ALINE DO AMARAL LEITE

SOLUBILIZATION AND MINERALIZATION OF PHOSPHORUS BY SELECTED BACTERIAL STRAINS IN BIOCHAR ENRICHED WITH ROCK PHOSPHATE

LAVRAS – MG

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> Dissertation presented to the Federal University of Lavras, as part of the requirements of the graduate Program in Soil Science, area of concentration in Soil Fertility and Plant Nutrition, to earn the title of Master.

Prof. Dr. Leônidas Carrijo Azevedo Melo

Advisor

Profª Dr. Fatima Maria de Souza Moreira

Co-advisor

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Dr. Ederson da Conceição Jesus   EMBRAPA AGROBIOLOGIA

Dr. Teotônio Soares de Carvalho   UFLA

Dr. Fatima Maria de Souza Moreira   UFLA

Dr. Leônidas Carrijo Azevelo Melo

Advisor

Dr. Fatima Maria de Souza Moreira

Co-advisor

LAVRAS – MG

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To God, for everything I have achieved and overcome in life;

To my parents, Amélia S. Amaral. and Aurelino A. Leite and my brother João Paulo A. Leite, for the love and support in all moments;

To my stepfather, for the unconditional support.

I DEDICATE.

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GENERAL ABSTRACT

Biochar-based fertilizers (BBF) are nutrient enriched biochar that may increase nutritional efficiency of plants, especially in tropical soils. Biomass impregnated with phosphates sources prior to pyrolysis increase biochar yield and carbon stability. However, when rock phosphate is added as a phosphorus (P) source, P solubility is very low and may not supply plant requirement. An environmentally friendly alternative to increase its solubility is to use selected bacterial strains that solubilize or mineralize phosphates, which has not been explored in these type of materials so far. The aim of this study was investigate solubilization and mineralization of P in BBF enriched with Bayóvar rock phosphate by selected bacterial strains. We also aimed to evaluate the agronomic efficiency of BBF in maize (*Zea mays*) growth under bacterial inoculation. The BBF was characterized and its P solubility was studied in an *in vitro* assay using five selected strains: UFLA 03-09 (*Acinetobacter* sp.), UFLA 04-155 (*Burkholderia funforum*), UFLA 03-10 (*Paenebacillus kribbensis*), UFLA 03-116 (*Paenibacillus* sp.) and UFPI B5-8A (*Pseudomonas* sp.). Based on this assay, three strains (UFLA 03-10, UFLA 04-155 and UFPI B5-8A) were chosen to test the agronomic efficiency of BBF in maize. Five doses of P (0; 50; 100; 200 and 400 mg kg^{-1} based on total P) from the BBF were applied. A positive control (200 mg kg^{-1} P as triple superphosphate) was also included. Shoot dry matter, P accumulation in shoot and agronomic efficiency index were evaluated. After cultivation, soil pH, available P and activity of acid and alkaline phosphatase in soil were determined. Bacterial strains solubilized P in the *in vitro* assay, which was closely associated with a pH decrease and the types of organic acids produced. A significant interaction between P doses and bacterial inoculation was observed for available P in soil. Inoculation increased shoot dry matter and P doses increased shoot dry matter, P accumulation in shoot, soil pH and phosphatase activity in soil. Positive control presented higher agronomic efficiency index than BBF. These results indicate that inoculation with selected bacterial strains combined with BBF fertilization is an inexpensive and sustainable strategy for improving maize growth and enhancing available P in soil. However, further studies must be carried out to improve the P use efficiency from low availability P sources.

Key words: Bayóvar. Biochar-based fertilizer. *Zea mays*.

RESUMO GERAL

Fertilizantes à base de biocarvão (BBF) são biocarvões enriquecidos em nutrientes que podem aumentar a eficiência nutricional das plantas, especialmente em solos tropicais. A biomassa impregnada com fontes de fosfatos antes da pirólise aumenta o rendimento do biocarvão e a estabilidade do carbono. No entanto, quando o fosfato de rocha é adicionado como uma fonte de fósforo (P), sua solubilidade é muito baixa e pode não suprir a necessidade da planta. Uma alternativa ambientalmente amigável para aumentar sua solubilidade é o uso de selecionadas estirpes bacterianas que solubilizam ou mineralizam os fosfatos, que até agora não foram explorados neste tipo de material. O objetivo deste estudo foi investigar a solubilização e mineralização de P em BBF enriquecido com fosfato de rocha de Bayóvar por estirpes bacterianas. Objetivou-se também avaliar a eficiência agronômica do BBF no crescimento de milho (*Zea mays*) sob inoculação. O BBF foi caracterizado e sua solubilização de P foi estudada em um ensaio in vitro utilizando cinco estirpes selecionadas: UFLA 03-09 (*Acinetobacter* sp.), UFLA 04-155 (*Burkholderia funforum*), UFLA 03-10 (*Paenebacillus kribbensis*), UFLA 03-116 (*Paenibacillus* sp.) e UFPI B5-8A (*Pseudomonas* sp.). Com base neste ensaio, três estirpes (UFLA 03-10, UFLA 04-155 e UFPI B5-8A) foram escolhidas para testar a eficiência agronômica do BBF no cultivo de milho. Cinco doses de P (0; 50; 100; 200 e 400 mg kg^{-1} calculadas com base no P total) foram aplicadas via BBF. Um controle positivo (200 mg kg^{-1} P fornecido como superfosfato triplo) também foi incluído. A matéria seca da parte aérea, o acúmulo de P na parte aérea e o índice de eficiência agronômica foram avaliados. Avaliou-se a matéria seca da parte aérea, o acúmulo de P na parte aérea e o índice de eficiência agronômica. Após o cultivo, determinou-se o pH do solo, a disponibilidade de P e atividade da fosfatase ácida e alcalina no solo. As cepas bacterianas solubilizaram o P no ensaio in vitro, que esteve intimamente associado à diminuição do pH e aos tipos de ácidos orgânicos produzidos. Houve interação significativa entre as doses de P e a inoculação bacteriana para o P disponível no solo. A inoculação aumentou a matéria seca da parte aérea e as doses de P aumentaram a matéria seca da parte aérea, o acúmulo de P na parte aérea, o pH do solo e a atividade da fosfatase no solo. O controle positivo apresentou maior índice de eficiência agronômica que o BBF. Estes resultados indicam que a inoculação com estirpes bacterianas combinadas com a fertilização com BBF é uma estratégia barata e sustentável para melhorar o crescimento do milho e aumentar o P disponível no solo. No entanto, mais estudos devem ser realizados para melhorar a eficiência do uso de P de fontes de baixa disponibilidade.

Palavras-chave: Bayóvar. Fertilizantes a base de biocarvão. *Zea mays.*

SUMMARY

GENERAL INTRODUCTION

Phosphorus (P) is a major growth-limiting nutrient for food production. The main P sources for phosphate fertilizers production are phosphate rocks, which are finite, scarce and cannot be replaced (SCHOLZ et al., 2013). P has a complex dynamic in soils, mainly in tropical soils due to acidity and high levels of Fe and Al oxides, which causes high P sorption rendering low availability to plants (ABDALA et al., 2015; NOVAIS; SMYTH, 1999). Thus, constant and high fertilization rates are necessary in order to achieve good yields and to compensate the low P residual effect and the amount exported by the crops.

In Brazil, intensive cultivation systems are increasing, requiring greater amounts of soluble phosphate fertilizers which presents a low P recovery efficiency. Thereby, phosphate fertilizers, particularly in the soluble form, are required in high doses to achieve production similar to temperate regions (ROY et al., 2016; ZAPATA; ROY, 2004). To increase P use efficiency and to minimize P losses, it is necessary to develop fertilizers that present a greater residual effect.

To overcome this problem, recycling P from residues, such as compost may be interesting as a P source besides reducing the input of chemical phosphate fertilizers (BUSATO et al., 2012). Composts generated from animal manures can present a relatively high level of P and when incorporated to soil it can improve soil physical, chemical and biological properties and provide restoration of organic carbon (DARBY et al., 2016; SINGH; NAIN, 2014; WEI et al., 2015). However, despite the advantages of compost addition to soils, some negative impacts can occur in the environment mainly related to substantial amounts of greenhouse gases release during its production and use (DARBY et al., 2016; FERREIRA et al., 2018; YUAN et al., 2017).

Lately, many studies had been conducted to produce more stable materials such as biochar which feature less environmental problems (LI et al., 2014; SINGH; COWIE; SMERNIK, 2012). Biochar has been shown to be a promising alternative for P supply, increasing nutrient use efficiency and crop production, furthermore, presenting good agronomic efficiency in soils with low natural fertility (CHEN et al., 2016; DING et al., 2010; LEHMANN et al., 2011; PUGA et al., 2015). However, its influence on soil and nutrient availability depends mainly on the feedstock and the pyrolysis conditions used (SILVA et al., 2017). Biochar improves a wide variety of soil properties such as pH, aggregation, water retention capacity and nutrient availability and increase stable forms of C in soil (SINGH; COWIE; SMERNIK, 2012; VANEK; LEHMANN, 2015).

Many studies have already been carried out aiming to understand the transformations provided by biochar use in soil. These transformations are mainly related to the improvement of soil conditioning that leads to changes in physical-chemical and biological characteristics and better structuring of the microbial community, which provides greater nutrients availability to plants (FOX et al., 2016). It has also been demonstrated that biochar addition resulted in increasing of microbial biomass, causing greater P availability, mostly due to P mineralization and solubilization (FOX et al., 2014; LIU et al., 2017; LU et al., 2015).

Biochars, in general, present low P concentration as compared to conventional soluble P sources. Besides, P release is slow and provide a low P availability to plant uptake (BORNØ; MÜLLER-STÖVER; LIU, 2018; NELSON et al., 2011). Many mechanisms are responsible for the slow release of P, but mainly the low solubility P forms after pyrolysis and the high pH when added to the soil (BRUUN et al., 2017). Therefore, most studies focus on the use of biochar as a soil amendment, which requires application of high doses, and its fertilizer effect is considered as secondary (BAIAMONTE et al., 2015; DING et al., 2016; JIANG et al., 2012; LIU et al., 2012). This high dose requirement makes biochar unfeasible at large scale due to the relatively high costs of biochar production (VOCHOZKA et al., 2016).

One alternative to overcome this situation is to enrich the biomass prior to pyrolysis with plant nutrients aiming to apply doses as low as 500 kg ha^{-1} of biochar (JOSEPH et al., 2013). It has been shown that P enrichment in the biomass prior to pyrolysis with soluble P sources (e.g. phosphoric acid, triple superphosphate or monoammonium phosphate - MAP) causes an increase in the carbon stability and yield of the resulting biochar (CARNEIRO et al., 2018; ZHAO et al., 2014, 2016). Lustosa Filho et al. (2017) reported that phosphate-enriched biochar, giving rise to biochar-based fertilizers (BBF) has the potential to be used as a slow-release fertilizer providing similar effects as soluble phosphate fertilizers. However, biochar-based rock phosphate fertilizer can be attractive from an environmental point of view because it is a raw material, but still not well understood and explored so far. Fertilization promoted by rock phosphate sources presented favorable residual effects consistent with the nutritional demands of crops in succession (MARTINS et al., 2017). However, in order to improve rock phosphate efficiency, reduced particle sizes combined with microorganisms constitute promising strategies for P solubilization, even in a short period of time (KLAIC et al., 2017).

Bayóvar rock phosphate is formed by the deposition and decomposition of marine animals remains and comes from the region of Bayóvar (Sechura) in Peru. Currently, Bayóvar rock phosphate is mainly used for the manufacturing of soluble phosphate fertilizers. The raw material receives treatment in order to increase the concentration of P$_2$O$_5$, posteriorly receives

the sulfuric acid attack arising the highly soluble phosphate fertilizers (MATTIELLO et al., 2016). In previous studies, rice crop inoculated with phosphate-solubilizing bacteria in an Oxisol fertilized with Bayóvar rock phosphate, presented a significant P availability in soil comparable to soluble-P sources and likewise resulting in a considerable nutrient accumulation in the plant tissues (COSTA et al., 2015). Therefore, it becomes an economically cheaper combination that is efficient and sustainable, capable of increasing plant growth and soil fertility and also as an alternative to soluble phosphate fertilizers (LAVAKUSH et al., 2014).

Several microbial groups have the ability to solubilize inorganic phosphates, which vary according to the microbial community and the culture medium used. Some bacteria genera, such as *Bacillus*, *Pseudomonas* and *Enterobacter* are reported to be efficient in solubilizing inorganic P, increasing its availability, leading to a higher crop yield (KHAN et al., 2009). In addition, rock phosphate fertilization also provides changes in the microbial community, stimulating P cycling (TRABELSI et al., 2017). Several mechanisms explain the solubilization of inorganic P and mineralization of organic P by the microbial community. Organic acid production and extrusion of protons are noteworthy for the solubilization of inorganic P. The excreted protons remain surrounding the cell surface, which cause a decrease of pH (SCHOLZ et al., 2013). Simultaneous production of distinct organic acids of low molecular mass may contribute to a greater potential for solubilization. However, despite a decrease in pH influenced by the production of organic acids, acidification may not be directly related to phosphate solubilization and other mechanisms may be involved (MARRA et al., 2015).

Enzymatic activity is involved in P cycling as a mineralization mechanism. Microbial, plant and animal cells may release enzymes after cell death and lysis or due to physiological reasons, such as hydrolysis of polymers in oligomers or monomers (GIANFREDA; RUGGIERO, 2006). Phosphatase is related to P mineralization and can be increased in the rhizosphere in the presence of microorganisms, providing higher availability of soluble P (YOUNG et al., 2013). It is reported that bacteria is able to secrete various phosphatases in response to conditions of low soluble P content. However, the activity of acid and alkaline phosphatase may vary among bacteria genera or even among species of the same genera (NAHAS, 2002). Inoculated corn treatments with bacteria under conditions of P deficiency or in the presence of insoluble phosphate sources, presented higher activity of the enzyme phosphatase and increased P in the plant tissue as compared with control without inoculation (IBARRA-GALEANA et al., 2017; YOUNG et al., 2013).

Biochar addition to soil positively influences the plant growth and its use as biofertilizer can possibly become a promising technique for improving plant growth (SAXENA; RANA;

PANDEY, 2013). Recently, biochar associated with selected bacterial strains has been shown to be a favorable technique due to the increase in P availability under application of insoluble phosphate fertilizers (BEHESHTI; ETESAMI; ALIKHANI, 2017). Increased growth and nutrient accumulation (e.g. N, P and K) was also observed in maize inoculated in soil amended with biochar (RAFIQUE et al., 2017).

In general, there are few studies reporting on combined application of biochar and bacterial strains. To the best of our knowledge, there are no records on the application of BBF (enriched, prior to pyrolysis, with Bayóvar rock phosphate) as slow release P fertilizer in tropical soils, combined with selected bacterial inoculation. Thus, evaluating the agronomic efficiency as well as unraveling the mechanisms of P release is needed for this type of material. The advantage is its low production cost and addition of stable and functional carbon to the soil.

We hypothesized that selected bacterial inoculation will increase P solubilization supplied as BBF enriched with Bayóvar rock phosphate, resulting in higher plant yields and available P in soil. In the second part of this dissertation are presented in detail the results obtained with laboratory and greenhouse experiments performed to test this hypothesis.

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SECOND PART

ARTICLE

Selected bacteria enhance P availability in an Oxisol fertilized with biochar-based rock phosphate fertilizer

Preliminary Version

ABSTRACT

Co-pyrolysis of biomass and phosphates generates biochar-based fertilizer (BBF), which may increase plant phosphorus (P) use efficiency, especially in tropical soils, by preventing P fixation on oxides. However, when rock phosphate is used in co-pyrolysis, P release rate is slow and may not supply plant requirement. Therefore, bacterial inoculation may increase P release through solubilization and mineralization mechanisms. The aim of this study was investigate the mechanisms associated with the solubilization and mineralization of P in BBF enriched with Bayóvar rock phosphate under selected bacterial inoculation. Was also aimed to evaluate the agronomic efficiency of BBF under maize (*Zea mays*) inoculation. BBF was characterized and its P solubility and mineralization profile was studied in an *in vitro* assay using five selected bacterial strains: UFLA 03-09 (*Acinetobacter* sp.), UFLA 04-155 (*Burkholderia funforum*), UFLA 03-10 (*Paenebacillus kribbensis*), UFLA 03-116 (*Paenibacillus* sp) and UFPI B5-8A (*Pseudomonas* sp.). Later, the strains (UFLA 03-10, UFLA 04-155 and UFPI B5-8A) were chosen to test the agronomic efficiency index of BBF under greenhouse conditions using maize (*Zea mays*) as a plant test. Five doses of P (0; 50; 100; 200 and 400 mg kg^{-1} based on total P) from the BBF were applied. A positive control (200 mg kg^{-1} P as triple superphosphate) was included. Shoot dry matter yield, P accumulation in shoot and the agronomic efficiency index were evaluated. Soil was analyzed after cultivation for pH, available P and activity of acid and alkaline phosphatase. Bacterial inoculaton were effective in solubilizing P in the *in vitro* assay, which was closely associated with a pH decrease, likely due to the types of organic acids released. A significant interaction between P doses and bacteria inoculation was observed for available P in soil. Bacteria inoculation increased shoot dry matter and P doses increased shoot dry matter, P accumulation in shoot, soil pH and phosphatase activity in soil. Positive control presented higher agronomic efficiency index than the BBF in the same dose. These results indicate that bacterial inoculation and BBF fertilization is an inexpensive and sustainable strategy for improving maize plant growth and enhancing available P in soil. However, further studies must be carried out to improve P use efficiency from low availability P sources

Keywords Biochar. Rock phosphate. Plant growth

1 Introduction

Phosphorus (P) is one of the most limiting nutrients for crop development in tropical soil due to its strong interaction with aluminum and iron oxides. This soil may contain large amounts of total P in both organic and inorganic forms, however, it has a complex dynamic. Acidity and oxidic mineral composition results in low available P fraction for plant uptake (ABDALA et al., 2015; NOVAIS; SMYTH, 1999) rendering low efficiency of phosphate fertilizers, that must be compensated with high and constant application rates for profitable agricultural yields.

Acidified phosphate fertilizers, which present high water-soluble P content, are the main P sources used in agriculture (CHIEN et al., 2011) and are favorable to P losses by leaching in sandy soils or fixation in clay soils. However, due to the constant use of mineable rock phosphate reserves in the world, it is mandatory to optimize P recovery efficiency (CORDELL; DRANGERT; WHITE, 2009). The exploitation of P-rich organic wastes, such as poultry litter, could be an alternative P source to replace conventional phosphate fertilizers. Poultry litter is produced in large amounts in Brazil and worldwide and it is often misused, posing risks to the environment (ABDALA et al., 2012).

The conversion of poultry litter into biochar through pyrolysis is a promising alternative to sanitize, stabilize the carbon and cause a slow release of P (LUSTOSA FILHO et al., 2017; SINGH; COWIE; SMERNIK, 2012; WANG et al., 2015; ZHAO et al., 2016), which might improve its soil conditioning traits and fertilizer value. Application of biochar to soil improves several of its properties, such as pH, aggregation, water retention capacity and nutrient availability (VANEK; LEHMANN, 2015). Furthermore, it is also reported that biochar leads to changes in physical, chemical and biological characteristics and due to its porous condition it likely influences the structuring of the microbial community, resulting in greater nutrient availability to plants (FOX et al., 2016).

Generally, large amounts of biochar are used as soil conditioning, which might not be feasible at large scale use due to high costs (VOCHOZKA et al., 2016). To overcome this limitation, the enrichment of biomass with minerals prior to pyrolysis originates the biochar-based fertilizers (BBF), which has potential to be used as slow-release and high-agronomic performance fertilizers (LUSTOSA FILHO et al., 2017; ZHAO et al., 2016). Also Phosphate impregnation prior to pyrolysis has been shown to increase biochar yield and improve carbon stability measured by chemical and thermal oxidation methods (CARNEIRO et al., 2018). Available P fraction from BBFs is essential for defining the doses to be applied for crop

production (VANEECKHAUTE et al., 2016). BBFs dissolve more slowly than soluble phosphate fertilizers, and the dissolution rates varies among the types of phosphates used for biomass enrichment (LUSTOSA FILHO et al., 2017).

Biochar enrichment with reactive rock phosphate is an option to produce BBF, since it is a raw material and might be a relatively low cost P source. Bayóvar rock phosphate is a sedimentary rock that contains approximately 28% of P_2O_5 and 31% of calcium; it is insoluble in water and approximately 13% of P_2O_5 is soluble in citric acid (DIAS et al., 2014). Fertilization promoted by rock phosphate sources presented favorable residual effects consistent with the nutritional demands of crops in succession (MARTINS et al., 2017). However, due to its low solubility, an important method for increasing the utilization efficiency of rock phosphates is the use of phosphate-solubilizing microorganisms such as bacterial strains that could potentially increase the amount of available P to plant uptake (OLIVEIRA-LONGATTI et al., 2013, 2014; ESTRADA et al., 2013; PEREIRA; CASTRO, 2014).

Some bacteria genera, such as *Pseudomonas*, *Bacillus*, *Rhizobium*, *Agrobacterium*, *Burkholderia* and *Erwinia* are reported to be efficient in increasing P availability, leading to higher crop yields (KHAN et al., 2009; RODRÍGUEZ; FRAGA, 1999; YU et al., 2011). Bacterial strains can convert insoluble phosphates into available forms through the mineralization of the organic forms and solubilization of the inorganic P forms (JORQUEIRA et al., 2008; LIU et al., 2014; SHARMA et al., 2013). The abilities of solubilization and mineralization can coexist in the same bacterial strain (TAO et al., 2008).

Few studies on the combined use of biochar and bacterial inoculation have already been carried out (GŁODOWSKA et al., 2016; RAFIQUE et al., 2017; WEI et al., 2016). None of these studies, however, explored the effect of biochar-based Bayóvar rock phosphate fertilizer under maize plant inoculation in tropical soils. Additionally, it is important to unravel the mechanisms of P solubilization and mineralization in order to take advantage of this low cost and environmentally friendly technology.

This study aimed to evaluate: 1- the ability of five selected bacterial strains to solubilize and mineralize P from BBF produced from poultry litter enriched with Bayóvar rock phosphate in an *in vitro* assay. 2- the agronomic efficiency of BBF and inoculation with selected bacterial strains on maize cultivated in an Oxisol under greenhouse conditions.

2 Material and Methods

2.1 Biomass preparation and biochar-based fertilizer production

Poultry litter was collected from a farm near Lavras, Minas Gerais, Brazil (915 m altitude, 21°13'34" S and 44°58'31" W). The sample was air-dried at room temperature, passed through a 20-mesh sieve (1.00 mm) and thoroughly mixed with Bayóvar rock phosphate in powder form at the ratio of poultry litter residues/rock phosphate 1:0.5 (W/W), according to a previous study (LUSTOSA FILHO et al., 2017). Subsequently, the blend was moistened overnight to ensure greater uniformity and was oven-dried at 60 ºC to a constant mass prior to pyrolysis. The pre-treated sample was placed in a muffle furnace equipped with steel cylinders (10.6 cm diameter and 42 cm height), sealed and the temperature was raised to 500 °C at a heating rate of 10 °C min^{-1}, maintaining the target temperature for 2 h to allow sufficient time for complete carbonization (ZHAO et al., 2014). The condensable gases were collected and stored. BBF was sieved (< 2.0 mm) and characterized (TABLE 1). pH and electrical conductivity was determined according to Rajkovich et al. (2012) and cation exchange capacity according to Gaskin et al. (2013). The C content was determined in an automatic analyzer. Citric acid-soluble P_2O_5 was extracted by stirring the mixtures with a 2% citric acid solution. Neutral ammonium citrate solution (pH 7) was used to extract NAC-soluble P_2O_5. These analytical procedures were performed according to the official Brazilian fertilizer methods, Brasil (2014). Total Ca and Mg contents were determined by ICP-OES (Sprecto Analytical Instruments, Kleve, Germany) after sample preparation according to Enders and Lehmann (2012).

Table 1 - Selected properties of the biochar-based fertilizer.

pH	10.52 ± 0.03
EC (dS m^{-1})	2.1 ± 0.07
CEC (cmol$_c$ kg^{-1})	9.66 ± 1.27
C (%)	22.95 ± 0.04
P$_2$O$_5$ total (g kg^{-1})	265 ± 2.67
P$_2$O$_5$ NAC soluble (g kg^{-1})	58 ± 0.29
P$_2$O$_5$ citric acid (g kg^{-1})	59 ± 1.20
Ca (g kg^{-1})	273.60 ± 3.85
Mg (g kg^{-1})	6.88 ± 0.15
K (g kg^{-1})	20.85 ± 0.75
Al (g kg^{-1})	0.35 ± 0.01
Fe (g kg^{-1})	5.38 ± 0.06

*Mean value ± Standard Deviation. EC, electrical conductivity; CEC, cation-exchange capacity; NAC, Neutral ammonium citrate. C and CEC (n = 2), pH, EC, P, Ca, Mg, K, Fe and Al (n=3).
Source: From the author (2019).

2.2 Bacterial strains

Five selected bacterial strains belonging to the collection of the Laboratory of Soil Microbiology of the Department of Soil Science (Federal University of Lavras) were evaluated. *Paenibacillus kribbensis* (UFLA 03-10), *Pseudomonas* sp. (UFPI B5-8A), *Burkholderia fungorum* (UFLA 04-155), *Paenibacillus* sp. (UFLA 03-116) and *Acinetobacter* sp. (UFLA 03-09). Was accessed the origin, accession numbers of the 16S rRNA gene sequence, the *in vitro* solubilization capacity and plant growth promoting characteristics of theses strains (TABLE 2).

Table 2 – Origin, solubilization of $CaHPO_4$, $Al(H_2PO_4)_3$ and $FePO_4.2H_2O$ and plant growth promoting characteristics of the strains evaluated.

Strains	Origin	Identification	Accession No. In GenBank of the 16S rRNA sequences (NCBI)	Solubilization in solid* and liquid** medium [b,c,e]						Plant growth promoting characteristics [b,e]	
				$CaHPO_4$		$Al(H_2PO_4)_3$		$FePO_4.2H_2O$		IAA ($\mu g\ mL^{-1}$)	
										Tryptophan	
				*	**	*	**	*	**	+	-
UFLA 04-155	AM[a,b]	*Burkholderia fungorum*	GU144370	+	ND	+	ND	ND	ND	4.53	6.29
UFLA 03-10	MG[c]	*Paenebacillus kribbensis*	JQ041885	-	-	-	-	-	+	ND	ND
UFPI B5-8A	PI[d,e]	*Pseudomonas* sp.	Kj979613	+	ND	-	ND	-	ND	9.71	5.40
UFLA 03-116	MG[c]	*Paenibacillus* sp.	JQ041897	-	-	-	-	-	+	ND	ND
UFLA 03-09	MG[c]	*Acinetobacter* sp.	JQ041884	+	+	-	-	-	-	ND	ND

AM –Amazonas state; MG – Minas Gerais state; PI – Piauí state; ND – Not determined; IAA - Indole-3-acetic acid in DYGS medium without (-) and with (+) Tryptophan.
Source: [a] Silva et al. (2012); [b] Oliveira-Longatti et al. 2013; [c] Marra et al. 2012; [d] Costa et al. 2015; [e] Costa et al. 2016.

2.3 P Solubilization from BBF by bacterial strains

The capacity of the bacterial strains to solubilize P from the BBF was evaluated using a modified NBRIP culture medium (NAUTIYAL, 1999) with the following composition per liter of solution: 10 g of glucose; 5g de $MgCl_2$ $6H_2O$; 0.25 g de $MgSO_4$ 7H2O; 0.2 g de KCl; 0.1 g de $(NH_4)_2$ SO_4. The pH was adjusted to 7.0 before autoclaving.

Before inoculation the strains were cultured in liquid medium 79 (FRED; WAKSMAN, 1928) under stirring of 110 rpm at 28 °C until reaching the cell density of 1 x 10^8 per mL. Then, 1 mL of inoculum was added into 50 mL of NBRIP medium, containing 100 mg L^{-1} of P concentration from powder BBF. After, the flasks were incubated for 10 days at 28 °C at 120 rpm. Culture medium without inoculation was used as control. The assay was carried out in a completely randomized design, with six treatments and six replicates. At the end of the incubation period, samples were centrifuged at 3,000 rpm for 15 min and the supernatant was filtered through a 0.45 µm cellulosic membrane filter, being analyzed for pH and soluble P (ICP-OES). Part of the extract was stored at -80 °C for identification and quantification of organic acids and another part was used to determine the activity of the acid phosphatase.

The soluble P concentration was submitted to a linear correlation analysis with pH values, acid phosphatase activity and organic acids production. At the end of the experiment three strains were chosen to inoculate *Zea mays* under greenhouse condition in a P deficient Oxisol fertilized with BBF.

2.3.1 Identification and quantification of low molecular mass organic acids

An aliquot of 100 µL of the supernatant prepared as described above was used for identification and quantification of organic acids. The analyzes were performed using high performance liquid chromatography (HPLC) (Agilent 1220 Infinity). The samples were injected in a chromatographic column, model Synergil Hydro-RP 80A ($250 \times 4,6$ nmid; 4 mm). The run time was 20 min, at the flow rate of 0.7 mL min^{-1} at a wavelength of 220 nm. The mobile phase used was a KH_2PO_4, 20 mM L^{-1}, pH 2.9, used according to the analytical procedure indicated by the column manufacturer for the identification of organic acids.

Certified samples of acetic, formic, malonic, oxalic, quinic, shikimic, D-malic, maleic, succinic, citric and fumaric acids were used as analytical standards. The organic acids in the samples were quantified by calibration curves constructed with the analytical standards, relating the concentration of each acid with its respective area of absorption. Three replicates were used.

The identified molecules and standard mean retention times for the acid are as follows: oxalic (3.60 min), quinic (4.28 min), D-malic (5.28 min), acetic (7.31 min) succinic (11.26), fumaric (9.64 min), shikimic (6.19 min), maleic (8.55), citric (8.40 min), salicylic (3.70 min), propionic (17.29) and malonic (5.4 min) acids.

2.3.2 Acid phosphatase activity in liquid NBRIP medium

The activity of acid phosphatase was determined using the methodology proposed by Juma and Tabatabai (1988). One hundred microliters of the supernatant obtained as described previously were incubated at 37 ºC with 100 µL (25 mM) of ρ-nitrophenyl phosphate and 400 µL of modified universal buffer (pH 6.5.) After 1 h, the reaction was paralyzed by 400 µL NaOH (0.5 M). The absorbance of the yellow color developed after the incubation period was read in a spectrophotometer at 410 nm using a standard calibration curve of ρ-nitrophenol.

2.4 Greenhouse experiment

An experiment was carried out under greenhouse conditions at the Department of Soil Science of the Federal University of Lavras - UFLA, located at 21°14' 00" S and 45°00' W, with an average altitude of 918 m.

The experiment was conducted in a 5 x 4 + 1 factorial scheme, with 5 P concentrations supplied as BBF (0, 50, 100, 200 and 400 mg kg^{-1} P - based on total concentration), 4 types of inoculation (UFLA 03-10, UFLA 04-155 and UFPI B5-8A and without inoculation) plus a positive control of 200 mg kg^{-1} P supplied as triple superphosphate. Three bacterial strains were selected based on *in vitro* solubilization, as described previously. The experiments were conducted in a randomized block design, with four replicates.

Pots were filled with 3 kg of an Oxisol, collected from the 20-60 cm layer in Itumirim, Minas Gerais (21º 17' 16'' S e 44º 48' 07" W). The soil was air dried, sieved (<2.0 mm), homogenized, and chemically characterized (TABLE 3), according to the Brazilian routine soil testing. Briefly, P, Na, K and micronutrients were extracted by Mehlich-1, pH was determined in water, Ca, Mg and Al concentrations were extracted by KCl 1 mol L^{-1} and the organic matter content was determined by oxidation with $Na_2Cr_2O_7$ and H_2SO_4.

Table 3 - Soil characteristics before the experiment.

pH	P	K$^+$	Ca^{2+}	Mg^{2+}	Al^{3+}	H + Al	TB	t	T	V	m
	--- mg dm^{-3} ---		----------------------- cmol$_c$ dm^{-3} --------------------						--------------- % ----------------		
4.6	0.08	32.6	0.24	0.12	0.68	2.62	0.44	1.12	3.06	14.5	61
OM	P-rem	Zn	Fe	Mn	Cu	B	S	Sand	Silt	Clay	
- g kg^{-1} -	- mg L^{-1} -	----------------------------- mg dm^{-3} ----------------------------						---------- g kg^{-1} -----------			
7.9	32.5	0.53	46.6	1.81	0.39	0.04	2.68	730	40	230	

pH (H$_2$O); P (Mehlich-1); TB – Total base cations; t – Effective cation exchange capacity; T – Potential cation exchange capacity; V – index of base saturation; m – index of exchangeable acidity; MO – Organic matter; P-rem – remaining P.
Source: From the author (2019).

Liming was performed to increase the base saturation to 60% using calcium carbonate (CaCO$_3$) and magnesium carbonate (MgCO$_3$) at a 3:1 molar ratio. The soil was incubated for 20 days with a moisture of approximately 70% of field capacity. The following fertilization was performed in each pot (in mg kg^{-1}) applied as solution: 200 of N, 200 of K and 40 of S, provided as potassium nitrate (KNO$_3$), ammonium nitrate (NH$_4$NO$_3$) and ammonium sulphate [(NH$_4$)2SO$_4$)], and 1.5 of Cu, 3.5 of Mn, 5.0 of Zn, 0.8 of B, 0.1 of Mo and 3.0 of Fe, provided as copper sulfate (CuSO$_4$.5H$_2$O), manganese chloride (MnCl$_2$.4H$_2$O), zinc sulphate (ZnSO$_4$.7H$_2$O), boric acid (H$_3$BO$_3$), sodium molybdate (Na$_2$MoO$_4$.2H$_2$O) and iron chloride (FeCl$_3$), respectively (MALAVOLTA et al., 1997). The nitrogen and potassium fertilization were divided into 3 applications via fertigation at planting and 15 and 30 days after planting.

Maize (*Zea mays*) seeds were sterilized using 98% ethanol (30 seconds), 2% sodium hypochlorite (2 minutes), and successive washes in distilled water. After, the seeds were placed in sterilized Petri dishes with moistened cotton and filter paper, where they remained 72 hours at 28 °C until the emergence of rootlets. Four seeds were sown per pot and each seed received two mL of bacterial culture with approximately 2x10^8 CFU, grown in liquid medium 79, as described previously. After six days only one plant was left in each pot.

Soil moisture was kept at approximately 70% of field capacity and water was replaced daily by weight. After 45 days of planting, shoots were harvested and placed in paper bags and oven dried at 65 ºC for 72 hours. After, shoot dry matter was weighed and milled for chemical analysis.

2.4.1 Counting of phosphate solubilizing microorganisms

After lime reaction, a single soil sample was collected from several pots to form a composite sample in order to estimate the number of phosphate-solubilizing microrganisms present in the soil. Ten grams of soil obtained from the composite sample was diluted in 90 mL

of saline (8.5 g L^{-1} NaCl) to form a homogeneous suspension. From this suspension serial dilutions (10^{-1} to 10^{-10}) were performed, and 100 µL of each dilution was plated in the solid NBRIP medium. The plates were incubated at 28 °C and colony counts were periodically conducted until the end of the incubation period (10 days) (FIGURE 1).

Figure 1 - Petri dishes with liquid NBRIP medium inoculated with the soil suspension (dilution of 10^{-1}).
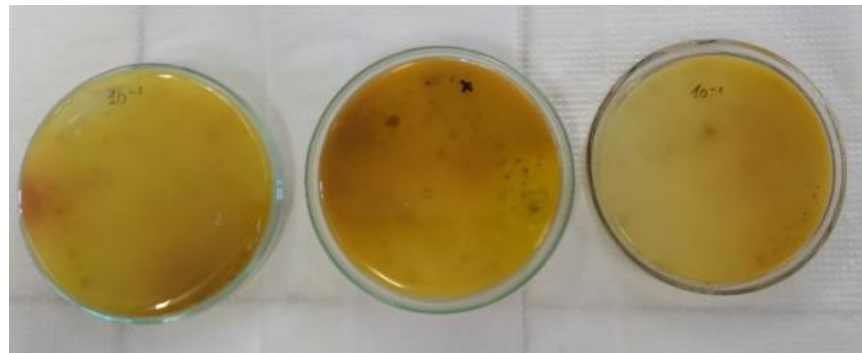
Source: From the author (2019).

Several microbial groups were observed in the 10^{-1} dilution, but no phosphate-solubilizing microrganisms were identified since it was not observed the presence of a transparent halo, which is characteristic of solubilization.

2.4.1 Plant analysis

Shoot dry matter and the P accumulation in shoots were evaluated (MALAVOLTA et al., 1997). Shoot tissues were digested in a block digestion system using concentrated nitric-perchloric acid solution (2 mL perchloric acid + 4 mL nitric acid) and P concentration was measured in ICP-OES. P accumulation in shoots was obtained by multiplying the P concentration by shoot dry matter production.

The agronomic efficiency index was calculated as described by Resende et al. (2006). Shoot dry matter of each treatment in the dose of 200 mg kg $^{-1}$ P minus shoot dry matter in the control (without inoculation and without P addition) divided by shoot dry matter in the positive control minus shoot dry matter in the control, according to equation 1.

$$AEI = \frac{(SDM\ 200\ mg\ kg^{-1} + inoculation\ type) - (SDM\ Control)}{(SDM\ TSP) - (SDM\ Control)} \times 100 \qquad (1)$$

2.4.2 Soil Chemical Analysis

Immediately after harvesting, a soil sample from the center of each pot at 10 cm depth was collected to determine acid/alkaline phosphatase activity. Also, rhizosphere soil (adhered to roots) and bulk soil samples were taken from each pot for chemical analyzes, including pH (H_2O) and available P by anion exchange resin. These samples were air-dried, sieved (< 2.0 mm) and homogenized prior to analysis.

2.4.3 Acid/Alkaline phosphatase activity

The enzymatic activity in soil samples was determined by the method described by Tabatabai (1994). This method is based on the colorimetric determination of ρara-nitrophenol (yellow color) formed after the addition of specific substrates for acid and alkaline phosphatase. Two analytical replicates and two controls were performed for each soil sample.

Phosphatase activity was determined using ρara-nitrophenyl phosphate as the analogue substrate. 1.0 g of soil sample was incubated with 4 mL of a buffer solution (modified universal buffer) pH 6.5 for the acid phosphatase assay, or pH 11 for the alkaline phosphatase assay and 1 mL of ρara-nitrophenyl phosphate (0.025 M). The samples were incubated at 37 ºC for 1 h, then 1 mL of $CaCl_2$ (0.5 M) and 4 mL of NaOH (0.5 M) were added to stop the reaction. The absorbance of the yellow color developed after the incubation period was measured in a spectrophotometer at 410 nm using a standard calibration curve of ρara-nitrophenol. Phosphatase activity was expressed as μg of ρara- nitrophenol released per gram of dry soil per hour.

2.5 Data Analyses

All experimental data were checked for normality by Shapiro–Wilk test prior to other data analysis. Data from the *in vitro* experiment were submitted to analysis of variance (PROC ANOVA) and the means of the treatments were grouped by the Scott–Knott clustering algorithms ($p \leq 0.05$). Data from the greenhouse experiment were submitted to analysis of variance (PROC ANOVA) and when the interaction of P doses × inoculation was significant ($p \leq 0.05$), regression models (PROC REG) were adjusted. When the interaction of P doses × inoculation was not significant ($p > 0.05$), the factors were analyzed separately. Regression models were adjusted as a function of P doses. Inoculation types were compared by the Scott–

Knott test ($p \leq 0.05$). The additional control was compared with the factorial mean (5 x 4) by the F test ($p \leq 0.05$). Data were analyzed using the software SAS® v. 9.3 (Statistical Analysis System, Cary, North Carolina).

3 Results and discussion

3.1 *In vitro* experiment:

3.1.1 Soluble-P and pH in the liquid NBRIP medium

All strains evaluated the strains were able to solubilize phosphates from BBF *in vitro* (FIGURE 2). P concentration was low and pH was high in control (uninoculated treatment) at the end of incubation period. A significant drop in the pH of the medium was observed ($p < 0.05$) consequently an increase in the amount of soluble-P. The soluble phosphate ranged from 9.3 to 41.4 mg L^{-1} and showed the following order: UFLA 03-09 > UFLA 04-155 = UFPI B5-8A > UFLA 03-10 = UFLA 03-116 > control (FIGURE 2A).

The maximum drop in pH was recorded for the strain UFLA 04-155 (2.95), followed by UFLA 03-09 (3.55) and UFPI B5-8A (3.80) (FIGURE 2B). Therefore, a strong negative correlation (r = - 0.915**) was observed between pH and soluble-P concentration in liquid NBRIP medium ($p < 0.05$) (FIGURE 3).

Figure 2 - Soluble P (A) and pH (B) in the liquid NBRIP medium containing biochar enriched with Bayóvar rock phosphate (100 mg L^{-1} of P) ten days after inoculation of phosphate-solubilizing bacteria. Control: uninoculated treatment. Means followed by the same letter do not differ among themselves by the Scott–Knott test ($p<0.05$). Error bars represent the standard deviations of the treatment mean replicates (n=6).
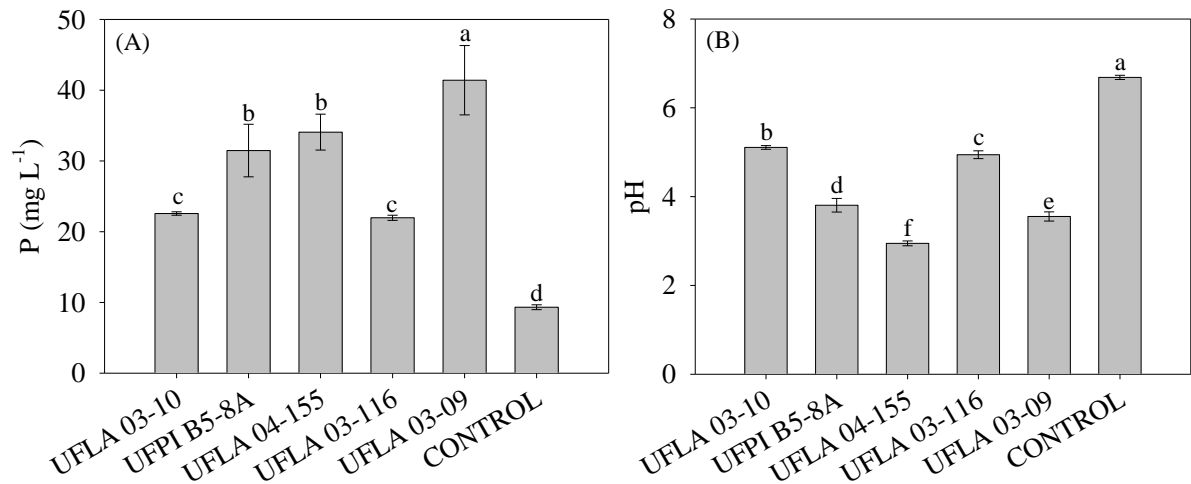
Source: From de author (2019).

Figure 3 – Correlation between pH and soluble-P concentration in liquid NBRIP medium containing biochar enriched with Bayóvar rock phosphate (100 mg L^{-1} of P) ten days after inoculation of phosphate-solubilizing bacteria. ** significant at $p<0.05$.
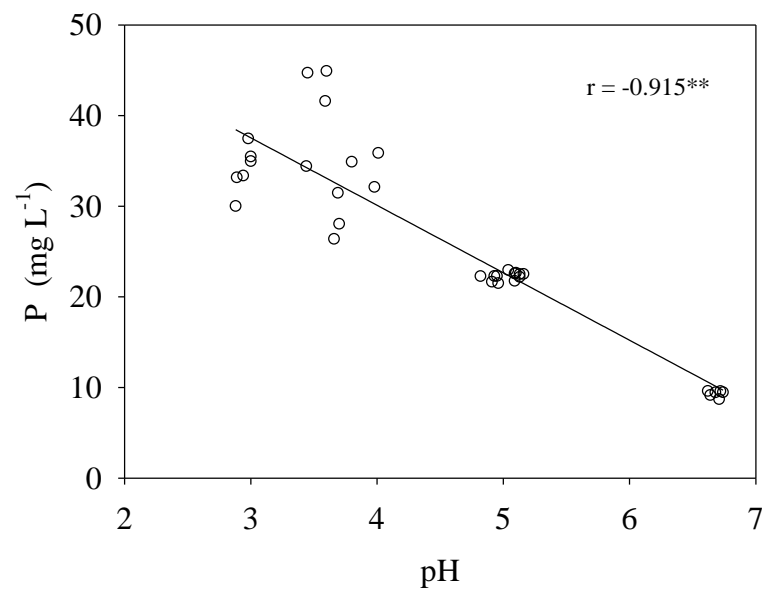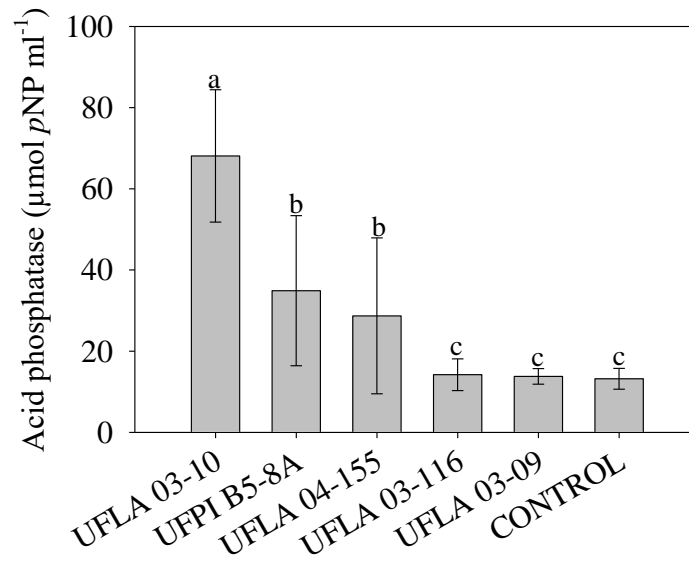

Source: From de author (2019).

The decrease in liquid NBRIP medium pH resulted in P release, similarly as reported by Yu et al. (2012). The ability of these strains in releasing P supplied by BBF had never been previously tested. But, phosphate solubilization by these strains has already been reported (COSTA et al., 2016; MARRA et al., 2012; OLIVEIRA-LONGATTI et al., 2013). The strain UFPI B5-8A was able to solubilize Ca phosphate and the strain UFLA 04-155 was able to solubilize both Ca and Al phosphate in solid medium (COSTA et al., 2016; OLIVEIRA-LONGATTI et al., 2013). The strain UFLA 03-09 was able to solubilize Ca phosphate in solid and in liquid medium, while the strains UFLA 03-116 and UFLA 03-10 were able to solubilize only Fe phosphate in liquid medium (MARRA et al., 2012). Furthermore, media composition , likely influence on P solubilization (MARRA et al., 2012). In this regard, glucose as the main C-source in liquid medium results in greater P solubilization than in the presence of arabinose, fructose, galactose, mannitol, maltose, sucrose, or xylose (NAUTIYAL, 1999). Thus, the amount of P solubilization depends mainly on the strain, the carbon source, the production of organic acids and the types of insoluble phosphates provided (ALEXANDER et al., 1961; MARRA et al., 2019).

3.1.2 Acid phosphatase activity

In general, bacterial strains produced low amounts of acid phosphatase in liquid NBRIP medium (FIGURE 4). The strains UFLA 03-09, UFPI B5-8A and UFLA 04-155 differed significantly from control. There was no correlation between the soluble-P and acid phosphatase activity at the end of incubation (r = -0.01 ns) ($p > 0.05$). However, acid phosphatase analysis was performed only at the end of incubation, not being possible to detect its maximum activity during this period indicating that soluble P may come from both solubilization and mineralization process.

Figure 4 - Acid phosphatase activity in liquid NBRIP medium containing biochar enriched with Bayóvar rock phosphate (100 mg L^{-1} of P) ten days after inoculation of phosphate-solubilizing bacteria. ρNP: *para*-nitrophenyl phosphate. Means followed by the same letter do not differ among themselves by the Scott–Knott test ($p<0.05$). Error bars represent the standard deviations of the treatment mean replicates (n=6).

Source: From the author (2019).

Acid phosphatase activity mainly participates in the mineralization of organic phosphates (RICHARDSON, 2002; RICHARDSON et al., 2009). The lack of correlation between P released and the acid phosphatase activity may indicate that other mechanisms are involved, or particularly, this enzyme activity may have decreased over time not been detected at the end of the assay. Another explanation for the low acid phosphatase activity is that its synthesis is stimulated only when the soluble inorganic P level is limited (DICK; DOS-SANTOS; MEYER-FERNANDES, 2011), and, in this case, the amount of soluble-P quantified after incubation may have inhibited its activity.

3.1.3 Identification and quantification of organic acids

The ability of bacterial strains to produce organic acids was further investigated in liquid NBRIP medium (TABLE 4). All bacterial strains produced different organic acids at different concentrations. The following organic acids were detected: oxalic, quinic, d-malic, succinic, citric and lactic. Quinic acid was produced by all strains, followed by oxalic acid produced by UFPI B5-8A, UFLA 04-155, UFLA 03-116 and UFLA 03-09 strains, while lactic acid was

produced only by UFLA 04-155 strain. However, the type of organic acids is more important than its total concentration and its acidity level (pKa) might explain better P solubilization.

It is reported that phosphate-solubilizing activity is associated with the release of organic acids produced, as well as the proton extrusion (cellular respiration and ammonium absorption), (CHEN et al., 2016; CHEN et al., 2006; ILLMER; SCHINNER, 1992; MARRA et al., 2019). The oxidation of glucose to organic acids results in acidification around bacteria cell, favoring phosphate solubilization (KPOMBLEKOU; TABATABAI, 1994).

In previous studies the strain UFLA 03-09 released tartaric acid, the strain UFLA 03-10, tartaric and citric acids, while the strain UFLA 03-116 did not release organic acids in the medium containing glucose as the carbon source and Ca phosphate as the P source. These same strains produced other types of organic acids when the medium composition (GELP-SYLVESTER-BRADLEY et al., 19820) was changed (MARRA et al., 2019).

Table 4 - Concentration of low molecular mass organic acids in the liquid NBRIP medium containing biochar enriched with phosphate Bayóvar (100 mg L^{-1} of P) in function of the inoculation of phosphate-solubilizing bacteria.

Organic acids*	UFLA 03-10	UFPI B5-8A	UFLA 04-155	UFLA 03-116	UFLA 03-09	CONTROL
	μmol L^{-1}					
Oxalic (1.23)**		763±14.1	1618±52.7	185±22.8	1082±395	149±36.9
Quinic (3.46)	1297±66.7	446±121	969±149	173±102	600±126	261±99.0
D-malic (3.40)				431±248		
Acetic (4.76)	59.6±4.1			61.5±1.80		
Succinic (4.16)	676±393	140±19.5		395±16.7		
Citric (3.14)	84.1±16.6					20.0±3.70
Lactic (3.08)			353.6±63.7			
Total	2116±481	1350±154	2941±265	1245±391	1683±521	431±139

*Mean value ± standard deviation; ** pKa – constant of acidity.
Source: From the author (2019).

In this assay the strains UFLA 04-155, UFPI B5-8A and UFLA 03-09, acidified the medium and high amount of soluble P was quantified, thus, P was released mainly by acidification (organic acids or proton extrusion). Other researchers also found an inverse relationship between pH and soluble P, indicating that acidification could possibly facilitate P solubilization (PARK et al., 2011a). These strains released the highest concentrations of oxalic acid (Table 4), which has the lowest pKa of the organic acids detected rendering the lowest pH, explaining better P release for these strains. However, the strains UFLA 03-10 and UFLA 03-116 hold the media pH at around 5, indicating that acidification was not the main mechanism

used to promote solubilization, and the acids may be present in anionic forms playing a major role in Ca^{2+} chelation rather than acidification of the medium (JONES, 1998; MARRA et al., 2011; PARK et al., 2011b). Thus, other solubilization mechanisms, such as, siderophores and exopolysaccharide production may be indirectly involved (HAMDALI et al., 2008; YI; HUANG; GE, 2008). Besides, the strain UFLA 3-116 and UFLA 03-10 was reported as producers of large amounts of exopolysaccharide and low amounts of organic acids (MARRA et al., 2012, 2019).

Therefore, based on the results reported, the strains UFLA 03-10, UFLA 04-155 and UFPI B5-8A were selected to test the agronomic efficiency of BBF on maize cultivation in an Oxisol under greenhouse conditions. Although the strain UFLA 03-09 was verified as the most efficient in P release, it was not chosen due to its potential as a human pathogen.

3.2 Greenhouse experiment

3.2.1 Shoot dry matter and P uptake

P doses increased linearly shoot dry matter and P accumulation in shoots. No P application (0 mg kg^{-1} P) restricted plant growth, which shows the strong P deficiency in this soil (FIGURE 4). The interaction of P doses and bacterial inoculation was not significant for shoot dry matter and P accumulation in shoots ($p > 0.05$). However, inoculation significantly increased maize shoot dry matter by 8% ($p < 0.05$) (TABLE 5).

Figure 4 - Maize shoot dry matter (A) and P accumulation in shoot (B) in function of P doses (biochar enriched with Bayóvar rock phosphate). ** significant at $p < 0.05$.
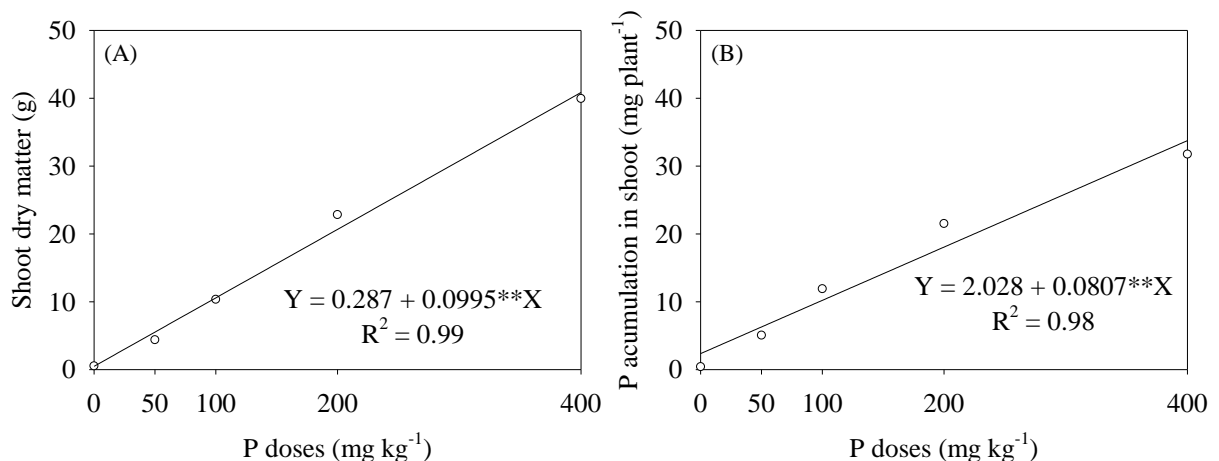
Source: From the author (2019).

BBF fertilization and selected bacterial inoculation constitutes an important strategy for providing sustainable alternatives to the highly water soluble phosphate fertilizers such as triple superphosphate, which has a high energy cost for its production. This combination aiming to achieve plant growth has been reported as a promising approach for various crops (RAFIQUE et al., 2017). Indigenous isolated bacterial strains increased nutrient uptake by french beans, maize and rice plants (DEB et al., 2016; RAFIQUE et al., 2017; SAXENA; RANA; PANDEY, 2013). The variety of nutrients present in biochar, its surface area and its highly porous nature reflects on its ability to act as a safe environment for microorganisms, which is one of the main reasons for the changes in soil properties and the increase of nutrient uptake by plants (NIGUSSIE et al., 2012), showing more efficiency in plant growth and nutrient uptake than conventional chemical fertilizers.

A difference between the mean of positive control and the factorial mean was observed for shoot dry matter and P accumulation in shoots ($p<0.05$). Plants in the positive control showed higher values of shoot dry matter and P accumulation in shoots, with an increasing of 73 and 82%, respectively (TABLE 5). Nevertheless, maize plants would probably respond to higher P doses from BBF than those tested, producing higher values of shoot dry matter and accumulating more P in the shoot.

Table 5 - Acid phosphatase activity (AP), alkaline phosphatase activity (ALP), rhizosphere soil pH (pH R), bulk pH (pH B), shoot dry matter (SDM), P accumulation in maize shoots (PAS) as a function of phosphate-solubilizing bacteria inoculation.

Inoculation Type	AP	ALP	pH R	pH B	SDM	PAS
	μg-ρNP g^{-1} soil h^{-1}				g pot^{-1}	mg plant^{-1}
NI	5517a	1288a	5.28a	5.80a	14.8b	8.69a
UFLA 03-10	5972a	1295a	5.51a	5.77a	16.0a	12.6a
UFPI B5-8A	6502a	1335a	5.55a	5.82a	16.0a	10.5a
UFLA 05-155	5811a	1186a	5.52a	5.71a	15.5a	12.2a
Mean	5950	1276	5.46	5.77	15.5	11.3
TSP	4671 ns	956**	5.50 ns	5.43**	57.2**	62.5**

Means followed by the same letter in the columns do not differ by the Scott–Knott test ($p<0.05$). NI: no inoculation. ns: not significant. TSP: positive control (200 mg kg^{-1} P) provided as triple superphosphate without inoculation. ρNP: *para*-nitrophenyl phosphate. **Positive control mean differs from factorial mean by F test ($p<0.05$).
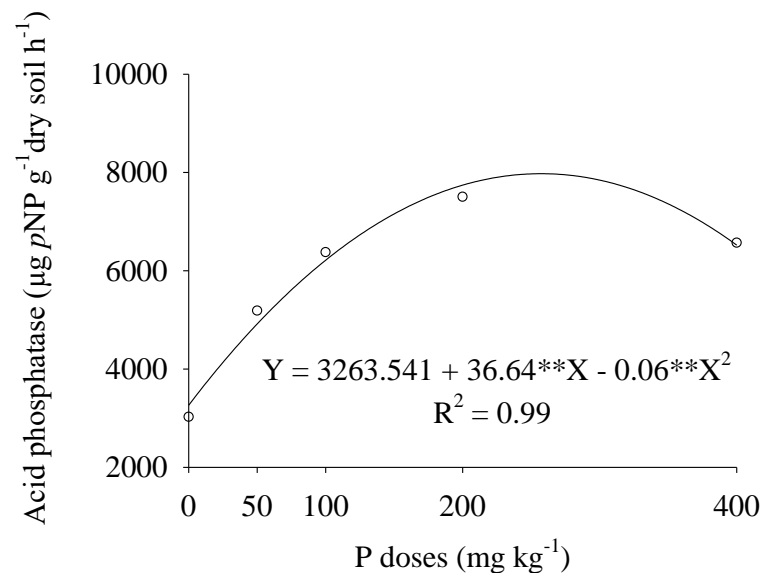Source: From the author (2019).

The shoot dry matter increase with the inoculation of UFLA 03-10, UFLA 04-155 and UFPI B5-8A strains is closely related to a better absorption of soluble phosphate from soil

amended with BBF, due to the increase of P labile fractions in soil (WEI et al., 2016), or other plant growth promoting mechanisms not studied here. Other studies have demonstrated that strains belonging to the genera *Pseudomonas* are effective in enhancing growth and P uptake by maize plants (KAUR; REDDY, 2014; PEREIRA; CASTRO, 2014). However, plant growth promotion by bacterial inoculation is not constantly followed by an increase on P accumulation in plant tissues (PEREIRA; CASTRO, 2014). Additional processes that promote plant growth can be attributed as the ability to inhibit the growth of phytopathogens and the production of plant growth hormones (OLIVEIRA-LONGATTI et al., 2013).

3.2.2 Acid/Alkaline phosphatase and bulk and rhizosphere soil pH

Among the factors studied, only P doses was significant for acid phosphatase activity, bulk and rhizosphere soil pH ($p<0.05$). The highest acid phosphatase activity was estimated around 305 mg kg^{-1} (FIGURE 5).

Figure 5 - Acid phosphatase activity in function of P doses (biochar enriched with Bayóvar rock phosphate). ρNP: *para*-nitrophenyl phosphate. ** significant at $p<0.05$.

$$Y = 3263.541 + 36.64**X - 0.06**X^2$$
$$R^2 = 0.99$$

Source: From the author (2019).

Biochar amended soil has a tendency to increase alkaline phosphatase activity and decrease acid phosphatase activity due to the increase of pH in treatments with biochar (BERA et al., 2016; BORNØ et al., 2018). In our study, it was observed a decrease in acid phosphatase activity at the highest P dose applied as BBF. However, no increase in alkaline phosphatase

activity was observed, because the P doses applied as BBF did not raise the soil pH higher than 9 to activate and cause a higher activity of this enzyme (EIVAZI; TABATABAI, 1977; HERBIEN; NEAL, 1990). Acid and alkaline phosphatases can coexist but within different ranges of soil pH. In tropical soil, naturally acidic soils, acid phosphatase is more representative, while the alkaline phosphatase is typically measured at high pH, remote from the natural soil pH values typically found (MARGALEF et al., 2017).

The control (without P) did not decrease the pH after cultivation. A low biological activity, as seen in acid phosphatase activity, may have constrained root growth. Conversely, higher BBF doses exhibited an increase in pH both in bulk and rhizosphere soil. (FIGURE 6 A and B), while at lower doses a decrease in pH was observed. Increasing P doses favored biological activity and nutrient supply, improving the root system activity and leading to a decrease in soil pH. However, due to its alkaline nature, BBF application tends to promote a natural correction of soil exhibiting higher pH values.

Figure 6 - Rhizosphere soil pH (A) and bulk soil pH (B) in function of P doses (biochar enriched with Bayóvar rock phosphate). Means followed by the same letter do not differ among themselves by the Scott–Knott test ($p<0.05$). Error bars represent the standard deviations of the treatment mean replicates (n=4).
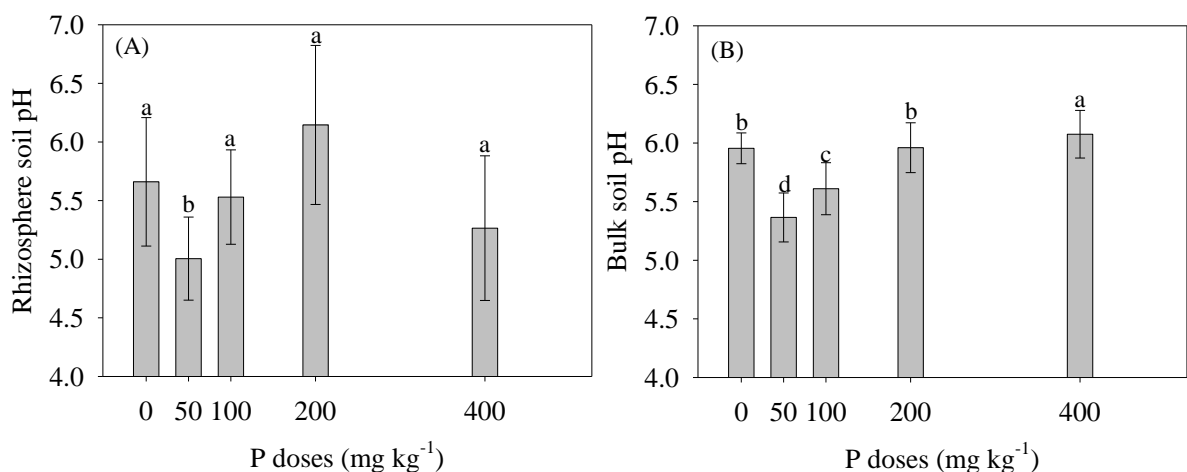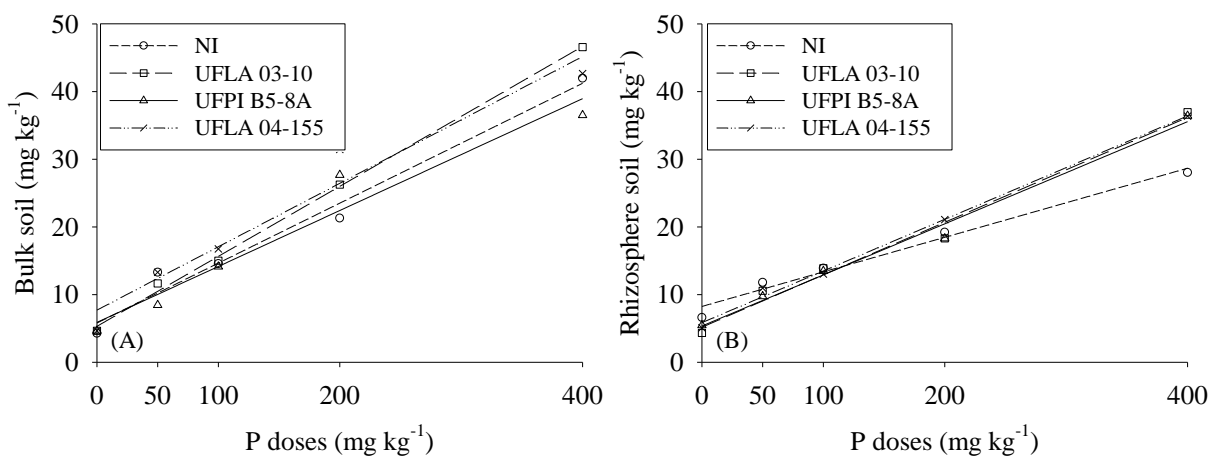

Source: From the author (2019).

A difference between the mean of the positive control and the factorial was observed only for alkaline phosphatase activity and bulk pH ($p<0.05$). Alkaline phosphatase activity and bulk pH decreased in positive control in the range of 25% and 5%, respectively (TABLE 5).

3.3.3 Available P in soil

The interaction of P-BBF doses and inoculation was significant for available P in bulk and rhizosphere soil ($p<0.05$). Bacterial strains increased P availability linearly in both (FIGURE 7 A and B). In the bulk soil P availability was increased by 2% compared to uninoculated treatment in the dose of 400 mg kg^{-1} P (FIGURE 7A). Bacterial inoculation and root activity significantly increased available P by 30% in rhizosphere soil at the same dose (FIGURE 8B). Rhizosphere soil is highly influenced by root activity and bacterial colonization is positively affected by plant root exudates ensuring its survival and performing better in P solubilization.

A difference between the mean of available P from positive control and the available P in the factorial was observed both bulk and rhizosphere soil ($p<0.05$). The factorial mean compared to the mean of the positive control for available P in bulk and rhizosphere soil are 50% and 63% lower, respectively (TABLE 5).

Figure 7 - Available P in bulk soil (A) and in the rhizosphere soil (B) in function of P doses (biochar enriched with Bayóvar rock phosphate) fertilization in maize in function of phosphate solubilizing bacteria inoculation. SI: without inoculation. ** significant at $p<0.05$.

Bulk Soil	Rhizosphere Soil
UFLA 03-10: $5.52 + 0.102^{**}X$ ($R^2 = 0.99$)	UFLA 03-10: $5.46 + 0.075^{**}X$ ($R^2 = 0.99$)
UFLA 04-155: $6.81 + 0.099^{**}X$ ($R^2 = 0.97$)	UFPI B5-8A: $5.42 + 0.079^{**}X$ ($R^2 = 0.99$)
NI: $6.65 + 0.082^{**}X$ ($R^2 = 0.98$)	UFLA 04-155: $5.95 + 0.075^{**}X$ ($R^2 = 0.99$)
UFPI B5-8A: $6.48 + 0.079^{**}X$ ($R^2 = 0.97$)	NI: $8.28 + 0.050^{**}X$ ($R^2 = 0.99$)

Source: From the author (2019).

It should be mentioned that *in vitro* activity will not always correlate with *in vivo* effects on plant development and available P. In our study, for example, the strain UFLA 03-10 was less efficient in releasing P *in vitro* than it was *in vivo*. One of the reasons can be attributed to the carbon source present in the liquid NBRIP medium, which provides only one carbon source and it may not be the major source required by this strain to incorporate into its microbial biomass and operate better in P solubilization. In *in vivo* promotes a different environment for microbial colonization, most probably by root exudates and the variety of carbon sources, (JACOBY et al., 2017), furthermore, the same result in both assays cannot be expected.

The capacity of inoculated strains to provide available P in soil should be emphasized, since both P sources were supplied based on the total P concentration, whereas P from BBF is up to five times less soluble than the positive control. However, P solubilization was not necessarily reversed in shoot dry matter production. Maize plants require constant P supply during the initial stage of growth, however, bacterial solubilization may have been limited by the ability of short-term solubilization or a dilution effect could have occurred, since BBF was entirely mixed in soil and inoculation was depended of the root system growth to access this source and perform solubilization.

Also, it is well known that biochar addition works as a soil conditioner by improving moisture content and nutrient availability (CHEN; SHINOGI; TAIRA, 2010; NGUYEN et al., 2017; NIGUSSIE et al., 2012). Besides, several authors have suggested that biochar addition to soil promotes an increasing of phosphate-solubilizing bacteria genera through changes in C fluxes and for providing pores to accommodate these microorganisms, protecting them against predators in soil (ANDERSON et al., 2011; FOX et al., 2016; WARNOCK et al., 2007; ZHANG et al., 2018).

3.3.4 Agronomic efficiency index

The BBF showed around 40% of agronomic efficiency index as compared to positive control. at the same dose (200 mg kg^{-1}). However, the P doses in both BBF and positive control were based on the total P, being the later ten times more soluble than the former. Although, inoculation did increase the P availability in soil amended with BBF, this was not enough to provide P to regular maize growth in this short-term experiment.

Figure 8 - Agronomic efficiency index (AEI) after biochar enriched with Bayóvar rock phosphate fertilization in maize in function of phosphate-solubilizing bacteria inoculation. NI: no inoculation. Means with the same letter do not differ from each other, according to the Scott–Knott test ($p<0.05$). *200 mg kg^{-1} of P-BBF (biochar-based fertilizer, corresponding to 44 mg of soluble-P in NAC) and TSP (triple superphosphate as positive control, corresponding to 200 mg of soluble-P in NAC) without inoculation. NAC: Neutral ammonium citrate.
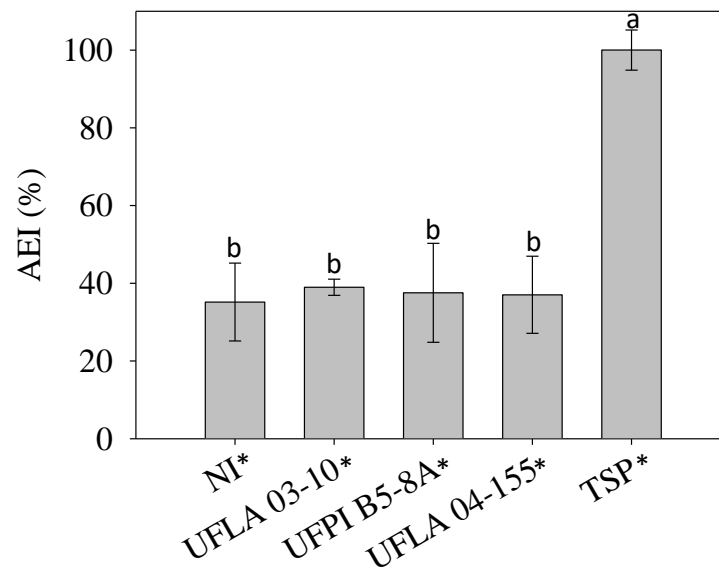
Source: From the author (2019).

4 Environmental implications

In this study, BBF and the strains evaluated as inoculants had the potential to significantly enhance available P in a deficient P Oxisol, hence, they show a profitable combination to be used for sustainable agriculture. The current study provides evidences that it is possible to replace gradually from chemical fertilizers to biofertilizers, however optimizations in the cultivation system must be better defined.

BBF produced from poultry litter provides a better final destination for this residue for its nutrients reutilization as well as for reducing the environmental impact of an incorrect disposal. Besides, its production uses a cheaper technology, and when applied to soil, due to its slow nutrient release, it reduces P losses by fixation in highly weathered and clay soils or by leaching in sandy soils. Moreover, the use of BBF improves C retention, supporting the sequestration o stable and functional C in soil.

Although inoculation increased soil P availability, for annual crops BBF efficiency would be limited, since are required large amounts of readily available P, which is not supplied

by the BBF due to its slow-release nature. However, for perennial crops the chemical fertilizers could be partially or entirely substituted, since a residual effect is essential as the culture remains longer in the area.

5 Conclusions

Selected bacterial strains were able to release soluble P in the liquid NBRIP medium, however, the strains UFLA 03-09, UFLA 04-155 and UFPI B5-8A were the most efficient. For these three, organic acids production, mainly oxalic acid, may explain part of the soluble P quantified. While the strains UFLA 03-116 and UFLA 03-10, the mechanisms evaluated were not sufficient to fully elucidate the amounts of soluble P quantified.

Possibly, inoculation of UFLA 03-10, UFLA 04-155 and UFPI B5-8A increased maize shoot dry matter and available P in soil. However, compared to positive control, BBF treatments presented lower agronomic efficiency.

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Supplementary material

Table S1. Analysis of variance for acid phosphatase (AP), pH of the supernatant (pH) and soluble phosphorus (P) as a function of *in vitro* inoculations in liquid NBRIP medium containing Bayóvar rock phosphate enriched biochar.

		MS		
VS	**DF**	**P**	**pH**	**AP**
Dose	5	671.167330**	10.61914275**	0.00260600**
Residue	28	6.237085ns	0.00761369ns	0.00017874ns
CV (%)		9.63	1.91	44.9

MS: Mean square; SV: source of variation; CV: coefficient of variation; DF: Degrees of freedom.
** Significant at 5% probability by F test.
Source: From the author (2019).

Table S2 - Analysis of variance for acid phosphatase (AP), alkaline phosphatase (ALP), shoot dry matter (SDM) and P accumulation in the shoots (PAS) of maize as a function of bacterial inoculation and P doses (Bayóvar rock phosphate enriched biochar).

		MS			
SV	**DF**	**AP**	**ALP**	**SDM**	**PAS**
Dose	4	41427578.0**	73018.241ns	3465.31452**	2415.209440**
Inoculation	3	3624979.9ns	27163.894ns	55.58300**	4.737869ns
Dose × Inoculation	12	958942.2ns	88927.084ns	0.00000ns	8.551309ns
Block	3	11352787.1**	31817.871ns	35.11888**	47.699767**
Residue	55	2078559.0	68966.654	9.56563	10.11804
CV (%)		23.8	20.8	21.4	23.1
SFT	1	4167204.9**	655315.06**	6709.200599**	7027.75208**

MS: Mean square; SV: source of variation; CV: coefficient of variation; DF: Degrees of freedom.
**Significant at 5% probability by F test.
Source: From the author (2019).

Table S2 - Analysis of variance for bulk soil pH (B pH), rhizosphere soil pH (R pH), available P in bulk soil (BP) and available P in rhizosphere soil (RP) of maize as a function of bacterial inoculation and P doses (Bayóvar rock phosphate enriched biochar).

		MS			
FV	**GL**	**B pH**	**R pH**	**BP**	**RP**
Dose	4	1.39036502**	1.17120750**	3317.36070**	1960.948295**
Inoculation	3	0.05724221ns	0.20937333ns	31.47527ns	8.364946ns
Dose × Inoculation	12	0.05390365ns	0.22523583ns	37.03671**	20.624864**
Block	3	0.00998045ns	1.85799000**	83.69328**	11.506521ns
Residue	55	0.03664643	0.21972333	18.33213	8.176964
CV (%)		3.31	8.58	21.52	16.97
SFT	1	0.2823167	0.01030095	2982.73996	10690.6699

MS: Mean square; SV: source of variation; CV: coefficient of variation; DF: Degrees of freedom.
**Significant at 5% probability by F test.
Source: From the author (2019).