

Natural rock phosphate	% Total P
Argelia	13.50
Bayovar	11.80
Catalão	16.20
Crandalita	3.90
Gafsa	13.50
Patos de Minas	14.40

RPs solubilization kinetics was studied following a stirred-flow technique (Figure 1) [Strawn and Sparks, 2000] with extractant solutions of citric and oxalic acids at 10 mmol L^{-1} tested separately. These solutions had a pH of 2.0 and 2.6 respectively. Treatments corresponding to the combination of citric and oxalic acids, at a final concentration of 10 mmol L^{-1} (5 mmol L^{-1} for each acid) with pH of 2.25, and deionized water acidified to pH 2.0 and 7.0 with HCl 1 mol L^{-1}, were also included as controls. The extractant solution was maintained in a continuous flow of 1 mL min^{-1} for 160 min and stirred at 600 rpm in the reactor chamber. Ten milligrams of RP were placed in the reactor chamber. The samples were collected every 2 min in a fraction collector and released P was determined by the method of Murphy and Riley (1962). A total of 40 P measurements were done for each replication. Accumulated total solubilized P was determined by the following adapted equation from Sparks (1998):

$$q(ti) = \{[P_i J \Delta t] + (ti)V\} / m$$

where:

$q(t_i)$ is the amount of P desorbed at time t.

P is the concentration of phosphorus in the collected fraction.

p is the phosphorus concentration in the chamber.

t_i is time at the end of sample collection.

Δt is the length of the collection period.

J is the flow rate.

V is the volume of solution in the chamber (17 ml).

m is the mass of 75-µm fractions of the RP in the chamber.

The minimum and maximum desorption rate (MIDR and MADR, respectively), desorption rate stabilization time (DRET), and P desorption stabilization rate (SR) for the natural RP fractions were determined observing the behavior from the data obtained.

The experiments were conducted in a randomized block design with three replications in time. The obtained data were submitted to ANOVA, F test ($p < 0.05$), and processed with the R Studio software, using the ExpDes package. The significant interactions were employed, and the averages compared by the Tukey test ($p < 0.05$).

RESULTS

Oxalic acid was the tested organic acid that showed the highest maximum solubilization rate (MASR) for all RPs tested and in the shortest time. For the phosphate rocks of Catalão and Patos de Minas RP it was more efficient when compared to citric acid in final percentage of solubilized P (Figure 2; Table 2). The maximum percentages of P solubilization with oxalic acid were 31.74 % and 44.67 %, for these RP respectively, at the concentration of 10 mmol L^{-1} (Figure 2).

Argélia, Bayóvar, Catalão, Crandalita, Gafsa and Patos de Minas RP treatment with oxalic acid led to higher solubilization P than those obtained with water at pH 2.0 and water pH 7. Treatment with water pH 2 resulted in P solubilization percentages equal to 13.31 % for Argélia, 14.80 % for Bayóvar, 7.69 % for Catalão, 1.40 % for Crandalita, 12.11 % for Gafsa and 7.10 % for Patos de Minas.

Solubilization percentages obtained with water pH 7.0 were Argélia 3.43 %, Bayovar 3.57 %, Catalão 0.89 %, Crandalita 1.38 %. Gafsa 3.20 % and Patos de Minas 0.95 %.

Citric acid presented higher percentages of P solubilized for Argélia, Bayovar and Gafsa RP when compared to oxalic acid. The maximum percentages of P solubilization with citric acid were 43.91, 49.78 and 37.85 % for these RP respectively, at the concentration of 10 mmol L^{-1} (Figure 2).

Oxalic and Citric acids together at final concentration of 10 mmol L^{-1} (5 mmol L^{-1} for each component) was the treatment that resulted in higher solubilization percentages for Catalão and Patos de Minas RP, when compared to the other treatments. The percentages were 44.2 e 44.67 %, respectively.

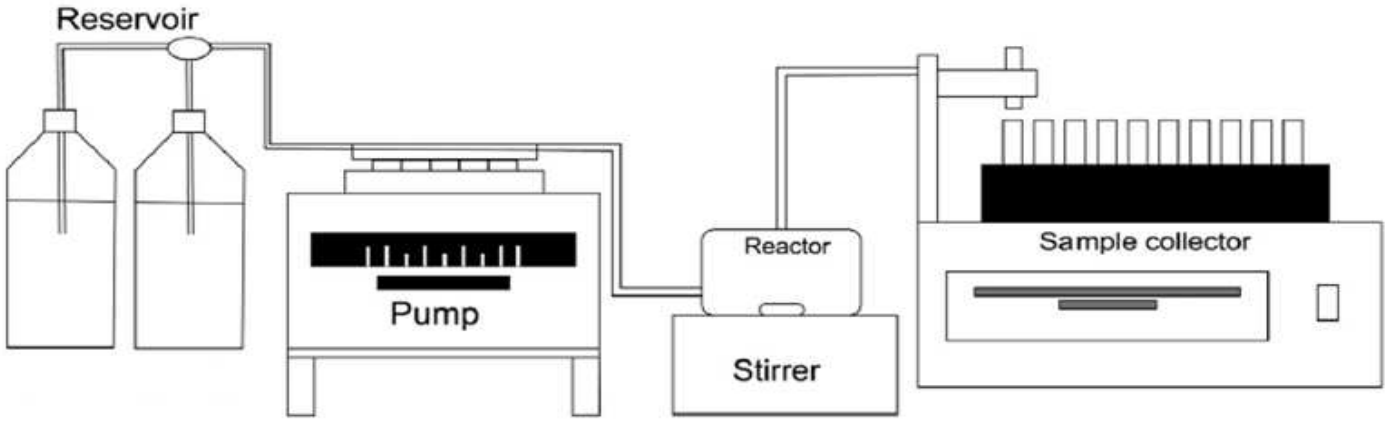

Figure 1. Stirred-flow tecnique: extractant solution in the reservoir is pumped into the reactor chamber where it gets into contact with the particulate material under study (rock phosphate). The suspension formed in the chamber is constantly agitated at 600 rpm. After passing through the reaction chamber at 1 mL min^{-1}, the extractant solution containing solubilized materials is collected in Eppendorff tubes in a sample collector.
Fonte: Guedes *et al.* (2016)

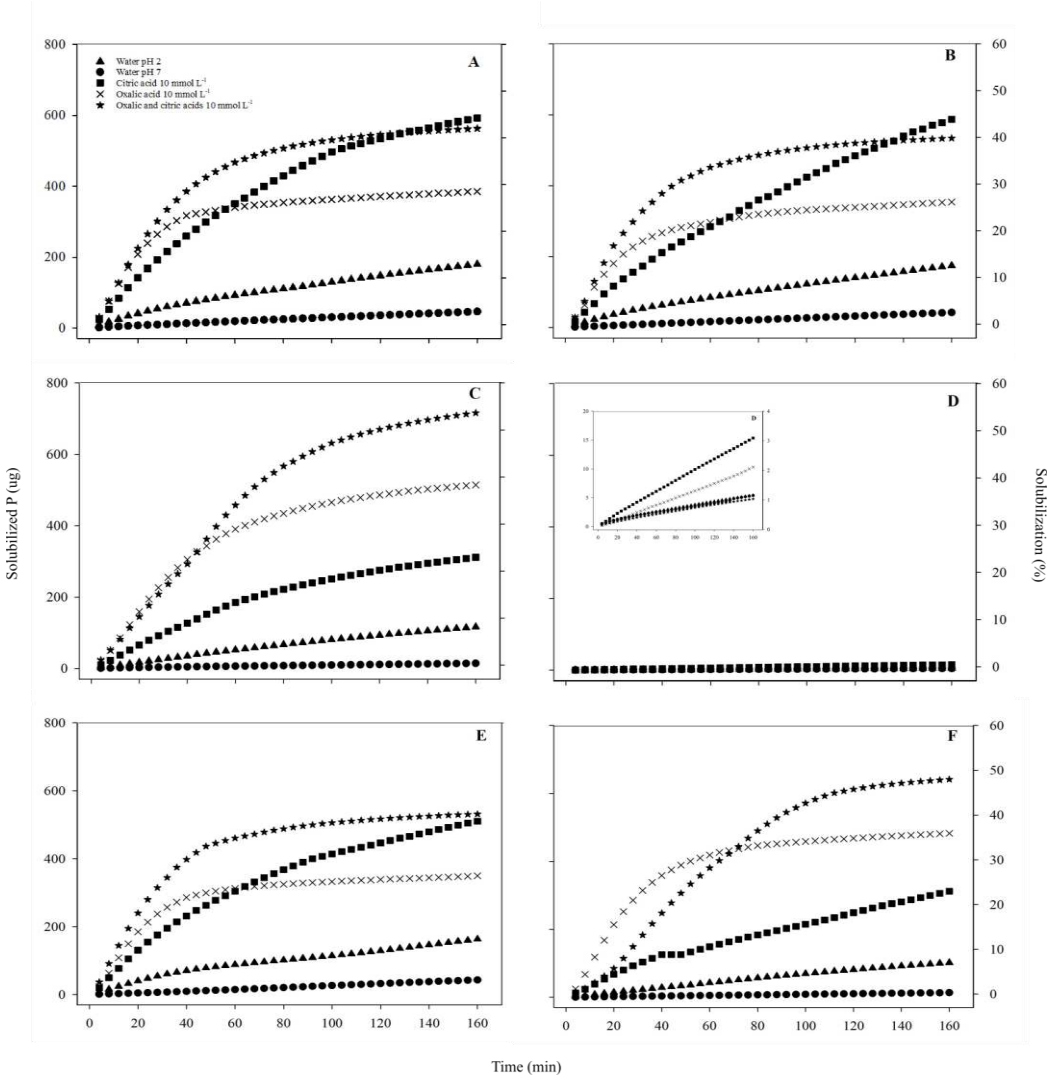

Figure 2. Solubilized P and percentage of solubilization of rock phosphate of Argelia (A), Bayovar (B), Catalão (C), Crandalita (D), Gafsa (E) e Patos de Minas (F) treated with oxalic acid, citric acid, at the final concentrations of 10 mmol L^{-1} and their combination at the concentrations of 5 + 5 mmol L^{-1} and water at pH 2.0 and 7.0, in a stirred-flow system with a continous flow of 1 mL min^{-1} and 600 rpm, at room temperature.

Table 2. P solubilized, solubilization percentage, minimun (MISR) and maximum (MASR) solubilization rate, solubilization rate stabilization time (SRET) and stabilization rate (SR) for rock phosphates treated with by citric and oxalic acid, separately, oxalic, and citric acid together, water pH 2.0, and water pH 7.0 in a stirred-flow system with a continous flow of 1 mL min^{-1} and 600 rpm, at room temperature.

ROCK PHOSPHATES	EXTRACTANT SOLUTION	TOTAL P SOLUBILIZED (µg)	SOLUBILIZATION (%)	MISR (µg min^{-1})	MASR (µg min^{-1})	SRET (min)	SR (µg min^{-1})
Argelia	Citric acid 10 mmol L^{-1}	592.83 a	43.91 a	2.42	15.42	74	2.90
	Oxalic acid 10 mmol L^{-1}	385.35 b	28.54 b	0.65	25.36	58	0.77
	Oxalic and Citric acid 5 + 5 mmol L^{-1}	562.94 a	41.70 a	0.66	26.07	76	0.72
	Water pH 2.0	179.69 c	13.31 c	1.48	4.37	60	1.75
	Water pH 7.0	46.19 d	3.43 d	0.50	0.78	-	0.54
Bayovar	Citric acid 10 mmol L^{-1}	587.35 a	49.78 a	2.74	15.05	74	3.97
	Oxalic acid 10 mmol L^{-1}	354.29 b	30.02 b	0.63	25.08	70	0.87
	Oxalic and Citric acid 5 + 5 mmol L^{-1}	534.10 a	45.26 a	0.54	28.19	64	0.90
	Water pH 2.0	174.66 c	14.80 c	1.66	4.08	78	1.87
	Water pH 7.0	42.18 d	3.57 d	0.48	0.64	-	
Catalão	Citric acid 10 mmol L^{-1}	311.35 c	19.22 c	1.60	7.40	68	1.90
	Oxalic acid 10 mmol L^{-1}	514.26 b	31.74 b	1.04	18.58	74	1.24
	Oxalic and Citric acid 5 + 5 mmol L^{-1}	716.06 a	44.20 a	1.81	21.65	78	1.83
	Water pH 2.0	116.57d	7.69 d	1.1	2.35	76	1.13
	Water pH 7.0	14.31 d	0.89 d	0.15	0.81	-	0.15

Continue

Table 2. P solubilized, solubilization percentage, minimun (MISR) and maximum (MASR) solubilization rate, solubilization rate stabilization time (SRET) and stabilization rate (SR) for rock phosphates treated with by citric and oxalic acid, separately, oxalic, and citric acid together, water pH 2.0, and water pH 7.0 in a stirred-flow system with a continous flow of 1 mL min-1 and 600 rpm, at room temperature.

ROCK PHOSPHATES	EXTRACTANT SOLUTION	TOTAL P SOLUBILIZED (µg)	SOLUBILIZATION (%)	MISR (µg min^{-1})	MASR (µg min^{-1})	SRET (min)	SR (µg min^{-1})
Crandalita	Citric acid 10 mmol L^{-1}	15.34 a	3.87 a	0.17	0.30	78	0.17
	Oxalic acid 10 mmol L^{-1}	10.32 a	2.66 a	0.11	0.15	68	0.11
	Oxalic and Citric acid 5 + 5 mmol L^{-1}	4.79 a	1.23 a	0.04	0.11	76	0.04
	Water pH 2.0	5.46 a	1.40 a	0.09	0.26	68	0.1
	Water pH 7.0	5.37 a	1.38 a	0.01	0.22	-	0.01
Gafsa	Citric acid 10 mmol L^{-1}	510.92 a	37.85 a	2.28	14.88	70	2.81
	Oxalic acid 10 mmol L^{-1}	349.75 b	25.91 b	0.58	22.82	70	0.55
	Oxalic and Citric acid 5 + 5 mmol L^{-1}	531.75 a	39.23 a	0.54	27.23	72	0.70
	Water pH 2.0	163.46 c	12.11 c	1.21	4.22	72	1.75
	Water pH 7.0	43.32 d	3.20 d	0.50	0.65	-	1.1
Patos de Minas	Citric acid 10 mmol L^{-1}	328.03 c	23.52 c	2.31	7.59	70	2.61
	Oxalic acid 10 mmol L^{-1}	482.98 b	33.54 b	0.67	25.4	62	0.83
	Oxalic and Citric acid 5 + 5 mmol L^{-1}	641.80 a	44.67 a	0.96	19.23	78	1.02
	Water pH 2.0	102.30 d	7.10 d	0.98	1.63	62	1.08
	Water pH 7.0	13.62 d	0.95 d	0.15	0.23	-	0.15

Lines with the same small letter are not statistically different by the Tukey test (p<0.05).

None of the treatments tested showed a significant difference for the percentage of solubilization of Crandalite RP. For this phosphate the solubilization average was 4.6 % which is equivalent to 8.26 µg.

All RP solubilization with the organic acids tested in the stirred-flow system varied with time until stabilization (Table 2). The stabilization of the solubilization rates occurred at different times depending on the acid used and its concentration (Table 2). The lowest solubilization rates recorded corresponded to those obtained after stabilization of P release (Table 2).

DISCUSSION

In general, RPs are sensitive to the decrease in the pH of the medium and are partially solubilized by acidification (Nautiyal et al, 2000; Mendes et al., 2014). As water at pH 2.0 was not as effective as citric and oxalic acids in solubilizing RPs, acidification alone has a limited contribution to the solubilization of these rocks. It was observed that the organic acids tested, in addition to promoting the acidification of the medium, used another mechanism to increase the solubilization of phosphate rocks. Organic acids are reported as chelating agents and this may have occurred during the process for the RP solubilization products, i.e., Ca^{2+}, shifting the reaction equilibrium towards the release of P and the accompanying cation (Kpomblekou-A and Tabatabai, 1994). In addition, the agitated flow technique used in our work allows the removal of all soluble products of the reaction, preventing the accumulation of compounds that can inhibit the continuity of solubilization (Sparks, 1998). In this way, it was possible to obtain information throughout the process.

Among the organic acids tested in our work, oxalic acid at 10 mmol L^{-1} showed the highest MASR for all RPs tested. In fact, oxalic acid has been repeatedly shown to be one of the most efficient metabolites in RP solubilization (Mendes et al., 2013; Duarte, 2017; Mendes et al., 2020) due to its structural characteristics, such as the number of carboxyl groups. Despite being efficient in the first minutes of contact with the rock, this efficiency was lost during the process for Argélia, Bayovar and Gafsa RP, resulting in lower solubilization percentages when compared to citric acid. The highest solubilization percentages were observed in phosphates from Catalão and Patos de Minas RP. This was also the behavior observed for RP Araxá in which the use of oxalic acid 10 mmol L^{-1} led to a solubilization of 43 % of P and when combined with citric acid this efficiency was increased to 71 % (no published data).

These data show that there is a tendency for oxalic acid to be better in the solubilization process for low reactivity RP such as Araxá and Catalão (igneous origin) and Patos de Minas (metamorphic origin). Our hypothesis is that the degree of isomorphic substitution of the rock may be related to this behavior. Reactive RP have greater isomorphic substitution between phosphate and carbonate. This substitution leads to less unstable bonds with the calcium present in these materials, that is, in reactive phosphates there is a greater amount of calcium available. RP low-reactive, on the other hand, have less isomorphic substitution and, therefore, bonds with calcium in minerals are more established, which makes it difficult for calcium to leave the mineral matrix. When oxalic acid gets on the RP reactive, due to the greater availability of calcium, there is an immediate precipitation of oxalate minerals on the mineral particle while phosphorus is solubilized in solution. This precipitation can prevent oxalic acid from continuing to act directly on the mineral and reduce its effectiveness in the process. On the other hand, when oxalic acid gets on RP low-reactive, calcium availability is lower and the reaction to form calcium oxalate is slower. We believe that for this reason oxalic acid is more effective in solubilizing these materials.

This hypothesis can be confirmed when observing the results of solubilization percentages with citric acid. This acid acted as the best solubilizing agent especially in the reactive RP, such as, Argélia, Bayovar and Gafsa. Citric acid, like oxalic acid, has a chelating function and acts by chelating released elements such as Ca^{2+}. The big difference is that the complex formed, in this case the citrate is calcium, is soluble and therefore is removed from the medium by the stirred flow system. Thus, the reaction is not discontinued, and the mineral is always in contact with citric acid directly, explaining its greater efficiency when compared to oxalic acid.

We also observed that when citric and oxalic organic acids were combined, there was an increase in the values observed for the percentage of solubilized P. In terms of concentration the two acids were reduced by half, however we observed better efficiency in the final process. For the reactive phosphates, we observed that the percentage values of solubilized P were the same obtained when citric acid was used at twice the concentration. And yet the MARS was kept the same as oxalic acid. This shows a joint action of these acids in this process, increasing the efficiency of use of each one. Oxalic acid initially reaches the maximum solubility for these materials. The fact that oxalic acid to react more quickly with calcium in the rock causes it to reduce the amount of calcium even before it diffuses into solution. Citric acid acts less aggressively and complements the solubilization activity initially exerted more significantly by oxalic acid.

When we looked at the data on the percentage of P solubilized in the low reactivity phosphates, we observed that the combination of these acids led to higher values than those observed with the treatment of the acids separately. Even being used concentration by half. Once again, we have confirmation that the formation process with a greater or lesser degree of isomorphic substitution can influence the efficiency of the acids used for the solubilization process.

This synergistic action may reflect a complementary solubilization mechanism acting on very complex RPs, both in chemical and mineralogical composition, as exemplified in the work by Mendes et al. (2014). The difference between organic acids and their combinations in solubilizing RP may be related to other chemical properties of the organic acids tested that may have an impact on the solubilization efficiency of each compound, such as the number of carboxyl groups, spatial shape of the molecule, molecular mass, and others. Physicochemical properties that affect the interaction of organic acid with the surface of RP particles. As far as we know, this is the first work that describes the kinetics of RP solubilization by different organic acids. More studies need to be carried out to understand how this interaction occurs and which rock minerals are attacked by each of these organic acids.

The solubilization rates of RPs varied according to the type of acid used alone or in combination and the pH of the water. Generally, solubilization rates decreased with time until reaching a stage of stability. At this time, the release of P from RPs could still be observed, but at solubilization rates very close to zero. From this stage, the remaining P-containing minerals would be depleted in quite a long time. The higher rates of RP solubilization at the beginning of the experiments in the agitated flow system can possibly be explained by the immediate dissolution of more reactive phosphate minerals and the removal of soluble P.

CONCLUSION

Oxalic acid is the most efficient organic acid tested in the P solubilization from Argélia, Bayovar, Catalão, Gafsa and Patos de Minas RP in stirred flow system. Improvements in solubilization efficiency can be obtained by combining this compound with citric acid for low-reactivity phosphate rocks. More studies should be carried out to identify what may decrease the efficiency in the oxalic acid solubilization process in reactive rocks, since it has a high rate in a short reaction time, but then this process is impaired.

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Capítulo III

ROCK PHOSPHATE SOLUBILIZATION BY ORGANIC ACIDS: II - MORPHOLOGICAL AND MINERALOGICAL CHANGES

ROCK PHOSPHATE SOLUBILIZATION BY ORGANIC ACIDS: II - MORPHOLOGICAL AND MINERALOGICAL CHANGES

ABSTRACT

Oxalic acid and citric acid are low molecular weight organic acids produced by *Aspergillus* and *Penicillium* species. These metabolites are efficient in solubilizing phophatic rocks. oxalic acid has excelled in research tests being as effective as sulfuric acid at the same concentration to solubilize P contained in these materials. Interestingly, the effectiveness of oxalic acid in the percentage of total P solubilization is variable according to the reactivity of the RP. In studies carried out in continuous flow system, which allows the removal of the soluble products formed in the reaction, it was observed that the process for this treatment was effective in the initial moments, but later interrupted by unknown factors. Visualizing what happens in the process is important to propose new strategies that optimize RP solubilization. Thus, the work aims to study the morphological, chemical, and mineralogical transformations of RPs after contact with organic acids. In 250 mL Erlenmayer flasks, 0.3 g of the following RP were added separately: Argélia, Bayóvar, Catalão, Crandalita, Gafsa, Patos de Minas RP and mixed with 100 ml of solutions: 1) oxalic acid at 10 mmol L^{-1}; 2) citric acid at 10 mmol L^{-1}; 3) oxalic acid + citric acid 10 mmol L^{-1} (5 mmol L^{-1} for each component) and 4) water pH 2.0 adjusted with 1 % HCl. The flasks were incubated at 28 °C and 150 rpm for 48 hours. After drying, the material was transferred to eppendorf tubes and taken for analysis. The material was submitted to scanning electron microscopy (SEM) with EDS coupling and X-ray diffraction (XRD). In SEM it was observed that treatments with oxalic acid led to the formation of new minerals. The combination of oxalic and citric acids led to the formation of oxalate minerals in smaller amounts when compared to the acid alone. In the EDS analysis, it was observed that the treatment that caused the greatest changes in the relative chemical compositions of the elements was with oxalic acid 10 mmol L^{-1}. The main element that suffered a decrease compared to the control was P. XRD analyzes confirmed the identity of the newly formed calcium oxalate minerals (whewelite, wedelite and kaoxite). In addition, it was also possible to observe a significant decrease in the apatite peak in the treatments applied compared to the control. No significant differences were observed in the treatments in relation to the control for the RP of Crandalite in any of the analysis performed. The formation of a protective layer over the reactive RP particles reduces their effectiveness

in the percentage of P solubilization of these materials. We believe that the concentration of calcium and oxalic acid in the solution and the rate of reaction of this element with oxalic acid is the explanation for the pattern of formation of different calcium oxalates and in different ways, depending on the reactivity of the RP under study.

Keywords: Oxalic acid. Citric acid. Whewellite. Wedelite. Kaoxite. Complexation.

INTRODUÇÃO

Rocks phospahtes (RP) present different natural reactivities depending on their origin as igneous, sedimentary, or metamorphic (Correa et al., 2005). Although they have varied composition in relation to the ores present, texture and geological origin, most RP have minerals of the apatite group in common (Kaminski and Peruzzo, 1997).

RP of igneous origin are rich in silica and have a fine texture. RP this origin is considered low reactivity or “hard”. Fluorapatite is the main mineral that composes the phosphates of Jacupiranga and Catalão, located in Brazil, and the phosphate of Tennessee, in the United States, for example (Kaminski and Peruzzo, 1997). The RPs of sedimentary geological origin are more complex and varied. The main minerals that are part of the composition are apatites with a high degree of isomorphic substitution between phosphate and carbonate, and for this reason they are considered RP of high reactivity or “soft”. However, these RPs may also contain detritus, chemical precipitates, and fossil apatite. The North Carolina RP in the US, Gafsa RP in Tunisia, Sechura RP in Peru, and Arad RP in Israel are examples of these rocks. These RPs are often used directly in agriculture because they have this characteristic (Leon et al., 1986). Metamorphic RP origin are an intermediate classification between igneous and sedimentary. They are hard rocks and have in their composition several minerals besides apatite. The RP of Patos de Minas in Brazil is an example (McClellan and Kauwenberg, 1990). To produce soluble phosphates, chemical processes are commonly used. For example, the use of strong acids, such as sulfuric acid, has been widely used (Goldstein et al., 1993). It promotes the destabilization of apatite structure, leading to increased available P content, Also, when in the soil, the susceptibility to react with other ions is facilitated (Goldsteins et al., 1993; Novais et al., 2007).

In addition to inorganic acids, there are some organic acids that can also act in the solubilization of natural phosphates. These acids, in general, are microbial metabolites. Several organic acids are reported in the literature acting on this process (Kpomblekou-A and

Tabatabai, 1994; Mendes et al., 2014, Nascimento et al., 2018; Mendes et al., 2020). The mains are oxalic and citric acids. Oxalic acid has proven effectiveness in the solubilization of natural phosphates, mainly for low reactivity RP.

Its performance is so high that it can be even more effective than sulfuric acid at same concentration, the standard inorganic acid used in the industrial process (Kpomblekou-A e Tabatabai., 1994; Mendes et al., 2020). With these results, oxalic acid presents itself as an effective alternative for the treatment of RP that have low P content and are of low quality.

The concentration of oxalic acid is important for successful P solubilization. Excessive oxalic acid can cause calcium oxalate to build up on the surface of RP particles (Nascimento et al., 2018). Complete solubilization is achieved when using the stoichiometric ratio between apatite and oxalic acid. This process happens quickly, in about 1 hour 75 % of the P contained in Pratápolis RP was released. The calcium released in the process is precipitated with oxalic acid, forming a poorly soluble compound, calcium oxalate. The most observed forms of calcium oxalate were whewelite and wedelite (Nascimento et al., 2018; Mendes et al., 2021).

Despite knowing this potential of oxalic acid, the changes that are promoted in the RP during the solubilization process remain little known. Knowledge of changes in RP morphology, chemical composition, and mineralogy during solubilization by organic acids is important to understand how this process occurs and then propose strategies to optimize RP solubilization. It is also not reported in the literature about the effectiveness of oxalic acid in solubilizing P to be the same in RP of different reactivity. In our previous chapter, we observed that some process related to the reactivity of rocks causes oxalic acid to have different results for these materials.

Objectives with this work was to evaluate the morphological, chemical, and mineralogical alterations of Argélia, Bayovar, Catalão, Crandalita, Gafsa and Patos de Minas RP caused by contact with organic acids involved in the phosphate solubilization process.

MATERIALS AND METHODS

250 mL Erlenmayer flasks were added with 0.3 g of the following RP separately: Argélia, Bayóvar, Catalão, Crandalita, Gafsa, Patos de Minas RP and mixed with organic acid solutions or pH water adjusted. The solutions used were 1) oxalic acid at 10 mmol L^{-1}; 2) citric acid at 10 mmol L^{-1}; 3) oxalic acid + citric acid 10 mmol L^{-1} (5 mmol L^{-1} for each component) and 4) water pH 2.0 ajusted with HCl 1 %. The flasks were incubated at 28 °C and 150 rpm for 48 hours (Nascimento et al., 2018).

After incubation the supernatant was discarded, and the RP residue was transferred to 50 ml falcon tubes. The tubes were centrifuged to eliminate the supernatant and the residue was washed 3 times with distilled water. Then the residues were transferred to Petri dishes and allowed to air dry. After drying, the material was transferred to eppendorf tubes and taken for analysis. The material was submitted to scanning electron microscopy (SEM) with a EDS coupled and X-ray diffraction (XRD).

For SEM-EDS, the procedure described by Haddad et al. (2007) was used. The residue was placed in stubs and left overnight in a silica desiccator. Then, a procedure for removing excess material from the carbon tape was carried out with the aid of N gas under pressure. The residue was metallized with a gold layer of 20 to 30 nm in a Quorum Q150R S metallizer. Residues of RP from Argelia, Bayovar and Crandalita were observed in a scanning electron microscope Leo 1430VP Scanning Electron Microscope. RP residues from Catalão, Gafsa and Patos de Minas were observed in JEOL Scanning Electron Microscope – JSM-6010LA. The elements and their relative amounts in the sample analyzed by EDS were carbon (C), oxygen (O), fluorine (F), aluminum (Al), silicon (Si), phosphorus (P), calcium (Ca), iron (Fe) and gold (Au). Gold was included in the element count because the samples were prepared with gold overlay.

X-ray diffractometry was performed using a scanning X'Pert PRO with Co (CoKa) radiation. The X-ray diffraction patterns were collected in the range of 10 to 70 ° 2θ at a scanning speed of 0.06 ° 2θ per second, with a potential generator of 40kV and a current generator of 40 mA.

RESULTS

Treatment with oxalic acid 10 mmol L^{-1} led to changes in the morphology, chemical composition, and mineralogy of the particles of all RPs tested. In all treatments with oxalic acid, the formation of calcium oxalate crystals is observed (Figure 1 – 6, D, E and F).

Citric acid at 10 mmol L^{-1} slightly altered the morphology of the RP particles, but no new precipitated mineral formation was observed for any of the RP tested (Figure 1 – 6, G, H, and I).

The combination of oxalic + citric acids 10 mmol L^{-1} (5 mmol L^{-1} for each component) also promoted morphological and chemical changes in the tested RPs, being possible to observe the changes in the SEM and EDS results. It was also possible to observe the formation of oxalate crystals, but in smaller proportions when compared to the treatment with

oxalic acid 10 mmol L^{-1} alone (Figure 1 – 6, J, K and L). The crystallographic patterns were the same observed for the treatment with oxalic acid 10 mmol L^{-1}, that is, the formation of whewellite was observed in the most reactive RP Argelia, Bayovar, Gafsa and Patos de Minas. Weddelite and kaoxite were observed in RP Catalão. Whewellite ($CaC_2O_4.H_2O$) is an oxalt monohydrate crystal that forms flat hexagons, styloids, cubes and their conglomerates (druses) (Figure 7 Aand B). Weddelite ($CaC_2O_4.2H_2O$) is an oxalate dihydrate crystal that is pyramid-shaped and bipyramidal prisms (Figure 7 C). Kaoxite ($CaC_2O_4.3H_2O$) is a rarer and very unstable calcium oxalate mineral (Figure 7 D).

With the SEM analysis, it was possible to observe that the calcium oxalate layer around the particle is permeable. It is also possible to observe a "gap" between the structure and the RP particle (Figure 8 A and B). It was also possible to see that in some RP particles the calcium oxalate layer was breaking down, leaving the particle free again (Figure 8 C and D).

The contact of RP with water at pH 2.0 did not change the morphology of the mineral particles (data no showed).

A tendency towards the formation of whewellite, the most stable form of calcium oxalate, was observed in the more reactive RP Argelia, Bayovar, Gafsa and Patos de Minas. Weddelite and kaoxite, more unstable forms of calcium oxalate, were observed in low-reactivity RP such as Catalão. The identity of these minerals was later confirmed by XRD analyses (Figure 15).

From the analysis of the EDS, it is noted that the treatment that most altered the chemical composition of the RPs was oxalic acid 10 mmol L^{-1}. There is a significant decrease in the phosphorus signal in relation to the control for all RP studied. The calcium signal had a slight tendency to become more distributed. Silicon did not show major changes in any of the treatments when compared to the control (Figure 9– 14).

In the EDS analysis, the main elements, C, O, F, Al, Si, P, Ca, and Fe, present in the studied RPs, were analyzed. All the analyzed elements suffered variations in their relative composition in the samples in all the treatments tested when compared to the control. The treatment that caused the greatest variation, generally decreasing the relative amount, was with of oxalic acid 10 mmol L^{-1} (Table 1).

In general, it is possible to notice from the SEM analysis that the treatment with oxalic acid, whether applied alone or together with citric acid, that for Argélia, Bayovar and Gafsa RP there is a decrease in the general size of the particles. For Patos de Minas and Catalão RP, no decrease in the size of the minerals is observed.

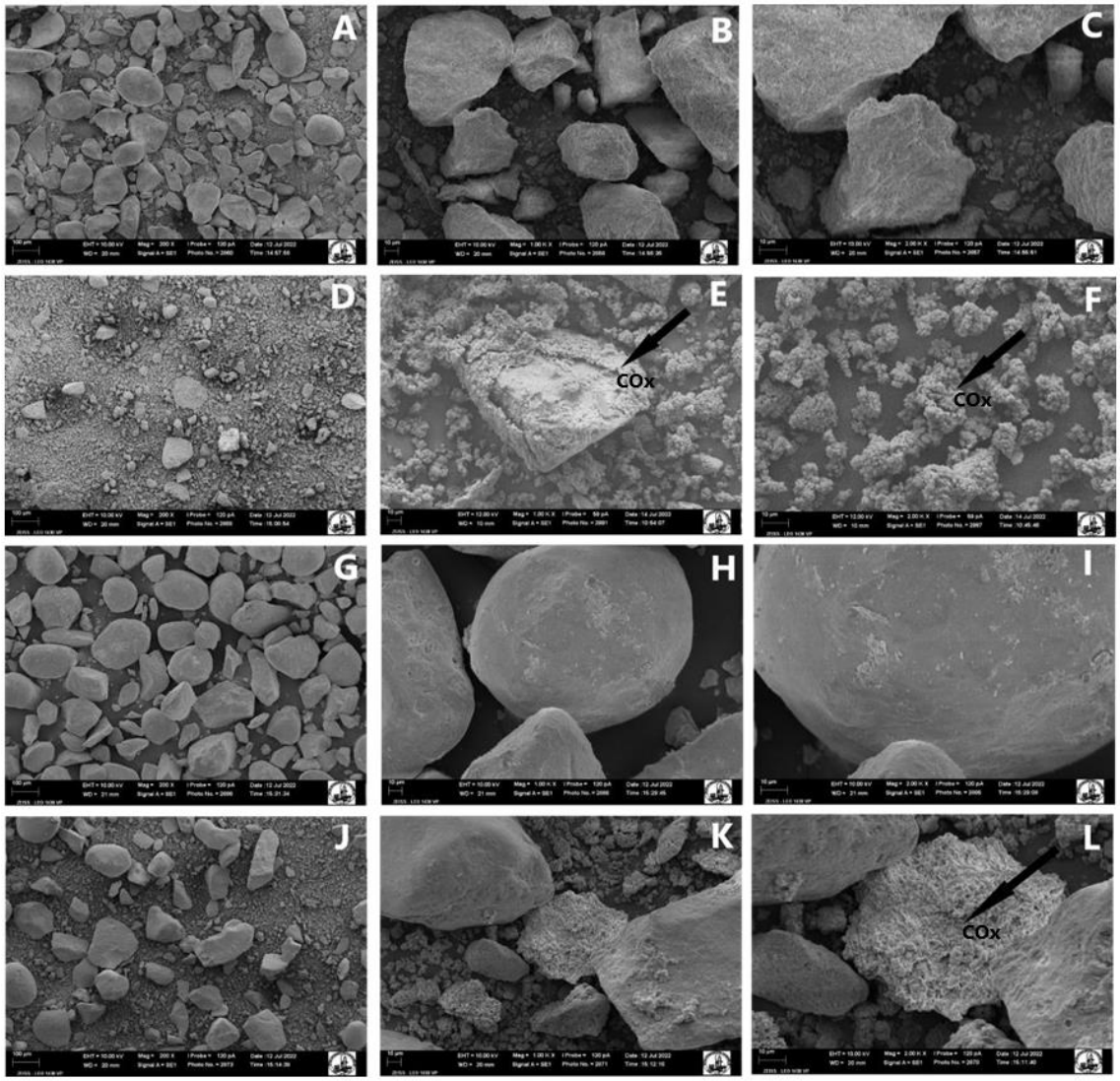


Figure 1 – Morphological changes in Argelia RP particules by organic acids. (A), (B) and (C) Argelia RP without treatment (control). (D), (E) and (F) Oxalic acid 10 mmmol L^{-1} treatment. (G), (H) and (I) Citric acid 10 mmmol L^{-1} treatment. (J), (K) and (L) Oxalic + Citric acids 10 mmmol L^{-1} treatment. In the first column, 200x magnification, second column 1000x magnification, 2000x magnification of the third column. Arrows highlight forms of calcium oxalate. Note the decrease in particle size on treatment with oxalic acid 10 mmol L^{-1} (more pronounced) or combined with citric acid (D and J). Note the difference of the particle surface when compared to the control (F and C). Formation of oxalate crystals (F). RP particle protecting by calcium oxalate layer (E).

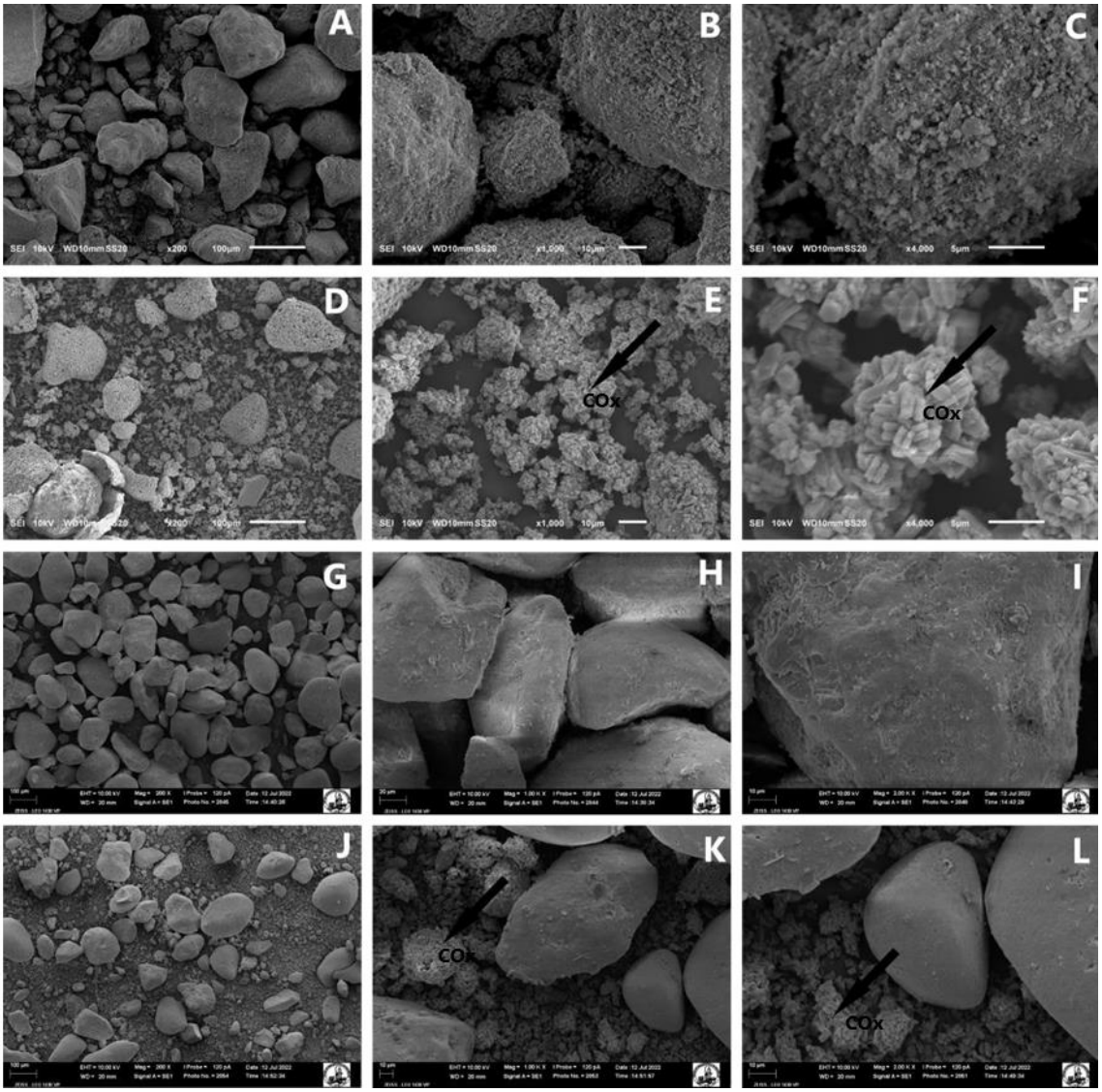


Figure 2 – Morphological changes in Bayovar RP particules by organic acids. (A), (B) and (C) Bayovar RP without treatment (control). (D), (E) and (F) Oxalic acid 10 mmmol L^{-1} treatment. (G), (H) and (I) Citric acid 10 mmmol L^{-1} treatment. (J), (K) and (L) Oxalic + Citric acids 10 mmmol L^{-1} treatment. In the first column, 200x magnification, second column 1000x magnification, 4000x magnification of the third column. Arrows highlight forms of calcium oxalate. Note the decrease in particle size on treatment with oxalic acid 10 mmol L^{-1}or combined with citric acid (D and J). Note the difference of the particle surface when compared to the control (F and C). Formation of oxalate crystals (F). Protection of RP particles by a structured (E) or destabilized (D) calcium oxalate layer.

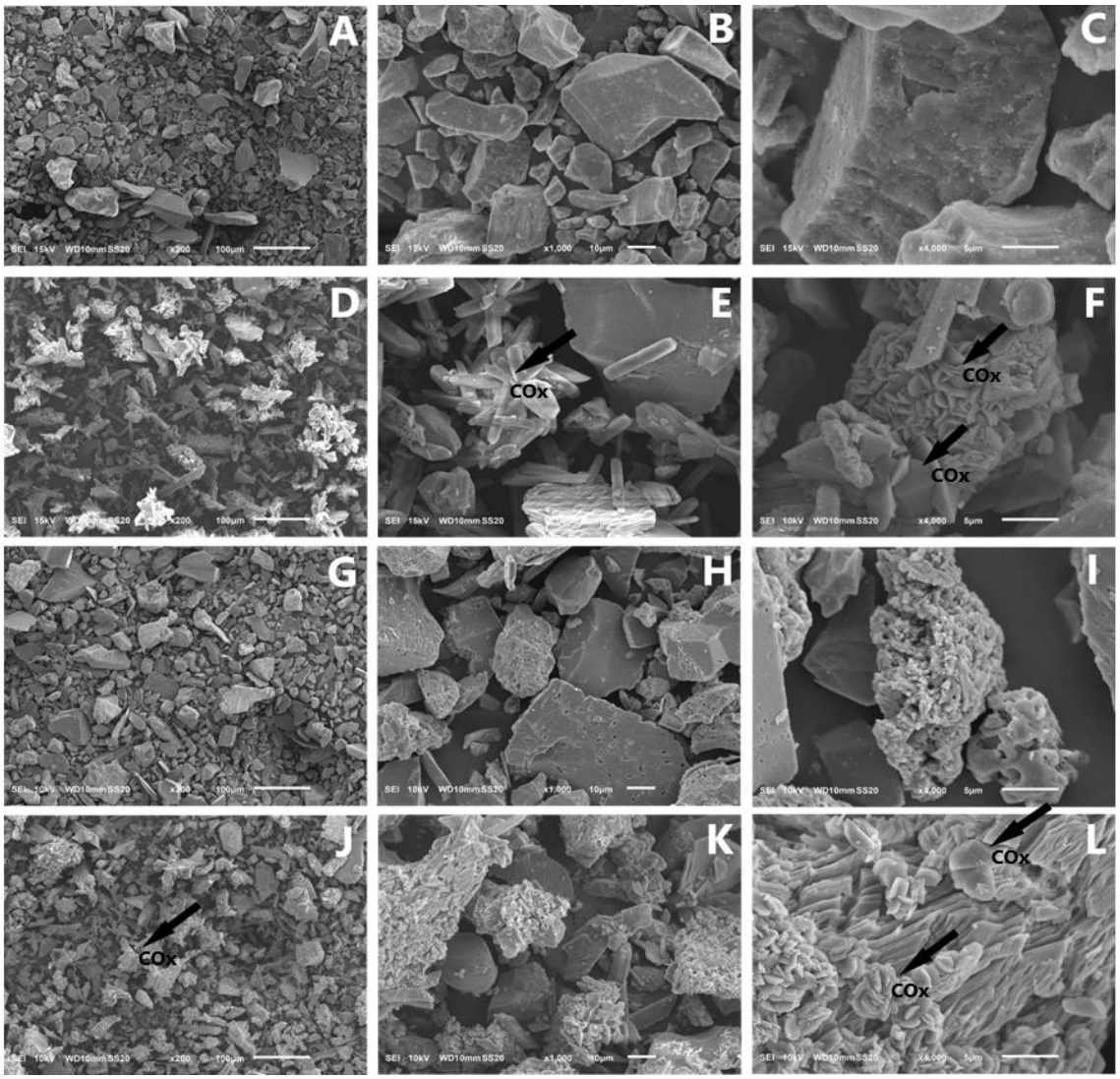


Figure 3 – Morphological changes in Catalão RP particules by organic acids. (A), (B) and (C) Catalão RP without treatment (control). (D), (E) and (F) Oxalic acid 10 mmmol L^{-1} treatment. (G), (H) and (I) Citric acid 10 mmmol L^{-1} treatment. (J), (K) and (L) Oxalic + Citric acids 10 mmmol L^{-1} treatment. In the first column, 200x magnification, second column 1000x magnification, 4000x magnification of the third column. Arrows highlight forms of calcium oxalate. Note the formation of calcium oxalate having different shapes and sizes in the treatment with oxalic acid (E and F). Pattern of calcium oxalate formation more distributed in the sample (D).

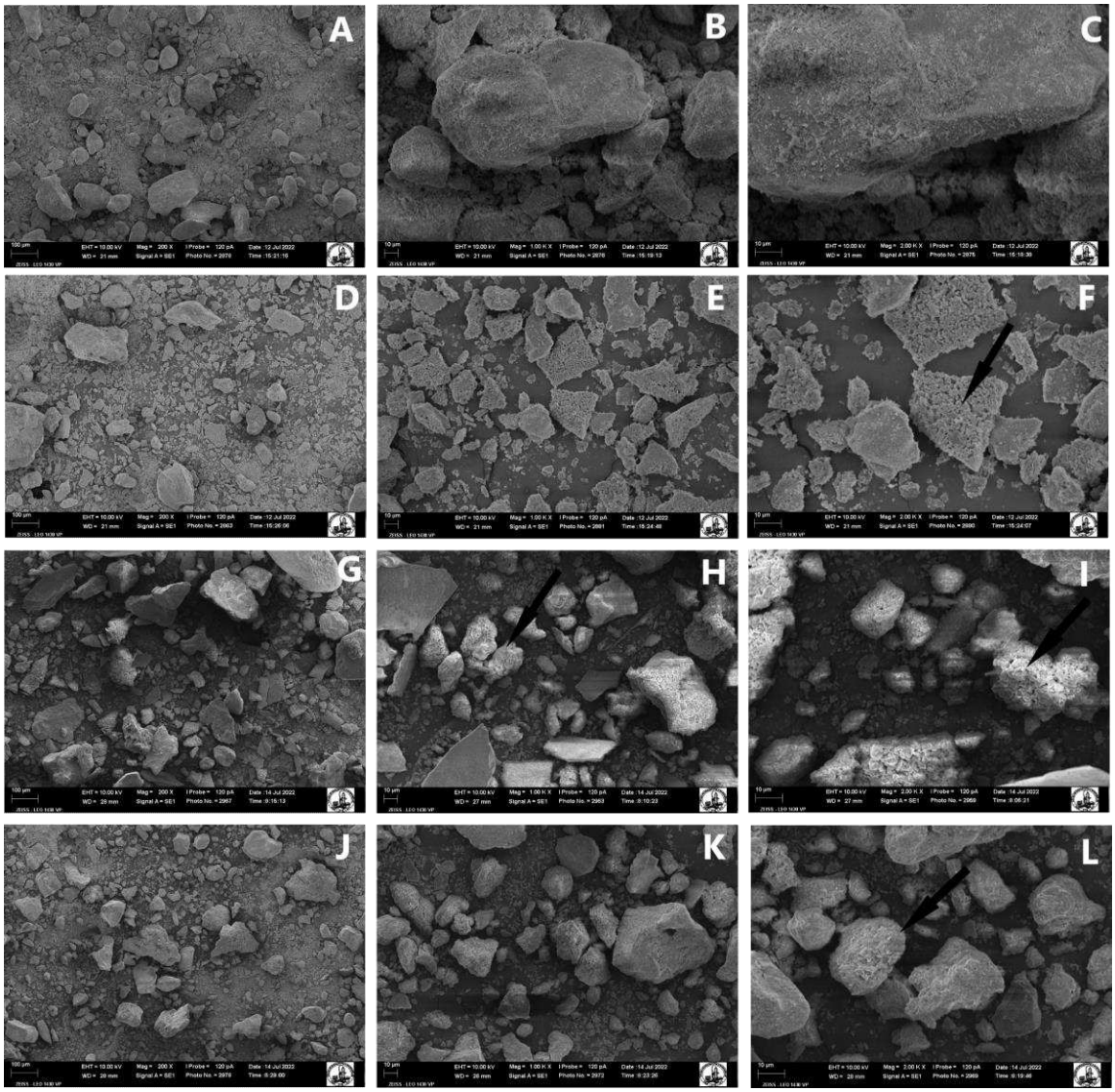


Figure 4 – Morphological changes in Crandalita RP particules by organic acids. (A), (B) and (C) Crandalita RP without treatment (control). (D), (E) and (F) Oxalic acid 10 mmmol L^{-1} treatment. (G), (H) and (I) Citric acid 10 mmmol L^{-1} treatment. (J), (K) and (L) Oxalic + Citric acids 10 mmmol L^{-1} treatment. In the first column, 200x magnification, second column 1000x magnification, 2000x magnification of the third column. Arrows highlight changes in the particle surface caused by oxalic acid treatment.

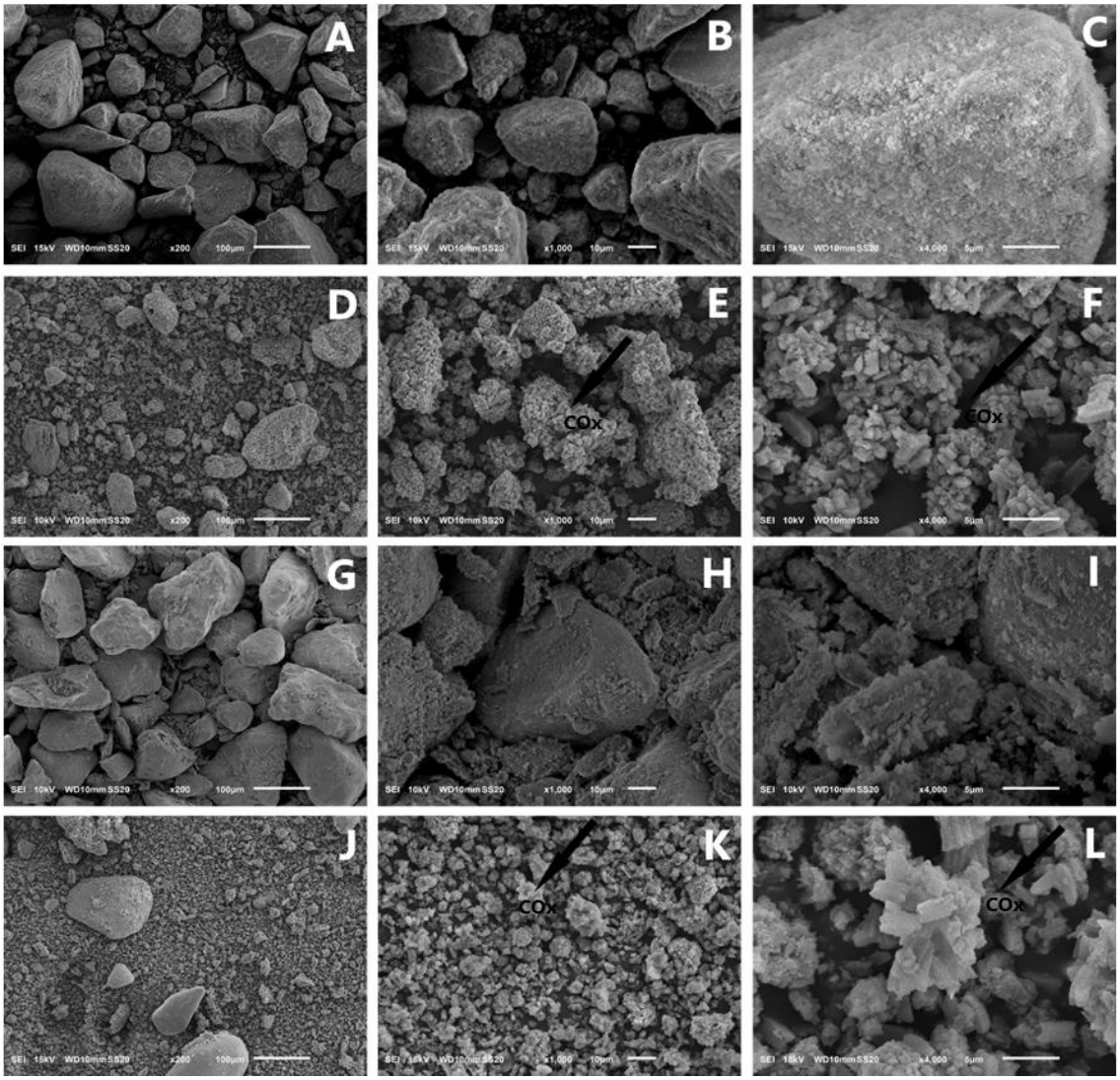


Figure 5 – Morphological changes in Gafsa RP particules by organic acids. (A), (B) and (C) Gafsa RP without treatment (control). (D), (E) and (F) Oxalic acid 10 mmmol L^{-1} treatment. (G), (H) and (I) Citric acid 10 mmmol L^{-1} treatment. (J), (K) and (L) Oxalic + Citric acids 10 mmmol L^{-1} treatment. In the first column, 200x magnification, second column 1000x magnification, 4000x magnification of the third column. Arrows highlight forms of calcium oxalate. Note the decrease in particle size on treatment with oxalic acid 10 mmol L^{-1} or combined with citric acid (D and J). Note the difference of the particle surface when compared to the control (F and C). Formation of oxalate crystals (F).

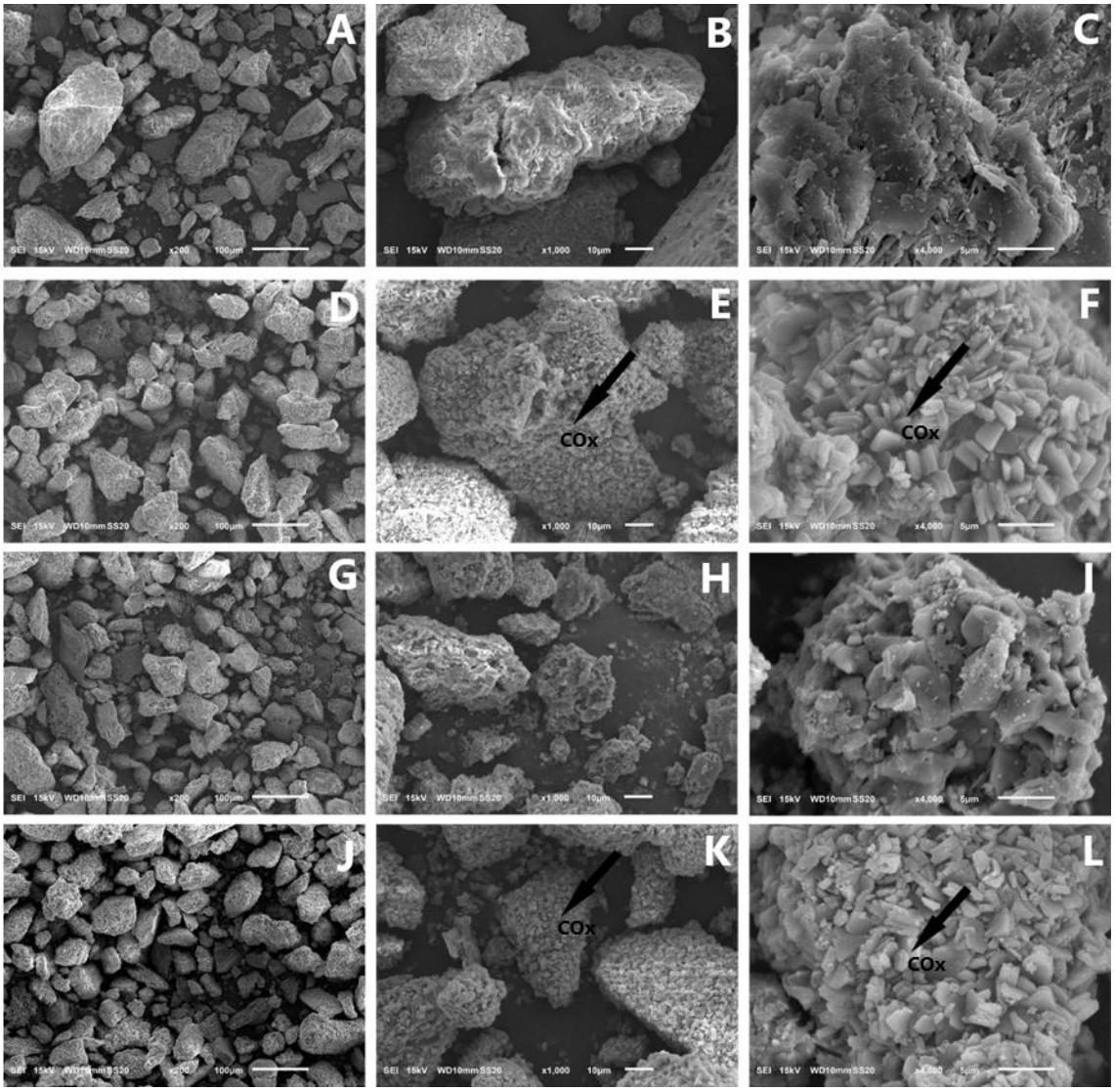


Figure 6 – Morphological changes in Patos de Minas RP particules by organic acids. (A), (B) and (C) Patos de Minas RP without treatment (control). (D), (E) and (F) Oxalic acid 10 mmmol L^{-1} treatment. (G), (H) and (I) Citric acid 10 mmmol L^{-1} treatment. (J), (K) and (L) Oxalic + Citric acids 10 mmmol L^{-1} treatment. In the first column, 200x magnification, second column 1000x magnification, 4000x magnification of the third column. Arrows highlight forms of calcium oxalate. Note the difference of the particle surface when compared to the control (F and C). Formation of oxalate crystals (F). RP particle protecting by calcium oxalate layer (E).

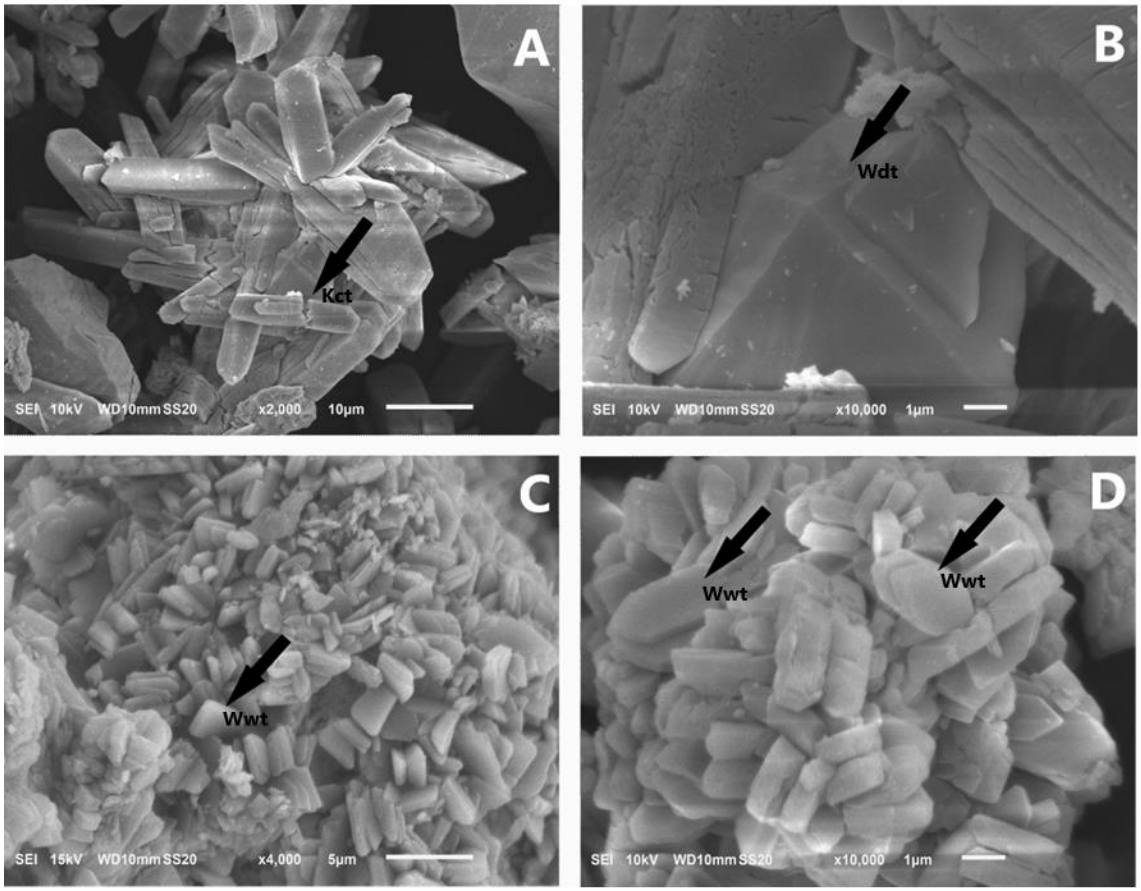


Figure 7 – Crystallographic forms of calcium oxalate formed in oxalic acid treatments observed by scanning electron microscopy (SEM). A – Kaoxite (Kct) ($CaC_2O_4.3H_2O$), calcium oxalate trihydrate, observed in Catalão RP. B - Weddelite (Wdt) ($CaC_2O_4.2H_2O$), calcium oxalate dihydrate, observed in Catalão RP. A and B - Forms also observed in Araxá RP. C e D – Whewellite (Wwt) ($CaC_2O_4.H_2O$), calcium oxalate monohydrate, observed in Argélia RP. Forms also found in Bayovar, Gafsa and Patos de Minas RP.

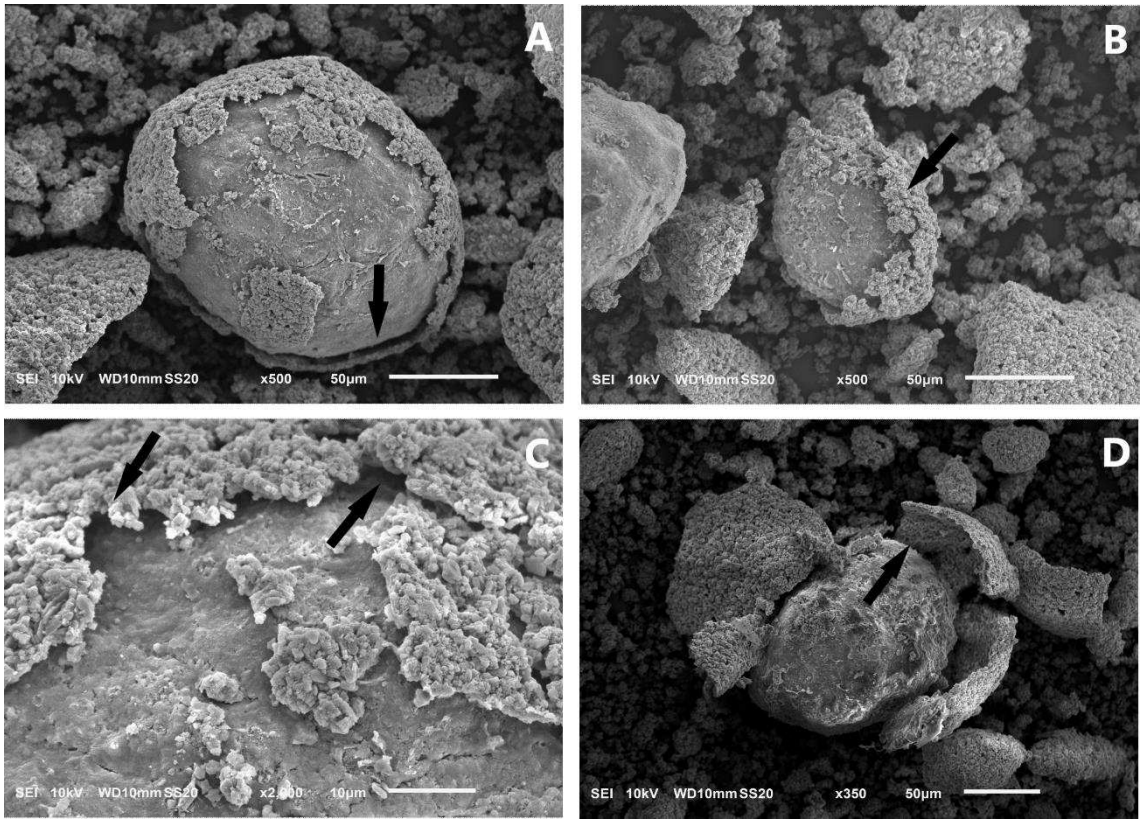


Figure 8 – Bayovar RP particle coated with calcium oxalate layer. A and B – Permeable calcium oxalate layer surrounding the particle but showing a "gap" between the structure and the RP particle. Indication that this layer is permeable to the oxalic acid solution. The process of P solubilization is delayed by the physical barrier formed but is not prevented from happening. C - D – Calcium oxalate layer that surrounded the RP particle disengaging. Note that the particle size within the calcium oxalate layer formed is decreased. Indication of the continuity of the P solubilization process by oxalic acid.

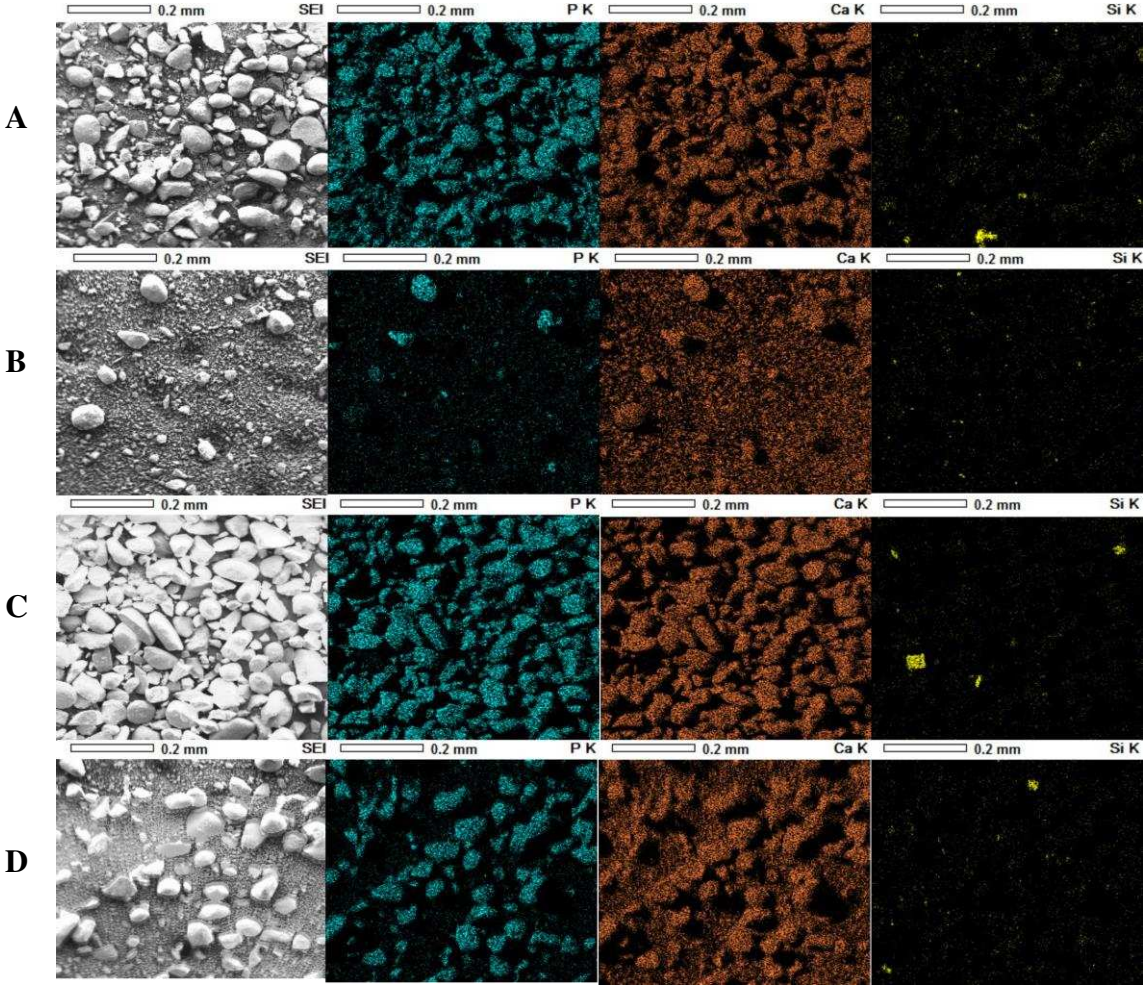


Figure 9 - Change in chemical composition observed in Argelia RP particles by organic acids. (A) Argelia RP particles without treatment (control). (B) Oxalic acid 10 mmmol L^{-1} treatment. (C) Citric acid 10 mmmol L^{-1} treatment. (D) Oxalic + Citric acids 10 mmmol L^{-1} treatment. The gray image represents the area where the analysis was performed. The blue color represents the P element. The orange color represents the Ca element, and the yellow color represents the Si element. Notice that the more intense the color, greater the presence of the element.

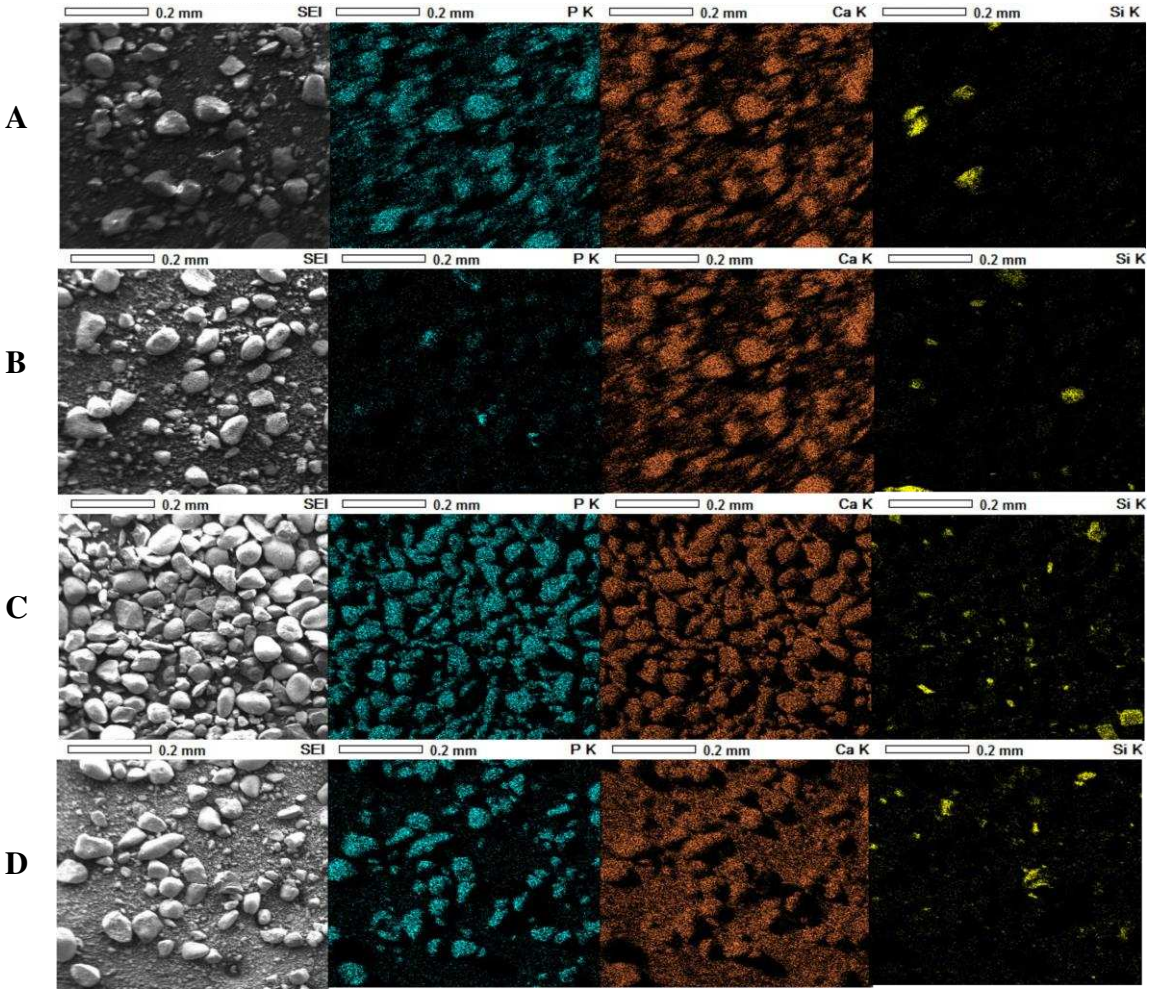


Figure 10 - Change in chemical composition observed in Bayovar RP particles by organic acids. (A) Bayovar RP particles without treatment (control). (B) Oxalic acid 10 mmmol L^{-1} treatment. (C) Citric acid 10 mmmol L^{-1} treatment. (D) Oxalic + Citric acids 10 mmmol L^{-1} treatment. The gray image represents the area where the analysis was performed. The blue color represents the P element. The orange color represents the Ca element, and the yellow color represents the Si element. Notice that the more intense the color, greater the presence of the element.

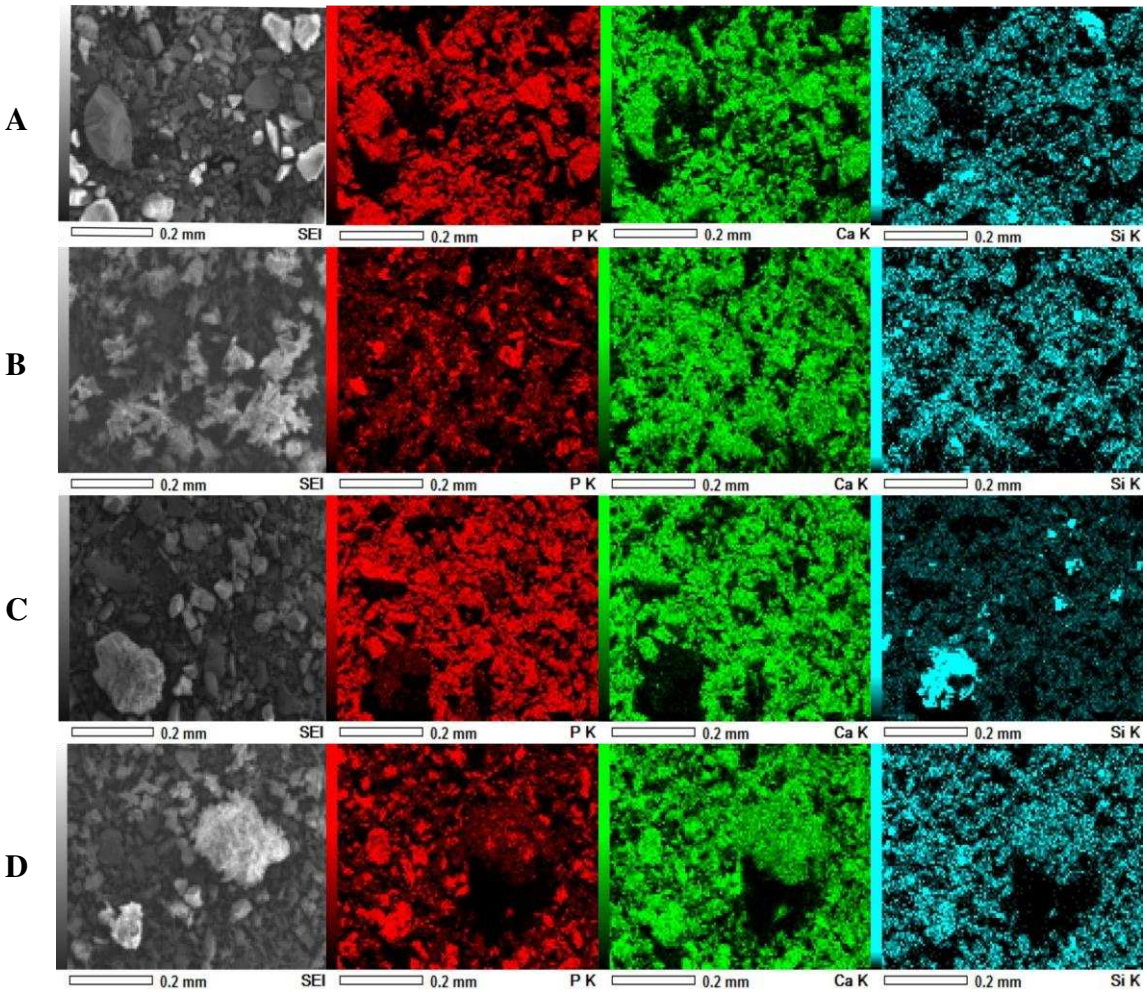


Figure 11- Change in chemical composition observed in Catalão RP particles by organic acids. (A) Catalão RP particles without treatment (control). (B) Oxalic acid 10 mmmol L^{-1} treatment. (C) Citric acid 10 mmmol L^{-1} treatment. (D) Oxalic + Citric acids 10 mmmol L^{-1} treatment. The gray image represents the area where the analysis was performed. The red color represents the P element. The green color represents the Ca element, and the blue color represents the Si element. Notice that the more intense the color, greater the presence of the element.

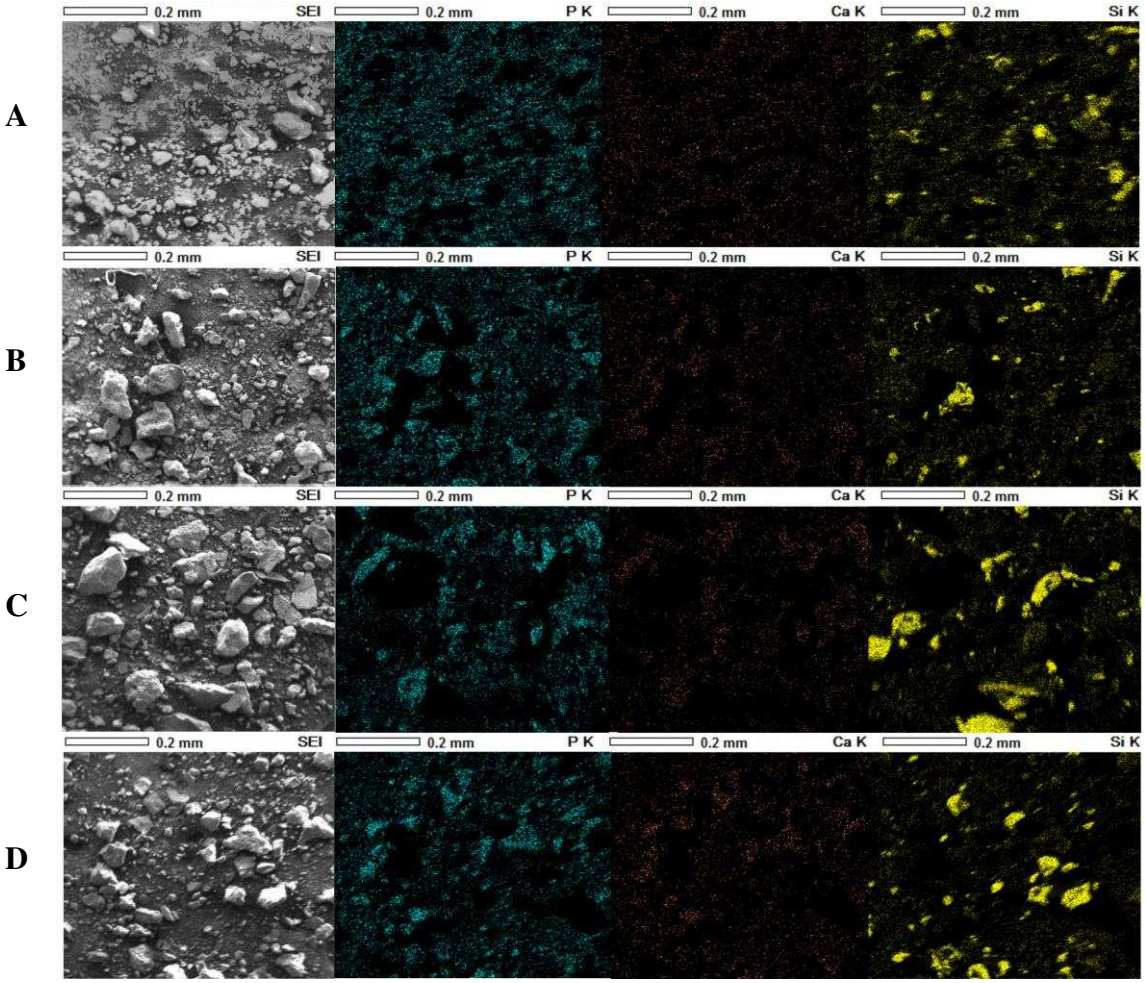


Figure 12 - Change in chemical composition observed in Crandalita RP particles by organic acids. (A) Crandalita RP particles without treatment (control). (B) Oxalic acid 10 mmmol L^{-1} treatment. (C) Citric acid 10 mmmol L^{-1} treatment. (D) Oxalic + Citric acids 10 mmmol L^{-1} treatment. The gray image represents the area where the analysis was performed. The blue color represents the P element. The orange color represents the Ca element, and the yellow color represents the Si element. Notice that the more intense the color, greater the presence of the element.

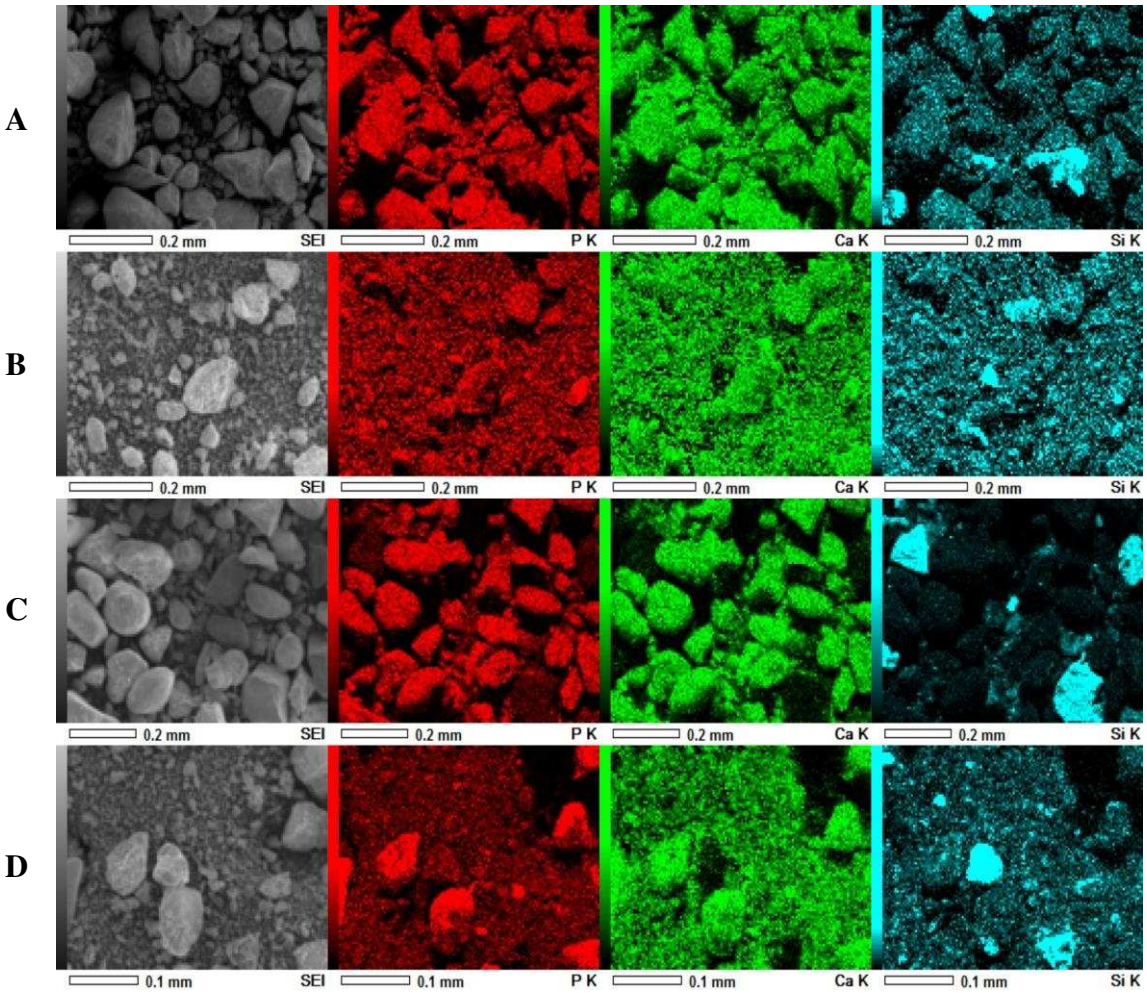


Figure 13 - Change in chemical composition observed in Gafsa RP particles by organic acids. (A) Gafsa RP particles without treatment (control). (B) Oxalic acid 10 mmmol L^{-1} treatment. (C) Citric acid 10 mmmol L^{-1} treatment. (D) Oxalic + Citric acids 10 mmmol L^{-1} treatment. The gray image represents the area where the analysis was performed. The red color represents the P element. The green color represents the Ca element, and the blue color represents the Si element. Notice that the more intense the color, greater the presence of the element.

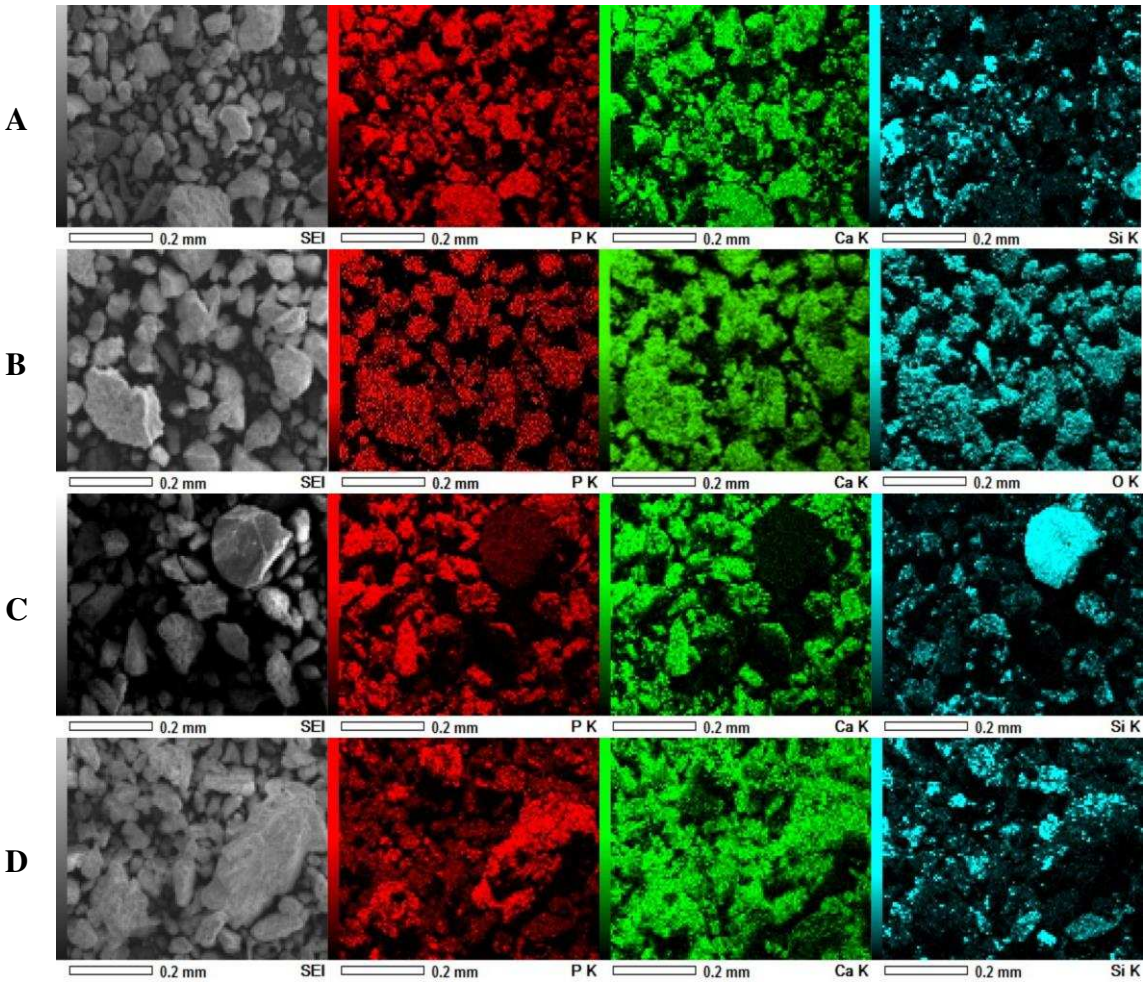


Figure 14 - Change in chemical composition observed in Patos de Minas RP particles by organic acids. (A) Patos de Minas RP particles without treatment (control). (B) Oxalic acid 10 mmmol L^{-1} treatment. (C) Citric acid 10 mmmol L^{-1} treatment. (D) Oxalic + Citric acids 10 mmmol L^{-1} treatment. The gray image represents the area where the analysis was performed. The red color represents the P element. The green color represents the Ca element, and the blue color represents the Si element. Notice that the more intense the color, greater the presence of the element.

XRD analyzes showed overall decreases in apatite peaks in treatments with oxalic and citric acids, alone or in combination. In all treatments with oxalic acid a remarkable formation of calcium oxalate crystals was detected. Whewellite peaks were
observed in greater abundance in Argelia, Bayovar, Gafsa and Patos de Minas RP. And weddelite and kaoxite peaks present in Catalão RP. No marked changes were observed for Crandalite RP in any of the treatments and in any of the analyses.

DISCUSSION

In view of these results, we propose of natural RP solubilization model based on the reactivity of these rocks (Scheme 1). For reactive RPs, like Argélia, Bayovar and Gafsa RPs, and of medium reactivity like Patos de Minas RP, we have that the apatite in contact with oxalic acid is solubilized, mainly in the initial minutes. During the process, calcium oxalate precipitates on the surface of the particles, forming a protective layer that surrounds the particle. This physical barrier prevents the oxalic acid solubilization
process from continuing at the same rate and therefore, over time, the acid solubilization of P becomes less effective. This reaction is rapid and there is no time for the calcium released from the material to diffuse into the solution. Thus, the solution has a low calcium/oxalate ratio. An environment is formed in which the concentration of oxalic acid is greater than the concentration of calcium.

In conditions of medium with high concentration of oxalate the formation of whewelite is favored. The formation of whewelite is dependent on the concentration of oxalic acid in the solution (Scheme 1 - A).

The data shown indicate that for the RP of low reactivity, such as the Catalão and Araxá RP, the solubilization process by oxalic acid is different. When the process of P solubilization of these rocks is initiated, there is no precipitation of calcium oxalate on the particles. This precipitation pattern occurs more distributed in the sample and almost never on the particle surface. The reaction between oxalic acid and the low-reactive RP occurs more slowly. This lower speed is due to the lower degree of isomorphic substitution of these materials, causing the release of P to be slower. Therefore, calcium is released more slowly from the material and ends up diffusing into the solution. This explains the pattern of calcium oxalate formation being more distributed in the sample.

Table 1 - Change in chemical composition of the elements C, O, F, Al, Si, P, Ca, Fe observed in Argelia, Bayovar, Catalão, Crandalita, Gafsa and Patos de Minas RP particles induced by treatments with oxalic and citric acids.

Chemical formula	**Argelia RP**			
	Control	Oxalic acid 10 mmol L^{-1}	Citric acid 10 mmol L^{-1}	Oxalic + Citric acids 10 mmol L^{-1}
	Mass (%)	Mass (%)	Mass (%)	Mass (%)
C	5.72	11.79	5.99	9.02
O	17.5	20.28	15.83	18.87
F	1.66	0.53	1.63	1.16
Al	1.23	1.04	1.22	1.13
Si	1.74	1.28	1.59	1.47
P	8.29	1.34	8.64	5.29
Ca	23.48	21.06	23.07	22.43
Fe	1.41	1.41	1.31	1.35
Au	38.97	41.27	40.71	39.28
Total	100.00	100.00	100.00	100.00
Chemical formula	**Bayovar RP**			
	Control	Oxalic acid 10 mmol L^{-1}	Citric acid 10 mmol L^{-1}	Oxalic + Citric acids 10 mmol L^{-1}
	Mass (%)	Mass (%)	Mass (%)	Mass (%)
C	16.89	11.38	6.36	8.75
O	20.67	30.47	16.47	19.48
F	1.78	0.53	1.29	0.78
Al	0.99	1.26	1.73	1.42
Si	1.71	2.09	3.01	2.63
P	7.75	1.18	7.15	4.07
Ca	20.53	23.83	20.52	21.37
Fe	1.14	1.8	1.49	1.60
Au	28.54	27.89	40.99	39.89
Total	100.00	100.00	100.00	100.00
Chemical formula	**Catalão RP**			
	Control	Oxalic acid 10 mmol L^{-1}	Citric acid 10 mmol L^{-1}	Oxalic + Citric acids 10 mmol L^{-1}
	Mass (%)	Mass (%)	Mass (%)	Mass (%)
C	8.21	13.98	6.42	12.13
O	26.03	33.54	28.55	32.16
F	1.09	Nd	0.76	0.11
Al	0.41	0.29	0.47	0.21
Si	0.52	0.332	1.62	0.11
P	12.42	2.44	12.62	5.96
Ca	32.60	29.96	31.59	30.90
Fe	3.80	3.05	5.85	3.21
Au	14.91	16.41	12.12	15.21
Total	100.00	100.00	100.00	100.00

Continue

Table 1 – Change in chemical composition of the elements C, O, F, Al, Si, P, Ca, Fe observed in Argelia, Bayovar, Catalão, Crandalita, Gafsa and Patos de Minas RP particles induced by treatments with oxalic and citric acids.

Chemical formula	**Crandalita RP**			
	Control	Oxalic acid 10 mmol L^{-1}	Citric acid 10 mmol L^{-1}	Oxalic + Citric acids 10 mmol L^{-1}
	Mass (%)	Mass (%)	Mass (%)	Mass (%)
C	6.98	10.50	10.56	13.84
O	23.42	22.17	28.94	28.47
F	0.14	0.16	0.17	0.35
Al	10.04	9.17	10.19	9.65
Si	7.78	6.74	10.13	8.12
P	4.06	3.85	4.65	4.37
Ca	2.92	2.78	3.11	2.91
Fe	4.28	3.39	3.81	3.48
Au	40.38	41.23	28.44	28.84
Total	100.00	100.00	100.00	100.00
Chemical formula	**Gafsa RP**			
	Control	Oxalic acid 10 mmol L^{-1}	Citric acid 10 mmol L^{-1}	Oxalic + Citric acids 10 mmol L^{-1}
	Mass (%)	Mass (%)	Mass (%)	Mass (%)
C	8.99	14.95	8.64	17.21
O	28.63	33.98	29.64	31.69
F	2.20	0.02	1.36	0.27
Al	0.35	0.45	0.91	0.49
Si	1.28	1.20	6.37	2.46
P	10.60	0.98	9.18	2.67
Ca	32.91	27.63	26.78	23.73
Fe	0.40	0.47	0.73	0.52
Au	14.66	20.32	16.38	20.96
Total	100.00	100.00	100.00	100.00
Chemical formula	**Patos de Minas RP**			
	Control	Oxalic acid 10 mmol L^{-1}	Citric acid 10 mmol L^{-1}	Oxalic + Citric acids 10 mmol L^{-1}
	Mass (%)	Mass (%)	Mass (%)	Mass (%)
C	7.97	16.90	13.09	11.37
O	31.06	39.28	28.23	32.33
F	0.77	0.27	0.50	Nd
Al	2.37	0.49	1.33	1.06
Si	5.72	1.62	8.66	4.33
P	10.38	0.85	8.05	3.95
Ca	26.10	10.42	20.34	25.43
Fe	2.85	0.21	1.83	1.53
Au	18.50	2.21	17.96	20.00
Total	100.00	100.00	100.00	100.00

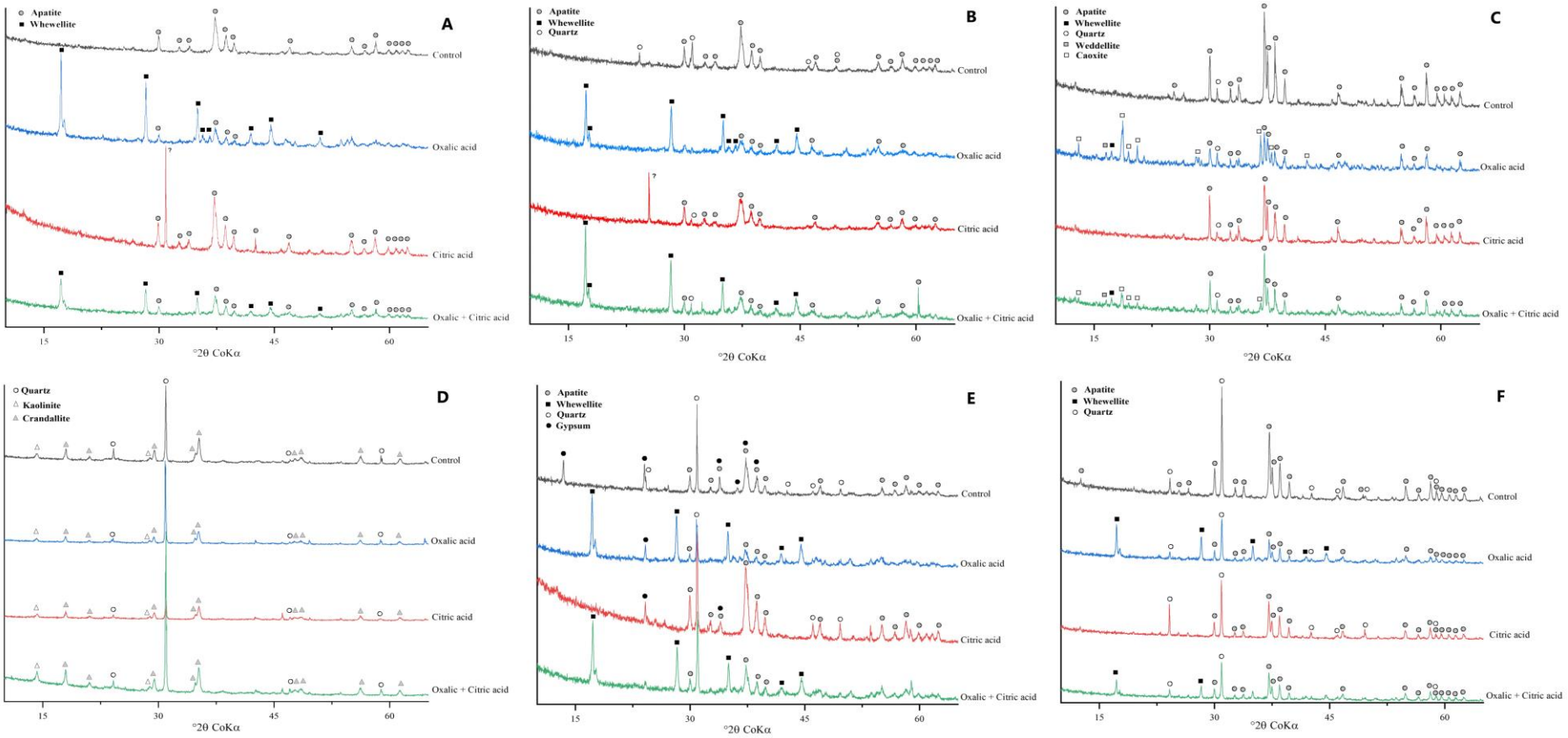
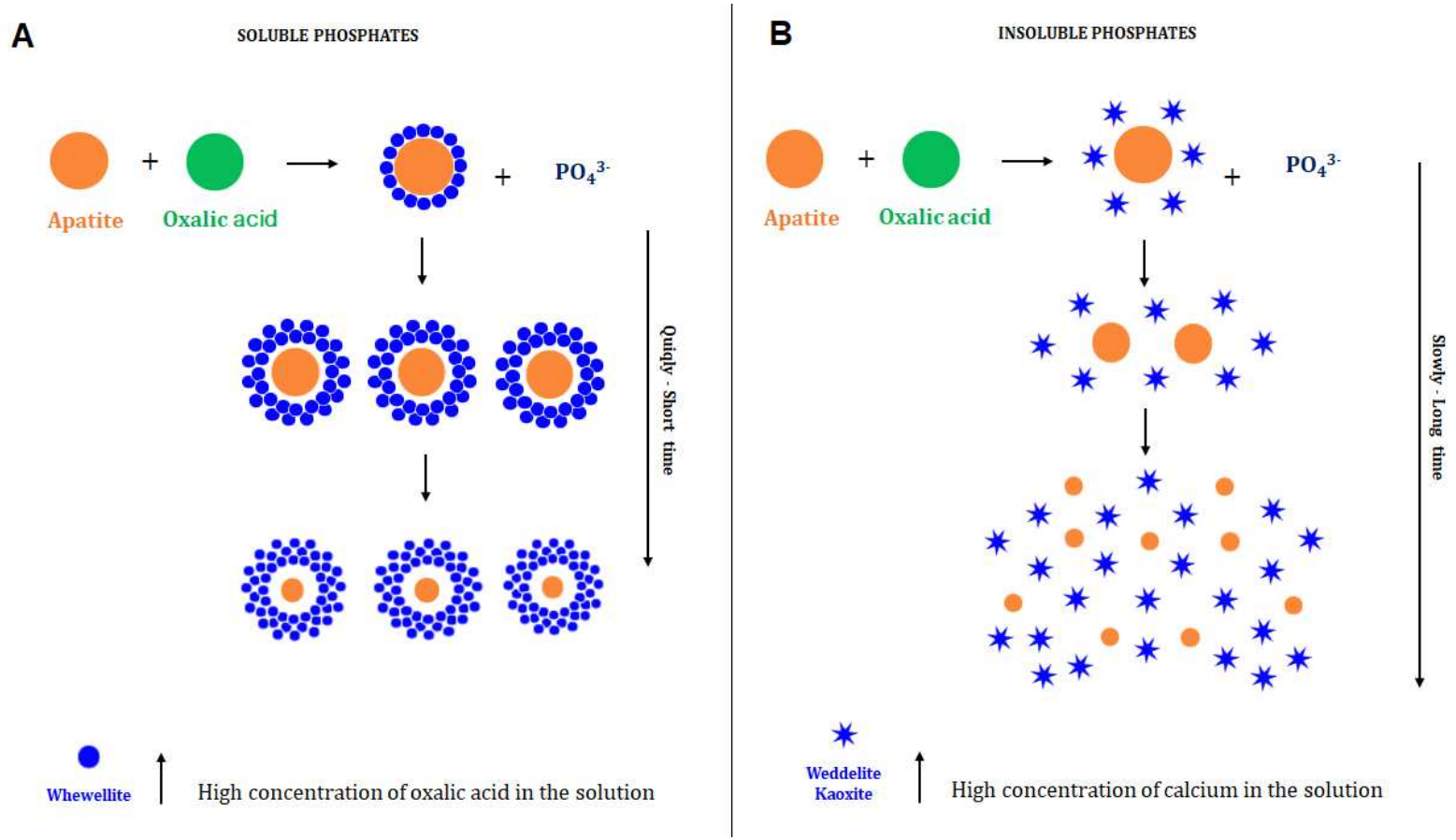


Figure 15 – X-ray diffraction pattern of Argélia (A), Bayovar (B), Catalão (C), Crandalita (D), Gafsa (E) e Patos de Minas (F) RP treated with different organic acids. Black curve – Control (without treatment). Red curve - Oxalic acid 10 mmol L^{-1}. Blue curve – Citric acid 10 mmol L^{-1}. Green curve – Oxalic + Citric acid 10 mmol L^{-1} (5 mmol L^{-1} for each component).



Schemee 1 – Schematic drawing illustrating the steps of solubilization of phosphate rocks by oxalic acid, and calcium oxalates formation. A – Model proposed for solubilization of soluble (more reactive) RP. The formation of oxalate minerals is rapid, in a short period of time, forming more crystalline and less hydrated minerals (eg. Whewellite). In these rocks, oxalate minerals tend to precipitate on the surface of the particles forming a protective layer that is not impermeable. This causes the oxalic acid to continue operating, but at a slower rate due to a physical barrier, over the mineral and continue the solubilization process. B – Model proposed for solubilization of insoluble (less reactive) RP. Here, oxalate minerals precipitate more distributed in the medium, and the presence of a physical barrier is not observed. Mineral formation is a slower process that allows the formation of larger minerals with a higher degree of hydration (eg. Weddellite and Kaoxite).

This diffusion of calcium into the medium keeps the calcium/oxalate ratio higher. Environments with a high concentration of calcium favor the formation of forms of calcium oxalate such as wedelite and caoxite (Scheme 1 - B).

Our work shows that RP with greater reactivity tend to form calcium oxalate precipitates on the phosphate particle. At first, we believed that this protection formed was impermeable (Nascimento et al., 2018). SEM results showed us that for RP with higher reactivity it is not true, and we also verified that it tends to destabilize as the process occurs (Figure 15).

This incrustation delays the process because the protective layer of calcium oxalate formed on the mineral particle works as an obstacle for the action of oxalic acid on the rock. We can also observe that this layer is not impermeable and tends to destructure as the mineral goes through the process of solubilization with oxalic acid (Figure 15). Our data support this hypothesis because all maximum solubilization rates are achieved with oxalic acid treatment, however there was some factor preventing the process from continuing (See chapter II – Table 1).

The pattern of calcium oxalate formation is different depending on the RP reactivity. Reactive and medium reactivity RP form calcium oxalate caps that surround the particle while low reactivity RP tend to form calcium oxalates that are more spread over the sample. We hypothesized that because there is a higher degree of isomorphic substitution of these rocks, the concentration of calcium in the medium is low, leading to a very fast reaction time with the oxalate and the precipitate is formed on the particle. To form whewellite, the reaction is dependent on the oxalate concentration and only crystallizes in an environment where the calcium/oxalate ratio is low, that is, in a solution with a high concentration of oxalate (Harrache et al., 1997; Daudon, 2004; Silva et al., 2009). Thus, the chelation reaction that occurs with oxalic acid is almost instantaneous and, therefore, the formation of calcium oxalate cap on the particulate is observed in these phosphates. In addition, it is possible to observe the expressive presence of small clumps of oxalate crystals forming in the sample. This behavior was observed for Argélia, Bayovar, Gafsa and Patos de Minas RP.

We believe that for less reactive natural phosphates the process is different. The low degree of isomorphic substitution between phosphate and carbonate in these minerals cause less calcium release and therefore this calcium is released more slowly. The concentration of calcium in the solution in the process of solubilization of these phosphates is higher. As the concentration of calcium in solution is higher, the reaction rate with oxalic acid is also slower,

requiring more time for this process. Thus, precipitation mainly occurs outside the surface of the particle. For the formation of weddelite, the medium must have a high calcium/oxalate ratio, that is, the calcium concentration in the solution must be greater than the oxalate concentration (Harrache et al., 1997; Daudon, 2004; Silva et al., 2009). The precipitation observed by SEM is more homogeneous in the sample and mineral particles do not appear with the calcium oxalate mineral coating. This behavior was observed for Catalão and Araxá RP (data no showed), suggesting that there is a different pattern of behavior that varies with the type of rock studied.

Whewellite ($CaC_2O_4.H_2O$) is an oxalate monohydrate crystal that forms flat hexagons, styloids, cubes and their conglomerates (druses) (Frey-Wyssling 1981) It is the most found form in nature and the most stable crystallographic form of calcium oxalate (Ruiz-Agudo et al., 2017). Wedelite ($CaC_2O_4.2H_2O$) is an oxalate dihydrate crystal that is pyramid-shaped and bipyramidal prisms (Frey-Wyssling 1981). It is a more unstable mineral, tending, depending on the conditions, to transform into whewellite by dehydration (Thongboonkerd et al., 2006; Izatulina et al., 2018). Kaoxite ($CaC_2O_4.3H_2O$) is a rarer and very unstable calcium oxalate mineral (Conti et al., 2014). It's a thermodynamically metastable form of kaoxite tends, spontaneously, although slowly, to transform whewelite by the dehydration process (Frey-Wyssling 1981; Modenesi et al. 2001; Izatulina et al., 2018). The process of transformation of calcium oxalate minerals and the variables that can interfere and command this process are not yet clarified. Factors such as temperature, umidity, molar concentration, buffering, pH and the presence of some inhibitory agents such as citric acid are studied, but this matter remains inconclusive (Thongboonkerd et al., 2006; Conti et al., 2014; Izatulina et al., 2018; Valido et al., 2020; Hsu et al.,2021).

The formation of new structures on the mineral surface is a factor that decreases and slows down the action of oxalic acid in the P solubilization of more reactive rocks (Nascimento et al., 2018; Mendes et al., 2021). Our data confirm that the formation of oxalate crystals on the rock surface or not it may be linked to the reactivity of the RPs. In a study carried out by Nascimento et al., 2018, treatment with oxalic acid at a concentration of 5 and 10 mmol l^{-1} in Araxá RP showed that a concentration of 5 mmol released P in lesser amounts when compared to 10 mmol ($p<0.05$) This result indicates that the concentration of 10 mmol would be the most adequate to reach higher solubilized P values. In fact, the precipitation of oxalate minerals interferes with the speed of the solubilization process, however, we believe,

based on our results, that this is not only due to excess oxalic acid concentration but also varies according to the reactivity of these materials.

EDS analysis confirmed that treatments that have oxalic acid have a notable decrease of P in the mineral composition when compared to untreated rock for all phosphates studied. Indicating that this metabolite is, among those tested, the one that most depletes the RP in relation to the amount of P solubilized. These results are observed for all RP tested, except for Crandalita RP.

XRD analyzes confirm that the crystallinity of the studied RPs is different. RPs such as Argélia, Bayovar and Gafsa present more open peaks, which indicate lower crystallinity of the material (Schwertmann et al., 1985). This characteristic can be related to a greater tendency to solubilization. The treatment with 10 mmol L^{-1} oxalic acid was what most altered the mineralogical structure of these RPs, causing a decrease in apatite peaks and the appearance of calcium oxalate minerals. In the case of reactive RP, most of them are whewellite formation. The presence of gypsum was observed in the Gafsa RP. This mineral is a solubilized by treatment with citric acid, but apparently does not interfere with the solubilization of P. For the RP of Catalão it is possible to observe that the peaks formed are thinner and well defined, which indicates higher degree of crystallinity of the mineral (Schwertmann et al., 1985). Apatite and quartz are the main RP constituents. Pattern that is also observed for the RP of Patos de Minas, despite being considered a medium reactivity rock. Here, the treatment with oxalic acid was also the one that caused the most mineralogical changes, where all types of calcium oxalate were formed, most of which were wedelite and caoxite. this same behavior was observed for the analyzes carried out in Araxá RP (Nascimento et al., 2018). Crandalite RP is the only aluminum RP that has been studied. It has in its constitution the mineral crandalite identified in the XRD analysis. In general, there were no changes observed in the mineralogy of RP Crandalite. It is possible to notice a small tendency towards P solubilization of this material if we compare the relative intensities of the main peak of crandalite with the main peak of quartz. In the control diffractogram, the ratio of the two intensities was 3.17. When RP was treated with oxalic acid alone or in combination with citric acid the ratio was 6.14. Considering that quartz was not influenced by the treatment, the decrease in the relative intensity of crandalite spikes can be attributed to the solubilization of this mineral. However, the P solubilization of Crandalite RP is considered low (See chapter II - Table 1). This is the first study that describes the process of P

solubilization, in terms of morphology and mineralogy, between natural phosphate rocks of different degrees of reactivity and organic acids.

CONCLUSION

The formation of a protective layer on the PR particles reduces the effectiveness of oxalic acid on the P solubilization percentage of these materials. The formed calcium oxalate layer is not intact and may become destabilized during the solubilization process. The nutrient that suffers the most decrease in all PR studied is P. There is a different pattern of calcium oxalate formation according to the reactivity of the PR studied.

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Capítulo IV

Aspergillus niger IMPROVES P UPTAKE BY Eucalyptus grandis IN A HIGHLY WEATHERED P-FIXING TROPICAL SOIL

Aspergillus niger IMPROVES P UPTAKE BY Eucalyptus grandis IN A HIGHLY WEATHERED P-FIXING TROPICAL SOIL

ABSTRACT

Phosphorus (P) can undergo adsorption, precipitation and soil fixation reactions that reduce the availability of this nutrient to the plants. When fixed to iron and aluminum oxides and hydroxides, the P starts to form the solid phase of the soil, being difficult to make available to crops. Some microrganisms and organic acids, can extract P fixed in weathered highlith soil *in vitro*. These compounds can be produced by plants and microorganisms as a strategy to acquire P for growth. It is already known that *Aspergillus niger* FS1 and its metabolites, especially oxalic acid, can release fixed P in highly weathered soils *in vitro*. However, it is not yet known whether this microorganism can help in the growth and development of plants grown under conditions of low available P. The objectives of this work were to evaluate the P release by *A. niger* FS1 in highly weathered soil and the contribution of this microorganism to the nutrition and growth of eucalyptus plants. The P amount adsorbed to the soil corresponded to 5, 10, 15, 20 e 25 % of the maximum P adsorption capacity (MPAC) and it was incubated for 40 days. These percentages are equivalent to 70, 140, 210, 280 e 350 mg kg^{-1} de P. In addition, treatment composed by fertilization at the time of planting with $Ca_2(H_2PO_4)_2$ and Araxá rock phosphate at a dose of 350 mg kg^{-1}. *Aspergillus niger* FS1 significantly increased the root dry mass of eucalyptus plants. The fungus also helped the accumulation of macronutrients significantly higher than the control. The accumulation of nutrients in relations to the control in the shoot was 234 % for P. In the root, this increase was even greater, up to 650 % for P. *Aspergillus niger* FS1 was able to reverse the P adsorption process to the soil, effectively contributing to the growth and nutrition of eucalyptus. Therefore, these results opening prospects for accessing the P fixed to agricultural soils.

Keywords: Oxisol. Adsorption. Phosphate solubilizing microorganisms. MPAC.

INTRODUTION

The current consumption of phosphate fertilizers is high and show trends of increase in the coming years. Worldwide consumption of P_2O_5 is about 41 million tons. In Brazil, 5.6 million tons were consumed in 2018, of which 50 % came from imports (FAO, 2020). In addition to importing phosphate rock (PR) to produce soluble phosphate fertilizers, Brazil also imports sulfur to produce sulfuric acid, an input necessary to treat natural phosphates and transform them into soluble fertilizers. About five countries in the world control 90 % of the most reactive P reserves and 8 countries produce about 45 % of all global sulfuric acid (Bruijne et al., 2009). As a large part of inputs necessary to produce phosphate fertilizers is imported and given that Brazilian economy is based mainly on agriculture, geopolitical instabilities in these countries could strongly affect agricultural production in Brazil.

Most of the world's arable land is composed of oxisols with a high degree of weathering and of a predominantly oxidic nature, generating a high capacity for adsorption of anions (Novais et al., 2007; Roy et al., 2016; Eduah et al., 2019). These soils act as a phosphorus (P) drain, decreasing the concentration of the nutrient in the soil solution. P adsorption to mineral particles leads to fixation. The nutrient becomes strongly retained, making it unavailable to plants (Novais and Smith, 1999). To circumvent this problem, it is common to apply phosphate fertilizers beyond the crop needs (Syers et al., 2009; Roy et al., 2016). Saturation with P has been an agricultural strategy used to maintain ideal concentrations of this element in the solution and meet the nutritional requirements of plants (Fox and Kamprath 1970). This practice, although effective, becomes economically unfeasible, since, in general, only a small fraction of what is applied is effectively absorbed by the crops (Batten, 1992). In addition, phosphate fertilization done in this way consumes a large part of the available P reserves resulting in a worldwide problem (Roy et al., 2016).

In this context, alternatives to access residual P could contribute to reducing the demand for phosphate fertilizers in addition to saving PR, which is a finite natural resource (Sattari et al., 2012; Mew, 2016). The agricultural model practiced in tropics needs to be reformulated according to its particularities to become more efficient and sustainable (Whiters et al., 2015; Roy et al., 2016; Whiters et al., 2018). One of the paths that can be followed is based on the use of microorganisms that can improve the efficiency of nutrient use and promote plant growth.

There are many reports on microorganisms that can solubilize less reactive P sources (Mendes et al., 2014). The solubilization of these rocks is done through fungal metabolism mechanisms such as the decrease in pH and the production of organic acids (Mendes et al., 2014; Prabhu et al., 2019). Oxalic acid, one of the organic acids produced by these microorganisms, is more efficient in the solubilization of low reactivity PRs than sulfuric acid, widely used by the industry. (Mendes et al., 2020).

These phosphate solubilizing microorganisms, as well as their metabolites, may act in the desorption and release of P from the soil *in vitro* too (Nascimento et al., 2021). *Aspergillus niger* FS1 and its metabolites, mainly oxalic acid, partially reversed the fixation process, between 18 to 32 % of highly weathered soils releasing P into the solution (Nascimento et al., 2021). Therefore, the present work aims at evaluating P release by *A. niger* FS1 in the soil weathered with fixed P and the contribution of this microorganism to nutrition and growth of eucalyptus plants.

MATERIALS AND METHODS

The experiment was conducted at the greenhouse, under the coordinates 20º 45’ 29.5” S 42º 52’ 13.8” W, of Departamento de Microbiolgia and at the Laboratório de Ecologia Microbiana of Universidade Federal de Viçosa, Minas Gerais, Brazil.

The fungus *A. niger* FS1 belongs to the collection of Phosphate Solubilizing Fungi of the Laboratório de Ecologia Microbiana. The fungus was chosen owing of its capacity to solubilize rock phosphates (RPs) and promote the release of soil-fixed P (Mendes et al., 2014; Nascimento et al., 2021). The fungus was maintained in potato-dextrose-agar (PDA) at 30 °C and transferred to fresh PDA every seven days.

Soil collection and preparation

The B horizon Oxisol was collected in the Viçosa region, Minas Gerais, Brazil, under the coordinates 20º 46’ 00.9” S 42º 52’ 43.4” W. The Oxisol was sieved through a 4 mm sieve. After this procedure, 3.5 kg were weighed and packed in plastic bags. Chemical and physical analysis of the soil were carried out according to EMBRAPA (1997) (Table 1).

The maximum phosphorus absorption capacity (MPAC) of the soil was determined following the method described by Alvarez and Fonseca (1990) and the maximum value corresponds to 1.3 g kg^{-1} P (Figure 1).

The data obtained were submitted to ANOVA ($p < 0.05$) and means were used to adjust the Langmuir model (Figure 1). Soil field capacity was determined using the moisture equivalent method according to Ruiz *et al*. (2003).

Seedling production

Seeds were disinfected to produce the *Eucaliptus grandis* seedlings. The seeds were superficially disinfected by immersion in 70 % ethanol for one minute followed by immersion in 20 % hydrogen peroxide (H_2O_2) for seven minutes (Santos, 2011). After this time, the seeds were washed five times in autoclaved distilled water. After disinfestation, the seeds were sown in autoclaved sand arranged in a tray and covered with a plastic film until they germinated. The trays were kept at a temperature of 24 °C for approximately seven days. After this period, the seedlings were taken to the greenhouse, acclimatized, and maintained for 45 days. Every week the seedlings were fertilized with 100 ml of Clark's nutrient solution (Clark, 1975).

Plant growth promotion by *Aspergillus niger* in Eucalyptus cultivated in highly weathered soil with fixed P

For the incubation of soil with P, 100 ml solutions of $Ca(H_2PO_4)_2$ were prepared in the doses of 0.86, 1.72, 2.58, 3.44 and 4.31 g for each 3.5 kg of soil. Values that correspond to 5, 10, 15, 20, and 25 % of the MPAC. The Oxisol was stored for 40 days. Field capacity was maintained at 80 % during this period.

After this period, the bags were opened, and the soil exposed to air for complete drying. Then the clods were broken, and the soil was placed in pots to proceed with the planting of the seedlings.

The seedlings were transplanted to the pots after 45 days of growth. The experiment was conducted in a completely randomized design in an 5x2 factorial scheme with four replications, totalizing 40 pots. The first factor corresponded to P fertilization doses at MPAC of 5, 10, 15, 20 and 25 %. And the second factor was the fungus *A. niger* FS1 and a control (without fungus).

To carry out the transplant, the seedlings were removed from the tray with sand, and the roots were washed with distilled water in a very delicate way, avoiding causing damage to the roots.

Table 1. Physical and chemical characteristics of the B horizon of Oxisol collected in Viçosa, MG, Brazil.

Soil	pH H_2O	P	K	Ca^{2+}	Mg^{2+}	Al^{3+}	H + Al	SB	CEC (t)	CEC (T)	V	M	OM	N	P-rem	Clay	Silt	Sand
		mg dm^{-3}		$cmol_c$ dm^{-3}							%		dag kg^{-1}		mg L^{-1}	%		
B horizon	4.92	0.4	0	0.22	0.03	0.39	3.4	0.25	0.64	3.65	6.8	60.9	1,6	0.056	6,8	72.4	4.2	23.3

pH in H_2O – Relation 1:2.5; P e K – Extractant Mehlich 1; Ca – Mg – Al – Extratant: KCl – 1mol L^{-1}; SB – Exchangeable Bases Sum; CEC (t) = Effetive cation exchange capacity; CEC (T) = Cation exchange capacity at pH 7.0; V = Saturation by Bases Index; m = Aluminium Saturation Index; OM – Organic Matter – C.org * 1.724 – Walkley-Black; P-rem = Remaining phosphorus.

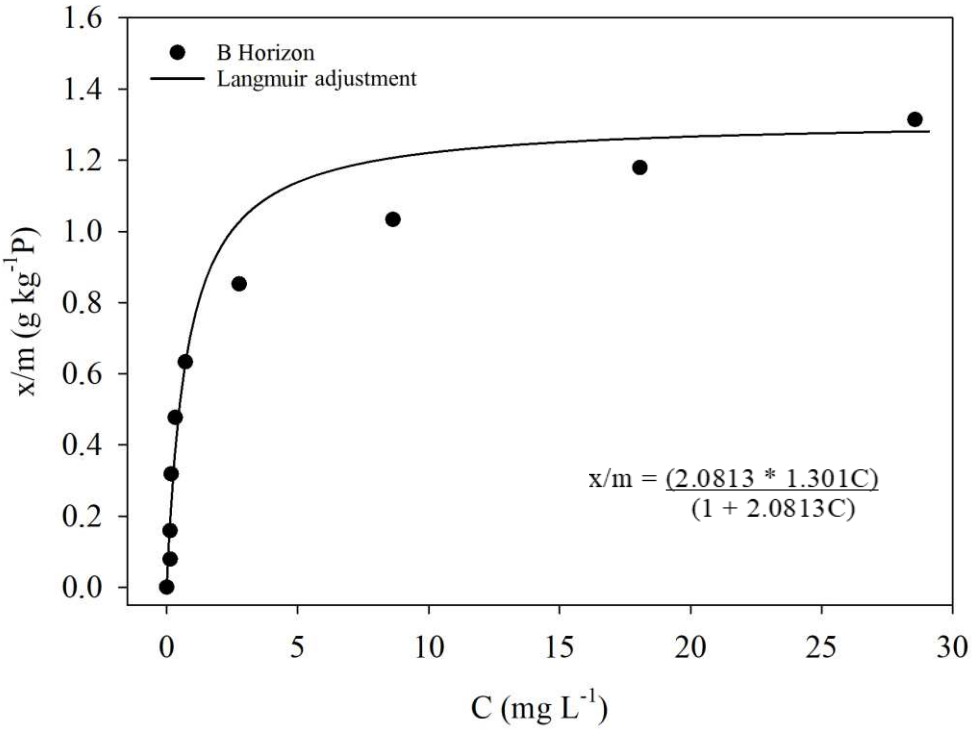

Figure 1. Maximum P adsorption capacity in B horizons of Oxissol from Viçosa, MG, Brazil.

After washing, the apical root was cut to stimulate their growth and development. Perforations were made in the soil according to the size of the roots, and 3 disks of 3 mm of fungal cultures were added to the roots. The potted were maintained at 70 % of the field capacity and the humidity was controlled by weighing a pot sample (Novais et al. 1991). Fertilization the other nutrients of the experiment was carried out as described by (Novais et al. 1991) for greenhouse experiments excluding the source of P.

Eucalyptus cultivated under different phosphate fertilizers inoculated with *Aspergillus niger*

For the incubation of soil with P, solution of $Ca(H_2PO_4)_2$ was prepared in the dose of 4.31 g for each 3.5 kg of soil. Values that correspond 25 % of the MPAC. The Oxisol was stored for 40 days. Field capacity was maintained at 80 % during this period. The soil that received phosphate fertilization with $Ca(H_2PO_4)_2$ and Araxá RP at planting were also incubated in the same conditions without the presence of $Ca(H_2PO_4)_2$. After this period, the bags were opened, and the soil exposed to air for complete drying. Then the clods were broken, and the soil was placed in pots. Fertilization with 350 mg kg^{-1} with Araxá RP (insoluble phosphate) or $Ca(H_2PO_4)_2$ (soluble phosphate) applied at planting.

The seedlings were transplanted to the pots after 45 days of growth. The experiment was conducted in a completely randomized design in an 3x2 factorial scheme with four replications, totalling 24 pots. The first factor corresponded to P fertilization types. And the second factor was the fungus *A. niger* FS1 and a control (without fungus).

Perforations were made in the soil according to the size of the roots, and 3 disks of 3 mm of fungal cultures were added to the roots. The potted were maintained at 70 % of the field capacity and the humidity was controlled by weighing a pot sample (Novais et al. 1991). Fertilization the other nutrients of the experiment was carried out as described by (Novais et al. 1991) for greenhouse experiments excluding the source of P.

The experiments were harvested after 60 days. The plants were removed from the pots and separated into shoots and roots. The roots were removed from the pots, washed and air dried. The samples of shoot and roots were placed in an oven with forced air circulation at 70 °C and dried to constant weight. Then, the samples were ground in stainless steel knife mills and packed in paper bags. The samples were weighed on a precision balance in the glass flasks used for nitric perchloric digestion. The variables analyzed were height (cm), dry mass of shoot and roots (g), analysis of N, P, K, Ca, and Mg content in tissues. The Kjedahl method

Table 2. Dry mass, nitrogen, phosphorus, potassium, calcium and magnesium content of the shoot, root and total of *Eucaliptus grandis* cultived during 60 days in association with by *Aspergillus niger* FS1 on Oxisol previously adsorved with doses P different by 40 days.

P doses (mg kg^{-1})	**MCAP (%)**	**Shoot**		**Root**		**Total**	
		A. niger FS1	Control	*A. niger* FS1	Control	*A. niger* FS1	Control
				Dry mass (g)			
70	5	1.68	2.37	0.52 ns	1.10 ns	2.20	3.47
140	10	5.49	3.19	2.14 A	0.92 B	7.62	4.11
210	15	4.43	3.22	2.80 A	1.25 B	7.23	4.48
280	20	17.52	18.44	4.35 ns	5.17 ns	21.87	23.61
350	25	24.0	22.96	9.31 A	6.10 B	33.31	29.06
Media		10.62 ns	10.04 ns	3.82	2.91	14.45 ns	12.95 ns
				N content (mg)			
70	5	27.77 ns	36.74 ns	5.10 ns	2.29 ns	54.92 ns	66.70 ns
140	10	121.25 A	35.80 B	22.60 A	2.89 B	249.83 A	59.06 B
210	15	100.54 ns	72.73 ns	25.32 A	9.38 B	235.40 A	147.99 B
280	20	299.81 ns	301.60 ns	40.12 ns	42.04 ns	576.58 ns	530.95 ns
350	25	395.78 A	310.79 B	73.88 A	49.60 B	813.84 A	631.75 B
				P content (mg)			
70	5	0.83 ns	1.15 ns	0.26 ns	0.23 ns	2.19 ns	2.65 ns
140	10	3.74 A	1.11 B	1.36 A	0.18 B	10.08 A	2.22 B
210	15	3.86 B	6.37 A	2.16 A	0.76 B	12.40 ns	11.42 ns
280	20	17.23 ns	15.74 ns	3.41 ns	2.93 ns	38.74 A	30.58 B
350	25	24.16 A	21.10 B	8.30 A	4.76 B	63.22 A	49.62 B
				K content (mg)			
70	5	15.30 ns	18.23 ns	3.84 ns	3.72 ns	34.97 ns	42.83 ns
140	10	59.19 A	15.19 B	18.69 A	2.34 B	150.50 A	29.97 B
210	15	53.48 ns	39.08 ns	26.50 A	8.87 B	163.06 A	98.32 B
280	20	135.07 ns	157.77 ns	31.08 A	23.97 B	325.05 ns	319.92 ns
350	25	158.96 ns	147.31 ns	49.86 A	36.99 B	398.80 ns	364.64 ns
				Ca content (mg)			
70	5	5.42 ns	7.48 ns	1.48 ns	0.72 ns	10.45 ns	14.66 ns
140	10	25.77 A	7.99 B	3.89 A	0.55 B	50.17 A	12.75 B
210	15	28.22 ns	18.25 ns	6.63 A	2.25 B	66.74 A	38.19 B
280	20	76.95 ns	88.92 ns	11.07 ns	10.56 ns	152.38 ns	166.23 ns
350	25	121.68 ns	113.72 ns	30.70 A	18.37 B	279.96 ns	232.81 ns
				Mg content (mg)			
70	5	2.29 ns	2.75 ns	0.48 ns	0.54 ns	4.86 ns	6.35 ns
140	10	9.89 A	2.45 B	2.68 A	0.33 B	23.81 A	4.60 B
210	15	10.26 ns	6.63 ns	3.73 A	1.13 B	27.43 A	15.05 B
280	20	29.51 ns	27.12 ns	4.11 ns	4.76 ns	57.52 ns	49.47 ns
350	25	41.97 A	31.87 B	13.32 A	5.22 B	107.03 A	65.50 B

Within each fertilization, lines with same uppercase letter are not statistically different by Tukey test ($p < 0.05$). ns: not significant by the F test ($p < 0.05$).

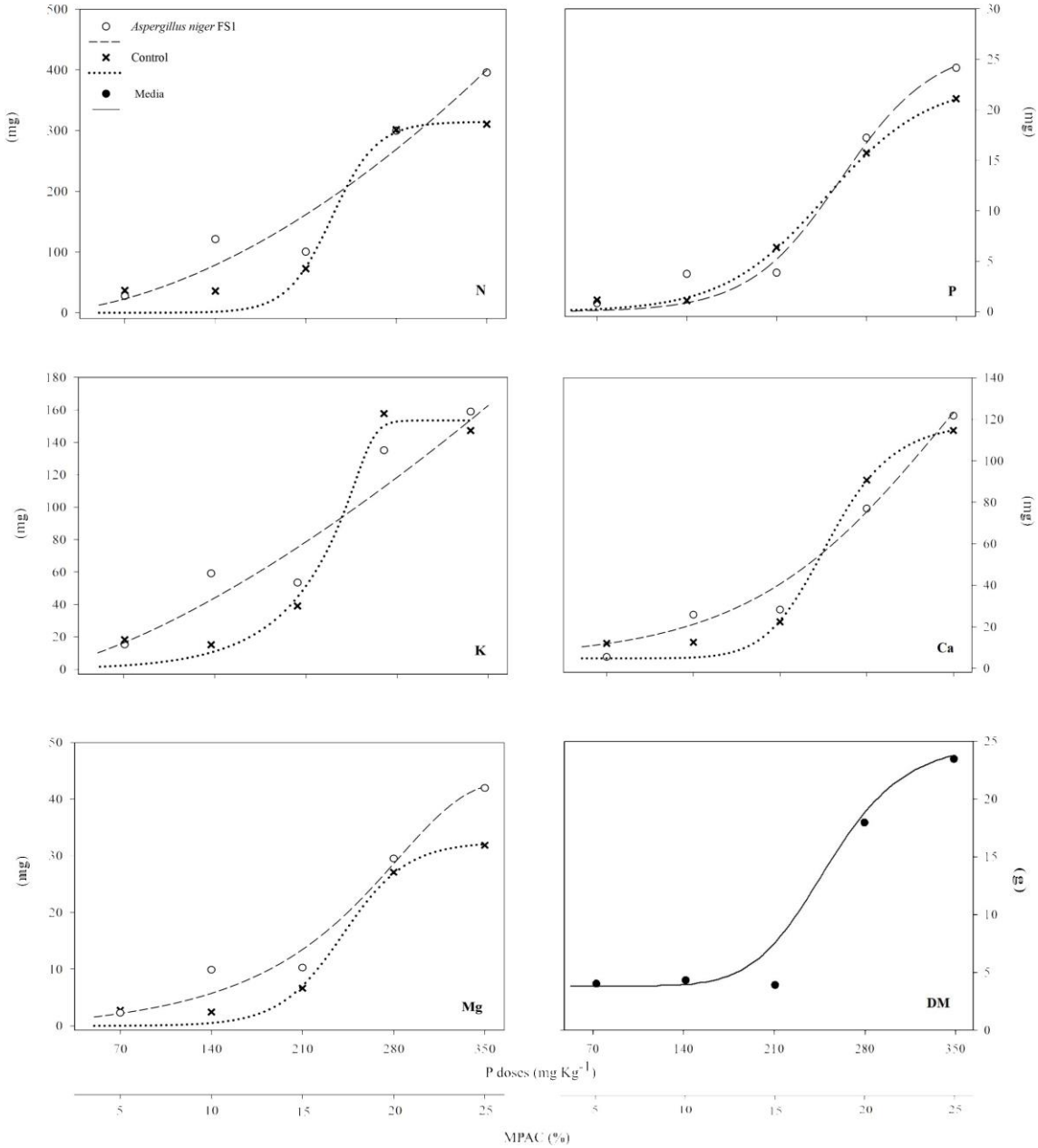

Figure 2 – Nitrogen (N), phosphorus (P), potassium (K), calcium (Ca) and magnesium (Mg) content and dry mass (DM) of the shoot of *Eucaliptus grandis* cultived during 60 days in association with by *Aspergillus niger* FS1 on Oxisol previously absorved with doses P different by 40 days.

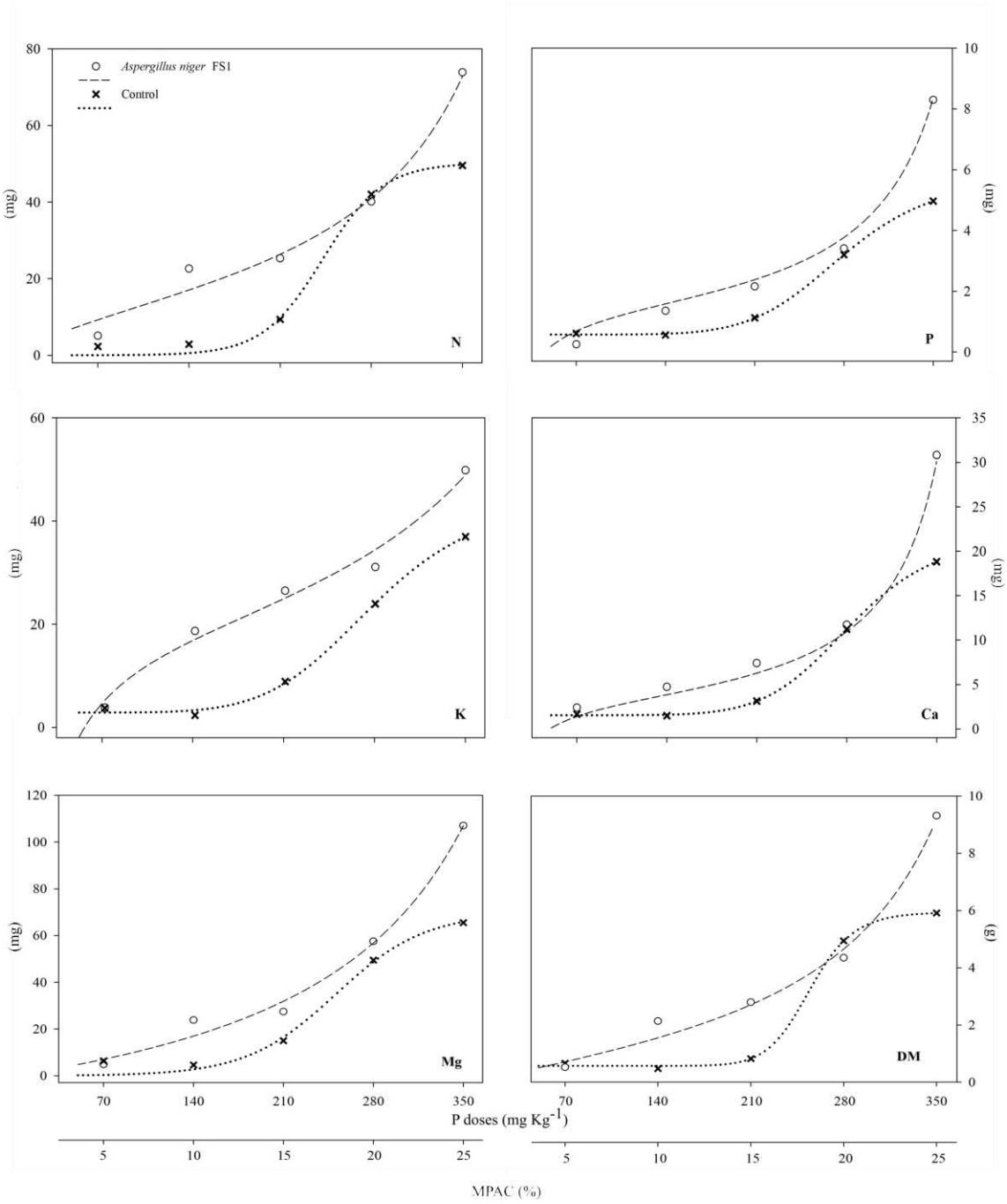

Figure 3 – Nitrogen (N), phosphorus (P), potassium (K), calcium (Ca) and magnesium (Mg) content and dry mass (DM) of the root of *Eucaliptus grandis* cultived during 60 days in association with by *Aspergillus niger* FS1 on Oxisol previously absorved with doses P different by 40 days. Regression equations see supplementary table 2.

was used to determine the N contents, phosphorus contents by colorimetry (Murphy and Riley, 1962), Ca and Mg by atomic absorption (Horwitz and Latimer, 2010) and K contents by flame photometry (Horwitz and Latimer, 2010).

The total nutrient content plant tissues (mg) were calculated by multiplying the concentrations of N, P, K, Ca and Mg by the dry matter.

The data was subjected to analysis of variance – ANOVA. Means were compared by Tukey's test ($p < 0.05$). When appropriate, regression analyses were done. The R Studio Software ExpDes package was used.

RESULTS

Plant growth promotion by *Aspergillus niger* in Eucalyptus cultivated in highly weathered soil with fixed P

There was no statistically significant difference between the treatments at the different doses tested for the following variables: height, shoot dry mass and total dry mass (Table 2). All variables showed interaction between the factors. The general means obtained for height was 40.6 cm. For shoot dry mass and total dry mass were 10.3 and 13.7 g (Table 2).

For the dose of 70 mg kg^{-1}, no significant difference was observed between the means of the variables studied in any treatment and in none of the doses tested (Table 2). Generally, the means associated with inoculation of *A. niger* FS1 were better for all variables studied at the dose of 140 mg Kg^{-1} (Figure 2 and 3). For the dose of 280 mg kg^{-1}, the inoculation with *A. niger* FS1 presented significant and statistically superior means to the control in the potassium content in the root dry matter, 31.1 mg, and in the phosphorus content in the dry mass total matter, 38.8 mg (Tables 4 and 5).

In the root dry mass, a significant difference was observed at doses of 140, 210 and 350 mg Kg^{-1}, the means highest observed in treatments inoculated with *A. niger* FS1. The means were 2.14, 2.8 and 9.3 mg in the presence of *A. niger* FS1 (Table 2).

The macronutrient accumulation at shoot and root increased as the P dose in the soil also increased. For the content of macronutrients N, P, K, Ca and Mg in the shoot, *A. niger* FS1 was shown to be efficient in increasing the accumulation of these nutrients in the tissues (Table 2).

Significant means at macronutrient content in the shoot dry mass were observed for the treatment inoculated with the fungus at doses of 140 mg kg^{-1}, for all macronutrients, and 350 mg kg^{-1} for nitrogen, phosphorus, and magnesium (Table 2).

The content of all macronutrients in the root dry mass was higher ($p < 0.05$) in the presence of *A. niger* FS1 at doses of 140, 210 and 350 mg Kg^{-1} (Table 2).

A niger FS1 showed significant and statistically higher ($p < 0.05$) means in total dry mass when compared to the control at a dose of 140 mg Kg^{-1} for all macronutrients (Table 2). At the dose of 210 mg Kg^{-1}, the means were significant and statistically higher for the macronutrient nitrogen, potassium, calcium, and magnesium (Table 2). At the dose of 350 mg Kg^{-1} *A. niger* FS1 presented significant and statistically superior means to the control for the following macronutrients: nitrogen, phosphorus, and magnesium, being the means 813.8, 63.2 and 107 mg, respectively (Table 2).

Eucalyptus cultivated under different phosphate fertilizers inoculated with *Aspergillus niger*

No interaction was observed between the factors studied in the different types of fertilization applied for the variable height, shoot, root, and total dry mass (Table 4). For height, no significant difference was identified between the means of the treatments, the general means being equal to 55.8 cm. For shoot, root and total dry mass, the treatment that was inoculated with *A. niger* FS1 showed higher ($p < 0.05$) means than the non-inoculated treatment (control) (Table 4).

Among the applied fertilizations, statistically higher means were observed for $Ca(H_2PO_4)_2$ applied at planting, being height = 66.8 cm, shoot dry mass = 30.5 g and total dry mass = 36.7 g. For root dry mass, no statistical difference was observed between the application of $Ca(H_2PO_4)_2$ 40 days before or at the time of planting (Table 4). The lowest means were obtained with the application of Araxá RP for all the variables described above (Table 4).

For the macronutrient content, there was an interaction between the factors for the nitrogen and phosphorus in the shoot, root and total mass, and, for the magnesium content in the total mass (Table 4). All others do not show significant interaction between the factors (Table 4).

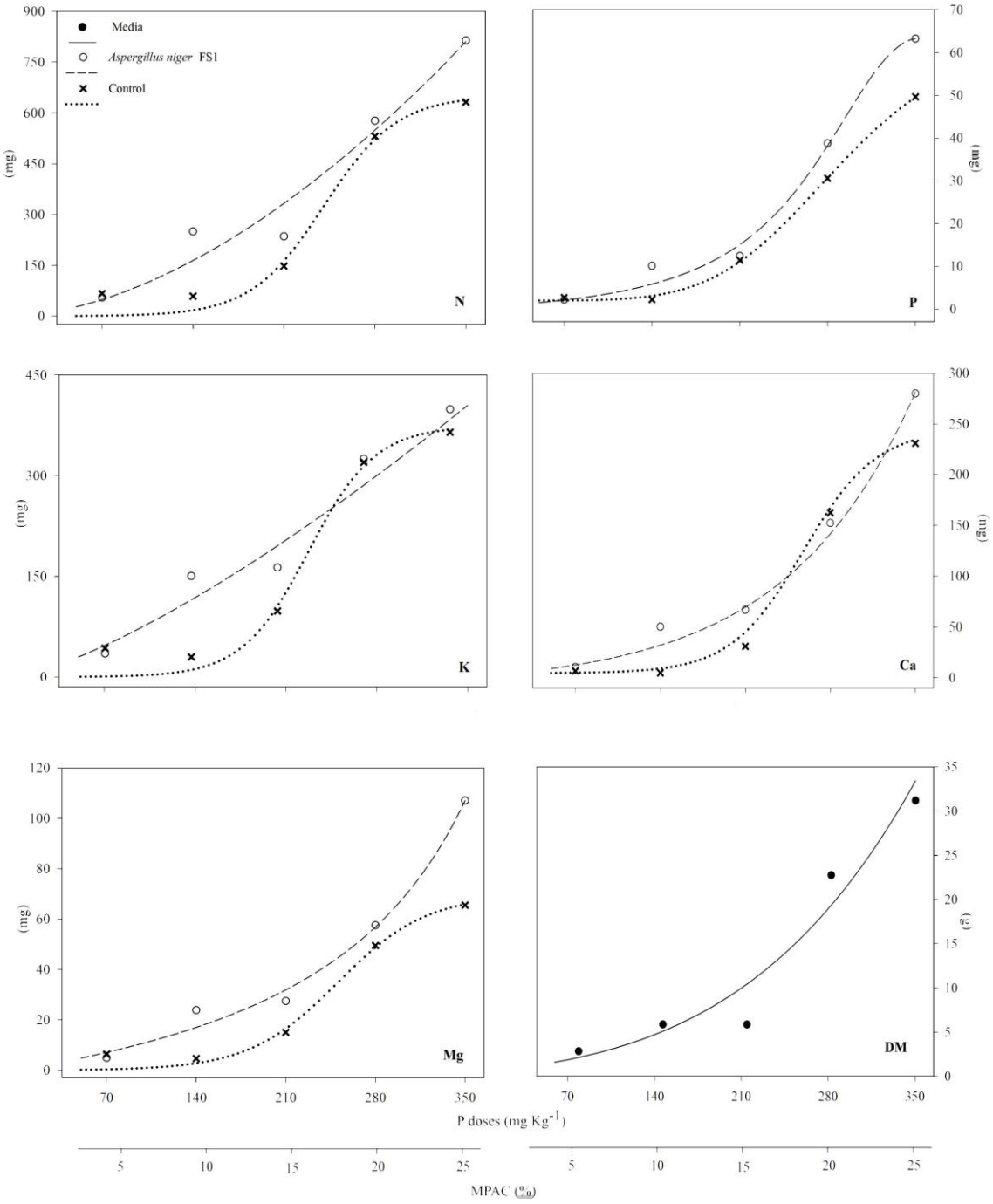

Figure 4 – Nitrogen (N), phosphorus (P), potassium (K), calcium (Ca) and magnesium (Mg) content and dry mass (DM) of the total mass of *Eucaliptus grandis* cultived during 60 days in association with by *Aspergillus niger* FS1 on Oxisol previously absorved with doses P different by 40 days. Regression equations see supplementary table 2.

The nitrogen content in the shoot mass was higher in the treatment inoculated with *A. niger* FS1. The nitrogen accumulation in the shoot was statistically equal when compared $Ca(H_2PO_4)_2$ 40 days before or applied at planting, both inoculated (Table 4). The phosphorus content in the shoot was higher in fertilization with $Ca(H_2PO_4)_2$ at planting. The inoculated treatment presented a higher ($p < 0.05$) media than the control in the fertilization with Araxá RP, obtaining a media higher of 9.1 mg (Table 4). *Aspergillus niger* FS1 was the best treatment for shoot dry mass potassium accumulation with significant mean of 135.3 mg (Table 4). Calcium and magnesium content in shoot dry mass did not present significant means between treatments (Table 4).

In the root mass, the content of nitrogen and phosphorus was higher ($p < 0.05$) when *A. niger* FS1 was inoculated in the fertilization with $Ca(H_2PO_4)_2$ 40 days before planting, with means of 73.9 and 8.3 mg, respectively (Table 4). The P content in the root was significative in the treatment inoculated in the fertilization with Araxá RP, with a means of 3.6 mg (Table 4). For potassium content, treatment means did not differ statistically. For this macronutrient, the highest means observed was in fertilization with $Ca(H_2PO_4)_2$ 40 days before planting, 43.4 mg (Table 4). For the macronutrient calcium and magnesium, the treatments with the inoculation of *A. niger* FS1 were superior to the control 22.3 and 9.4 mg, respectively. Fertilization with $Ca(H_2PO_4)_2$ both applied 40 days before and applied at the time of planting showed statistically equal means (Table 4).

The nitrogen and phosphorus content in the total dry mass was significative in treatments inoculated under fertilization with $Ca(H_2PO_4)_2$ applied 40 days before planting and with Araxá RP applied at planting (Table 4). The observed means were 873.2 mg and 304.6 mg for nitrogen content and 66.0 mg and 25.2 mg for phosphorus, respectively. No statistical difference was observed between inoculated treatments fertilized 40 days before or at the time of planting with $Ca(H_2PO_4)_2$ for nitrogen content (Table 4). The potassium and shoot and root calcium content were higher in the treatment inoculated with the fungus (Table 4). The magnesium total content in the inoculated treatment was statistically superior to the control under the fertilization with $Ca(H_2PO_4)_2$ 40 days before planting (Table 4).

DISCUSSION

Our results indicate that *A. niger* FS1 was efficient in releasing fixed P in highly weathered soil, contributing to the nutrition of eucalyptus plants grown in soils with low P

availability. In our experiment, it was assumed that over the time of 40 days of soil incubation, P would be retained with greater strength as described in the literature (Barrow, 1985; Novais and Smith, 1999). In highly weathered soils, much of the available P is adsorbed quickly, within 100 minutes after P contact with the soil (Guedes et al., 2016). After this period, approximately 70 % of the P loses balance with the P of the solution, being fixed by the soil matrix (Guedes et al., 2016).

Inoculation with *A. niger* FS1 promoted increased root growth of *E. grandis* cultivated in soils with fixed P. The increase in roots with fungal inoculation was up to 132 % (Figure 2). A more developed root system results in greater absorption of nutrients and water (Sukumar et al., 2013). Characteristic that helps the plant to better withstand possible stress conditions, in addition to allowing the plant to exploit a greater volume of soil (Sukumar et al., 2013). As these plants were cultivated under P limitation, this characteristic becomes essential for greater efficiency in nutrient absorption. Significant increases in root dry mass indicate that the fungus produces phytohormones that are important for plant growth and development, such as indoleacetic acid, auxins, and gibberellins (Salas-Marina et al., 2011; Sukumar et al., 2013; Lubna et al., 2018).

There is a report in the literature that clones of *E. grandis* x *E. urophylla* are excellent in producing greater volume and wood quality and resistance to diseases, however, they have difficulty in rooting (Prado et al., 2019). Although we did not use cloned plants in this work, our results show that this fungus has the potential to induce greater root development as an alternative to this problem.

Our data show that the fungus plays an important role in the acquisition of nutrients by eucalyptus plants under conditions of P limitation. *Aspergillus niger* FS1 can act on the forms of P fixed in the soil, making this nutrient available again in the solution (Nascimento et al. 2021), therefore, this nutrient is not more limiting for the development of plants.

Aspergillus niger FS1 was the best treatment at a dose of 140 mg kg^{-1} for all variables analyzed (Table 2). Indicating that in some fertilization ranges the presence of the microorganism is essential for the best performance of the plant. In ranges where the concentration of P can be limiting to the growth, the plant associated with the fungus had a better performance in the acquisition of P due to the increase in the mass of the roots. With a greater volume of roots, the plant can better exploit the soil, increasing the contact surface with the soil particles and, consequently, absorbing a greater amount of P.

Table 4. Dry mass, nitrogen, phosphorus, potassium, calcium and magnesium content of the shoot, root and total of *Eucaliptus grandis* cultived during 60 days in association with by *Aspergillus niger* FS1 on Oxisol previously adsorved with 350 mg kg^{-1} P by 40 days and oxisol fertilized at the time of planting with the same dose mentioned above of Araxá phosphate rock (insoluble) and $Ca(H_2PO_4)_2$ (soluble).

Fertilizers	**Shoot**			**Root**			**Total**		
	A. niger FS1	Control	**Media**	*A. niger* FS1	Control	**Media**	*A. niger* FS1	Control	**Media**
					Dry mass (g)				
$Ca(H_2PO_4)_2$ 40 days	24.00	22.84	23.42 B	8.82	6.10	7.46 A	32.82	28.95	30.88 B
$Ca(H_2PO_4)_2$	31.43	29.57	30.50 A	7.31	5.02	6.16 A	38.74	34.58	36.66 A
Araxá RP	7.89	3.79	5.84 C	3.10	1.26	2.18 B	10.99	5.05	8.24 C
Media	21.57 a	19.14 b		6.41 a	4.13 b		27.51 a	22.86 b	
					N content (mg)				
$Ca(H_2PO_4)_2$ 40 days	395.78 Aa	310.79 Bb	353.29	73.88 Aa	49.60 Ab	61.74	873.10 Aa	631.75 Bb	752.42
$Ca(H_2PO_4)_2$	455.63 Ans	520.75 Ans	488.19	56.93 Bns	51.53 Ans	54.23	862.68 Ans	890.49 Ans	876.58
Araxá RP	155.07 Bns	109.11ns	132.09	24.72 Cns	17.26 Bns	20.99	304.56 Ba	205.84 Cb	255.20
Media	335.49	313.55		51.85	39.47		680.11	576.02	
					P content (mg)				
$Ca(H_2PO_4)_2$ 40 days	24.16 Bns	21.10 Bns	22.63	8.30 Aa	4.76 Bb	6.53	65.98 Ba	49.62 Bb	57.80
$Ca(H_2PO_4)_2$	41.94 Ans	49.95 Ans	45.94	6.93 Bns	6.55 Ans	6.74	88.27 Ab	99.99 Aa	94.13
Araxá RP	9.08 Ca	5.37 Cb	7.22	3.61 Ca	1.87 Cb	2.74	25.24 Ca	13.69 Cb	19.47
Media	25.06	25.47		6.28	4.40		59.82	54.14	
					K content (mg)				
$Ca(H_2PO_4)_2$ 40 days	158.96	147.31	153.13 A	49.86	37.00	43.42 A	418.02	364.64	391.43 A
$Ca(H_2PO_4)_2$	173.62	153.31	163.47 A	37.47	33.21	35.34 B	412.09	386.78	399.43 A
Araxá RP	73.18	46.76	59.97 B	25.50	16.92	15.22 C	191.57	88.66	140.12 B
Media	135.25 a	115.79 b		37.61 ns	25.05 ns		340.56 a	280.03 b	
					Ca content (mg)				
$Ca(H_2PO_4)_2$ 40 days	121.69	113.72	117.70 B	30.70	18.37	24.54 A	306.16	232.81	269.49 B
$Ca(H_2PO_4)_2$	182.91	163.38	173.15 A	25.86	23.07	24.47 A	362.56	336.78	349.67 A
Araxá RP	42.71	25.78	34.24 C	10.45	6.52	8.49 B	96.32	53.19	74.76 C
Media	115.77 ns	100.96 ns		22.34 a	15.98 b		255.02 a	207.59 b	
					Mg content (mg)				
$Ca(H_2PO_4)_2$ 40 days	41.97	31.87	36.92 B	13.32	5.22	9.27 A	124.08 Aa	65.50 Bb	94.79
$Ca(H_2PO_4)_2$	50.15	51.73	50.94 A	8.78	7.91	8.34 A	108.15 Ab	107.38 Aa	107.76
Araxá RP	12.32	8.74	10.53 C	6.06	2.90	4.48 B	38.11 Cc	22.13 Cc	30.12
Media	34.81 ns	30.78 ns		9.39 ns	5.34 ns		90.12	65.00	

Within each fertilization, columns with same lowercase letter are not statistically different by Tukey test (p<0.05). Within each treatment lines with the same uppercase letter are not statistically different by the Tukey test (p<0.05). ns: not significant by the F test (p<0.05).

As the P dose increased in the soil, the fungus did not present significant effects for the most variables when compared to the control. The plant-microorganism association is stronger when there is a deficiency of some factor that may impair plant development (Broeckling et al., 2008; Hodge and Fitter, 2013). Thus, under these conditions, the plant releases compounds into the rhizosphere that will attract and keep microorganisms that can help with the problem. As the plant becomes established, it controls rhizodeposition and the association with microorganisms becomes weaker, as it can have a high energy cost for the plant (Broeckling et al., 2008; Hodge and Fitter, 2013; Massenssini et al., 2008; Hodge and Fitter, 2013; Massenssini et al., 2008; al., 2014).

The accumulation of nutrients in the shoot, in addition to P (234 %), shows that *A. niger* FS1 was not only acted in the desorption of P from the soil, but also in the acquisition of other nutrients as N, K, Ca e Mg. *A. niger* FS1 allowed the plant to accumulate a greater amount of N in the order of 238 %, K of 44 %, Ca 223 % e Mg 313 %. In the root, this increase was even greater, up to 679 % for N, 650 % for phosphorus, 713 % for potassium, 680 % for Ca and 800 % for Mg.

There are reports in the literature on *Aspergillus* species involved in plant growth promotion such as *Brassica chinensis* Linn., *Arabidopsis thaliana*, eucalyptus clones (*Eucalyptus grandis* x *Eucalyptus urophylla*) and coffee varieties (*Coffea arabica* L.) through the production of phytohormones (Chuang et al., 2007; Salas-Marina et al., 2011; Prado et al., 2019; Araújo et al., 2020). Our work shows that *A. niger* FS1 can promote the growth of *E. grandis* plants. Due to the restrictions of phosphate fertilization to which the fungus was submitted, there is great potential of this microorganism to survive, colonize the rhizosphere and still promote growth allowed under extreme conditions of P limitation.

Aspergillus sp. has been cited in the literature as a multifunctional growth promoting fungus. When present in the rhizosphere, this fungus can bring several advantages. They cooperate with other microorganisms present and establish a mycorrhizosphere that can aid in nitrogen fixation, solubilize phosphates, produce phytohormones and siderophores, in addition to acting in the biocontrol of phytopathogens (Salas-Marina et al., 2011).

The response of the plants varied depending on the fertilizer used in the nutrition of the plants and the form of application (40 days before or at the time of planting). The application of $Ca_2(H_2PO4)_2$ at planting time led to better plant performance in general (Table 4). Expected result due to greater availability of P in the soil solution at the time of plant

transplant, furthermore this condition also neutralizes stress and therefore results better results for the variables. On the other hand, surprisingly, the accumulation of P in the root dry mass was higher where there was fertilization with $Ca_2(H_2PO4)_2$ 40 days before planting. This can be explained by the balance that the fungus promotes between P forms in the soil solution, accessing P forms that the plant would not be able to obtain. The fungus acts by reversing the fixation process and makes small amounts of P available in the solution. Thus, the plant has constant amounts of P available in the solution throughout the cultivation. In addition, the P incorporated in the fungal biomass also functions as a reserve of organic P that can be accessed. In this way, the fungus maintains a synergistic relationship with the plant and acts as a modulator in the availability of P.

The performance of plants fertilized with Araxá RP was the lowest observed (Table 4). In general, the results were better for plants grown in $Ca_2(H_2PO_4)_2$ 40 days before planting than with the Araxá RP on the day of planting. Due to the very low reactivity that this phosphate presents, it was expected that the plant would present low growth rates. These indices were improved with the inoculation of *A. niger* FS1 indicating that the process of solubilization of this phosphate occurred in the presence of the fungus, which resulted in the availability of P in the soil solution.

Aspergillus niger FS1 is described in the literature for its great ability to solubilize phosphates with very low reactivity (Mendes et al., 2014). The production of essential metabolites for this process, such as organic acids, such as oxalic, citric, and malic, are the main way these microorganisms act to solubilize P. The desorption process of P fixed in the soil was described *in vitro* for this fungus by Nascimento et al. (2021). However, there was no information that this fungus would also be able to act in the soil in the presence of plants and improve the accumulation of P and other nutrients. Our results confirm the ability of *A. niger* FS1 not only to provide soil-fixed P in the solution, but also to promote the growth of *E. grandis* plants.

CONCLUSION

Aspergillus niger FS1 promotes eucalyptus plant growth in addition to and positively contributes to phosphate nutrition. P accumulation can increase up to 600 % in the presence of the fungus. Most likely by the desorption process, providing P fixed to the soil. *Aspergillus niger* FS1 also helps the plant to accumulate macronutrients like N, K, Ca, and Mg.

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CONCLUSÕES GERAIS

Oxalic acid is the most efficient organic acid tested in the solubilization of P from Argelia, Bayovar, Catalão, Gafsa and Patos de Minas RP in an agitated flow system. Improvements in solubilization efficiency can be obtained by combining this compound with citric acid for low-reactivity phosphate rocks. More studies should be carried out to identify what may decrease the efficiency in the oxalic acid solubilization process in reactive rocks, since it has a high rate in a short reaction time, but then this process is impaired.

The formation of a protective layer over the reactive RP particles reduces their effectiveness in the percentage of P solubilization of these materials. The concentration of calcium and oxalic acid in the solution and the rate of reaction of this element with oxalic acid is the explanation for the pattern of formation of different calcium oxalates and in different ways, depending on the reactivity of the RP under study. More studies should be carried out to decipher and understand which factors determine the formation of different types of calcium oxalate in different types of RP.

Aspergillus niger FS1 promotes eucalyptus plant growth in addition to and positively contributes to phosphate nutrition. P accumulation can increase up to 600% in the presence of the fungus. Most likely by the desorption process, providing P fixed to the soil. *Aspergillus niger* FS1 also helps the plant to accumulate macronutrients like N, K, Ca and Mg.

SUPPLEMENTARY MATERIAL

Chapter IV

Table S1. Regression equations of the shoot dry matter (SDM), root dry matter (RDM), total dry matter (TDM) and height (HGT) of *Eucaliptus grandis* cultived during 60 days in association with by *Aspergillus niger* (FS1) on Oxisol previously absorved with doses P different by 40 days.

MENSURED VARIABLE	***Aspergillus niger* (FS1)**	**R²**	**Control**	**R²**
SDM (g)	$\hat{y} = 0.000082*x^{2.153013}$	0.9330	$0.000028*x^{2.330936}$	0.9015
RDM (g)	$\hat{y} = \frac{X}{(100.328583+(-0.00062)*x^{1.966213})}$	0.9892	$0.000049*x^{2.011176}$	0.8784
TDM (g)	$\hat{y} = 0.940637* \frac{1+(-0.200088)*x}{(-66.861981))^{(-1/(-0.200088))}}$	0.9661	$0.000054*x^{2.261820}$	0.8981
HGT (cm)	$\hat{y} = \frac{1}{(0.036741+(-0.000052)*x)}$	0.9469	$26.184475*1.003602^{x}*x^{(-0.077638)}$	0.9811

Table S2. Nitrogen (N), phosphorus (P), potassium (K), calcium (Ca) and magnesium (Mg) content of the shoot dry matter (SDM), root dry matter (RDM) and total dry matter (TDM) of *Eucaliptus grandis* cultived during 60 days in association with by *Aspergillus niger* (FS1) on Oxisol previously absorved with doses P different by 40 days.

	Aspergillus niger (FS1)	R^2	Control	R^2
		SDM		
N (mg)	$0.012402*x^{1.772232}$	0.9303	$\hat{y} = \frac{311.150998}{(1+EXP(245.374612-0.869837*x))^{(1/48.959671)}}$	0.9853
P (mg)	$\hat{y} = \frac{26.373707}{(1+1412.380231*2.72^{(-0.027842*x)})}$	0.9741	$\hat{y} = \frac{22.725091}{(1+506.220488*2.71^{(-0.025131*x)})}$	0.9972
K (mg)	$0.039068*x^{1.422831}$	0.9206	$\hat{y} = \frac{118.35238}{(1+(x/249.838999)^{(-9.594)})}$	0.9888
Ca (mg)	$\hat{y} = \frac{236.313504}{(1+74.437014*2.72^{\wedge(-0.01252*x)})}$	0.9802	$\hat{y} = \frac{(872.60*284.41 + 4.52x^{91.49})}{(284.41 + x^{91.49})}$	0.9998
Mg (mg)	$\hat{y} = \frac{1}{(4.097559+(-1.037158)*LN(x)+0.009958*(LN(x))^3)}$	0.9736	$\hat{y} = \frac{32.424395}{(1+18833.105092*2.72^{(-0.040765*x)})}$	0.9859
		RDM		
N (mg)	$\hat{y} = \frac{X}{(6.005431+0.028427x+(-0.000091)x^2)}$	0.9811	$\hat{y} = \frac{50.228477}{(1+36366.105952*2.72^{(-0.043229x)})}$	0.9949
P (mg)	$\hat{y} = \frac{((-4012098.004184)+70752.054609x)}{(1+51452.846468x+(-126.561155)x^2)}$	0.9991	$\hat{y} = \frac{0.193023+(5.390523(x^{7.562348})}{(278.853418\wedge7.562348+x^{7.562348})}$	0.9998
K (mg)	$\hat{y} = \frac{((-321494706.428064)+5684111.730361x)}{(1+269772.864676x+(-492.221398)x^2)}$	0.9840	$\hat{y} = \frac{2.889935+(41.918772(x^{6.486125})}{(279.098673\wedge6.486125+x^{6.486125})}$	0.9978
Ca (mg)	$\hat{y} = \frac{((-8339505.659295)+171008.018063x)}{(1+44912.770903x+(-114.343913)x^2)}$	0.9999	$\hat{y} = \frac{0.608807+20.830235x^{8.260137}}{(283.013934\wedge8.260137+x^{8.260137})}$	0.9999
Mg (mg)	$\hat{y} = \frac{1}{(3.291301+(-0.548957)*LN(x))}$	0.9696	$\hat{y} = \frac{0.432969+4.815087*x^{13.786367}}{(239.049988^{13.786367}+x^{13.786367})}$	0.9989
		TDM		
N (mg)	$0.030554*x^{1.738667}$	0.9524	$\hat{y} = \frac{650.83333184}{1+5218.269476*2.72^{(-0.035527*x)}}$	0.9888
P (mg)	$\hat{y} = \frac{1}{(4.378371+(-1.114684)*LN(x)+0.010781(LN(x))^3)}$	0.9900	$\hat{y} = \frac{1.983355+65.542241x^{5.482571}}{(292.953721\wedge5.482571+x^{5.482571})}$	0.9992
K (mg)	$0.156826*x^{1.341054}$	0.9589	$\hat{y} = \frac{373.832754}{(1+6051.609794*2.72^{(-0.037006x)})}$	0.9779
Ca (mg)	$\hat{y} = 10.597861* EXP \frac{(-x)}{(-106.71837)}$	0.9926	$\hat{y} = \frac{244.093975}{(1+5446.723336*2.72^{(-0.033357x)})}$	0.9932
Mg (mg)	$\hat{y} = \frac{(-42.330751)x}{((-488.489619)+x)}$	0.9887	$\hat{y} = \frac{69.658654}{(1+1352.505009*2.72^{(-0.028749x)})}$	0.9863