PATRÍCIA CARDOSO MATIAS

PRODUCTION AND AGRONOMIC EVALUATION OF BIOGENIC PHOSPHATE AND POTASSIUM FERTILIZERS

Thesis submitted to the Soil Science and Plant Nutrition Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Doctor Scientiae*.

Adviser: Edson Marcio Mattiello

Co-advisers: Jorge Luis Badel Pacheco
Víctor Hugo Alvarez V.
(*in memoriam*)

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APPROVED: April 28, 2021.

Assent:

Pcmatias

Patrícia Cardoso Matias
Author

Edson Marcio Mattiello
Adviser

To my parents, Sandra and Ronaldo;
To my grandparents, Maria do Carmo (*in memoriam*),
José Ladeira (*in memoriam*), Vicente Matias (*in memoriam*)
and Maria da Silva. To my aunt Eny (*in memoriam*).

I dedicate.

"We are what we repeatedly do.

Excellence, then, is not an act, but a habit."

Aristotle

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BIOGRAPHY

PATRÍCIA CARDOSO MATIAS, daughter of Vicente Ronaldo Matias and Sandra Cardoso da Silva Matias, was born in Viçosa – MG on August 5th, 1992. In March 2010, she joined the Universidade Federal de Viçosa (UFV), Viçosa – MG, to study Agronomy, graduating on January, 2015. In March 2015, she began her Master's studies in the Graduate Program in Soil Science and Plant Nutrition at UFV, under the advisory of Prof. Edson Marcio Mattiello, having defended her dissertation on February 14th, 2017. In March 2017, she joined the PhD Program in Soil Science and Plant Nutrition at UFV, and defended her thesis under the guidance of Prof. Edson Marcio Mattiello and co-advisory of Prof. Jorge Luis Badel Pacheco and Prof. Víctor Hugo Alvarez V. (*in memoriam*).

ABSTRACT

MATIAS, Patrícia Cardoso, D.Sc., Universidade Federal de Viçosa, April, 2021. **Production and agronomic evaluation of biogenic phosphate and potassium fertilizers**. Adviser: Edson Marcio Mattiello. Co-advisers: Jorge Luis Badel Pacheco and Víctor Hugo Alvarez V. (*in memoriam*).

Solubilization of rocks using sulfuric acid produced by oxidizing bacteria from elemental S is an attractive alternative for the production of phosphate and potassium fertilizers. This process can be used with low and high solubility rocks, as it is simple, efficient, of low cost and environmentally friendly. The present study aimed to evaluate the production of biogenic acid by *Acidithiobacillus thiooxidans*. Chemical solubilization of phosphate rocks from Morocco (M-PR) and Serra Salitre (SS-PR) phosphate rocks, and of K silicate minerals verdete (Ve) and phonolite (Fo) rocks using commercial or biogenic sulfuric acid was evaluated and compared with the rocks *in natura*. Chemical, mineralogical and morphological changes were evaluated in the biogenic fertilizers produced using biogenic acid. Agronomic performance of the Morocco and Serra Salitre biogenic P-fertilizers, and verdete and phonolite biogenic K-fertilizers was also assessed in a successive corn-soybean-millet cultivation. The acidification of the rocks with sulfuric acid increased the content of extractable P and K forms in water and in 2% citric acid as well as of P in ammonium acid citrate pH 3, in relation to their *in natura* forms. In addition, biogenic sulfuric acid caused equal or higher solubilization P or K than commercial acid when used at a 1:5 rock:acid ratio. A reduction in intensity and/or disappearance of some phases of P or K in the X-ray diffraction, infrared and Raman spectra of biogenic fertilizers was observed with respect to those of rocks without acidulation. Dry matter yield mass as well as absorbed and accumulated P or K during corn-soybean-millet cultivation varied according to the fertilizer source. In fact, biogenic fertilizers provided more P or K to the crops than the rocks *in natura*; they also provided S in soluble and elemental forms. Within each P or K source, acidulation of SS-PR and Ve resulted in notorious increases in the variables under study.

Keywords: Biogenic acid. Fertilizers. Phosphate rock. Potassium silicates.

RESUMO

MATIAS, Patrícia Cardoso, D.Sc., Universidade Federal de Viçosa, abril de 2021. **Produção e avaliação agronômica dos fertilizantes fosfatados e potássicos biogênicos**. Orientador: Edson Marcio Mattiello. Coorientadores: Jorge Luis Badel Pacheco e Víctor Hugo Alvarez V. (*in memoriam*).

A solubilização de rochas, utilizando ácido sulfúrico produzido por bactérias oxidantes do S, se apresenta como uma alternativa atraente para a produção de fertilizantes fosfatados e potássicos. Esse processo é aplicável para rochas de baixa e alta solubilidade, por ser simples, eficiente, de baixo custo e ambientalmente correto. O presente trabalho teve como objetivo avaliar a produção de ácido biogênico por *Acidithiobacillus thiooxidans*. A solubilização química de fosfatos naturais de Marrocos (M-PR) e Serra Salitre (SS-PR), e de minerais silicatados de K a partir das rochas verdete (Ve) e fonolito (Fo), usando o ácido sulfúrico biogênico ou comercial foi avaliada e comparada com as rochas *in natura*. As alterações químicas, mineralógicas e morfológicas foram avaliadas nos fertilizantes biogênicos, produzidos usando ácido biogênico. A performance agronômica dos fertilizantes fosfatados biogênicos de Marrocos e Serra Salitre, e fertilizantes potássicos biogênicos do verdete e fonolito em cultivo sucessivo milho-soja-milheto também foi avaliada. A acidificação das rochas com ácido sulfúrico incrementou o teor das formas extraídas de P e K em água e em ácido cítrico 2%, e também do P em citrato ácido de amônio pH 3, em relação às suas formas *in natura*. Além disso, o ácido sulfúrico biogênico solubilizou igual ou mais P ou K que o ácido comercial quando utilizado na proporção rocha:ácido 1:5. Observou-se uma redução na intensidade e, ou, desaparecimento de algumas fases de P ou K nos espectros da difração de raio X, infravermelho e Raman dos fertilizantes biogênicos em relação aos das rochas sem acidulação. A massa de matéria seca e o P ou K absorvido e acumulado pelas plantas durante o cultivo sucessivo milho-soja-milheto foi variável com as fontes de fertilizante. De fato, os fertilizantes biogênicos forneceram mais P ou K para as culturas que as rochas *in natura*; além de fornecer S nas formas solúvel e elementar. Dentro de cada fonte de P ou K, a acidulação de SS-PR e Ve resultou em um incremento notório nas variáveis em estudo.

Palavras-chave: Ácido biogênico. Fertilizantes. Fosfatos naturais. Silicatos de potássio.

SUMMARY

GENERAL INTRODUCTION

Phosphorus and potassium are essential elements for all organisms and thus, play important roles in crop production (Calderini, 1995). Potassium participates in the activation of many enzymes, osmoregulation processes as well as in respiratory and photosynthetic metabolism. Furthermore the P is a structural component of nucleic acids and is implicated in energy and carbohydrate transfer in leaf cells (Hawkesford et al., 2012). However, there is a need for fertilization of crops with high-quantity P and K sources due to the use of low-fertility and acidic soils in the Brazilian agriculture. Brazil stands out on the world stage as the fourth largest consumer of fertilizers, accounting for about 8% of global consumption. However, in 2020, it is estimated that the country imported more than 80% of fertilizers to meet its domestic demand, with national production accounting for only 20% (ANDA, 2021). The high dependence on external sources reinforces the need to conduct research aimed to seek alternative sources and routes for the production of potassium and phosphate fertilizers in Brazil.

Among the unconventional and widely occurring materials in the state of Minas Gerais (MG), verdete and phonolite stand out as sources of K. As for P sources, significant reserves of natural phosphates occur in Brazil, but they have low P contents and exhibit poor agronomic efficiencies. Verdete rock occurs in extensive areas in Serra da Saudade, Central Minas Gerais. It is composed of micas such as glauconite, illite and muscovite as well as feldspars, as mineral sources of K (Toledo Piza et al., 2011), with K-K_2O content reaching up to 9% (Santos et al., 2015). Phonolite, on the other hand, is a silicate rock that occurs in the region of Poços de Caldas-MG, which is primarily rich in potassium feldspars as sources of K, but also has traces of micas containing around 8% K-K_2O (Fernandes et al., 2010). Apatite deposits are also significantly abundant in Minas Gerais, which are currently used for the production of soluble phosphate fertilizers, thermophosphates or are not commercially used due to their low industrial and agronomic qualities.

The low solubility in water or weak acids of potassium silicate sources, such as verdete and phonolite; and sources of, such apatites (igneous or metamorphic) that occur in the Brazilian territory confers to these materials a low efficiency as fertilizers (Eichler and Lopes, 1983; Santos et al., 2017), limiting their use *in natura* (Leite, 1985). On the other hand, the use of thermal or chemical processes to solubilize these potassium silicates has been shown to be efficient (Santos et al., 2017, 2015; Varadachari, 1992). However, the need for high temperatures or high amounts of acidic/basic solutions in these processes can economically limit the production of fertilizers from these sources.

Acid leaching using the bacterium *Acidithiobacillus thiooxidans* is an important biotechnological process for the recovery and concentration of elements such as copper, zinc, nickel, uranium and gold, from ores with low concentrations of these elements (Fowler and Crundwell, 1999). The bacterium is a chemilithoautotroph, uses CO_2 as a C source and obtains energy through the oxidation of reduced forms of Fe or S, which generates acidity as a product of the catalyzed reactions (Masau et al., 2001). This acidity can be exploited for the solubilization of potassium silicate rocks and natural phosphates with potential for commercial use (Basak and Biswas, 2009).

Recent studies carried out at the Department of Soils-UFV have shown the solubilization potential of verdete rock using *A. ferrooxidans* (Santos, 2012) and *A. thiooxidans* (Matias et al., 2019) using elemental sulfur as an energy source for bacterial metabolism. However, further studies are needed to assess the solubilization of rocks that have elements of agricultural interest using the biogenic acid produced by *A. thiooxidans* and the agronomic performance of fertilizers produced using the biogenic acid.

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Paper 1

DISSOLUTION OF K-SILICATE ROCKS WITH A BIOGENIC ACID: CHARACTERIZATION AND AGRONOMIC PERFORMANCE OF THE END-PRODUCTS

Abstract

Solubilization of plant nutrients from silicate rocks, using acids produced by microorganisms has emerged as a both environmentally friendly and economically viable process for fertilizer production. In this work, we evaluated the production of biogenic acid by *Acidithiobacillus thiooxidans* and the chemical solubilization of K from verdete (Ve) and phonolite (Fo) rocks were contrasted each other using biogenic and commercial sulfuric acid. Furthermore, after the acid solubilization, the rocks were characterized. Agronomic performances of biogenic K-fertilizers were evaluated in a sequential corn-soybean-millet cropping system. Acid type and rock:acid ratio influenced the solubilization of K species from the rocks. Overall, biogenic acid led to a higher solubilization of K species in water and in citric acid 2% than commercial sulfuric acid. The amount of solubilized K was higher under a 1:5 (rock:acid) ratio. Total dry matter mass and total K uptake by the crops increased under K rates and varied between K sources. Ve or Fo biogenic K-fertilizers were more effective than they are *in natura* forms (rocks), providing higher amount of available K to the crops. However, the acidulation effect was more notorious for verdete rock, significantly increasing the value of its acidified product as a fertilizer. Thus, our data demonstrate that biogenic acid produced by *A. thiooxidans* using elemental sulfur is a promising alternative to leach out K from silicate rocks.

Key-words: solubilization, potassium, verdete, phonolite, *Acidithiobacillus thiooxidans*

1. Introduction

Potassium is an essential element, required in large amounts for plant nutrition. It plays a pivotal role in diverse physiological process, such as activation of cellular enzymes, synthesis of proteins, and resistance to abiotic and biotic stresses (Amtmann and Armengaud, 2007; Epstein and Bloom, 2005; Hastings and Gutknecht, 1978). In general, highly weathered soils, which are poorly fertile and acidic, have a low K availability (Bernardi et al., 2002). Thus, this nutrient must be supplied through fertilization to ensure or maintain an adequate supply of K available for crops. Potassium chloride, extracted from deep deposits of soluble salts, is the main K source used as fertilizer worldwide (Trivedi et al., 2017; Zörb et al., 2014).

The deposits of mined salts used to produce K fertilizers, especially sylvinite, $KCl + NaCl$, and carnallite, $KMgCl_3.6(H_2O)$, (Ciceri et al., 2015) are not globally distributed; 90% of K is produced in Canada, Russia, Belarus and Germany. Brazil is one of the major importers of K-fertilizers due to the limited national reserves occurrence of soluble K minerals; imported K-fertilizers accounts for about 96% of the K domestic demand (FAO, 2020). Thus, it is highly strategical for this country to develop technologies to produce K-fertilizers from alternative potassium sources, such as silicate rocks.

The search of alternative K sources from silicate minerals began a long time ago; the first work on potassium-rich silicate rocks as a source of potassium was reported in 1847 by Tilghmann (Ciceri et al., 2015). However, the high cost of rock treatment, using acidic solutions or high temperatures, makes the processes economically unfeasible (Ciceri et al., 2015). Potassium species, which occur in various types of silicate minerals, present in igneous, metamorphic or sedimentary rocks, could potentially be used as K-fertilizers (Rosa-Magri et al., 2012). Nevertheless, the long time required to release potassium from these minerals, due to their both low solubility or reactivity, and consequently, to provide the available K for plant nutrition, makes the effective use of silicate rocks as fertilizers in agriculture to be questioned (Harley and Gilkes, 2000; Hinsinger et al., 1995).

Biotechnological processes offer novel approaches for the use of alternative K sources, such as micas or K-feldspars minerals and also represents an unconventional alternative for K fertilizers production. Bioleaching has been applied to recover metals from low-grade ores and to concentrate materials that cannot be processed by conventional and economically feasible methods (Bosecker, 1997). This occurs because bioleaching is more cost-efficient, simpler and more environmentally friendly than conventional methods of acidification (Asghari et al., 2013; Mishra et al., 2008; Santhiya and Ting, 2005).

Acidithiobacillus thiooxidans, an obligately acidophilic and aerobic bacteria, utilizes reduced sulfur compounds to support its autotrophic growth (Kelly and Wood, 2000) and is the most important microorganism used worldwide in bioleaching processes (Asghari et al., 2013; Deshpande et al., 2018; Yin et al., 2014). This bacterium obtains energy from the oxidation of reduced S-compounds, such as elemental S and sulfide species. Sulfate and sulfuric acid formed as by-products of these reactions create an acidic environment suitable for rocks bioleaching (Bhatti and Yawar, 2010; Deshpande et al., 2018).

The goal of the present study was to evaluate the production of a biogenic acid produced by *A. thiooxidans* and its effect on K-silicate rocks solubilization. In addition, the agronomic performance of K-fertilizers, end-products of the solubilizing, in a successive cropping system was investigated.

2. Materials and Methods

2.1. Bacterial growth and production of biogenic acid

The *A. thiooxidans* isolate FG-01 was first obtained by Garcia Jr. (1991) from an acid effluent produced by a uranium mine in Brazil. In this study, we used an isolate descendant from the initial one, which is deposited at the Instituto de Química of Universidade Estadual Paulista Júlio de Mesquita Filho under the care of Dra. Denise Bevilaqua.

The biogenic acid was produced by growing the *A. thiooxidans* isolate in 1000 mL Erlenmeyer flasks containing 500 mL of modified 9 K medium (Silverman and Lundgren, 1959), adding 5.0 g of powder elemental sulfur-S^0 (CAS 7704-34-9) and 5% (v/v) bacterial inoculum. Due to its high consumption, 1 g of S^0 was also added at 18 and 27 days after inoculation (dai). The Erlenmeyer flasks were maintained at 30 °C in an orbital shaker at 150 rpm during 30 d. Samples of the culture medium were collected every 3 days and their pH and hydrogen ion concentration (acid–base titration) determined. The free SO_4^{2-} concentration was determined by turbidimetric analysis using the 4500-SO_4^{2-}-E method (Clesceri et al., 1998).

2.2. Solubilization of potassium rocks with commercial or biogenic acid

Representative samples of verdete (Ve) and phonolite (Fo) rocks were used in this trial. Prior to conducting the chemical analysis and the trial, rock samples were ground and passed through a 75 µm sieve (200 mesh). The total K content was determined as described by the Brazilian Ministry of Agriculture, Livestock, and Food Supply, Normative Instruction Nº 28 (Brasil, 2007).

In order to evaluate the acids effect on rock dissolution, two rocks (Ve and Fo) were treated with two types of acids (commercial sulfuric acid with analytical purity and a biogenic acid produced by *A. thiooxidans*; both at 0.9 mol L^{-1} H^+) and two rock:acid ratios (1:2 and 1:5 w/v). This trial was performed in Becker flasks containing the rock samples and acids at the ratios aforementioned. The mixture was homogenized and kept at rest at room temperature for 7 d. After this, the acidulated rocks were dried in a beating stove at 50 °C.

The solubility of these acidulated rocks and their *in natura* forms were assessed by determining K soluble contents in water (water-K) and citric acid 2% (CA-K) (Brasil, 2007). The K concentration in the extracts was measured by flame emission spectrophotometry.

2.3. Production of biogenic K-fertilizers

The production of biogenic K-fertilizers was carried out in two steps. Firstly, the biogenic acid, which is the resulting bacteria's culture medium, was produced by *A. thiooxidans*, and then, this acid was used to solubilize the silicate rocks.

The biogenic K-fertilizers were produced from Ve and Fo rocks, in their as *in natura* forms, using the biogenic acid at a 1:5 (w/v) rock:acid ratio, based on preliminary results from 2.2 trial. Appropriate proportions of silicate rocks and biogenic acid (pH 0.66; 1.58 mol L^{-1} H^+) to obtain the desired ratio were added into a glass jar. The mixture was left to settle at room temperature for 7 d shaking twice a day. Then, the biogenic K-fertilizers as powder form from verdete (Ve-BKF) and phonolite (Fo-BKF) were oven-dried at 100 °C for 24 h, ground and homogenized. The total concentrations of K, Si, Fe, S, Ca, Al, Mn, Zn and Cu present in the *in natura* and biogenic K-fertilizers were measured by Total Reflection X-ray Fluorescence (TXRF). Extractable contents of K in water and in citric acid 2% were measured as described by the Brazilian Ministry of Agriculture, Livestock, and Food Supply, Normative Instruction No. 28 (Brasil, 2007). Additionally, the rocks (*in natura* and biogenic K-fertilizers) were characterized by X-ray diffraction (XRD), attenuated total reflectance Fourier transform infrared spectroscopy (FTIR-ATR), Raman spectroscopy, and scanning electronic microscopy (SEM) coupled to energy-dispersive spectrometry (EDS).

The TXRF analyses were performed in a Shimadzu EDX-720; and the XRD analyses, in a Shimadzu XRD-6000 diffractometer, using a graphite crystal monochromator to select CoKα radiation ($\lambda = 1.7889$ Å) at a rate of 1.2° 2θ min^{-1} and a 2θ range between 5° to 80°. Powder mounts were prepared by packing ground (< 75 µm) samples into Al holders. The FTIR-ATR spectra were recorded with a Perkin Elmer, model Spectrum 1000, modulo ATR, in the range from 400 to 4000 cm^{-1}, at 4 cm^{-1} resolution and 32 scans. Raman spectra were obtained using a Raman Renishaw spectrometer. The samples were measured with 514 nm laser line, 20 accumulations, exposure time of 10 s and 20 mW of power. For SEM and EDS analyses, a JEOL model JSM-6010-LA microscope integrated with an EDS probe operating at 6 kV was used to obtain secondary electron images of the samples, with magnification levels at 500x. An EDS operating at 10 kV was used to obtain the elementary chemical composition of verdete and phonolite rocks.

2.4. Effect of biogenic K-fertilizers on crop development in the greenhouse

A sequential corn-soybean-millet cropping system was performed to evaluate the agronomic performance of the K sources at short and medium terms. Seeds of simple hybrid corn BM 709 PRO 2, soybean BMX BÔNUS IPRO and millet BRS 1502 were used.

A K-deficient soil was collected in the municipality of Viçosa, state of Minas Gerais, Brazil, from the 10–40 cm layer, air-dried and sieved to obtain < 2 mm particles prior to conduct chemical characterization and experiments in the greenhouse. The soil is classified as a

Latossolo Vermelho-Amarelo, LVA (Oxisol), according to the Brazilian Soil Classification system (Santos et al., 2018). Samples of 2 dm^3 of the Oxisol were placed in pots inside plastic bags. Lime ($CaCO_3$ and $MgCO_3$ at a 4:1 Ca:Mg ratio) was applied aiming to increase the soil base saturation to 60% supplying Ca and Mg, and to neutralize Al^{3+} species. The soil was wetted to 80% of field capacity and incubated for 30 d at room temperature. Subsequently, the soil of each pot was air-dried and homogenized.

After incubation with lime, the soil presented a pH (H_2O) of 5.9 (at a ratio of 1:2.5 v/v); available P and K (Mehlich-1 extractor) were 0.1 and 7.0 mg dm^{-3}, respectively; exchangeable Ca^{2+} and Mg^{2+} (KCl 1.0 mol L^{-1} extractor) of 2.92 and 0.62 $cmol_c$ dm^{-3}, respectively; potential acidity (H+Al) of 3.9 $cmol_c$ dm^{-3} (calcium acetate 0.5 mol L^{-1} pH 7); soil organic C of 16 g kg^{-1} (Walkley and Black, 1934); remaining P of 8.5 mg L^{-1} (Alvarez V. et al., 2000) and 750 g kg^{-1} of clay (Claessen, 1997).

The experiment was conducted in a factorial scheme (5×4) +1, including five fertilizers: potassium chloride (KCl), verdete rock (Ve), phonolite rock (Fo), verdete (Ve-BKF) and phonolite (Fo-BKF) biogenic K-fertilizers; four K rates (25, 50, 100 and 200 mg dm^{-3}); and a control treatment without K application. The experiment was carried out in a randomized block design with four replications. The biogenic K-fertilizers were produced as mentioned in item 2.3 and their *in natura* forms, both the powder forms, together with 300 mg dm^{-3} P (MAP), were thoroughly mixed with soil volumes to obtain the desired doses in the pots.

For the first cropping, six seeds of corn were sown in each pot at a depth of 2 cm. At five days after seedlings emergence, they were thinned to obtain three uniform plants per pot. Total rate of nutrients applied via nutrient solution, in installments at 7, 14 and 21 days after planting were: 200 mg dm^{-3} N (urea), 50 mg dm^{-3} S (ammonium sulfate), 3 mg dm^{-3} Zn (zinc sulfate), 1.0 mg dm^{-3} B (boric acid), 1.3 mg dm^{-3} Cu (copper sulfate), 7.2 mg dm^{-3} Mn (manganese sulfate), 3.12 mg dm^{-3} Fe (ferric chloride) and 0.15 mg dm^{-3} Mo (ammonium molybdate). At 32 d after planting, plants were harvested by cutting the stems at the soil surface.

Two days after corn harvesting, six seeds of soybean were sown at 2 cm depth in the undisturbed soil. After seedling emergence, the three most homogeneous seedlings were left in each pot and were inoculated with commercial *Bradyrhizobium* in order to fix and provide N to the plants. Micronutrients were applied as described above for corn cropping whereas for macronutrients only 50 mg dm^{-3} S (ammonium sulfate) was applied at 10, 20 and 30 d after planting. After 40 d after planting, the soybean plants were harvested by cutting the stems at the soil surface.

Then, twenty seeds of millet were sown at 1 cm depth and after their emergence, fifteen plants were kept in each pot. At 21 d after sowing, 50 mg dm^3 N (urea) was applied. After 30 d of sowing, millet plants were harvested at the soil surface.

Water availability was controlled daily by maintaining the soil near 80% of field capacity throughout the experimental period. Plant tissues were oven-dried at 70 °C for 72 h, weighed and milled. Then, they were digested in an open-vessel-digestion system, using a 3:1 nitric-perchloric solution (v/v) (Miller, 1998). The K and S concentrations in the plant extracts were measured by inductively coupled plasma atomic emission spectroscopy (ICP-AES; Perkin Elmer 8300 DV, US).

Soil samples were collected in all soil volume between the cultivation of soybean and millet and after millet cultivation at the end of the experiment. For that, the whole volume of soil from the pot was collected and passed through a sieve with a mesh of 2.0 mm for analysis of available K by Mehlich-3 (Mehlich, 1984). The K concentration was determined by flame photometry.

2.5. Data analysis

Statistical analysis was carried out by two-way analysis of variance (ANOVA). The 2.2 item we analyzed as a randomized block design in double factorial scheme (3 × 2) where the differences among means of the treatments (acidification and rock) were analyzed within each rock:acid ratio (1:2 and 1:5 w/v), and the means were compared by Tukey's test ($p = 0.05$). For 2.4 item differences among K rate treatments for each K fertilizers were determined by regression analysis. All analysis were carried out using the R software version 3.6.3 (R Core Team, 2020).

3. Results

3.1. Production of biogenic acid by Acidithiobacillus thiooxidans

The *A. thiooxidans* potential for biogenic acid production was assessed by monitoring the SO_4^{2-}-S and H^+ concentrations and the pH in the bacterial culture. A linear increase in SO_4^{2-}-S concentration was observed during bacterial growth up to 30 days of incubation (dai), when the assay was terminated.

The initial SO_4^{2-}-S concentration of 5 154 mg L^{-1}, increased to 14 500 mg L^{-1} at 6 dai, and reached 84 236 mg L^{-1} after 30 dai (Fig. 1a). The concentration of H^+ was initially 0.0002 mol L^{-1} and increased at a constant rate of 0.04 mol L^{-1} per day, reaching 1.2 mol L^{-1} at 30 dai

(Fig. 1b). Moreover, the pH decreased at an exponential rate from 1.61 to 0.4 at 30 dai (Fig. 1c).

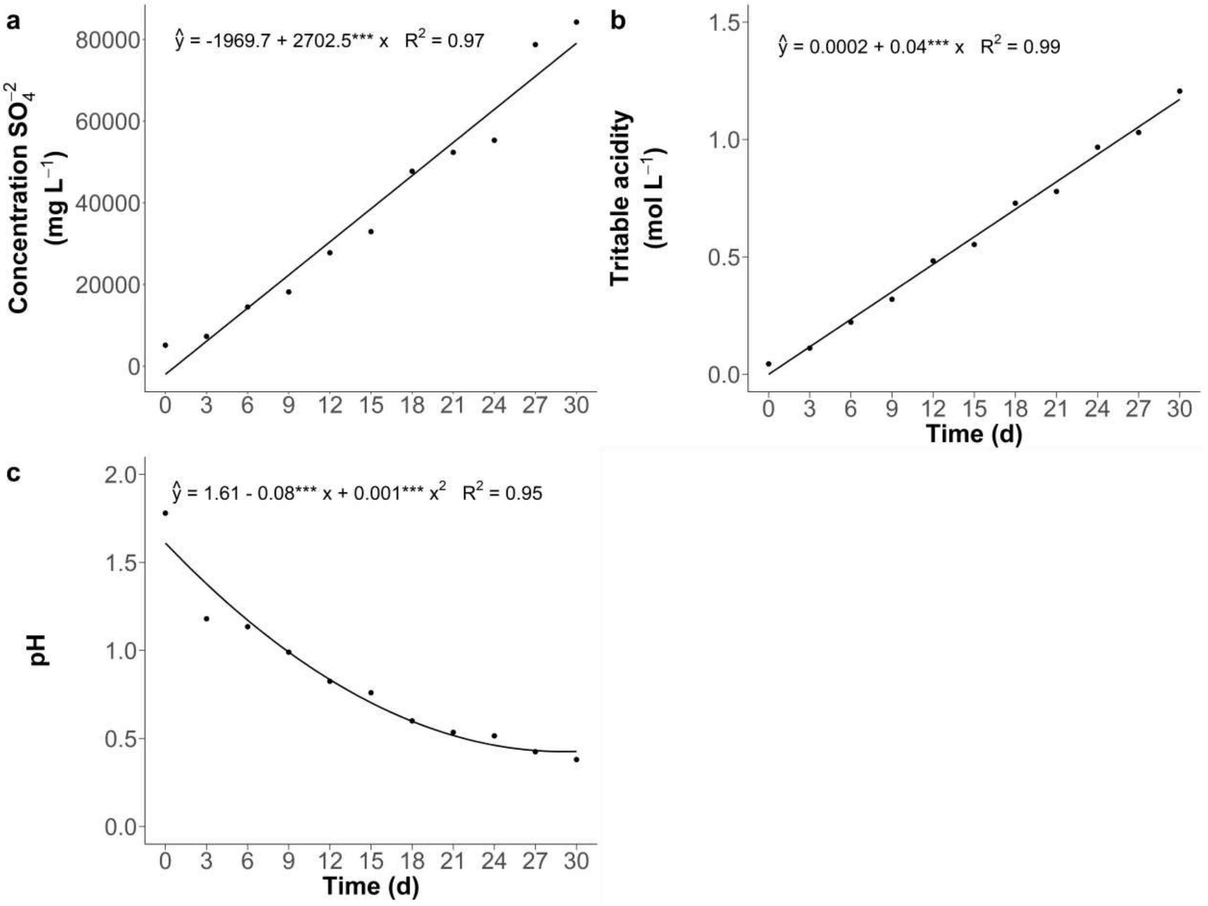

Fig. 1. Effect of *Acidithiobacillus thiooxidan* on sulfate production over time (a), titratable acidity (b), and pH (c) of 9K modified culture medium. ***, All regression coefficients are significant (t-*test*, $p < 0.01$).

3.2. Treatment of silicate rocks with commercial and biogenic acids

In order to define the best rocks:acid ratio to be used in the experiments (1:2 or 1:5), a previous study was performed (Fig. 2). K-rocks proved to be poorly soluble in water, with extractable water-K lower than 0.2% (Figs. 2a and b). In citric acid, the soluble K contents were contrasting between rocks, highlighting the Fo as a more soluble rock, about 12-fold higher than Ve (Figs. 2 c and d).

Treatment of Ve and Fo rocks with sulfuric or biogenic acid increased the fertilizer value of the end-products as indicated by the increasing extractable amounts of water-K, and CA-K (Fig. 2). On average, the water-K of Fo and Ve treated with biogenic acid at a 1:5 w/v rock:acid ratio was 50- and 52-fold higher than those of the rocks *in natura*, respectively (Fig. 2b).

Interestingly, overall biogenic acid was more effective than sulfuric acid in leaching K into water or citric acid from both rocks. For Fo, using the 1:2 rock:acid ratio, the treatment with biogenic acid increased 1.5-fold water-K, and 1.3-fold CA-K in relation of the commercial acid (Figs. 2a and c). For Ve, the effect of the biogenic acid was 1.2-fold higher than commercial, in either water or citric acid, in the 1:5 rock:acid ratio.

Considering these results of rock acidifications with commercial and biogenic acids and the better performance of the latter acid observed in this study, *in natura* rocks forms treated with biogenic acid (1:5, w/v) were chosen for further production and characterization of K-fertilizers, and to be used in greenhouse experiments.

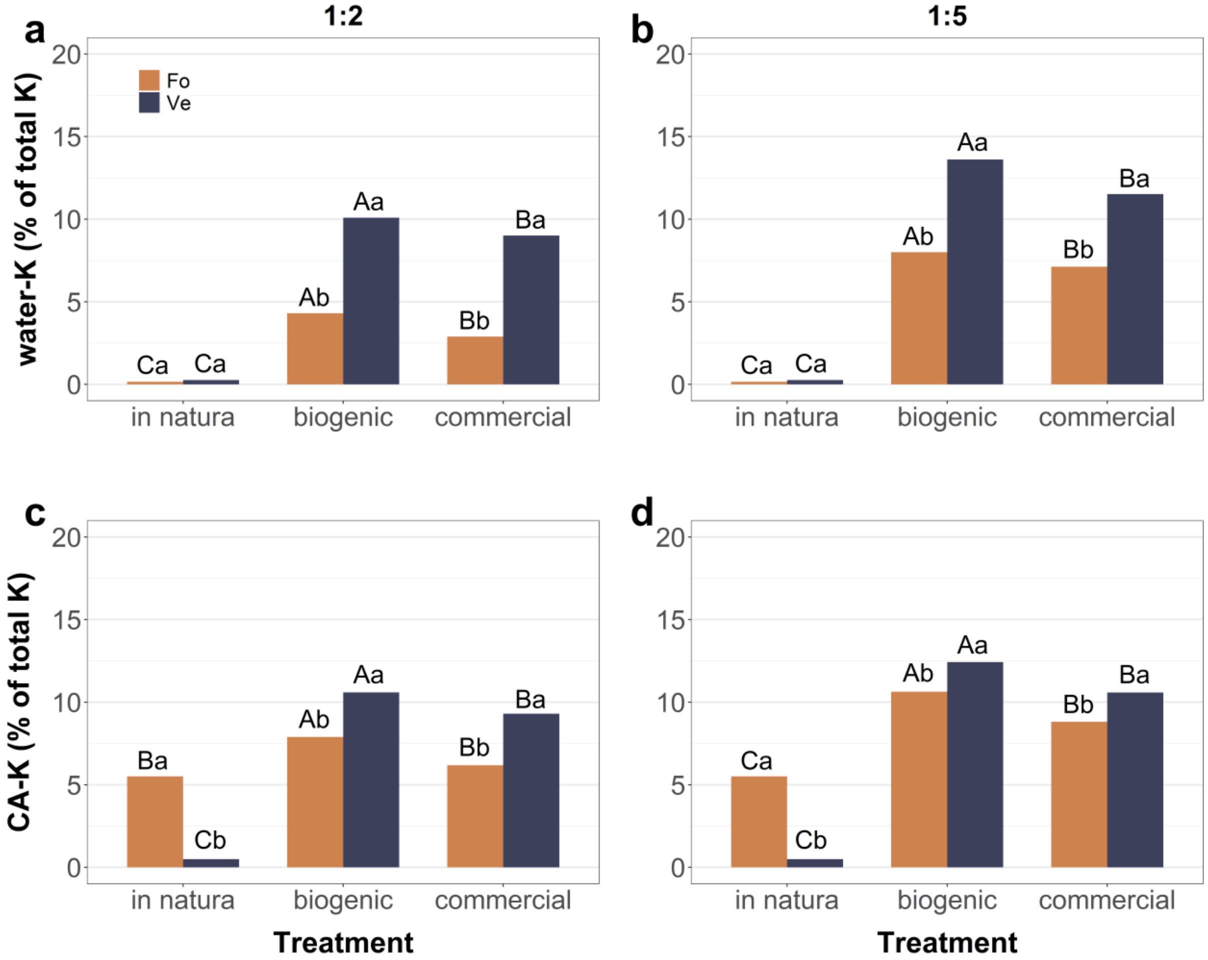

Fig. 2. Extractable K in water (water-K) and 2% citric acid (CA-K) in verdete (Ve) and phonolite (Fo) rocks, *in natura* forms and acidulated with biogenic acid or commercial sulfur acid, using rock:acid ratios of 1:2 and 1:5 (w/v). Different letters on bars indicate significant differences according to Tukey test ($p \leq 0.05$). Uppercase letters compare mean treatments within each rock and lowercase letters compare between rock types within the same treatment.

3.3. Chemical characterization of potassium fertilizers

The chemical characterization of Ve and Fo rocks (Ve and Fo) and acidulated products from biogenic acid-biogenic K-fertilizers (Ve-BKF and Fo-BKF), is shown in Table 1. The total K concentration determined by X-ray fluorescence spectroscopy in Ve and Fo were 9.2 and 7.0 wt%, respectively. Lower total-K concentrations in biogenic K-fertilizers is due the mass dilution. We detected high Si and Fe contents in Fo, Fo-BKF and in Ve-BKF. The rocks also contained micronutrients, such as Mn, Zn and Cu. Sulfur was also detected in Ve and Fo biogenic K-fertilizers.

The nutrients Si, Fe, S and Ca were not detected on Ve sample. After acid dissolution, the mineral structure changed and possibly the nutrients were more available, and thus, could be detected by fluorescence on Ve-BKF.

Table 1. Total concentration of chemical elements, extractable K in water (water-K) and in citric acid 2% (CA-K) in verdete (Ve) and phonolite rocks (Fo) and biogenic K fertilizers from verdete (Ve-BKF) or phonolite (Fo-BKF).

Fertilizer	**Total concentration**									**Water-K**	**CA-K**
	K	**Si**	**Fe**	**S**	**Ca**	**Al**	**Mn**	**Zn**	**Cu**		
	------------- dag kg^{-1} -------------						---- mg kg^{-1} ----			---- % of total K------	
Ve	9.2	nd	nd	nd	nd	8.0	310	95	93	0.73	0.83
Ve-BKF	5.1	17	1.4	4.9	nd	nd	193	85	69	29.38	39.33
Fo	7.0	25	1.3	nd	0.7	10.8	1355	179	58	0.18	7.40
Fo-BKF	4.1	15	0.7	3.5	0.5	3.5	809	129	46	11.91	14.8

nd: not detected. Soluble K in water and 2% citric acid were measured as described by the Brazilian Ministry of Agriculture, Livestock, and Food Supply, Normative Instruction No. 28 (Brasil, 2007).

3.4. Morphological and crystal alterations caused by rock acidulation

X-ray diffraction analysis provided evidence that potassium silicates are present in verdete and phonolite rocks (Fig. 3). Glauconite ($(K,Ca,Na)_{0.84}(Al_{47}Fe_{0.66}Mg_{0.4})(Si,Al)_4O_{10}(OH)_2$ – d = 0.100, 0.450, 0.258, 0.331, 0.239, 0.213 nm), Silicon oxide (SiO_2 – d = 0.429, 0.337, 0.229, 0.182 nm), and Orthoclase ($K(Al,Fe)Si_2O_8$ d = 0.324, 0.378, 0.300, 0.347, 0.258 nm) were identified in Ve. Treatment with biogenic acid caused a change in the rock mineral composition, with disappearance of the Glauconite and Orthoclase peaks. Likewise, Silicon sulfide (SiS_2 –d = 0.427, 0.336, 0.246, 0.182, 0.213 nm) and Silicon oxide (SiO_2 –d = 0.429, 0.337, 0.182 nm) were found in Ve-BKF.

The mineral species identified in Fo rock were: Orthoclase ($K(Al,Fe)Si_2O_8$ –d = 0.423, 0.378, 0.362, 0.347, 0.332, 0.324, 0.300, 0.258 nm), Sanidine ($KAlSi_3O_8$ –d = 0.378, 0.345, 0.299, 0.288, 0.258, 0.217, 0.332 nm), Nepheline ($K(Na,K)_3Al_4Si_4O_{16}$ – d = 0.421, 0.387, 0.329, 0.30, 0.290, 0.259, 0.251, 0.235, 0.217, 0.213, 0.180 nm), Microcline ($KAlSi_3O_8$ –d = 0.422, 0.379, 0.347, 0.329, 0.324, 0.300, 0.289, 0.277 nm) and Analcite ($NaAl(SiO_3)_2H_2O$ – d = 0.566, 0.343, 0.294, 0.270, 0.250, 0.174 nm).

Acid treatment of Fo rock resulted in partial dissolution of its mineral species. In fact, the peaks of Microcline ($KAlSi_3O_8$ –d = 0.422, 3.47, 0.329, 0.24, 0.297, 0.289, 0.260, 0.216 nm), Sanidine ($KAlSi_3O_8$– d = 0.424, 0.346, 0.322, 0.299, 0.289, 0.276, 0.260, 0.217 nm) and Orthoclase ($K(Al,Fe)Si_2O_8$– d = 0.423, 0.378, 0.347, 0.332, 0.324, 0.300 nm) remained after the acid treatment, but with minor intensity (Fig. 3).

Overall, after treating the rocks with biogenic acid, the Bragg peaks decreased in intensity and became broader, probably due to changes in the crystallinity of partially dissolved minerals (Fig. 3).

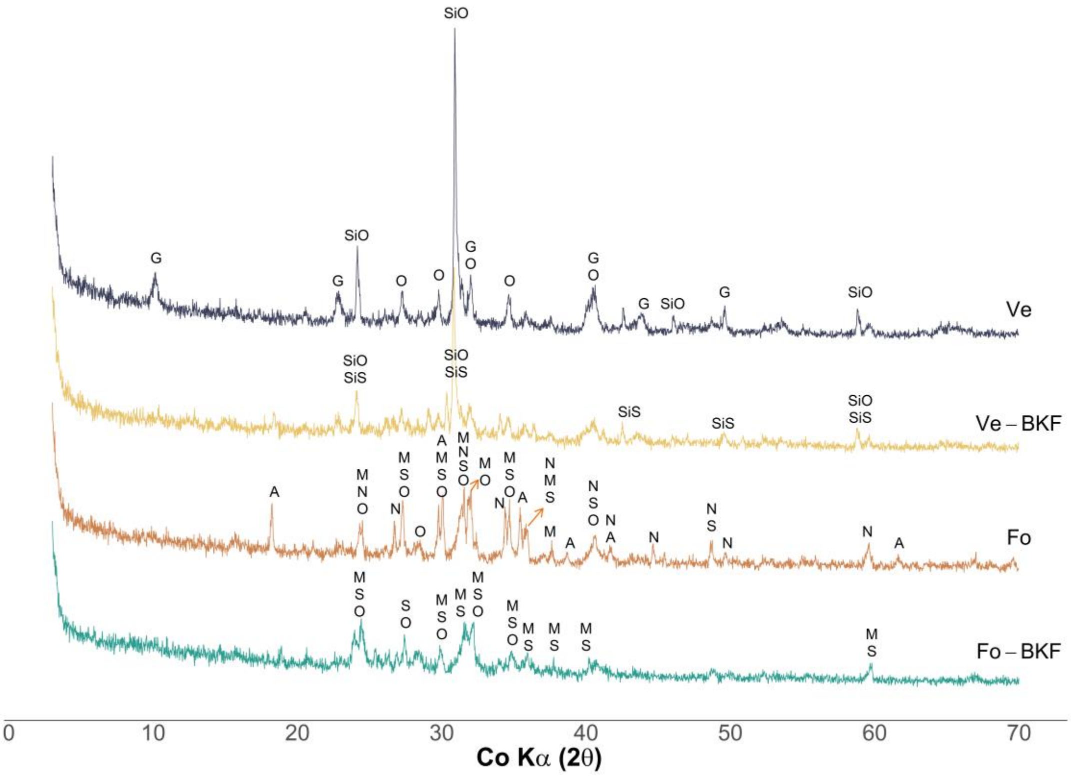

Fig. 3. X-ray diffraction patterns from verdete rock (Ve), verdete biogenic K-fertilizer (Ve-BKF), phonolite rock (Fo) and phonolite biogenic K-fertilizer (Fo-BKF). G: Glauconite; SiO_2: Silicon oxide; O: Orthoclase; SiS: Silcon Sulfide; A: Analcite; M: Microcline; N: Nepheline; S: Sanidine.

Biogenic K-fertilizers and *in natura* rocks were also analyzed by Infrared spectroscopy (Fig. 4). Ve exhibited two intense bands, one at 445,8 cm^{-1} related to Si-O bending mode (Chattoraj et al., 2018) and another at 981 cm^{-1} Si-O-Si stretching vibration mode (Odin, 1989). Furthermore, weak features at 2357 and 1993 cm^{-1} were also observed. On the other hand, Ve-

BKF showed two additional absorptions, a stretching mode at 3251 cm^{-1} and a bending mode at 1638 cm^{-1}, likely due to stretching mode of -OH and molecular H_2O (Chattoraj et al., 2018). In addition, the bands at 445.8 and 1002 cm^{-1} became broader and another band at 1098 cm^{-1} was likely present, which was assigned to SiO_2 out-of-plane vibration (Hassan and Baioumy, 2006).

For Fo, intense bands were present at 424, 534, 717.8 and 989 cm^{-1} as well as a feature at 1072 cm^{-1}. The bands at 424, 534 and 717 cm^{-1} were related to Si-O vibration and that at 989 cm^{-1} could be ascribed to Si-O-Si in plane stretching vibration (Odin, 1989). Furthermore, small features were also present at 2006 and 2352 cm^{-1} due to the presence of CO_2.

After biogenic acid treatment, the bands from Fo-BKF became broader and the band at 1072 cm^{-1} increased in intensity, likely due to SiO_2 stretching vibration. Moreover, a water absorption band at 1642 cm^{-1} and bands at 2896 and 3356 cm^{-1} related to OH stretching vibrations were also present. The small features detected in the Fo rock were also present in Fo-BKF.

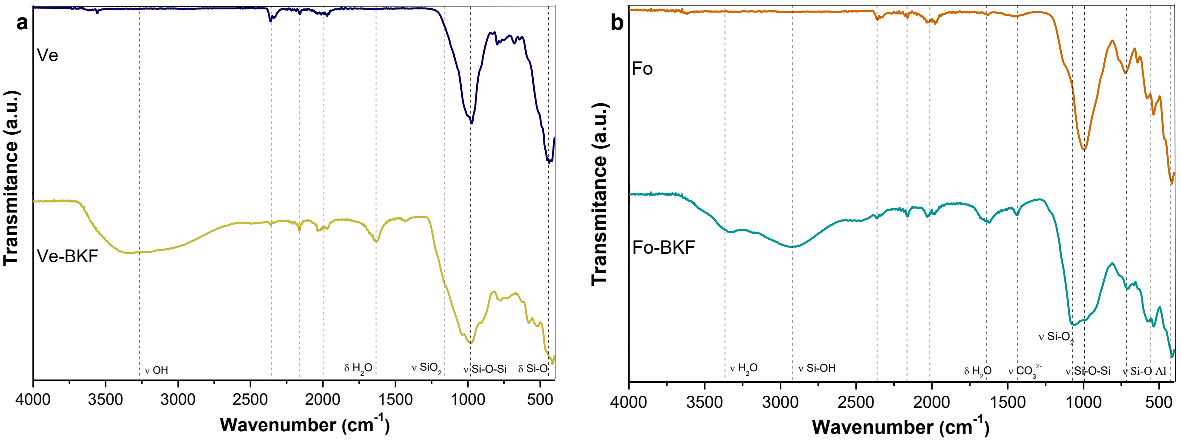

Fig. 4. Infrared spectra of verdete rock (Ve) and verdete biogenic K-fertilizer (Ve-BKF) (a); and phonolite rock (Fo) and biogenic K-fertilizer (Fo-BKF) (b).

Raman analysis (Fig. 5) of verdete showed four small bands, at 140.9 cm^{-1} assigned to Si-O stretching from orthoclase phase (Freeman et al., 2008), and at 408, 513 and 1327 cm^{-1} attributed to Si-O stretching from glauconite (Košařová et al., 2013). This bands could be also associated with FeO stretching (López and Frost, 2015). After the acid treatment, no band was found, due to the high florescence observed during the measurement.

Phonolite presented three bands, one at 146 cm^{-1} related to orthoclase phase and also at 512.7 and 969 assigned to Si-O stretching from orthoclase/sanidine and the last that could be related to orthoclase/ nepheline/sanidine (Freeman et al., 2008; McCloy et al., 2015). The Fo-

BKF on the other hand, also showed a feature at 150 cm^{-1} related to orthoclase (Freeman et al., 2008) and 3 additional peaks, at 216.7, 470 and 989 cm^{-1}, assigned to Si-O stretching from microcline (Sharma et al., 2003), and the last one related to orthoclase phase, respectively. Likely, a partial dissolution of some phases occurred, and microcline became more prominent.

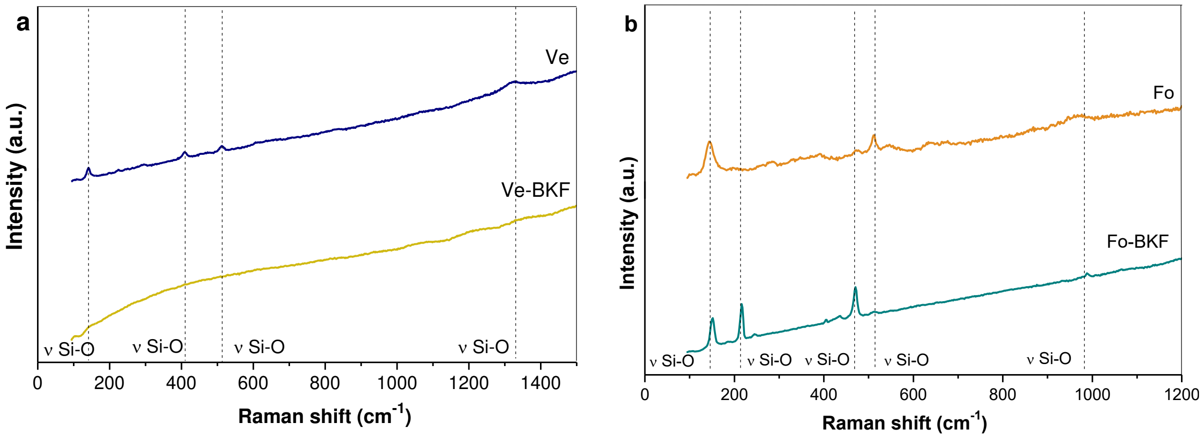

Fig. 5. Raman spectra of verdete rock (Ve), biogenic K-fertilizer (Ve-BKF) (a), phonolite rock (Fo) and biogenic K-fertilizer (Fo-BKF) (b).

The rock morphology and biogenic K-fertilizer forms of Ve and Fo were analyzed by SEM/EDS (Fig. 6). The rocks *in natura* had a more defined structure, more angular shape, with particles of different sizes and crystalline morphology (Figs. 6a and c), as was seen in XRD. After rock treatment with biogenic acid the shape of mineral constituents was modified, as revealed by the presence of more spherical particles. This sphericity was likely associated with congruent dissolution, without preference for a specific plane and with precipitation of new minerals. Furthermore, the particle became smaller (Figs. 6b and d). EDS spectra (Fig. 6 insets) revealed that before the acid treatment no S species could be detected. On the other hand, after treatment with biogenic acid, a sulfur peak could be detected.

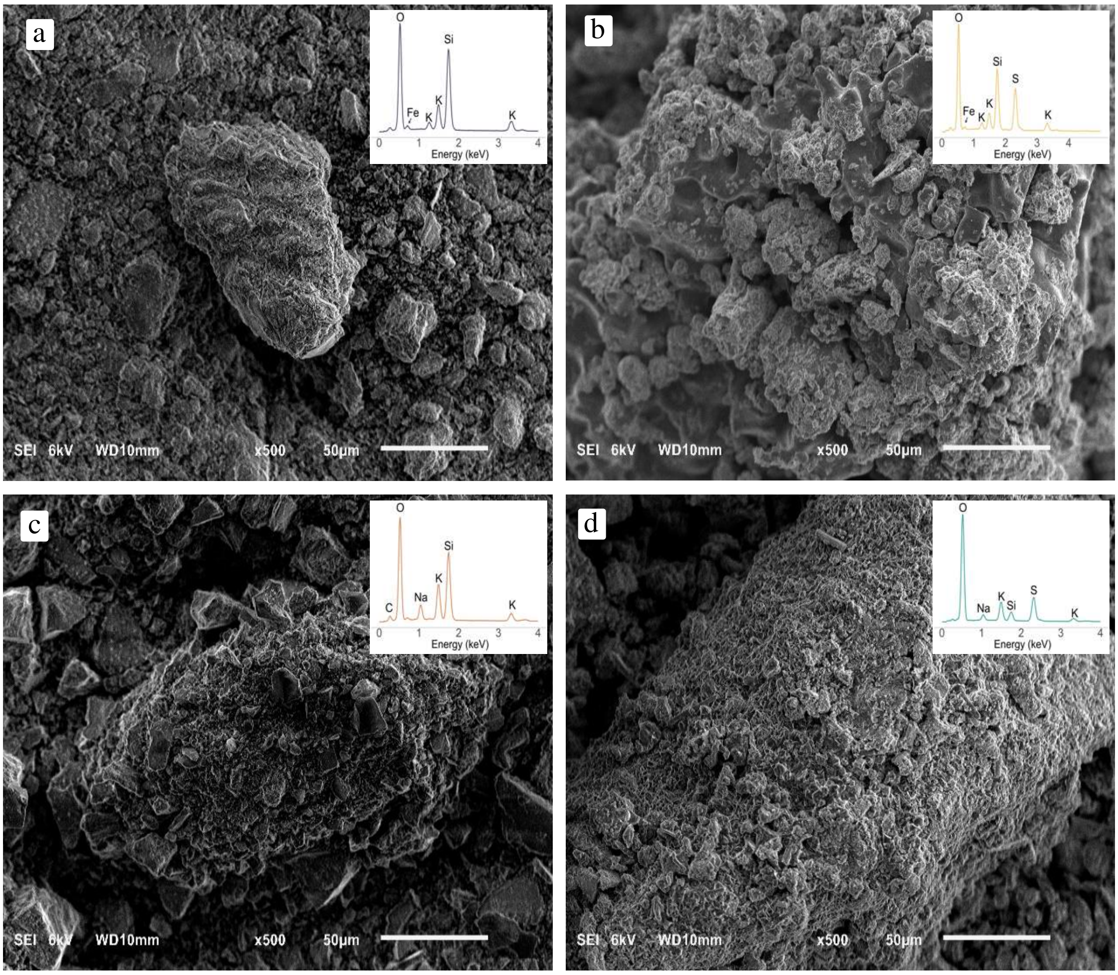

Fig. 6. Scanning electron microscopy (SEM) and energy-dispersive spectrometry (EDS) of verdete rock (a), verdete biogenic K-fertilizer (b), phonolite rock (c) and phonolite biogenic K-fertilizer (d).

3.5. Agronomic performance of K-fertilizers in greenhouse experiments

Significant effects of K fertilizer treatments on the dry matter yield and K uptake were observed for corn, soybean and millet crops (Fig. 7, Fig. A1). When corn was cropped, a direct and linear relation of dry matter production with increase of K rate was observed for Ve-BKF, Fo-BKF, and Fo (Fig. 7a). Indeed, rock acidulation resulted in increases in both dry matter production and K uptake, and this effect was more pronounced for Ve rock. Ve-BKF at a 200 mg dm^{-3} K rate caused a 3.2- and 3.6-fold increase in dry matter production and K uptake, respectively, when compared to verdete; the values for Fo-BKF, were 2.1- and 1.9-fold, respectively, with respect to its *in natura* counterpart. At rate of 200 mg dm^{-3} K, dose at which the maximum dry matter mass was obtained with KCl use, corn plants treated with Ve-BKF and Fo-BKF produced 24 and 3% more dry matter than KCl, respectively (Fig. 7a). In contrast, plants treated with *in natura* Fo and Ve produced only 45 and 32% of the dry matter produced by plants treated with KCl, respectively. As for K uptake, higher values were observed in plants treated with KCl in comparison with those treated with the other fertilizers (Fig. 7b). When the rate at 200 mg dm^{-3} K was considered, plants treated with Ve-BKF had 28% and those treated Fo-BKF had 19% the total K uptake with respect to plants treated with KCl. These values represented increases of 4.5- and 2.5-fold in relation to Ve and Fo, respectively.

Phonolite rock led to a high production of dry matter by soybean, with a value that corresponded to 91% of the maximum production obtained when KCl was applied at a dose of 190 mg dm^{-3} K (Fig. 7c). On the other hand, the value for Fo biogenic K-fertilizer was 76% in relation to KCl. Soybean plants treated with Ve-BKF produced 75% of dry mass in relation to those treated with KCl; for Ve it was only 66%. The contents of K in shoots of soybean (Fig. 7d) were similar for Ve-BKF and both forms of phonolite, which exhibited about 53% in relation to the maximum K uptake obtained with KCl application. Ve rock provided less K to the plant, while KCl application resulted in higher values of dry matter and K uptake at all K rates evaluated (Figs. 7c and d).

When millet was cropped the dry matter production and K uptake as function of K sources and rates presented different trends (Figs. 7e and f). These values exhibited small increases with an increase in the K rate, except for KCl. At 172 mg dm^{-3} K (a rate at which KCl caused the highest dry matter production) plants with Fo application produced 81% dry matter when compared with those that received KCl. Nonetheless, this dry matter production was higher than that obtained with Ve rock. On the other hand, dry matter yield by plants with Ve-BKF application was higher (83% compared to KCl) than Ve rock (71% compared to KCl). K uptake of millet was higher when the plants received Ve-BKF and Fo, 58% and 55%, respectively, when compared to that absorbed and accumulated by plants with KCl application.

At completion of the cropping system, total dry matter and K uptake were influenced not only by K addition in the soil but also by the K source utilized (Figs. 7g and h). Among the K-sources, at a dose of 180 mg dm^{-3} of K, verdete biogenic K-fertilizer caused higher dry matter production and K uptake, followed by Fo-BKF, phonolite and verdete rocks. The effect of acidulation was more pronounced for verdete rock, which resulted in higher values in relation to Ve. However, higher values of dry matter production and K uptake by the crops were obtained when KCl was the K source. Plants with Ve-BKF application produced 87% dry matter when compared to the maximum observed with plants that received KCl; the value for the *in natura* Ve form was only 60%. With respect to K uptake, 35 and 15% was obtained for the biogenic K-fertilizer and *in natura* forms of verdete in comparison to KCl.

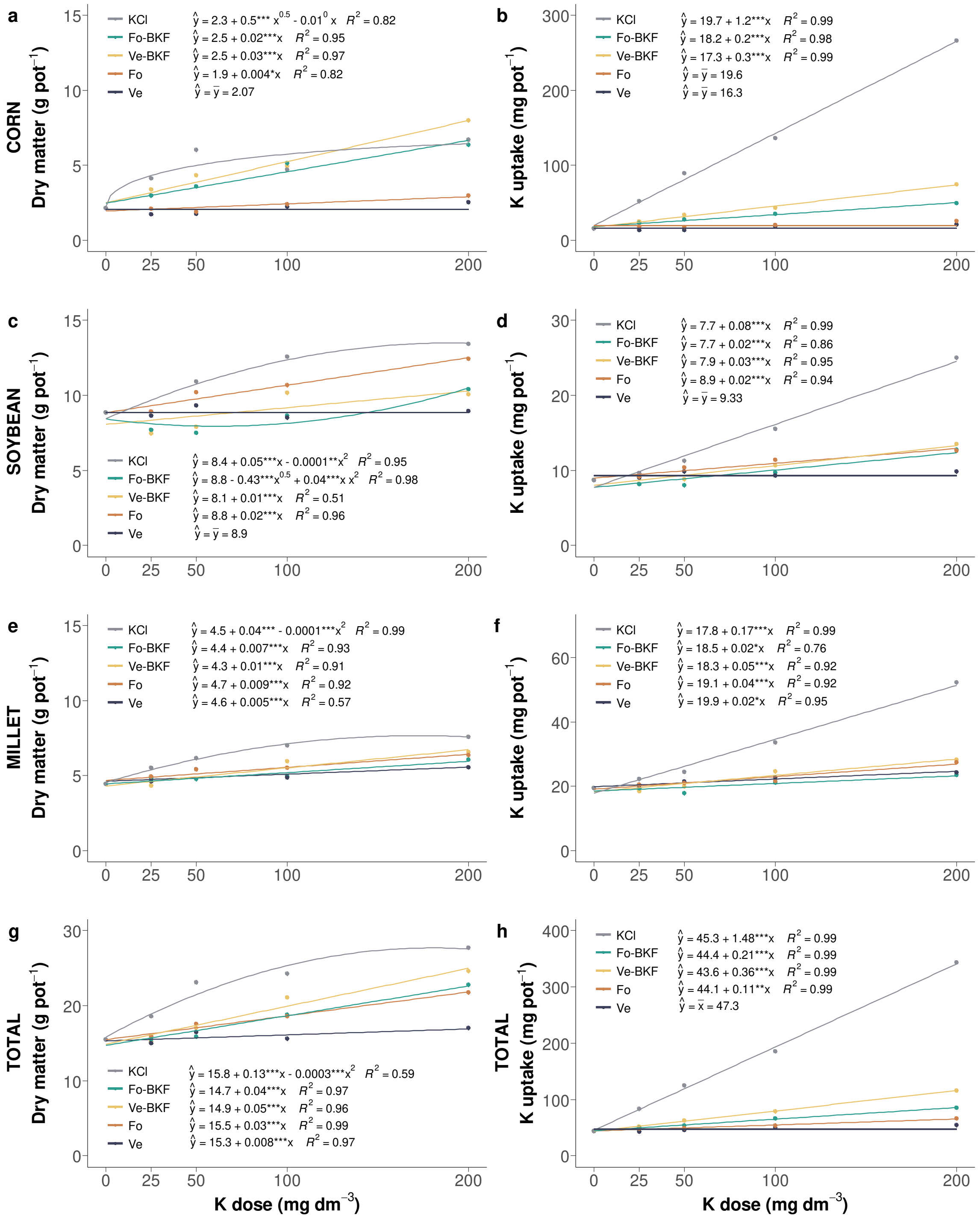

Fig. 7. Effects of K sources (KCl, potassium chloride; Fo-BKF, phonolite biogenic K-fertilizer; Ve-BKF, verdete biogenic K-fertilizer; Fo, phonolite rock; Ve, verdete rock) over different K doses on dry matter production (a, c, e, g) and K uptake (b, d, f, h) by corn (a, b) soybean (c, d), millet (e, f), and total accumulated at the end of sequential crop cultivation (g, h). ^{0}, *, **, ***: significant at 10, 5, 1, and 0.1% by t-test, respectively.

It was observed that KCl treatment exhibited the highest value of K recovery rate (Krr), followed by Ve-BKF (Table A1). Krr was 61% of the total applied to corn as KCl, whereas the value for Ve-BKF was 14%, and for Fo-BKF was only 8%. For soybean, the Krr for KCl was

14%, and for the other sources, except Ve rock, was 1%. For millet, the Krr for KCl was lower (8%) than those of the previous crops, but higher than those for Ve and Fo in their different forms. In regards to total Krr at the end of the cropping system, 74% K from KCl, 18% from Ve-BKF, 10% from Fo-BKF and 5% from Fo rock was uptaken by the plants. Uptake of K by plants applied with verdete rock did not differ from that of control plants without K application.

The acidulated rocks produced by the use of biogenic acid present in their composition soluble forms of S and elemental sulfur that were not totally oxidized by *A. thiooxidans*. Consistently, treatment with Ve-BKF and Fo-BKF resulted in the highest values of S uptake by corn, soybean and millet (Figs. 8a, b and c). The total mean S uptake at the K rate of 200 mg dm^{-3} were 86.4 mg pot^{-1} S for Ve-BKF and 71.3 mg pot^{-1} for Fo-BKF.

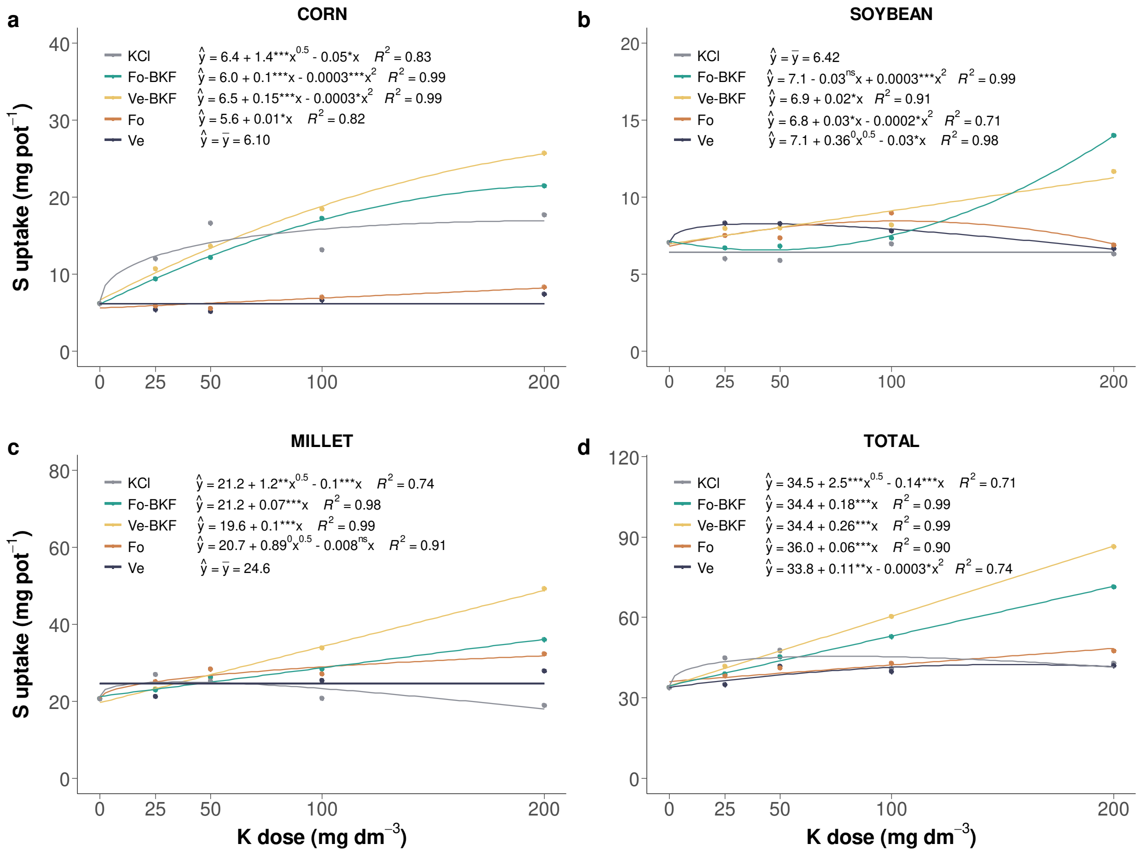

Fig. 8. Effects of rate of K fertilizers with different content of S (KCl, potassium chloride; Fo-BKF, phonolite biogenic K-fertilizer; Ve-BKF, verdete biogenic K-fertilizer; Fo, phonolite rock; Ve, verdete rock) on S uptake by corn (a), soybean (b), millet (c), and total accumulated after the successive crop cultivation (d). ^{0}, *, **, ***: significant at 10, 5, 1, and 0.1% by t-test, respectively.

There were different trends for K extracted from soil by Mehlich-3 as function of K sources and rates as well as of sampling time (Figs. 9a and b). For the first sampling, available K content in soil increased according to the following order: KCl, followed by Fo rock, Ve-BKF and Fo-BKF. Higher K rates caused an increase in available K contents for plants, an effect that was observed even after corn and soybean cultivation (Fig. 9a). The highest increases occurred when KCl was used, followed by Fo rock, Ve-BKF and Fo-BKF. After millet cultivation (Fig. 9b), the available K content was lower (< 4 mg dm^{-3}), and in this case, Fo rock application resulted in a similar available amount of K to that observed for KCl.

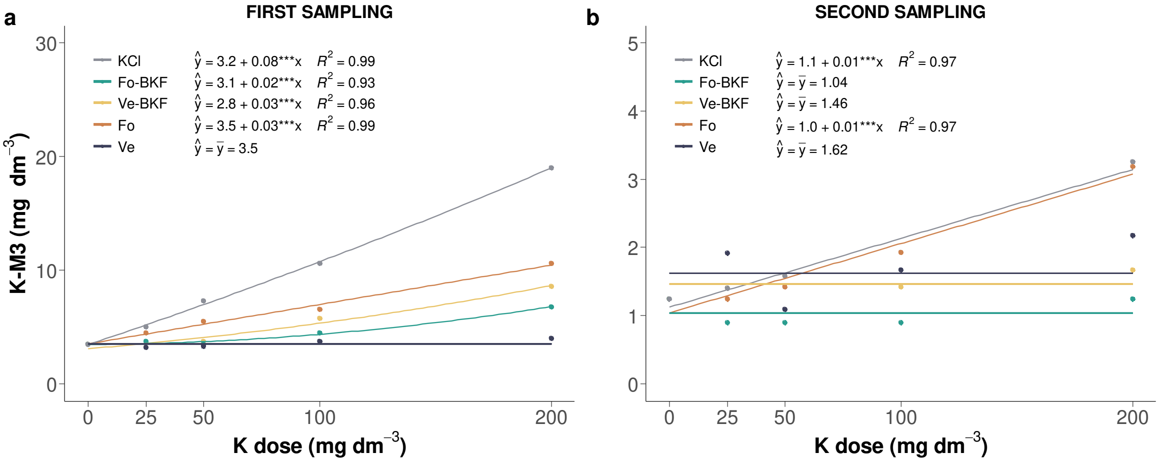

Fig. 9. Available K content in soil as a function of K rate when KCl (potassium chloride), phonolite (Fo-BKF) or verdete (Ve-BKF) biogenic K fertilizer, phonolite (Fo) or verdete (Ve) rock were used for successive corn, soybean and millet cultivation. (a) After corn and soybean; (b) after millet cropping at the end the experiment. ***: significant at 0.1% by t-test, respectively.

4. Discussion

4.1 Ac. thiooxidans as a producer of biogenic acids

We detected the oxidation of elemental sulfur in our assay by an increase of sulfate concentration in the culture medium, a primary metabolite of *A. thiooxidans*. The occurrence of this reaction reflects bacterial active metabolism, which is supported by the medium acidic condition. At the end of the assay, we found the pH 0.4 and the concentration of H^+ 1.2 mol L^{-1}. These results indicates that *A. thiooxidans*, a sulfur-oxidizing bacteria, uses reduced inorganic sulfur species as an energy source (Friedrich et al., 2005; Ghosh and Dam, 2009), converting elemental sulfur to sulfate form, with concomitant acidity production (Eq. 1). Thus, due to its oxidation activity and high capacity of acidity production, this bacterium is used as a catalyst in solubilization processes (Chen and Lin, 2004; Kelly and Wood, 2000).

$$2\,S^0 + 3\,O_2 + 2\,H_2O \rightarrow 2\,H_2SO_4 \quad \text{(Eq. 1)}$$

In this study, rocks solubilization was performed in two steps. Firstly, the bacterium was grown in culture media until attaining the desired condition, and then, the culture medium, containing the biogenic acid, was added to rocks and mixed for five minutes. This process, carried out in this way, has proven to be efficient, since there are no restrictions on bacterial growth (Mishra et al., 2008). This strategy made possible to use silicate rocks in our study, which may have elements in concentrations that are toxic to the bacterium. In addition, these rocks do not contain sufficient quality and quantity of mineral components necessary to sustain viable bacterial populations. Furthermore, the process described here is suitable for large-scale production, which is desirable, considering that agricultural inputs are used in large amounts of K fertilizers.

4.2 Biogenic acid inducting changes in extractable K from Ve or Fo rocks

The chemical solubilization with inorganic acid is a typical way to produce soluble fertilizers (Nasri et al., 2014). In this study, sulfuric acid caused an increase in verdete and phonolite rocks solubility resulting in the formation of more soluble K species in water and citric acid. Importantly, the biogenic acid produced here caused higher solubilization than commercial acid, and consequently, higher K contents were solubilized from the rocks in water and citric acid. This observation suggests that there may be some components, such as additional metabolites that contributes to a better performance of biogenic acid in K solubilization. Similar results were found by Calle-Castañeda et al. (2018) and Bosio et al. (2008) using biogenic acid from *A. thiooxidans* to recover phosphorus and nickel from phosphate rock and spent material from the hydrogenation of vegetable oil, respectively. Mishra et al. (2008) concluded that additional metabolites are responsible for providing additional effects of biogenic solutions. These metabolites can be organic acids excreted by potassium-solubilizing bacteria, including *Pseudomonas* spp., *Acidithiobacillus ferrooxidans*, *Bacillus mucilaginosus*, *B. edaphicus* and *B. megaterium*, which are able to solubilize powders of K rocks such as micas, illite and orthoclases (Friedrich et al., 1991; Meena et al., 2014; Ullman et al., 1996; Zhang and Kong, 2014).

4.3 Biogenic acid inducting changes in morphological and mineral proprieties

Acid attack caused morphological and crystal alterations in the minerals. This information is in agreement with XRD, which showed that after acid treatment, some phases presented partial dissolution and new phases were formed, altering the rock structure. Furthermore, the samples colors also changed after acidification (Fig. A2), which corroborated the data discussed before. From infrared spectroscopy, it was verified that after acid treatment, the band at 1080 cm^{-1} (assigned to SiO_2 stretching) for both rock types increased in intensity, which indicated the collapse of some silane groups, Si-O-Si, and the replacement of SiO_2 vibrations. Furthermore, water and OH stretching vibrations were also found, likely due to water adsorption and to SiOH production. The acid attack promoted the bands broadening, which is also an evidence that a change is occurring in the material structure. The Si-O linkages from both materials was also verified in Raman analysis, which after the acid treatment, the bands disappear for Ve-BKR, due to minerals dissolution as showed by XRD and FTIR. For the materials *in natura,* no S was verified on EDS spectra. On the other hand, after acid dissolution, the S was present and a modified structure was observed by SEM. Thus, the rocks treated with acid are more susceptible to reactions that take place in the soil than the ones *in nature*, and may release nutrients still present in the rock.

We observed that K content in water and in citric acid, as well as the agronomic performance were different for the rock types under study. Although both rock types are silicates, there is a difference in solubility between verdete and phonolite, and consequently the availability of their nutrients to plants is dependent on their mineralogical composition and reactivity. Verdete has glauconite as its main K-mineral constituent whereas phonolite has feldspars. In these minerals, K is strongly retained in their structures, forming bonds with oxygen in SiO_4 and AlO_4 tetrahedrons (Curi et al., 2005). Thus, silicates exhibit a slow release of elements, limiting their direct use in some applications (Leite, 1985; Valarelli, 1993). In fact, we observed a low efficiency of the silicate rocks used in this work as a K-fertilizer when it was used in its *in natura* form as compared to its acidified form and the soluble source KCl. In contrast, Fo rock showed higher levels of CA-K than Ve rock, and caused a higher dry matter production in soybean. This is most likely due to the fact that phonolite it is constituted of nepheline, a mineral that has a high dissolution rate (Blum and Stillings, 1995) and also because phonolite presented a lower crystallinity than verdete. Hence, the presence of this mineral in the rocks should be considered when choosing alternative K sources (Manning, 2010).

The minerals which are components of verdete and phonolite rocks, after undergoing changes in their structures, release K and other elements, such as Ca, Fe, Al and Si (Meurer, 2006; Resende et al., 2007). The release of K occurs due to an exchange reaction between cations in an acidic medium, as shown for feldspar in Equation 2 (Curi et al., 2005).

$$KAlSi_3O_8 + H^+ \rightarrow HAlSiO_8 + K^+ \quad \text{(Eq. 2)}$$

This reaction causes a change in the mineral structure, increasing the availability of nutrients to plants, and consequently increasing the production of dry matter and K uptake. This effect was more pronounced for verdete rock, in which the acidulated form caused a significative increase in dry matter production and K uptake in a K-dose dependent manner. In addition, acidified rocks are a source of sulfur for plants.

4.4 Acid treatments increasing fertilizer values

The rocks solubilization using biogenic acid is a technology with considerable potential for use in the fertilizers industry. It allows fertilizer production from rocks that have elements of agricultural interest but that exhibits low solubility, with attractive environmental and cost-benefit advantages. The current high cost of conventional potassium fertilizers, together with the increased demand of agricultural production, justify the strategic investment on this type of technology. Nonetheless, further studies aimed to optimize fertilizes solubilization from rocks are still necessary to gain an efficient translation to agriculture applications. In addition, studies that could characterize the additional metabolites present in the *A. thiooxidans* culture medium are required.

5. Conclusions

Treatment of verdete and phonolite rocks with biogenic acid produced by *A. thiooxidans* caused an increase of their solubility as well as of extractable K in water and citric acid. XRD, SEM, FTIR and Raman confirmed the solubilization effectiveness and revealed a change in rock structure and morphology due to the chemical action of acidity. Furthermore, the acidulated rocks showed a greater agronomic efficiency, providing higher amount of available K to cultivated plants, compared to their *in natura* counterparts. In addition, the biogenic K-fertilizers are also a source of S, adding more value to the end products.

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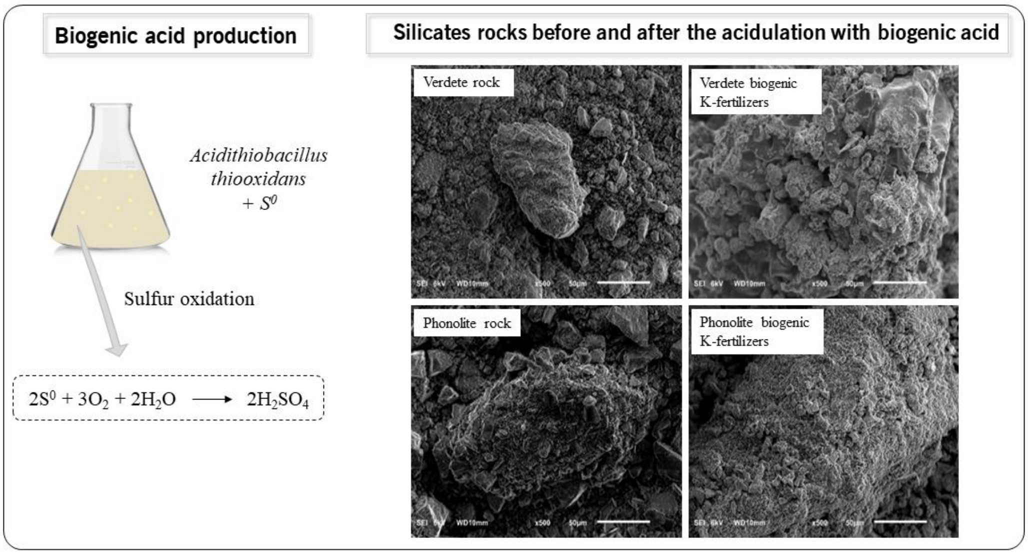
Graphical abstract



Supplementary material

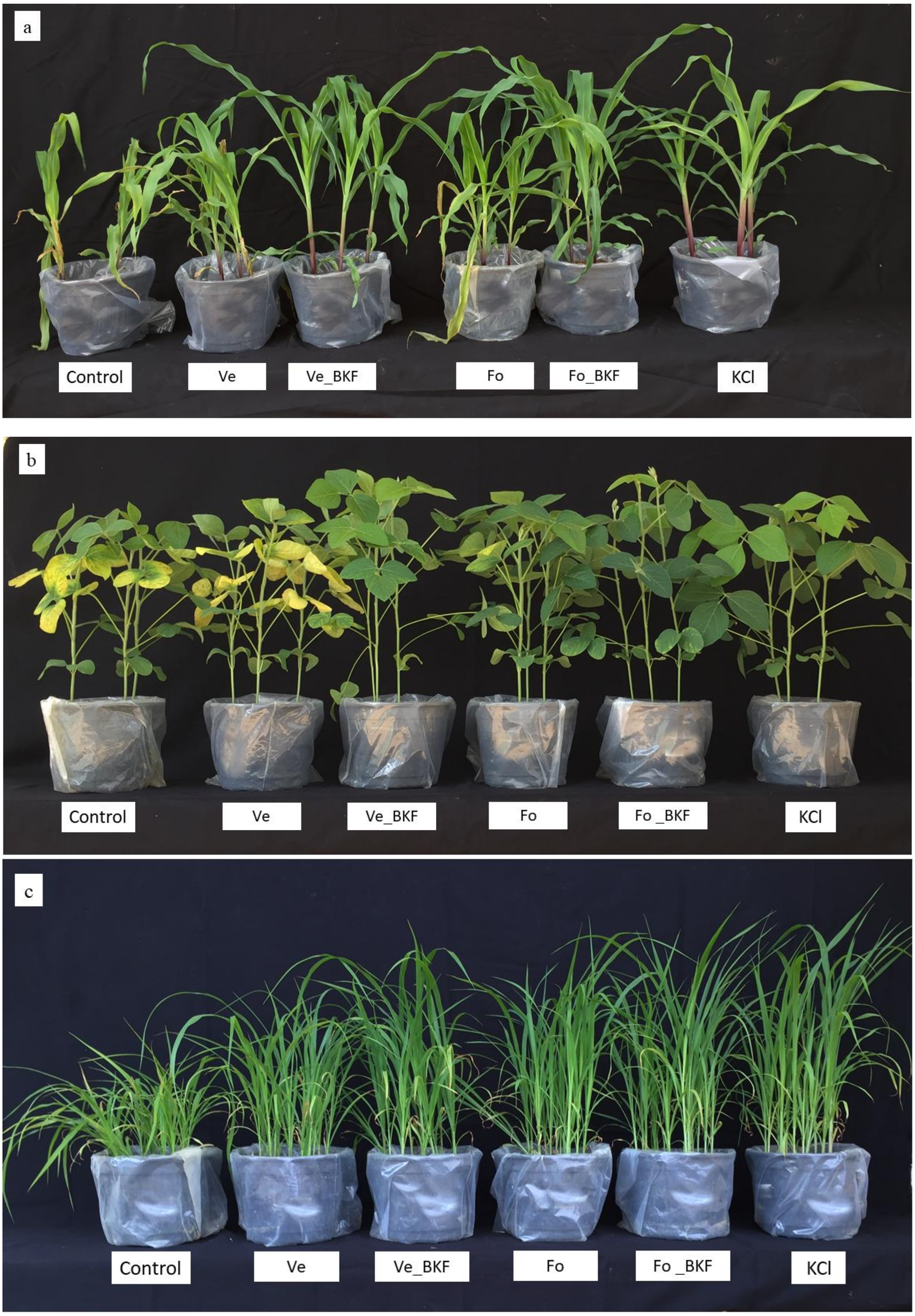


Fig. A1. Response of corn (a), soybean (b) and millet (c) plants to application of verdete (Ve) and phonolite (Fo) rock, and verdete (Ve-BKF) and phonolite (Fo-BKF) biogenic K-fertilizer and KCl at a dose of 200 mg dm^{-3} K, compared to control plants without K aplication. Pictures were taken at 32, 40 and 30 days after sowing corn, soybean and millet, respectively.

Table A1. Equations of recovered K contents by crop (ŷ, mg dm^{-3}), according to the K doses (x, mg dm^{-3}) of each fertilizer (KCl, verdete (Ve) and phonolite (Fo) rock, and verdete (Ve-BKF) and phonolite (Fo-BKF) biogenic K-fertilizers, coefficient of determination (R²) and K recovery rate (Krr).

Fertilizer	Equation	R^2	Krr
CORN			
KCl	$\hat{y} = 9.87 + 0.61$ x	0.99	0.61
Fo-BKF	$\hat{y} = 9.09 + 0.08$ x	0.98	0.08
Ve-BKF	$\hat{y} = 8.65 + 0.14$ x	0.99	0.14
Fo	$\hat{y} = \bar{y} = 9.79$		
Ve	$\hat{y} = \bar{y} = 8.14$		
SOYBEAN			
KCl	$\hat{y} = 3.87 + 0.14$ x	0.99	0.14
Fo-BKF	$\hat{y} = 3.87 + 0.01$ x	0.86	0.01
Ve-BKF	$\hat{y} = 3.99 + 0.01$ x	0.95	0.01
Fo	$\hat{y} = 4.50 + 0.01$ x	0.94	0.01
Ve	$\hat{y} = \bar{y} = 4.67$		
MILLET			
KCl	$\hat{y} = 8.92 + 0.08$ x	0.99	0.08
Fo-BKF	$\hat{y} = 9.25 + 0.01$ x	0.76	0.01
Ve-BKF	$\hat{y} = 9.17 + 0.03$ x	0.92	0.03
Fo	$\hat{y} = 9.55 + 0.02$ x	0.94	0.02
Ve	$\hat{y} = 9.93 + 0.01$ x	0.95	0.01
TOTAL			
KCl	$\hat{y} = 22.67 + 0.74$ x	0.99	0.74
Fo-BKF	$\hat{y} = 22.21 + 0.10$ x	0.99	0.10
Ve-BKF	$\hat{y} = 21.82 + 0.18$ x	0.99	0.18
Fo	$\hat{y} = 22.04 + 0.05$ x	0.99	0.05
Ve	$\hat{y} = \bar{y} = 23.6$		

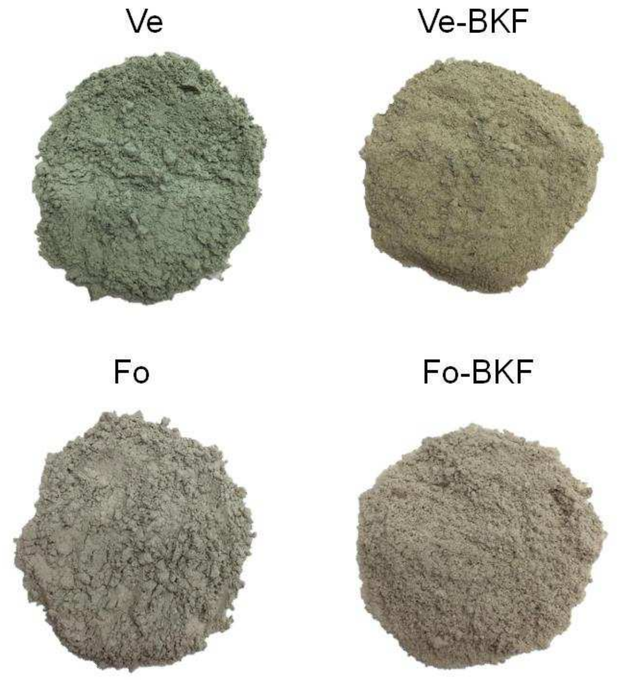


Fig. A2. Macroscopic appearance of verdete (Ve) and phonolite (Fo) rock and after acidulation (BKF) with biogenic acid. BKF = biogenic K-fertilizer.

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Paper 2

BIOGENIC SULFURIC ACID FOR PHOSPHATE FERTILIZERS PRODUCTION

Abstract

The development of alternative methods for phosphate fertilizer production, using acid produced by S-oxidizing bacteria, and applicable to both high- and low-quality phosphates, is important because is simple, cost-efficient and environmentally friendly. In this work, we evaluated the effect of a biogenic acid produced by *Acidithiobacillus thiooxidans* on solubilization of P species from Morocco (M-PR) and Serra Salitre (SS-PR) phosphate rocks. Furthermore, the agronomic performances of the end-products from the biological route were contrasted with those from commercial sulfuric acid and with rocks *in natura* through a sequential corn-soybean-millet cropping system. When used at a 1:5 rock:acid ratio (w/v), the biogenic sulfuric acid promoted similar to higher content of soluble P in water, 2% citric acid (CA-P) and acid ammonium citrate (AAC-P) than commercial acid for both PRs. For M-PR, using a 1:2 rock:ratio, treatment with biogenic acid caused higher CA-P and AAC-P contents compared to the commercial acid, while for SS-PR the opposite was observed. A decrease in intensity of peaks and bands associated with P species and appearance of S species were detected respectively by X-ray diffractionand FTIR spectroscopy of Morocco and Serra Salitre rocks acidulated with biogenic acid. The Morocco (M-BPF) and Serra Salitre biogenic (SS-BPF) P-fertilizers lead to higher amount of plant dry matter and P uptake than phosphate rocks in the cropping system evaluated; an effect that was more pronounced for SS-BPF. Thus, treatment of PR with biogenic acid as a route for phosphate fertilizer production efficiently provided P to plants in a successive corn-soybean-millet cropping system.

Keywords: biogenic acid, P-fertilizers, *Acidithiobacillus thiooxidans*, phosphate rock

1. Introduction

The increase of food production required to support the demands of global population is dependent on fertilizer supply (Godfray et al., 2010; Gregory and George, 2011). Agriculture is responsible for the consumption of approximately 90% of all P in the world (Brunner, 2010; Pantano et al., 2016).

The basic raw material used in the phosphate fertilizer industry is apatite, a low water-soluble calcium phosphate, which forms predominantly (~95%) of the P found in nature (Smil, 2000). Apatites vary widely in physical, chemical, and crystallographic properties (Lehr et al., 1967), depending on their origin (Hammond et al., 1986).

There are mining problems to obtain P ores due limitations in their accessibility and good quality of phosphate rock deposits, which increase the extraction cost, and consequently the production cost of P fertilizers (Mendes et al., 2013; Pantano et al., 2016). In addition, more than 70% of the worldwide P reserve are concentrated in Morocco and Western Sahara (U.S. Geological Survey, 2019). Brazil is the third largest consumer of phosphate products in the world, importing approximately 60% of its internal demand (IFA, 2018). This situation is economically and strategically undesirable, but encourages the use of alternative methods to produce fertilizers from non-conventional sources.

Bioleaching is a process of mobilizing chemical elements from low-soluble ores (Rohwerder et al., 2003), which generally involves acidification by bacterial metabolism products or the oxidation by leaching bacteria (Chen and Lin, 2004). In general, these processes are simpler, of low cost and less energy intensive than conventional hydrometallurgical methods (Xin et al., 2009). Besides, it is an efficient and environmentally friendly process which needs few requirements (Mishra et al., 2008). This process has been widely developed recently and is successfully applied to copper sulfide and uranium ores as well as for pre-oxidation of refractory gold ores (Tang et al., 2000).

Chemolithotroph microorganisms, such as *Acidithiobacillus thiooxidans*, *Acidithiobacillus ferrooxidans*, *Acidithiobacillus caldus*, *Leptospirillum ferrooxidans*, *Leptospirillum ferriphilum* and *Acidiphilium* ssp., play an important function in elements bioleaching from rocks, ores or wastes (Coram and Rawlings, 2002; Deshpande et al., 2018). Among them, the most active bioleaching bacteria belong to the genus *Acidithiobacillus* (Asghari et al., 2013; Bosecker, 1997).

The species *A. thiooxidans* is extremely acidophilic (Yin et al., 2014) because sulfuric acid, the main inorganic acid formed in the leaching environment, is produced by the oxidation of reduced inorganic sulfur (Mishra et al., 2005). This bacterium has been well studied for industry applications and for metals bioleaching, such as iron, arsenic, magnesium, zinc and copper (Hocheng et al., 2014; Huang et al., 2013; Lee et al., 2015; Wang et al., 2009). Efforts have been made to use this acidophilic bacterium of phosphate rock (Besharati et al., 2007; Bhatti and Yawar, 2010; Calle-Castañeda et al., 2018a, 2018b; Priha et al., 2014; Xiao et al., 2011). Calle-Castañeda et al. (2018b) performed bioleaching of PR in a two-step process: first growing the microorganism and recovering the acid produced and then treating the phosphate rock with it, thus, promoting the solubilization of 100% of P.

Although these studies demonstrated the potential of phosphate rock solubilization by microorganisms, there are few works reaching end products for commercial purposes.

Similarly, there are few works evaluating the agronomic performance and residual effects of such fertilizers. Therefore, the goal of this study was the development of a new route to produce more soluble phosphate fertilizer using a biogenic sulfuric acid from *A. thiooxidans*.

2. Materials and Methods

2.1. Solubilization of phosphate rocks with commercial or biogenic acids

The effect of acidity on P solubilization was evaluated in commercial samples of Morocco (M-PR) and Serra Salitre (SS-PR) phosphate rocks. To do so, rock samples were first ground and passed through a 75-µm sieve (200 mesh) for total P determination (Brasil, 2007) to be used in the trial.

The rocks M-PR and SS-PR were treated with two acids (0.9 mol L^{-1} H^+): commercial sulfuric acid, with analytical purity, and biogenic acid produced by *A. thiooxidans*, using two rock:acid ratios (1:2 and 1:5 w/v). The rock samples and acids were added in 50 mL becker flasks, homogenized and kept at room temperature for 7 days. Then, the whole mixtures were oven-dried at 50 °C; and chemical analyses were performed.

For water-soluble P (water-P) determination, 0.20 g of the fertilizers and 30 mL deionized water were added in centrifuge tubes. The tubes were placed in a vertical shaker at 60 rpm for 12 h. After this time, the suspensions were filtered, and the filtrates were used to determine P concentrations. The extractable P in 2% citric acid (CA-P), and total P were measured as described by the Brazilian Ministry of Agriculture, Livestock, and Food Supply, Normative Instruction N° 28 (Brasil, 2007). The soluble P species in acid ammonium citrate pH 3 (AAC-P) were determined using centrifuge tubes containing 0.50 g of fertilizer and 30 mL of acid ammonium citrate extractor, prepared as described by Chien and Hammond (1978). The mixture was shaken in a vertical shaker at 60 rpm for 1 h, then filtered and stored. The P concentration in the extracts was measured by molecular absorption spectrophotometry (Braga and Defelipo, 1974).

2.2. Production of biogenic phosphate fertilizers

The Morocco and Serra Salitre biogenic P-fertilizers (M-BPF and SS-BPF, respectively) were produced using a two-step biotechnological process. In the first step, the biogenic sulfuric acid was produced by *A. thiooxidans*; and in the second step, the solubilization of M-PR and SS-PR was performed using this acid. For this, phosphate rocks and biogenic acid (pH 0.66; 1.58 mol L^{-1} H^+) were added into a glass jar, rock:acid ratio of 1:5 (w/v). The suspensions were manually mixed twice a day during 7 d and left to settle at room temperature. Then, the materials were oven-dried at 100 °C for 24 h and homogenized. Chemical, mineralogical and morphological analyses of phosphate rocks and biogenic P-fertilizers were performed.

Total P and extractable P in 2% citric acid were determined according to Brasil (2007). The total concentration of Si, Fe, S, K, Zn and Cu was measured by total X-ray fluorescence spectroscopy (EDX-720; Shimadzu). Mineralogical analysis by X-ray diffraction (XRD) was performed using a Shimadzu XRD-6000 diffractometer, from 5° to 80° 2θ range with CoKα radiation ($\lambda = 0.179$ nm) at a rate of 1.2° 2θ min^{-1}. Powder mounts were prepared by packing ground (< 75 μm) samples into Al holders. The spectroscopy measurements in the infrared region with a Fourier Transform Infrared Spectroscopy (FTIR-ATR) were performed on a Perkin Elmer spectrometer, model Spectrum 1000, module ATR, in the range of 4000 to 400 cm^{-1}, at 4 cm^{-1} resolution and 32 scannings. Raman spectra were obtained using a Raman Renishaw spectrometer. The Morocco phosphate samples were measured with 785 nm laser line, 10 accumulations, exposure time of 5 s and 0.005% of power. Serra Salitre phosphate samples were measured with 785 nm laser line, 5 accumulations, exposure time of 10 s and 0.05% of power. Scanning Electronic Microscopy (SEM) and Energy Dispersive Spectrometry (EDS) analyses were conducted in a JEOL microscope model JSM-6010-LA integrated with an EDS probe operating at 4 or 10 kV, according to the characteristics of the material analyzed, at a 500x magnification. EDS operating at 10 kV was used to investigate the elemental chemical composition of the phosphate rocks and biogenic P-fertilizers.

2.3. Agronomic efficiency of P sources

A sequential cultivation of corn-soybean-millet was utilized to evaluate the agronomic performance of biogenic P-fertilizers produced using the biogenic acid and the residual effect of P sources in the soil. For that, a soil sample from a 10-40 cm depth layer of an Oxisol (*Latossolo Vermelho-Amarelo*) was collected in the municipality of Viçosa, state of Minas Gerais, Brazil. Samples were air-dried, homogenized, sieved (< 2 mm) and submitted to chemical and physical analyses. Lime ($CaCO_3$ and $MgCO_3$ at a Ca:Mg ratio of 4:1) was applied aiming to neutralize Al^{3+} and supply Ca^{2+} and Mg^{2+}. Soil samples (2 dm^3) were wetted to 80% field capacity and incubated for 30 d at room temperature. After incubation with lime, the soil presented the following properties: 75% of clay (Claessen, 1997), remaining P of 8.5 mg L^{-1} (Alvarez V. et al., 2000), available P of 0.1 mg dm^{-3} (Mehlich-1), pH (H_2O) of 5.9 (at a ratio of 1:2.5 v/v), Ca^{2+} and Mg^{2+} of 2.92 and 0.62 mmol$_c$ dm^{-3}, respectively (KCl 1.0 mol L^{-1} extractor), soil organic C of 16 g kg^{-1} (Walkley and Black, 1934).

A factorial scheme (5×4)+1 was established with five P sources: single superphosphate (SSP), Morocco (M-PR) and Serra Salitre (SS-PR) phosphate rocks, Morocco (M-BPF) and Serra Saltire (SS-BPF) biogenic P-fertilizers; four doses of P (50, 100, 200, 300 mg dm^{-3}); and a control treatment without P application. The experimental design was a randomized complete block with four replications. Soil (2 dm^3) and fertilizers were homogenized in plastic bags and placed in pots.

Six seeds of corn (simple hybrid BM 709 PRO 2) were sown per pot at a depth of 0.02 m. After five days of emergence, the seedlings were thinned to the three most uniform plants in each pot. Nutritive solutions were added at 7, 14, and 21 d after planting, resulting in a total basal nutrient dose per dm^3 soil of 100 mg of N (urea), 150 mg K (KCl), 50 mg S ($(NH_4)_2SO_4$), 3.0 mg Zn ($ZnSO_4$), 1.0 mg B (H_3BO_3), 1.3 mg Cu ($CuSO_4$), 7.2 mg Mn ($MnSO_4$), 3.12 mg Fe ($FeCl_3$) and 0.15 mg Mo ($(NH_4)_6Mo_7O_{24}$). The pots were wetted daily by weight with distilled water to maintain the soil near 80 % field capacity. At 32 d after planting, plants were harvested by cutting the stems at the soil surface. Collected plant tissue was oven-dried at 65 °C until a constant weight was obtained.

Two days after corn harvesting, the second cultivation was started. For this, six seeds of soybean (BMX BÔNUS IPRO) were sown in the undisturbed soil of the pots at a depth of 2 cm. After plant emergence, each pot was thinned to the three most homogeneous seedlings and were inoculated with commercial *Bradyrhizobium* in order to fix and provide N to the plants. For this crop, a second application of micronutrients at the same concentrations as used for corn was performed and only 100 mg dm^{-3} K (KCl) and 50 mg dm^{-3} S ($(NH_4)_2SO_4$) of macronutrients were applied at 10, 20 and 30 d after planting. After 40 d, the soybean plants were harvested by cutting the stems at the soil surface and oven-dried as described for corn. After the second cultivation, the soil of each pot the soil was air-dried, homogenized and a sample was collected to determine the P availability.

Next, for the first cultivation of millet, twenty seeds of cultivar BRS 1502 were sown at a depth of 2 cm. After the emergence, fifteen plants were kept in each pot. Application of 50 mg dm^{-3} N (urea) was performed at 21 days after sowing. Millet aerial parts were harvested at 30 days after sowing and left for regrowth. For the second cultivation of millet, four plants were kept in each pot and left them to grow for 30 d. Then, the plants were harvested by cutting the stems at the soil surface and oven-dried at 65 °C until a constant weight was obtained. At the end of cultivation, second soil samples were collected from all soil volume of the pot.

The plant tissues were ground and analyzed according to method described by (Embrapa, 2009). The P and S concentration in the plant extracts were measured by inductively coupled plasma atomic emission spectroscopy (ICP-AES; Perkin Elmer 8300 DV, US). The soil samples were passed through a sieve with a 2.0-mm mesh for analysis of available P by Mehlich-3 (Mehlich, 1984). The P concentration was determined by molecular absorption spectrophotometry using ascorbic acid according to Braga and Defelipo (1974).

2.4. Data analysis

Data were subjected to a one-way analysis of variance (ANOVA). For 2.1 item, the P soluble contents in water, CA and AAC of the acidulated rocks and their *in natura* forms (control) were analyzed utilizing a randomized block design in a triple factorial scheme (3×2×2). The factors acid (without acid - control, biogenic and commercial acid) and rocks (M-PR and SS-PR). For 2.3 item, differences among P rate treatments for each P fertilizers were determined by regression analysis followed a general linear model. The R software version 3.6.3 (R Core Team, 2020) was used to perform the statistical analyses.

3. Results

3.1. Solubilization of phosphate rocks with biogenic and commercial sulfuric acid

Morocco PR (M-PR) is a reactive rock, based on its native solubility in 2% citric acid and its higher than 30% total P (Brasil, 2016), which represented 11.2 % of total P. In contrast, Serra Salitre (SS-PR) is a non-reactive phosphate rock (< 30% of extractable P in 2 % citric acid) containing a total P of 8.17%. As expected, acid treatment of these rocks caused significant increases in the extractability of P for all extractors, except at a 1:2 ratio for M-PR in water using either acid and for SS-PR in CA-P using biogenic acid (Fig. 1). When 1:5 ratio was used, water-P (Fig. 1b) was higher in the acidified products of both SS-PR and M-PR, regardless of the acid origin. At this ratio, the biogenic acid caused an increase of 1.5- and 1.2-fold in water-P for M-PR and SS-PR, respectively, in relation to the commercial acid.

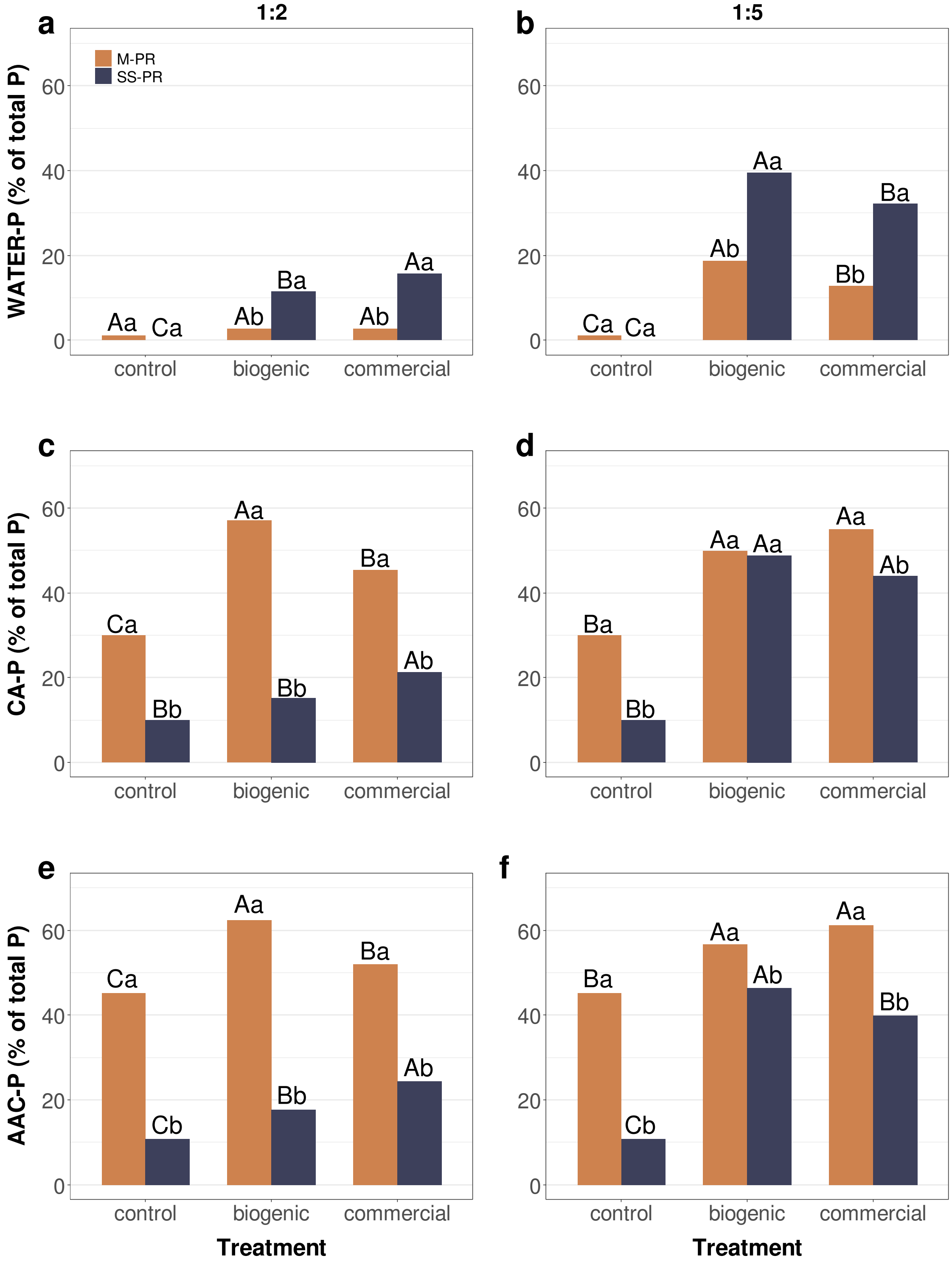

Fig. 1. Extractable P species in water (water-P) (a, b), 2% citric acid (CA-P) (c, d) and acid ammonium citrate pH 3 (AAC-P) (e, f) from the Morocco (M-PR) or Serra Salitre (SS-PR) phosphate rocks, without acid treatment (control) or treated with biogenic or commercial sulfuric acid, using rock:acid ratios of 1:2 and 1:5 (w/v). Different letters on each bar indicate significant differences (p=0.05) according to Tukey test. Uppercase letters compare treatment means within each rock and lowercase letters compare rock means within each treatment.

The amount of extractable P in 2% citric acid (CA-P) for M-PR was higher than that of SS-PR for all treatments, except for biogenic acid at a 1:5 ratio (Fig. 1c and d). For M-PR at a 1:2 ratio (Fig. 1c), the biogenic acid caused an increase in extractable CA-P of about 1.3-fold in comparison to commercial acid, while the opposite effect was observed for SS-PR. At a 1:2 ratio for the biogenic acid, the amount of the CA-P for M-PR was 57% whereas for SS-PR was only 15%, which represents an about 4-fold difference. On the other hand, when used at a1:5 ratio (Fig. 1d), the effects of biogenic and commercial acid on CA-P were similar for both rocks, but the amount of CA-P in the acidulated forms was significantly higher than those of their *in natura* forms.

Rock acidification caused an increase of AAC-P regardless the rock type or acid origin (Fig. 1e and f). Treatment of M-PR with biogenic acid at a 1:2 ratio caused a 1.2-fold increase in AAC-P with respect to commercial acid. At this ratio, 62.5% of P was extracted from M-PR using biogenic acid, while only 17.7% of P from SS-PR was soluble, which represents a 3.5-fold difference in the amount AAC-P. However, at a 1:5 ratio, similar effects on AAC-P were observed for biogenic and commercial acids when M-PR was treated. As for SS-PR, biogenic acid caused the extraction of higher amounts of AAC-P when compared to commercial acid (Fig. 1f).

3.2. Characterization of the phosphate fertilizers

Chemical, mineralogical and morphological characterization of phosphate rocks (M-PR and SS-PR) and their derived biogenic phosphate fertilizers (M-BPF and SS-BPF) was performed. Results of P sources chemical characterization is shown in Table 1. The total P concentration was similar for M-PR and SS-PR. However, a lower concentration of these elements was found in the acidulated fertilizers due to mass dilution by acid inputs. Serra Salitre PR carried higher contents of Si, Fe and K. Furthermore, due to acidification, the presence of S was detected only in SS-BPF and M-BPF. Also, a notorious increase on CA-P was observed in the rock-derived from biogenic fertilizers.

Table 1. Total P content, and extractable P forms in 2% citric acid (CA-P), and Si, Fe, K, S, Zn and Cu concentrations in Morocco (M-PR) and Serra Salitre (SS-PR) phosphate rocks and their corresponding derived biogenic P-fertilizers (M-BPF and SS-BPF, respectively).

Fertilizer	**P total**	**CA-P**	**Total concentration**					
			Si	**Fe**	**K**	**S**	**Zn**	**Cu**
	---------------------- dag kg^{-1}					----------------------	-- mg kg^{-1} --	
M-PR	12.52	31.7	8.3	0.1	nd	nd	270	87
M-BPF	7.54	73.0	2.3	0.1	nd	11	187	66
SS-PR	14.63	4.1	13.2	0.8	0.8	nd	nd	nd
SS-BPF	8.42	59.3	8.5	0.5	0.4	12	nd	55

nd (non-detected).

The X-ray diffraction analysis (Fig. 2) showed that Hydroxyapatite (Ha) and Fluorapatite (Fa) were the main minerals constituents of both Morocco and Serra Salitre PRs. In M-PR, Ha was $(Ca, Pb)_{10}(PO_4, CO_3)_6(OH, F, Cl)_{2.561.5}H_2O$ – JCPDS Card Nº 47-1758 and Fa was $Ca_5(PO_4)_3F$ – JCPDS Card Nº 15-876. In Serra Salitre PR, Fa was $Ca_5F(PO_4)_3$ – JCPDS Card Nº 12-261 and Ha was $Ca_5(PO_4)_3(OH)$ – JCPDS Card Nº 9-432.

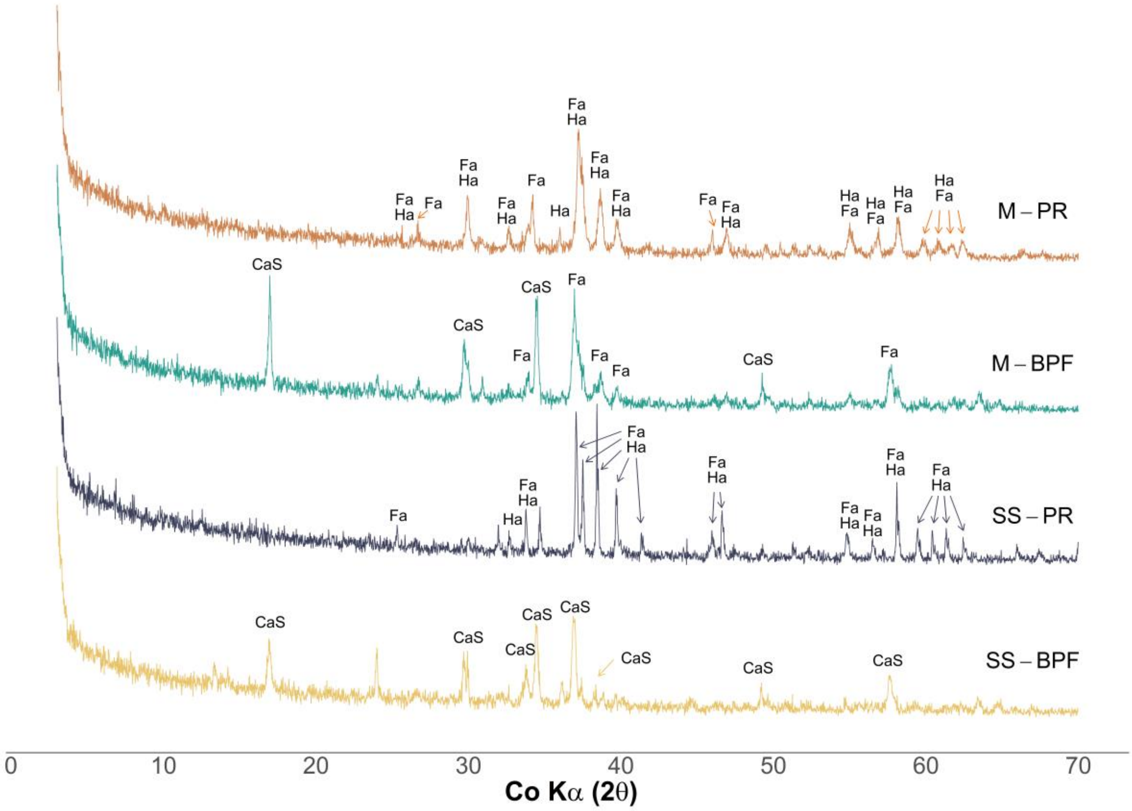

Fig. 2. X-ray diffraction patterns from Morocco (M-PR) and Serra Salitre (SS-PR) phosphate rocks, Morocco (M-BPF) and Serra Salitre (SS-BPF) biogenic phosphate fertilizers. Fa: fluorapatite; Ha: hydroxyapatite; CaS: calcium sulfate hydrate.

Treatment with biogenic sulfuric acid altered considerably the mineralogical rocks structures. M-BPF XRD revealed the presence of some Fa peaks ($Ca_5(PO_4)_3F$ – JCPDS Card N° 15-876), disappearance of Ha peaks, and appearance of a new phase, corresponding to Calcium Sulfate Hydrate, CaS (JCPDS Card No 45-848 – $CaSO_{4\ 0.15}H_2O$). On the other hand, for SS-BPF, only the peaks associated with CaS ($CaSO_{4\ 0.15}H_2O$ – JCPDS Card No 45-848) were identified. The P solubilization of Fa and Ha phases was highly correlated with phosphate acidulation and with a change in the rocks color, as shown in Figure A2.

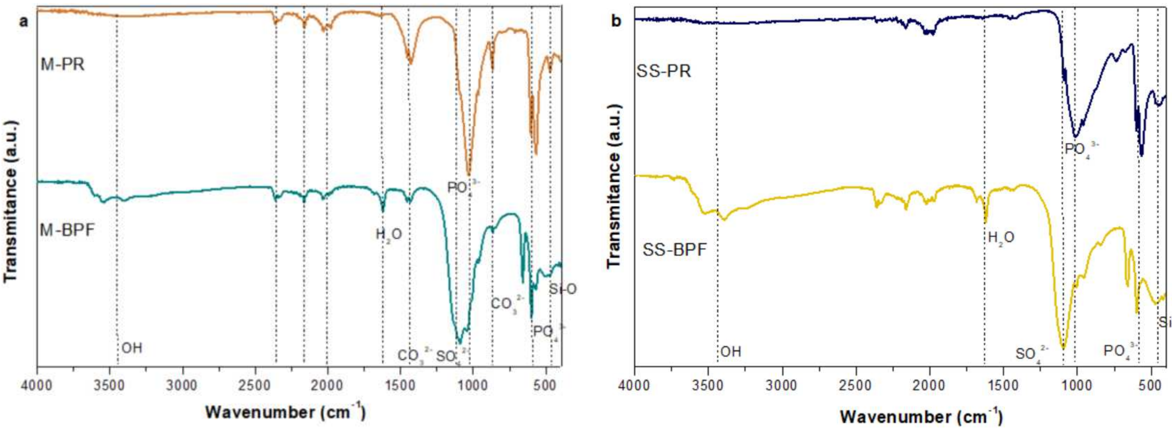

Fig. 3. Infrared spectrum from (a) Morocco (M-PR) and (b) Serra Salitre (SS-PR) phosphate rocks and their derived biogenic P-fertilizers (M-BPF and SS-BPF, respectively).

Figure 3 shows the FTIR spectra of the phosphate rocks without and after acidification with biogenic acid (biogenic P-fertilizer). In the spectra of both phosphate rocks (M-PR and SS-PR), bands characteristic of phosphate phases were found at ~1010, ~665 and ~589 cm^{-1}, which were attributed to PO_4^{3-} vibrational modes (Paz et al., 2012). Absorption bands related to Si-O were also observed at 451-464 cm^{-1} (Saikia et al., 2008). In addition, CO_3^{2-} signature was present in M-PR at 873 cm^{-1} (Veerasingam and Venkatachalapathy, 2014).

After biogenic acidulation of the rocks, the PO_4^{3-} band decreased in intensity and a IR-active mode of sulfate species (SO_4^{2-}) at 1087-1109 cm^{-1} appeared (Prasad, 2005), which was related to the sulfuric acid action which led to the formation of calcium sulfate species, as described previously for XRD results. Furthermore, for M-BPF and SS-BPF, absorption bands of OH and water species were also found at 3448 and 1613 cm^{-1}, respectively (Saikia and Parthasarathy, 2010). Indeed, in the bacterial medium water could be adsorbed on the materials surface. And we observed the decreased of the band from carbonate species in the M-BPF.

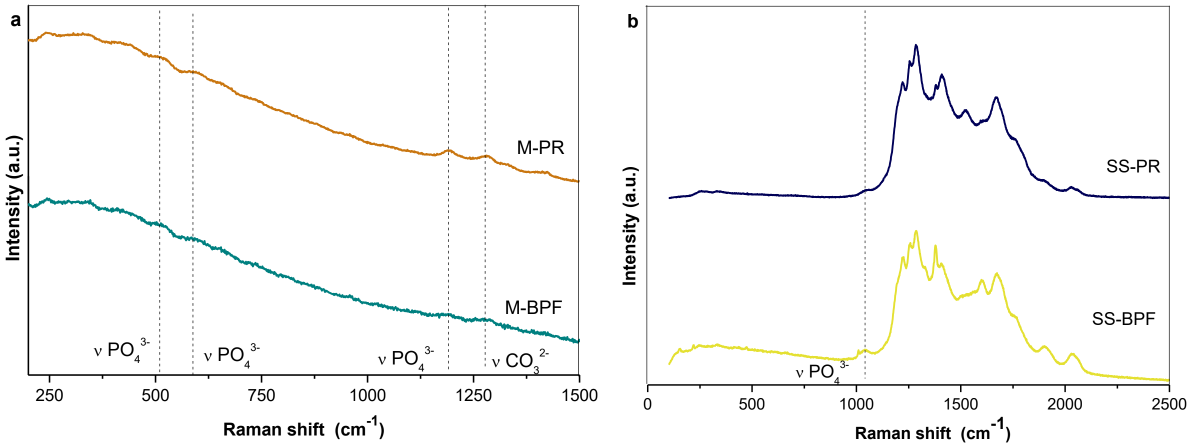

Fig. 4. Raman spectra of (a) Morocco phosphate rock (M-PR) and its derived biogenic phosphate fertilizer (M-BPF), and (b) Serra Salitre phosphate rock (SS-PR) and its derived biogenic phosphate fertilizers (SS-BPF).

Morocco PR exhibited few features in the Raman spectra. Two bands detected at 515 and 590 cm^{-1} were assigned to phosphate stretching from apatite phase (Awonusi et al., 2007). Other two bands at 1190 and 1280 cm^{-1} were related to Raman-active modes of phosphate and carbonate (Rehman and Bonfield, 1997). A decrease in intensity of bands at 1190 and 1280 cm^{-1} were observed in the Raman spectra of M-BPF, which were likely related to the sulfuric acid action (Fig. 4a). The Raman spectra of SS-PR and SS-BPF showed a small shoulder at 1045 cm^{-1} related to ν_3 phosphate stretching, occurring in the same region where carbonate stretching (ν_1) was expected (Awonusi et al., 2007). Although hydroxyapatite bands in the 200-800 cm^{-1} region associated with P-O stretching were not observed for neither SS-PR or SS-BPF, likely, the sample is associated with Fe species (Fig. 4b).

The morphology ofphosphate rocks and their derived biogenic phosphate fertilizers was investigated by SEM/EDS (Fig. 5). Before acid treatment, the rocks had smooth surfaces (Figs. 5a and c) and well-defined and compact forms, especially SS-PR. After acidulation. disintegration of some particles and a more porous and prismatic surface was observed in the biogenic P-fertilizers (Figs. 5b and d), which confirmed the surface effect of the acid treatment. SS-PR possessed a well-crystalline, nonporous apatite crystal structure, and showed more alterations in its structure after acid treatment (Figs. 5c and d). These structural changes corroborated by alterations in XRD, FTIR, the color (Fig. A2) and density.

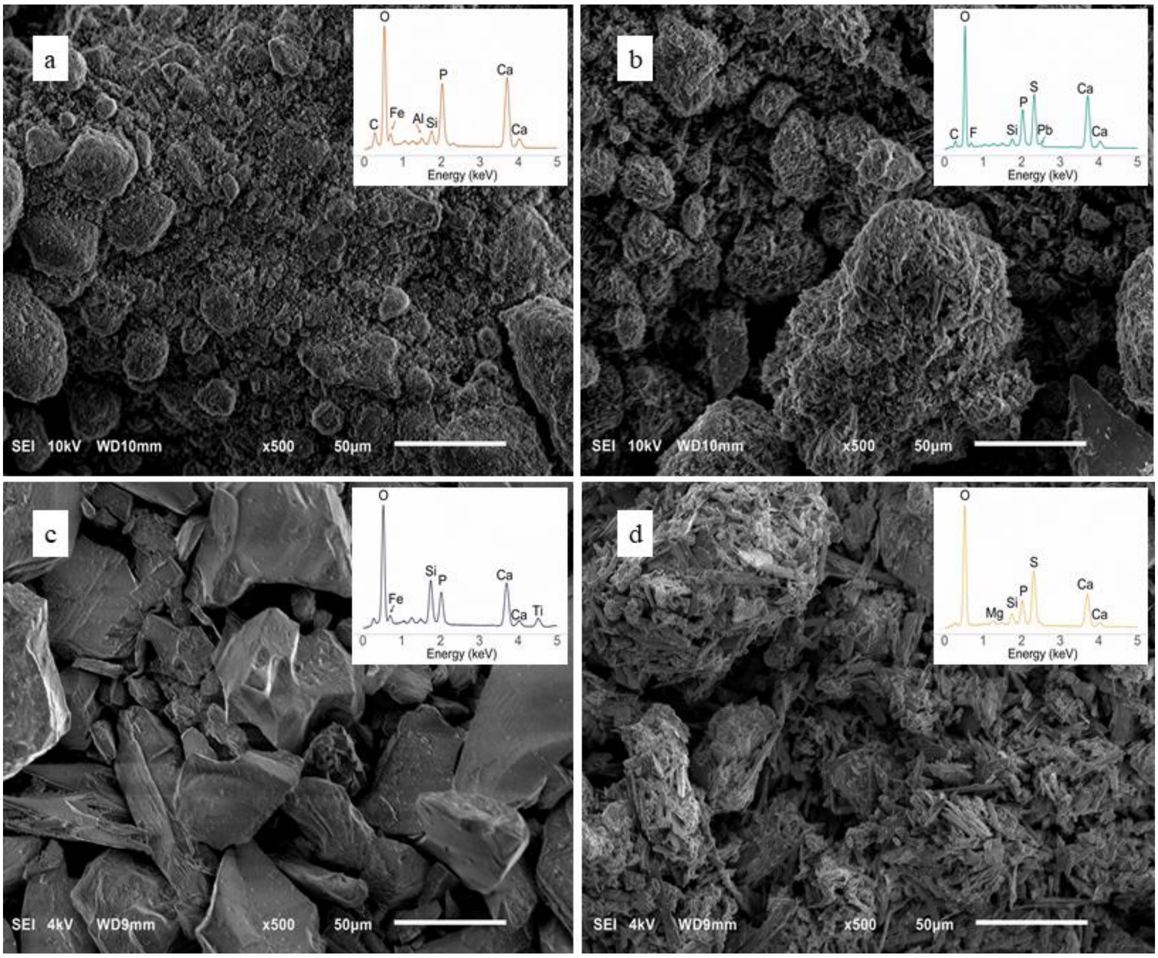

Fig. 5. Scanning electron microscopy and energy-dispersive spectrometry of (a) Morocco (M-PR) and (c) Serra Salitre (SS-PR) phosphate rocks, and (b) Morocco (M-BPF) and (d) Serra Salitre (SS-BPF) biogenic P-fertilizers.

3.3. Agronomic performance of P-sources in greenhouse experiments

The P sources evaluated presented different performances as fertilizers with regard to dry matter production and P uptake by corn, soybean and millet crops (Fig. 6, Table A2). In the cultivation of corn, a direct relationship and a linear increase of the evaluated variables with the P dose was observed, except SS-PR. SSP caused greater dry matter production and P uptake by the plants compared with other fertilizers evaluated (Figs. 6a and b). In fact, PR acidulation caused a significant increase in dry matter production and P uptake compared to non-acidulated P sources; such an effect that was more pronounced for SS-PR. When applied a dose of 300 mg dm^{-3} P, SS-BPF caused an increase of 7.4- and 19.6-fold in dry matter production and P uptake, respectively, when compared to the SS-PR (Figs. 6a and b, A1). Application of SS-BPF presented a more pronounced effect than other treatments, except SSP; it caused the production of 77.2% dry matter and 80.9% of P uptake of the maximum obtained with SSP, when 300 mg dm^{-3} P dose was used (Table A1). The P uptake when M-BPF, M-PR and SS-PR were applied corresponded to 51.4, 26.9 and 4.5%, respectively, of the maximum P absorbed and accumulated when using SSP.

In the soybean crop, M-BPF and SS-BPF also showed lower agronomic performances than SSP, but were better than their corresponding non-acidulated phosphate rocks (Figs. 6c and d). Dry matter and P uptake caused by M-PR exhibited a similar trend to the one observed for M-BPF, with overall smaller values. For SS-PR, no significant increase in the evaluated variables was observed regardless the P dose utilized. M-BPF lead to higher increases in dry matter and P uptake than all other fertilizers tested, except by SSP, which caused an increase corresponding to 85.6 and 83.2%, respectively, of dry matter and P uptake obtained with SSP (155.9 mg dm^{-3} P).

In the millet cultivation, dry matter production was similar for all fertilizers, except for SS-PR, which did not cause any increase when compared to the control treatment without P application (Fig. 6e). SS-BPF caused the highest dry matter production (100.4% when compared to the maximum observed with SSP at 300 mg dm^{-3} P), followed by M-PR (91.4%), M-BPF (88.1%) and SS-PR (10.4%) (Table A1). With respect to P uptake, the linearly increased with an increase in the P dose utilized. SS-BPF lead to the highest P uptake (70.6% compared to SSP) whereas the treatment with SS-PR did not differ from the control without fertilizer application (0.9% in comparison to SSP) (Fig. 6f). M-BPF caused the second largest increased in P uptake (52.6% with respect to SSP).

Overall, at the end of cropping system, the acidulated P-fertilizers, M-BPF and SS-BPF, caused similar increases in accumulated dry matter of corn, soybean and millet (Fig. 6g). However, application of SSP resulted in the highest increase in dry matter production by all three crops when compared to other fertilizers evaluated. For the *in natura* sources, M-PR performed better than SS-PR, to which the dry matter production by the crops was similar to that of the control treatment without P application. At P dose of 300 mg dm^{-3}, the acidulated rocks presented similar effects on dry matter production; for SS-BPF the value was 66.9% and for M-BPF 64.1% in comparison to that of SSP (Table A1). While M-PR caused 49.3%, SS-PR caused 13.4% of the maximum dry matter production obtained when SSP was applied. The accumulated P uptake (Fig. 6h) showed a linear response with the increase of P in soil, except for SS-PR, which did not cause any P uptake increase with an increase in the P dose applied. Rocks acidulation with biogenic acid caused 16.5- and 1.4-fold increases in P uptake in relation SS-PR and M-PR were used, respectively. In addition, SS-BPF (75.6% when compared to SSP at 300 mg dm^{-3} P) performed better than M-BPF (50.9%) when P uptake by the crops was considered.

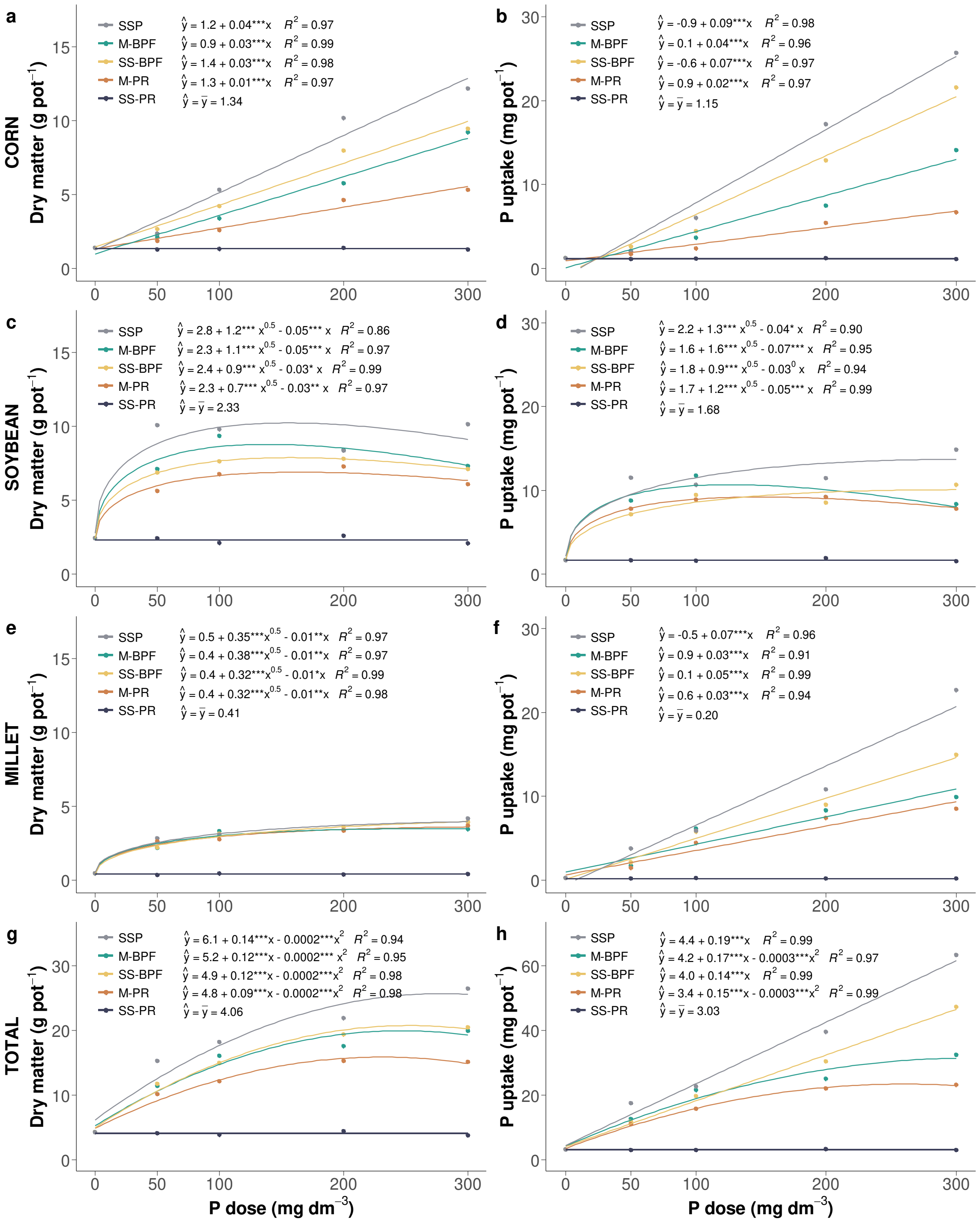

Fig. 6. Effect of P fertilizers of different solubility (SSP, single superphosphate; M-BPF, Morocco biogenic P-fertilizer; SS-BPF, Serra Salitre biogenic P-fertilizer; M-PR, Morocco phosphate rock; SS-PR, Serra Salitre phosphate rock) at different P doses on dry matter production and P uptake by corn (a, b), soybean (c, d) and millet (e, f) crops, and total accumulated after sequential crop cultivation (g, h). ^{0}, *, **, ***: significant at 10, 5, 1, and 0.1 % (t-test), respectively.

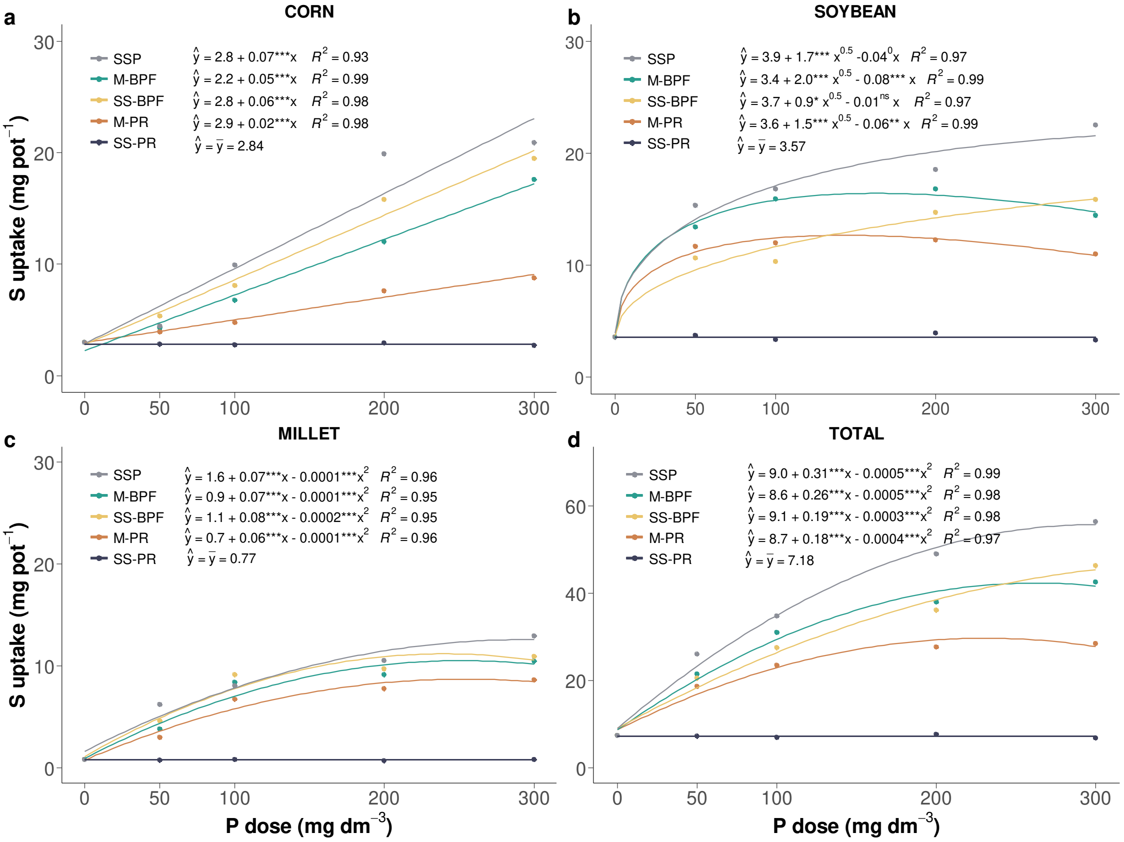

Fig. 7. Effects of rate of P fertilizers with different content of S (SSP, single superphosphate; M-BPF, Morocco biogenic P-fertilizer; SS-BPF, Serra Salitre biogenic P-fertilizer; M-PR, Morocco phosphate rock, SS-PR, Serra Salitre phosphate rock) on the S uptake by corn (a), soybean (b) and millet (c) crops and total accumulated after the sequential crop cultivation. ^{0}, *, **, ***: significant at 10, 5, 1, and 0.1 % (t-test), respectively.

The P sources used in this study possessed different S contents, which was reflected in the S uptake by the crops (Fig. 7). The cumulative S uptake was higher when SSP was used, followed by the biogenic P-fertilizers and M-PR; application of SS-PR did not cause alterations in S uptake by corn, soybean and millet.

The highest P content in soil after corn and soybean cultivation (Fig. 8) was obtained with the M-PR treatment, followed by its acidulated form and residual effects for the next crop were observed. In contrast, SS-PR did not cause any alteration in the soil P content throughout the time of cultivation of the first two crops.

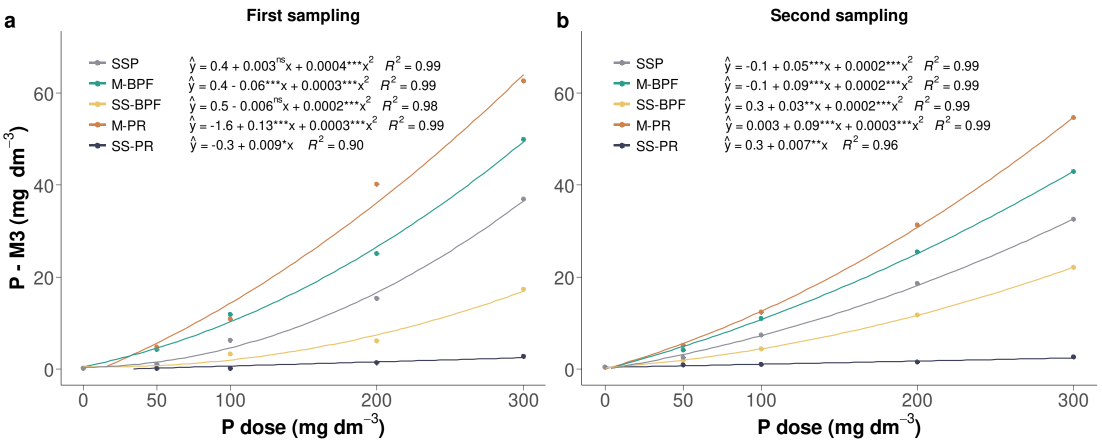

Fig. 8. Available P content in the soil as a function of the P dose, when SSP (single superphosphate), Morocco (M-BPF) and Serra Salitre (SS-BPF) biogenic P-fertilizers, and Morocco (M-PR) and Serra Salitre (SS-PR) phosphate rocks were used for successive corn, soybean and millet cultivation. (a) After corn and soybean; (b) after millet cropping at the end the experiment. ^{0}, *, **, ***: significant at 10, 5, 1, and 0.1 % (t-test), respectively.

4. Discussion

4.1. Effect of biogenic and commercial sulfuric acid on phosphate rocks dissolution

During P fertilizer production, PR are usually acidified with sulfuric acid using a high-cost technology (Nahas et al., 1990). This chemical process is applied to rocks with relatively high P content (Priha et al., 2014). However, biotechnological processes, involving the use of biogenic sulfuric acids produced by *A. thiooxidans* represent a more economical and environmentally friendly route for P-fertilizers production from rocks with low P content. Indeed, it is well known that this bacterium has the ability to cause the oxidation of reduced sulfur species, generating acidity as a product (Lee et al., 2000; Pepper and Miller, 1978; Rawlings et al., 2003; Zhang et al., 2016).

The effects of acid treatment on dissolution of apatite sources are well known. Calcium phosphate species are highly soluble in acid solutions (Becker, 1989; Chien et al., 2011), as demonstrated by Santos et al. (2016) from Leikam and Achorn (2005) data in the production of single superphosphate (Eq 1). The amounts of extractable P in water for both PRs tested here was low and statistically not different, because of the predominance of low water soluble P species in these rocks, such as tricalcium phosphate, fluorapatite and hydroxyapatite (Dorozhkin, 2011; Haynes, 1982). The higher soluble CA-P and AAC-P contents of the M-PR and SS-PR compared to water-P were expected due to the proton supply by the acid extractors.

$$2\,Ca_5(PO_4)_3F + 7\,H_2SO_4 + 3H_2O \rightarrow 7CaSO_4 + 3Ca(H_2PO_4)_2.H_2O + 2HF \quad \text{(Eq 1)}$$

The higher solubility of M-PR and its acidulated products treated with acid extractors (CA and AAC) as compared to SS-PR-based products is supported by some properties of these rocks. Brazilian phosphate deposits, such as those of SS-PR, are predominantly of igneous origin, and exhibit low reactivity due to the high crystallinity of their apatites (XRD data) (Abram et al., 2011). In contrast, the high reactivity of M-PR, which has an influence on extractable P forms, can be attributed the lower crystallinity of its apatites (Lehr and McClellan, 1972; Rajan et al., 1996). High levels of isomorphic replacement of phosphate by carbonate anions in apatites from M-PR and high porosity of their structures are key attributes for increasing the reactivity of this phosphate source (Lehr and McClellan, 1972; Rajan et al., 1996).

Superior performance of biogenic compared to commercial sulfuric acid for some treatments in terms of extractable P species can be attributed to the production of unknown bacterial metabolites, which may provide additional effects on the phosphates dissolution. Similar results were found by Calle-Castañeda et al. (2018b) using biogenic acid from *A. thiooxidans* to recover phosphorus from a phosphate rock.

4.2. Effect of acid treatments on phosphorus species

Production of biogenic P-fertilizers using a two-step biotechnological process is viable and eliminates physical-chemical relationships that could influence the growth of the acidophilic bacteria during solubilization; for example, the increase in the pH of the culture medium due to the presence of carbonates contained in the M-PR (U.S. Geological Survey, 2003).

Mineralogical changes in the biogenic P-fertilizers were observed with respect to their source of phosphate rocks. Decreases in intensity of peaks and bands associated with phosphate species and alterations in surface properties of the rocks were caused by the acid attack (Niu et al., 2014). However, no monocalcium or dicalcium phosphate species was detected in XRD, likely because these species are formed with a low degree of structural organization, and are not detectable by the techniques here utilized. During the acidulation process of reactive and non-reactive phosphates, the formation of more soluble Ca phosphate species occurs, such as amorphous Ca phosphate, monocalcium and dicalcium phosphates, detected by Santos et al. (2016) using the X-ray absorption near edge structure.

The presence of sulfur forms in the P-fertilizers was detected in the analyses performed. Besides, for M-BPF, the intensity of a band from carbonate species decreased since in the acid medium, most of the carbonate was solubilized, which agrees with previous results of a two-step process using biogenic acid (Calle-Castañeda et al., 2018b).

4.3. Plant response to P sources

The higher agronomic efficiency of M-PR when compared to SS-PR is attributed to a less crystalline apatite in the former (Lehr and McClellan, 1972; Rajan et al., 1996), which makes it more soluble in weak acids, such as 2% citric acid. Besides, M-PR has higher reactivity, a factor that determines the effectiveness of applying non-acidulated PR directly to the soil (Chien and Hammond, 1978; Lehr and McClellan, 1972), and consequently, its higher agronomic performance (Chien, 1977; Chien and Black, 1976). On the other hand, the low reactivity of the SS-PR rock was not favorable to cause increases in dry matter production and P uptake by corn, soybean and millet, making it relatively unsuitable as a P-supply when directly applied in its non-acidulated form.

The biogenic P-fertilizers exhibited better agronomic performances than the phosphate rocks due to their increased solubility promoted by the acid attack, which was reflected in higher extracted CA-P (Table 1), dry matter production and P uptake (Fig. 6). As expected, the CA-P in 2% citric acid of M-BPF was higher than that of SS-BPF due the characteristics of the rock, as aforementioned. However, the effect of acidulation in increasing P available for the crops, as estimated by the amount of CA-P, was more notorious for SS-PR; the increase was 14.4-fold for SS-PR whereas for M-PR it was only 2.3-fold with respect to their acidulated fertilizers. Nonetheless, the CA-P for M-BPF was 1.2-fold higher than that for SS-BPF.

The small difference in CA-P and the similar agronomic efficiency of the biogenic P-fertilizers produced from the Serra Salitre and Morocco rocks are attributed to the greater acidity available to solubilize the apatite of the former. PR from sedimentary origin contain calcareous ores as major impurities (U.S. Geological Survey, 2003). Thus, part of the acidity added may have been consumed by carbonates, reducing the number of protons available to attack the mineral structure and interfering with the solubilization of Morocco PR minerals. Hence, an additional amount of acid is required to overcome the effect of free carbonates in this rock during the industrial manufacture of phosphoric acid and superphosphates (Gharabaghi et al., 2010). Also, the gypsum formed during the reaction of the PR with sulfuric acid can coat the PR particles, affecting the acidulation rate, influencing the quality of the products and reducing the agronomic performance (Al-Fariss et al., 1991; Léon et al., 1986).

Biogenic phosphate fertilizers produced from Morocco and Serra Salitre PR using acid from *A. thiooxidans* displayed better agronomic performances than their source phosphate rocks; the most pronounced effect was obtained with SS-PR. An important consideration in relation to biogenic P-fertilizers is the amount of P forms that is readily available to the plants and also in the forms of less solubility. This is important in soil conditions with high P-fixing capacity in which soluble P fertilizers have low agronomic efficiency (McLean and Logan, 1970). Some studies have demonstrated equal or greater efficiency of partially acidulated phosphate rocks in relation to superphosphates under conditions of acidic soils with high P-fixation capacity (McLean and Balam, 1967; McLean and Logan, 1970; McLean and Wheeler, 1964). To increase the fertilizations efficiency it is necessary to consider other factors besides higher solubility of P fertilizers, such as management practices and species selection, which also influence the availability of nutrients for the plants (Bolan et al., 1990; Chien and Menon, 1995).

The P uptake by the crops decreased the soil availability of this nutrient after the successive corn, soybean and millet cropping. However, a residual effect of the fertilizers was verified. At the end of the experiment, M-PR, followed by M-BPF, were the sources that promoted the highest contents of P in the soil. M-PR did not supply as much P to the crops as the soluble and partially acidified sources, but it increased the amount of available P in soil over time due the continuous dissolution of the PR (Sale and Mokwunye, 1993). Thus, this fertilizer may have agronomic importance when application frequency is considered.

Our work shows a new potential of biogenic acid to solubilize apatite-based P sources with significant economic and environmental advantages, which can be used in the production of fertilizers. It is especially important for ores of low technological quality, as many deposits that occur in Brazil, are characterized by the presence of high concentrations of Si and Fe. Developing bioreactor systems and assaying different atmosphere conditions to optimize acid production are some examples of future investigation with the final goal to set up an industrial process. On the other hand, to determine the stoichiometry of the reaction for a desirable degree of solubilization is needed, considering that a partially solubilization can be enough to get an phosphate fertilizers efficiently produced by the biological process.

5. Conclusions

Biogenic sulfuric acid from *A. thiooxidans* offers an attractive alternative pathway to increase phosphate rocks solubility and can be used to produce acidulated phosphate fertilizers. The biogenic acid changed the rocks structure and mineralogical properties as observed by SEM, XRD, FTIR; it also changed the rocks color. Furthermore, the biogenic acid was efficient to increase water- or citric acid-soluble P to an extent similar to those obtained with traditional chemical processes. The biogenic phosphate fertilizers provided P and S for a successive corn-soybean-millet cultivation. The biotechnological process here described offers novel pathways to achieve a more sustainable agriculture through the use of nonconventional phosphorus resources and environmentally friendly fertilizer production routes.

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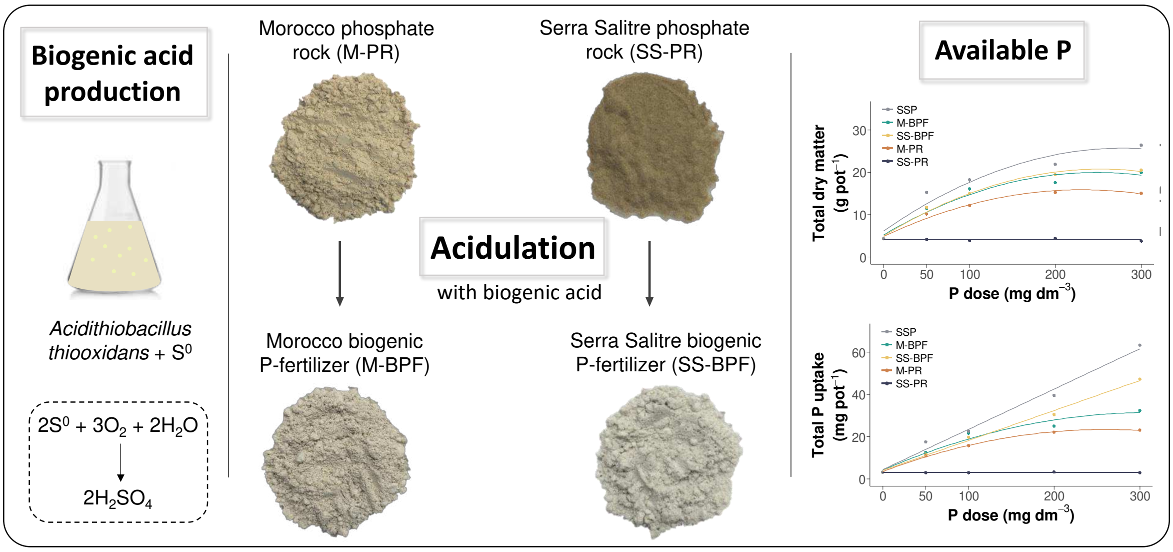
Graphical abstract



Supplementary material

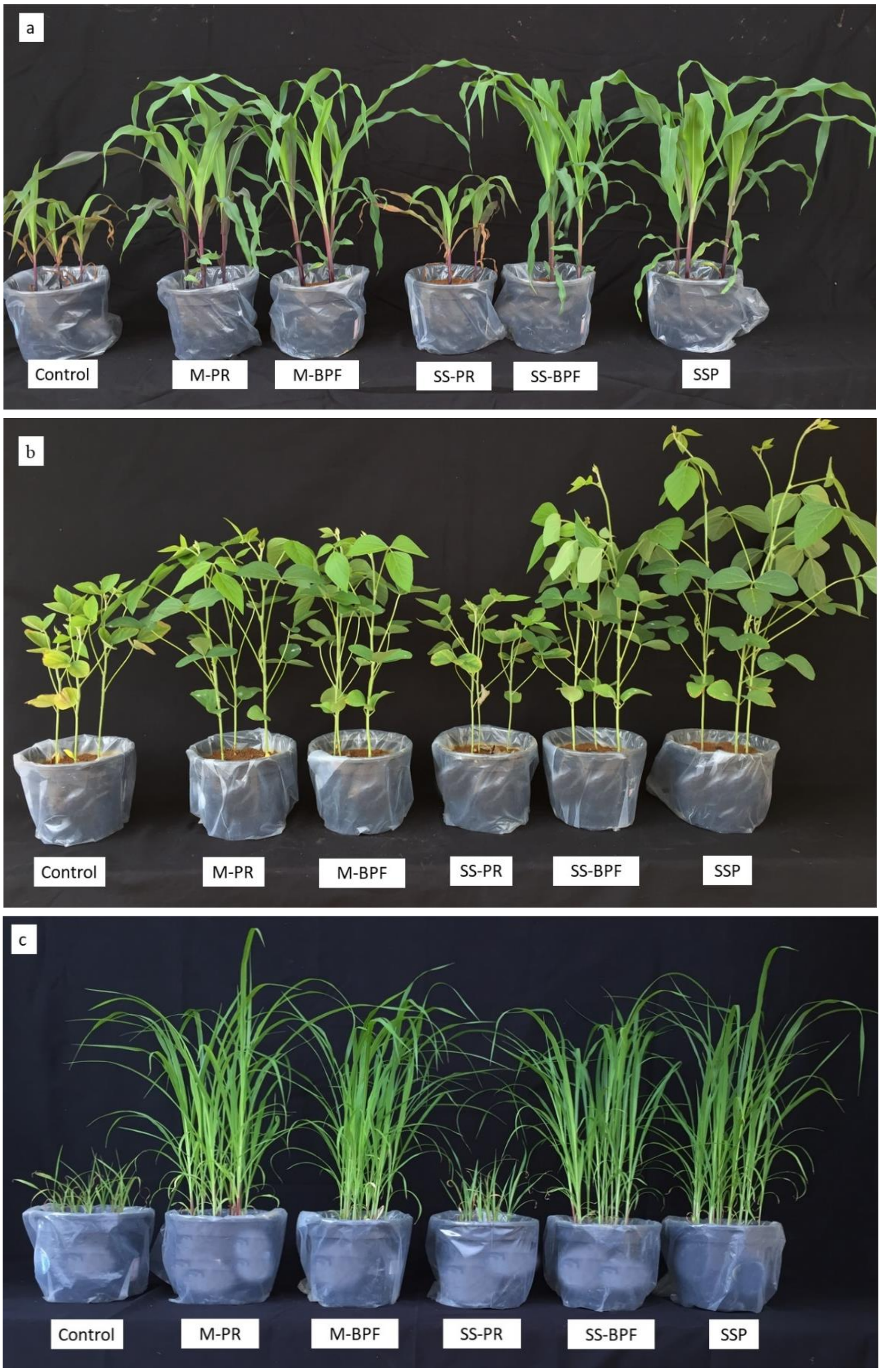


Fig. A1. Response of corn (a), soybean (b) and millet (c) plants to application of Morocco (M-PR) and Serra Salitre (SS-PR) phosphate rocks, Morocco (M-BPF) and Serra Salitre (SS-BPF) biogenic P-fertilizers, and single superphosphate (SSP) to the soil at a dose of the 300 mg dm^{-3} P. The control treatment received no P aplication. Pictures were taken at 32, 43 and 30 days after sowing for corn, soybean and millet, respectively.

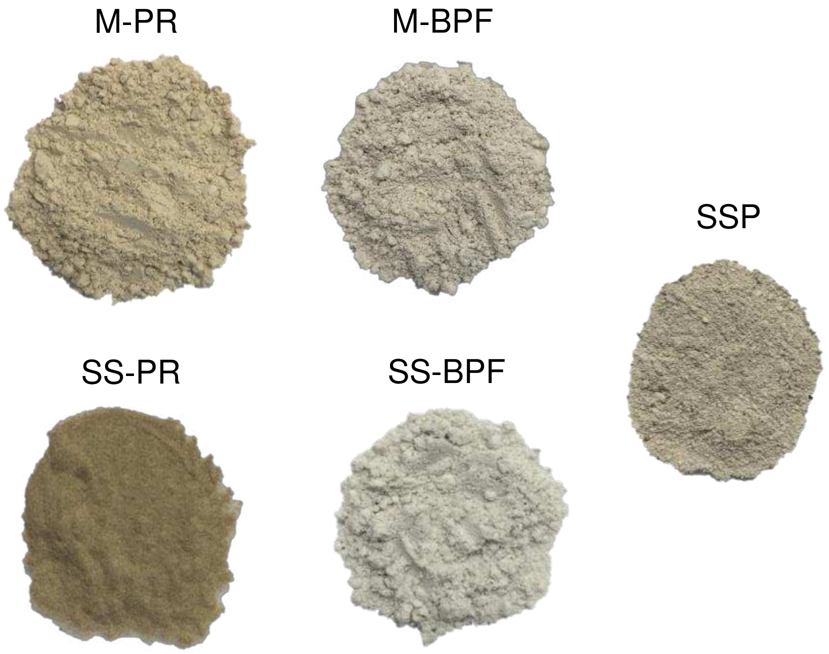


Fig. A2. Macroscopic appearance of Morocco (M) and Serra Salitre (SS) phosphate rocks before (PR) and after acidulation (BPF) with biogenic acid, and single superphosphate (SSP). BPF = biogenic P-fertilizer.

Table A1. Percentage of dry matter production and P uptake obtained for Morocco PR (M-PR) and its derived biogenic P-fertilizer (M-BPF); Serra Salitre PR (SS-PR) and its derived biogenic P-fertilizer (SS-BPF) in relation to single superphosphate (SSP), at the fertilizer dose of maximum dry matter production.

Fertilizer	Dry matter	P uptake
	------------- % -------------	
	CORN	
SSP	100	100
M-BPF	68.4	51.4
SS-BPF	77.2	80.9
M-PR	43.1	26.9
SS-PR	10.4	4.5
	SOYBEAN	
SSP	100	100
M-BPF	85.6	83.2
SS-BPF	77.1	74.2
M-PR	67.4	72.4
SS-PR	22.8	13.2
	MILLET	
SSP	100	100
M-BPF	88.1	52.6
SS-BPF	100.4	70.6
M-PR	91.4	45.3
SS-PR	10.4	0.9

	TOTAL	
SSP	100	100
M-BPF	64.1	50.9
SS-BPF	66.9	75.6
M-PR	49.4	37.9
SS-PR	13.5	4.9

Table A2. Summary of the analysis of variance (*p*-value) for dry matter mass (DM), phosphorus accumulated plant (K upt.) and S (S upt.) uptake for corn, soybean and millet.

Source of variation	DF	CORN			SOYBEAN		
		DM	P upt.	S upt.	DM	P upt.	S upt.
Block	3	0.007	0.044	0.005	0.094	0.001	0.055
Fertilizer	4	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Dose	3	< 0.001	< 0.001	< 0.001	0.608	0.418	0.090
Fertilizer × Dose	12	< 0.001	< 0.001	< 0.001	0.829	0.407	0.450
Additional × Factorial	1	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Residuals	60						
CV (%)		31.05	20.97	16.17	34.18	31.25	11.88

Source of variation	DF	MILLET			TOTAL		
		DM	P upt.	S upt.	DM	P upt.	S upt.
Block	3	< 0.001	0.005	< 0.001	0.346	0.032	0.026
Fertilizer	4	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Dose	3	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Fertilizer × Dose	12	0.1047	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Additional × Factorial	1	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Residuals	60						
CV (%)		55.99	44.84	35.13	14.68	6.90	6.46

Table A3. Summary of the analysis of variance (p-value) for available P content in soil after corn and soybean cultivation (first sampling) and after millet cropping at the end the experiment (second sampling).

Source of variation	DF	First sampling	Second sampling
Block	3	0.456	0.168
Fertilizer	4	< 0.001	< 0.001
Dose	3	< 0.001	< 0.001
Fertilizer × Dose	12	< 0.001	< 0.001
Additional × Factorial	1	< 0.001	< 0.001
Residuals	60		
CV (%)		17.10	12.38

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GENERAL CONCLUSIONS

The acidulation process of phosphate and potassium rocks with sulfuric acid increased the levels of P soluble in water, citric acid and ammonium acid citrate.

The biogenic sulfuric acid produced by *A. thiooxidans* increased the reactivity of natural phosphate and silicate rocks.

The agronomic performance of P and K biogenic fertilizers supplied P or K to the plants, increasing dry matter mass production as well as absorption and accumulation of these elements by corn, soybean and millet plants.

The alternative route for P fertilizers production using biogenic acid to increase the solubility of low- and high-reactivity rocks was efficient and has potential for application on an industrial scale.