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Sustainable Improvement of Fertility of Acid Arenosols: Application of Phosphate Rock, Dolostone and Biochar

Author

Rogério Borguete Alves Rafael

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Sustainable Improvement of Fertility of Acid Arenosol: Application of Phosphate Rock, Dolostone and Biochar

PhD Candidate: **RAFAEL, Rogério Borguete Alves**

Supervisor: **Prof. CORTI, Giuseppe (PhD)**

Co-Supervisors: **Prof. FERNÁNDEZ-MARCOS, Maria L. (PhD) & Dr. COCCO, Stefania (PhD)**

Referees

Prof. FERNÁNDEZ-SANJURJO, Maria José (PhD)

Prof. WEINDORF, David (PhD)

The Coordinator of the PhD Program

Prof. MEZZETTI, Bruno (PhD)

November 30th, 2017

This thesis is dedicated to my family: my sons ("*Dhara Audrey Padil Rafael* - Dharusca" and "*Aaron Afro Padil Rafael*") and wife ("*Afiza Irene Salimo Padil Rafael*")

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List of publications

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International Conferences/Workshops with Peer Review

1. Rogério Borguete Alves RAFAEL; Maria Luisa FERNANDEZ-MARCOS; Stefania COCCO; Maria Letizia RUELLO; Flavio FORNASIER, Giuseppe CORTI (2017). Benefits of biochars for soil quality, nutrient use efficiency and cowpea growth in acid Arenosol. 1st World Conference on Soil and Water Conservation under Global Change (CONSOWA) – Lleida, Catalonia, Spain, from 12-16 June 2017.
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Others

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Abstract

Tropical acid soils are infertile and low nutrient content. In Mozambique the use of lower fertilizer rates, shifting cultivation, and rain fed agriculture lead to food insecurity and soil deterioration. Implementation of practical, low-cost and result oriented long-term strategies that address the need of poor farmers and for sustainable long-term care of the soil are of paramount importance. Soil application of rock fertilizers/amendments has the advantage of slow nutrient release with benefit of liming. Biochar as organic amendment can improve soil properties and reduce trace metal availability. In this study, we aim to: i) investigate the feasibility of phosphate rock and dolostone; ii) assess the effects of simultaneous application of phosphate rock, dolostone, and biochar on soil fertility improvement; and iii) assess the role of biochars in improving NPK fertilizer efficiency. Laboratory and pot experiments were established to assess the reactivity, nutrients release, and agronomic performance of rocks and biochar with an acid Arenosol. The soils were separated in rhizosphere and bulk soils to understand nutrients and metal dynamics in the soil-plant systems. Soil and plant parameters were determined following standard methods.

These rocks are slow release fertilizer and potential source of P, Ca, and Mg with benefit of liming in acid soils. Higher pH in phosphate rock and biochar treatment increased activities of C, N, and P related enzymes. We demonstrated that rhizosphere is an active environment that regulates nutrient availability through activation of extracellular enzymes. Application of phosphate or dolostone to acid Arenosols fails to improve soil fertility, but it dramatically improves if applied in combination with biochar. Therefore, corn and cowpea yield increased dramatically. Cowpea yield was the same with full or half dose of NPK for each biochar type. Yield increase reflect increased profit and food security, but further evaluation on field conditions assessing both agronomic effectiveness and economic benefit is necessary to validate these results.

Sommario

I suoli tropicali sono poco fertili a causa della loro acidità e del basso contenuto di nutrienti. In Mozambico il ridotto uso di fertilizzanti, l'agricoltura itinerante e la non irrigazione riducono le rese e deteriorano il suolo. È di primaria importanza intraprendere pratiche a basso costo, orientate verso strategie a lungo termine di un uso sostenibile del suolo. L'applicazione di rocce fosfatiche e dolomite come fertilizzanti/ammendanti ha il vantaggio di un lento rilascio di nutrienti, mentre il biochar è un ammendante organico che può migliorare le proprietà del suolo e ridurre la disponibilità di metalli pesanti. In questo studio, gli scopi sono stati: i) investigare l'impiego di roccia fosfatica e dolomite; ii) valutare l'effetto di una applicazione simultanea di roccia fosfatica, dolomite e biochar sul miglioramento della fertilità del suolo; iii) valutare il ruolo del biochar sul miglioramento dell'efficacia di fertilizzanti NPK. Esperimenti di laboratorio e in vaso hanno permesso di valutare reattività, rilascio di nutrienti e performances agronomiche di rocce e biochar su suoli acidi.

Il suolo su cui sono stati svolti gli esperimenti è stato anche separato in bulk e rizosfera per comprendere le dinamiche di nutrient e metallic pesanti nel Sistema suolo-pianta. Le rocce rilasciano lentamente nutrienti quali P, Ca e Mg, e aumentano il pH di suoli acidi. Incrementi di pH e aggiunte di biochar aumentano l'attività di enzimi coinvolti nei cicli di C, N e P. La rizosfera, inoltre, regola la disponibilità di nutrienti attivando enzimi extracellulari. Al contrario, l'applicazione di roccia fosfatica o dolomite non migliora la fertilità del suolo se non in combinazione con biochar. I test sono stati effettuati su mais e fagiolo dall'occhio, le cui rese sono sensibilmente aumentate. Il fagiolo dall'occhio mostra un uguale rendimento con dose intera o dimezzata di NPK per ogni tipo di biochar. L'aumento delle rese si traduce in maggiori profitti e una maggiore sicurezza alimentare. Ulteriori valutazioni in campo sono necessarie per validare i risultati.

Contents

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CHAPTER 1 - INTRODUCTION

1.1. Background and context

The increase of food production is paramount to eradicate food insecurity in developing countries (Agegnehu and Amede, 2017). Therefore, exploring natural resources such as soils is the immediate alternative to fulfill this goal (Nkonya and Mirzabaev, 2016). Soils can provide food and fiber, but also a wide range of other essential ecosystem services, such as carbon sequestration, water purification, etc. (Materechera, 2001; Wang et al., 2014; Nkonya and Mirzabaev, 2016). However, soil deterioration due unsustainable practices can lead to decrease of soil fertility and functions (Materechera, 2001; Sosibo et al., 2017). For example, excess of ploughing and hoeing can accelerate organic matter decomposition, losses of nutrients and soil particles through increased erosion and leaching (Materechera, 2001). Loss of organic matter can occur if agriculture is practiced on poor infertile soils without sufficient application of manure or fertilizer, leading to soil deterioration and decreased production (Jones et al., 2013). The impact of soil deterioration depends on type of soil and farming system (Kassa et al., 2017).

Tropical sandy soils are generally infertile due to the low water retention capacity, soil organic matter, and nutrients (Lahmar et al., 2012; Fujii et al., 2017). These soils are susceptible to further acidification due to their low acid neutralization capacity (Sposito, 2008). Soils with sandy texture have inherently low available water capacity and high air capacity, and are not conducive for crop production (Jeffery et al., 2015; Obia et al., 2016). Arenosols also has low aggregate stability (Molnár et al., 2015). In Mozambique, these types of soil cover ~ 40% of South region (Gouveia and Azevedo, 1949). Cultivation of annual crops under these sandy soils would require management of inputs that generally are not economically justifiable (FAO, 2015), but in large areas they are the only soils available for agriculture.

In Mozambique, the use of fertilizers by farmers is low and has been estimated by TIA[1] to be, on average, 3 kg ha^{-1} per Mozambican farmer (Benson et al., 2012; Rafael and Nhancale, 2013). This value is lower than the average fertilizer dose in African countries (e.g. 8 kg ha^{-1}) and even much lower than the target value set by the African Union in the Abuja Declaration (12 June 2006) to increase average rate to at least 50 kg ha^{-1} in African countries by 2015 (NEPAD[2], 2011; Zandamela, 2013). Inorganic fertilizers are one of the production factors that boost agricultural productivity (Stancheva et al., 2004; Karaivazoglou et al., 2007; Zahoor et al., 2017). In this context, the

[1] TIA – Agrarian Inquiry Survey

[2] NEPAD – New Partnership for Africa Development

production process should include this input to ensure a good harvest. Low fertilizer rate, shifting cultivation, and rain-fed agriculture in Mozambique stressed cultivation conditions leading to failure of the agriculture system, and increases the risk of malnutrition and food insecurity (Jones et al., 2016). For instance, corn *(Zea mays* L.*),* rice *(Oryza sativa* L.), and bean (*Vigna unguiculata)* yields in the ranges ~ 0.7-1.3 ton ha^{-1}, 0.6-1 ton ha^{-1} and 0.3-0.6 ton ha^{-1}, which have potential yields above 4.5 ton ha^{-1}, 4.5 ton ha^{-1} and 1 ton ha^{-1} respectively, to farming families that own properties with 3 hectares (Cunguara, 2013; TIA, 2007).

Therefore, the continuous exportation of essential nutrients through the harvest of food crops leads to soil nutrient depletion and acidification (Agegnehu and Amede, 2017). Also, some soils have inherently low fertility because overly "infertile" rock formations (Van Straaten, 2006). Since agriculture is the basic activity and sustains 90% of the Mozambican population (TIA, 2007), the country is dependent on the discovery of low-cost technologies that can boost agricultural production (Van Straaten, 2006). Soil fertility restoration must be the starting point to reverse both the current trend of pressure on land and soil degradation (Bekunda et al., 2002; Malavolta, 2006; Van Straaten, 2006; Prochnow, 2010; Van Raij, 2011).

1.2. Agro-geological practices as sustainable fertilizer or amendments sources

Implementation of practical, low-cost and result-oriented long-term strategies that address the needs of poor farmers and for sustainable long-term care of the soil are of paramount importance (Van Straaten, 2006). The application of agro-geological practices is one of the biophysical tools that can tackle long-term soil related problems (Van Straaten, 2002; 2006). Tropical soils are mainly depleted of macronutrients such as N, P, and K (Buresh et al., 1997). Therefore, while N can be obtained from the air by biological nitrogen fixation (BNF) and organic sources, P and K and others plant nutrients must be provided from rocks and minerals (Zahran, 1999; Salvagiotti et al., 2008). For instance, feldspars are alkali aluminosilicates and important sources of K, Ca and Na (Lee et al., 1998), while mica group minerals also can be a source of K, Mg, Zn and Mn (Gilkes et al., 1972). Apatite minerals are a source of P and Ca to plants (Dorozhkin, 2002; 2012). Conversely, limestone and dolomite are source of Ca and Mg to plants (Liu et al., 2005). These phosphate rock fertilizers has liming effect in acid soils (Harley and Gilkes, 2000; Van Straaten, 2006). Unfortunately, phosphate rocks are not abundant among soil parent materials and are not evenly distributed throughout the world; therefore, phosphorus has to be applied in the form of fertilizer in most

agricultural soils.

Phosphate rocks are more effective in tropical environments due the high temperatures and moisture regime which play a key role in rock dissolution; but, at the same time, the soils are depleted, high rates of weathering and leaching, highly receptive addition of nutrients (Gilman et al., 2000; 2002). Rock fertilizers have the advantages of providing a large number of macronutrients (e.g. P, K, Ca, and Mg), micronutrients (e.g. Cu, Zn, and Mn) and beneficial elements (e.g. Si) (Epstein, 1999; Van Straaten, 2006). However, the effectiveness of rock fertilizers in agriculture has been questioned due to conflicting experimental data, and the generally low reactivity and subsequent low availability of nutrients to plants, as well as the practicability of applying large amounts of ground rock to soils (Bolland and Baker, 2000; Harley and Gilkes, 2000).

In soils the mineral dissolution is enhanced by disequilibrium between the soil solution and the mineral phases, resulting from removal by processes such as leaching and nutrient uptake by plants (Hinsinger et al., 1995). In addition, rhizosphere processes and other biological activity may further increase mineral dissolution through the release of H^+ ions and complexing organic compounds, which react with mineral surfaces (Hinsinger, 1998; Harley and Gilkes, 2000). Therefore, understanding these surface reactions may lead to preparative methods to enhance nutrient release mineral surface. The mineralogical and geochemical processes determining the pathways and the rate limiting reactions that Control nutrient release from rocks are well documented (Harley and Gilkes, 2000; Davis et al., 2007; Dorozhkin, 2012; Oliva et al., 2012) however, limited information has been reported regarding nutrient release to plants from rock powders applied to tropical acid soils.

Rock fertilizers represent inexpensive and environmentally sound fertilizer options for farmers in areas of the world with infertile soils and suitable climates (Khasawneh and Doll, 1979; Appleton, 2002). Therefore, with the right choice of locally available rock materials for the right soils, these materials have to local agriculture, especially when modified or blended with locally available organic resources (Van Straaten, 2006; Biswas, 2010).

1.2.1. Phosphate rock as slow release fertilizer

Phosphate rock is the major raw material used to produce all water soluble phosphate fertilizers (Van Kauwenbergh, 2001a). Direct application of phosphate rock to soils has the advantage of slow nutrient release to plants due its low reactivity (Msolla et al., 2005; Biswas, 2010), in addition to the considerable savings involved. Depending on the origin and its geological history, apatite minerals have widely differing chemical and mineralogical characteristics and physical properties (Bao et al.,

2016; Kirkland et al., 2017; Prokopyev et al., 2017). The mineralogical, chemical, and textural characteristics of phosphate ores and concentrates determines the suitability the ore and suitability for use as direct application fertilizer (Reijnders, 2014). For instance, phosphate rock from igneous deposit are low in grade and contain apatite of fluoroapatite ($Ca_{10}(PO_4)_6F_2$) and hydroxyapatite ($Ca_{10}(PO_4)_6OH_2$) varieties that are relatively unreactive and must be ground very finely for direct application (Van Kauwenbergh, 2001a, b). Conversely, sedimentary deposits are high grade, reactive, and most suitable for direct application (Msolla et al., 2007). Sedimentary phosphate rock that has been subjected to a deep pressure and perhaps shearing forces and /or heat maybe further form metamorphic phosphate rock, which assume most of the characteristics of igneous phosphate rock (Van Kauwenbergh, 2001a). High temperature igneous minerals are associated with igneous apatite deposits, while clastic sedimentary minerals such as quartz and clays or biological or chemical precipitates such as carbonates are associated with sedimentary phosphate rock (Van Kauwenbergh, 2001a).

Ground rock has been recommended as slow release fertilizer for highly weathered soils and leaching environments where soluble fertilizer may be easily removed (Maene, 2001; Van Straaten, 2006). Direct application of ground phosphate rock is suitable on tropical acid soils as low-cost alternative where local deposit of phosphate are located (Chien, 2001) Several studies have reported the increased agricultural yield by direct application of phosphate rock fertilizers (Vanlauwe et al., 2000; Weil, 2000; Msolla et al., 2005; Odongo et al., 2007; Chien et al., 2010). Husnain et al. (2014) reported an improvement of soil fertility and corn yield by direct application of Moroccan phosphate rock to an Ultisol under field conditions. Szilas et al. (2007) demonstrated that Minjingu phosphate rock from Tanzania can replace the triple superphosphate (TSP) in acid, severely weathered tropical soils low to very low available P and exchangeable Ca. Biswas (2010) showed an improvement of soil fertility and potatoe yield after application of compost and low grade phosphate rock in an Inceptisol. These results demonstrated the opportunity of making use of this resource for direct application in acid tropical soils, mainly for poor farmers that cannot afford water-soluble fertilizers. Low grade phosphate rock can be modified to increase agronomic effectiveness (Weil, 2000; Van Straaten, 2006).

Low cost modification of low grade phosphate rock include fine grinding, blending, and phospho-composting (Van Straaten, 2006). Blending phosphate rock with locally produced fertilizer, pelleting, combining with cattle manure and composting showed an increase of corn yield in Zimbabwe (Dhliwayo, 1999). In Kenya combining Minjingu phosphate rock with an organic source enriched

with N and K such as shrub *Tithonia diversifolia* obtained similar yields as those found with urea and TSP (Sanchez et al., 1997). Promising results on phosphate fertilizer production were found by Biswas and Narayanasamy (2006), who produced an organic compost enriched with high reactivity phosphate rock. Odongo et al. (2007) demonstrated that the application of organic compost enriched with phosphate rock provided an increase in available phosphorus and crop yield in different soils, with higher performance than TSP. It is possible to increase the reactivity of phosphate rock through bio-solubilisation with bacteria and /or fungi inoculation (phosphate rock solubilizing microorganisms (PRSM)) (Van Straaten, 2006). For instance, Nahas et al. (1990) succeeded in the production of phosphate biological fertilizers (PBF) through solubilisation of natural phosphates with *Aspergillus niger* combined with bagasse. Also, Ghani et al. (1994) produced PBF 15 times more soluble than phosphate rock through solubilisation with elemental sulphur and *Thiobacilli* bacteria population in growth medium. Kaur and Reddy (2015) demonstrated that the production of PBF is a more cost effective alternative compared with the use of diammonium phosphate (DAP) and can replace DAP in sustainable production systems.

The mechanism behind the increase in PBF reactivity was reported by Kpomblekou and Tabatabai (2003), Goyne et al. (2006; 2010) and Calvaruso et al. (2013), who explained that the increase in PBF reactivity is associated with production of rhizospheric acids (low molecular weight organic acids) by the phosphate solubilizing microorganisms. Other examples of success have been found by Asomaning et al. (2009) through production of phosphate fertilizers by 50% partial acidification of phosphate rock with sulphuric acid, resulting in phosphate fertilizer with efficiency equivalent to TSP. This combinations of modified P sources and organic sources provide a slow release fertilizer and multi-nutrient input to a deficient acid soil (Van Straaten, 2006; Odongo et al., 2007). There is a synergy to combine organic resources with phosphate rock, resulting in an organic fertilizer with better properties when compared to their individual sources (Nishanth and Biswas, 2008). This practice of using locally available organic sources in combination with P sources including available phosphate rock brought approximately 150000 families in Western Kenia out of poverty (Sanchez, 2002).

In Mozambique, apart from numerous small guano deposits, there are large phosphate deposits of metamorphic, igneous, and sedimentary origins (Appleton, 2002; Van Straaten, 2002). The large phosphate deposit is located in Monapo district, in Evate, 100 km east of Nampula province close to Nacala Port (Van Straaten, 2002). The Evate apatite/magnetite/biotite occurs in the oval-shaped Monapo structure composed of a metasedimentary sequence of biotite and graphitic gneiss with two

separate marble horizons (Van Straaten, 2002). Preliminary reserve estimates indicate a resource of ~ 155,413,000 tonnes of apatite ore with 9.32% P_2O_5, 5.76% Fe, and 1.12% TiO_2 (Ministry of Mineral Resources and Energy, 1997). The reserve was calculated to be at 100 m depth above sea level, and with ~14.5 million tonnes of P_2O_5, being one of the largest phosphate deposit in east-central Africa (Appleton, 2002; Van Straaten, 2002). Sedimentary phosphate deposits have been reported near Magude, 29 km northwest of Maputo province on the left bank of Incomati river (Manhiça, 1991; Van Straaten, 2002). Guano deposits also have been reported in several regions of the country, the Cheringoma (Sofala province) deposit the largest guano accumulations with ~ 600,000 tonnes (5.14% P_2O_5, 2.74% NO_3, and 1.37% K_2O) estimated reserve, followed by Buzi (Sofala province) with ~ 132,700 tonnes (3.88% P_2O_5, 3.26% NO_3, and 1.52% K_2O), and Vilanculos (Inhambane province) deposits with ~ 22,000 tonnes (8 to 13% P_2O_5) (Manhiça, 1991). The use of local natural rocks for direct application can also be less costly and sustainable alternative to reduce the dependence on imported soluble fertilizers (Sanchez, 2000). These resources are not being used for agriculture purposes, neither been reported studies assessing their agronomic effectiveness in Mozambique.

1.2.2. Calcareous rock as amendment to acid soils

Calcareous rock has been considered a viable strategy to increase crop productivity in acid soils (Anikwe et al., 2016; Carmeis Filho et al., 2017). Application of calcareous rocks to acid soils can correct soil acidity and consequently increase crop yield (Flower and Crabtree, 2011; Raboin et al., 2016). There are two main types of carbonates rocks, which are dolomites ($CaMg(CO_3)_2$) and limestones ($CaCO_3$) (Bucher and Grapes, 2011). These rocks normally occurs evaporite environments particularly in phanerozoic sediments (e.g. limestones and siliceous dolomites) or metamorphic origin, widely associated with orogenic belts (e.g. dolomitic marbles and calcitic marbles) (Bucher and Grapes, 2011). Dominant mineralogy of sedimentary carbonates rocks include mainly dolomite, calcite, and quartz, while metamorphic ones have also dolomite or calcite marbles with water bearing minerals in the pores (Bucher and Grapes, 2011; Liu et al., 2005). Generally, dolomite forms through the chemical alteration of calcite or aragonite as these minerals are thermodynamically unstable (Al-Awadi et al., 2009). Higher porosity and permeability are expected to be preserved in dolostone than in limestone because the supporting framework of dolomite crystals provides greater compressive strength, therefore the limestone is more susceptible to compaction (Gillott, 1963; Churcher et al., 1991; Al-Awadi et al., 2009; Luhmann et al., 2014).

The reactivity of calcareous rock is affected by the heterogeneous surface reaction of the rock and

mass transport of ions from the rock surface to solution via diffusion (Liu et al., 2005). The dissolution of dolomite is more homogeneous determined by surface reaction and is lower than limestone (Davis et al., 2007; Pokrovsky et al., 2009; Yasuda et al., 2013). A thorough understanding of carbonate rock dissolution is paramount to the successful application to the soil as amendment, with significant environmental and geochemical implications (Xu et al., 2013). Application of calcareous rock to acid soils is the most feasible cost-effective strategy for alleviating soil acidity, and for correcting chemical deficiencies (Carmeis Fihlo et al., 2016). In countries that cannot afford costly fertilizers, the exploitation of their own rock outcrops is a possible alternative (Appleton 2002; Van Straaten 2002; 2006; Odongo et al., 2007).

In Mozambique, limestone/dolomite resources are sedimentary, metamorphic and igneous (Van Straaten, 2002). Extremely large sedimentary deposits of limestones are found in Cheringoma south of Inhaminga (Sofala province) with at least 100 millions tonnes, followed by Salamanga (South of Maputo), and near Nacala along the coast between Tanzania border and Pemba (Van Straaten, 2002). Metamorphic limestone and dolomite resource are mainly found in north and central Mozambique, 50 km north of Lichinga (Niassa province) (Pinna et al., 1993), north and northwest of Tete province in the Massamba and Monte Muande area, in Monapo structures (Nampula province), and at Lurio river near Namapa (Van Straaten, 2002). The main igneous limestone/dolomite are found at Muande (Tete province) and Xiluvo (Sofala province), with complex of carbonate rock (Gittins, 1966; Markwich, 1996). These calcareous resources are mainly used by cement industry, and limited information is available regarding the use in agriculture. Application of limestone/dolomite to acid soils has advantage of increasing soil pH, resulting in precipitation of trace metals, and availability of other nutrients and crop yield (Van der Perre et al., 2012).

1.3. Biochar as organic amendment

Biochar is a solid product obtained through pyrolysis of biomass at temperatures between 300 to 900 ^{o}C intended to be used as soil amendment (Lehmann et al., 2011; Enders et al., 2012). Biochar can be produced via slow pyrolysis (SP), fast pyrolysis (FP), and gasification (G), in which feedstock biomass is combusted from minutes to h at 350-800 ^{o}C in absence of oxygen, for two seconds at 425-550 ^{o}C in absence of oxygen, and from seconds to h at > 800 ^{o}C in presence of oxygen, for SP, FP, and G respectively (Hussain et al., 2017). Recently biochar has been proposed as soil amendment to increase soil fertility and mitigate climate change (Chan et al., 2007; Lehmann et al., 2011; Wang et

al., 2015).

Application of biochar to soil can increase soil C storage (Steinbeiss et al., 2009; Yao et al., 2015), and improve soil physical (aggregation, porosity, water retention), chemical (pH, cation exchange capacity (CEC), nutrients sorption and desorption, etc.), soil biological (soil microbial biomass, root nodulation, mycorrhiza colonization, etc.) and biochemical (enzyme activities) properties, and reduce trace metal availability (Warnock et al., 2007; Xu et al., 2014; Wiszniewska et al., 2016; Ajayi and Horn, 2017; Hussain et al., 2017). Also, the efficiency of fertilizer may be enhanced by biochar application, due sorption properties of biochar, and subsequent reduction of N, P, and K leaching (Borchard et al., 2014). Recently it has been reported biochar application leads to immobilization of soil contaminants, thereby limiting their bioavailability and phytotoxicity (Wiszniewska et al., 2016). The greatest chemical difference between biochar and other organic matter is the much larger proportion of aromatic C and the occurrence of condensed aromatic structures (Lehmann et al., 2011).

These properties of biochar strongly depend on pyrolysis conditions and types of feedstock (Jindo et al., 2014; Purakayastha et al., 2015). Brunn et al. (2012) found lower pH and particle size, higher surface area in wheat straw biochar produced by FP compared with SP. Biochar produced by SP and FP differ in their physicochemical properties and different behaviour in soil (Brewer et al., 2009; Hussain et al., 2017). SP is a conversion technique that maximizes the yield of biochar per unit of feedstock (Borchard et al., 2014). The temperature of pyrolysis also affects biochar properties (Hussain et al., 2017). Rafiq et al. (2016), demonstrated that the C concentration in corn stover biochar increased from 45 to 65% with increasing of pyrolysis temperatures (from 300 to 500 ^{o}C), and higher stability of biochar was found at 500 ^{o}C. Higher pyrolysis temperatures causes higher ash content, pH, surface basicity, specific surface area, and mineral element concentrations, while lower surface acidity, CEC and nitrogen concentrations (Zhang and Wang, 2015). Particle size of biochar decreases with the increase of pyrolysis temperatures from 450-700 ^{o}C (Downie et al., 2009). Karunanithi et al. (2015) found higher total N, organic carbon, but low C/N ratio and total P and K concentrations in biochar produced at low temperature (300 ^{o}C).

The type of feedstock used for producing biochar may also affect overall properties of biochar in soils (Farrell et al., 2013; Jindo et al., 2014). Different biochars can induce different soil chemical and microbial community structure changes (Muhammad et al., 2014). Naisse et al. (2014) found very low proportion of rye grass (*Dactylus glomerata*) biochar mineralized compared with plant material. Similar found by Calderón et al. (2015) using corn residue biochar. Soil CEC of manure

based biochar is higher compared with wood (*Eucalyptus*) biochar (Singh et al., 2010) however, woodchip biochar application in soil results in higher hydraulic conductivity than manure biochar (Lei and Zhang, 2012). Enders et al. (2012) reported that wood biochar has higher volatile matter than non-wood biochars. The biochar derived from rice material normally has high ash content at all temperature ranges probably due the high Si concentration in rice plants, which is strongly related with ash in biochar (Jindo et al., 2014). Purakayastha et al. (2015) reported that corn biochar enhanced N and P concentration in soil, wheat biochar increased available K in soil, and rice husk biochar being relatively labile in soil fuelled the proliferation of microbial biomass, and thereby enhanced the physiological efficiency of microbes measured in terms of dehydrogenase activity. Therefore, biochar properties must be well understood as a function of pyrolysis conditions and type of feedstock in order to match the soil needs with the appropriate biochar type.

1.4. Methods to study phosphate rock, calcareous rock and biochar

Several analytical methods are used to evaluate rock samples in order to provide an integrated approach (Rajan et al., 1992; Szilas et al., 2008). Microscopic analytical methods include optical microscopy and scanning electron microscopy to identify mineral species, type of mineral particles, and examine the texture of consolidated rocks and grain size of various components (Pecher et al., 2003; Szmaja, 2013). These techniques are used to identify minerals that have very fine crystal structures (Chien, 2001). Conversely, mineral phases can be identified using X ray diffraction (XRD) spectroscopy, which is broadly used for qualitative and semi-quantitative mineral identification (Das and Hendry, 2011; Louër, 2017). Copper Kα radiation is often used to irradiate powder packs of samples and the diffracted X-rays are analyzed in terms of the angles at which they are emitted and intensity (number of counts at angular increments) (Szilas et al., 2008; Louër, 2017). Mineral phases in biochar can also be determined by XRD (Domingues et al., 2017). In case minerals cannot be identified using the previous methods, scanning electron microscopy method (SEM) can be very useful when coupled with energy dispersive X –ray analysis (EDX) (Korte et al., 2017; Šorša et al., 2017). Electron beam can be focused on small particles in order to generate X-ray, which in turn are analyzed to give a qualitative or semi-quantitative estimate of the chemical composition of rock sample (Korte et al., 2017). Elemental analysis in rocks can be determined using wet extraction methods or portable X-ray fluorescence (PXRF) procedure as reported by Weindorf and Chakraborty (2016).

Phosphate rock reactivity (solubility) is determined by extracting P concentration using neutral ammonium citrate (NAC), 2% citric acid, and 2% formic acid (Rajan et al., 1992; Chien et al., 2011). Citric acid can underestimate phosphate rock reactivity if the phosphate rock contains significant amount of free carbonates (calcite/dolomite) resulting in Ca common ion depressive effect (Chien et al., 2011). The suitability of phosphate rock for direct application can be determined using these extractants (Rajan et al., 1992). The methods vary depending on the extraction media used and the specific extraction procedure, and the variables in the extraction procedure may include size fractions, amount of sample, temperature of shaker bath, length of time the sample is agitated in shaker bath (Chien et al., 2011). A single extraction dissolution method generally does not assess the mid to long-term reactivity of phosphate rock (Chien et al., 2011). In addition, the reactivity feedback of rock particle size during a single extraction procedure most related with presence of easily dissolvable mineral surfaces probably activated during the grinding process (Truong and Fayard, 1995; Zapata and Roy, 2004), which could overestimate the nutrient release efficiency. Because of this, the use of dissolution experiments could be the most appropriate for assessing the mid to long-term reactivity of phosphate rock for direct applications, but is time consuming (Truong and Fayard, 1995).

Total carbonates determination is based on neutralization of samples by titrated acid, and all present minerals (calcite, dolomite, magnesite, etc.) in the samples will be dissolved (Pansu and Gautheyrou, 2006). The reactivity of calcareous rock can also be determined by using low molecular weight organic acids (LMWOA) such as acetic acid, citric acid, formic acid, etc. (Sengul et al., 2006). These LMWOAs are produced in soil as plant exudates from dead or living cells or microbial metabolites (Kpomblekou-A and Tabatabai, 2003; Wei et al., 2011). Therefore, the LMWOAs play a paramount role in plant rhizosphere processes (Sokolova, 2015). Thus, the reactivity of calcareous rock in LMWOAs helps to predict possible rhizosphere processes (Hinsinger et al., 2006). Calcareous rock reactivity in soil is determined by measuring the active carbonate by titration of excess of ammonium oxalate remaining after the attack of samples with ammonium oxalate under standard time conditions for attack and agitation (Pansu and Gautheyrou, 2006). The application of LMWOAs and dissolution trials to assess the mid to long-term reactivity represents a new perspective in calcareous rock research.

Biochar total C, O, H, N, S concentration can be determined by elemental analyzer equipments (e.g.: Thermo Flash EA-1112; Thermo Flash EA-1110, Carlo Erba Instruments, etc.) at 1020 ^{o}C (Graber et al., 2010; Jeffery et al., 2015; Raya-Moreno et al., 2017). Easily oxidizible organic C (EOOC) can be determined by using the Walkey and Black procedure (Agegnehu et al., 2016; Raya-

Moreno et al., 2017). Inorganic C in biochar can be determined by titration with acid (Wang et al., 2014), and the recalcitrant organic C in soil can be estimated by subtracting the EOOC from TOC (Suárez-Abelenda et al., 2015). Biochar reactivity can be determined by using LMWOAs as in the case of rock materials (Zhang et al., 2016). Biochar physical attributes (ash and volatile matter) contents are determined by ASTM[3] D 1762-84 protocol, in which samples are dried at 105 ^{o}C, then volatilized at 950 ^{o}C, before finally being combusted at 750 ^{o}C (Jindo et al., 2014; Jeffery et al., 2015). Volatile matter refers to an ASTM standard methodology that was developed to evaluate the quality of coals as fuel, and is only beginning to be evaluated as a material property with explanatory value for biochar stability (Lehmann et al., 2011). Methods for biochar characterization still need standardization as in the case of soil or plants samples (Raya-Moreno et al., 2017). Other technics such as thermogravimetric analysis (TGA) and nuclear magnetic resonance (^{13}C - NMR) analysis can be applied to understand the thermal decomposition and mass loss at different temperatures and the type of functional groups of organic matter in biochar, respectively (Jindo et al., 2014; Antunes et al., 2017). These technics are useful to identify and quantify the organic compounds responsible for biochar recalcitrance (Zhang et al., 2015).

1.5. Research questions, objectives and hypothesis

1.5.1. Research questions

1. How the reactivity of phosphate and calcareous rock with different grain sizes and acid-neutralising agents during the selected reaction times as long-term estimation?

2. What combination phosphate rock, calcareous rock, and biochar improve the fertility of an acid Arenosol and P availability to corn?

3. What is the implication of application of high rates of phosphate rock to an acid Arenosol microbial community and trace metal availability to different crops, and how biochar help prevent potential negative effects?

4. How different feedstocks affect biochar properties, and how these biochars contribute for acid Arenosol fertility improvement and reducing inorganic fertilizer?

[3] Standard Test Method for Chemical Analysis for Wood Charcoal

1.5.2. Objectives

The final objective of this study is to increase the productivity of corn and cowpea in Mozambique by improving the fertility of acid Arenosol by applying local phosphate, calcareous rocks, and biochar, contributing to the improvement of livehood of rural population.

1. To investigate the feasibility of using phosphate rock and dolostone from local sources in Mozambique, ground to different grain sizes (0.063-0.25, 0.25-0.5, and 0.5-2 mm), as sources of P, Ca, and Mg;

2. To assess in pot experiments the effects of the simultaneous application of phosphate rock (as sparingly soluble P source), dolostone (as liming material), and biochar (contributing soluble P and retaining capacity) from local sources in Mozambique on acid Arenosol chemical and biochemical improvement, phosphorus flows and uptake, growth and yield of corn (*Zea mays* L.);

3. To assess the contribution of pH and root-induced changes to alteration in soil biochemical and biological properties mediated by phosphate rock and biochar application to acid Arenosol;

4. To determine in pot experiments the effect of biochars in combination with NPK fertilizer on acid Arenosol chemical and biochemical improvement, nutrients use efficiency, growth and yield of cowpea (*Vigna unguiculata* L.).

1.5.3. Hypothesis

1. In the first specific objective we hypothesize that both rocks will provide substantive nutrient release in support of agronomic production and that finer particle size will facilitate enhanced solubility.

2. In the second specific objective: a) Since in acid Arenosol crop yields are low, the use of phosphate and calcareous rocks and biochar should improve soil chemical and biochemical properties and, as a result, corn yield will increase. In fact, the overall increase of pH and improvement of soil properties should directly or indirectly increase nutrient availability and decrease nutrient leaching (because of biochar), so to improve soil fertility; b) The response of the soil enzyme activity should be enzyme specific, as reported by Hussain et al. (2017); c) Since plants can exudate phosphatases and organic molecules to mobilize organic P and increase the availability of P from phosphate rock and biochar (Marschner et al., 2004; Hinsinger et al., 2006; Neumann and Römheld 2012), the rhizosphere should affect differently than the bulk soil the enzyme activity patterns to increase availability of P.

3. In the third specific objective we hypothesized that simultaneous application of phosphate rock and biochar can improve soil biochemical quality, and biochar addition would synergistically reduce the negative impact of high dose of phosphate rock; response of soil enzyme activity, respiration, and microbial C will be crop specific and that rhizosphere processes would affect differently enzyme activity patterns;

4. In the fourth specific objective we hypothesized that biochars will affect significantly soil properties which will increase NPK fertilizer efficiency and cowpea yield, while reducing environmental problems due the leaching of nutrients. Nutrient contents and recalcitrance of biochars will determine their interaction with soil and influence on plant growth.

1.6. Thesis structure

The following thesis chapters (5) report the research made during years of PhD course which was prepared as independent manuscript. These chapters refer to:

- ✓ *Chapter 2* – Reports the activities made to respond the specific objective 1. Here we focused on laboratory characterization of both phosphate and calcareous rock;
- ✓ *Chapter 3* – Reports the activities made on specific objective 2. Soil fertility improvement and agronomic effectiveness of various combination of phosphate and calcareous rock and biochar assessed in pot trials with corn (*Zea mays* L., cultivar: MRI594 - Hybrid white early maturity);
- ✓ *Chapter 4* – Reports the activities made on specific objective 3. Soil biological and biochemical response due the application of high rate of phosphate rock, biochar, as well the corn (*Zea mays* L., cultivar: MRI594 - Hybrid white early maturity) and cowpea (*Vigna unguiculata*, cultivar: IT98K-1105-5 early maturity cultivar) yield response is assessed in pot trials;
- ✓ *Chapter 5* – Reports the activities made on specific objective 4. Soil fertility improvement and agronomic effectiveness of different biochars and inorganic fertilizer is assessed in pot trials with cowpea (*Vigna unguiculata*, cultivar: IT98K-1105-5 early maturity cultivar);
- ✓ *Chapter 6* – Reports the final consideration of the research made summarizing the main findings from chapters 2, 3, 4, and 5.

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CHAPTER 2 - PHOSPHATE AND CALCAREOUS ROCK ASSESSMENT[4]

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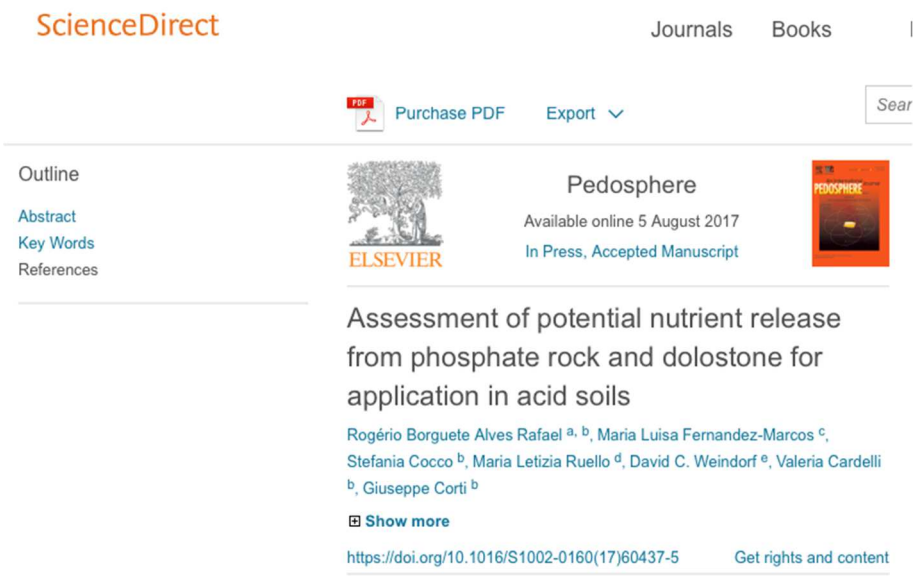
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Assessment of potential nutrient release from phosphate rock and dolostone for application in acid soils
Rogério Borguete Alves Rafael [a, b], Maria Luisa Fernandez-Marcos [c], Stefania Cocco [b], Maria Letizia Ruello [d], David C. Weindorf [e], Valeria Cardelli [b], Giuseppe Corti [b]
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Abstract

The aim of this study was to evaluate the feasibility of using phosphate rock and dolostone as fertilizer and amendment, respectively, for application in tropical acid soils. The dissolution of different particle-size fractions by water and citric acid was studied. Laboratory column experiments were run following a completely randomized design, by using 0.063 – 0.25, 0.25 – 0.5, and 0.5 – 2 mm particle-size fractions of both rocks. Each rock particle-size was subjected to exhaustive dissolution with distilled water, citric acid solution at pH 4, and citric acid solution at pH 2, with the following extraction times: 1, 3, 5, 7, 12, 24, 72, 144, 240, and 360 h. The dissolution of both rocks depended on particle-size, leaching solution and extraction time. The dissolution rate of rock-forming minerals augmented as the specific surface area increased, corresponding to a decrease in particle-size. In all cases, the kinetics of release was characterized by two phases: *1*) a first stage of rapid release that lasted 24 h, which would ensure short-term nutrient release, and *2*) a second stage of slow release (after 24 h), representing the long-term nutrient release efficiency. Both rocks are suitable as slow release fertilizers in strongly acid soils and would ensure the replenishment of P, Ca, and Mg. A combination of fine and medium particle-size fractions should be used to ensure high nutrient release efficiency. Much work has to be done to assess the overall impact of considerable amounts of fresh rocks in soils.

Key words: citric acid, soil fertility, rock weathering, nutrient release

1. Introduction

Worldwide acid soils are mainly present into two belts: 1) humid northern temperate zones, which are mostly occupied by coniferous forest, and 2) humid tropics, which host savannah systems and tropical rainforests (FAO, 2016). Acid soils are deficient in plant nutrients, while having increased availability of phytotoxic elements (van Straaten, 2002; 2006; Caires et al., 2005; van Raij, 2011; FAO, 2016). These deficient elements (with exception of N) are mostly derived from rocks and minerals (Chien et al., 2011).

Since soil acidification strongly reduces crop production, amelioration of acid soils is paramount for food security and agriculture sustainability. Application of fertilizers can satisfy the nutrient deficiency in acid soils (Chien et al., 2011), but the cost of fertilizers, particularly of P fertilizers, has increased substantially worldwide (van Straaten, 2002; 2006; Chien et al., 2011). Therefore, finding alternative local sources of plant nutrients which could supply P or other elements for sustainable crop production is a practical, low-cost, long-term strategy that addresses the need of poor farmers (Appleton, 2002; van Straaten, 2002; 2006; Zapata and Roy, 2004).

Apatites and calcareous rich rocks have been tested for their application in agricultural acid soils. For instance, it is known that the reactivity of apatites can be increased through grinding, and this increases P availability and produces similar yields responses and agronomic effects to those of water soluble fertilizer (van Straaten, 2002; 2006; Szilas et al., 2008).

Thus the application of limestone, possibly with a certain amount of dolomite, can mitigate soil acidity, prevent Al and Mn toxicity (Álvarez et al., 2012; van Straaten, 2002; 2006; 2007), and provide Ca and Mg to the soil. Consequently, food production and quality are enhanced (Maria and Yost, 2006). In addition, both apatitic and calcareous rocks may contain a wide rage of minor chemical elements, some of which are beneficial plant nutrients (IAEA, 2002; Szilas et al., 2007; van Straaten, 2006).

In Mozambique, acid soils cover ~70% of the country (Gouveia and Azevedo, 1949). Here, two important rock outcrops merit consideration: a metamorphic phosphate rock and a dolostone. Mineralogically, these two rocks are extremely relevant for mitigating acidity and replenishing P, Ca, and Mg. Their importance is even more significant considering that African countries cannot afford to purchase expensive water soluble phosphatic fertilizers, but could exploit their own rock outcrops, with reduced and Controlled environmental risks (Khasawneh and Doll, 1978; Appleton, 2002; van Straaten, 2006; 2007).

Phosphatic rocks and dolostones vary widely in their mineralogical and physic-chemical properties. Consequently, their reactivity and agronomic potential also vary (IAEA, 2002; Szilas, 2002; Szilas et al., 2007; 2008). Thus, it is prudent to evaluate their fertility value in terms of nutrient release to assess their possible use for crop production while minimizing fertilizer requirements (van Straaten, 2002; Zapata and Roy, 2004).

The dissolution rate of rocks tested for their possible use in agriculture mainly depends on their mineral composition as well as on the solubilizing acid and pH of the extracting solution (Goyne et al., 2006; Calvaruso et al., 2013). Low molecular weight organic acids (LMWOAs) are preferred to others, citric and oxalic acids being the most used (IAEA, 2002). In fact, citric acid originates in soil from the secretions of plant roots and soil microorganisms like fungi, lichens, and prokaryotes (Olsson and Wallander, 1998; Goyne et al., 2006; Joergensen and Wichern, 2008; Klugh-Stewart and Cumming, 2009; Goyne et al., 2010), and can simulate the weathering occurring in the rhizosphere (Turpault et al., 2009; Calvaruso et al., 2013).

For instance, when apatites are exposed to citrate and oxalate ligands, the release of P and Ca is the highest (Goyne et al., 2006, 2010). Instead, the dissolution of calcareous minerals such as calcite ($CaCO_3$) and dolomite [$CaMg(CO_3)_2$] depends on solution pH, pCO_2, temperature, and mineral properties such as crystallinity (Yasuda et al., 2013) as well as specific surface area (van Straaten, 2002, 2006, 2007). Dolomite dissolution rates are usually lower than those of calcite (Liu et al., 2005; Dewaide et al., 2014).

This study aims to investigate the feasibility of using a phosphate rock and a dolostone from local sources in Mozambique, ground to different grain sizes (0.063-0.25, 0.25-0.5, and 0.5-2 mm), as sources of P, Ca, and Mg. Further, we seek to assess their effectiveness as acid-neutralising agents, by testing their solubility in water and different concentrations of citric acid solutions in open-system columns under Controlled conditions. We hypothesize that both rocks will provide substantive nutrient release in support of agronomic production and that finer particle size will facilitate enhanced solubility.

2. Materials and Methods

2.1. Sample preparation

Both studied rocks are from Mozambique. The phosphate rock was collected from the Nampula Province deposit in the Evate district, while dolostone was collected from the Mount Muambe deposit located in the Tete Province. Blocks of rocks were fragmented by using a grinding press; then the rock fragments were ground using an agate mortar. For both rocks, three size-fractions were obtained by dry sieving: 0.063-0.25, 0.25-0.5 and 0.5-2 mm.

2.2. General characterization

The mineralogical composition was determined on powdered rocks by X-ray diffraction with a Philips PW 1830 diffractometer, using the Fe-filtered Co Kα1 radiation (35 kV and 25 mA); the step size was 0.02° 2θ and the scanning speed was one second per step. A semi-quantitative estimation was obtained after the identification of the minerals on the basis of their characteristic peaks as reported by Dixon and Schulze (2002), and MINCRYST database (http://database.iem.ac.ru/mincryst, accessed on 22th June 2016).

The elemental analysis of both rocks was obtained using DP-6000 Delta Premium portable X-ray fluorescence (PXRF) spectrometer (Olympus, Waltham, MA, USA) accordingly to Weindorf and Chakraborty (2016). The instrument features a Rh X-ray tube operated at 15–40 kV with quantification via ultra-high resolution (165 eV) silicon drift detector. The analysis was firstly conducted in "Soil Mode" (three beams of 30 s each), and secondly in "Geochem Mode" (two beams of 30 s each). Therefore, the contents of Al, Si, P, Mg, Ca, and S were validated using "Geochem Mode" readings, and the remaining elements were validated using "Soil Mode" (Weindorf and Chakraborty, 2016).

Total C concentration in dolostone was determined using CHNS-O analyser (EA1110, Carlo Erba Instruments, Milan, Italy). The fractions of phosphate rock were analysed for water soluble P, P extractable with neutral ammonium citrate (NAC-P), P extractable with 2% citric acid solution (citric-P), and P extractable with 2% formic acids solution (formic-P) as per Rajan et al., (1992). For each size- fraction of both rocks, 10 g of sample was added to 25 mL of distilled water, and the abrasion pH was measured after 6 min of solid – liquid contact (Romero et al., 1987). To measure the pH at 1.5 h, the suspension was stirred for 1 h on an oscillating table and left to rest for 30 min before

repeating the pH measurement; in a similar way, pH was measured at 24 h (Pansu and Gautheyrou, 2006).

2.3. Leaching experiment

To assess the long-term dissolution (reactivity) of the different size-fractions of both rocks, two sets of column experiments were conducted using a completely randomized design. Samples of the three particle-size fractions (0.063 – 0.25, 0.25 – 0.5 and 0.5 – 2 mm) of both rocks were treated for 15 d with different leaching solutions (distilled water, citric acid solution at pH 4 and citric acid solution at pH 2); leachates were retrieved at the following extraction times: 1, 3, 5, 7, 12, 24, 72, 144, 240, and 360 h. Similarly, percolations without rock sample were conducted as Controls. The experimental design consisted of three replicates for each fraction and leaching solutions.

The experiments were conducted in an isolated room at a temperature of 25 ± 1ºC. The leaching solutions were prepared 24 h prior to use by dissolving monohydrate citric acid crystals (AR manufactured by Carlo Erba Reagents) in distilled water. The citric acid solution at pH 4 ($\approx 10^{-4}$ M) was considered representative of the soil solution conditions of acid soils from tropical environments, while the citric acid solution at pH 2 ($\approx 10^{-1}$ M) was taken as an example of extreme acidity conditions that only rarely can occur in soil, even in the rhizosphere.

Each column (diameter x length of 30 x 400 mm) of 200 mL capacity was fitted with 0.3 g of fiberglass at the bottom to prevent rock fragments loss. Thus, 1.000 g of each rock size-fraction was put into the column, and 100 mL of leaching solution added (solid:liquid ratio of 1:100, w:v); At each extraction time, the liquid was allowed to percolate at a constant rate (100 mL h^{-1}). Thus, 100 mL of fresh leaching solution was added to each column.

The elements measured in the leachate of both rocks were Ca, Mg, K, Na, Ba, Al, Fe, Mn, Zn, Cd, Ni, and Pb; only in those from phosphate rock fractions was P also determined. The analyses of leachates were carried out the same day they were collected. The cations were measured by an inductively coupled plasma optical emission spectrometer (Perkin Elmer Optima 8300) as described by Boss and Fredeen (1997), while a simple colorimetric method based on ascorbic acid reduction of the ammonium phosphomolybdate complex (Kuo, 1996) was used to measure P in the leachates.

2.4. Kinetic analysis

The Langmuir (1997) equation below was used to determine the release rate using the data obtained by the leaching experiment, assuming that the dissolution of minerals in phosphate rock and dolostone were controlled by surface reaction:

$$dC_{(t)}/dt = k; \qquad (1)$$

$$\text{Integrated rate law: } C = C_o + kt, \qquad (2)$$

where $C_{(t)}$ (mol L^{-1}) is the concentration of released species in the bulk solution at the time t; C (mol L^{-1}) is the concentration of released species in the leachate after the release, C_o (mol L^{-1}) is the initial concentration of the species in bulk solution before the release starts; k (mol L^{-1} s^{-1}) is the constant rate.

2.5. Statistical analysis

R version 3.1.2 (2014-10-31) was used for statistical analysis. Single extraction data were analysed for analysis of variance (ANOVA) after a *boxcox* transformation (Meloun et al., 2005) of the data to perform parametric tests (Shapiro-Wilk normality test and Bartlett test of homogeneity of variances). A multiple comparison Tukey test (at 5% significance) was used to compare the means. Similarly, data obtained from the leaching experiments were analysed using parametric tests, but they revealed a non-normal distribution and heteroscedasticity. Therefore, summary statistics (mean, variance, standard error and deviation) were used whenever possible.

3. Results and discussion

3.1 Mineralogical and chemical composition of the two rocks

X-ray diffraction analysis showed that the phosphate rock was mainly made of fluoroapatite, while the dolostone was made of dolomite (Fig. 1). Both rocks contained traces of phyllosilicates. The major constituent elements of the phosphate rock were Ca and P, followed by Si, Al, Fe, K, Sr, and Ba; all the other elements were present in concentrations close to or <1000 mg kg^{-1} (Table 1).

The rock P content was ~24%, equivalent to 55% of P_2O_5, which satisfies the European legislation in terms of total P content to be used for direct application to the soil, as it exceeds the threshold value of 25% of P_2O_5 (European Commission, 2000). The concentrations of trace elements were similar to those commonly reported for phosphate rocks (Adriano, 2013). The concentrations of As and Hg

were moderately high, but they should not be problematic for the application of phosphate rock to soils at moderate rates.

The major elements in the dolostone were Ca, Mg, and C, followed by Si, P, Al, Fe, K, Mn, Ba, S, Sr, and Ti, with the other elements present in amounts <40 mg kg^{-1} (Table 1). The richness in Ca, Mg, and C was ~ 29.4, 18.2, and 8.2%, respectively, equivalent to ~ 41, 30, and 41% of the respective oxides. The Ca:Mg molar ratio of 1:1 confirmed the mineralogical observation indicating that the main rock forming mineral was dolomite (Al-Awadi et al., 2009). This dolostone can be considered completely safe given its low concentration of potentially hazardous trace elements.

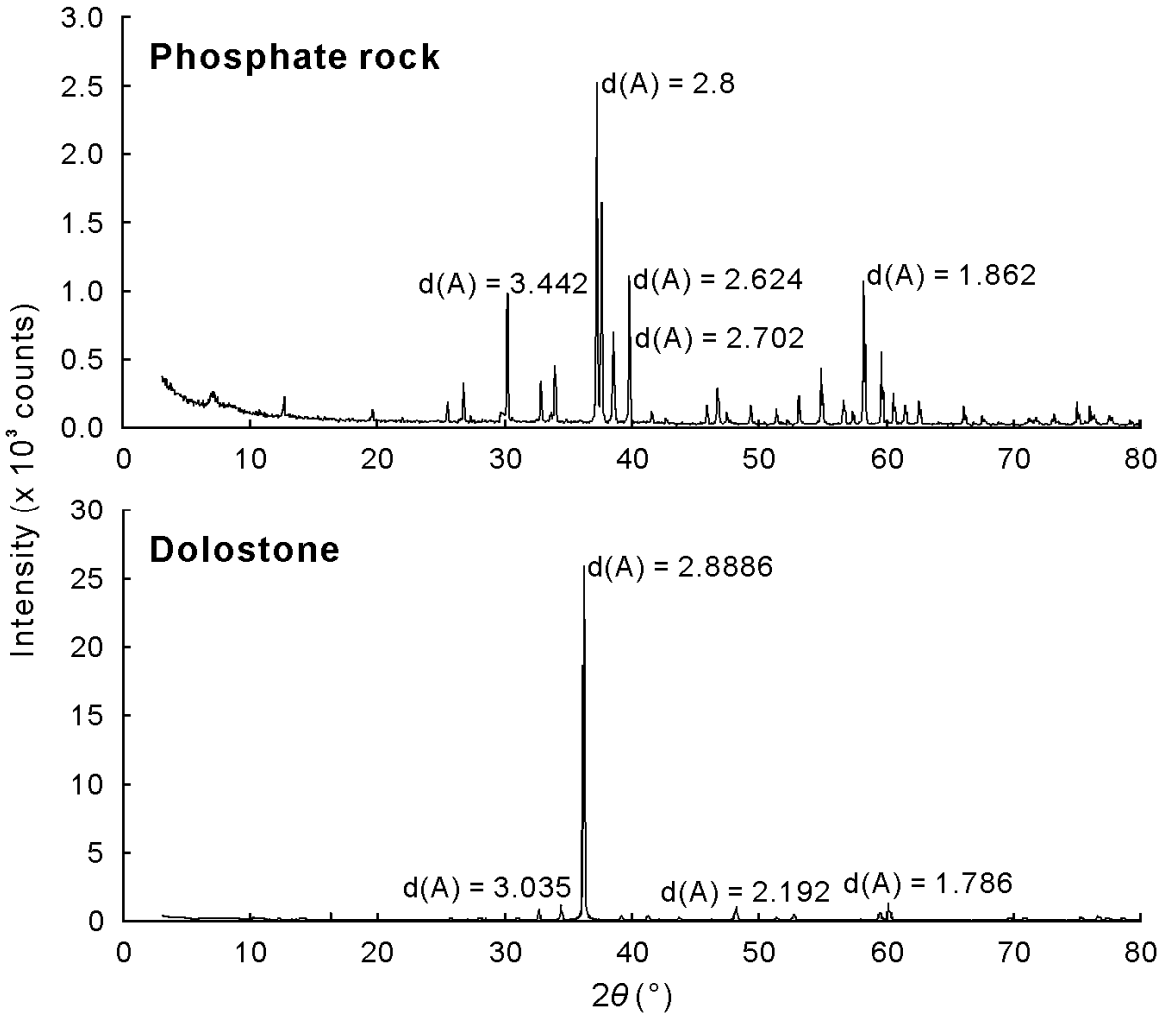

Fig. 1 X-ray diffractogram, using Co $K\alpha 1$ radiation, of phosphate rock and dolostone obtained from Mozambique, indicating a composition mostly made of fluoroapatite and dolomite, respectively, with traces of phyllosilicates. d(A) represents the d spacing expressed in Ångström (1 Å = 10^{-10} m).

Table 1
Elemental analysis and corresponding composition in oxides of the phosphate rock and dolostone from Mozambique used for the trials.

Phosphate rock				Dolostone			
Elements	**mg kg^{-1}**	**Oxides**	**mmol kg^{-1}**	**Elements**	**mg kg^{-1}**	**Oxides**	**mmol kg^{-1}**
Si	20,160.96 (240.21)[a)]	SiO_2	717.98 (8.55)	Si	12,941.70 (242.05)	SiO_2	460.89 (8.62)
C	< LOD	CO_2	< LOD	C	82,121.7 (3,249.59)	CO_2	6,843.42 (270.80)
Al	7,684.33 (484.64)	Al_2O_3	142.83 (9.01)	Al	2,438.91 (1,241.23)	Al_2O_3	45.33 (23.07)
Fe	7,378.00 (63.00)	Fe_2O_3	66.11 (0.56)	Fe	1,410.50 (19.00)	Fe_2O_3	12.64 (0.17)
P	239,666.10 (689.95)	P_2O_5	3865.58 (11.13)	P	2,801.29 (160.30)	P_2O_5	45.18 (2.59)
Ca	362,922.97 (5,277.00)	CaO	9073.07 (131.93)	Ca	293,958.27 (1,751.26)	CaO	7,348.96 (43.78)
Mg	<LOD	MgO	<LOD	Mg	181,719.405 (4508.33)	MgO	7,478.16 (185.53)
K	3,275.00 (123.00)	K_2O	41.99 (1.58)	K	695.00 (67.00)	K_2O	8.91 (0.86)
S	<LOD	SO_3	<LOD	S	314.88 (30.66)	SO_3	9.84 (0.96)
Ba	2,004.00 (112.00)	BaO	14.59 (0.82)	Ba	332.00 (56.00)	BaO	2.42 (0.41)
Sr	2,572.00 (39.00)	SrO	29.36 (0.45)	Sr	285.00 (4.00)	SrO	3.25 (0.05)
Cl	1,254.00 (171.00)	ClO_2	35.37 (4.82)	Cl	<LOD	ClO_2	<LOD
Ti	1,227.91 (22.00)	TiO_2	25.66 (0.46)	Ti	114.00 (8.00)	TiO_2	2.38 (0.17)
Mn	498.00 (11.00)	MnO_2	9.07 (0.20)	Mn	448.00 (8.00)	MnO_2	8.16 (0.15)
V	120.00 (4.00)	V_2O_5	2.36 (0.08)	V	4.40 (1.20)	V_2O_5	0.09 (0.02)
Cr	129.00 (6.00)	Cr_2O_3	2.48 (0.12)	Cr	<LOD	Cr_2O_3	<LOD
Ni	58.00 (7.00)	NiO	0.98 (0.12)	Ni	36.00 (5.00)	NiO	0.61 (0.08)
Cu	17.00 (3.00)	CuO	0.27 (0.05)	Cu	<LOD	CuO	<LOD
Zn	36.00 (2.00)	ZnO	0.55 (0.03)	Zn	12.05 (1.20)	ZnO	0.09 (0.01)
As	36.90 (2.00)	As_2O_3	0.49 (0.03)	As	<LOD	As_2O_5	<LOD
Rb	21.60 (1.10)	Rb_2O	0.25 (0.01)	Rb	3.30 (0.50)	Rb_2O	0.02 (0.00)
Cd	<LOD	CdO	<LOD	Cd	<LOD	CdO	<LOD
Y	152.77 (1.65)	Y_2O_3	0.86 (0.01)	Y	<LOD	Y_2O_3	<LOD
Zr	51.00 (6.00)	ZrO_2	0.28 (0.03)	Zr	11.40 (1.60)	ZrO_2	0.13 (0.02)
Hg	11.70 (1.90)	HgO	0.03 (0.00)	Hg	<LOD	HgO	<LOD
Mo	9.70 (1.20)	MoO_3	0.05 (0.01)	Mo	<LOD	MoO_3	<LOD
W	24.45 (4.05)	WO_3	0.07 (0.01)	W	<LOD	WO_3	<LOD
Se	6.10 (0.80)	Se_2O_3	0.04 (0.01)	Se	1.70 (1.40)	Se_2O_3	0.01 (0.01)
Pb	26.00 (2.00)	PbO_2	0.13 (0.01)	Pb	4.00 (1.00)	PbO_2	0.02 (0.00)

[a)]Numbers in parentheses are the standard deviation (n=3). LOD - Limit of detection.

3.2. Phosphate rock

3.2.1. General properties

For the phosphate rock, the abrasion pH decreased significantly ($P < 0.05$) with contact time for all fractions, ranging from 9.36 for the finest fraction at 6 min of solid-liquid contact, to 7.81 for the coarsest fraction after 24 h (Table 2). As expected, the finest fraction presented higher pH values ($P < 0.05$) compared with the other fractions. This was ascribed to the higher specific surface area of this fraction. Newly formed mineral surfaces like those obtained by grinding are able to release alkaline and alkaline-earth elements and adsorb H^+, so to induce an abrasion pH that lasts until other factors perturb the suspension (Grant, 1969; Romero et al., 1987). The temporal decrease of abrasion pH was ascribed to the dissolution of atmospheric CO_2 (Grant, 1969) and possibly to surface passivation (Cuniglio et al., 2009).

Single extractions of P by water, NAC, 2% citric acid solution and 2% formic acid solution showed that the 2% formic acid solution was able to extract the highest ($P < 0.001$) amount of P (Table 3). The increase of extractable P with the grinding for all the extractants except water was explained with the highest reactivity of the finest fraction of this rock (Rajan et al., 1992). Therefore, taking 2% formic acid solution as the best predictor of agronomic effectiveness of phosphate rock (Rajan et al., 1992), the finest size fraction features high potential ($P < 0.001$) for a direct application in strongly acid soils (Ghani et al., 1994; Rajan et al., 1996; Zapata and Roy, 2004).

3.2.2. Long-term release in column experiments

(i) Cumulative releasing pattern of P, Ca, and Mg

The cumulative release of P, Ca, and Mg in water and in citric acid solutions at pH 4 and pH 2 from different particle sizes of phosphate rock calculated for the different extraction times. Other elements such as K, Na, Ba, Al, Fe, Mn, Zn, Cd, Ni, and Pb were leached in concentrations always lower than 100 mmol kg^{-1}. The cumulative release of P, Ca and Mg showed an increasing trend for all leaching solutions (Fig. 2). The rate of release was relatively high in the first 24 h of leaching and decreased thereafter, indicating that most of the extractable nutrients were released within 24 h. For all nutrients and leaching solutions, the highest cumulative release was obtained for the finest fraction ($P < 0.01$), followed by the medium and, then, by the coarse fraction (Table 4).

Table 2
Abrasion pH at different contact times of different particle size fractions of phosphate rock and dolostone from Mozambique and analysis of variance (ANOVA) for extraction time, particle-size fraction, and their interaction

Contact time	Particle size fractions			ANOVA		
(h)	0.063 – 0.25 mm	0.25 – 0.5 mm	0.5 – 2 mm	Extraction time	Particle-size fraction	Extraction time × Particle-size fraction
			Phosphate rock			
0.1	9.36 (0.01) [a] aA [b]	8.63 (0.23) bA	8.66 (0.02) bA			
1.5	8.24 (0.07) aB	7.79 (0.01) bB	7.69 (0.01) bB	***	***	**
24	8.23 (0.08) aB	7.98 (0.03) bB	7.81 (0.03) bB			
			Dolostone			
0.1	9.36 (0.04) aA	9.57 (0.14) aA	9.50 (0.11) aA			
1.5	8.73 (0.10) aB	8.81 (0.05) aB	8.66 (0.03) aB	***	*	**
24	8.29 (0.03) aC	8.27 (0.03) aC	8.25 (0.03) aC			

*, **, *** Significant at $P = 0.05$, $P = 0.01$ and $P = 0.001$, respectively. [a] Means (standard deviations, n=3); [b] Means followed by the same lowercase letter within each row are not significantly different at $P < 0.05$ by multiple-comparison Tukey test and those followed by the same uppercase letter within each column for a given rock are not significantly different at $P < 0.05$ by multiple-comparison Tukey test.

Table 3
P extraction (expressed as P_2O_5 content) by water, neutral ammonium citrate (NAC), 2% citric acid solution (CA), and 2% formic acid solution (FA) in different particle-size fractions of phosphate rock collected from Mozambique and analysis of variance (ANOVA) for particle-size fraction, extractant, and their interaction.

Particle-size fractions	Extractant				ANOVA
	Water	NAC	2% citric acid	2% formic acid	
0.063 – 0.25 mm	0.14 (0.01) [a] dA [b]	1.90 (0.42) cA	8.74 (0.04) bA	13.34 (1.33) aA	
0.25 – 0.5 mm	0.11 (0.01) dA	0.63 (0.13) cB	5.34 (0.26) bB	7.19 (0.26) aB	***
0.5 – 2 mm	0.08 (0.00) cA	0.37 (0.25) bB	4.35 (0.37) aB	5.42 (0.63) aC	

*** Significant at $P = 0.001$. [a]Means (standard deviations, n=3). [b]Means followed by the same lowercase letter within each row are not significantly different at $P < 0.05$ by multiple-comparison Tukey test and those followed by the same uppercase letter within each column are not significantly different at $P < 0.05$ by multiple-comparison Tukey test.

Table 4
Cumulative release of P, Ca, and Mg in water, citric acid solution (CA) at pH 4, and CA at pH 2 during the extraction time between 24 and 360 h from different particle-size fractions of phosphate rock collected from Mozambique and analysis of variance (ANOVA) for leaching solution, particle-size fraction, and their interaction.

Elements	Leaching solutions	Particle-size fractions			ANOVA		
		0.063 - 0.25 mm	0.25 - 0.5 mm	0.5 – 2 mm	Rock size fraction	Leaching solution	Rock size fraction vs. Leaching solution
P	Water	11.8 (3.1) [a] aA [b]	6. 7 (0.5) bA	7.7 (0.6) bA	***	***	**
	Citric acid at pH 4	67.6 (2.3) aB	49.3 (5.9) bB	61.6 (4.9) aB			
	Citric acid at pH 2	1874.7 (23.8) aC	1826 (35) aC	1702 (118) aC			
Ca	Water	4.9 (1.6) aA	2 (0.6) bA	1.1 (0.1) bA	***	***	**
	Citric acid at pH 4	16.3 (0.8) aB	4.8 (0.3) bB	8.2 (0.8) cB			
	Citric acid at pH 2	1367 (95) aC	1244 (124) aC	1308 (212) aC			
Mg	Water	1.7 (0.7) aA	0.9 (0.43) bA	0.6 (0.1) bA	***	***	**
	Citric acid at pH 4	0.8 (0.2) aB	0.6 (0.2) aA	1.2 (0.0) aB			
	Citric acid at pH 2	14.2 (1.3) aC	27.4 (2.4) bB	25.0 (5.4) bC			

**, ***Significant at $P = 0.01$ and $P = 0.001$, respectively. [a]Means (standard deviations, n=3). [b] Means followed by the same lowercase letter within each row are not significantly different at $P < 0.05$ by multiple-comparison Tukey test and those followed by the same uppercase letter within each column for a given element are not significantly different at $P < 0.05$ by multiple-comparison Tukey test.

The citric acid solution at pH 2 leached the highest amount of nutrients. For instance, at the end of the experiment, citric acid at pH 2 had leached from the finest fraction 27 and 86 times the amount of P obtained with the citric acid at pH 4 and water, respectively. The amounts of P and Ca extracted by the leaching experiment were higher than those extracted by single extraction in both water and 2% citric acid solution (Table 5).

For Ca there was no difference between the coarse and medium size-fractions in water and citric acid solution at pH 4 and for all the extraction times. The same was true for Mg in the finest and medium size-fractions in the case of citric acid solution at pH 2 during the first 72 h of extraction; thereafter the medium size-fraction presented a slightly higher cumulative release than the fine size fraction.

(ii) Release kinetics of P and Ca

The release kinetics was determined for both P and Ca, the most important nutrients in the phosphate rock fractions. During the leaching period the release rate decreased sharply up to 24 h, to steadily decrease until reaching values close to zero (Fig. 3). The finest fraction showed a higher release rate than the other particle-size fractions, which did not differ significantly between them, indicating that the kinetics was Controlled by superficial processes (Dorozhkin, 2012).

The citric acid solution at pH 2 showed the highest release rate, followed by citric acid solution at pH 4. The cumulative curves showed two leaching stages: 1) a rapid release during the first 24 h of leaching ascribed to the presence of easily dissolvable mineral surfaces probably activated during the grinding process, which could be taken as the short-term nutrient release efficiency (Truong and Fayard, 1995; Zapata and Roy, 2004; Gholizadeh et al., 2009), and 2) a slow release after 24 h of leaching, where the dissolution of the bulk mineral begins representing the medium- to long-term nutrient release efficiency, which is agronomically relevant.

Most of the solid had not dissolved when the near-zero release rate was reached, and therefore only the thin layer of superficial, very fine, poorly crystalline mineral material had dissolved. The amount of P and Ca extracted in this stage was similar among the particle-size fractions ($P < 0.01$) for citric acid at pH 2 (Table 4).

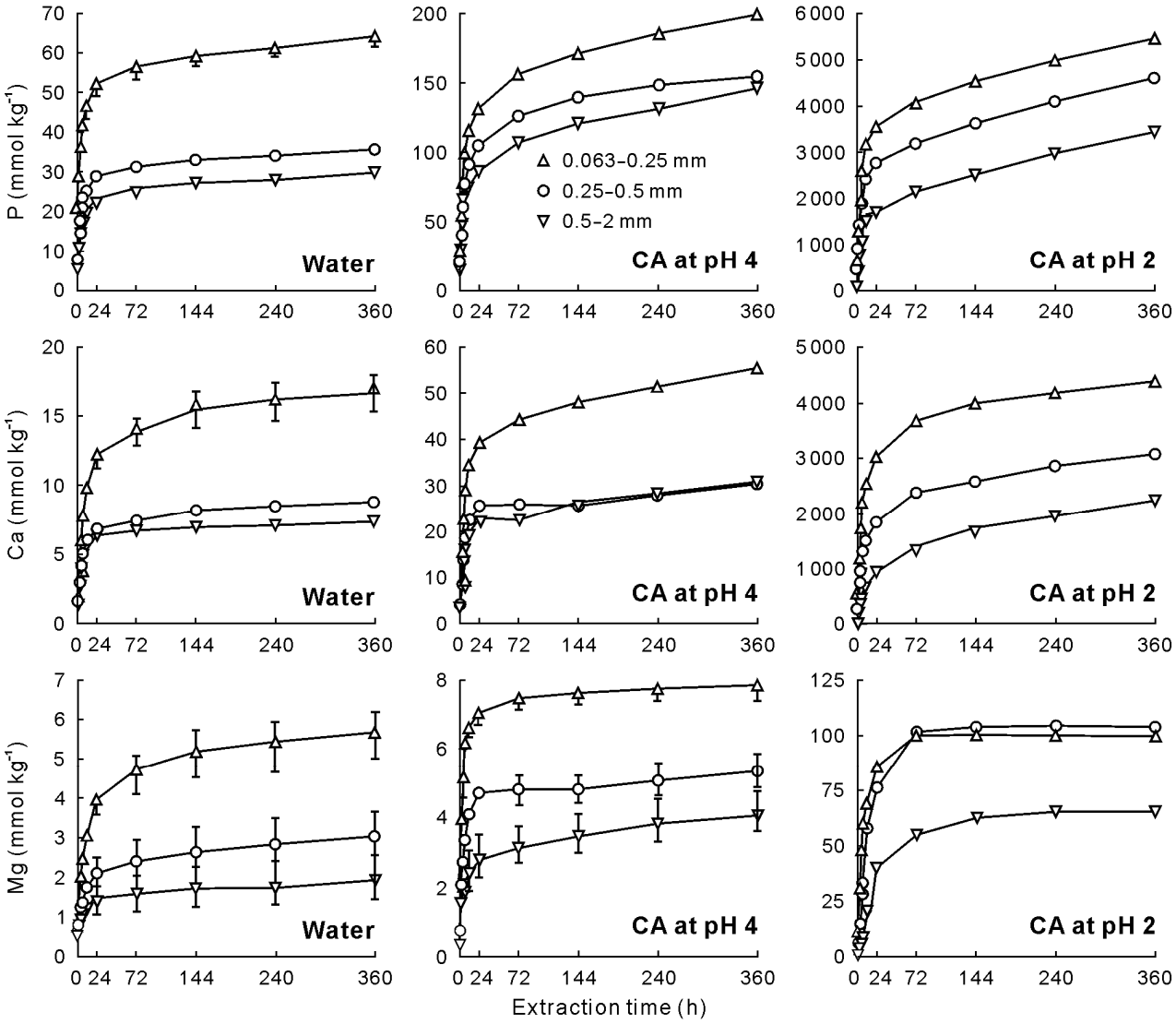

Fig. 2 Cumulative release of P, Ca, and Mg in water, citric acid solution (CA) at pH 4, and CA at pH 2 during the extraction time of 360 h from different particle-size fractions of phosphate rock collected from Mozambique. Vertical bars indicate standard errors of the means (n = 3).

Table 5
Amounts of P and Ca released in water and 2% citric acid solution (CA) during single extraction and leaching experiments from different size fractions of phosphate rock collected from Mozambique and analysis of variance (ANOVA) for extraction type, particle-size fraction, and their interaction.

Elements	Extractants	Extraction type	Particle-size fractions			ANOVA
			0.063 – 0.25 mm	0.25 – 0.5 mm	0.5 – 2 mm	
P	Water	Single	19.7 (1.5) [a)] aA [b)]	15.0 (1.7) bA	11.0 (0.0) cA	***
		Leaching	62.7 (4.6) aB	35.3 (3.2) bB	30.7 (2.1) bB	
	2% Citric acid	Single	1231 (5) aA	751.7 (36.1) bA	613.0 (52.0) bB	
		Leaching	5375 (135) aB	4590 (197) aB	3492 (296) bB	
Ca	Water	Single	1.0 (0.0) aA	1.0 (0.0) aA	1.0 (0.0) aA	***
		Leaching	16.7 (2.5) aB	9.0 (0.0) bB	7.7 (1.5) bB	
	2% Citric acid	Single	485.3 (18.5) aA	219.3 (17.4) bA	165.7 (4.6) bA	
		Leaching	4328 (263) aB	3094 (192) bB	2285 (320) bB	
Ca:P	Water	Single	0.05 (0.03) aA	0.07 (0.01) aA	0.09 (0.01) aA	***
		Leaching	0.27 (0.06) aB	0.25 (0.02) aB	0.25 (0.03) aB	
	2% Citric acid	Single	0.39 (0.02) aA	0.29 (0.01) aA	0.26 (0.02) aA	
		Leaching	0.81 (0.05) aB	0.67 (0.02) aB	0.65 (0.05) aB	

*** Significant at $P = 0.001$. [a)]Means (standard deviations, n=3). [b)]Means followed by the same lowercase letter within each row are not significantly different at $P < 0.05$ by multiple-comparison Tukey test and those followed by the same uppercase letter within each column for a given element and a given extractant are not significantly different at $P < 0.05$ by multiple-comparison Tukey test.

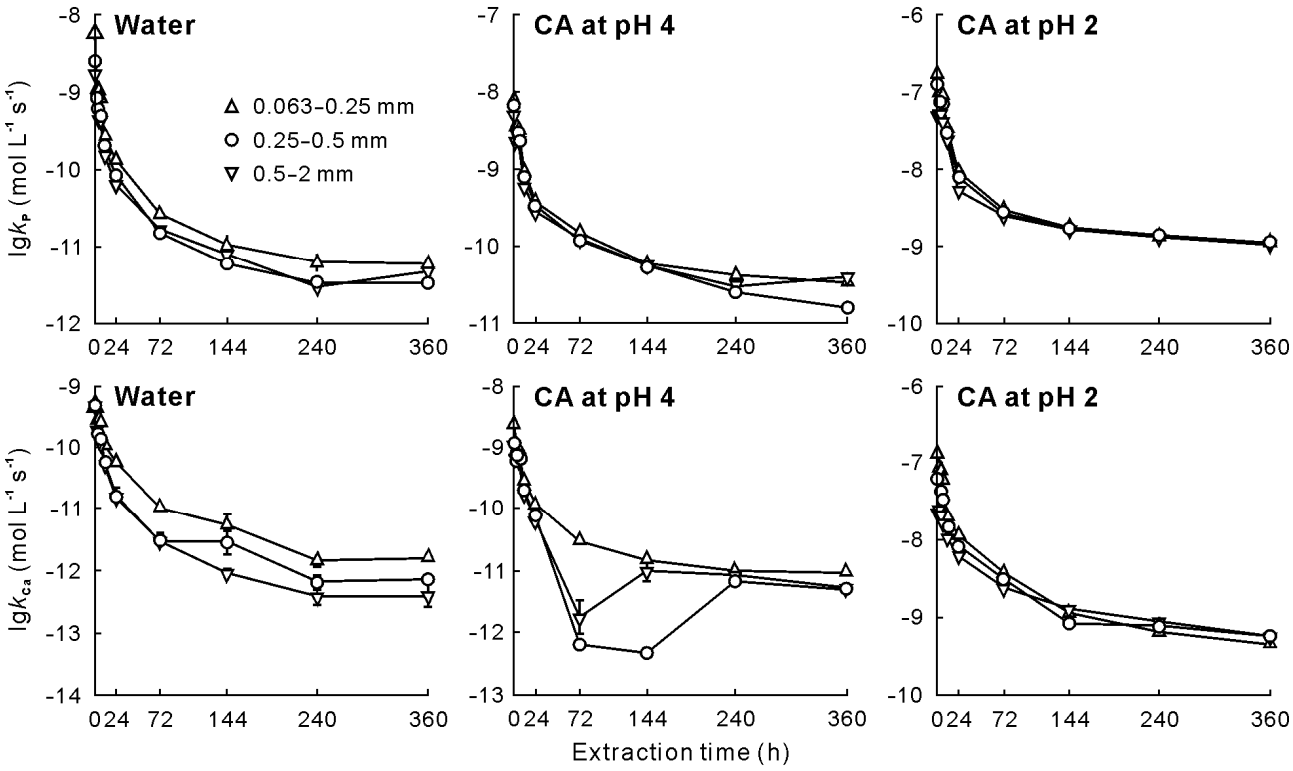

Fig. 3 Logarithms of dissolution rates (k) of P and Ca (k_P and k_{Ca}, respectively) in water, citric acid solution (CA) at pH 4, and CA at pH 2 during the extraction time of 360 h from different particle-size fractions of phosphate rock collected from Mozambique. Vertical bars indicate standard errors of the means ($n = 3$).

The bulk mineral in the second stage was approaching the equilibrium by reaching the metastable conditions characterized by the persistence of apatite crystals with a passivated surface that reduces the reactivity of the mineral grain (Cuniglio et al., 2009). Because of this, mineral grains in contact with unsaturated solutions release lesser amounts of ions than expected from the mineral formula (Dorozhkin, 2012).

In our trial we demonstrated that the size of rock fragments and the leaching solution strength were the determinant factors for the release rates. The ideal (congruent) dissolution of fluoroapatite promoted by an organic acid is given below (adapted from Calvaruso et al., 2013):

$$Ca_5(PO_4)_3F + 6\ AH \leftrightarrows 5\ Ca^{2+} + 6A^- + 3\ H_2PO_4^- + F^-, \qquad (3)$$

where A^- represents the organic anion

An appropriate supply of protons (H^+) and the removal of the reaction products (Ca^{2+}, $H_2PO_4^-$, and F) are necessary for the reaction to proceed forward (Robarge, 1999). The open system used in this study (columns) ensured the removal of the reaction products through solution percolation, while the citric acid solutions supplied H^+ so the release could go forward. The mechanism of dissolution of fluoroapatite by LMWOAs such as citric acid is ascribed to the supply of H^+, the formation of surface complexes that weaken and break the bonds among metals and lattice oxygen (Goyne et al., 2006; Dorozhkin, 2002; 2012), and the complexation of metals (Ca, Fe, and Al) by carboxyl ligands (-COOH). All of these reactions entail the release of phosphorus ($H_2PO_4^-$) from the mineral (Goyne et al., 2006; Calvaruso et al., 2013).

The phosphate rock had a molar Ca/P ratio of ~ 1.2 (Table I). During the leaching in water and citric acid solution at pH 4, the molar Ca/P ratio in solution did not reach 0.5 (Fig. 4). The much lower Ca/P ratio in the leachates indicates the occurrence of an incongruent dissolution of fluoroapatite because P was being released preferentially over Ca. However, with citric acid solution at pH 2, the fluoroapatite dissolution seemed to be incongruent too, even though between 24 and 72 h of leaching the Ca/P ratio reached values close to the Ca/P ratio in the initial solid phase. Yet, the finest size-fraction always displayed the highest Ca/P ratio.

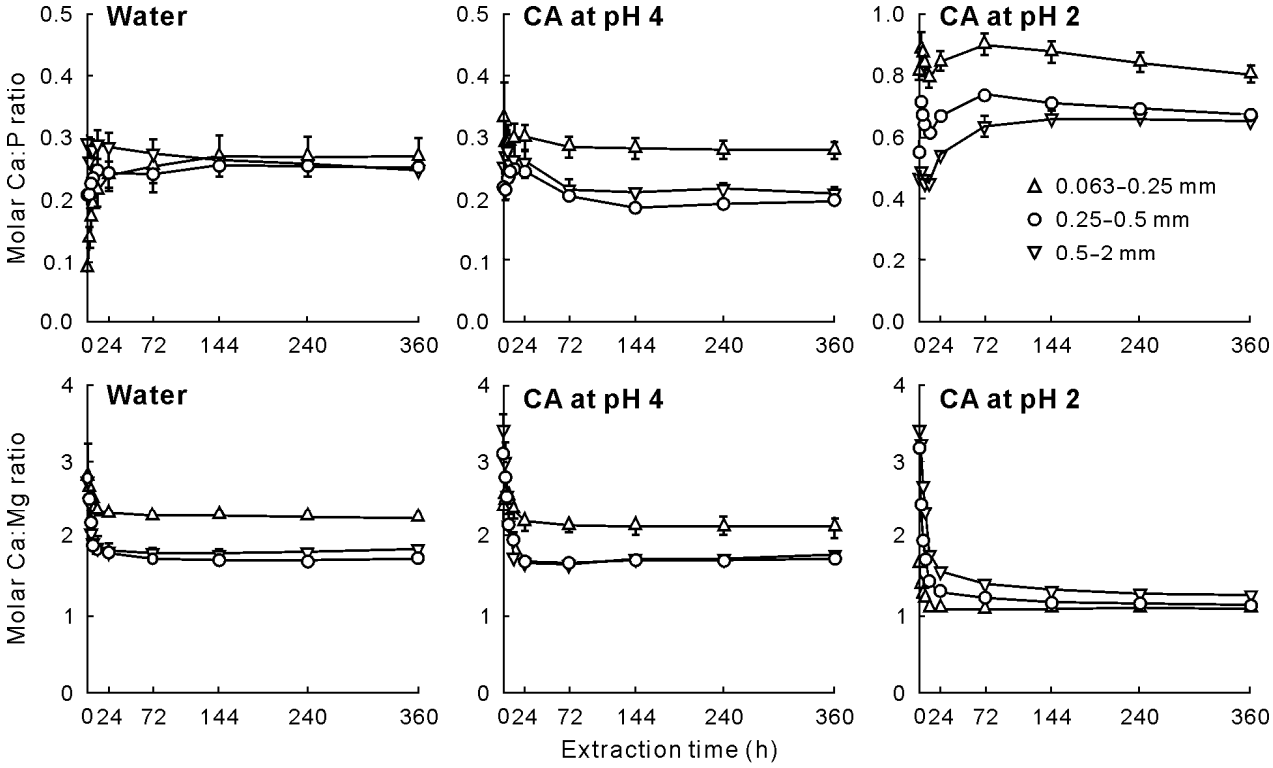

Fig. 4 Molar Ca:P and Ca:Mg ratios in water, citric acid solution (CA) at pH 4, and CA at pH 2 during the extraction time of 360 h in different particle-size fractions of phosphate rock and dolostone collected from Mozambique. Vertical bars indicate standard errors of the means (n = 3).

According to Harouiya et al., (2007), Dorozhkin (2002; 2012), and Crundwell (2014; 2015; 2016) Ca-apatites in acid solutions dissolve by ionic detachment of Ca and orthophosphate ions from the solid toward the solution. In our case, the formation of a Ca-rich layer (self – inhibition model) in both water and citric acid solution at pH 4, probably was determinant in decreasing the dissolution rate of apatite in the second stage.

Hence, the diffusion of Ca and orthophosphate ions occurred from the surface layer formed, so leading to incongruent dissolution (Dorozhkin, 2002; 2012). The Ca rich layer was probably thicker in the second stage of leaching because of the major contact time between the unsaturated solutions and apatite crystals. Because of this, the dissolution of fluoroapatite in citric acid solution at pH 2 proceeded at a certain rate in first 72 h of extraction, and decreased thereafter due the lack of removal of the Ca rich layer formed during the longer periods of solid-solution contact.

The citrate anion (and others such as oxalic, formic, etc.) have higher affinity for Ca than orthophosphate (Goyne et al., 2006; Dorozhkin, 2002; 2012) and play a considerable role in the dissolution of phosphate rock (Wei et al., 2011; Calvaruso et al., 2013). Therefore, the citric acid used in the leaching experiments may allow predicting the dissolution of phosphate rocks in acid soils.

However, the LMWOAs produced in the soil rhizosphere by root exudates and microbial activity (Marschner et al., 2011; Wei et al., 2011) have the capability to sparingly solubilize phosphatic particles and increase the availability of P (Kpomblekou and Tabatabai, 2003; Li et al., 2011; Cocco et al., 2013; Gómez and Carpena, 2014; De Feudis et al., 2016).

The dissolution of phosphate rock fractions can be increased by the removal of reaction products, in the plant-soil system and, even more, in the rhizosphere. Thus, Ca uptake may increase rock particle solubilization and, consequently, the availability of P for plants and microrganisms (Marschner et al., 2011; Panhwar et al., 2014).

3.3. Dolostone

3.3.1. General properties

Similar to phosphate rock, the abrasion pH of dolostone decreased significantly with contact time for all the particle-size fractions ranging from 9.57 at 6 min for the medium size fraction (0.25-0.5 mm) to 8.25 after 24 h for the coarse size fraction (Table 2). There were no significant differences among the size fractions during the contact time. As in the case of the phosphate rock fractions, the temporal decrease of abrasion pH can be attributed to the dissolution of atmospheric CO_2.

3.3.2. Long-term release in column experiments

(i) Cumulative releasing pattern of Ca, Mg, and K

The Ca and Mg were the major elements leached during the extraction period. Other elements such as Na, Ba, Zn, Cd, Ni, and Pb were present in concentrations close to 5 mmol kg^{-1}. Despite elements such as Fe, Mn, and Al being leached in concentrations lower than 30 mmol kg^{-1}, namely higher than K, here we discuss K together with Ca and Mg as plant macronutrients. The cumulative release of Ca, Mg, and K showed an increasing trend in all leaching solutions (Fig. 5). The release rate for Ca and Mg was relatively high in the first 24 h of leaching, and decreased thereafter. Among the particle-size fractions, the finest one showed the maximum cumulative release, followed by the medium size for Ca and Mg.

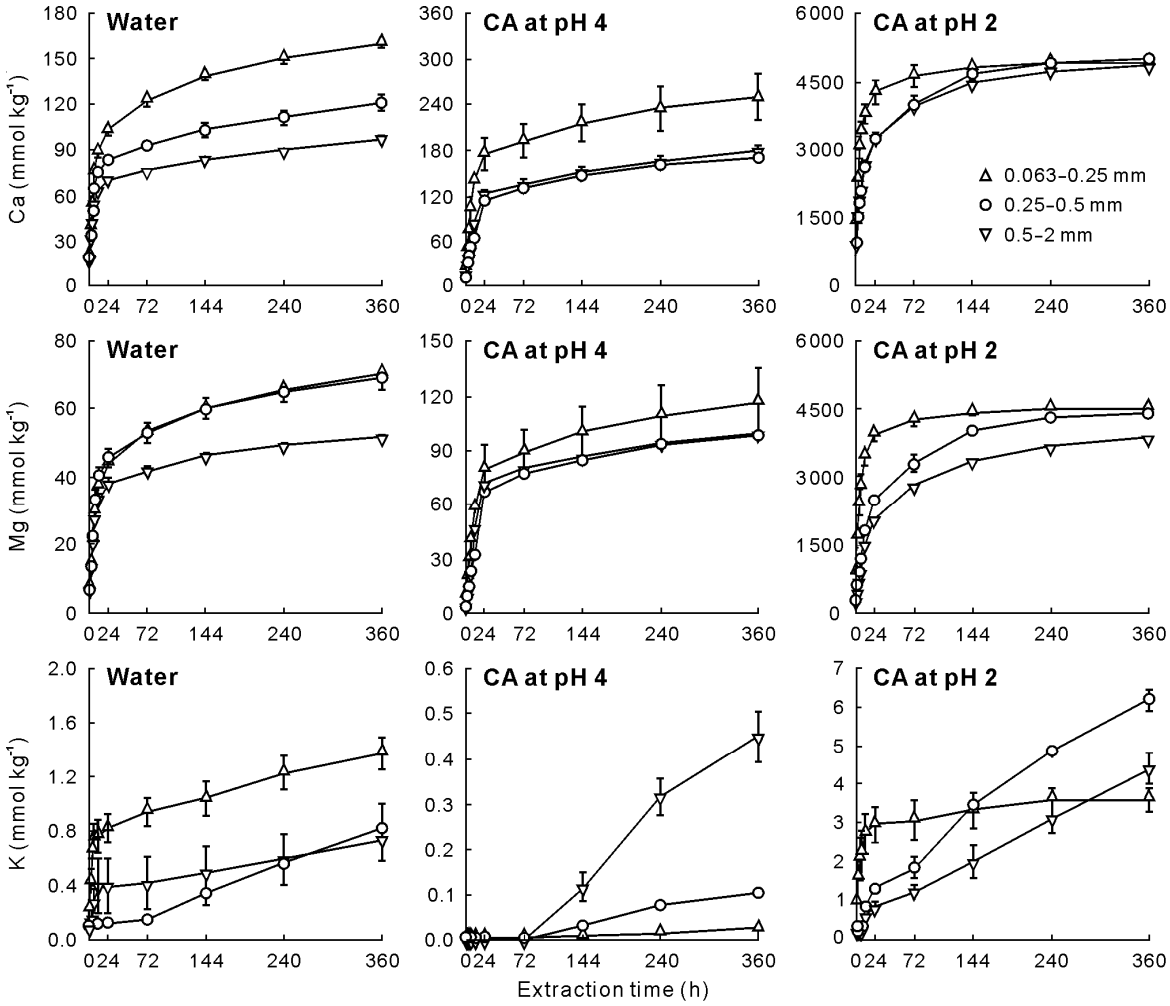

Fig. 5 Cumulative release of Ca, Mg, and K in water, citric acid solution (CA) at pH 4, and CA at pH 2 during the extraction time of 360 h from different particle-size fractions of dolostone collected from Mozambique. Vertical bars indicate standard errors of the means (n = 3).

As expected, the citric acid solution at pH 2 extracted substantively more nutrients than the other leaching solutions. The cumulative pattern of K differed from other nutrients as the medium and coarse size-fractions showed a linear trend after 72 h of leaching for both citric acid solutions at pH 4 and 2. Interestingly, more K dissolved from the finest fraction by water, not by citric acid at pH 4.

(ii) Release kinetics of Ca and Mg

The kinetics of release was assessed for both Ca and Mg as they were the most important nutrients in dolostone. During the leaching period the release rate decreased sharply up to 24 h, followed by a steady decrease until reaching values close to zero (Fig. 6). The finest size fraction showed a slightly higher release rate than the other fractions. There were no significant differences between the medium

and coarse sizes. The citric acid solution at pH 2 showed much higher release rates than the other leaching solutions.

Two stages of release were evident for all leaching solutions: 1) a stage of fast release in the first 24 h, and 2) a stage of slow release after 24 h for all leaching solutions, probably due to the precipitation of calcium citrate during the long period of solid/liquid contact (Al-Khaldi et al., 2007). During this second stage, probably characterized by incongruent dissolution, the release rate curves of the different size fractions became much closer, indicating a negligible effect of the particle size. The amounts of Ca and Mg leached during the second stage were higher for medium and coarse particle-size fractions ($P < 0.01$) in citric acid solution at pH 2, while there was not much difference among particle size fractions in water and citric acid solution at pH 4 (Fig. 6, Table 6).

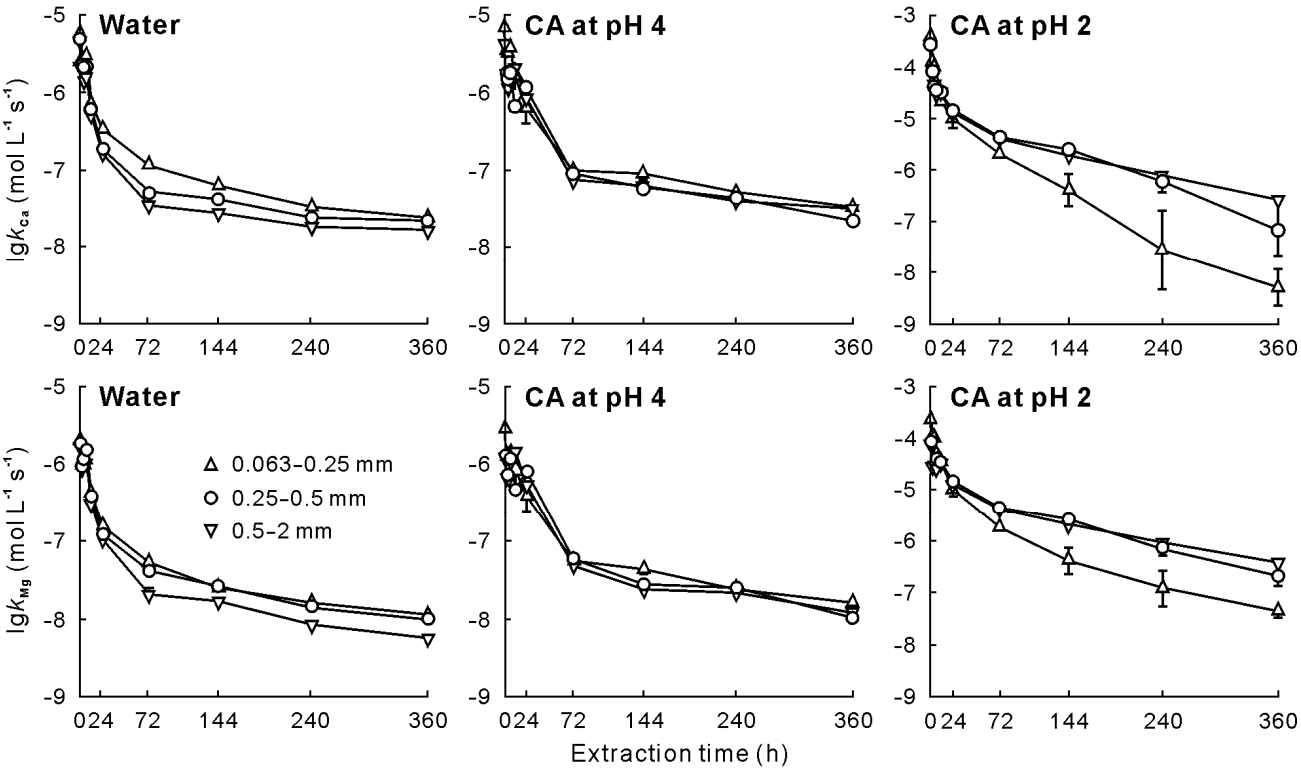

Fig. 6 Logarithms of dissolution rates (k) of Ca and Mg (k_{Ca} and k_{Mg}, respectively) in water, citric acid solution (CA) at pH 4, and CA at pH 2 during the extraction time of 360 h from different particle-size fractions of dolostone collected from Mozambique. Vertical bars indicate standard errors of the means (n = 3).

Table 6
Cumulative release of Ca, Mg, and K in water, citric acid solution (CA) at pH 4, and CA at pH 2 during the extraction time between 24 and 360 h from different particle-size fractions of dolostone collected from Mozambique and analysis of variance (ANOVA) for leaching solution, particle-size fraction, and their interaction

Elements	Leaching solutions	Particle-size fractions			ANOVA
		0.063 - 0.25 mm	0.25 - 0.5 mm	0.5 – 2 mm	
Ca	Water	57.7 (0.7) [a)] aA [b)]	37.9 (4.3) bA	26. 9 (1.6) cA	***
	Citric acid at pH 4	73.2 (16.0) aA	55.8 (1.2) aA	56.3 (4.6) bB	
	Citric acid at pH 2	643.0(413.1) aB	1747 (293) bC	1600 (64) bC	
Mg	Water	26.5 (0.4) aA	23.6 (3.6) aA	13.72 (2.61) bA	***
	Citric acid at pH 4	37.1 (9.0) aB	30.8 (2.2) aA	27.4 (1.7) aB	
	Citric acid at pH 2	579. 9 (327.0) aC	1913 (261.0) bB	1781.7 (59.1) bC	
K	Water	0.6 (0.0) aA	0.7 (0.3) aA	0.4 (0.2) bA	**
	Citric acid at pH 4	0.0 (0.0) aB	0.1 (0.0) aB	0.5 (0.1) bA	
	Citric acid at pH 2	0.8 (0.1) aC	4.9 (0.5) bC	3.6 (0.5) bB	

**, ***Significant at $P = 0.01$ and $P = 0.001$, respectively; [a)]Means (standard deviations, n=3). rock size fraction; and interaction: leaching solutions x rock size fraction; [b)]Means followed by the same lowercase letter within each row are not significantly different at $P < 0.05$ by multiple-comparison Tukey test and those followed by the same uppercase letter within each column for a given element are not significantly different at $P < 0.05$ by multiple-comparison Tukey test

The release rate of Ca was twice those of Mg in both water and citric acid solution at pH 4, and similar for both elements in citric acid solution at pH 2 because of the considerable amounts of Mg dissolved by this leaching solution. In water and citric acid solution at pH 4, Ca dissolved preferentially compared to Mg during most of the extraction time and the releasing was non-stoichiometric (Fig. 4). In citric acid solution at pH 2, after a short period of preferential release of Ca, the molar Ca/Mg ratio dropped to values close to 1, the Ca/Mg ratio in dissolved rock, because of the low pH (Pokrovsky et al., 2009). The ideal (congruent) dissolution of calcareous minerals due to an organic acid is given below (adapted from Pansu and Gautheyrou, 2006):

$$CaCO_3 + 2AH \leftrightarrows Ca^{2+} + 2A^- + H_2O + CO_2 \text{ (fast reaction)} \quad (4)$$

$$MgCO_3 + 2AH \leftrightarrows Mg^{2+} + 2A^- + H_2O + CO_2 \text{ (slow reaction)} \quad (5)$$

where A^- represents the organic anion

The dissolution rate of dolomite generally slower than that of calcite and in acid solution is affected by the transport rate of the reactants, surface reaction, and transport rate of products away from the surface (Yasuda et al., 2013). The same as in the phosphate rock, the open system of columns used in this study ensured the removal of products and the supply of H^+ by citric acid solutions, so favouring the dissolution of dolostone.

This explanation is in accordance with the sharp decrease of Ca/Mg ratio in the first 24 h of leaching, which was due to the increase of Mg release. In citric acid solution at pH 2, the steady decrease of Ca/Mg ratio after 24 h of leaching was ascribed to higher dissolution of the remaining carbonates (enriched in Mg). The higher affinity of the citrate anion for Ca over Mg (Dorozhkin, 2002, 2012) and the leaching of the reaction products were the driving force of dolostone dissolution. The high citrate concentration in citrate solution at pH 2 favored the complexation of Mg, rendering the dissolution congruent in the later periods of leaching. Thus, this type of rock can be used for pH correction and Ca plus Mg replenishment for plant uptake, while mitigating Al toxicity (Conyers et al., 1996; Conyers, 2003; Viadé et al., 2011; Bothe, 2015; da Costa and Crusciol, 2016; Tiritan et al., 2016).

The LMWOAs such as citric acid promote dolostone dissolution by complexing both Ca and Mg from the rock surface (Manahan, 2000; Zimdahl, 2015) resulting in an increase of nutrients (Panhwar et al., 2014; da Costa and Crusciol, 2016). Based on leaching curves, the dolostone will have a much higher impact in strongly acid soils, even though the slower release rate of dolomite within the dolostone will ensure the long-term efficiency of nutrient release.

4. Conclusions

The leaching experiments run in an open-system (columns) under Controlled conditions showed that the nutrient release from phosphate rock and dolostone fraction was mainly a function of particle-size, strength of the leaching solution, and time. Increased concentrations of citric acid solution resulted in a greater dissolution. The application of the tested rocks fractions to strongly acid soils from tropical areas is suitable as they behave like slow release fertilizers that are able to replenish the soil with P, Ca, and Mg with the benefit of liming.

Application of the coarse size fraction (0.5 – 2 mm) of both rocks demands less energy inputs for crushing and grinding than other sizes; however, the reactivity of this size fraction is low, and its application to the soil would be much less effective than finer size-fractions. The application of both fine and medium grained materials might represent a good balancing to ensure a short and medium-term release of nutrients and alkalizing species. Managing the rhizosphere activities to promote the production of LMWOAs will be a good strategy to improve nutrient release by these rock fractions in acid soils. Much work has to be done to transfer these lab results to the field, in particular on the overall impact of considerable amounts of fresh rocks added to tropical soils.

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CHAPTER 3 - PHOSPHATE ROCK, DOLOSTONE, AND BIOCHAR APPLICATION TO AN ACID ARENOSOL

Increased phosphorus availability to corn as a result of the simultaneous application of phosphate rock, dolostone and biochar to an acid Arenosol

[ab,★]Rafael, Rogério Borguete Alves; [cf]Fernández-Marcos , Maria Luisa; [b]Cocco, Stefania; [d]Ruello, Maria Letizia; [e]Fornasier, Flavio; [b]Corti, Giuseppe

[a]Department of Rural Engineering, Faculty of Agronomy and Forestry Engineering, Universidade Eduardo Mondlane, 257, Maputo, Mozambique

[b]Department of Agriculture, Food and Environmental Sciences, Università Politecnica delle Marche, 60131 Ancona, Italy

[c]Department of Soil Science and Agricultural Chemistry, Universidad de Santiago de Compostela, 27002 Lugo, Spain

[d]Department of Materials, Environmental Sciences and Urban Planning, Università Politecnica delle Marche, 60131 Ancona, Italy

[e]Consiglio per la Ricerca e la Sperimentazione in Agricoltura, Centro di Ricerca per lo Studio delle Relazioni tra Pianta e Suolo (C.R.E.A.-R.P.S.), Gorizia, Italy

[f]Instituto de Biodiversidade Agraria e Desenvolvemento Rural, Universidad de Santiago de Compostela, 27002 Lugo, Spain

★Corresponding author: rogerborguete@gmail.com; rogerio.rafael@uem.mz

Other authors: mluisa.fernandez@usc.es; s.cocco@univpm.it; m.l.ruello@univpm.it; flavio.fornasier@crea.gov.it; g.corti@univpm.it

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Abstract

Phosphorus (P) deficiency is one of the main constrains for crop production in acid soils, including many Arenosols. The aim of this study is to investigate the chemical and biochemical responses of an acid Arenosol treated with phosphate rock, dolostone, and biochar, and implication in corn yield. The experiment used a surface soil (0-20 cm) from Marracuene district, Mozambique. The following treatments were assessed, in which corn was grown for 110 d: Control, WSP (zinc phosphite as soluble P), PR (phosphate rock), WSP+D (calcareous rock), WSP+BC (biochar), WSP+D+BC, PR+BC, and PR+D+BC. Biochar was produced by pyrolysis of babycorn peels for 4 h at 450ºC and applied at 1.1 % (w:w) rate. The soil pH_{H2O} was highest in the PR+D+BC treatment, but no significant difference among WSP+D, WSP+D+BC and PR+BC treatments was found. Easily oxidizable organic C (EOOC), CEC, base saturation, and available P were higher in treatments including biochar compared to Control. Treatments containing dolostone and/or biochar led to the highest activity of alkaline phosphomonoesterase, phosphodiesterase and α-glucosidase, increasing phosphorus availability, and gave the highest biomass and yields. Biochar supplied most P in treatments including PR, and enabled P adsorption and consequently slowed P release in treatments including WSP.

Key words: enzyme activity; soil fertility; fertilizers; Arenosols

1. Introduction

Phosphorus, being a scarce element in most natural soils, is one of the nutrients that most often limit agricultural yields. In agricultural soils, P must generally be supplied through fertilizers (Holford, 1997). In most developed countries agriculture contributes a net excess of P, which leads to an increase of soil P stock and the risk of exportation of this element to water bodies, in developing countries soils are often P-deficient (McDowell et al., 2016).

Acid Arenosols are among the most problematic soils for crop growth because of their acidity and low cation exchange capacity, water holding capacity and organic matter content (Molnár et al., 2015; Price et al., 2015), as well as deficiency of plant essential nutrients (van Straaten 2002; 2006; van Raij, 2011). Phosphorus deficiency in particular, especially in tropical and sub-tropical areas, is critical (Zhang et al., 2016). Liming has often been used to increase P availability as it can improve the fertility of soils that have P in unavailable forms (Bhat et al., 2010). However, the amounts of both total and available P in acid Arenosols are usually so low that the addition of P fertilizers is the only practice that may ensure sufficient yields. Unfortunately, in many areas with acid Arenosols the high cost of commercial fertilizers represents an insurmountable obstacle.

Therefore, finding alternative local organic or inorganic resources that can supply nutrients for sustainable crop production is a practical, low-cost and long-term strategy that addresses the needs of poor farmers (Haque et al., 1999; Odongo et al., 2007; Asomaning et al., 2009). In countries that cannot afford costly fertilizers, the exploitation of their own rock outcrops is a possible alternative (Appleton, 2002; Van Straaten, 2002; 2006; Odongo et al., 2007). Phosphate and calcareous rocks can be considered suitable for direct application as P fertilizer and liming material, respectively, in acid soils (Szilas et al., 2008; Rafael et al., *in press*). Application of phosphate rock to acid soils can increase soil pH as well as crop yields (Weil, 2000). Application of lime to acid soil can improve significantly soil physic-chemical properties, reducing Al and Mn toxicity (Alvarez et al., 2009), improving nutrient uptake, aeration and P availability (Viadé et al., 2011) and leading to optimum pH for crop growth (Anikwe et al., 2016).

Also, the application of biochar to degraded, poor, tropical soils has positive effects on soil properties (Dari et al., 2016; Hussain et al., 2017). In acid Arenosols, biochar can improve soil physic-chemical properties by boosting the soil organic C pool, water holding capacity, nutrient availability and adsorption through CEC improvement (Lehmann et al., 2011; Głąb et al., 2016; Obia et al., 2016). Biochar in soil can act as phosphate adsorbent and/or source of available P (Alburquerque et al., 2014;

Qayyum et al., 2015; Dari et al., 2016; Zhang et al., 2016). Atkinson et al. (2010) and Zhang et al. (2016) stated that the increase of P availability in soil after biochar application was due to the increase of anion exchange capacity of the soil. Other researchers attributed the increase of P availability in soil amended with biochar to the increase of pH (Alling et al., 2014; Ding et al., 2016; Berihun et al., 2017), or to the competition between phosphate and negatively charged organic species dissolved from biochar for the adsorption sites on colloids (Warnock et al., 2007; Soinne et al., 2014).

Some types of biochars (ex.: Pinus biochar) can also promote the growth of phosphate solubilizing bacteria (Anderson et al., 2011), which play a key role in the dissolution of sparingly soluble P fertilizers such as phosphate rocks. Biochar produced from corn straw has been reported to improve the quality of acid soils by raising soil pH, exchangeable cations, base saturation and CEC, while reducing Al activity, besides contributing available P and P adsorbing capacity and improving soil physical properties. So, corn straw biochar improves agricultural yields (Chintala et al., 2014a and b; Kauffman et al., 2014; Wan et al., 2014; Bai et al., 2017; Sandhu et al., 2017; Zhang et al., 2017). It has also been reported that corn biochars influence microbial activity in soils with low organic matter content (Khadem and Raiesi, 2017).

Soil enzyme activity has been used as indicator of biological equilibrium and soil quality (Gil-Sotres et al., 2005; Shukla and Varma, 2011). Biochar may affect availability of nutrients as it is able to increase several soil enzyme activities, in particular those involved in the C, N, and P cycles (Shukla and Varma, 2011; Quilliam et al., 2013; Pandey et al., 2016). However, biochar nutrients content and properties are mostly defined by feedstock source and pyrolysis conditions (Alburquerque et al., 2014; Ding et al., 2016). Notwithstanding the knowledge amassed on the effect of phosphate rock, calcareous rock, and biochar on soil, as far as we know there is no published research on the synergies between biochar and rocky materials, and modifications of the soil chemical and biochemical properties due to their simultaneous application in tropical acid soils. Therefore, more information on these subjects are needed to increase crop production while protecting the environment.

The aim of this study is to investigate the chemical and biochemical responses of an acid Arenosol treated with phosphate rock, dolostone, and babycorn peel biochar. Specifically, we aim to assess in pot experiments the effects of the simultaneous application of phosphate rock (as sparingly soluble P source), dolostone (as liming material), and biochar (contributing soluble P and retaining capacity)

on: a) soil chemical and biochemical (enzyme activity) properties, b) corn growth and yield, and c) P flows and uptake.

The hypotheses tested were: 1) Since in acid Arenosols of disadvantaged countries crop yields tend to reduce, the use of phosphate and calcareous rocks and biochar should improve soil chemical and biochemical properties and, as a result, corn yield increase. In fact, the overall increase of pH and improvement of soil properties should directly or indirectly increase nutrient availability and decrease nutrient leaching (because of biochar), so to improve soil fertility. 2) The response of the soil enzyme activity should be enzyme specific, as reported by Hussain et al. (2017). 3) Since plants can exudate phosphatases and organic molecules to mobilize organic P and increase the availability of P from phosphate rock and biochar (Marschner et al., 2004; Hinsinger et al., 2006; Neumann and Römheld, 2012), the rhizosphere should affect differently than the bulk soil the enzyme activity patterns to increase availability of P.

2. Materials and Methods

2.1. Soil sampling and inputs preparation

The A horizon (0-20 cm thickness) of an albic Arenosol was collected in an open scrubland located in southern Mozambique, Marracuene district (25.72577º S, 032.64835º E). Prior to use, the soil was air dried and sieved at 2 mm to remove gravels and roots. As water soluble P fertilizer a commercial zinc phosphite solution (28% of P_2O_5 and 8% Zn, w:w) was used. A nutrient solution with the following composition was also prepared (adapted from Vanek and Lehmann, 2014): 2 mmol L^{-1} of NH_4NO_3, 2.56 mmol L^{-1} of KCl, 1 mmol L^{-1} of $MgSO_4$, 25 µmol L^{-1} of H_3BO_3, 2 µmol L^{-1} of $MnSO_4$, 2 µmol L^{-1} of $ZnSO_4$, 0.5 µmol L^{-1} of $CuSO_4$, 0.5 µmol L^{-1} of $(NH_4)_6Mo_7O_{24}$. In the treatments where zinc phosphate was applied, the nutrient solution did not include $ZnSO_4$ to avoid excess of Zn application.

Both rocks (phosphate and calcareous) from Mozambique. The phosphate rock (PR) was collected from the Evate district (Nampula Province), while the calcareous rock, a dolostone (D), was collected from Mount Muambe (Tete Province). Blocks of both rocks were fragmented by using a grinding press, and the fragments were then ground using an agate mortar. Both rock powders were reduced to two particle-size fractions: 0.063-0.25 mm and 0.25-0.5 mm. The dimensions of the fractions were obtained as per Rafael et al. (2017). Babycorn peel was used as feedstock for biochar production using a low-cost home-made pyrolyser made of two concentric cylinders. The babycorn

peel was placed into the inner cylinder, while firewood was burnt in the outer cylinder providing energy to heat the inner cylinder. The babycorn peel was pyrolysed for 4 h and the temperature reached about 450ºC. After the carbonisation process, the biochar was washed twice with distilled water to eliminate the excess ash and was then dried in an oven for 48 h at 60 ºC. Prior to be analysed and used, the biochar was ground using a mortar until the particles were in the range 0.063 – 0.25 mm.

2.2. Pot experiments

In the facility of the Faculty of Agronomy and Forest Engineering (Eduardo Mondlane University, Maputo, Mozambique), a pot trial was designed as a completely random test with eight treatments: 1) Control soil, with no addition; 2) soil with water soluble zinc phosphite fertilizer (WSP); 3) soil with ground phosphate rock (PR); 4) soil with WSP and ground dolostone (WSP+D); 5) soil with WSP and biochar (WSP+BC); 6) soil with WSP, D, and BC (WSP+D+BC); 7) soil with PR and BC (PR+BC); 8) soil with PR, D, and BC (PR+D+BC). For each treatment tree replications were applied. The use of zinc phosphite was preferred over other water soluble P bearing fertilizers as it is highly soluble and, once in soil, phosphite is gradually transformed to phosphate (Adams, 1953). We did not includ biochar or dolostone treatments alone since it has been widely documented that the application of biochar alone may fail to increase crop production (Gaskin et al., 2010; Spokas, 2010; Calderón et al., 2015; Hussain et al., 2017), and similarly may happen with applications of calcareous rock alone to acid Arenosol in the short-term (Musharo and Nyamangara, 2011; Rothwell et al., 2015).

The pots were prepared with 17 L plastic buckets with 3 small holes of 10 mm in diameter at the bottom to avoid stagnant water. Each pot was prepared with a soil layer of 6 kg on the bottom, and then a layer of 4 kg of the same soil homogenized with the inputs so to make a 15 cm thickness with an approximate bulk density of 1.50 kg dm^{-3}. Dolostone was applied as a 1:1 mixture of both 0.063-0.25 and 0.25-0.5 mm fractions, at a rate of 5 g pot^{-1} each, equivalent to about 7.5 ton ha^{-1} of dolostone (considering a soil depth of 20 cm with a bulk density of 1.5 kg dm^{-3}). Phosphate rock was applied as a mixture made of 9 g pot^{-1} of the 0.063-0.25 mm fraction, and 26 g pot^{-1} of the 0.25-0.5 mm fraction, so as to provide 140 mg pot^{-1} of available P. These amounts were equivalent to about 26.25 ton ha^{-1} of phosphate rock and 105 kg ha^{-1} of available P (20 cm soil depth, bulk density of 1.5 kg dm^{-3}) predicted by neutral ammonium citrate extraction (Rafael et al., *in press*). In the treatments without PR, WSP was used as a P source and was applied at a rate of 1.14 g pot^{-1} so as to provide 140 mg pot^{-1} of available P, which is equivalent to 105 kg ha^{-1}. Biochar was applied at 1.1 % (w:w)

rate, equivalent to about 33 ton ha^{-1} (for a soil depth of 20 cm with a soil bulk density of 1.5 kg dm^{-3}).

The soil in the pots was pre-incubated for 7 d to allow an initial dissolution of both dolostone and phosphate fragments, and to avoid possible negative effects on plant growth due to biochar volatile substances (Vanek and Lehmann, 2014; Dutta et al., 2017). During the pre-incubation, each pot was irrigated three times per week with 500 mL of distilled water. After this period, 3 corn seeds per pot (MRI594 - Hybrid white early maturity cultivar) were sown (March 28th, 2016), but only one emerged plant per pot was left. During the growing period (90 d) the soil temperature ranged from 24.0 ± 2.0 to 28.0 ± 1.2ºC, and all the pots were watered with 500 mL of distilled water once per week, and fertirrigated every two d with 300 mL of nutrient solution (NS). Fertirrigation was made during the first 70 d, while watering lasted growing period. The watering frequency was increased to three times per week due the high water demand of corn plants in vegetative stage, starting from 6 weeks after the emergence.

Pests were Controlled by an alternating weekly distribution of fungicide Amistar Top (active substances: azoxystrobin + difenoconazole) + insecticide/acaricide Agromectin 1.8 EC (active substance: abamectin) with fungicide Ridomil Gold (active substances: metalaxyl + mancozeb) + HITCEL 44% EC (active substances: profenofos + cypermethrin). Plants were harvested 90 d after germination, during flowering. Dry (60 ºC) total biomass and shoot weight were determined. Soil samples were collected separating the rhizosphere from the bulk soil so as to assess the P flux between these two soil compartments. The soil adhering to the roots was considered as rhizospheric soil and separated according to Corti et al. (2005).

2.3. Soil, plant, and biochar analysis

The soil particle-size distribution was determined by the pipette method (Day, 1965), after maintaining the samples submerged in deionized water for 24 h. The sand fraction was retrieved by wet sieving at 0.05 mm, while silt was separated from the clay by sedimentation after dispersion in 0.01 M NaOH. The pH was determined potentiometrically on dry soil (1:2.5 w:v) and biochar (1:100 w:v) in both water and 1M KCl solution. For biochar, the suspensions were heated to 90°C in a water bath, stirred for 20 min to allow dissolution of the soluble components, and the pH determined after cooling to room temperature (Ahmedna et al., 1997).

Total carbon, total nitrogen (TN) and total sulphur (TS) in soil, plant portions (roots, shoots, and cobs), and biochar were determined using a CHNS analyser (EA1110, Carlo Erba Instruments, Milan,

Italy). Since the soil had an acid pH, TC was considered as total organic C (TOC). The total contents of P, Ca, Mg, and K in plant portions and biochar were determined by heating 0.5000 g of sample at 500 ºC in a muffle furnace for 16 h, and dissolving the ashes in 5M HCl solution (Lambert, 1976). A simple colorimetric method based on ascorbic acid reduction of the ammonium phosphomolybdate complex was used to measure P in the solutions (Kuo, 1996). The concentration of Ca, Mg, and K were measured by an inductively coupled plasma optical emission spectrometer (ICP-OES Perkin Elmer Optima 8300). The Walkey-Black method was used to estimate the easily oxidizable organic C (EOOC) in soil and biochar. The recalcitrant organic carbon in soil was estimated by subtracting the EOOC from TOC (Suárez-Abelenda et al., 2014).

The Mehlich-3 protocol (Mehlich, 1984) was used to estimate available P in soil samples. In the biochar, extractable P was determined by water, 2% citric acid solution, and 2% formic acid solution using 0.1000 g of sample and 30 mL of extractant (Zhang et al., 2016). The suspension was shaken (120 rpm) for 48 h in centrifuge tubes, centrifuged (≈2700 g, 15 min), and the supernatant filtered through Whatman 42 filter paper before the colorimetric determination (Zhang et al., 2016). Cation exchange capacity and exchangeable cations (Ca, Mg, K, Na, and Al) in soil and biochar were determined by the $BaCl_2$ method (Gillman and Sumpter, 1986; Agnelli et al., 2017). Biochar volatile matter was determined by weight loss after heating the muffle furnace to 950 ºC, then heated for 2 min on the outer ledge of the furnace (300 ºC) with the door open, and then for 3 min on the edge of furnace (500 ºC) (Zhao et al., 2015); the biochar ash content was determined as the weight remaining at 750 ºC (Jindo et al., 2014).

Enzyme activities in soil were determined as per Fornasier and Margon (2007), who desorbed enzymes by heteromolecular exchange using an excess of exogenous protein. Thus, 250 mg of samples were placed in 2-mL Eppendorf tubes with glass beads and 1 mL of 50 mM tris–HCl buffer solution at pH 7.5, containing lysozyme as desorbing protein. The tube was subjected to bead-beating (3 min, 30 strokes s^{-1}) using a Retsch MM400 mill, then centrifuged for 5 min at 20,000 g. The enzymatic activity was assayed fluorometrically in microplates using 4-methyl-umbelliferyl and L-leucine-7-amino-4-methylcoumarine derivatives (Fornasier and Margon, 2007). The activities of acid phosphomonoesterase, alkaline phosphomonoesterase, α-glucosidase, arylsulfatase, ß-glucosidase, phosphodiesterase, chitinase, nonanoate-esterase, pyrophosphatase-phosphodiesterase, glucuronidase, and xylosidase were determined in 200 mM morpholineethanesulfonic acid solution at pH 6, while the activity of leucine-aminopeptidase was assessed in 50 mM tris–HCl buffer solution at pH 7.5. Double-strand DNA (dsDNA) was determined through aliquots of 500 mg of sample placed

in 2-mL Eppendorf tubes glass beads and 1 mL of 0.12 M sodium phosphate buffer (pH 8.0) solution. The tubes were submitted to bead-beating (2 min, 30 strokes s^{-1}) and then centrifuged for 5 min at 20,000 g. The dsDNA content was quantified on aliquots of the supernatant by fluorimetry using PicoGreen1 reagent (Life Technologies) according to the instructions of the manufacturer.

2.4. Calculation of phosphorus use efficiency

Nutrients taken up (mg pot^{-1}) by roots, shoots (stem + leaves), and cobs were calculated by multiplying the nutrient concentration (mg kg^{-1}) by the respective biomass weight. Phosphorus harvest index (PHI) was calculated as follows (Agegnehu et al., 2016a):

$$PHI\ (\%) = \frac{GPU}{TPU} \times 100$$

where: GPU is P in the cobs (mg pot^{-1}), and TPU is total P in the plant (cob + shoot) (mg pot^{-1}).

Fertilizer P use efficiency was expressed by the following components:

i) Agronomic efficiency (AE) is defined as the grain production per unit of P applied, and was calculated as:

$$AE\ (g\ g^{-1}) = \frac{GY_{f-}\ GY_u}{P_a}$$

where: GY_f is the cob yield of the fertilized pot (g), GYu is the cob yield of the not fertilized pot (g), and P_a is the quantity of P applied as biochar and/or P fertilizer (g) (Agegnehu et al., 2016a).

ii) Apparent P recovery efficiency (ARE), or P uptake efficiency, was calculated as:

$$ARE\ (\%) = \frac{P_f - P_u}{P_a} \times 100$$

where: P_f is the P in cob + shoot of the fertilized pot (g), P_u is the P in cob + shoot of the not fertilized pot (g), and P_a is the quantity of P applied (g) (Agegnehu et al., 2016a).

iii) Physiological efficiency (PE), which expresses the ability of the plant to transform P absorbed from fertilizers into grain yield, was calculated as:

$$PE\ (gg^{-1}) = \frac{GY_f - GY_u}{P_f - P_u}$$

where: GY_f is the cob yield of the fertilized pot (g), GYu is the cob yield of the not fertilized pot

(g), P_f is the P in cob + shoot of the fertilized pot (g), P_u is the P in cob + shoot of the not fertilized pot (g) (Agegnehu et al., 2016a).

iv) The P translocation factor (TF) was calculated as:

$$TF = \frac{P_{shoots}}{P_{roots}}$$

where: P_{shoots} and P_{roots} are the P concentration (mg kg^{-1}) in shoots and roots, respectively. TF > 1 indicate that the plant translocated P effectively from the roots to the shoots (stem, leaves, and cobs), TF < 1 indicate that translocation is ineffective (Liu et al., 2014).

2.5. Statistical analysis

R version 3.1.2 (2014-10-31) was used for statistical analysis. The experimental data were analysed by analysis of variance (ANOVA) after a *boxcox* transformation (Meloun et al., 2005) of the data to perform parametric tests (Shapiro-Wilk normality test and Bartlett test of homogeneity of variances) when necessary. A multiple comparison Duncan test at 5% significance level was used to compare the means. Principal components analysis (PCA) was performed on the response variables data to assess the correlation among enzymatic activities, chemical properties, and corn yield determined as response of treatments and rhizosphere processes.

3. Results and discussion

3.1. Characteristics of soil and inputs used in pot experiment

The soil used in the experiment was strongly acid (Table 1). This condition, together with the sandy texture, the low concentrations of EOOC and nutrients, and the low CEC (Table 1) may severely reduce the crop yield (Ritchey and Carter Jr., 1993; Bhat et al., 2010). The soil used in the experiment had a generalized low activity for all the enzymes considered (Table 1), and this was probably due to the low EOOC content (e.g. Bastida et al., 2006; Hannachi et al., 2014; Godin et al., 2015). Since the soil microbial community produces enzymes in reply to any soil change that modifies the organic substrate or other soil factors (Sinsabaugh et al., 2002), the enzyme activity can be used as an indicator of soil changes (Schloter et al., 2003; Allen et al., 2011; Jain et al., 2016).

Both phosphate rock and dolostone showed alkaline pH and contained high amounts of P, Ca, and Mg (Table 1). Because of this, these rocks were considered as suitable to be applied to the soil so to increase the pH (Oliveira and Pavan, 1996) and to provide nutrients (Chien et al., 2010; Rafael

et al., *in press*). Babycorn peel biochar displayed a strong alkaline pH, and high concentrations of TOC, EOOC and TN (Table 1). Because of this, as also reported by Lehmann et al. (2011), it is expected that the application of biochar to the soil will increase both pH and CEC. This biochar can also be a potential source of available P and K.

3.2. Soil properties after the experiment

3.2.1. Chemical properties

The treatments had a significant impact on almost all the parameters considered, except exchangeable Na and Al (Table 2). Soil pH, EOOC, CEC, and available P were higher in the treatments including rocks and/or biochar. The highest increase of pH_{H2O} was observed in treatments in which both dolostone and biochar were applied, probably because the liming properties of the two amendments add up. Similar results were often reported with the addition of these materials (e.g. Van Zwieten et al., 2010; Yuan et al., 2011; Valentinuzzi et al., 2015; Berihun et al., 2017). All treatments including biochar increased the content of both TOC and EOOC. Although biochar is usually considered to be mainly made of recalcitrant C, Skjemstad and Taylor (1999) reported that the Walkley-Black method can recover a substantial part of charcoal, particularly the finely fragmented charcoal. Calvelo Pereira et al. (2011) suggested that EOOC obtained by dichromate oxidation reflects the degree of carbonization of biochar, and can be used to estimate the labile C fraction in biochar. The EOOC accounted from 19 to 41% of TOC, with concentrations being higher in treatments including biochar. This suggests that biochar may have a priming effect on soil organic matter decomposition (Luo et al., 2011; Zimmerman et al., 2011).

Treatments including biochar or dolostone increased the exchangeable Ca and Mg compared to the Control, with the highest values in the treatments with dolostone. However, also the WSP treatment increased the exchangeable Mg compared to Control, and all treatments increased the exchangeable K, with the highest value in PR+BC treatment. The fact that CEC increased in all treatments including biochar or dolostone was ascribed to the increase of TOC and of the corresponding carboxylic groups that, at the highest pH values, are deprotonated and act as cationic exchangeable complex (Sposito, 2008).

The PR+BC treatment showed the highest concentration of available P, followed by PR+D+BC and WSP+BC, WSP showed an available P concentration significantly similar to that of the Control (Table 2). As reported by Liang et al. (2006), Lehmann et al. (2011), Jiang et al. (2015), Heitkötter and Marschner (2015) and Zhang et al. (2017), biochar may adsorb P, but it also provides additional

available P. In our experiments, biochar from baby corn peel, having a high concentration of soluble P, appeared to provide substantial amounts of available P to soil.

Zhang et al. (2016) also demonstrated that biochar can improve P availability through direct P supply of soluble P and P sorption from fertilizers, so that P is less leached and can be slowly released from the adsorbent. However, these capabilities depend largely on the type of biochar and, to a lesser extent, on the temperature of pyrolysis (Wang et al., 2012; Zhang et al., 2016; Berihun et al., 2017). The application of WSP alone was not effective to increase available P. Leaching of P up to 90% has been documented for water soluble P fertilizers applied to acid Arenosols (Chen et al., 2006). Although some water percolating out the bottom of the pots was observed in treatments WSP and Control during growing season, the amounts were too small to be sampled and analysed. Nevertheless, some P was probably leached during the growing period.

Another possible explanation is that phosphite did not oxidize into phosphate during the trial, so it was not measured by the spectrophotometric method used. However, WSP and biochar showed a synergy when applied together, probably because the biochar retained water and adsorbed phosphate (and phosphite) groups, preventing their leaching (Dari et al., 2016; Zhang et al., 2016) while increasing soil biological activity. The application of phosphate rock alone was less effective than together with biochar, probably because of the beneficial effect of biochar in facilitating root and microorganism colonization of biochar that resulted in an increase of phosphate rock reactivity (Vanek and Lehmann, 2014). Hence, as also reported by Molnár et al. (2015), because of its reactivity and implication in many chemical processes, biochar represents an important potential amendment for acid Arenosols.

Table 1
Physical, chemical, and biological properties of soil, and physicochemical properties of phosphate rock, calcareous rocks, and biochar used in the pot experiment with acid Arenosol from Mozambique.

Parameters	Soil before the trial	Phosphate rock	Calcareous rock	Babycorn peel biochar
pH_{H2O}	4.3 (0.2)[a)]	8.2 (0.1)	8.3 (0.0)	9.6 (0.1)
pH_{KCl}	4.3 (0.0)	-	-	8.9 (0.2)
Sand (g kg^{-1})	937 (4)	-	-	-
Silt (g kg^{-1})	47 (9)	-	-	-
Clay (g kg^{-1})	16 (5)	-	-	-
EC (dS m^{-1})	0.0 (-)	-	-	0.86 (0.0)
TOC (g kg^{-1})	22.4 (0.9)	-	-	579.4 (102.6)
EOOC (g kg^{-1})	3.1 (0.8)	-	-	289.7 (27.0)
TN (g kg^{-1})	< dl	-	-	27.1 (1.1)
TS (g kg^{-1})	< dl	-	-	53.9 (6.9)
TP (g kg^{-1})	-	239 (1)	-	7.9 (1.0)
Total Ca (g kg^{-1})	-	-	294 (2)	-
Total Mg (g kg^{-1})	-	-	182 (5)	-
Water-P (mg kg^{-1})	-	1400 (100)	-	5425 (421)
2% citric-P (mg kg^{-1})	-	87 400 (400)	-	2513 (133)
2% formic-P (mg kg^{-1})	-	133 400 (13 300)	-	7998 (301)
Mehlich 3-P (mg kg^{-1})	6.6 (0.9)	-	-	-
Exchangeble Ca ($cmol_c$ kg^{-1})	0.1 (0.0)	3.5 (0.8)	32.4(3.7)	2.8 (0.2)
Exchangeble Mg ($cmol_c$ kg^{-1})	0.1 (0.0)	1.8 (0.2)	2.8 (0.4)	4.7 (0.4)
Exchangeble K ($cmol_c$ kg^{-1})	0.1 (0.0)	1.1 (0.2)	0.4 (0.0)	170.2 (17.3)
Exchangeble Na ($cmol_c$ kg^{-1})	0.0 (0.0)	9.2 (3.7)	3.1 (4.8)	1.4 (0.1)
Exchangeble Al ($cmol_c$ kg^{-1})	0.3 (0.0)	-	-	0.0 (-)
CEC ($cmol_c$ kg^{-1})	0.5 (0.1)	-	-	179.0 (17.9)
Base saturation (%)	40.8 (10.9)	-	-	100 (0)
AcP (nmol g^{-1} h^{-1})	16.2 (2.5)	-	-	-
AlkP (nmol g^{-1} h^{-1})	4.5 (0.4)	-	-	-
α-G (nmol g^{-1} h^{-1})	0.5 (0.1)	-	-	-
AryS (nmol g^{-1} h^{-1})	0.9 (0.1)	-	-	-
β-G, (nmol g^{-1} h^{-1})	4.8 (0.5)	-	-	-
BisP (nmol g^{-1} h^{-1})	1.5 (0.2)	-	-	-
Chit (nmol g^{-1} h^{-1})	2.4 (0.4)	-	-	-
Leu (nmol g^{-1} h^{-1})	13.5 (1.4)	-	-	-
Nona (nmol g^{-1} h^{-1})	28.9 (3.7)	-	-	-
Pyro (nmol g^{-1} h^{-1})	0.3 (0.1)	-	-	-
Uroni (nmol g^{-1} h^{-1})	-	-	-	-
Xylo (nmol g^{-1} h^{-1})	1.2 (0.2)	-	-	-
dsDNA (μg g^{-1})	< dl	-	-	-

[a)]Numbers in parentheses are the standard deviation (n=3). < dl: below the detection limit. EC: electrical conductivity; TOC: total organic carbon; EOOC: easily oxidizable organic carbon; TN: total nitrogen; TS: total sulphur; TP: total phosphorus; Water-P: phosphorus extracted by distilled water; 2% citric-P: phosphorus extracted by 2% citric acid; 2% formic-P: phosphorus extracted by 2% formic acid; Mehlich 3-P: phosphorus extracted by Mehlich 3 procedure; CEC: cation exchange capacity; AcP: acid phosphomonoesterase; AlkP: alkaline phosphomonoesterase; α-G: α-glucosidase; AryS: arylsulfatase; β-G: β-glucosidase; BisP: phosphodiesterase; Chit: chitinase; Leu: leucine aminopeptidase; Nona: nonanoate esterase; PyroP: pyrophosphate phosphodiesterase; Uroni: glucuronidase; Xylo: xyloxidase; dsDNA: double-strand DNA.

Table 2
Chemical and biochemical properties of acid Arenosol from Mozambique after the pot experiment. Means followed by different lowercase letters significantly differed among the treatments at $P < 0.05$ by Duncan multiple mean comparison test.

Soil parameters	Treatments								ANOVA
	Control	WSP	PR	WSP+D	WSP+BC	WSP+D+BC	PR+BC	PR+D+BC	
pH_{H2O}	4.54 (0.18) [a)] c	4.46 (0.18) c	4.55 (0.24) c	6.61 (0.47) ab	5.82 (0.21) b	6.96 (0.29) a	6.16 (0.84) b	7.38 (0.31) a	***
pH_{KCl}	4.09 (0.08) c	4.07 (0.06) c	4.12 (0.07) c	6.20 (0.41) a	4.61 (0.11) b	6.15 (0.35) a	4.74 (0.16) b	6.57 (0.24) a	***
TOC (g kg^{-1})	19.75 (0.66) b	18.17 (1.58) b	20.22 (1.39) b	19.55 (1.44) b	22.38 (0.92) a	22.64 (1.41) a	22.98 (0.75) a	23.18 (0.97) a	***
EOOC (g kg^{-1})	4.78 (0.30) c	3.83 (1.34) c	3.83 (0.70) c	4.76 (1.28) c	9.17 (0.94) a	7.31 (1.27) b	8.72 (0.48) ab	7.66 (0.83) ab	***
Recalcitrant C (g kg^{-1})	14.97 (0.94)	14.33 (2.04)	16.39 (2.08)	14.79 (0.33)	13.21 (1.13)	15.33 (2.67)	14.26 (0.69)	15.51(2.04)	NS
Mehlich 3-P (mg kg^{-1})	11.1 (5.2) d	7.8 (2.0) d	43.7 (6.9) c	48.9 (28.8) bc	76.2 (18.5) b	63.7 (12.1) bc	104.3 (10.6) a	72.3 (16.6) b	***
Exchangeble Ca ($cmol_c$ kg^{-1})	0.05 (0.01) c	0.05 (0.02) c	0.06 (0.02) c	0.38 (0.03) a	0.10 (0.01) b	0.34 (0.05) a	0.12 (0.03) b	0.37 (0.02) a	***
Exchangeble Mg ($cmol_c$ kg^{-1})	0.03 (0.00) f	0.06 (0.01) e	0.03 (0.01) f	0.26 (0.01) ab	0.14 (0.01) d	0.25 (0.04) b	0.19 (0.03) c	0.30 (0.03 a	***
Exchangeble K ($cmol_c$ kg^{-1})	0.06 (0.01) d	0.19 (0.03) b	0.11 (0.02) c	0.20 (0.02) b	0.25 (0.08) b	0.19 (0.05) b	0.43 (0.07) a	0.24 (0.03) b	***
Exchangeble Na ($cmol_c$ kg^{-1})	0.09 (0.06)	0.11 (0.09)	0.07 (0.06)	0.09 (0.03)	0.12 (0.09)	0.15 (0.02)	0.09 (0.07)	0.14 (0.00)	NS
Exchangeble Al ($cmol_c$ kg^{-1})	0.03 (0.00)	0.16 (0.00)	0.15 (0.04)	0.03 (0.02)	0.02 (0.03)	0.05 (0.04)	0.02 (0.01)	0.12 (0.02)	NS
CEC ($cmol_c$ kg^{-1})	0.40 (0.03) d	0.52 (0.12) cd	0.42 (0.07) d	0.96 (0.10) ab	0.63 (0.11) c	0.98 (0.14) ab	0.85 (0.02) b	1.06 (0.07) a	***
Base saturation (%)	57.5 (5.6) c	78.8 (8.2) b	64.3 (15.4) b	96.9 (1.6) a	96.8 (1.6) a	94.9 (2.8) a	97.6 (4.3) a	99.1 (0.8) a	***
AcP (nmol g^{-1} h^{-1})	9.64 (1.63) a	6.65 (3.69) ab	6.03 (1.36) b	2.36 (0.54) c	3.00 (1.27) c	1.01 (0.98) c	2.75 (0.63) c	0.92 (0.82) c	***
AlkP (nmol g^{-1} h^{-1})	1.42 (0.67) bc	1.95 (3.37) c	0.52 (0.56) c	13.94 (7.89) a	9.74 (4.00) a	10.77 (6.07) a	7.96 (2.73) ab	11.58 (8.79) a	**
α-G (nmol g^{-1} h^{-1})	0.03 (0.05) c	0.09 (0.16) bc	0.00 (-) c	0.26 (0.03) a	0.16 (0.07) ab	0.19 (0.03) a	0.26 (0.03) a	0.41 (0.17) a	***
AryS (nmol g^{-1} h^{-1})	0.31 (0.25)	0.34 (0.38)	0.00 (-)	0.25 (0.04)	0.35 (0.12)	0.30 (0.08)	0.42 (0.08)	0.23 (0.06)	NS
β-G, (nmol g^{-1} h^{-1})	1.63 (0.19)	1.28 (0.91)	0.92 (0.18)	0.97 (0.27)	1.26 (0.30)	1.23 (0.08)	1.21 (0.28)	0.90 (0.06)	NS
BisP (nmol g^{-1} h^{-1})	0.53 (0.46) abc	0.36 (0.63) bc	0.00 (-) c	1.43 (0.63) a	0.84 (0.83) ab	1.12 (0.32) ab	0.86 (0.05) ab	1.17 (0.68) ab	*
Chit (nmol g^{-1} h^{-1})	0.51 (0.08)	0.58 (0.51)	0.36 (0.11)	0.41 (0.07)	1.05 (0.87)	0.50 (0.16)	0.78 (0.51)	0.65 (0.41)	NS
Leu (nmol g^{-1} h^{-1})	2.55 (1.72)	2.93 (2.46)	1.25 (0.27)	3.08 (0.64)	2.77 (0.99)	2.95 (1.27)	3.82 (0.49)	2.57 (0.99)	NS
Nona (nmol g^{-1} h^{-1})	6.50 (5.64)	11.01 (8.58)	7.88 (10.65)	9.40 (6.28)	7.98 (3.93)	11.99 (2.94)	10.10 (3.93)	6.30 (6.96)	NS
Pyro (nmol g^{-1} h^{-1})	0.03 (0.05)	0.10 (0.18)	0.00 (-)	0.36 (0.25)	0.09 (0.16)	0.13 (0.15)	0.09 (0.09)	0.16 (0.14)	NS
Uroni (nmol g^{-1} h^{-1})	0.00 (-)	0.00 (-)	0.00 (-)	0.00 (-)	0.00 (-)	0.05 (0.09)	0.00 (-)	0.06 (0.11)	NS
Xylo (nmol g^{-1} h^{-1})	0.40 (0.11) a	0.37 (0.32) ab	0.23 (0.04) abc	0.11 (0.09) c	0.20 (0.04) abc	0.09 (0.09) c	0.27 (0.09) abc	0.12 (0.05) bc	*
dsDNA (μg g^{-1})	1.86 (0.49)	1.95 (0.65)	1.83 (0.08)	1.49 (0.16)	1.54 (0.29)	1.47 (0.09)	1.11 (0.16)	1.61 (0.27)	NS

NS, *, **, *** Not significant, significant at $P = 0.05$, $P = 0.01$ and $P = 0.001$, respectively. [a)]Numbers in parentheses are the standard deviation (n=3).

TOC: total organic carbon; EOOC: easily oxidizible organic carbon; Mehlich 3-P: phosphorus extracted by Mehlich 3 procedure; CEC: cation exchange capacity; AcP: acid phosphomonoesterase; AlkP: alkaline phosphomonoesterase; α-G: α-glucosidase; AryS: arylsulfatase; β-G: β-glucosidase; BisP: phosphodiesterase; Chit: chitinase; Leu: leucine-aminopeptidase; Nona: nonanoate esterase; Pyro: pyrophosphate-phosphodiesterase; Uroni: glucuronidase; Xylo: xylosidase

3.2.2. Enzyme activities

The overall impact of the treatments on enzyme activities was rather limited (Table 2), with significant differences among the treatments only for acid phosphomonoesterase ($P<0.001$), alkaline phosphomonoesterase ($P<0.01$), α-glucosidase ($P<0.001$), phosphodiesterase ($P<0.05$), and xylosidase ($P<0.05$). The lowest activities of acid phosphomonoesterase and xylosidase occurred in treatments including biochar and/or dolostone, those able to induce relatively higher pH values. On the contrary, the activities of alkaline phosphomonoesterase, α-glucosidase, and phosphodiesterase were the highest in the treatments including biochar and/or dolostone, since these enzyme activities increased with pH.

Application of biochar to the soil can influence the soil biota and consequently increase or decrease enzyme activities (Gianfreda and Rao, 2014). However, the effect of biochar on soil enzymes is variable and depends on the interaction between biochar and enzymes' target substrate (Bailey et al., 2011; Lu et al., 2015). For example, Wang et al. (2015a) found an increase of activity of extracellular enzymes involved in the C and S cycles (excluding xylosidase) with the lower application rate of corn biochar (0.5% w/w). Instead, Lone et al. (2015) obtained an increase of phosphatases activity, which are responsible for catalizing the release of available P from P sources through interfering on P cycle (Eivazi and Tabatabai, 1977; Turner, 2010; Kumar et al., 2013). Thus, Wang et al. (2015b) observed a general increase of activity of enzymes related to utilization of N and P with application of biochar. As the optimum pH range of acid phosphomonoesterase and xylosidase is from ~ 4 to 5 (Gianfreda and Rao, 2014) and, in our experiments, the treatments including biochar and/or dolostone led to a soil pH 5, this pH increase could have contributed to depress activities of the two enzymes.

Contrariwise, in the treatments with relatively higher pH values, the alkaline phosphomonoesterase, α-glucosidase, and phosphodiesterase activities were high possibly because they have their optimum pH at values close to 5 (Ekenler and Tabatabai, 2013; Gianfreda and Rao, 2014; Lu et al., 2015). Also Huang et al. (2017) found an increase of alkaline phosphomonoesterase with application of rice straw biochar. Since alkaline phosphomonoesterase and phosphodiesterase are involved in the P cycle and α-glucosidase in the C cycle, it is plausible that their increased activity contributed to the increase of available P and C mineralization (Shukla and Varma, 2011). The high activity of acid phosphatase in our Control and WSP treatment must be related not only to low pH,

but also to P deficiency under these treatments (Wallenstein et al., 2009; Alster et al., 2013; Wen et al., 2017).

3.3. Corn growth, yield, and nutrient uptake

The effect of treatments was significant for corn growth and yield parameters. The treatments with biochar showed the highest ($P < 0.001$) production of corn dry biomass, root, shoot, and cob, and the largest plant diameter and height (Table 3). The PR and WSP+D treatments did not result in any significant increase of yield parameters. The application of WSP alone even depressed corn yield relative to Control. The use of phosphite as foliar fertilizer has been demonstrated to cause toxicity to plants (Thao et al., 2008; Zambrosi et al., 2017), while its direct application to soil has given contrasting results as it may increase, decrease or have null effect on growth and yield of vegetables (Moor et al., 2009; Thao and Yamakawa, 2009; Gómez-Merino and Trejo-Téllez, 2015).

An explanation to these conflicting results can be found in Bertsch et al. (2009) and Thao and Yamakawa (2009), who referred that phosphite applications to soil in presence of sufficient amounts of available phosphate may induce a synergic effect between phosphite and phosphates, so to promote P absorption into plants. Since plants cannot convert phosphite into phosphate, and accumulation of phosphite in the cells may have negative effects on the plants (Varadarajan et al., 2002; Loera-Quezada, 2015). Thus, the phosphite effect strongly depends on the P status of the plant (Thao and Yamakawa, 2009; Gómez-Merino and Trejo-Téllez, 2015).

Finally, as the plant P status mostly depends on the amount of soil available P, the depressed corn yield obtained with the WSP treatment was ascribed to the very low content of available P in the soil used, which has probably imbalanced the absorption of phosphite and phosphate so to induce negative effects to corn. The corn yield in all treatments including biochar, along to phosphate was attributed to the improvement of both soil chemical and biochemical properties. The decrease of soil acidity together with an increase of available P affected directly the corn yield, while CEC influenced the corn yield indirectly by retaining nutrients. Improving the soil physical properties such as water retention, which have not been measured in this study, may have contributed to the good performance of the treatments including BC. The inefficiency of treatments like PR, WSP+D and, especially, WSP must be related to their limitation to improve soil chemical parameters, particularly pH and CEC in the case of PR, and pH, CEC and available P in the case of WSP.

Table 3
Treatment effect on growth and yield of corn on acid Arenosol from Mozambique. Means followed by different lowercase letters significantly differed among the treatments at $P < 0.05$ by Duncan multiple mean comparison test.

Corn growth and yield parameters	Treatments								ANOVA
	Control	WSP	PR	WSP+D	WSP+BC	WSP+D+BC	PR+BC	PR+D+BC	
Dry biomass, g pot^{-1}	2.2 (0.6) [a)] b	0.8 (0.2) c	5.3 (1.4) b	3.3 (2.9) b	52.6 (25.2) a	89.1 (18.2) a	51.1 (36.2) a	70.6 (35.8) a	***
Dry root, g pot^{-1}	0.4 (0.1) c	0.2 (0.0) c	0.7 (0.2) b	0.6 (0.3) b	4.2 (2.1) a	6.0 (1.2) a	3.5 (1.8) a	7.0 (4.4) a	***
Dry shoot[b)], g pot^{-1}	1.8 (0.7) b	0.6 (0.2) c	4.4 (1.2) b	2.2 (1.8) b	37.6 (18.5) a	45.8 (7.1) a	34.0 (18.5) a	54.9 (31.8) a	***
Dry cob, g pot^{-1}	NA (-)	NA (-)	0.2 (0.3) b	0.5 (0.8) b	10.8 (5.9) a	37.3 (13.6) a	13.6 (17.5) a	8.7 (7.8) a	***
Plant diameter, cm	7.8 (2.3) cd	5.4 (0.2) d	8.7 (1.3) bcd	9.0 (1.3) bc	11.7 (2.0) ab	12.9 (1.8) a	13.6 (0.8) a	13.8 (3.1) a	***
Plant height, cm	30.4 (10.8) b	26.5 (9.9) b	46.4 (4.9) b	38.6 (27.9) b	154.7 (71.1) a	158.0 (24.3) a	134.3 (68.2) a	171.3 (16.5) a	***

*** Significant at $P = 0.001$. [a)] Numbers in parentheses are the standard deviation (n=3). [b)] Shoot: stem + leaves; NA: Not available because there was not cob production.

The WSP+D treatment did improve pH, CEC and available P, but its major drawback seemed to be the inability to increase soil organic C. The application of phosphate rock combined with an organic source (e.g. biochar) can increase corn yield (Husnain et al., 2014). A principal component analysis (PCA) was run assessing the variation of soil properties and plant yield parameters as affected by the treatments, which contributed to 62 % of the variation (Fig. S1). PC1 and PC2 were responsible for ~ 51 and 11 % of the variation, respectively. The PCA biplot showed variations of the soil properties and plants yield parameters between the treatments. Many soil and plant parameters showed positive correlation with PC1 (Dim1), contributing for ~ 86% of variability (Table S1). The parameters involved belonged to both chemical (pH_{H2O}, pH_{KCl}, TOC, EOOC, available P, exchangeable Ca, Mg, K, and Al, base saturation, CEC, and Al saturation), and biochemical (α-glucosidase and alkaline phosphomonoesterase) properties, but also to yield issues such as dry biomass, root, shoot, cob, leaves, plant height and diameter, P agronomic efficiency, P apparent recovery, P physiologic efficiency, and P harvest index. Other parameters such as dsDNA, phosphodiesterase, acid phosphomonoesterase, leucine-aminopeptidase, chitinase, xylosidase, ß-glucosidase, and arylsulfatase showed positive correlation with PC2 (Dim2), contributing ~ 82% of variability.

Treatments where biochar, phosphate rock and dolostone were applied showed higher pH, available P, EOOC, CEC, alkaline phosphomonoesterase, phosphodiesterase, dry biomass, root, cob, and P agronomic efficiency. These results suggest the occurrence of synergies between rocks (phosphate and dolostone) and biochar. Herrero et al., (1998), Pancholy et al., (1975), and Schulten and Schnitzer (1998), asserted that the enzyme activities are more closely related to plant production under native condition or highly disturbed soil than in agricultural systems. In our case, enzyme activities responded mostly to the treatments including biochar, phosphate and dolostone rocks, probably for the change of soil pH.

Inorganic fertilizers alone did not improve corn yield compared with Control. This implied that application of biochar is of paramount importance to increase fertility of this poor sandy soil, possibly because it enhances the effect of inorganic fertilizers via the increase of CEC and soil organic matter content. For the nutrient concentration in plant tissues, ANOVA revealed the existence of significant interactions between plant portion (roots, shoots and cobs) and treatments for C, S, K, Ca, and Mg (Table 4). Treatments like WSP+D+BC, PR+D+BC, and PR+BC caused the highest accumulation of nutrients in shoots and, then, cobs. An effective translocation of nutrients from the roots to shoots and cobs was found in the treatments with biochar. Nutrients uptake showed a biomass-dependent

characteristic, as reported by Tao et al. (2005). This showed again the importance of biochar to improve crop production in nutrient-poor soils.

3.3.1. Phosphorus uptake

The interaction between plant portions (roots, shoots, and cobs) and treatments was significant for P content (mg pot^{-1}) ($P < 0.05$) (Fig. 1a). In addition, also a significant effect of individual factors (treatments or plant portion) on P content was found ($P < 0.001$). The WSP+BC, WSP+D+BC, PR+BC, and PR+D+BC treatments induced higher contents of P in shoots and/or cobs, while the difference between roots and cobs was significant only in WSP+D+BC (Fig. 1a). The treatment WSP resulted in the highest concentrations of P in roots and shoots, even though they were not statistically different from those of the Control (Fig 1b). In the case of roots, PR+BC and PR+D+BC treatments displayed the lowest P concentration (Fig. 1b). The high P concentration in the shoots in the WSP treatment contrasted with the respective very low P content (Fig. 1a).

The contrast was explained by the low yield obtained with this treatment, which evidently produced a concentration effect in the small biomass produced. Nutrient concentration may rise for plants under nutrient starvation (Barker and Pilbeam, 2007), as we observed in WSP and Control treatments. Despite phosphate and phosphite uptake and mobility similar, treatments with WSP indicated that corn cannot utilize phosphite as a sole P source (Varadarajan et al., 2002; Thao et al., 2008). Ticconi et al. (2001) and Zambrosi et al. (2017) reported that fertilizations with phosphite decreased yield, despite significant increase of P found in plant tissues, since phosphite cannot play the physiological role of phosphate. The treatments including WSP generally presented higher P concentration in both roots and shoots probably because phosphite accumulated in these plant portions without being converted into phosphate (Varadarajan et al., 2002; Loera-Quezada, 2015).

In addition, WSP+D+BC, PR+BC, and PR+D+BC treatments induced an efficient P translocation from the roots to the cobs (Fig. 1c). The higher P content and translocation in shoots and cobs corresponded to the higher concentration of soil available P, except for WSP+BC, with high values of available P and P content in the shoots, but relatively low P content in the cobs and translocation factors. The P content in plant portions was lowest in all treatments without biochar (Fig. 1a), and pH was lowest in Control, WSP, and PR (Table 2). It is noteworthy that P deficiency inhibits anion uptake, so fostering soil acidification because of unbalanced absorption between cations and anions that reduces the release of OH^- by the roots (Shen et al., 2005).

Table 4
Amounts of C, N, S, K, Ca, and Mg taken up (mg pot^{-1}) in corn portions after the pot experiment with acid Arenosol from Mozambique. Means followed by different lowercase letters significantly differed among the treatments at $P < 0.05$ by Duncan multiple mean comparison test.

Nutrient		Treatments								ANOVA
		Control	WSP	PR	WSP+D	WSP+BC	WSP+D+BC	PR+BC	PR+D+BC	
C	Roots	121.8 (46.0) [a)] d	57.6 (2.0) d	229.6 (69.0) d	1427.6 (688.1) d	1427.6 (688.1) d	2185.7 (302.3) d	1343.6 (720.9) d	2444.8 (1465.0) d	
	Shoot[b)]	644.4 (230.8) d	194.0 (55.1) d	1693.2 (362.1) d	14483.8 (7042.7) b	14483.8 (7042.7) b	19695.0 (2528.8) a	14499.9 (7613.8) b	22508.0 (12304.4) a	***
	Cob	NA [c)]	NA	55.5 (96.6) d	3747.4 (2032.2) d	3747.4 (2032.2) d	14 294.8 (4592.6) c	8089.5 (5095.2) cd	4764.7 (230.1) cd	
N	Roots	4.3 (2.2) c	2.6 (0.1) c	9.9 (4.6) c	0.0 (-) c	0.0 (-) c	0.0 (0.0) c	19.7 (34.2) c	0.0 (0.0) c	
	Shoot	29.4 (2.3) c	16.8 (7.3) c	75.9 (15.1) c	359.9 (232.6) bc	359.9 (232.6) bc	470.6 (59.7) ab	669.7 (824.2) a	54.5 (94.3) c	*
	Cob	NA	NA	1.2 (2.0) c	73.5 (39.5) c	73.5 (39.5) c	361.3 (139.8) bc	186.4 (117.6) c	103.6 (2.2) c	
S	Roots	15.1 (5.1) f	6.6 (0.3) f	27.3 (6.0) f	165.0 (85.9) f	165.0 (85.9) f	260.3 (34.0) f	165.6 (107.7) f	282.1 (150.5) f	
	Shoot	83.8 (31.8) f	25.5 (9.9) f	224.1 (65.3) f	1682.5 (819.5) cd	1682.5 (819.5) cd	2437.3 (642.5) ab	2156.5 (1419.0) bc	2965.1 (1600.8) a	***
	Cob	NA	NA	7.2 (12.4) f	437.4 (238.1) f	437.4 (238.1) f	1831.5 (497.1) de	993.2 (631.3) ef	671.4 (24.0) ef	
K	Roots	2.8 (1.1) b	0.7 (0.2) b	16.6 (12.1) b	69.0 (43.2) b	69.0 (43.2) b	63.8 (17.8) b	66.8 (42.0) b	71.1 (48.3) b	
	Shoot	16.5 (12.3) b	11.8 (7.0) b	104.1 (14.9) b	880.7 (538.5) a	880.7 (538.5) a	923.5 (133.6) a	1004.0 (1127.2) a	795.5 (437.5) a	**
	Cob	NA	NA	1.9 (3.3) b	46.7 (71.4) b	46.7 (71.4) b	234.6 (128.2) b	185.1 (119.6) b	95.8 (33.1) b	
Ca	Roots	0.4 (0.1) c	0.2 (0.2) c	0.6 (0.2) c	0.6 (0.2) c	0.6 (0.2) c	4.0 (3.1) c	1.0 (0.6) c	4.1 (3.3) c	
	Shoot	1.4 (1.4) c	1.2 (0.8) c	8.1 (1.8)	27.5 (22.6) b	27.5 (22.6) b	56.1 (4.9) a	22.6 (18.2) b	57.2 (30.9) a	***
	Cob	NA	NA	0.0 (0.0) bc	0.4 (0.6) b	0.4 (0.6) b	5.7 (3.0) a	1.4 (0.9) b	2.9 (0.3) a	
Mg	Roots	0.3 (0.1) b	0.2 (0.0) b	1.6 (0.7) b	2.6 (1.3) b	2.6 (1.3) b	4.1 (2.1) b	4.5 (3.4) b	3.2 (2.1) b	
	Shoot	2.7 (1.6) c	2.2 (1.4) c	12.3 (2.0) b	56.8 (31.1) a	56.8 (31.1) a	67.6 (6.1) a	55.8 (46.6) a	82.3 (50.5) a	**
	Cob	NA	NA	0.2 (0.3) b	7.6 (11.8) a	7.6 (11.8) a	40.1 (3.3) a	20.3 (12.9) a	14.1 (3.3) a	

*, **, *** Significant at $P = 0.05$, $P = 0.01$ and $P = 0.001$, respectively. [a)] Numbers in parenthesis are standard deviation (n=3). [b)] Shoot = stem + leaves; [c)] NA: not available.

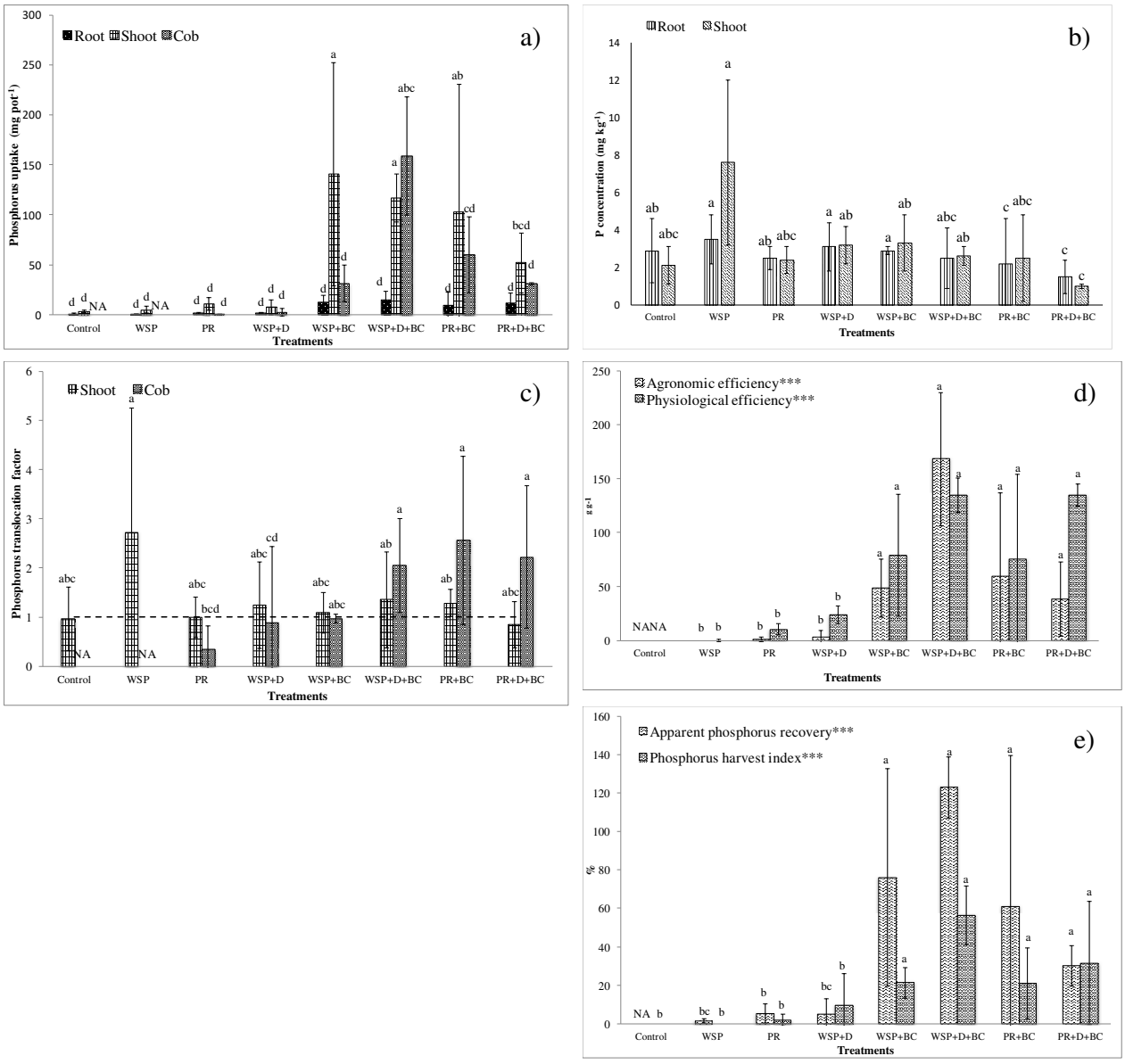

Fig. 1 Phosphorus uptake (a), phosphorus concentration (b), translocation factor (c), phosphorus agronomic and physiological efficiencies (d), and apparent phosphorus recovery, and phosphorus harvest index (e) by corn after the pot experiment with acid Arenosol from Mozambique. Means followed by different lowercase letters significantly differed among the treatments at $P < 0.05$ by Duncan multiple mean comparison test. The dashed line represents the limit of Translocation Factor (TF); TF > 1 indicates effective translocation, whereas TF< 1 indicates not effective translocation. The whiskers indicate the standard deviation (n=3). NA – Not available data. *** Significant at $P = 0.001$.

Application of biochar increased ($P < 0.001$) the agronomic and physiological efficiency of P, apparent nutrient recovery, and harvest index (Fig. 1d,e). The higher agronomic and recovery efficiency in biochar, phosphate rock and dolostone treatments indicated that crop nutrition was optimized by the application of both rocks and biochar (Agegnehu et al., 2016b). Also, the fraction

of P converted into cob, assessed as physiological efficiency (Fig. 1d), was higher in the treatments where biochar was applied together with WSP, phosphate rock or dolostone ($P < 0.001$). These results demonstrated the positive effect of biochar and both rocks, which included the supply of adequate levels of P. P deficiency was observed on treatments such as Control and WSP alone. In the WSP+BC treatment, biochar may have reduced the negative effect of phosphite by supplying P in form of phosphate.

3.4. Rhizosphere effect on soil properties

3.4.1. Rhizosphere effect on soil chemical properties

Available P concentration in soil differed from rhizosphere to bulk soil ($P < 0.05$), with higher content in the bulk than in the rhizosphere (Fig. S2). Sas et al. (2001), among others, asserted that roots are able to mobilize sparingly soluble P amounts through exudation of large amounts of organic anions. Because of this, generally, the highest concentrations of soluble P have been found in the rhizosphere (Gerke et al., 1994; Zhao et al., 2010). Instead, in our experiments, the reverse occurred probably because of the higher P absorption induced in the corn rhizosphere by the treatments (Sas et al., 2001; Shen et al., 2005; Marschner et al., 2011). However, Maltais-Landry et al. (2014) found similar available P content in rhizosphere and bulk soil, while Valentinuzzi et al. (2015) argued that uptake of nutrients around rhizosphere contributes to the decrease of available of nutrients in this soil portion. There was no significant effect of rhizosphere on soil pH (in both H_2O and KCl), EOOC, CEC, and exchangeable Ca, Mg, and K (Fig. S2). Principal component analysis (PCA) made on rhizosphere and bulk soil chemical data (Fig. S3a) indicated that a strong and positive correlation occurred between all soil chemical parameters, except exchangeable Al. Also, there was an evident overlapping between rhizosphere and bulk soil clusters, indicating a similarity between the two soil fractions in terms of soil chemical properties. This result reinforces what the univariate analysis has highlighted.

3.4.2. Rhizosphere effect on soil enzyme activities

No interaction was observed between rhizosphere and treatments for α-glucosidase, arylsulfatase, acid phosphomonoesterase, nonanoate-esterase, and leucine aminopeptidase activities, a significant rhizosphere effect ($P < 0.05$) was found, with higher activities in the rhizosphere than in the bulk except for leucine aminopeptidase for which the reverse was true (Fig. 2). No rhizosphere effect was found for the other enzymes.

While the activity of acid phosphomonoesterase was higher in the rhizosphere than in the bulk (Fig. 2), the concentration of available P show the opposite result. Wen et al. (2017) reported that, under P deficiency, corn roots are able to exudate acid phosphatases into the rhizosphere. Further, plants and microorganisms can increase the solubility of sparingly soluble inorganic P and mineralize organic P by releasing protons, organic acids and several phosphatases (Marschner et al., 2011). Our results demonstrated the occurrence of a corn rhizosphere effect, which included increase of rhizodeposition (protons, organic anions, enzymes), and accelerated nutrient uptake to facilitate the plant growth. Therefore, with the increase of rhizodeposition, microbial biomass also may increase, so leading to the increase of overall enzymes activities and respiration (Burns et al., 2013).

The interaction between rhizosphere and treatments was significant ($P < 0.001$) only for glucuronidase. The highest glucuronidase activities were found in the rhizosphere of the WSP+BC, WSP+D+BC, PR+BC, and PR+D+BC treatments (Fig. 3). Glucuronidase activity can provide a valuable tool for detecting and monitoring specific fungus strains like *Trichoderma harzianum* in the soil (Arora, 2003), and is correlated with fungal biomass especially in plant infections (Thomma et al., 1999) but here we considered that the aforementioned treatments have promoted fungus colonization of the rhizosphere. Fiorito et al. (2008) reported that this enzyme can be adsorbed onto clay minerals, but in our case the enzyme might have been stabilized by the biochar via adsorption mechanisms.

Negative and strong correlation was found between alkaline phosphomonoesterase *vs.* acid phosphomonoesterase, β-glucosidase nonanoate esterase, and xylosidade; in contrast, positive and strong correlations were found between alkaline phosphomonoesterase and other enzymes such as phosphodiesterase and pyrophosphate-phosphodiesterase (Fig. S3b). Despite an overlapping between rhizosphere and bulk soil, the rhizosphere showed a pattern oriented to the positive part of PC1, where higher activities of α-glucosidase and arylsulfatase were found. The same result was found using univariate analysis.

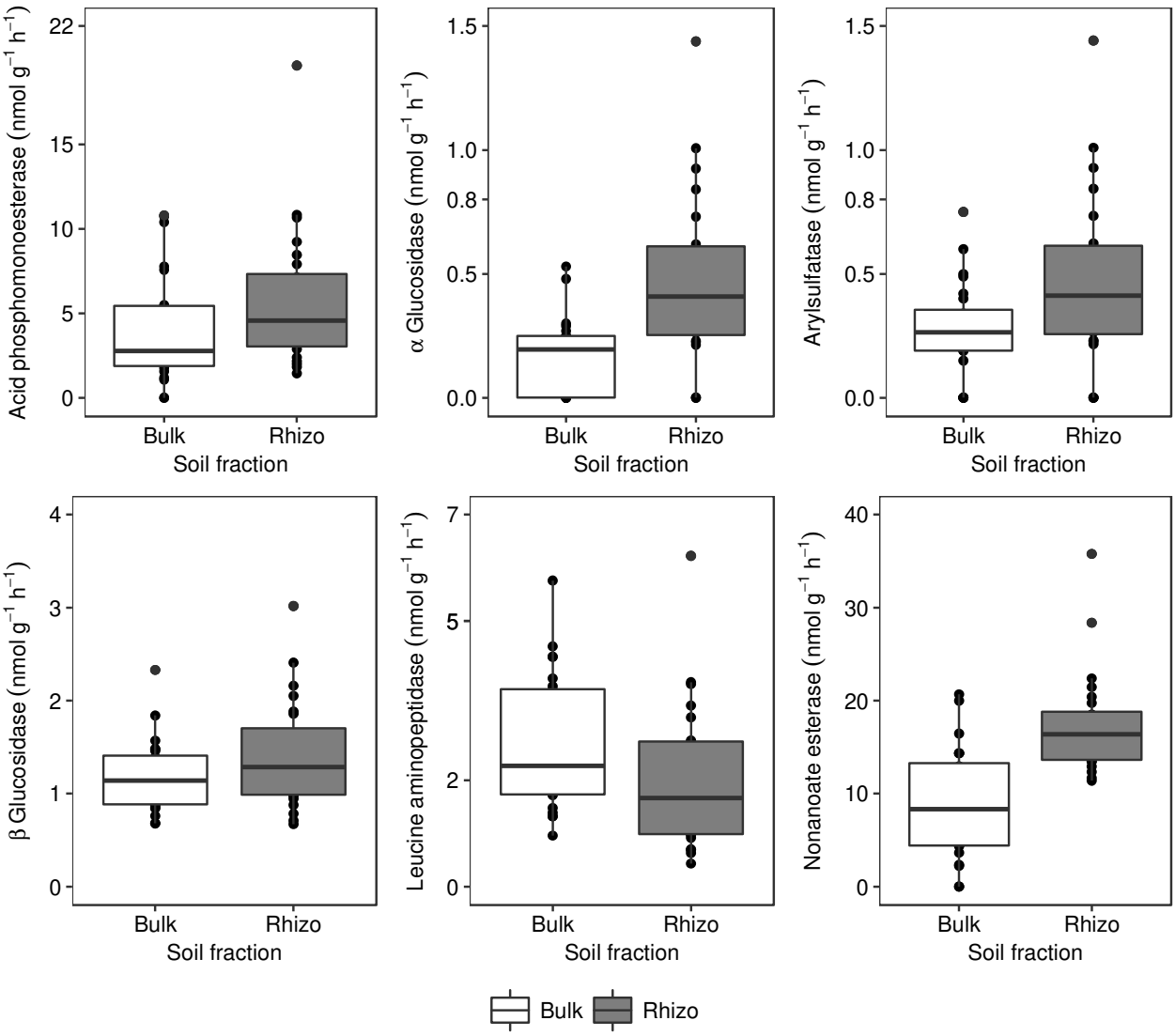

Fig. 2 Rhizospheric effect on soil acid phosphomonoesterase (P = 0.01), α-glucosidase (P = 0.001), arylsulfatase (P = 0.05), β-glucosidase (P = 0.001), leucine aminopeptidase (P = 0.05), nonanoate esterase (P = 0.001) activities after the pot experiment with acid Arenosol from Mozambique.

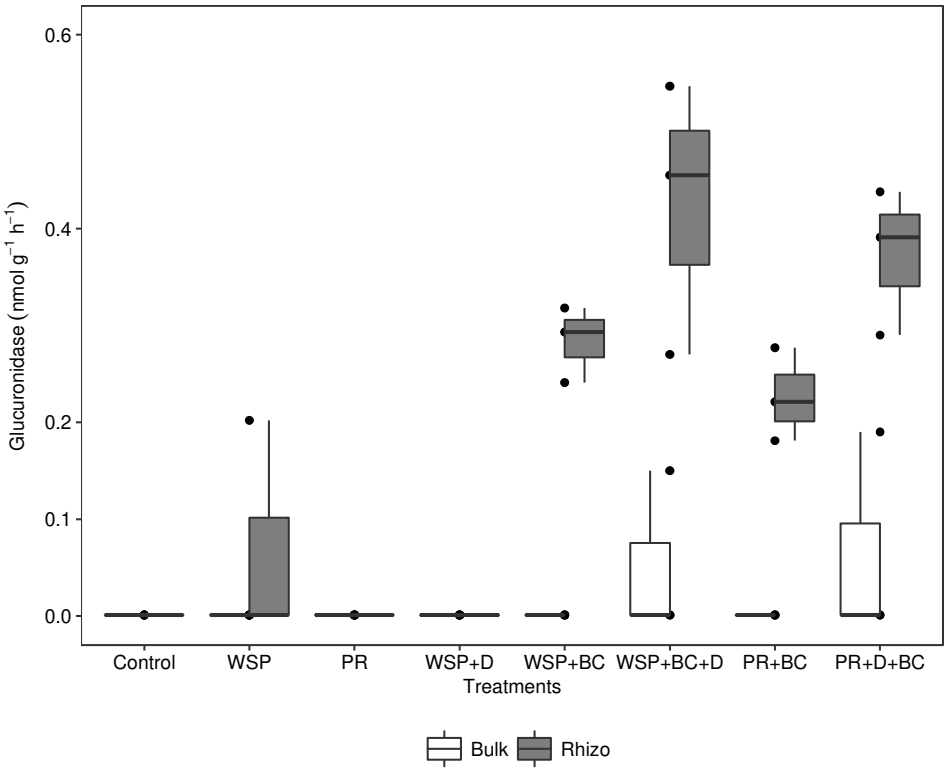

Fig. 3 Effect of treatments as affected by corn rhizosphere on soil glucuronidase ($P = 0.001$) activity after pot experiment with acid Arenosol from Mozambique.

4. Conclusions

Simultaneous application of phosphate rock, dolostone, and biochar improved soil properties. Soil pH, CEC, EOOC and available P were the most important in improving soil chemical fertility, but also the increased activities of alkaline phosphomonoesterase, α-glucosidase, and phosphodiesterase improved the soil quality. Phosphate rock alone failed to increase soil pH, but combined with dolostone or biochar was more effective on soil pH correction. Biochar supplied considerable amounts of P and facilitated P sorption, so limiting P leaching on WSP treatments. Zinc phosphite can not be used as sole P fertilizer due to the risk of toxicity to the plants, especially in available P deficient soils.

P uptake appeared to depend on soil treatment. Application of biochar together with phosphate rock/zinc phosphate and/or dolostone increased corn growth and yield. These treatments and corn rhizosphere effect promoted the increase of available P through the increase of exudation of extracellular enzymes. The P agronomic efficiency and P uptake were also high in treatments including biochar.

Application of phosphate rock as source of P is not a new practice, and it is considered agronomically effective and environmentally friendly. Combination of this rock with biochar notably improves P uptake. The combination of phosphate rock, biochar and dolostone is less effective than phosphate rock and biochar, since phosphate rock hardly dissolves at high pH. Further investigation under field condition is needed to validate these results and quantify the long-term benefit.

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Supplementary materials

Table S1
Percentage contribution of variables to the PCA.

Variables[a)]	PC1	PC2
	%	
Arylsulfatase	0.20	**17.73**
α-glucosidase	**2.86**	1.37
β-glucosidase	0.30	**14.75**
Xylosidase	1.67	**13.79**
Glucuronidase	0.85	0.44
Chitinase	0.28	**6.95**
Leucine-aminopeptidase	0.39	**16.52**
Acid phosphomonoesterase	3.57	**4.86**
Pyrophosphate-phosphodiesterase	0.60	1.87
Phosphodiesterase	2.02	**3.73**
Alkaline phosphomonoesterase	**2.62**	0.96
Nonanoate esterase	0.03	1.73
dsDNA	1.52	**3.20**
pH_{H2O}	**4.32**	0.25
pH_{KCl}	**3.27**	1.30
Total organic carbon	**3.28**	1.46
Easily oxidizible organic carbon	**2.97**	1.02
Phosphorus extracted by Mehlich 3 procedure	**3.23**	0.01
Exchangeable Ca	**2.54**	1.98
Exchangeable Mg	**4.05**	0.55
Exchangeable K	**2.17**	0.56
Exchangeable Na	0.41	0.58
Exchangeable Al	**4.05**	0.80
Cation exchange capacity	**3.96**	0.53
Base saturation	**3.94**	0.39
Aluminum saturation	**3.94**	0.39
Biomass	**4.08**	0.10
Root	**3.01**	0.03
Shoot	**3.87**	0.12
Cob	**3.57**	0.27
Leaves	**3.90**	0.02
Diameter	**3.59**	0.12
Plant height	**3.90**	0.67
Phosphorus harvest index	**4.10**	0.23
Phosphorus agronomic efficiency	**3.57**	0.26
Apparent phosphorus recovery efficiency	**3.19**	0.17
Phosphorus physiological efficiency	**4.22**	0.31

[a)]Numbers of variables in bold contributes most to PC1 or PC2

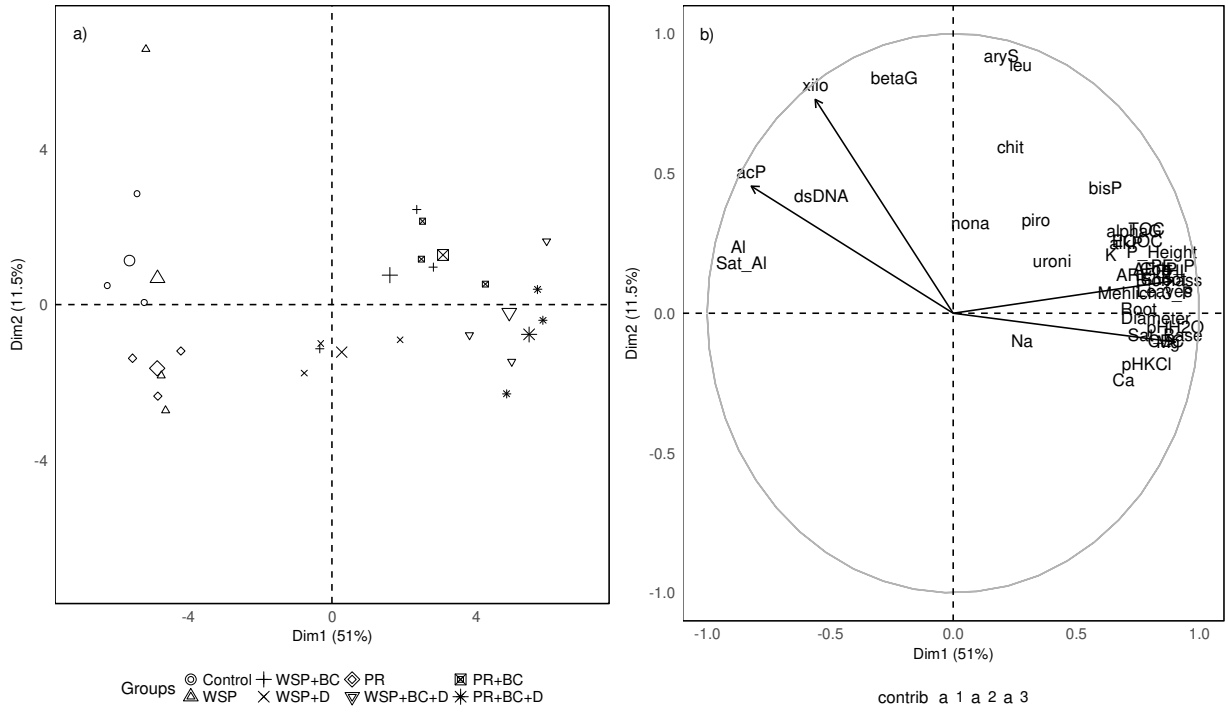

Fig. S1 Principal component analysis of soil and corn growth and yield parameters[5] as affected by the treatments of acid Arenosol from Mozambique [contrib. = percentage contribution of variables to the principal components (Dim1 - PC1, Dim2 - PC2)].

(a) graph of individuals (symbols), in which individuals close to each other have similar properties, whereas individuals far apart differ strongly in soil and corn growth and yield parameters. Symbols with bigger size represent the main effect of the group. The values on the axes refer to the percent of total variance explained by the axes.

b) graph of contribution (%) of variables to PCA; contrib.: Intensity of arrows (variables) in scale from 1-3%, in which arrows with color tending light grey has less contribution to PCA, whereas arrows with color tending to black has higher contribution to PCA.

[5]TOC: total organic carbon; EOOC: easily oxidizible organic carbon; Mehlich3_P: phosphorus extracted by Mehlich 3 procedure; CEC: cation exchange capacity; SatAl: aluminium saturation; AcP: acid phosphomonoesterase; AlkP: alkaline phosphomonoesterase; alphaG: α-glucosidase; AryS: arylsulfatase; betaG: β-glucosidase; BisP: phosphodiesterase; Chit: chitinase; Leu: leucine-aminopeptidase; Nona: nonanoate esterase; Pyro: pyrophosphate-phosphodiesterase; Uroni: glucuronidase; Xylo: xylosidase; dsDNA: double-strand DNA; P_Height: plant height.

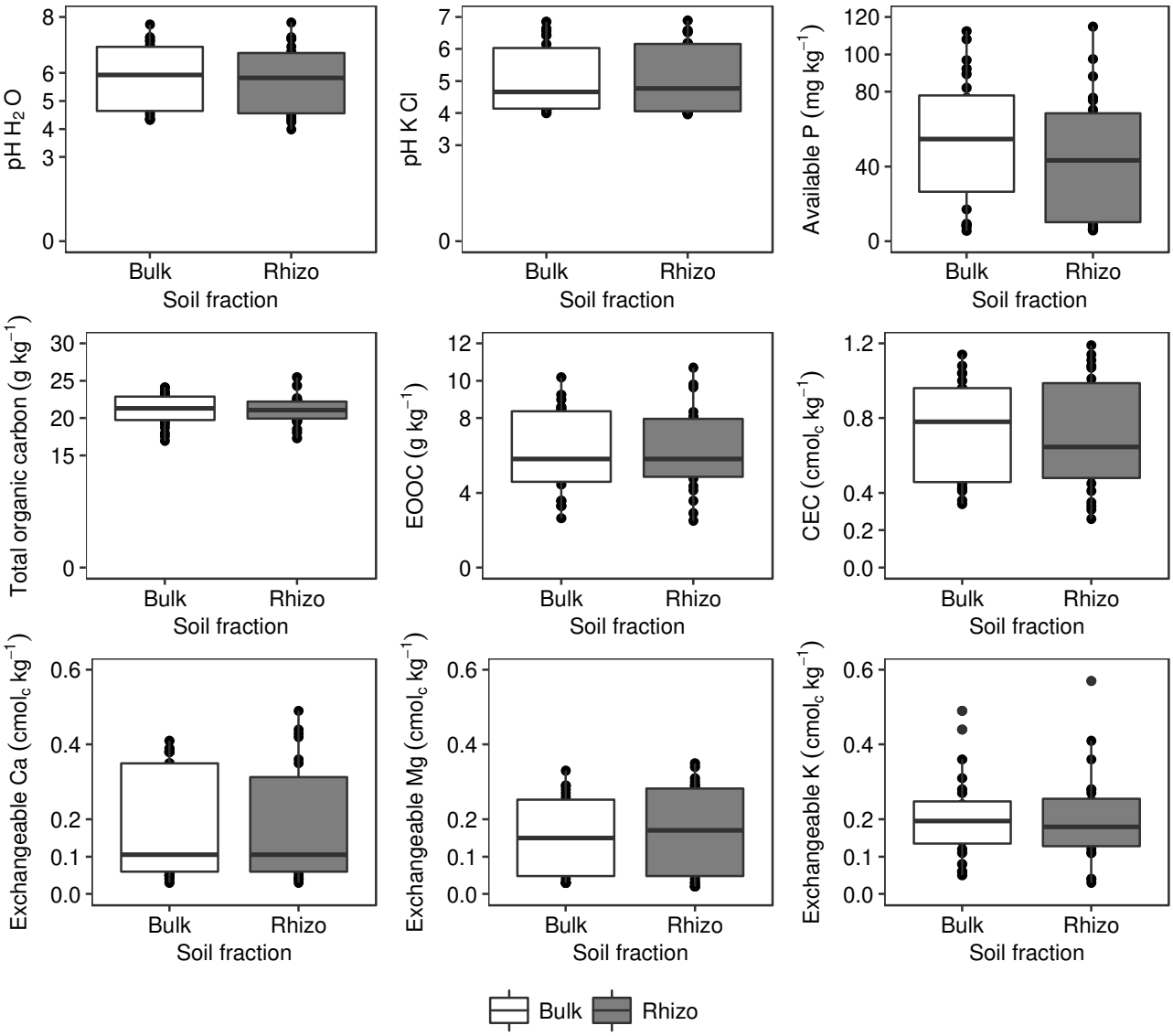

Fig. S2 Rhizospheric effect on soil pH_{H2O} (not significant, $P > 0.05$), pH_{KCl} (not significant, $P > 0.05$), available P ($P = 0.05$), total organic carbon (not significant, $P > 0.05$), easily oxidiziable organic C (EOOC) (not significant, $P > 0.05$), cation exchange capacity (CEC) (not significant, $P > 0.05$), exchangeable Ca, Mg and K (not significant, $P > 0.05$) after pot experiment with acid Arenosol from Mozambique.

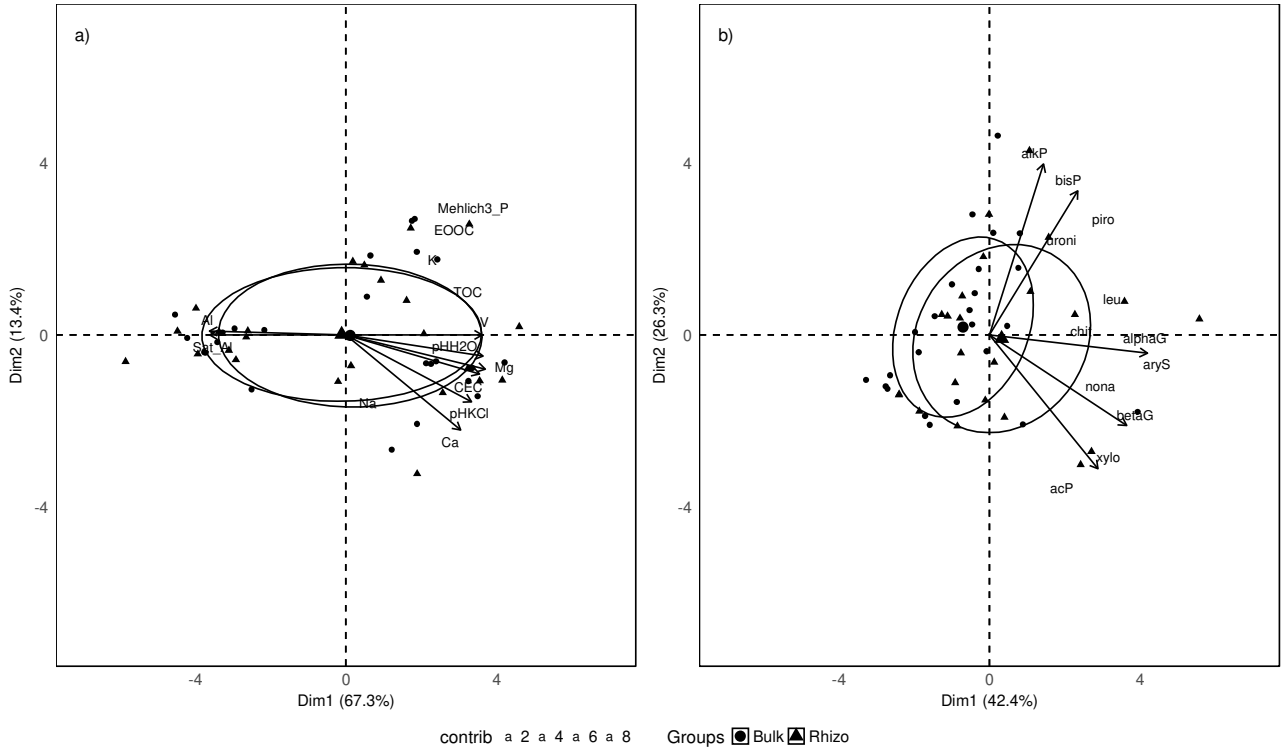

Fig. S3 Principal component analysis of soil chemical parameters (a) and enzyme activities (b) as affected by the corn rhizosphere[6] after the pot experiment with acid Arenosol from Mozambique [contrib. = percentage contribution of variables to the principal components (Dim1 - PC1, Dim2 - PC2)]. The values on the axes (graph a and b) are refer to the percent of total variance explained by the axes.

a) graph of individuals (symbols) and variables (arrows) for chemical parameters; b) graph of individuals (symbols) and variables (arrows) for enzyme activities. Individuals close to each other have similar properties, whereas individuals far apart differ strongly. Symbols with bigger size represent the main effect of the group (Bulk or Rhizo). The grey circle surrounds bulk samples, the light grey circle surrounds rhizosphere soil samples; Contrib.: Intensity of arrows (variables) in scale from 2-8%, in which arrows with color tending to light grey has less contribution to PCA, whereas arrows with color tending to black has higher contribution to PCA.

[6]TOC: total organic carbon; EOOC: easily oxidizable organic carbon; Mehlich3_P: phosphorus extracted by Mehlich 3 procedure; CEC: cation exchange capacity; Sat_Al: aluminium saturation; AcP: acid phosphomonoesterase; AlkP: alkaline phosphomonoesterase; alphaG: α-glucosidase; AryS: arylsulfatase; betaG: β-glucosidase; BisP: phosphodiesterase; Chit: chitinase; Leu: leucine-aminopeptidase; Nona: nonanoate esterase; Pyro: pyrophosphate-phosphodiesterase; Uroni: glucuronidase; Xylo: xylosidase.

CHAPTER 4 - BIOCHAR AND PHOSPHATE ROCK – IMPLICATION ON SOIL BIOLOGICAL AND BIOCHEMISTRY PROPERTIES

Phosphate rock and biochar impact on soil quality, and implication on corn and cowpea yields in an acid Arenosol

Rogério Borguete Alves Rafael[ab,★]; Maria Luisa Fernández-Marcos[cf]; [b]Danielle Janaina Westphalen; Stefania Cocco[b]; Maria Letizia Ruello[d]; Flavio Fornasier[e], Giuseppe Corti[b]

[a]Faculty of Agronomy and Forestry Engineering, Universidade Eduardo Mondlane, 257, Maputo, Mozambique

[b]Department of Agriculture, Food and Environmental Sciences, Università Politecnica delle Marche, 60131 Ancona, Italy

[c]Department of Soil Science and Agricultural Chemistry, Universidad de Santiago de Compostela, 27002 Lugo, Spain

[d]Department of Materials, Environmental Sciences and Urban Planning, Università Politecnica delle Marche, 60131 Ancona, Italy

[e] Consiglio per la ricerca in Agricoltura e l'Analisi della Economia Agraria Centro di Ricerca Viticoltura ed Enologia, Gorizia, Italy

[f]Instituto de Biodiversidade Agraria e Desenvolvimento Rural, Universidad de Santiago de Compostela, 27002 Lugo, Spain

*Corresponding author: rogerborguete@gmail.com; rogerio.rafael@uem.mz

Other authors: mluisa.fernandez@usc.es; daniellejanaina76@gmail.com; s.cocco@univpm.it; m.l.ruello@univpm.it; flavio.fornasier@crea.gov.it; g.corti@univpm.it

Manuscript are in preparation for submission

Abstract

Phosphorus (P) is an essential plant nutrient that must be added as fertilizer to agricultural soils. Phosphate rock can be used as a slow release fertilizer in acid soils, contributing not only P, but also Ca and Mg. The low reactivity of most phosphate rocks compels farmers to apply high rates of ground phosphate rock to obtain yields similar to those obtained by application of water soluble P fertilizer. The use of high rates of phosphate rock may contribute environmental pollutants which could affect soil microorganisms and, alter soil fertility. Biochar has been reported to improve soil quality and biological function, ameliorating soil physical properties and increasing soil nutrient availability and enzyme activity. The aim of this study is to assess the application of phosphate rock and biochar to an acid Arenosol, the associated root-induced changes and the resulting impact on soil quality and crop yield. The experiment was run in pots, using a surface soil (0 – 0.2 m) collected from an open scrubland in Marracuene district, Mozambique. The pot trial was conducted in a completely random design, with two crops (corn and cowpea) and three treatments (i. soil only (Control); ii. soil with Phosphate rock (PR) at 30 g pot^{-1}; and iii. soil with phosphate rock at a rate of 30 g pot^{-1} and baby corn peel biochar (PR+BC) at 0.9% (w:w)). Three replicates of each treatment were performed in each of the cultures. Bulk and rhizosphere soils were sampled at the end of experiment. Soil chemical and biochemical properties as well as plant growth, yield and nutrient uptake were determined at the end of the experiment. Soil pH, available P, easily oxidizible carbon (EOOC), cation exchange capacity (CEC), exchangeable cations increased significantly ($P<0.05$) with application of PR+BC treatment compared with others. Biochar treatment failed to increase microbial biomass or soil respiration, but increased the activity of three enzymes of the carbon cycle (β-glucosidase, cellulase, xylosidase), one enzyme of the nitrogen cycle (leucine aminopeptidase) and three enzymes of the P cycle (alkaline phosphomonoesterase, phosphodiesterase, and pyrophosphatase-phosphodiesterase). Moreover, the application of biochar increased of nodulation in cowpea and crop yields. Rhizosphere soils of PR treatment showed increased acid phosphomonoesterase and xylosidase activities. Cowpea rhizosphere induced changes promoted faster mineralization of soil organic matter and N fixation as verified by higher activity of β-glucosidase and chitinase, and nodulation. The root-induced biochemical changes contributed to the improvement of soil quality and crop yields. Phosphate rock applied in combination with biochar to an acid Arenosol can be considered as a slow release fertilizer and increase nutrient recovery efficiency by plants.

Key words: enzyme activities; soil respiration; soil fertility; acid soil

1. Introduction

Phosphorus (P) is an essential plant nutrient that is scarce in most soil parent materials. Because of this, P from different sources must be added as fertilizer to agricultural soils (Holford, 1997). Phosphate rocks are a natural resource that can be used as a source of P in agriculture, particularly in acid soils, especially in countries that own these rocks. Along with P, phosphate rock can contribute to soil other nutrients such as calcium (Ca) and magnesium (Mg) (Rafael et al., *in press*). Direct application of ground phosphate rock to the soil is a good alternative for P supply in developing countries that can not afford phosphate commercial fertilizer, but instead could exploit their own rock outcrops (Sokoto and Singh, 2008; Rafael et al., *in press*).

The low reactivity of most phosphate rocks compared with inorganic P fertilizers compels farmers to apply high rates of ground phosphate rock to obtain yields similar to those obtained by using water soluble P fertilizer (Jemo et al., 2006). As a demonstration of this, Rafael et al. (*in press*) investigated in column experiments the feasibility of using phosphate rock as a fertilizer for direct application in tropical acid soils and found that high rates of ground rock are needed to provide adequate amounts of available P to soil. Despite improving crop yields, the application of high rates of phosphate rock may add contaminants on the soil and affect the soil quality in the medium to long-term (Zapata and Roy, 2004). These contaminants could also affect soil microorganisms and therefore alter soil fertility and sustainable agricultural productivity (Olaniran et al., 2013). Since microorganisms are key in maintaining soil quality and driving processes such as organic matter (SOM) decomposition, nutrient cycling, and plant productivity (Lone et al., 2015), it is important to evaluate the influence of ground phosphate rock on soil biological properties.

Biochar, a solid material obtained from thermochemical conversion of biomass in an oxygen-limited environment, has been reported to improve soil quality and biological functions, while enhancing C sequestration (Lone et al., 2015). Biochar can provide a favourable habitat to microorganisms, and this explains the higher microbial biomass found in biochar amended soils (Lehmann et al., 2011; Lone et al., 2015; Demisie et al., 2014). Lehman et al. (2011) and Lu et al. (2015) reported that biochar pH could affect total microbial abundance. However, Quilliam et al. (2013a) argued that biochar does not provide a significant habitat to soil microorganisms, but instead changes in soil physico-chemical properties and enriches the soil surrounding biochar of metabolically available labile compounds that significantly alter microbial structure and activity, and consequently, affect soil-plant-microorganism interactions. Similarly, Ameloot et al. (2014) reported

lower soil microbial activity, abundance and basal cumulative respiration in biochar amended soils. Castaldi et al. (2011) found minimal impact of biochar on soil microbial parameters.

Soil enzyme activity has been used as indicator of biological equilibrium and soil quality (Gil-Sotres et al., 2015; Shukla and Varma, 2011). Increased activity of soil β-glucosidase and dehydrogenase as a result of biochar application has been reported (Pandey et al., 2016). Quilliam et al. (2013b) reported an increase of nitrogenase activity in biochar amended soils. In contrast, Ameloot et al. (2014) and Elzobair et al. (2016a,b) reported a decrease of potential β-glucosidase and leucine-aminopeptidase activities, while phosphatase, xylosidase and glucosaminidase were not affected by biochar amendment. The effect of biochar was dependent on the type of enzyme as well as on the type of soil and biochar (Kelly et al., 2015).

Moreover, how does the crop rhizosphere respond to these changes? In the rhizosphere a competition for nutrients between plants and microorganisms occurs, with the latter being more competitive due their higher ability to decompose plant derived chelators and their proximity to the root surface (Marschner et al., 2011). But the competitiveness of microorganism depends strongly on C availability (Marschner et al., 2001). The type and quantity of C available in different root zones will determine the microbial growth and rhizosphere community structures (Yang and Crowley, 2000; Marschner et al., 2001). Plant species, root zone and soil type affects soil microorganisms structure, and in sandy soil the root zone is most relevant (Marschner et al., 2001). Microorganism community in the rhizosphere can be affected by changes in root exudates composition caused by changes in plant nutritional status (Yang and Crowley, 2000).

Various legumes, such as cowpea, are able to access non-labile P under P deficient conditions (Pypers et al., 2006). Exudation of organic anions or phosphatases by legumes and pH changes in the rhizosphere are responsible for P acquisition from sparingly soluble P sources such as phosphate rocks (Jemo et al., 2006). Nitrogen acquisition can be enhanced significantly by symbiotic N_2 fixation by legumes (Lambers et al., 2009). Release of organic compounds from root epidermal and cortical cells fosters the proliferation of microorganisms inside, on the surface and outside the roots (Lambers et al., 2009). Rhizodeposition promotes different chemical, physical, and biological characteristics in the rhizosphere compared with the bulk soil (Lambers et al., 2009; Bakker et al., 2015). The rhizosphere processes depend on soil type and plant species (Erel et al., 2017).

Research assessing biochar effect on soil enzymes and soil microorganism are inconsistent. Furthermore, to our knowledge there is no research appraising the effects of biochar combined with

phosphate rock in tropical soils. In a previous paper (Rafael et al., *in press*) we investigated the feasibility of using phosphate rock as a fertilizer for direct application in tropical acid soils using column experiments. We found that high rates of the tested phosphate rock are needed to provide adequate amounts of available P to soil. The application of such high rates of phosphate rock might have adverse effects on the soil and the environment (Zapata and Roy, 2004).

The aim of this study was to investigate soil physic-chemical, biochemical and biological response to the use of phosphate rock and biochar for crop growth purposes. Specifically, we aim to assess the contribution of pH and root-induced changes mediated by phosphate rock and biochar to modify soil physicochemical, biochemical and biological properties. We used an acid Arenosol as substrate for corn and cowpea growth. Acidity and nutrient (particularly P) deficiencies are among the major constraints to crop production in this soil. We hypothesized that, crop growth being meagre in this soil, the application of phosphate rock and biochar would benefit soil quality, while biochar would reduce the possible environmental negative impact of a high dose of phosphate rock and increase crop yield. We also hypothesized that the response of soil enzyme activities, respiration, and microbial C will be crop specific and that the rhizosphere processes would affect the enzyme activity patterns.

2. Materials and methods

2.1. Soil sampling and inputs preparation

An acid Arenosol, classified as albic Arenosol (IUSS, 2015), was used for the pot experiments. The soil was collected from the A horizon (0-0.2 m) of an open scrubland located in southern Mozambique (25.72577º S, 032.64835º E). Prior to use, the soil was air-dried and sieved to remove gravels and roots ≥ 2 mm size. Babycorn (*Zea mays* L.) peel was used as feedstock for biochar production using a low-cost home-made pyrolyser made of two concentric cylinders. The pyrolysable material was placed in the inner cylinder, while firewood was burnt in the outer cylinder providing energy to heat the inner cylinder. The pyrolysable material was heated for 4 h and the temperature reached 450ºC. After the combustion stopped, the biochar was washed twice with distilled water to eliminate excess ash, then oven dried for 48 h at 60ºC. The biochar was then ground using a mortar to obtain particles from 0.063 to 0.25 mm.

The phosphate rock was collected from the Evate district (Nampula Province, Mozambique). Blocks of rocks were fragmented by using a grinding press, and the fragments were then ground using

an agate mortar. Rock powder was reduced to particle-size fraction of 0.063-0.25 mm. The dimension of the fraction was obtained as per Rafael et al. (*in press*). A nutrient solution adapted from Vanek and Lehmann (2014) [1 mmol L^{-1} $MgSO_4$, 25 µmol L^{-1} H_3BO_3, 2 µmol L^{-1} $MnSO_4$, 2 µmol L^{-1} $ZnSO_4$, 0.5 µmol L^{-1} $CuSO_4$, 0.5 µmol L^{-1} $(NH_4)_6Mo_7O_{24}$] was used to replenish other essential nutrients.

2.2. Pot experiments

In the facility of the Faculty of Agronomy and Forest Engineering (Eduardo Mondlane University, Maputo, Mozambique), the pot trial was designed as a completely random test with two crops (corn and cowpea) and three treatments: 1) soil only (Control); 2) soil with phosphate rock (PR); 3) soil with PR and biochar (PR+BC). For each treatment tree replications were applied. The pots were 17-L plastic buckets, in the bottom of which small holes 10 mm in diameter were drilled to avoid stagnant water. Each pot was prepared with a soil layer of 6 kg on the bottom, and then a layer of 4 kg soil homogenized with the inputs so to make a 15 cm thickness with an approximate bulk density of 1.5 kg dm^{-3}. Biochar was applied at 0.9% (w:w), equivalent to 27 ton ha^{-1} (for a soil depth of 0.2 m with a bulk density of 1.5 kg dm^{-3}). Phosphate rock was applied at 30 g pot^{-1} to provide 249 mg pot^{-1} of available P. These amounts were equivalent to about 22.5 ton ha^{-1} of phosphate rock and 187 kg ha^{-1} of available P (20 cm soil depth, bulk density of 1.5 kg dm^{-3}) predicted by neutral ammonium citrate extraction (Rafael et al., *in press*).

The soil in the pots was pre-incubated for 10 d to allow an initial dissolution of phosphate powder, and to avoid possible negative effects on plant growth due to biochar volatile substances (Vanek and Lehmann, 2014, Dutta et al., 2017). During the pre-incubation, each pot was irrigated three times per week with 500 mL of distilled water. After this period, three corn (*Zea mays* L., MRI594 - Hybrid white early maturity cultivar) or cowpea (*Vigna unguiculata* (L.) Walp, IT98K-1105-5 early maturity cultivar) seeds per pot were sown for each experiment (on February 26th, 2016), but only one emerged plant per pot was left. During the growing period (90 and 60 d for corn and cowpea, respectively) the soil temperature ranged from 34.1 ± 5.1 to 32.1 ± 3.8 ºC, and all the pots were watered with 500 mL of distilled water once per week, and fertirrigated every two d with 300 mL of nutrient solution (NS). Fertirrigation was made during the first 70 and 40 d (for corn and cowpea, respectively), while watering lasted during growing period. The watering frequency was increased to three times per week due the high water demand of corn and cowpea plants in vegetative stage, starting from 6 and 4 weeks (for corn and cowpea, respectively) after the emergence.

Pest Control was made once per week by alternating fungicide Amistar Top (active substances: azoxystrobin and difenoconazole) + insecticide/acaricide Agromectin 1.8 EC (active substance: abamectin) with fungicide Ridomil Gold (active susbstances: metalaxyl + mancozeb) + insecticide/acaricide HITCEL 44% EC (active substances: profenofos + cypermethrin). Plants were harvested at 90 and 60 d after emergence (for corn and cowpea, respectively) when they were fruiting. Dry (60 ^{o}C) total biomass and shoot weight were determined. Then, soil samples were collected separating rhizosphere and bulk soil, considering as rhizosphere the soil adhering to the roots (Cocco et al., 2013).

2.3. Physic-chemical properties of soil, plant, and biochar

Prior to the experiments, the soil particle-size distribution was determined by the pipette method (Day, 1965) after the soil was disaggregated in distilled water for 24 h. Soil (1:2.5 w:v) and biochar (1:100 w:v) pH were determined potentiometrically in water and KCl (1 M solution) suspensions by a combined glass-calomel electrode. For biochar samples, the suspensions in water or KCl were heated in a water bath to 90 °C, stirred for 20 min to allow dissolution of the soluble components, and cooled to room temperature before pH determination (Ahmedna et al., 1997).

Total carbon (TC), total nitrogen (TN) and total sulphur (TS) in soil, biochar, and plant samples were determined using a CHNS-O analyser (EA1110, Carlo Erba Instruments, Milan, Italy). The total contents of P, Ca, Mg, K, Na, Fe, Mn and Cu in plants and biochar were determined by dry ashing 0.5 g plant or biochar at 500 ^{o}C for 16 h in a muffle furnace and dissolving the ash in a 5 M HCl solution (Lambert, 1976). A simple colorimetric method based on ascorbic acid reduction of the ammonium phosphomolybdate complex was used to measure P in the solutions (Kuo, 1996). The concentrations of Ca, Mg, K, Na, Fe, Mn, and Cu were measured by inductively coupled plasma optical emission spectrometry (ICP-OES) a Perkin Elmer Optima 8300 spectrometer as described by Boss and Fredeen (1997).

The Walkley-Black (Nelson and Sommers, 1996) method was used to estimate easily oxidisable organic C (EOOC) in soil and biochar (Pansu and Gautheyrou, 2006). The Mehlich 3 reagent (Mehlich, 1984) was used to extract available P from soil samples. Extractable P in biochar was determined by water, 2% citric acid, and 2% formic acid extractants using a suspension of 0.1 g of sample and 30 mL of extractant, shaken at 120 rpm for 48 h in 50 mL centrifuge tubes. The suspension was then centrifuged (~ 2700 *g*, 15 min) and the supernatant filtered using Whatman No. 42 filter paper prior to colorimetric determination (Zhang et al., 2016). Cation exchange capacity (CEC) and

exchangeable cations in soil and biochar were determined by the $BaCl_2$ method (Gillman and Sumpter, 1986).

2.4. Soil biochemical and biological properties

Potential enzyme activities in soil were determined as per Fornasier and Margon (2007). Enzymes were desorbed by heteromolecular exchange using an excess of exogenous protein. Thus, 250 mg soil samples were placed in 2-mL Eppendorf tubes with glass beads and 1 mL of 50 mM tris–HCl buffer solution at pH 7.5, containing lysozyme as the desorbing protein. The tube was subjected to bead-beating (3 min, 30 strokes s^{-1}) using a Retsch MM400 mill, then centrifuged for 5 min at 20,000 *g*. The enzyme activity was assayed fluorometrically in microplates using 4-methyl-umbelliferyl and L-Leucine-7-amino-4-methylcoumarine derivatives. The activities of arylsulfatase, chitinase, glucuronidase, α-glucosidase, β-glucosidase, cellulase, xylosidase, acid phosphomonoesterase, phosphodiesterase, pyrophosphatase-phosphodiesterase, and alkaline phosphomonoesterase were determined in 200 mM morpholineethanesulfonic acid solution at pH 6, and the activity of leucine aminopeptidase (leu) in 50 mM tris–HCl buffer solution at pH 7.5.

Double-strand DNA (dsDNA) was determined on 500 mg soil aliquots that were placed in 2-mL Eppendorf tubes and added of glass beads and 1 mL of 0.12 M sodium phosphate buffer (pH 8.0) solution. The tubes were submitted to bead-beating (2 min, 30 strokes s^{-1}) and then centrifuged for 5 min at 20,000 *g*. The dsDNA content was quantified on aliquots of the supernatant by fluorometry using PicoGreen1 reagent (Life Technologies) according to the instructions of the manufacturer. Root nodules visible at naked eye were counted per each plant.

Soil basal respiration was determined at the end of the experiment by static incubation of 50 g of soil samples of each treatment (made in duplicate) at 60% humidity with 10 mL of 1M NaOH inside 2-L jars at 25ºC as described by Vance et al. (1987). The CO_2 concentration produced was measured by titration with 0.5M HCl at the following times (h): 24, 48, 72, 96, 120, 168, 240, 336, 432, 528. Soil microbial biomass was determined at the end of the experiment by the fumigation-extraction (Vance et al., 1987). The quantification of the extracted C was made by the Walkley-Black method.

2.5. Calculation of phosphorus use efficiency

Nutrients taken up (mg pot^{-1}) by roots, shoots (stem + leaves), and cobs or pods were calculated by multiplying their nutrient concentration (mg kg^{-1}) by their weight. Phosphorus harvest index (PHI) was calculated as follows (Agegnehu et al., 2016):

$$PHI\ (\%) = \frac{GPU}{TPU} \times 100$$

where: GPU is P in the cobs or pods (mg pot^{-1}), and TPU is total P in the plant (cob or pod + stem + leaves) (mg pot^{-1}).

Fertilizer P use efficiency was expressed by the following components:

i) Agronomic efficiency (AE) is defined as the additional grain production per unit of P applied, and was calculated as:

$$AE\ (g\ g^{-1}) = \frac{GY_{f-}\ GY_u}{P_a}$$

where: GY_f is the cob (or pod) yield of the fertilized pot (g), GYu is the cob (or pod) yield of the not fertilized pot (g), and P_a is the quantity of P applied as biochar and/or P fertilizer (g) (Agegnehu et al., 2016).

ii) Apparent P recovery efficiency (ARE), or P uptake efficiency, was calculated as:

$$ARE\ (\%) = \frac{P_f - P_u}{P_a} \times 100$$

where: P_f is the P in cob (or pod) + stem + leaves of the fertilized pot (g), P_u is the P in cob (or pod) + stem + leaves of the not fertilized pot (g), and P_a is the quantity of P applied (g) (Agegnehu et al., 2016).

iii) Physiological efficiency (PE), which expresses the ability of the plant to transform P absorbed from fertilizers into grain yield, was calculated as:

$$PE\ (gg^{-1}) = \frac{GY_f - GY_u}{P_f - P_u}$$

where: GY_f is the cob (or pod) yield of the fertilized pot (g), GYu is the cob (or pod) yield of the unfertilized pot (g), P_f is the P in cob (or pod) + shoot of the fertilized pot (g), P_u is the P in cob (or pod) + shoot of the unfertilized pot (g) (Agegnehu et al., 2016).

2.6. Statistical analysis

R version 3.1.2 (2014-10-31) was used for statistical analysis. The experimental data were analysed by analysis of variance (ANOVA), after a *boxcox* transformation (Meloun et al., 2005) of the data to perform parametric tests (Shapiro-Wilk normality test and Bartlett test of homogeneity of

variances) when necessary. A multiple comparison Duncan test (at 95% significance level) was used to compare the means. A principal component analysis (PCA) was performed to identify the variables capable of explaining most of the variability and correlations among potential enzyme activities, chemical parameters and cowpea or corn yields determined as response to treatments and rhizosphere soil processes.

3. Results

3.1. Characteristics of soil and inputs used in the experiments

The soil used in the experiment was sandy, strongly acid, and low in organic carbon, available nutrients and cation exchange capacity (CEC). The deficiency of nutrients and the very low CEC and water retention capacity are the main cause of the infertility of such soils. The phosphate rock showed alkaline pH and contained high amounts of P (Table 1). The babycorn peel biochar displayed high base saturation, strongly alkaline pH, and contained considerable amounts of total organic C, N, S, total P, extractable P (water – P, 2% citric acid P, and 2% formic acid P), exchangeable K, and CEC.

3.2. Soil chemical properties at the end of the experiment

The treatments significantly affected pH in H_2O and KCl, EOOC, available P, CEC (only for cowpea), exchangeable Ca, Mg, K, and Al (only for corn), and base saturation (Table 2). On the contrary, the TOC and exchangeable Na were not affected. Soil pH, EOOC, available P, CEC, exchangeable Ca, Mg, K, and base saturation were higher and exchangeable Al was lower in PR+BC treatment than in the Control. In contrast, PR alone did not produce any significant effect on the soil pH, EOOC, and exchangeable Mg and K, but increased Mehlich-3 P and exchangeable Ca. PR+BC treatment supplied 20 and 44% more available P than PR treatment in corn and cowpea, respectively, as well as 40 and 100% more exchangeable Ca respectively for corn and cowpea. Biochar appeared to contribute significantly for both CEC and available P.

Table 1
Physic-bio-chemical properties of acid Arenosol from Mozambique, phosphate rock, and biochar used in the pot experiment.

Parameters	Soil before the trial	Phosphate rock[a)]	Babycorn peel biochar
pH_{H2O}	4.3 (0.1)[b)]	8.2 (0.1)	9.6 (0.1)
pH_{KCl}	4.3 (0.0)	6.5 (0.1)	8.9 (0.1)
Sand (g kg^{-1})	937 (2)	-	-
Silt (g kg^{-1})	47 (5)	-	-
Clay (g kg^{-1})	16 (3)	-	-
EC (dS m^{-1})	0.0 (-)	0.2 (0.0)	0.9 (0.0)
TOC (g kg^{-1})	22.4 (0.5)	-	579.4 (59.3)
EOOC (g kg^{-1})	3.1 (0.5)	-	289.7 (15.6)
TN (g kg^{-1})	< dl	-	27.1 (0.6)
TS (g kg^{-1})	< dl	-	53.9 (4.0)
TP (g kg^{-1})	-	239 (0.6)	7.9 (0.6)
Total Ca (g kg^{-1})	-	363 (3)	5.1 (0.2)
Total Mg (g kg^{-1})	-	<dl	6.6 (0.2)
Water-P (g kg^{-1})	-	1.4 (0.1)	5.4 (0.2)
2% citric-P (g kg^{-1})	-	87. 4 (0.2)	2.5 (0.1)
2% formic-P (g kg^{-1})	-	133. 4 (7.7)	8.0 (0.2)
Mehlich 3-P (mg kg^{-1})	6.6 (0.5)	-	-
Exchangeble Ca ($cmol_c$ kg^{-1})	0.1 (0.0)	3.5 (0.5)	2.8 (0.1)
Exchangeble Mg ($cmol_c$ kg^{-1})	0.1 (0.0)	1.8 (0.1)	4.7 (0.2)
Exchangeble K ($cmol_c$ kg^{-1})	0.1 (0.0)	1.1 (0.1)	170.2 (10.0)
Exchangeble Na ($cmol_c$ kg^{-1})	0.0 (0.0)	9.2 (2.1)	1.4 (0.1)
Exchangeble Al ($cmol_c$ kg^{-1})	0.3 (0.0)	-	0.0 (-)
CEC ($cmol_c$ kg^{-1})	0.5 (0.1)	-	179.0 (10.3)
Base saturation (%)	40.8 (6.3)	-	100 (0)
AcP (nmol g^{-1} h^{-1})	16.2 (1.4)	-	-
AlkP (nmol g^{-1} h^{-1})	4.5 (0.2)	-	-
Cell (nm $g^{-1}h^{-1}$)	0.2 (0.1)	-	-
α-G (nmol g^{-1} h^{-1})	0.5 (0.1)	-	-
AryS (nmol g^{-1} h^{-1})	0.9 (0.1)	-	-
β-G, (nmol g^{-1} h^{-1})	4.8 (0.3)	-	-
BisP (nmol g^{-1} h^{-1})	1.5 (0.1)	-	-
Chit (nmol g^{-1} h^{-1})	2.4 (0.2)	-	-
Leu (nmol g^{-1} h^{-1})	13.5 (0.8)	-	-
Nona (nmol g^{-1} h^{-1})	28.9 (2.1)	-	-
Pyro (nmol g^{-1} h^{-1})	0.3 (0.1)	-	-
Uroni (nmol g^{-1} h^{-1})	-	-	-
Xylo (nmol g^{-1} h^{-1})	1.2 (0.1)	-	-
dsDNA (µg g^{-1})	< dl	-	-

[a)] The Ca, Mg, K, Na, Al ($cmol_c$ kg^{-1}) in phosphate rock are cations extractable by $BaCl_2$. [b)]Numbers in parentheses are the standard errors (n=3). < dl: below the detection limit. EC: electrical conductivity; TOC: total organic carbon; EOOC: easily oxidizable organic carbon; TN: total nitrogen; TS: total sulphur; TP: total phosphorus; Water-P: phosphorus extracted by distilled water; 2% citric-P: phosphorus extracted by 2% citric acid; 2% formic-P: phosphorus extracted by 2% formic acid; Mehlich 3-P: phosphorus extracted by Mehlich 3 procedure; CEC: cation exchange capacity; AcP: Acid phosphomonoesterase; AlkP: Alkaline phosphomonoesterase; cell: Cellulase; α-G: α-Glucosidase; AryS: Arylsulfatase; β-G: β-Glucosidase; BisP: Phosphodiesterase; Chit: Chitinase; Leu: Leucine aminopeptidase; Nona: Nonanoate esterase; PyroP: Pyrophosphate-phosphodiesterase; Uroni: Glucuronidase; Xylo: Xyloxidase; dsDNA: double-strand DNA

No significant interaction was found between treatments and soil (bulk and rhizosphere) and crops (corn and cowpea) on soil chemical properties. Significant interaction between treatments and soil compartment was only found for exchangeable K, in which rhizosphere soil of PR+BC treatment had high concentration of exchangeable K (Fig. S1 of supplementary material). Also, there was significant interaction between crops and soil compartment, and between treatments and crops on soil pH (H_2O and KCl). Soil pH (H_2O and KCl) in cowpea bulk and corn rhizosphere soils was higher than corn bulk soil (Fig. S2 of supplementary material). Treatment PR in both crops and corn Control presented lower pH_{H2O}, while corn PR+BC treatment had higher pH_{KCl}, followed by cowpea PR+BC treatment.

3.3. Soil biochemical and biological properties at the end of the experiment

Significant ($P < 0.05$) interaction was found between the treatments and soil (bulk and rhizosphere) for acid phosphomonoesterase, alkaline phosphomonoesterase, phosphodiesterase, β-glucosidase, cellulase, leucine-aminopeptidase, pyrophosphatase-phosphodiesterase and xylosidase activities (Fig. 1). Although differences were not always significant, most enzyme activities were higher in rhizosphere than in bulk soil in Control and PR treatment. On the contrary, in treatment PR+BC most enzyme activities were higher in bulk soil than in rhizosphere.

In the rhizosphere soil, treatment PR resulted in the highest activities of acid phosphomonoesterase and xylosidase, while treatment PR+BC resulted in the highest activity of leucine aminopeptidase and the lowest activity of acid phosphomonoesterase; no effect of treatment was observed for the other determined enzyme activities. In the bulk soil, treatment PR+BC resulted in the highest activities of alkaline phosphomonoesterase, phosphodiesterase, β-glucosidase, cellulase, leucine aminopeptidase, pyrophosphatase-phosphodiesterase and xylosidase; no effect of treatment was observed for acid phosphomonoesterase activity. For each type of enzyme, there was not significant difference between bulk soils of treatments Control and PR.

Table 2
Treatments effect on soil (bulk and rhizosphere) chemical properties after the pot experiments with acid Arenosol from Mozambique. Means followed by different lowercase letters differed significantly among treatments at $P < 0.05$ by Duncan multiple mean comparison test.

Chemical parameters	Corn				Cowpea			
	Treatments			ANOVA	Treatments			ANOVA
	Control	PR	PR+BC		Control	PR	PR+BC	
pH_{H2O}	4.56 (0.13) [a)] b	4.43 (0.17) b	5.33 (0.11) a	**	5.09 (0.12) a	4.55 (0.05) b	5.00 (0.11) a	**
pH_{KCl}	4.04 (0.06) b	4.03 (0.04) b	4.49 (0.04) a	***	4.22 (0.02) b	4.08 (0.02) c	4.36 (0.04) a	***
TOC, g kg^{-1}	18.09 (1.14)	20.40 (0.66)	21.15 (0.99)	NS	18.86 (0.35)	18.90 (1.00)	21.12 (0.23)	NS
EOOC, g kg^{-1}	4.11 (0.36) b	4.01 (0.48) b	7.19 (0.44) a	***	3.99 (0.37) b	4.23 (0.37) b	6.67 (0.53) a	**
Mehlich 3 - P, mg kg^{-1}	4.15 (1.15) c	76.31 (2.37) b	90.72 (2.60) a	***	3.98 (0.99) c	79.28 (5.33) b	112.36 (4.19) a	***
CEC, $cmol_c$ kg^{-1}	0.43 (0.06)	0.49 (0.07)	0.66 (0.10)	NS	0.36 (0.08) b	0.52 (0.08) ab	0.75 (0.14) a	*
Exchange Ca, $cmol_c$ kg^{-1}	0.05 (0.01) b	0.10 (0.01) a	0.12 (0.01) a	***	0.04 (0.00) c	0.08 (0.01) b	0.12 (0.01) a	**
Exchange Mg, $cmol_c$ kg^{-1}	0.05 (0.00) b	0.05 (0.00) b	0.12 (0.01) a	***	0.05 (0.01) b	0.05 (0.01) b	0.15 (0.03) a	***
Exchange K, $cmol_c$ kg^{-1}	0.18 (0.03) b	0.15 (0.03) b	0.33 (0.07) a	*	0.16 (0.04) b	0.20 (0.04) b	0.39 (0.09) a	*
Exchange Na, $cmol_c$ kg^{-1}	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	NS	0.00 (0.00)	0.01 (0.00)	0.01 (0.01)	NS
Exchange Al, $cmol_c$ kg^{-1}	0.16 (0.02) a	0.19 (0.02) a	0.07 (0.01) b	**	0.12 (0.03)	0.19 (0.03)	0.09 (0.02)	NS
Base saturation, %	62.45 (1.78) b	60.47 (1.35) b	88.54 (0.39) a	***	67.96 (2.01) b	63.23 (2.12) c	87.09 (2.77) a	***

NS, *, **, *** Not significant, significant at $P = 0.05$, $P = 0.01$ and $P = 0.001$, respectively. [a)]Numbers in parentheses are the standard errors (n=6). TOC: total organic carbon; EOOC: easily oxidizable organic carbon; Water-P: water-extractable phosphorus; Mehlich 3-P: available phosphorus extracted by Mehlich 3 method; CEC: cation exchange capacity

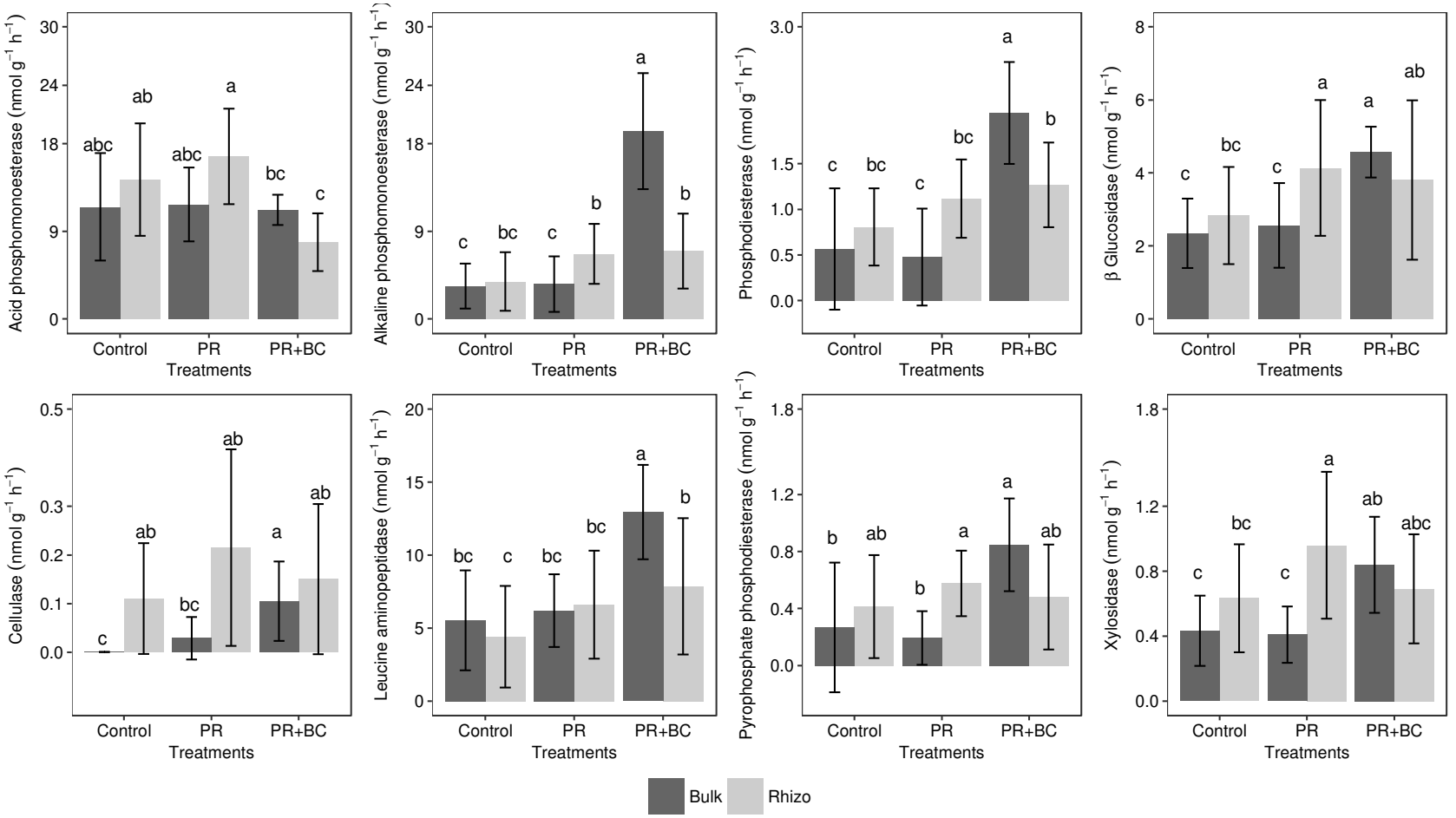

Fig. 1 Effect of interaction between treatments (Control, PR, and PR+BC) and soil compartiments (bulk and rhizosphere) on soil acid phosphomonoesterase (P = 0.05), alkaline phosphomonoesterase (P = 0.001), phosphodiesterase (P = 0.001), β-glucosidase (P = 0.05), cellulase (P = 0.05), leucine aminopeptidase (P = 0.05), pyrophosphate phosphodiesterase (P=0.05), and xylosidase (P=0.01) activities after the pot experiment with acid Arenosol from Mozambique in both corn and cowpea. Means followed by different lowercase letters differed significantly among treatments at $P < 0.05$ by Duncan multiple mean comparison test. The whiskers indicate the error bars (n=6).

Cowpea always presented higher ($P < 0.05$) activity of all enzymes (excluding Glucuronidase) than corn (Table S1 of supplementary material). In addition, significant ($P < 0.05$) interaction was found between crop type (corn and cowpea) and soil (bulk and rhizosphere) in β-glucosidase and chitinase (Fig. 2). The activities of β-glucosidase and chitinase were higher in rhizosphere than in bulk soil for cowpea, while in the case of corn there was no difference between rhizosphere and bulk soil. No significant impact of the treatments was found on double-strand DNA, soil microbial biomass (excluding corn), and soil basal respiration in 528 h (Table 3).

For corn, treatment PR presented lower soil microbial biomass than Control. In turn, cowpea nodulation in treatment PR+BC showed an increase of about 10 and 4 fold compared to the Control and PR treatments, respectively. Along the period of respiration measurement, for both crops soil basal respiration showed a dramatic increase from 24 to 168 h, thereafter started to increase slowly until 528 h (Fig. 3). There was not significant influence of treatment or soil (bulk and rhizosphere) for corn on soil basal respiration, while a significant ($P < 0.05$) influence of both factors was found for cowpea. For corn, the curves for different treatments overlapped during the whole period and the same did the curves for both soil compartments (Fig. 3).

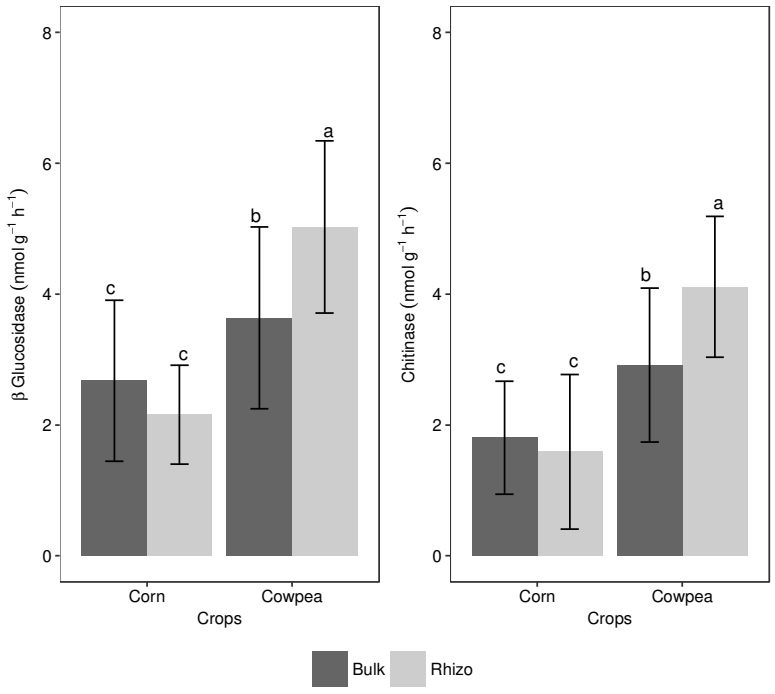

Fig. 2 Effect of interaction between crops (corn and cowpea) and soil compartment (bulk and rhizosphere) on soil β-glucosidase ($P = 0.01$) and chitinase ($P = 0.05$) activities after the pot experiment with acid Arenosol from Mozambique. Means followed by different lowercase letters differed significantly among treatments at $P < 0.05$ by Duncan multiple mean comparison test. The whiskers indicate the error bars (n=6).

Table 3
Treatments effect on soil (bulk and rhizosphere) biological properties after the pot experiment with acid Arenosol from Mozambique. Means followed by different lowercase letters differed significantly among treatments at $P < 0.05$ by Duncan multiple mean comparison test.

Biological parameters	Corn				Cowpea			
	Treatments			ANOVA[a)]	Treatments			ANOVA
	Control	PR	PR+BC		Control	PR	PR+BC	
dsDNA, µg g^{-1}	1.6 (0.2)[a)]	2.2 (0.2)	1.3 (0.3)	NS	2.5 (0.1)	3.3 (0.3)	3.2 (0.3)	NS
SMB, mg C kg^{-1}	17.1 (3.7) a	5.8 (1.0) b	10.0 (2.8) ab	*	6.7 (1.8)	3.3 (1.0)	7.5 (1.9)	NS
SR, mg CO_2 kg^{-1}	341.5 (37.8)	357.5 (17.6)	375.2 (26.2)	NS	343.6 (43.2)	390.2 (27.5)	337.5 (25.2)	NS
Nod. per plant[b)]	-	-	-	-	19.3 (3.7) b	50.0 (17.1) b	189.0 (40.7) a	**

NS, *, ** Not significant, significant at $P = 0.05$ and $P = 0.01$, respectively. [a)]Numbers in parentheses are the standard errors (n=6). [b)]Nod. - Nodules (Means of three repetitions); dsDNA - Double-stand DNA; SMB - Soil microbial biomass; SR - Soil respiration in 528 h;

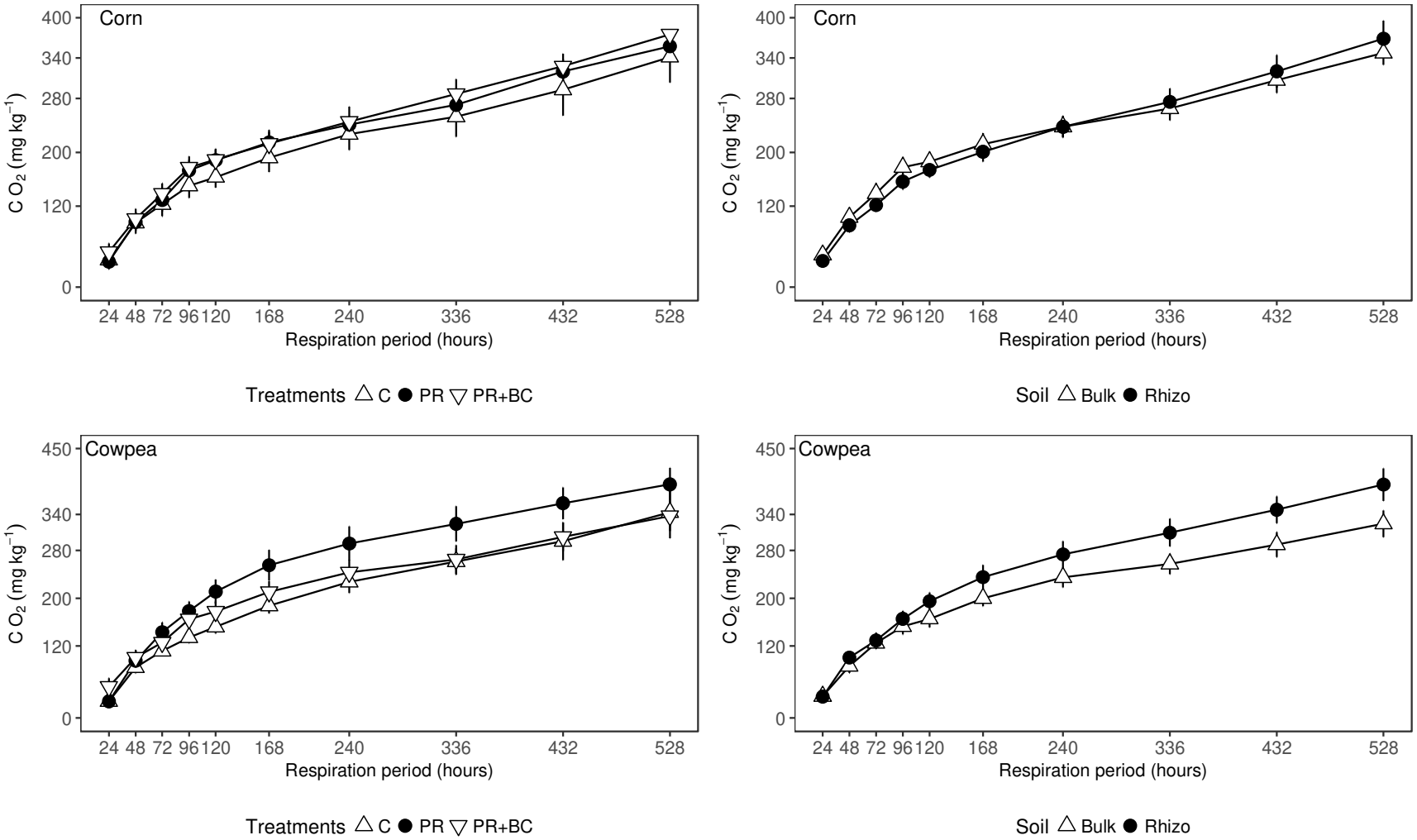

Fig. 3 Soil basal respiration (mg CO_2 kg^{-1}) in corn and cowpea as affected by treatment and soil compartment during the respiration period of 528 h at end of the pot experiment with acid Arenosol from Mozambique. The whiskers indicate the error bars (n=3).

In contrast, the cowpea soil basal respiration curves apparently showed higher CO_2 release for PR treatment and rhizosphere soil from 168 to 528 h. Moreover, significant interaction between both factors was observed in cowpea, the soil basal respiration being higher for rhizosphere than bulk soil in treatments Control and PR, but not significantly different in PR+BC treatment (Fig. 4). Principal component analysis (PCA) was run to assess the variation of soil chemical and biochemical properties as affected by soil compartment (rhizosphere and bulk), treatment (Control, PR, and PR+BC), and plant species (corn and cowpea). PC1 and PC2 explained 41.2 and 19.4% of the variation, respectively (Fig. 5).

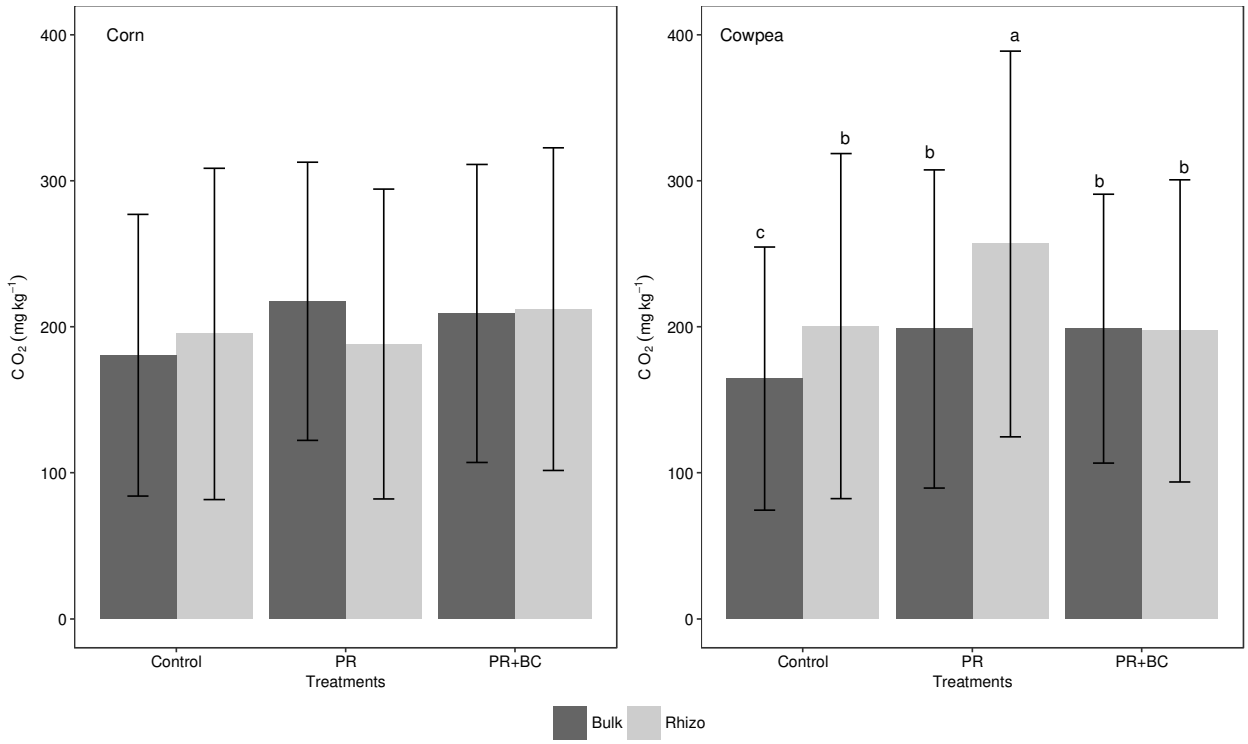

Fig. 4 Effect of interaction between treatments (Control, PR, and PR+BC) and soil compartments (bulk and rhizosphere) on soil basal respiration (mg CO_2 kg^{-1}) after 528 h in corn and cowpea at the end of the pot experiment with acid Arenosol from Mozambique. Means followed by different lowercase letters differed significantly among treatments at $P < 0.05$ by Duncan multiple mean comparison test. The whiskers indicate the error bars (n=3).

Several soil chemical and biochemical properties parameters showed positive correlation with PC1, contributing ~ 61.9% of the variability (Table S1 of Supplementary materials). These parameters were grouped into chemical (Available P) and biochemical (arylsulfatase, α-glucosidase, β-glucosidase, glucuronidase, chitinase, leucine aminopeptidase, pyrophosphate phosphodiesterase, phosphodiesterase and alkaline phosphomonoesterase activities). Conversely, parameters such as pH_{H2O}, pH_{KCl}, TOC, EOOC, exchangeable Ca, Mg, K, and Al, CEC, base saturation, cellulase, xylosidase, acid phosphomonoesterase, and nonanoate esterase activities showed positive correlation

with PC2 contributing ~ 87.4% of the variability (Table S2 of Supplementary materials). The PCA biplot grouped the samples according to treatment and plant species, but rhizosphere and bulk soil samples overlap almost completely (Fig. 5a, c, and d).

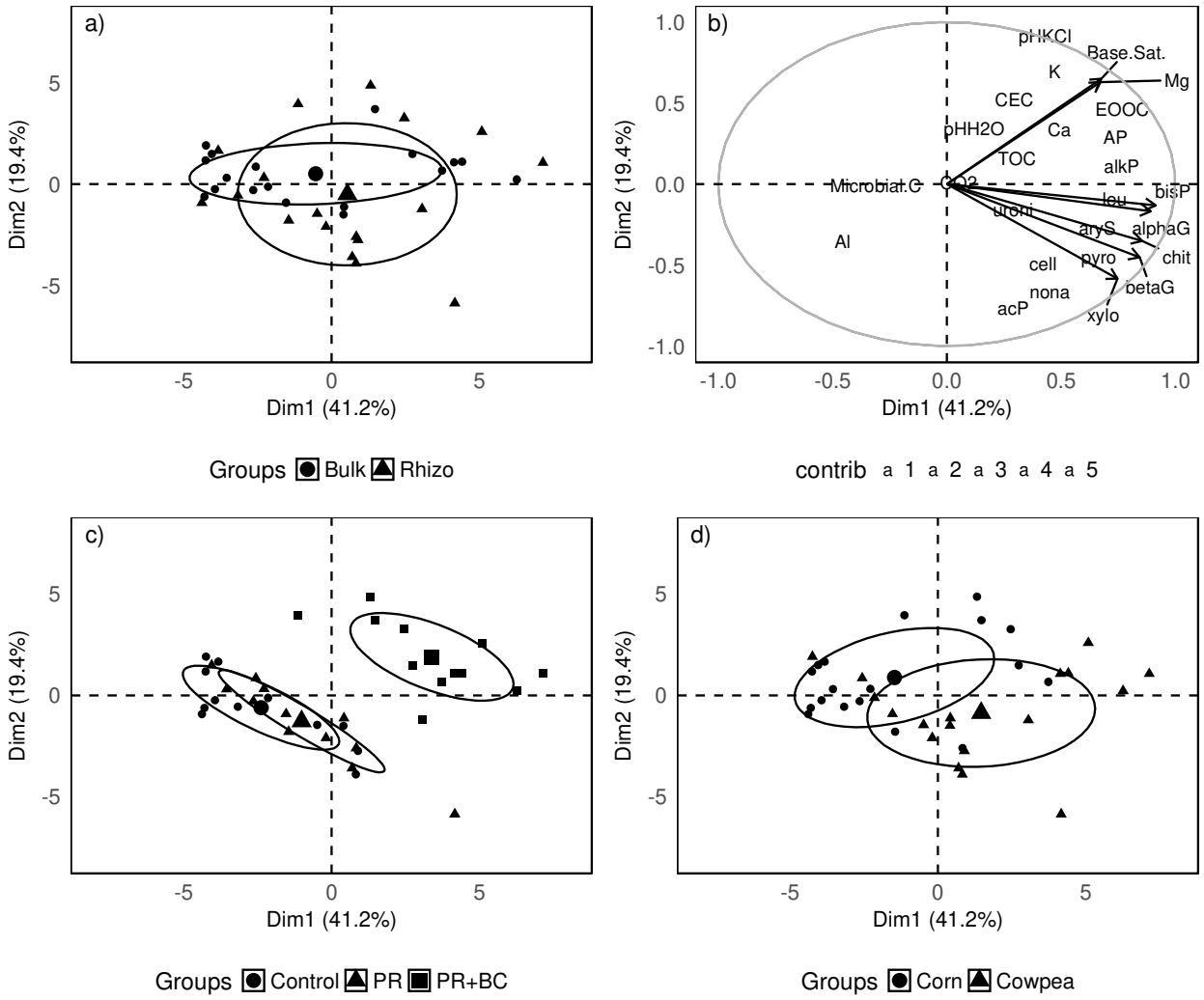

Fig. 5 Ordination plots (principal component analysis: Dim1 = PC1 and Dim2 =PC2) of chemical and biochemical properties after the pot experiment with acid Arenosol from Mozambique. (*a)* the circles surround samples from bulk and rhizosphere soils; *b)* graph of contribution (%) of variables to PCA; *c)* the circles surround samples from treatments Control, PR and PR+BC; and *d)* the circles surround samples from corn and cowpea). The values in the axis legend (graphs *a)*, *b), c)* and *d))* refer to % of total variance explained by the axis component. Individuals (symbols) close to each other have similar properties, whereas individuals far apart differ strongly in the chemical and biochemical properties. The main effect of each group (graphs a, c, and d) are represented by bigger symbols.

3.4. Crops growth and yield

Both PR and PR+BC treatments significantly improved corn and cowpea growth and yield parameters compared to Control (Table 4). It is worth noting that Control treatment in corn failed to produce cobs, while in cowpea only one out of three Control replicates produced pods. In general, the treatment PR+BC showed higher dry biomass, root, stem, leaves, and fruit (cobs or pods), plant height, and stem diameter than the Control for both corn and cowpea, but also higher dry biomass, root, stem, leaves and fruit (except in corn) yield than PR treatment. Application of PR+BC increased production of dry biomass by roughly 10 and 13 times compared to the Control, and ~ 90 and 74 % in relation to PR treatment, for corn and cowpea, respectively. In addition, cowpea dry pod yield increased by at least 1802% and 1268% with PR+BC and PR compared to the Control for corn and cowpea, respectively.

3.5. Macronutrient uptake by crops

Both treatment and plant portion (root, stem, leaves, and fruits (cobs or pods)) affected ($P < 0.05$) the elemental (C, N, S, P, K, Ca, and Mg) contents (mg pot^{-1}) in the vegetal tissues for both corn and cowpea (Table 5). Moreover, significant ($P<0.05$) interaction between plant portion and treatment was found (except for Ca in corn). In corn, the highest content of most macronutrients in each organ was found in treatment PR+BC, according to the highest yield. Concerning singular nutrients, C and S often accumulated in stem, the organ that accumulated most of plant biomass; N, K, Ca and Mg accumulated in leaves and P in stem, leaves or cob.

Also in cowpea the highest content of most macronutrients in each organ was found in treatment PR+BC. C, N, S, P and Mg in treatments PR and PR+BC accumulated in pods, which accounted for most of the plant biomass; in Control, where the stem accounted for most of the plant biomass, C, N and S accumulated in this organ while P and Mg accumulated in leaves. Ca accumulated in leaves in all treatments, and K accumulated in fruit, leaves or stem.

Table 4
Treatments effect on corn and cowpea yield and growth parameters after pot experiment with acid Arenosol from Mozambique. Means followed by different lowercase letters differed significantly among treatments at $P < 0.05$ by Duncan multiple mean comparison test.

Yield and growth parameters	Corn				Cowpea			
	Treatments			ANOVA	Treatments			ANOVA
	Control	PR	PR+BC		Control	PR	PR+BC	
Dry biomass, g pot^{-1}	9.8 (3.8)[a)] c	51.4 (7.2) b	97.6 (7.4) a	***	1.77 (0.10) c	12.95 (1.65) b	22.47 (1.16) a	***
Dry root, g pot^{-1}	1.3 (0.3) c	5.2 (1.1) b	9.4 (1.2) a	**	0.41 (0.06) b	0.62 (0.29) b	1.98 (0.36) a	*
Dry stem, g pot^{-1}	2.7 (1.4) c	17.8 (4.4) b	45.2 (7.1) a	**	0.71 (0.14) b	1.89 (0.20) b	4.52 (1.03) a	*
Dry leaves, g pot^{-1}	5.7 (2.1) c	14.4 (1.8) b	22.1 (2.1) a	**	0.46 (0.14) b	3.34 (1.08) ab	5.88 (1.19) a	*
Dry fruit, g pot^{-1}	NA (-)	14.0 (0.2) a	21.0 (3.9) a	**	0.56[b] (-) c	7.10 (0.19) b	10.09 (1.15) a	*
Plant height, cm	43.7 (13.6) b	146.0 (15.2) a	172.3 (7.7) a	***	21.00 (1.53) b	27.00 (1.53) a	26.67 (0.33) a	*
Diameter of stem, cm	0.6 (0.1) c	1.1 (0.1) b	1.3 (0.1) a	**	0.48 (0.04) b	0.47 (0.02) b	0.71 (0.02) a	**
Root length, cm	27.7 (3.8)	38.7 (3.5)	40.3 (4.3)	NS	26.33 (4.48)	35.00 (3.61)	26.67 (3.53)	NS

NS, *, **, *** Not significant, significant at $P = 0.05$, $P = 0.01$ and $P = 0.001$, respectively; NA – not available because this treatment did not produced cob; [a)] Numbers in parentheses are the standard errors (n=3). [b]Only one repetition produced pod

Table 5

Plant nutrient contents (mg pot^{-1}) in corn and cowpea after pot experiment with acid Arenosol from Mozambique. Means followed by different lowercase letters differed significantly among treatments at $P < 0.05$ by Duncan multiple mean comparison test.

Plant nutrient contents		Corn						Cowpea					
		Treatments			ANOVA			Treatments			ANOVA		
		Control	PR	PR+BC	Plant	Treat.	Interaction	Control[b)]	PR	PR+BC	Plant	Treat.	Interaction
C	Fruit	NA	5632.8 (74.0) [a)] bc	7971.7 (1454.0) b				187.1 (-) e	2668.9 (52.9) ab	3696.8 (488.2) a			
	Leaves	2045.5 (819.6) d	5840.5 (540.0) bc	8458.4 (731.5) b	**	***	***	123.0 (33.9) e	826.3 (435.4) cd	2028.7 (428.5) ab	**	***	***
	Stem	960.3 (516.5) e	6928.9 (1502.0) bc	16899.1 (2056.2) a				189.0 (42.4) e	687.2 (64.9) cd	1553.9 (343.6) bc			
	Roots	465.8 (139.4) e	2048.1 (390.2) d	3459.5 (379.9) cd				108.8 (9.6) e	224.1 (96.5) de	646.9 (96.0) cd			
N	Fruit	NA	209.4 (2.8) ab	436.7 (200.1) a				18.2 (-) cde	196.7 (4.4) a	280.8 (35.5) a			
	Leaves	98.1 (45.8) cd	192.2 (12.4) abc	250.5 (15.4) ab	***	***	***	15.7 (3.4) cde	70.8 (44.5) bc	161.4 (29.7) a	**	***	***
	Stem	32.3 (21.0) ef	155.1 (38.3) bcd	392.5 (64.7) a				22.0 (4.0) bcde	25.1 (5.0) bcde	61.9 (12.9) b			
	Roots	17.1 (3.2) f	58.2 (12.5) def	75.2 (15.5) de				10.2 (1.8) e	14.7 (7.8) de	49.7 (10.1) bcd			
S	Fruit	NA	1044.2 (27.7) bc	1335.5 (313.8) b				30.3 (-) def	502.8 (50.7) a	505.9 (118.0) a			
	Leaves	378.7 (154.9) de	933.2 (60.2) bc	1416.2 (164.0) b	***	***	***	21.6 (7.2) f	141.6 (78.7) bcd	307.2 (74.0) ab	**	***	***
	Stem	188.2 (94.7) ef	1233.7 (251.9) b	3015.7 (351.3) a				30.9 (5.7) def	103.1 (18.8) cde	228.8 (75.4) abc			
	Roots	79.5 (29.5) f	314.0 (53.4) de	509.5 (42.5) cd				14.8 (2.0) f	26.5 (9.0) ef	98.1 (18.9) cde			
P	Fruit	NA	20.8 (2.2) bcde	62.4 (10.8) ab				1.5 (-) cd	25.0 (2.5) ab	55.7 (14.3) a			
	Leaves	15.2 (4.2) cde	13.3 (1.5) cde	43.9 (11.3) bc	*	***	***	0.7 (0.3) d	14.7 (9.5) b	26.3 (9.7) ab	**	***	**
	Stem	11.6 (8.2) de	9.1 (1.1) cde	162.4 (96.9) a				0.5 (0.1) de	1.5 (0.8) cd	8.9 (3.7) b			
	Roots	8.1 (4.9) de	4.2 (1.1) e	34.0 (20.8) bcd				0.4 (0.1) de	1.4 (1.0) d	6.7 (1.6) bc			
K	Fruit	NA	125.9 (27.2) cd	304.1 (51.0) abc				5.6 (-) cd	85.2 (9.0) ab	132.4 (15.1) ab			
	Leaves	102.1 (50.5) d	184.7 (32.3) bcd	469.0 (54.7) a	***	***	**	10.6 (6.1) c	8.8 (0.3) c	200.9 (16.9) a	**	***	**
	Stem	34.2 (27.6) e	122.1 (56.4) d	447.7 (123.1) ab				10.5 (2.0) c	63.9 (7.2) b	239.7 (108.7) a			
	Roots	15.8 (7.7) e	67.4 (15.6) de	179.1 (39.4) bcd				5.5 (1.8) cd	6.4 (4.9) cd	49.9 (14.6) b			
Ca	Fruit	NA	5.8 (3.2)	5.2 (0.6)				0.5 (-) e	5.7 (0.6) bc	5.3 (0.7) bc			
	Leaves	16.2 (7.3)	36.0 (9.2)	38.9 (9.1)	***	***	NS	1.4 (0.5) cd	14.6 (4.7) b	40.3 (1.1) a	***	***	*
	Stem	1.7 (0.8)	8.0 (3.2)	9.8 (2.6)				0.7 (0.2) d	4.9 (0.8) bc	14.1 (6.5) b			
	Roots	1.0 (0.4)	5.3 (1.7)	9.3 (3.4)				0.4 (0.1) d	1.1 (0.5) d	5.2 (1.6) bc			
Mg	Fruit	NA	15.4 (5.0) b	26.8 (4.7) ab				1.6 (-) ef	12.9 (1.0) abc	23.8 (1.4) a			
	Leaves	16.3 (6.3) bc	25.8 (5.1) ab	44.2 (11.4) a	***	***	**	2.1 (0.6) def	3.6 (1.8) de	12.5 (0.9) abc	*	***	*
	Stem	3.6 (1.9) d	14.9 (4.6) c	43.8 (10.9) a				1.1 (0.3) ef	3.6 (0.8) cde	21.9 (11.5) ab			
	Roots	1.5 (0.3) d	4.7 (1.8) cd	10.8 (3.2) bc				0.4 (0.1) fg	0.7 (0.4) efg	6.9 (2.0) bcd			

NS, *, **, *** Not significant, significant at $P = 0.05$, $P = 0.01$ and $P = 0.001$, respectively. NA – not available because did not produced cob. [a)]Numbers in parentheses are the standard errors (n=3). [b)]Only 1 repetition produced pod.

As for macronutrient concentrations (g kg^{-1}), significant ($P < 0.05$) interaction was found between plant portion and treatments for C, N, S, P, K, Ca, and Mg concentration (mg kg^{-1}), except for K in corn and P and Ca in cowpea. Plant portion or treatment affected concentration ($P< 0.05$) of P, K, Ca and Mg in corn, and N, P, K, Ca and Mg in cowpea (Fig. 6 and 7). N and P concentrations in cowpea were highest in leaves or fruit. Higher N concentration in cowpea was found in Control (leaves, fruit and stem), while in corn was similar between treatments mostly in fruit, leaves and roots (except fruit in Control), resulting from a concentration effect (lower biomass yield); Not significant difference was found in P concentration between treatments and plant portions (excluding fruit in Control) in corn; however, in cowpea treatment PR+BC resulted in higher P concentration than PR and Control treatments.

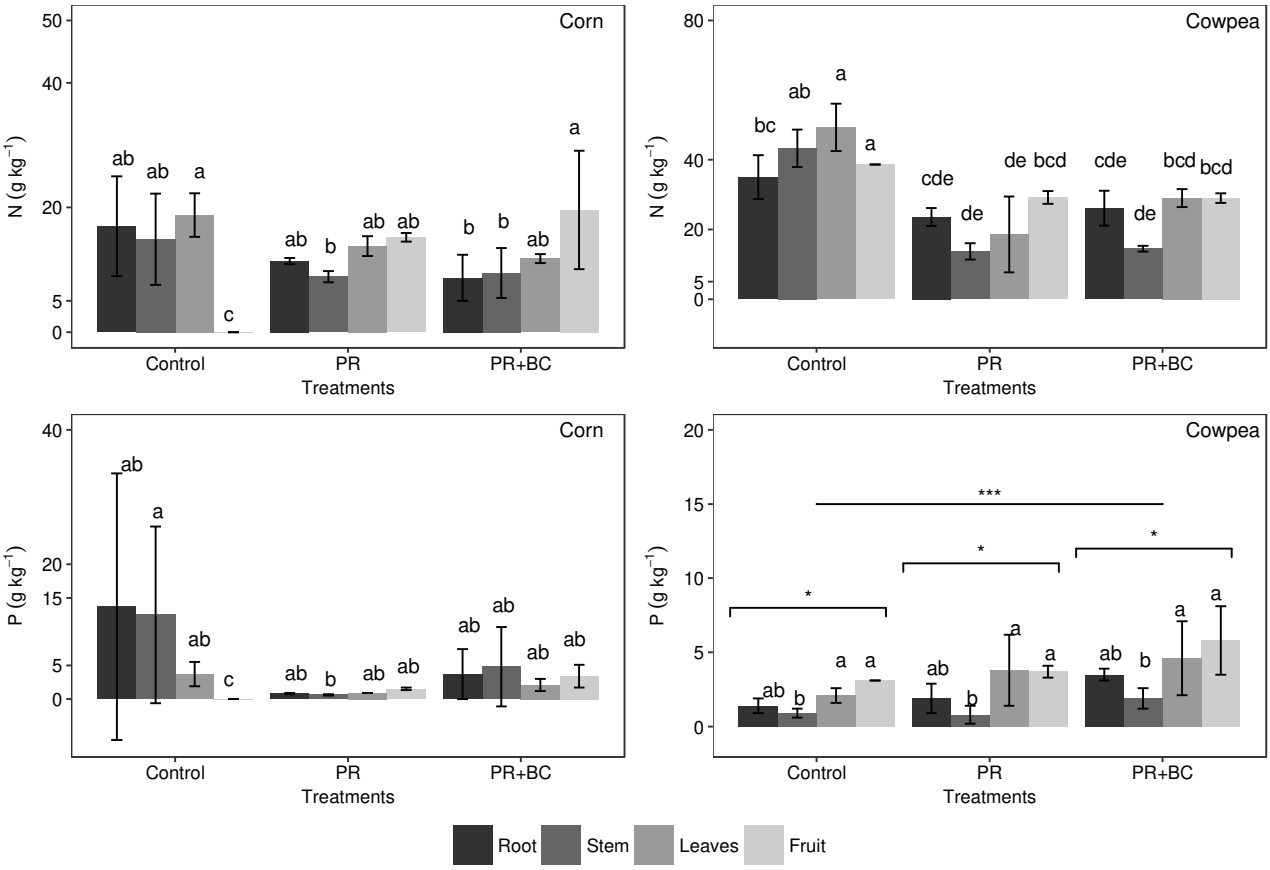

Fig. 6 Effect of interaction between treatments (Control, PR, and PR+BC) and plant organs (root, stem, leave, and fruit) on N and P concentration (g kg^{-1}) after the pot experiment with acid Arenosol from Mozambique. Symbols represent: *, **, *** significant at $P = 0.05$, $P = 0.01$ and $P = 0.001$, respectively for no interaction but significant effect of individual factors (treatments or plant organs). The whiskers indicate the error bars (n=3).

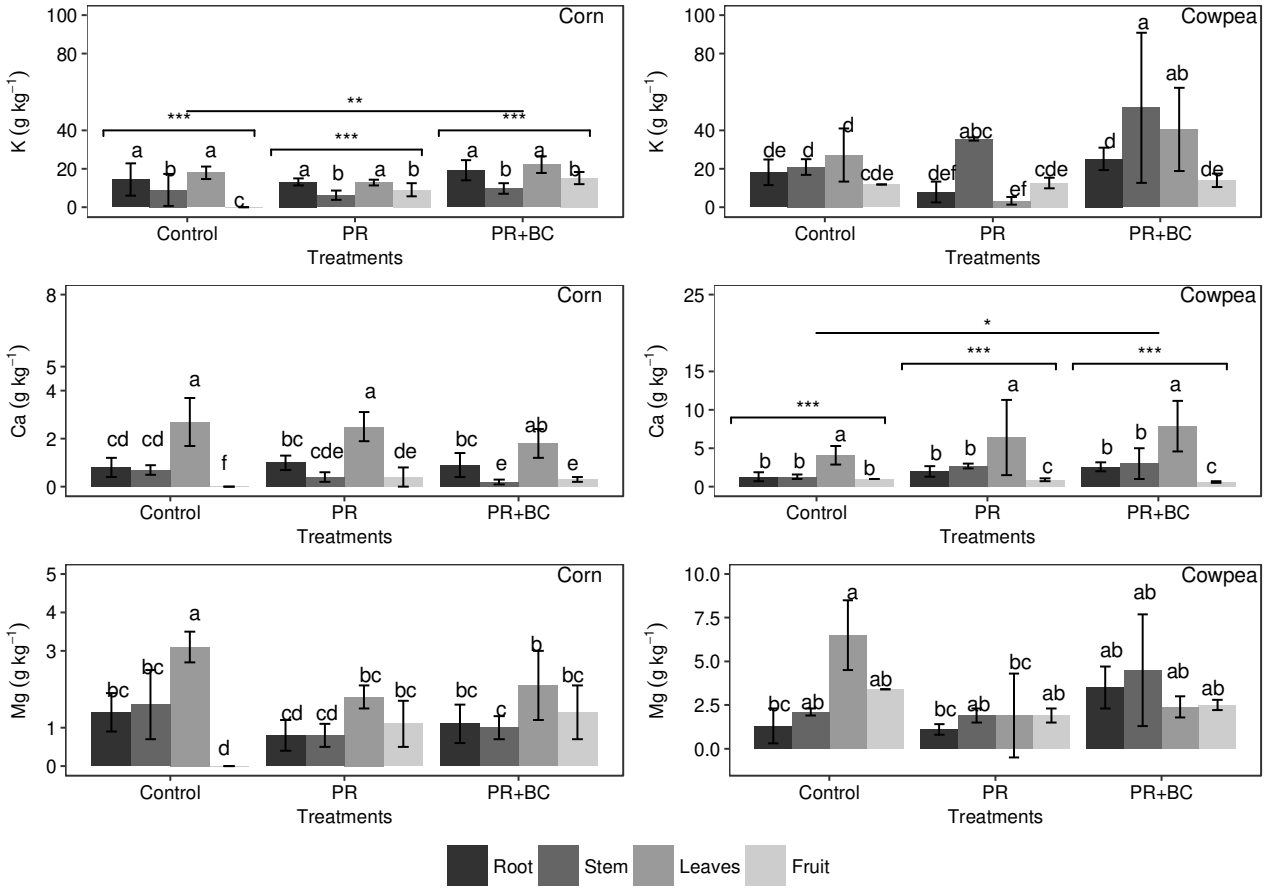

Fig. 7 Effect of interaction between treatment (Control, PR, and PR+BC) and plant organs (root, stem, leave, and fruit) on K, Ca, and Mg concentration (g kg^{-1}) after the pot experiment with acid Arenosol from Mozambique. Symbols represent: *, **, *** significant at $P = 0.05$, $P = 0.01$ and $P = 0.001$, respectively for no interaction but significant effect of individual factors (treatments or plant organs). The whiskers indicate the error bars (n=3).

K concentrations were higher in cowpea than in corn. K concentration in corn was highest in root and leaves, while in cowpea K concentration was highest in stem or leaves. In corn, higher K concentration was found in roots and leaves, and PR+BC treatments had higher K concentration; while in cowpea, there was higher K concentration in PR+BC treatment in stem and leaves, than in PR and Control treatments. Ca and Mg concentrations in both crops were highest in leaves. Ca concentration in corn was highest in leaves of all treatments, while in cowpea, PR+BC treatment had higher concentration. Mg concentration in corn was highest in Control leaves, resulting from a concentration effect, while in cowpea there was not significant difference between treatments or plant portions.

3.5.1. Phosphorus use efficiency

The P use efficiency by both crops was assessed as agronomic efficiency (AE), physiological efficiency (PE), apparent recovery efficiency (ARE), and harvest index (HI). The P AE and HI were affected by the crop but not by the treatment, the AE being higher in corn while the HI was higher in cowpea (Fig. 8). For the ARE and PE, a significant (P<0.05) interaction was found between crop and treatment. In both crops, the ARE was higher in treatment PR+BC than in PR; the PE was higher in treatment PR than in PR+BC in corn, while there was no significant difference between treatments in cowpea.

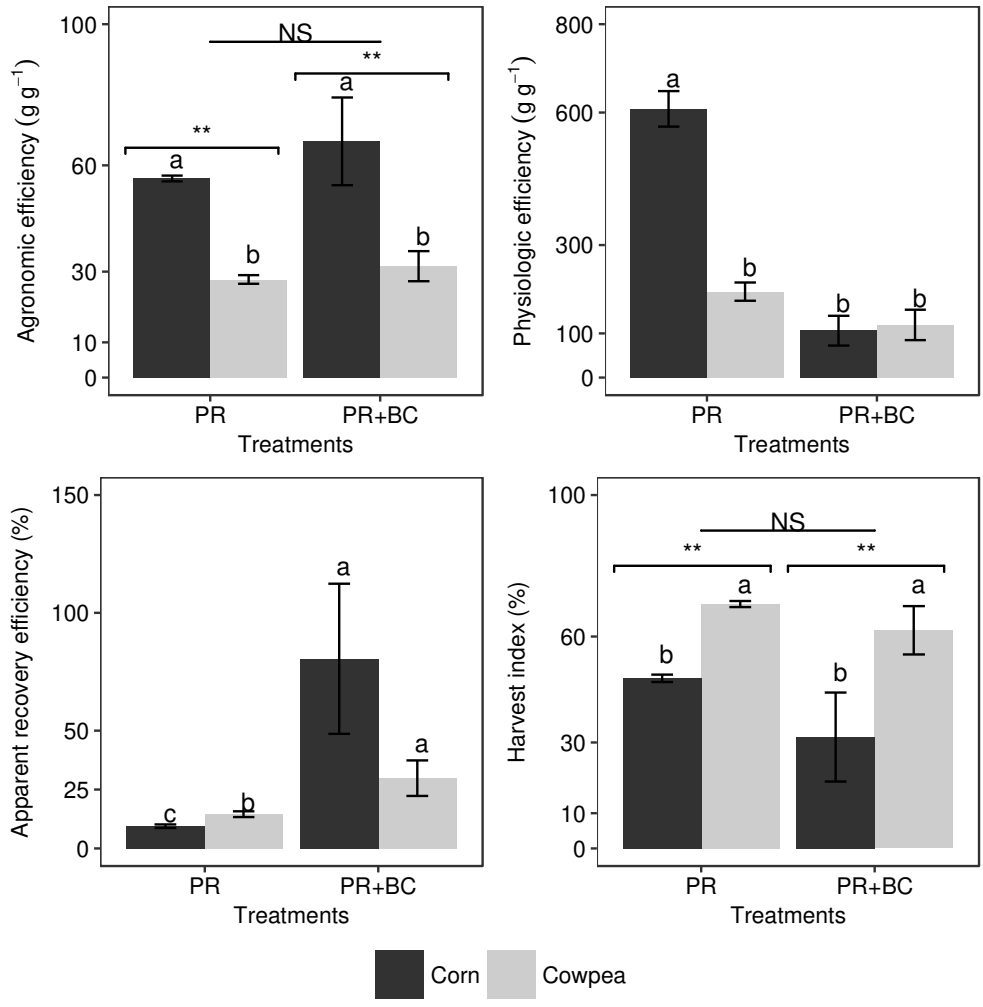

Fig. 8 Phosphorus agronomic (AE, g g^{-1}) and physiological (PE, g g^{-1}) efficiencies, apparent nutrient recovery (ARE, %), and harvest index (HI, %) by corn and cowpea after the pot experiment with acid Arenosol from Mozambique. Means followed by different lowercase letters differed significantly among treatments at $P < 0.05$ by Duncan multiple mean comparison test. Symbols represent: NS, *, **, *** Not significant, significant at $P = 0.05$, $P = 0.01$ and $P = 0.001$,

respectively for no interaction but significant effect of individual factors (treatments or crops). The whiskers indicate the standard error (n=3).

4. Discussion

4.1. Soil, phosphate rock and biochar physicochemical properties

The soil used in the trial had low fertility, and soil pH correction, replenishment of soil organic matter and plant nutrients are mandatory to increase the soil fertility (Kimetu et al., 2008). The phosphate rock is considered suitable to be applied to this soil, so to increase available P and other nutrients such as Ca and Mg (Chien et al., 2010; Rafael et al., *in press*). Biochar can be a source of both labile and recalcitrant C, cation exchange capacity (CEC), available nutrients, liming potential and water retention capacity, all of which will result in improving soil fertility and crop yield (Yuan et al., 2011).

Biochar application was responsible for the significant increase of soil pH, for both corn and cowpea cultivation, in treatment PR+BC. Lehmann et al. (2011) reported the ability of biochar to increase soil pH, CEC, and EOOC. In addition, biochar contributed to the increase of available P in PR+BC treatment because of the soluble P content in the biochar (Table 1), which is therefore a potential source of P (Wang et al., 2012; Zhang et al., 2016).

Our results indicate that soil rhizosphere pH (H_2O and KCl) in cowpea was not lower ($P<0.05$) than pH of bulk soils, while corn rhizosphere soils had higher pH (H_2O and KCl) than bulk soils (Fig. S2 of supplementary material). Plants can change the chemical conditions in the rhizosphere, in particular by releasing H^+/OH^- or organic anions, and influence the mobility of P from sparingly soluble P sources (Bertrand et al., 1999; Hinsinger et al., 2006). In our study root – induced alkalinisation in corn probably was due OH^-/HCO_3^- exudation by roots (Hinsinger, 2001), indicating higher uptake of anions (probably phosphate), which was promoted by PR+BC treatments. Rhizosphere acidification can increase under P deficiency (Zhou et al., 2009). The physic-chemical properties of biochar such as pH, CEC and C content influence nutrient availability and microbial community (Hussain et al., 2017).

According with Hinsinger (2001), acidification of the rhizosphere is expected to occur in the rhizosphere of N_2 fixing legumes particularly efficient at using P from phosphate, but in our study cowpea rhizosphere pH did not differ from bulk. The reason are because the H^+ released by roots are consumed in the dissolution of phosphate rock and do not contribute to any pH decrease in the rhizosphere (Hinsinger, 2001). Also, measuring only change in pH is rather poor indicator of H^+ or

OH^-/HCO_3^- actually released by roots (Hinsinger, 2001; Dakora and Philips, 2002; Neumann and Römheld, 2012).

4.2. Soil biochemical and biological properties

Since the soil microbial community produces enzymes in response to any soil change that modifies the organic substrate or other soil factors (Sinsabaugh et al., 2002), the enzymatic activity can be used as an indicator of soil changes (Schloter et al., 2003; Allen et al., 2011; Jain et al., 2016). The effect of treatments on enzyme activities was enzyme specific. Acid phosphomonoesterase and xylosidase activities were highest in rhizosphere soil of treatments Control and PR, probably due to the lower pH values (bulk and rhizosphere) compared with PR+BC treatment. PR treatment did not differ significantly from the Control for most enzymes assessed, excluding β-glucosidase and xylosidase in rhizosphere. The optimum pH range of acid phosphomonoesterase and xylosidase is from ~ 4 - 5 (Gianfreda and Rao, 2014). These enzymes are pH dependent, and in treatment PR+BC the pH (bulk and rhizosphere) was ~ 5, this could have depressed the activities of both enzymes.

However, in the PR + BC treatment most of the enzyme activities are higher in bulk soil; perhaps the biochar is preferentially concentrated in non-rhizospheric soil, creating a more favourable environment for biological activity. Biochar liming effect caused higher pH (but no significant difference was found between bulk and rhizosphere) on PR+BC treatment compared with Control and PR treatments, and increased the activity of these enzymes. Also, Jain et al. (2016) and Huang et al. (2017) found an increase of alkaline phosphomonoesterase with application of biochar ascribed to the increase of soil pH. These results suggest that biochar addition change soil properties, such as rhizosphere microorganism communities as reported by Lehmann et al. (2011). The effect of biochar on soil enzyme activity depends on soil type and the particular enzyme (Hussain et al., 2017).

Cowpea rhizosphere had higher activity of β-glucosidase (involved in C cycle) and chitinase (involved in both N and C cycles) than bulk soil, due the ability of legumes to release C compounds around the root zone and promote different chemical, physical, and biological characteristics in the rhizosphere compared with the bulk soil (Hinsinger et al., 2006; Lambers et al., 2009; Bird et al., 2011; Bakker et al., 2015). Legumes have rhizosphere processes than corn (Hinsinger, 2001). These results suggest that cowpea rhizosphere is able to decompose soil organic matter faster than corn rhizosphere. Cowpea was able also to significantly acquire N through symbiotic fixation, mainly in treatment PR+BC (Lambers et al., 2009; Laberge et al., 2011). Cowpea nodules enhance rhizosphere

priming effect by releasing rhizodeposits that accelerate soil organic matter decomposition (Zhu and Cheng, 2012).

There was not significant difference among treatments in soil microbial biomass in cowpea, but significantly higher soil basal respiration was found from 168 to 528 h in PR treatment than in Control or PR+BC. Conversely, higher acumulated soil basal respiration was found in cowpea rhizosphere of PR treatment than in bulk soil (Fig. 4). Marschner (2011), asserted that higher microbial activity in rhizosphere leads to higher soil basal respiration. Several factors can contribute to microbial composition in the rhizosphere, however, the plant itself exert highly selective effect that is greater than that of the soil (Marschner et al., 2001, 2004). Biochar is resistant to soil microorganism degradation and its recalcitrance makes reducing the release of CO_2 from the soil (Hussain et al., 2017; Zhu et al., 2017), in accordance with the respiration in PR+BC lower than in PR and not different from Control.

For corn, our results indicate that in the short term biochar did not increase soil microbial biomass or respiration; on the contrary, PR treatment reduced the total soil microbial biomass compared with Control. Similarly, Castaldi et al. (2011) and Ameloot et al. (2014) reported for field trials that, in the medium term, soil microbial activity was either not affected or inhibited to different extents in the biochar-amended plots. Lehmann et al. (2011) and Hussain et al. (2017), among others, reported that biochar has strong influence on soil microbial community composition and abundance, and C forms. The increase of soil microbial biomass may result in net nutrient immobilization, while a decrease can cause a net release of nutrients (Marschner, 2011).

The application of biochar did significantly increase most enzyme activities as well as nodulation in cowpea. Different from our results, in medium-term trials Quilliam et al. (2013) found no significant difference in the total number of nodules between biochar-amended and unamended soil. In this study the soil biological and biochemical properties were clearly affected by soil (bulk and rhizosphere), treatments and plant species. Enzymes such as phosphodiesterase, α-glucosidase, arylsulfatase, chitinase, leucine aminopeptidase, β-glucosidase, and alkaline phosphomonoesterase were significantly affected by both plant species and soil (bulk and rhizosphere), β-glucosidase and chitinase highest in cowpea rhizosphere. The increase of availble P significantly increased the activities of α-glucosidase, β-glucosidase, phosphodiesterase, pyrophosphate phosphodiesterase, and alkaline phosphomonoesterase (Fig. S3 of Supplementary materials). Similarly, the activities of these enzymes correlated to soil EOOC concentration (Fig. S4 of Supplementary materials). The activity

of acid phosphomonoesterase significantly decreased with increase of soil EOOC (Fig. S5 of Supplementary materials).

These enzymes are extracellular enzymes involved mostly in C, N, S and P cycles and can be released by plants (Shukla and Varma, 2011). For example, glucosidases are responsible for cellulose degradation, are useful as soil quality indicators, and are derived mostly from soil microorganism like fungi (Turner et al., 2002; Shukla and Varma, 2011), while arylsulfatase, that plays an important role in S cycle, mainly originates from fungi, bacteria and plants (Li and Sarah, 2003). Wang et al. (2015) reported an increase of soil enzymes related with N and P cycles after aplication of corn biochar. Biochar may be responsible for the stimulation of degradation of proteins and N rich substances and lead to the increase of leucine aminopeptidase (Wang et al., 2015). In addition, phosphatases are important for mineralization of P, and are also dependent on microbial activities (Jain et al., 2016). Biochar effects on soil enzyme activity are highly variable (Bailey et al., 2011). In this study we demonstrated that baby corn peel biochar significant affects enzymes related to C, N, and P cycles in an acid Arenosol. Plant rhizosphere played an important role influencing the enzyme activities.

4.3. Crops yield in response to the treatments

Both corn and cowpea yields increased dramatically in PR+BC and PR treatments compared with the Control. There has been reported an increase of yield by direct application of phosphate rock to acid soils (Vanlauwe et al., 2000; Somado et al., 2006; Sokoto and Singh, 2008; Chien et al., 2011). To date, no report regarding simultaneous application of phosphate rock and biochar to acid soils was found. The increase of soil available P affects crops yield and root weight (Edwards, 1991; McLaughlin and James, 1991; Wright et al., 1991). Plants with root well developed are able to absorb nutrients efficiently. In our study, dry root biomass was significantly higher in treatments with higher soil available P.

4.4. Nutrient uptake and concentration in corn and cowpea

Nutrient uptake (mg pot^{-1}) in both corn and cowpea were related to plant biomass, higher nutrient uptake being registered in PR+BC treatment because this treatment produced higher yield for both crops. Corn accumulated C, N, S, P, and Mg always in stem on treatment PR+BC, while cowpea always accumulated the same elements in pods for the same treatment. K and Ca always accumulated in leaves in both crops. The highest N, P, and K concentrations (mg kg^{-1}) in leaves, for both corn and cowpea, were found in Control, due the dilution effect in treatments PR and PR+BC, where pant growth was higher than in Control. N concentration in cowpea was higher than in corn, being in the

typical ranges for legumes and grasses, respectively (Barker and Bryson, 2007). P concentration in cowpea was within the sufficient range in PR and PR+BC treatments (Sanchez, 2007). P concentration in corn lay within the sufficient range in PR+BC treatment, but, quite surprisingly, was below this range in PR treatment. The higher P concentrations in cowpea than in corn in PR and PR+BC treatments, despite the similar values of soil Mehlich-3 P, are congruent with the dilution associated to higher biomass production in corn.

K concentration was also higher in cowpea than in corn. Highest concentrations in PR+BC treatment for both crops reflect the capacity of biochar to supply K and are consistent with the highest soil exchange K in this treatment. In both crops, K concentrations in leaves lay in the sufficient K ranges for corn and legumes (Mengel, 2007) in Control and PR+BC treatment and below this range in PR treatment. Ca concentrations were much lower in corn than in cowpea, in accordance with the general statement that monocotyledons contain much less Ca than dicotyledons (Pilbeam and Morley, 2007) and were allocated preferentially in leaves. In cowpea, the Ca concentrations were lowest in Control, in accordance with the lower soil exchange Ca. Also, Mg concentrations were lower in corn than in cowpea and were allocated preferentially in leaves, being within the reported sufficiency ranges (Merhaut, 2007).

4.4.1. Phosphorus use efficiency

High nutrient use efficiency by plants greatly enhances the efficiency of applied fertilizers, reducing costs and preventing nutrient losses to ecosystems and the concomitant degradation of soil and water quality (Baligar et al., 2001). The harvest index (HI) is a characteristic of the plant species or cultivar, being in the present case higher for cowpea than for corn, irrespective of treatment. Cowpea was more efficient than corn in using P from phosphate rock, as illustrated by the higher apparent recovery efficiency in treatment PR (Fig. 8). Cowpea has been reported to be able to access non-lablie P under P-deficiency conditions (Pypers et al., 2006). Krasilnikoff et al. (2003) found considerable activity of root-induced rhizosphere processes leading to higher P uptake from sparingly soluble P sources by cowpea. Due to N fixation, legumes take up small amounts of nitrate, which leads to an imbalance between anion and cation uptake with the subsequent excretion of H^+ ions, which acidify the rhizosphere and release P from phosphate rock (Weil, 2000; Neumann and Römheld, 2012).

The ability of the plant to transform P acquired from fertilizer into grain yield, assessed as physiological efficiency (PE), was higher for corn in PR treatment, and for this treatment was higher

in corn than cowpea. In addition, PR+BC treatment in both crops presented higher ARE, meaning that greater efficiency in P uptake was provided by biochar. This higher ARE may be related to the higher available P or higher pH (Table 2) or to higher phosphatase (alkaline phosphomonoesterase, phosphodiesterase and pyrophosphatase-phosphodiesterase) activities (Fig. 1); it might also be related to the water holding capacity contributed by biochar (not measured). Therefore, the application of phosphate rock in combination with biochar to and acid Arenosol can be considered as slow release fertilization, with the benefit of increasing P recovery efficiency.

Interaction of plants and soil microorganism has a great effect on nutrient use efficiency by plants (Baligar et al., 2001). Plants can also exude phosphatases that mobilize organic P, resulting in an increase of available P. Because the EOOC was higher in treatments including biochar, part of available P may have been provided by phosphatases activities. The root-induced change in soil pH plays a key role in the availability of several nutrients and enzyme activities (Duncan, 1994; Baligar and Fageria, 1997; Hinsinger et al., 2006; Neumann and Römheld, 2012). Sauerbeck et al. (1990) and Lambers et al. (1998), asserted that root-induced changes can affect nutrient availability in the rhizosphere through: i) modification of the rhizosphere pH; ii) exudation of organic acids, chelators, reductants and oxidants; iii) extracellular enzymes to turn over organically bound nutrients; and iv) provide subtract to microbial biomass. In our study, the most plausible mechanism in which phosphate rock and baby corn peel biochar increased P (and other nutrients) availability to corn and cowpea, was related with point *iii)*, with implication on increased yield, as verified by interaction between treatments and soil (bulk and rhizosphere) on activities of most of the enzymes measured.

Conclusions

Simultaneous application of phosphate rock and biochar increased soil pH, available P, EOOC, CEC, and alkaline cations in both corn and cowpea. The root-induced changes on soil chemical properties was not affected by treatments. Interaction between treatments *versus* soil compartments or crops *versus* soil compartments was enzyme specific. Rhizosphere soils where phosphate rock was applied alone increased acid phosphomonoesterase and xylosidase activities, both C and P cycle enzymes, while contribution of higher pH (bulk and rhizosphere) soils in phosphate rock and biochar treatment increased activities of C (β-glucosidase, cellulase), N (leucine aminopeptidase), and P (alkaline phosphomonoesterase, phosphodiesterase, and pyrophosphatase-phosphodiesterase) cycle enzymes. Cowpea rhizosphere induced changes contributed most for faster mineralization of soil

organic matter and N fixation as verified by higher activity of β-glucosidase and chitinase, and nodulation.

Biochar can minimize the negative impact of high dose of phosphate rock. Biochar treatment failed to increase microbial biomass due to recalcitrance of biochar. The biochemical root induced changes improved soil quality and increased crops yield. Application of phosphate rock in combination with biochar to acid Arenosol can be considered slow release fertilizer with the benefit to increase nutrient efficiency recovery by plants.

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Supplementary Material

Table S1
Effect of corn and cowpea on soil enzyme activities after pot experiment with acid Arenosol from Mozambique. Means followed by different lowercase letters differed significantly among crops at $P < 0.05$ by Duncan multiple mean comparison test.

Enzyme	Crops		ANOVA
	Corn	Cowpea	
Acid phosphomonoesterase (nmol g^{-1} h^{-1})	10.41 (0.87) [a)] b	14.06 (1.26) a	*
Alkaline phosphomonoesterase (nmol g^{-1} h^{-1})	5.25 (1.38) b	9.32 (1.61) a	***
α-Glucosidase (nmol g^{-1} h^{-1})	0.43 (0.09) b	0.84 (0.10) a	**
Arylsulfatase (nmol g^{-1} h^{-1})	0.52 (0.09) b	0.84 (0.08) a	***
β-Glucosidase (nmol g^{-1} h^{-1})	2.42 (0.24) b	4.33 (0.35) a	***
Phosphodiesterase (nmol g^{-1} h^{-1})	0.72 (0.15) b	1.38 (0.16) a	***
Cellulase (nmol g^{-1} h^{-1})	0.04 (0.02) b	0.17 (0.03) a	***
Chitinase (nmol g^{-1} h^{-1})	1.70 (0.24) b	3.51 (0.30) a	***
Leucine aminopeptidase (nmol g^{-1} h^{-1})	4.94 (0.83) b	9.57 (0.90) a	***
Nonanoate esterase (nmol g^{-1} h^{-1})	14.80 (2.05) b	26.79 (2.85) a	***
Pyrophosphate- phosphodiesterase (nmol g^{-1} h^{-1})	0.30 (0.07) b	0.63 (0.09) a	**
Glucuronidase (nmol g^{-1} h^{-1})	0.29 (0.06)	0.32 (0.07)	NS
Xyloxidase (nmol g^{-1} h^{-1})	0.48 (0.06) b	0.84 (0.09) a	***

NS, *, **, *** Not significant, significant at $P = 0.05$, $P = 0.01$ and $P = 0.001$. [a)]Number in parenthesis are standard error (n =3).

Table S2
Contribution of variables to PCA

Variables[a)]	PC1	PC2
pH_{H2O}	1.40	**3.51**
pH_{KCl}	2.80	**8.42**
TOC	1.17	**2.29**
EOOC	3.56	**6.08**
Available P	**3.94**	1.40
Exchange Ca	2.57	**5.05**
Exchange Mg	4.22	**7.86**
Exchange K	2.39	**5.72**
Exchange Al	1.39	**1.57**
CEC	1.87	**4.59**
Base Saturation	4.27	**8.35**
Arylsulfatase	**7.23**	0.48
α-Glucosidase	**7.44**	0.56
β-Glucosidase	**6.65**	4.05
Cellulase	4.24	**6.02**
Xylosidase	5.21	**6.76**
Glucuronidase	**2.26**	0.01
Chitinase	**6.83**	2.44
Leucine aminopeptidase	**6.97**	0.70
Acid phosphomonoesterase	0.41	**14.10**
Pyrophosphate phosphodiesterase	**5.96**	2.49
Phosphodiesterase	**7.80**	0.34
Alkaline phosphomonoesterase	**6.30**	0.01
Nonanoate esterase	2.64	**6.80**
Microbial biomass C	**0.46**	0.13
Soil respiration	0.00	**0.27**

[a)]Numbers of variables in bold contribute most to PC1 or PC2

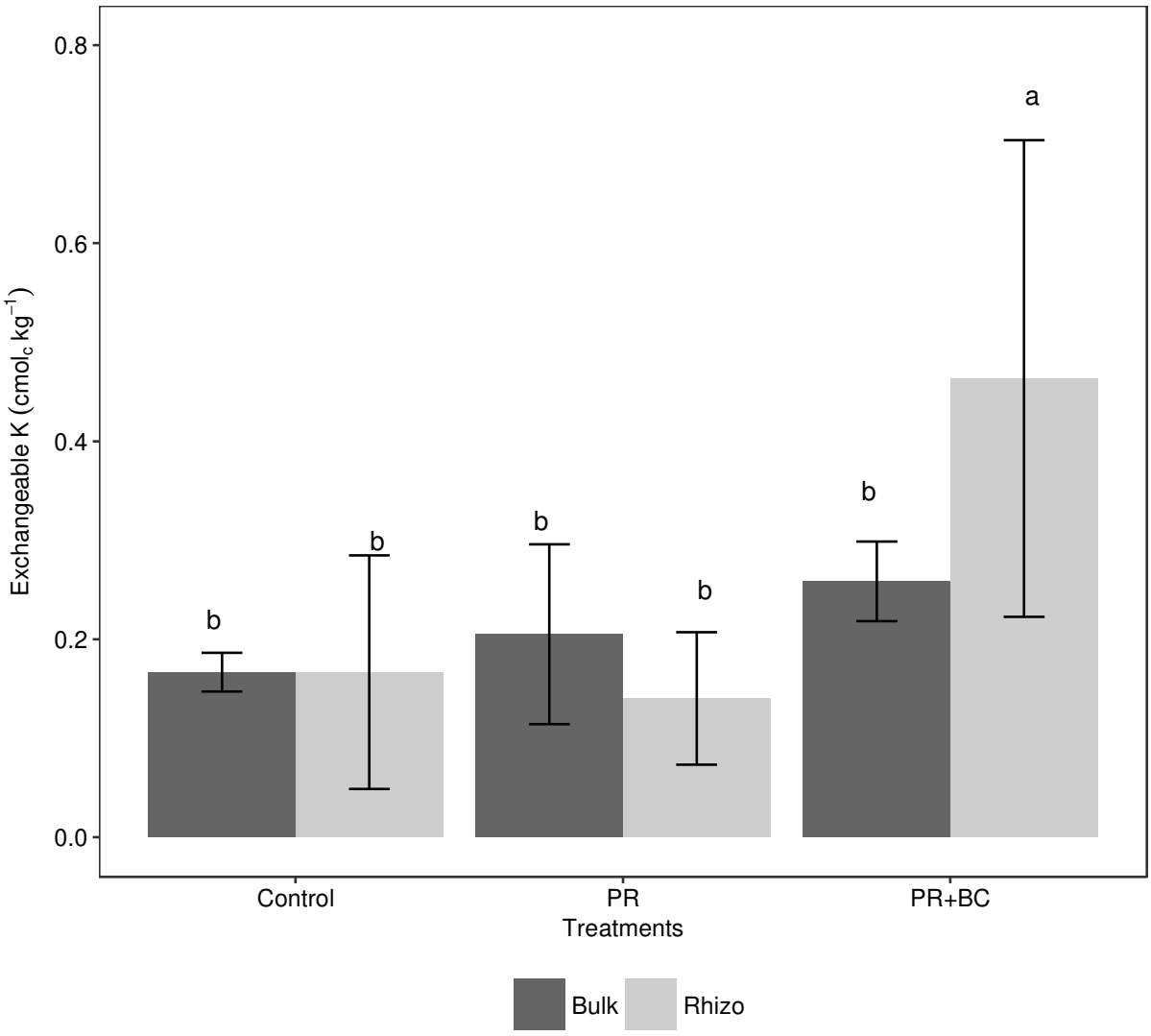

Fig. S1 Effect of interaction between treatments (Control, PR, PR+BC) and soil compartment (bulk and rhizosphere) on exchangeable K (P = 0.05) after the pot experiment with acid Arenosol from Mozambique. Means followed by different lowercase letters differed significantly among treatments at $P < 0.05$ by Duncan multiple mean comparison test. The whiskers indicate the standard error (n=6).

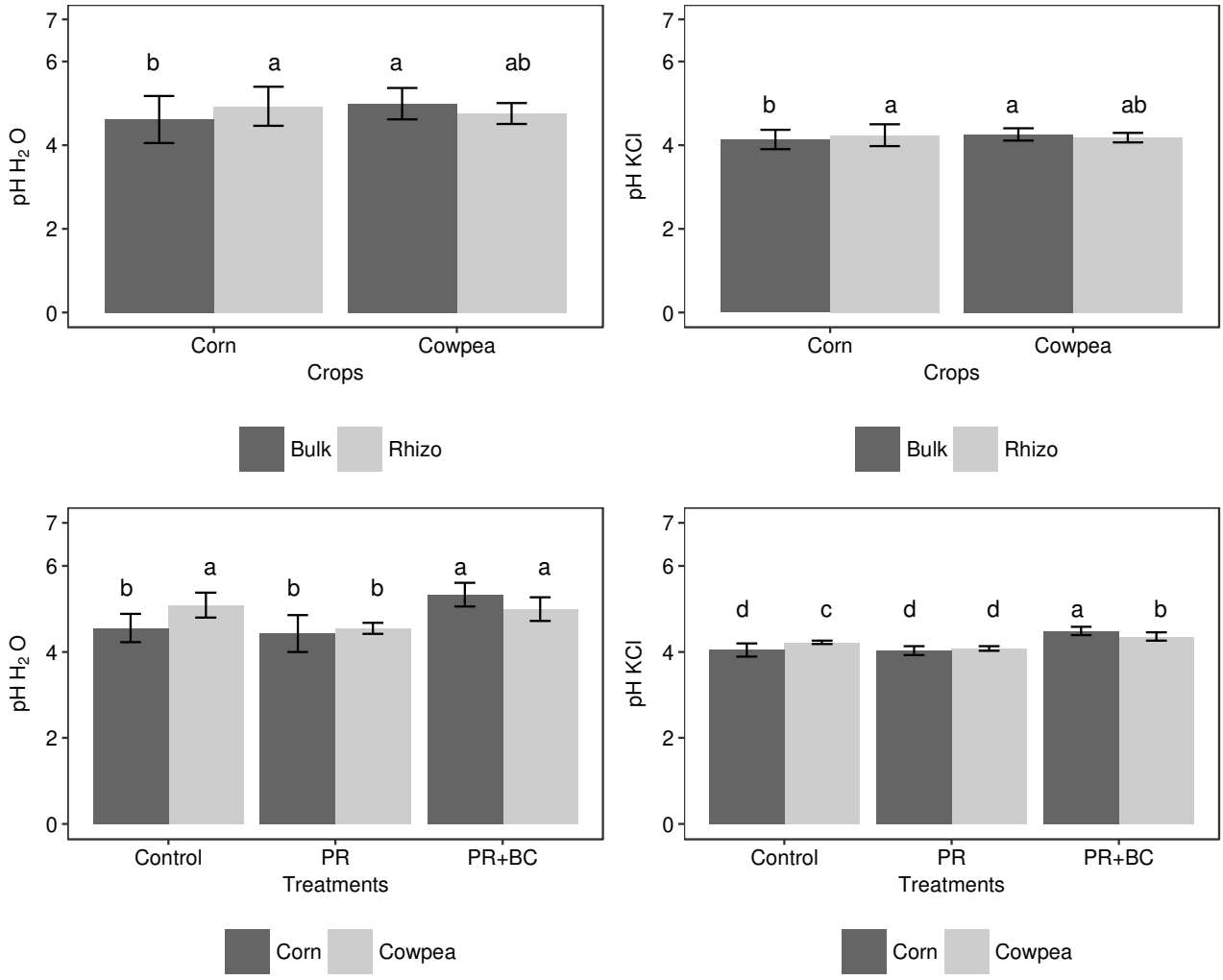

Fig. S2 Effect of interaction between crops (corn and cowpea) *versus* soil components (bulk and rhizosphere) and treatments (Control, PR, PR+BC) *versus* crops on soil pH (H_2O and KCl) after the pot experiment with acid Arenosol from Mozambique. Means followed by different lowercase letters differed significantly among treatments (or crops) at $P < 0.05$ by Duncan multiple mean comparison test. The whiskers indicate the error bars (n=6).

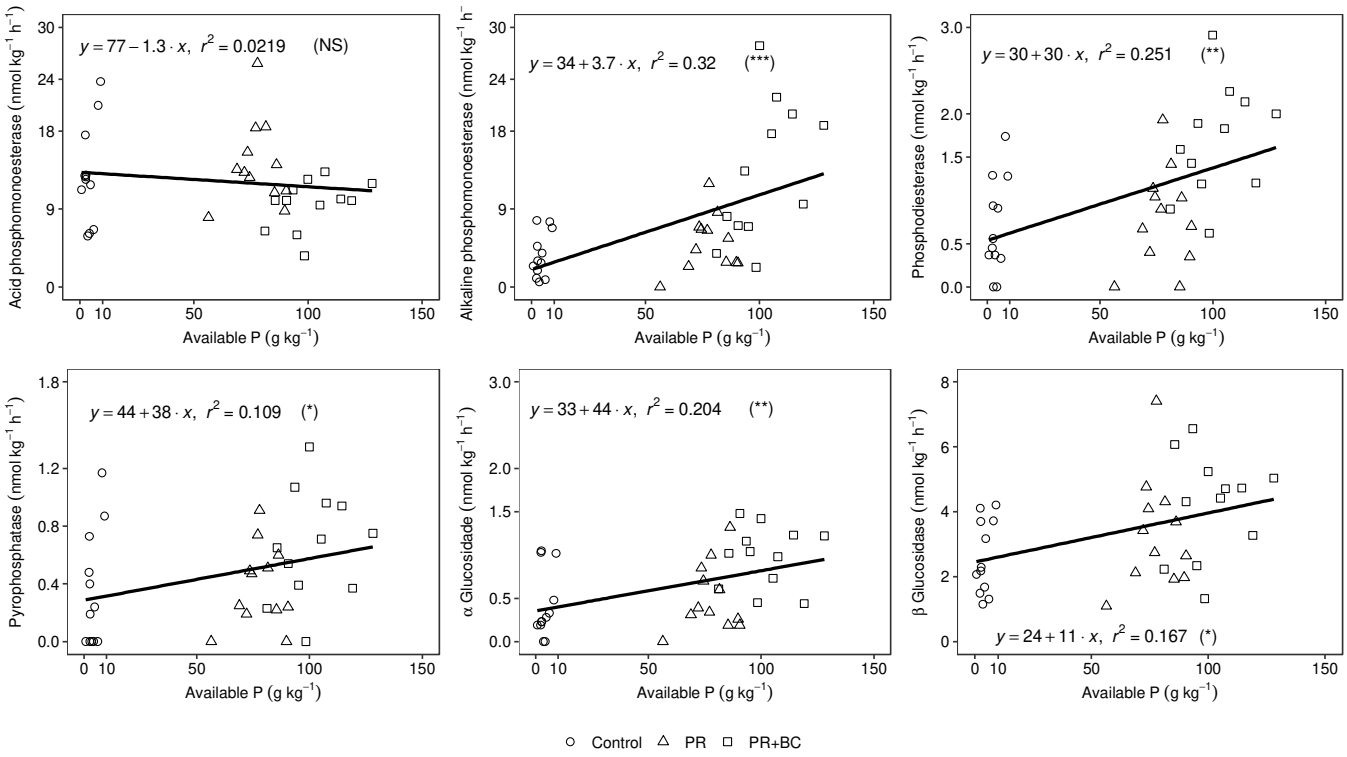

Fig. S3 Linear regression between available (Mehlich 3) P (mg kg^{-1}) and soil enzyme activities (nmol kg^{-1} h^{-1}) after the pot experiment with acid Arenosol from Mozambique.

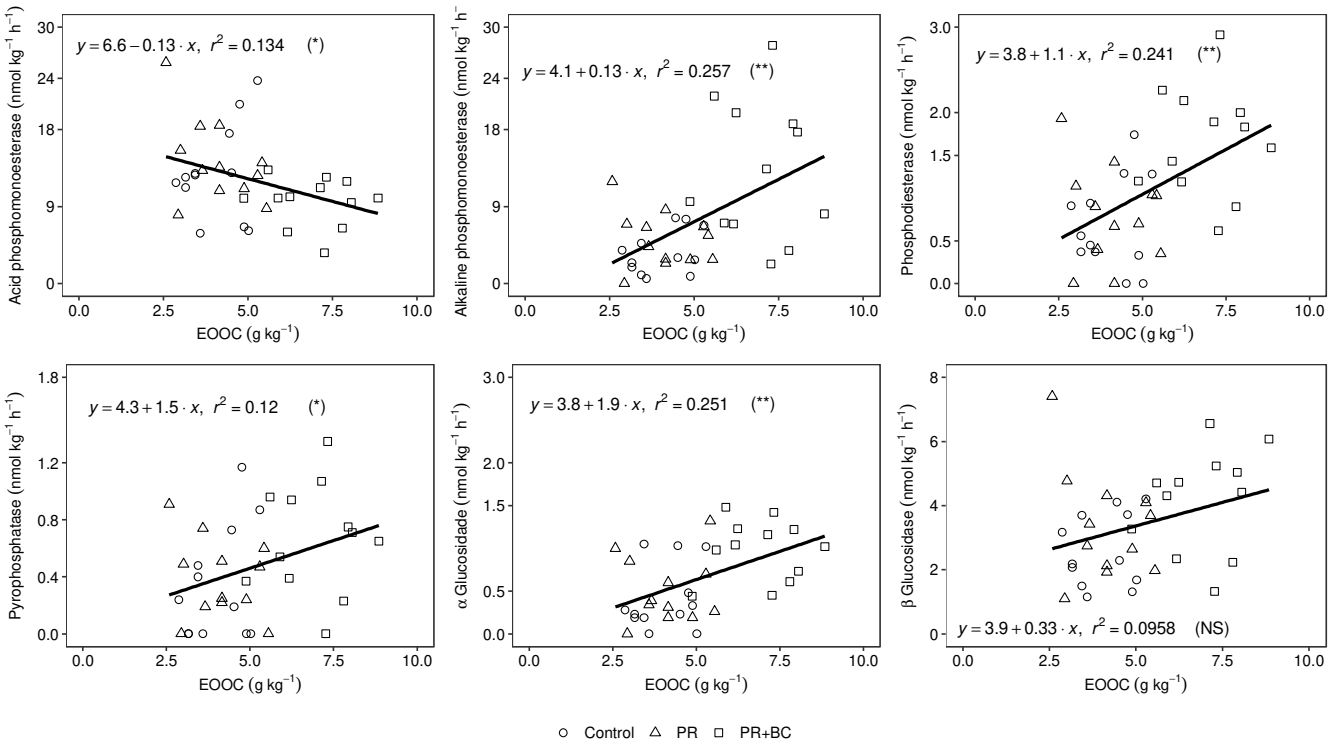

Fig. S4 Linear regression between EOOC (g kg^{-1}) and soil enzyme activities (mmol kg^{-1} h^{-1}) after the pot experiment with acid Arenosol from Mozambique.

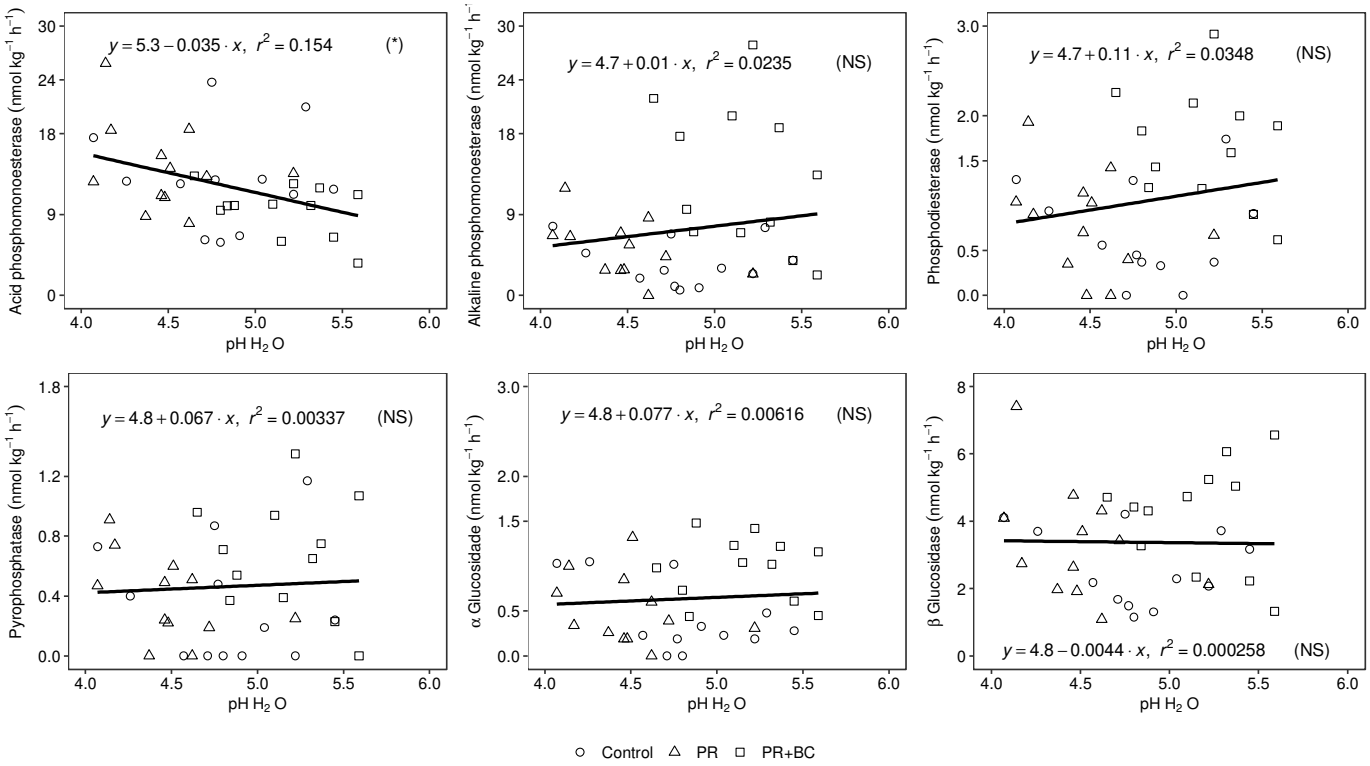

Fig. S5 Linear regression between pH_{H2O} and soil enzyme activities (mmol kg^{-1} h^{-1}) after the pot experiment with acid Arenosol from Mozambique.

CHAPTER 5 - BENEFITS OF BIOCHAR FOR SOIL QUALITY

Benefits of biochars and NPK fertilizer for soil quality, nutrient uptake and cowpea growth (*Vigna unguiculata L.*) in an acid Arenosol

Rafael, Rogério Borguete Alves[ab,★]; Fernández-Marcos, Maria Luisa[cf]; Cocco, Stefania[b]; Ruello, Maria Letizia[d]; Fornasier, Flavio[e], Corti, Giuseppe[b]

[a]Department of Rural Engineering, Faculty of Agronomy and Forestry Engineering, Universidade Eduardo Mondlane, 257, Maputo, Mozambique

[b]Department of Agriculture, Food and Environmental Sciences, Università Politecnica delle Marche, 60131 Ancona, Italy

[c]Department of Soil Science and Agricultural Chemistry, Universidad de Santiago de Compostela, 27002 Lugo, Spain

[d]Department of Materials, Environmental Sciences and Urban Planning, Università Politecnica delle Marche, 60131 Ancona, Italy

[e] Consiglio per la ricerca in Agricoltura e l'Analisi della Economia Agraria Centro di Ricerca Viticoltura ed Enologia, Gorizia, Italy

[f]Instituto de Biodiversidade Agraria e Desenvolvimento Rural, Universidad de Santiago de Compostela, 27002 Lugo, Spain

*Corresponding author: rogerborguete@gmail.com;

Other authors: mluisa.fernandez@usc.es; s.cocco@univpm.it; m.l.ruello@univpm.it; flavio.fornasier@crea.gov.it; g.corti@univpm.it

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Abstract

Fertilization is required for optimum plant growth, particularly in unfertile soils, while optimizing the nutrient use efficiency is an alternative to reduce inorganic fertilizer needs and reduce environment problems due nutrient leaching. This study investigated soil properties and cowpea yield responses to the application of biochars from different feedstocks in combination with NPK fertilizer. Eight treatments (Control; full dose of NPK (12-24-12) + urea (46% N) fertilizers; baby corn peel (BC1), tree branch (BC2), and rice husk (BC3) biochars applied at rate of 0.9 % (w:w); each type of biochar was combined with 1/2 and full dose of fertilizers) were assessed in a greenhouse experiment with an acid Arenosol for 70 d. Soil pH increased significantly in treatments with BC1 and BC2, while CEC and available P were higher in the treatments with BC1. BC1 and BC2 also induced higher activity of enzymes related with P and higher cowpea yield. Similar soil properties and cowpea yield parameters were obtained with the full and half dose of NPK fertilizer for each type of biochar. Biochar in combination with NPK fertilizer improved soil chemistry and enzymatic activities, allowing decrease fertilizer application and food production costs in acid soils.

Key words: Biochar, cowpea growth, acid soil, soil fertility, potential enzyme activity

1. Introduction

Acid Arenosols are problematic for crop growth due to their low nutrient content and retention capacity, and limited water holding capacity. Scarcity of organic matter combined with nutrient leaching and depletion are the main causes of the low quality in acid Arenosols (Atkinson et al., 2010; Agegnehu et al., 2016a). Given low physicochemical attributes in such soils, they require sustainable practices to improve medium to long term soil fertility (Syers et al., 2011; Marenya et al., 2012). Also substantial N, P, and K inputs are required to obtain optimal production (Martins et al., 2014). As these soils represent 22% of African soils (Jones et al., 2013) and are often used for agricultural production, improvement in their fertility is of paramount importance for regional food security.

Optimizing fertilizer use efficiency and developing more efficient fertilizing strategies, including the use of less expensive nutrient sources such as waste materials and biomass, while preventing environmental impacts due to groundwater leaching, is an alternative to reduce the need for inorganic fertilizers (Blackwell et al., 2015; Wen et al., 2016). Additionally, the introduction of biochar as an organic amendment in these systems would help to increase fertilizer efficiency and crop productivity (Waters et al., 2011; Dari et al., 2016), while contributing to long-term soil C sequestration (Atkinson et al., 2010; Ameloot et al., 2013; Bamminger et al., 2014; Awasthi et al., 2016).

Biochar is a solid product obtained by pyrolysis of biomass at 300 to 600 ^{o}C in an oxygen-lacking system. Biochar contains 40 to 80% C, which is mainly represented by aromatic compounds, and has high surface area, porosity, and variable charge (Anawar et al., 2015; Blackwell et al., 2015). Biochar is considered a valuable resource for problematic soils such as acid Arenosols, as it may help the soil to retain nutrients (Uchimiya et al., 2011; Anawar et al., 2015), improve soil biological properties (Bamminger et al., 2014; Chen et al., 2015), and increases soil pH (Atkinson et al., 2010; Anderson et al., 2011; Anderson et al., 2014). By increasing soil pH, biochar improves nutrient availability to plants (Agegnehu et al., 2015, 2016 a, b; Anawar et al., 2015). For instance, Molnár et al. (2015) reported an improvement of soil chemical and biological properties after application of biochar in combination with NPK fertilizer in an acid Arenosol. Rice husk biochar improved soil pH, available P, cation exchange capacity and exchangeable K and Ca in acid soil (Masulili et al., 2010). Agegnehu et al. (2016a) noted that the agronomic efficiency, apparent recovery efficiency, and physiological efficiency on barley (*Hordeum vulgare* L.) increased significantly upon application of biochar and NPK fertlizers in a Nitisol. Biochar applied together with inorganic fertilizers facilitated decrease application rates, improving plant growth and nutrition, and increasing the soil biological fertility

(Uchimiya et al., 2010; Blackwell et al., 2015; Wen et al., 2016). Most of the biochar properties depend upon the pyrolysis temperature and the type of feedstock (Atkinson et al., 2010; Anawar et al., 2015; Molnár et al., 2015), while the soil-biochar interactions depend on soil properties and biochar particle size (Anawar et al., 2015; Solomain and Anawar, 2015). Biochar from diverse feedstocks may differently affect soil properties, and subsequently the crop yield (Waters et al., 2011). Because of this, the extent to which biochars can reduce the inorganic fertilizer rate while obtaining similar yields to those obtained with recommend rates needs to be investigated, since a reduced fertilizer application has a positive impact on both environment and social issues, especially for poor countries and farmers.

The objective of this study was to determine the effect of biochars in combination with NPK fertilizer on: *i*) chemical and biochemical properties of an acid Arenosol; *ii*) N, P, and K uptake and use efficiency by cowpea (*Vigna unguiculata* L.); and *iii*) cowpea growth and yield. We hypothesized that biochars will affect soil properties which will increase NPK fertilizer efficiency and cowpea yield, while reducing environmental problems due the leaching of nutrients. Nutrient contents and recalcitrance of biochars will determine their interaction with soil and influence on plant growth.

2. Materials and methods

2.1. Soil sampling and inputs preparation

An acid Arenosol was used for the pot experiments. Soil was collected from the A horizon (0-0.2 m) of an open scrubland located in southern Mozambique (25.72577º S, 032.64835º E). Prior to use, the soil was air dried and sieved to remove gravels and roots ≥ 2 mm. Babycorn (*Zea mays* L.) peel, mango tree branch (*Mangifera indica* L.) and rice (*Oryza sativa* L.) husk were used as feedstocks for biochar production using a low-cost handmade pyrolyser made of two concentric cylinders. The pyrolysable material was placed in the inner cylinder, while firewood was burnt in the outer cylinder providing energy to heat the inner cylinder. The pyrolysable material was heated for 4 h, and the temperature reached 450ºC. After the combustion stopped, the biochar was washed out twice with distilled water to eliminate excess ash, then oven dried for 48 h at 60ºC. The biochar was then ground using a mortar to obtain particles from 0.063 to 0.25 mm in size.

NPK commercial fertilizer (12-24-12) was used as N, P, and K source for basal fertilization, and urea (46% N) was used for N top dressing fertilization. A nutrient solution adapted from Vanek and

Lehmann (2014) [1 mmol L^{-1} $MgSO_4$, 25 µmol L^{-1} H_3BO_3, 2 µmol L^{-1} $MnSO_4$, 2 µmol L^{-1} $ZnSO_4$, 0.5 µmol L^{-1} $CuSO_4$, 0.5 µmol L^{-1} $(NH_4)_6Mo_7O_{24}$] was used to replenish other essential nutrients.

2.2. Pot experiments

A greenhouse pot trial was carried out using a completely randomized design with eight treatments and three replicates. The following treatments were assessed: 1) Control, with no treatment (C); 2) NPK (12-24-12) fertilizer + urea (46%) at rates of 830 mg pot^{-1} (~ 400 kg ha^{-1}) and 217 mg pot^{-1} (~ 104.3 kg ha^{-1}), respectively; 3) baby corn peel biochar + 415 mg pot^{-1} of NPK + 108.5 mg pot^{-1} of urea (BC1+1/2NPK); 4) baby corn peel biochar + 830 mg pot^{-1} of NPK + 217 mg pot^{-1} of urea (BC1+NPK); 5) mango tree branch biochar + 415 mg pot^{-1} of NPK + 108.5 mg pot^{-1} of urea (BC2+1/2NPK); 6) mango tree branch biochar + 830 mg pot^{-1} of NPK + 217 mg pot^{-1} of urea (BC2+NPK); 7) rice husk biochar + 415 mg pot^{-1} of NPK + 108.5 mg pot^{-1} of urea (BC3+1/2NPK); 8) rice husk biochar + 830 mg pot^{-1} of NPK + 217 mg pot^{-1} of urea (BC3+NPK).

The pots were prepared with 17 L plastic buckets with a diameter of 30 cm. At the bottom three small holes of 10 mm diameter each were opened to avoid stagnant water. In each pot 6 kg of soil was placed at the base, and this was overlaid by a homogeneous mixture of 4 kg soil with the corresponding treatment. All biochars were applied at 0.9 % (w:w), equivalent to 27 ton ha^{-1} for a soil depth of 0.2 m with a bulk density of 1500 kg m^{-3}. The full dose of NPK fertilizer corresponded to 99.6 mg pot^{-1} N, 199.2 mg pot^{-1} P_2O_5, and 99.6 mg pot^{-1} K_2O, equivalent to 75 kg N ha^{-1}, 65 kg P ha^{-1}, and 62 kg K ha^{-1}, respectively.

The pots were pre-incubated for 10 d to avoid possible negative effects of biochar volatile substances on plant growth (Vanek and Lehmann, 2014, Dutta et al., 2016). During the pre-incubation period, each pot was irrigated five times with 500 mL of distilled water each time. After this period, basal fertilization with NPK was performed and three cowpea seeds (IT98K-1105-5 early maturity cultivar) were sown per pot on February 26th, 2016; after emergence, only one plant was left per pot. During the early growing stage (60 d) the soil temperature ranged from 32.0 ± 4.2 to 34.2 ± 5.6 ºC, and pots were watered with 500 mL of distilled water once per week, and ferti-irrigated every two d with 300 mL of nutrient solution. Thereafter, the watering frequency was increased to three times per week due the higher water demand by the plants in the growing vegetative stage. Pest Control was made once per week by alternating fungicide Amistar Top (active substances: azoxystrobin and difenoconazole) + insecticide/acaricide Agromectin 1.8 EC (active substance: abamectin) with fungicide Ridomil Gold (active susbstances: metalaxyl + mancozeb) + insecticide/acaricide HITCEL

44% EC (active substances: profenofos + cypermethrin). Plants were harvested 60 d after emergence when they were fruiting. Dry (60 ºC) total biomass and shoot weight were determined. Then, soil samples were collected separating rhizosphere and bulk soil, considering rhizosphere the soil adhering to the roots (Cocco et al., 2013).

2.3. Physicochemical properties of soil, plant, and biochar

Prior to the experiments, the soil particle-size distribution was determined by the pipette method (Day, 1965) after the soil was disaggregated in distilled water for 24 h. Soil (1:2.5 w:v) and biochar (1:100 w:v) pH were determined in water and KCl (1 M solution) suspensions by a combined glass-calomel electrode. For biochar samples, the suspensions in water or KCl were heated in a water bath to 90 °C, stirred for 20 min to allow dissolution of the soluble components, and cooled to room temperature before pH determination (Ahmedna et al., 1997).

Total carbon (TC), total nitrogen (TN) and total sulphur (TS) in soil, biochar, and plant samples were determined using a CHNS-O analyser (EA1110, Carlo Erba Instruments, Milan, Italy). The total content of P, Ca, Mg, K, Na, Fe, Mn and Cu in plants and biochar were determined by dry ashing 0.5 g plant or biochar at 500 ºC for 16 h in a muffle furnace and dissolving the ash in a 5 M HCl solution (Lambert, 1976). A simple colorimetric method based on ascorbic acid reduction of the ammonium phosphomolybdate complex was used to measure P in the solutions (Kuo, 1996). The concentration of Ca, Mg, K, Na, Fe, Mn, and Cu were measured by inductively coupled plasma optical emission spectrometry (ICP-OES) on a Perkin Elmer Optima 8300 spectrometer (in Ancona, Italy) as described by Boss and Fredeen (1997).

The Walkley-Black method was used to estimate easily oxidizable organic carbon (EOOC) in soil and biochar (Pansu and Gautheyrou, 2006). Mehlich 3 reagent (Mehlich, 1984) was used to extract available P from soil samples. Extractable P of biochar was determined by water, 2% citric acid, and 2% formic acid extractants using a suspension of 0.1000 g of sample and 30 mL of extractant, shaken at 120 rpm for 48 h in 50 mL centrifuge tubes. The suspension was then centrifuged (~ 2700 *g*, 15 min) and the supernatant filtered using Whatman No. 42 filter paper prior to colorimetric determination (Zhang et al., 2016). Cation exchange capacity (CEC) and exchangeable cations in soil and biochar were determined by the $BaCl_2$ method (Gillman and Sumpter, 1986). Biochar volatile matter was determined by weight loss after heating; the muffle furnace was set to 950 ºC, then samples were heated for 2 min on the outer ledge of the furnace (300 ºC) with the door open, and then for 3 min on the edge of the furnace (500 ºC) (Zhao et al., 2015). The biochar ash content was

determined as the remaining weight at 750 ºC (Jindo et al., 2014).

2.4. Soil biochemical and biological properties

Potential enzyme activities in soil were determined as per Fornasier and Margon (2007). Enzymes were desorbed by heteromolecular exchange using an excess of exogenous protein. As such, 250 mg soil samples were placed in 2-mL Eppendorf tubes with glass beads and 1 mL of 50 mM tris–HCl buffer solution at pH 7.5, containing lysozyme as the desorbing protein. The tube was subjected to bead-beating (3 min, 30 strokes s^{-1}) using a Retsch MM400 mill, then centrifuged for 5 min at 20,000 *g*. Enzyme activity was assayed fluorometrically in microplates using 4-methyl-umbelliferyl and L-Leucine-7-amino-4-methylcoumarine derivatives. The activities of arylsulfatase, chitinase, glucuronidase, α-glucosidase, β-glucosidase, cellulase, xylosidase, acid phosphomonoesterase, phosphodiesterase, pyrophosphatase-phosphodiesterase, and alkaline phosphomonoesterase were determined in 200 mM morpholineethanesulfonic acid solution at pH 6, and the activity of leucine aminopeptidase (leu) in 50 mM tris–HCl buffer solution at pH 7.5.

Double-strand DNA (dsDNA) was determined on 500 mg soil aliquots that were placed in 2-mL Eppendorf tubes glass beads and 1 mL of 0.12 M sodium phosphate buffer (pH 8.0) solution. The tubes were submitted to bead-beating (2 min, 30 strokes s^{-1}) and then centrifuged for 5 min at 20,000 *g*. The dsDNA content was quantified on aliquots of the supernatant by fluorometry using PicoGreen1 reagent (Life Technologies) according to the instructions of the manufacturer. Root nodules visible at naked eye were counted per each plant.

2.5. Calculation of N, P, and K use efficiency

Nutrients taken up by roots, shoots (stem + leaves), and pods are expressed in mg pot^{-1} and were calculated by multiplying nutrient concentrations (mg kg^{-1}) in roots, shoots and pods by their respective yield. Nitrogen, P, and K harvest indexes (HI) were calculated as follows:

$$HI\ (\%) = \frac{NPU}{TNU} \times 100,$$

where: NPU – nutrient uptake (mg pot^{-1}) in pods; TNU – total nutrient uptake (mg pot^{-1}) by the plant (pod + shoot) (Agegnehu et al., 2016a).

Fertilizer N-, P-, and K-use efficiency was expressed by four indexes:

i) Agronomic efficiency (AE) is defined as the grain production per unit of applied N, P and K, and was calculated as follows:

$$AE\ (g\ g^{-1}) = \frac{GY_{f-}\ GY_u}{N_a},$$

where: GY_f is the pod yield of the fertilized pot (g), GYu is the pod yield of the unfertilized pot (g) for each replicate, and N_a is the quantity of N, P, and K applied as biochar, NPK fertilizer, and urea (g) (Agegnehu et al., 2016a).

ii) Apparent N, P, and K recovery efficiency (ARE), also called the N, P, and K uptake efficiency, was calculated as follows:

$$ARE\ (\%) = \frac{N_f - N_u}{N_a} \times 100,$$

where: N_f is the N, P, and K uptake (pod + shoot) of fertilized pot (g), N_u is the N, P, and K uptake (pod + shoot) of the unfertilized pot (g) for each replicate, and N_a is the quantity of applied N, P, and K (g) (Agegnehu et al., 2016a).

iii) Physiological efficiency (PE) expresses the ability of the plant to transform N, P, and K absorbed from the fertilizer into grain yield, and was calculated as follows:

$$PE\ (gg^{-1}) = \frac{GY_f - GY_u}{N_f - N_u},$$

where: GY_f is the pod yield of the fertilized pot (g), GYu is the pod yield of the unfertilized pot (g) for each replicate, N_f is the N, P, and K uptake (pod + shoot) of fertilized pot (g), N_u is the N, P, K uptake (pod + shoot) of the unfertilized pot (g) for each replicate (Agegnehu et al., 2016a).

iv) The N, P, and K translocation factor defined as the ratio of N, P, and K concentration in the shoots (pod or leaves or stem) to those in the roots was calculated as follows (Liu et al., 2014):

$$TF = \frac{N_{shoots}}{N_{roots}},$$

where N_{shoots} and N_{roots} are N, P, and K concentration (mg kg^{-1}) in the shoots and roots, respectively. TF > 1 indicated that the plant translocates N, P, and K effectively from the roots to the shoots (pod or leaves or stem).

2.6. Statistical analysis

R version 3.1.2 (2014-10-31) was used for statistical analysis. The experimental data were analysed by analysis of variance (ANOVA), after a *boxcox* transformation (Meloun et al., 2005) of the data to perform parametric tests (Shapiro-Wilk normality test and Bartlett test of homogeneity of variances) when necessary. A multiple comparison Duncan test (at 95% significance level) was used

to compare the means. Principal components analysis (PCA) was performed to identify the variables capable of explaining most of the variability and correlation among potential enzyme activities, chemical parameters and cowpea yield determined response of treatments and rhizosphere soil processes.

3. Results

3.1. Characteristics of soil and inputs used in the experiments

The soil used in the experiment was sandy, strongly acid, and low in TOC content, available nutrients and CEC (Table 1). The deficiency of nutrients and the very low CEC and water retention capacity are the main cause of the infertility of such soils. The three biochars differed significantly for their physicochemical properties, except for base saturation that always ranged from 99 to 100% (Table I). Baby corn peel biochar (BC1) and mango tree branch biochar (BC2) displayed strongly alkaline pH, while rice husk biochar (BC3) had a pH close to neutrality. Among the biochars, BC2 showed a moisture higher than BC3 and the highest content of ash, while BC3 had the highest volatile matter content. BC1 and BC2 contained large amounts of TOC, but BC1 presented higher content of TOC, TN, TS, total phosphorus (TP), extractable P (water – P, 2% citric acid P, and 2% formic acid P), exchangeable K, and CEC.

3.2. Soil chemical properties after the treatments

The treatments significantly affected pH in H_2O and KCl, available P, CEC, exchangeable Ca, Mg, K, and Al, and base saturation (Table 2). Conversely, the TOC, EOOC, and exchangeable Na were not affected. Soil pH, available P, and CEC were higher and exchangeable Al was lower than the Control in all treatments with BC1 and BC2. In contrast, inorganic fertilization alone did not produce any significant effect on the soil, while combinations with BC3 affected only base saturation. For each type of biochar, both the full and half dose of NPK did not produce differences in terms of available macronutrients or CEC, while pH was lower in the BC1+NPK treatment than in BC1+1/2NPK. Both CEC and available P appeared to be directly supplied by BC1, whose application resulted in the highest CEC and available P, especially with the full dose of NPK fertilizer.

3.3. Soil biochemical and biological properties after the treatments

Significant impact of the treatments was only observed for xylosidase, acid phosphomonoesterase, alkaline phosphomonoesterase, phosphodiesterase, and pyrophosphate phosphodiesterase (Table 2). The effect of biochar depended on the enzyme activity considered and the type of biochar. The xylosidase activity was the lowest in the Control and in BC3+1/2NPK treatment, while application of NPK alone or treatments including tree branch biochar resulted in the highest activity of this enzyme. The highest acid phosphomonoesterase activity occurred in the NPK treatment, while the lowest activity of this enzyme occurred in both treatments with BC1 and in the BC3+1/2NPK treatment. For this enzyme, treatments including biochar did not differ significantly from the Control.

In contrast, the highest alkaline phosphomonoesterase and phosphodiesterase activities occurred in BC1+1/2NPK and BC2+NPK treatments and the lowest in BC3+1/2NPK treatment. The pyrophosphatase-phosphodiesterase activity was highest in BC2+NPK treatment and lowest in BC3+1/2NPK treatment. The dsDNA concentration was higher in BC3+NPK treatment than in all the other treatments with biochar (Table 2). With respect to the Control, cowpea nodulation showed an increase of about 4-7 fold in the treatments with biochar, while nodulation was not significantly improved by inorganic fertilizers alone.

3.4. Cowpea growth and yield

Almost all treatments significantly improved both cowpea growth and yield parameters (Table 3). Exceptions were the BC2+1/2NPK and BC3+1/2NPK treatments, which failed to produce pods. In general, the treatments with biochar showed higher dry biomass, root, shoot, and pods, number of leaves, plant height, and plant diameter than the Control, but also higher dry biomass and pod yield than NPK treatment. Application of BC1+NPK increased the production of dry biomass by roughly 4-5 times with respect to the Control, and 70% with respect to NPK treatment. In addition, dry pod yields increased by at least 783 and 121% with BC1 and BC2 (except BC2+1/2NPK) compared with the Control and NPK treatments, respectively. However, the NPK treatment increased the pod yield by 300% compared to the Control. Plant height, stem diameter, and number of leaves were similarly affected by all treatments. Apparently, these parameters respond positively to inorganic fertilization.

Table 1
Main physic-chemical and biological characteristics of acid Arenosol of Mozambique and biochars used in the experiment. Means followed by different lowercase letters differed significantly among biochars at $P < 0.05$ by Duncan multiple mean comparison test.

Parameters	Soil before the trial	Baby corn peel biochar (BC1)	Mango tree branches biochar (BC2)	Rice husk biochar (BC3)	ANOVA[a)]
Sand, g kg^{-1}	938.30 (3.55)[b)]	-	-	-	-
Silt, g kg^{-1}	45.50 (7.50)	-	-	-	-
Clay, g kg^{-1}	16.25 (6.25)	-	-	-	-
pH-H_2O	4.25 (0.21)	9.63 (0.07) a	9.26 (0.14) b	7.58 (0.06) c	***
pH-KCl	4.25 (0.04)	8.90 (0.17) a	8.70 (1.07) a	7.15 (0.03) b	**
Moisture, %	-	5.20 (1.67) ab	12.33 (7.09) a	3.40 (0.50) b	*
Volatile matter, %	-	78.00 (2.40) b	44.77 (9.09) c	90.10 (3.11) a	***
Ash, %	-	22.63 (2.66) b	52.77 (6.57) a	6.57 (2.70) c	***
EC, dS m^{-1}	0.03 (0.00)	0.86 (0.00) a	0.25 (0.00) b	0.16 (0.00) c	***
TOC, g kg^{-1}	22.35 (0.89)	579.37 (102.57) a	646.35 (36.39) a	372.62 (10.14) b	**
EOOC, g kg^{-1}	3.13 (0.78)	289.67 (27.04) a	132.70 (41.40) b	146.38 (4.99) b	***
TN, g kg-1	< LOD	27.08 (1.12) a	8.04 (0.45) b	6.04 (0.16) c	***
TS, g kg^{-1}	< LOD	53.90 (6.86) a	39.34 (2.22) b	19.47 (0.53) c	***
TP, g kg^{-1}	-	7.86 (0.97) a	3.35 (2.20) b	1.38 (0.19) c	***
Water-P, mg kg^{-1}	-	5425.72 (420.51) a	1088.76 (951.54) b	1313.61 (115.43) b	***
2% citric-P, mg kg^{-1}	-	2513.15 (132.96) a	213.67 (71.45) b	52.27 (3.95) c	***
2% formic-P, mg kg^{-1}	-	7997.70 (301.21) a	1321.50 (6.90) b	995.40 (82.03) c	***
Mehlich 3-P, mg kg^{-1}	6.59 (0.86)	-	-	-	-
Exch Ca, $cmol_c$ kg^{-1}	0.07 (0.01)	2.78 (0.22) b	14.31 (1.15) a	3.13 (1.00) b	***
Exch Mg, $cmol_c$ kg^{-1}	0.05 (0.02)	4.65 (0.37) a	2.13 (0.10) b	3.95 (1.11) a	***
Exch K, $cmol_c$ kg^{-1}	0.08 (0.03)	170.16 (17.33) a	23.69 (1.03) b	29.79 (7.72) b	***
Exch Na, $cmol_c$ kg^{-1}	0.00 (0.00)	1.41 (0.12) b	4.56 (0.19) a	1.64 (0.18) bc	***
Exch Al, $cmol_c$ kg^{-1}	0.29 (0.02)	0.0 (-) c	0.28 (0.21) a	0.03 (0.05) b	***
CEC, $cmol_c$ kg^{-1}	0.49 (0.06)	179.00 (17.95) a	44.97 (2.57) b	38.55 (9.90) b	***
Base saturation, %	40.82 (10.90)	100.00 (0.00)	99.37 (0.42)	99.93 (0.12)	NS
AcP (nmol g^{-1} h^{-1})	16.22 (2.47)	-	-	-	-
AlkP (nmol g^{-1} h^{-1})	4.53 (0.35)	-	-	-	-
Cell (nmol $g^{-1}h^{-1}$)	0.18 (0.11)	-	-	-	-
α-G (nmol g^{-1} h^{-1})	0.49 (0.08)	-	-	-	-
AryS (nmol g^{-1} h^{-1})	0.94 (0.09)	-	-	-	-
β-G, (nmol g^{-1} h^{-1})	4.78 (0.49)	-	-	-	-
BisP (nmol g^{-1} h^{-1})	1.52 (0.19)	-	-	-	-
Chit (nmol g^{-1} h^{-1})	2.28 (0.38)	-	-	-	-
Leu (nmol g^{-1} h^{-1})	13.48 (1.37)	-	-	-	-
Nona (nmol g^{-1} h^{-1})	28.93 (3.65)	-	-	-	-
Pyro (nmol g^{-1} h^{-1})	0.34 (0.12)	-	-	-	-
Uroni (nmol g^{-1} h^{-1})	0.0 (-)	-	-	-	-
Xylo (nmol g^{-1} h^{-1})	1.21 (0.16)	-	-	-	-
dsDNA (μg g^{-1})	1.35 (0.19)	-	-	-	-

NS, *, **, *** Not significant, significant at $P = 0.05$, $P = 0.01$ and $P = 0.001$, respectively. [a)]Analysis of variance was made just for biochars samples. [b)]Numbers in parentheses are the standard deviation (n=3). EC: electrical conductivity; TOC: total organic carbon; TN: total nitrogen; TS: total sulphur; TP: total phosphorus; EOOC: easily oxidizable organic carbon; Water-P: available phosphorus extracted by distilled water; Mehlich 3-P: available phosphorus extracted by Mehlich 3 method; 2% citric-P: available phosphorus extracted by 2% citric acid solution; 2% formic-P: available phosphorus extracted by 2% formic acid solution; CEC: cation exchange capacity; Exch Ca: exchangeable Ca; AcP: acid phosphomonoesterase; AlkP: alkaline phosphomonoesterase; cell: cellulase; α-G: α-glucosidase; AryS: arylsulfatase; β-G: β-glucosidase; BisP: phosphodiesterase; Chit: chitinase; Leu: leucine aminopeptidase; Nona: nonanoate esterase; PyroP: pyrophosphate phosphodiesterase; Uroni: glucuronidase; Xylo: xyloxidase; dsDNA: double-strand DNA.<LOD: below the limit of detection.

Table 2

Treatments effect on soil chemical and biochemical properties after the pot experiments with acid Arenosol of Mozambique. Means followed by different lowercase letters differed significantly among treatments at $P < 0.05$ by Duncan multiple mean comparison test.

Chemical and biochemical parameters	Treatments								ANOVA
	Control	NPK	BC1+1/2NPK	BC1+NPK	BC2+1/2NPK	BC2+NPK	BC3+1/2NPK	BC3+NPK	
pH-H_2O	4.3 (0.01) [a)] c	4.4 (0.1) c	5.7 (0.2) a	5.2 (0.4) b	5.5 (0.2) ab	5.7 (0.4) ab	4.5 (0.1) c	4.4 (0.2) c	***
pH-KCl	4.0 (0.1) c	4.0 (0.1) c	5.0 (0.4) a	4.5 (0.2) b	4.8 (0.1) ab	5.1 (0.4) a	4.0 (0.1) c	4.0 (0.1) c	***
TOC, g kg^{-1}	20.6 (1.2)	21.7 (2.5)	21.9 (1.7)	22.8 (3.1)	24.6 (4.0)	23.9 (2.4)	21.9 (2.1)	23.0 (2.3)	NS
EOOC, g kg^{-1}	4.2 (1.8)	4.2 (0.8)	6.9 (2.6)	6.7 (2.0)	6.9 (1.8)	5.5 (3.0)	5.2 (1.9)	4.8 (1.6)	NS
Mehlich 3 - P, mg kg^{-1}	14.7 (5.7) c	31.8 (18.8) bc	93.8 (9.7) a	107.8 (19.6) a	55. 7 (4.9) b	41.2 (18.6) bc	33.5 (15.8) bc	50.5 (15.4) bc	***
Ca, $cmol_c$ kg^{-1}	0.1 (0.0) c	0.1 (0.0) c	0.2 (0.1) ab	0.2 (0.1) b	0.3 (0.0) a	0.4 (0.1) a	0.1 (0.0) c	0.1 (0.0) c	***
Mg, $cmol_c$ kg^{-1}	0.0 (0.0) e	0.1 (0.0) de	0.3 (0.1) ab	0.3 (0.1) a	0.1 (0.0) c	0.1 (0.1) bc	0.1 (0.1) c	0.1 (0.0) cd	***
K, $cmol_c$ kg^{-1}	0.1 (0.1) c	0.1 (0.0) c	0.5 (0.5) ab	0.6 (0.3) a	0.1 (0.0) c	0.1 (0.0) c	0.1 (0.1) b	0.1 (0.0) c	**
Na, $cmol_c$ kg^{-1}	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0 .0(0.0)	0.0 (0.0)	NS
Al, $cmol_c$ kg^{-1}	0.3 (0.1) a	0.2 (0.1) ab	0.0 (0.0) e	0.1 (0.0) d	0.1 (0.0) d	0.0 (0.0) d	0.2 (0.1) bc	0.2 (0.0) c	***
CEC, $cmol_c$ kg^{-1}	0.5 (0.2) c	0.4 (0.1) c	1.0 (0.5) ab	1.1 (0.4) a	0.6 (0.1) b	0.6 (0.2) b	0.6 (0.2) bc	0.4 (0.1) c	*
Base saturation, %	41.9 (5.6) c	46.4 (15.2) c	97.1 (1.3) a	93.2 (2.3) a	91.6 (2.2) a	93.0 (0.9) a	60.5 (7.5) b	61.7 (1.4) b	***
AryS, nmol g^{-1} h^{-1}	0.37 (0.26)	0.81 (0.12)	0.86 (0.09)	0.67 (0.14)	0.88 (0.32)	1.00 (0.18)	0.23 (0.11)	0.51(0.64)	NS
α - G, nmol g^{-1} h^{-1}	0.10 (0.11)	0.65 (0.09)	0.70 (0.32)	0.48 (0.17)	0.56 (0.47)	0.88 (0.19)	0.00 (0.00)	0.53 (0.69)	NS
β - G, nmol g^{-1} h^{-1}	2.30 (0.21)	5.51 (0.94)	3.52 (0.72)	3.71 (0.66)	5.06 (3.84)	6.05 (0.39)	2.12 (1.30)	4.67 (4.51)	NS
Cell, nmol g^{-1} h^{-1}	0.03 (0.05)	0.25 (0.08)	0.05 (0.05)	0.12 (0.15)	0.17 (0.29)	0.27 (0.05)	0.00 (0.00)	0.15 (0.27)	NS
Xylo, nmol g^{-1} h^{-1}	0.46 (0.09) c	1.29 (0.20) a	0.66 (0.07) b	0.6 (0.2) b	1.53 (1.29) a	1.16 (0.07) a	0.4 (0.1) c	1.01 (1.02) b	*
Uroni, nmol g^{-1} h^{-1}	0.06 (0.10)	0.08 (0.13)	0.27 (0.06)	0.20 (0.03)	0.24 (0.24)	0.25 (0.04)	0.00 (0.00)	0.11 (0.20)	NS
Chit, nmol g^{-1} h^{-1}	1.32 (0.62)	3.37 (0.65)	2.92 (1.19)	2.59 (1.26)	2.50 (1.99)	3.44 (0.19)	0.82 (0.38)	3.75 (3.80)	NS
Leu, nmol g^{-1} h^{-1}	4.66 (2.89)	13.53 (2.61)	13.17 (4.58)	10.56 (6.52)	11.85 (7.39)	19.05 (5.64)	2.13 (0.80)	11.10 (13.33)	NS
AcP, nmol g^{-1} h^{-1}	11.66 (2.24) ab	18.25 (2.06) a	6.90 (0.25) b	4.73 (1.68) b	11.96 (6.69) ab	12.80 (1.52) ab	5.70 (2.81) b	13.14 (10.15) ab	*
BisP, nmol g^{-1} h^{-1}	0.49 (0.45) bc	1.67 (0.27) abc	2.44 (0.59) a	1.18 (0.23) abc	2.13 (1.00) ab	2.81 (0.72) a	0.12 (0.20) c	1.72 (2.26) abc	*
Pyro, nmol g^{-1} h^{-1}	0.31 (0.39) bc	0.61 (0.23) ab	1.06 (0.45) ab	0.25 (0.14) bc	1.39 (1.32) ab	2.36 (1.10) a	0.03 (0.05) c	1.14 (1.82) abc	*
AlkP, nmol g^{-1} h^{-1}	3.35 (2.61) cd	9.28 (2.28) abc	30.79 (14.21) a	10.86 (2.69) abc	20.26 (9.91) ab	25.78 (8.16) a	1.97 (1.27) d	11.03 (14.85) bcd	**
Nona, nmol g^{-1} h^{-1}	8.92 (1.08)	24.47 (5.11)	9.87 (1.78)	8.38 (6.72)	12.45 (9.40)	10.57 (1.47)	7.24 (3.28)	21.40 (20.43)	NS
dsDNA, µg g^{-1}	2.80 (0.34) abc	3.98 (1.39) ab	2.33 (1.26) bc	1.55 (0.55) c	2.06 (0.69) c	1.28 (0.25) c	2.32 (1.27) bc	4.24 (1.23) a	*
Number of nodules per pot	29.3 (13.2) b	109.7 (49.9) ab	170.7 (40.1) a	200.7 (25.0) a	151.7 (82.8) a	143.8 (8.0) a	132.8 (42.9) a	130.3 (94.5) a	*

NS, *, **, *** Not significant, significant at $P = 0.05$, $P = 0.01$ and $P = 0.001$, respectively. [a)]Numbers in parentheses are the standard deviation (n=3). TOC: total organic carbon; EOOC: easily oxidizable organic carbon; Water-P: available phosphorus extracted by distilled water; Mehlich 3-P: available phosphorus extracted by Mehlich 3 method; CEC: cation exchange capacity; AcP: acid phosphomonoesterase; AlkP: alkaline phosphomonoesterase; Cell: cellulase; α-G: α-glucosidase; AryS: arylsulfatase; β-G: β-glucosidase; BisP: phosphodiesterase; Chit: chitinase; Leu: leucine aminopeptidase; Nona: nonanoate esterase; Pyro: pyrophosphate phosphodiesterase; Uroni: glucuronidase; Xylo: xyloxidase; dsDNA: double-strand DNA.

Principal component analysis (PCA) was run to assess the variation of soil properties and plant yield parameters as affected by the treatments, which contributed to 57.2% of the variation (Fig. 1). PC1 and PC2 explained 35.9 and 21.3% of the variation, respectively. The PCA biplot showed variations of the soil properties and plant yield parameters between the treatments (Fig. 1a). Several soil and plant parameters showed positive correlation with PC1, contributing for ~ 71.2% of the variability (Table S1 of Supplementary materials).

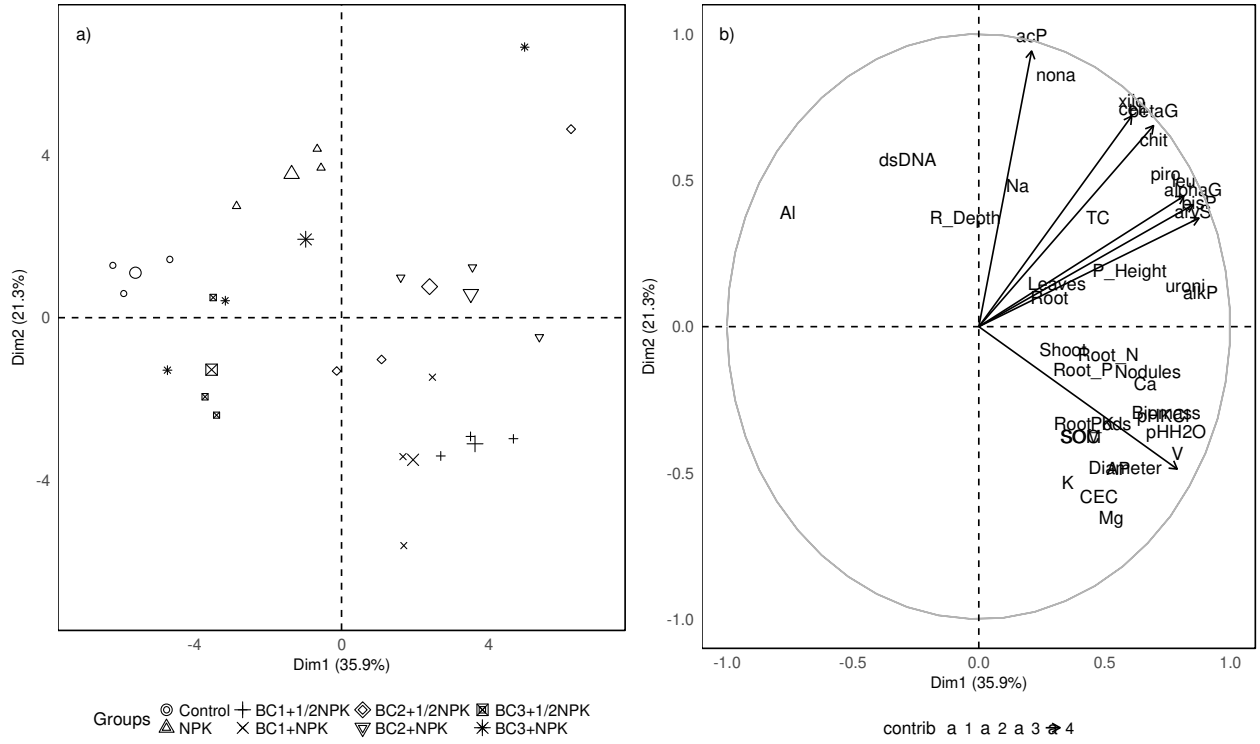

Fig. 1 Principal component analysis on soil and cowpea growth and yield parameters as affected by the treatments after the pot experiment with acid Arenosol of Mozambique; (a. Graph of individuals; b. Contribution of variables to PCA (Dim1 = PC1 and Dim2 = PC2)).

Table 3
Treatments effect on cowpea growth parameters and yield with acid Arenosol of Mozambique. Means followed by different lowercase letters differed significantly among treatments at $P < 0.05$ by Duncan multiple mean comparison test.

Cowpea yield	Treatments								ANOVA
	Control	NPK	BC1+1/2NPK	BC1+NPK	BC2+1/2NPK	BC2+NPK	BC3+1/2NPK	BC3+NPK	
Biomass, g pot^{-1}	6.5 (2.1) [a)] e	18.2 (1.5) d	29.5 (6.2) abc	32.1 (2.9) a	26.7 (2.9) abc	29.8 (6.6) ab	22.1 (4.5) cd	23.8 (0.6) bcd	***
Pod yield, g pot^{-1}	1.7 (0.5) c	6.8 (3.5) b	15.0 (5.1) a	19.5 (2.2) a	0.3 (0.3) c	16.7 (2.0) a	2.1 (2.7) c	11.4 (2.7) ab	***
Roots, g pot^{-1}	0.4 (0.1) c	1.7 (0.4) b	1.9 (0.7) b	1.8 (0.6) b	5.1 (0.2) a	2.6 (2.1) b	4.1 (1.8) ab	2.0 (0.9) b	***
Shoot[b)], g pot^{-1}	4.4 (2.3) c	9.7 (3.3) bc	12.5 (1.8) b	10.8 (1.1) bc	21.3 (2.7) a	10.5 (6.6) bc	16.0 (5.6) ab	10.3 (1.6) bc	***
Plant height, cm	20.3 (1.3) b	29.7 (5.7) a	28.3 (4.5) a	30.3 (2.5) a	29.7 (3.5) a	29.3 (0.6) a	26.3 (2.5) a	30.7 (3.8) a	*
Diameter of stem, cm	0.5 (0.0) b	0.6 (0.1) a	0.9 (0.1) a	0.9 (0.1) a	0.8 (0.1) a	0.7 (0.1) a	0.7 (0.1) a	0.7 (0.1) a	**
Number of leaves	12.0 (0.0) b	20.3 (5.0) a	27.0 (5.2) a	28.7 (4.0) a	21.7 (3.1) a	23.0 (8.2) a	21.7 (1.2) a	23.0 (4.4) a	*

*, **, *** Significant at $P = 0.05$, $P = 0.01$ and $P = 0.001$, respectively. [a)]Numbers in parentheses are the standard deviation (n=3). b) Shoot = stem + leaves.

These parameters were grouped into chemical (pH_{H2O}, pH_{KCl}, TOC, available P, exchangeable Ca and Al, and base saturation), biochemical (arylsulfatase, α-glucosidase, glucuronidase, leucine aminopeptidase, pyrophosphate phosphodiesterase, phosphodiesterase, alkaline phosphomonoesterase activities, and number of nodules), and cowpea yield (dry biomass, plant height, and N and P content in roots) properties. Conversely, parameters such as CEC, β-glucosidase, cellulase, xylosidase, chitinase, acid phosphomonoesterase, nonanoate esterase activities, and dsDNA showed positive correlation with PC2 contributing ~ 48.1% of the variability (Table S1 of Supplementary materials). Moreover, strong and positive correlation was found among available P, alkaline phosphomonoesterase and glucuronidase activities, dry biomass and pods, while negative correlation was found among available P, cellulase, xylosidase, acid phosphomonoesterase, nonanoate esterase activities, and dsDNA.

In addition, a strong and positive correlation was found among CEC, chitinase and xylosidase activities, while negative correlation was found among CEC, cellulase, β-glucosidase, acid phosphomonoesterase, nonanoate esterase activities, and dsDNA (Fig. 1b). These results suggested the occurrence of a synergistic effect between NPK fertilizer and biochar, in particular with BC1 and BC2, which showed higher values of the aforementioned parameters (Fig. 1a).

3.5. Macronutrient uptake by cowpea

Both treatment and plant portion (root, shoot or pod) affected (P<0.05) the elemental (C, N, P, S, K, Ca, and Mg) contents in the vegetal tissues (Table 4). Moreover, significant interaction between plant portion and treatment was found. For the Control, NPK, both BC3, BC1+1/2NPK and BC2+1/2NPK treatments, the highest contents of most macronutrients were found in shoots. In the BC1+NPK and BC2+NPK treatments, the highest contents of most macronutrients were found in pods. Concerning singular nutrients, N was often accumulated in pods, K and Mg in shoots, and Ca in shoots. Treatments significantly (P<0.01) influenced the contents of all macronutrients in cowpea shoots and pods, and all nutrients but C in roots. Inorganic fertilization led to the highest allocation of C, S, P, Ca, and Mg in shoots and, only for P, in roots. Treatments with BC3 were not significantly different from NPK contents for C, S, and Mg in shoots and for P in roots.

Table 4
Plant nutrient contents (mg pot^{-1}) in cowpea after greenhouse experiment with acid Arenosol of Mozambique. Means followed by different lowercase letters differed significantly among treatments at $P < 0.05$ by Duncan multiple mean comparison test.

Plant nutrient contents		Treatments								ANOVA		
		Control	NPK	BC1+1/2NPK	BC1+NPK	BC2+1/2NPK	BC2+NPK	BC3+1/2NPK	BC3+NPK	Plant	Treatment	Interaction
C	Pod	447 (172) de[a)]	2470 (1140) c	3842 (395) c	5786 (217) b	87 (150.3) f	4826 (181) b	735 (937) e	3353(210) c	***	**	***
	Root	711 (293) cd	1449 (396) c	1584 (182) c	953 (514) d	3775 (526) c	990 (1019) cd	1872 (584) c	1465 (618) c			
	Shoot[b)]	890 (45) d	10034 (3407) a	6005 (1219) b	2336 (275) c	4608 (878) b	3056 (1005) c	9935 (2869) a	7089 (950) ab			
N	Pod	29 (5) g	181 (78) d	370 (13) b	480 (47) ab	5 (9) h	362 (64) b	43 (49) g	246 (42) d	*	**	***
	Root	36 (19) gh	74 (10) d	111 (3) d	71 (36) de	198 (55) cd	60 (62) e	111 (22) d	79 (49) ef			
	Shoot	35 (18) gh	266 (181) d	300 (115) c	96 (22) d	272 (83) cd	98 (30) d	551 (300) a	144 (51) d			
S	Pod	77 (33) e	485 (232) cd	720 (107) c	1016 (101) ab	16 (27) g	865 (158) b	142 (187) f	662 (40) c	***	**	***
	Root	121 (57) ef	271 (34) cd	272 (32) cd	160 (83) d	709 (131) c	143 (156) de	332 (135) cd	255 (109) cd			
	Shoot	160 (18) d	1596 (526) a	1011 (210) ab	420 (68) cd	911 (97) b	505 (107) cd	1476 (462) a	1244 (189) a			
P	Pod	5 (2) ef	34(19) c	74 (5) ab	103 (7) a	1 (1) g	47 (7) b	10 (14) f	39 (4) b	*	***	***
	Root	5 (3) ef	21 (1) c	36 (7) c	15 (11) de	33 (10) c	10 (7) e	24 (8) c	20 (11) c			
	Shoot	2 (1) f	118 (100) a	93 (29) a	36 (20) c	30 (9) c	21 (15) c	58 (34) b	25 (12) c			
K	Pod	14 (13) g	52 (20) e	161 (60) c	134 (92) d	3 (6) j	145 (43) cd	20 (23) h	89 (76) e	***	***	***
	Root	17 (17) gh	19 (10) de	107 (35) d	24 (23) de	67 (25) ef	25 (23) de	50 (19) fg	21 (13) f			
	Shoot	21 (20) i	230 (73) b	642 (248) a	362 (132) b	103 (28) d	116 (33) d	163 (36) c	511 (46) ab			
Ca	Pod	2 (1) h	5 (1) gh	4 (1) gh	6 (6) fg	2 (3) i	21 (5) d	3 (3) gh	17 (4) de	***	***	**
	Root	4 (3) h	6 (5) gh	7 (1) gh	4 (3) fg	20 (13) de	16 (13) de	9 (3) de	6 (3) e			
	Shoot	18 (19) de	374 (135) a	65 (39) bc	39 (14) c	121 (62) b	180 (46) ab	61 (33) bc	203 (69) ab			
Mg	Pod	4 (3) ef	16 (4) c	39 (13) b	33 (22) bc	1 (2) g	37 (13) bc	7 (7) f	20 (12) c	***	**	***
	Root	4 (4) ef	8 (5) d	9 (2) d	4 (3) de	23 (7) c	10 (12) d	15 (6) c	7 (4) d			
	Shoot	10 (8) d	212 (80) a	44 (31) b	22 (9) c	46 (18) b	32 (12) bc	46 (21) b	82 (30) ab			

*, **, *** Significant at $P = 0.05$, $P = 0.01$ and $P = 0.001$, respectively. [a)]Numbers in parentheses are the standard deviation (n=3). [b)]Shoot = stem + leaves.

The highest contents of macronutrients in pods were found in treatments with BC1 or BC2. The highest contents of macronutrients in roots and shoots were found in the treatments with biochar. In most cases, the macronutrient contents in pods for the combined treatments (biochar + NPK) were higher in the treatments with the full NPK dose, and higher than for NPK alone, indicating positive effects of the biochar and NPK combination. Conversely, the macronutrient contents in shoots and roots often decreased with the full NPK dose; comparisons of Control, NPK, Biochar+1/2NPK, and Biochar+NPK suggested in some cases the occurrence of antagonism between BC1 or BC2 and NPK fertilization for macronutrient allocation in roots and shoots. For example, compared to the Control, N in shoots increased by 660% with the NPK treatment, by 757% with BC1+1/2NPK, and by only 174% with BC1+NPK.

The translocation of nutrients to pods is of paramount importance, as they are the edible plant parts. The BC1+NPK treatment, followed by BC2+NPK, gave the highest contents of C, N, S, and P in pods, while the BC1+1/2NPK treatment produced the highest contents of K and Mg in pods, and BC2+NPK gave the highest contents of Ca in pods. With respect to the other treatments, those including BC1 and BC2 showed higher accumulation of plant essential nutrients, particularly in pods, and this was likely favored by the higher dry biomass and root uptake stimulated by nodulation.

4. Discussion

4.1. Soil and biochar physicochemical properties

In this soil, liming and nutrient replenishment with inorganic fertilizers are useful to correct soil acidity and increase nutrient availability in the short term (Rosberg et al., 2006), but the problems of low CEC and water retention capacity remain. The application of biochar, besides providing certain nutrients, has a liming effect and gives rise to an increase of CEC and water retention capacity, all of which will increase crop yield. According to several authors, different properties among biochars depend on feedstock composition and pyrolysis conditions (Spokas et al., 2011; Enders et al., 2012; Lu et al., 2014; Fang et al., 2015). Since we used the same pyrolysis conditions throughout this study, differences among biochars were attributed to the nature of the feedstock. Enders et al. (2012) found that biochar produced using slow pyrolysis with corn stover feedstock at temperatures of 400 and 500 ºC had 18-20% ash, 45-63% TC, and 68-40% volatile matter, respectively; values are comparable to those found in our study. Volatile matter is related with stability to decomposition, as biochars with ~ 40% volatile matter can be decomposed in ~ 100 years, while those with volatile matter >80%

have zero C sequestration value (Enders et al., 2012). However, this parameter is highly variable and plays a role in plant and soil microoganism response to biochar amendment (Spokas et al., 2011). Following this rationale, BC3 has little use for C sequestration, BC1 is rather easily decomposable, and BC2 can be considered as the most recalcitrant biochar (Purakayastha et al., 2015).

Biochars were rich in C but also in plant nutrients, mainly P (Dari et al., 2016). In fact, biochar has high potential to release P to soil (Hall and Bell, 2015), but it can also increase soil N use efficiency (Zheng et al., 2013; Zhu et al., 2014; Agegnehu et al., 2016). The biochars obtained for this study represented sources of K and exchangeable sites (especially BC1), and because of this have great potential to increase soil CEC, improving soil nutrient retention (Liang et al., 2006). The reduction of soil acidity was ascribed to the alkalinity of the biochar, as reported by many authors (e.g. Chan et al., 2007; Zheng et al., 2013; Zhu et al., 2014; Dai et al., 2017), and attributed to carbonates and organic anions (Yuan et al., 2011a, b; Bera et al., 2016). An increase of available P and K by application of biochars to soil was previously reported by Tammeorg et al. (2014).

4.2. Soil biochemical and biological properties

This soil also showed generalized low potential enzyme activities, and this was likely due to the low EOOC content (Table I), as reported by many authors (e.g. Hannachi et al., 2015; Godin et al., 2015). However, even though most of the enzymes present in the soil are extracellular (Lu et al., 2015), it is well-known that the soil microbial community replies faster than other soil properties to any change able to modify the soil organic substrate or nutritional status (Sinsabaugh et al., 2002). Because of this, biochemical (potential enzyme activity and dsDNA content) and biological (cowpea nodulation) properties were used as indicators of soil changes (Allen et al., 2011; Bera et al., 2016).

Even though an improved soil potential enzyme activity via biochar amendment has been reported by many authors (e.g. Demisie et al., 2014; Lu et al., 2015; Pandey et al., 2016), in our case the overall impact of the treatment was limited. Bera et al. (2016) assessed biochar and manure effects together with NPK fertilizer and found a greater activity of acid phosphomonoesterase in NPK treatments without biochar application. The activities of phosphodiesterase, pyrophosphate phosphodiesterase, and alkaline phosphomonoesterase are well correlated with soil pH, as also reported by Chen et al. (2013), Purakayastha et al. (2015), and Bera et al. (2016). In fact, the lowest activity of these enzymes was found in the BC3+1/2NPK treatment, which had one of the lowest pH values in both H_2O and KCl, but the pyrophosphate phosphodiesterase and alkaline phosphomonoesterase activities were significantly lower in this treatment than in NPK treatment (with a similar pH).

The amount of dsDNA depends on soil microbial biomass content, and the addition of organic amendments (compost or manure) to soil has been reported to increase the content of dsDNA (Hannachi et al., 2015). In our study, the addition of BC1 and BC2 (the longer lasting biochars) reduced the dsDNA concentration, while the application of the easily decomposable biochar (BC3) with the full NPK dose maintained a dsDNA concentration similar to that of inorganic fertilizer and the Control. The different behaviour of the biochars was attributed to their labile C content. In fact, a lower content of labile C makes BC1 and BC2 less palatable for microorganisms because of the relatively high content of recalcitrant C, which has been demonstrated to be able to inhibit groups of the microbial community (Sun et al., 2014). Rice husk biochar is instead relatively labile and thereby enhances soil microbial activities (Purakayastha et al., 2015), so increasing the amount of dsDNA.

An increase of root nodulation in legumes by application of biochar has been previously reported by several authors (e.g. Agegnehu et al., 2015; Mete et al., 2015; Mollinedo et al., 2016). Several studies have also reported an increase of biological N fixation in the presence of biochar (e.g. Ogawa and Okimori, 2010; Lone et al., 2015). The mechanism responsible for the increase of nodulation in biochar amended soils depends on the finer structural pores of the biochar, which provide a favourable niche in which oxygen concentration declines and nitrogenase activity increases (Thies and Rilling, 2009). This hypothesis is supported by the high CEC of the biochar, which can exchange NH_4^+ with the soil solution, so modifying N availability to the plants and stimulating nodulation and fixation (Lone et al., 2015). Other mechanisms depend on the increase of available K (Lone et al., 2015), immobilization of inorganic N (Nelissen et al., 2012; Yang et al., 2017), and increase of P availability, which is correlated with the increase of pH in acid soils (Brewer et al., 2012; Lone et al., 2015).

The increase of pH in acid soils enhances nodulation and N fixation since *Rhizobium* increases its activity at neutral soil pHs (Lone et al., 2015). However, Revell et al. (2012) reported that a higher rate of biochar decreases nodulation efficiency. In our study, a significant correlation between Mehlich-3 P and the number of nodules was found, while for pH the correlation was not significant, even though the pH increase with respect to the Control and inorganic fertilizers was in parallel with the nodulation increase. Hence, the most plausible mechanisms able to explain the increased nodulation in all treatments with biochar was the improvement of the soil physicochemical conditions, which likely favoured the development of N-fixing bacteria (Mete et al., 2015; Lone et al., 2015). This may be ascribed to the favourable habitat for the growth of symbiont microorganisms provided by biochar (Lehman et al., 2011).

4.3. Cowpea yield in response to the treatments

Cowpea yields improved dramatically with biochar treatments. No differences in pod and root yields were found between complete and half rate NPK fertilizer in the BC1 treatments. BC1 and BC2 were mainly responsible for soil chemical and biochemical improvement, with the consequence on cowpea yields. Therefore, these results suggested that amendment with biochar together with chemical fertilizer offer an appropriate strategy to achieve higher crop productivity without compromising the soil quality (Pandey et al., 2016). Effects of the biochar application on crop yield have been reported previously by van Zwieten et al. (2010), Gregory et al. (2014), and Domene et al. (2015). This is likely due to the CEC generated by biochar, capable of retaining nutrients conveyed by inorganic fertilization. Also Mete et al. (2015) reported synergistic effects between biochar and NPK fertilizer on soybean yield.

Surprisingly, the BC2+1/2NPK treatment, which gave the highest shoot and root yields, failed to produce pods, indicating that nutrients were preferentially allocated in roots and shoots. The increase of cowpea yield with application of biochar is associated with the improvement of soil physicochemical and biochemical properties. The decrease of acidity together with the increase of available P and CEC were responsible for the increase of cowpea yield. However, water retention capacity, although not measured in this study, undoubtedly contributed to yield improvement. Pod yields were strongly and positively correlated with N, P, and K content, and root nodulation (Fig. 1b). Positive and strong correlations were also found among cowpea (dry biomass and pods) and soil parameters such as pH, CEC, exchangeable Ca, Mg, and K, available P, EOOC, glucuronidase, and alkaline phosphomonoesterase (Fig. 1b).

4.4. N, P, and K concentration and translocation by cowpea

The effect of treatments on N concentration of plant portions (root, shoot and pod) was not significant; however, significant interaction between treatments and plant parts was found. For P, there was a significant effect of treatment on plant concentration, but no significant effect of plant portion (root, shoot, and pod) was found. For K, the effect of treatment and plant portion was significant, but no significant interaction between treatment and plant part was found (Fig. 2). Higher P concentration was found in roots and pods of the treatments including BC1. The same treatments gave higher K concentration in plants. Choudhary and Kumar (2014) determined that N, P, and K uptake by cowpea is influenced by soil K and P levels, which induce root nodulation leading to more N uptake. BC1 featured high K and P concentrations which evidently contributed to N and P supply.

The translocation factors calculated for pods and shoots (stem + leaves) indicated effective translocation of N to pods in almost all treatments, and of K in some treatments, while P was effectively translocated to pods only in the Control (Fig. 3). In addition, an effective translocation was observed from roots to shoots for the three elements. High concentration of K in shoots can be considered as important to facilitate the translocation of other nutrients from the roots to the plant top (Conti and Geiger, 1982). Higher concentration of K in shoots indicates higher K uptake efficiency (Samal et al., 2010).

4.5. N, P, and K use efficiency by cowpea

The effect of treatments was significant (P<0.001) for N, P, and K agronomic efficiency, physiological efficiency, apparent recovery efficiency, and harvest index (with exception of K). Treatments including BC1 presented higher N agronomic efficiency, physiological efficiency, apparent recovery efficiency, and harvest index (Fig. 4). A higher N apparent recovery efficiency was found in the BC1+1/2NPK treatment. Higher P harvest index was found in the Control, BC1+NPK, and BC2+NPK treatments. Treatments like BC1, BC2+NPK, and BC3+NPK presented higher P agronomic efficiency, physiological efficiency, and apparent recovery efficiency. The BC1, BC2+NPK, BC3+NPK, and NPK treatments showed higher K physiological efficiency, while the BC2+NPK, BC3+NPK, and NPK treatments showed higher K agronomic efficiency and apparent recovery efficiency.

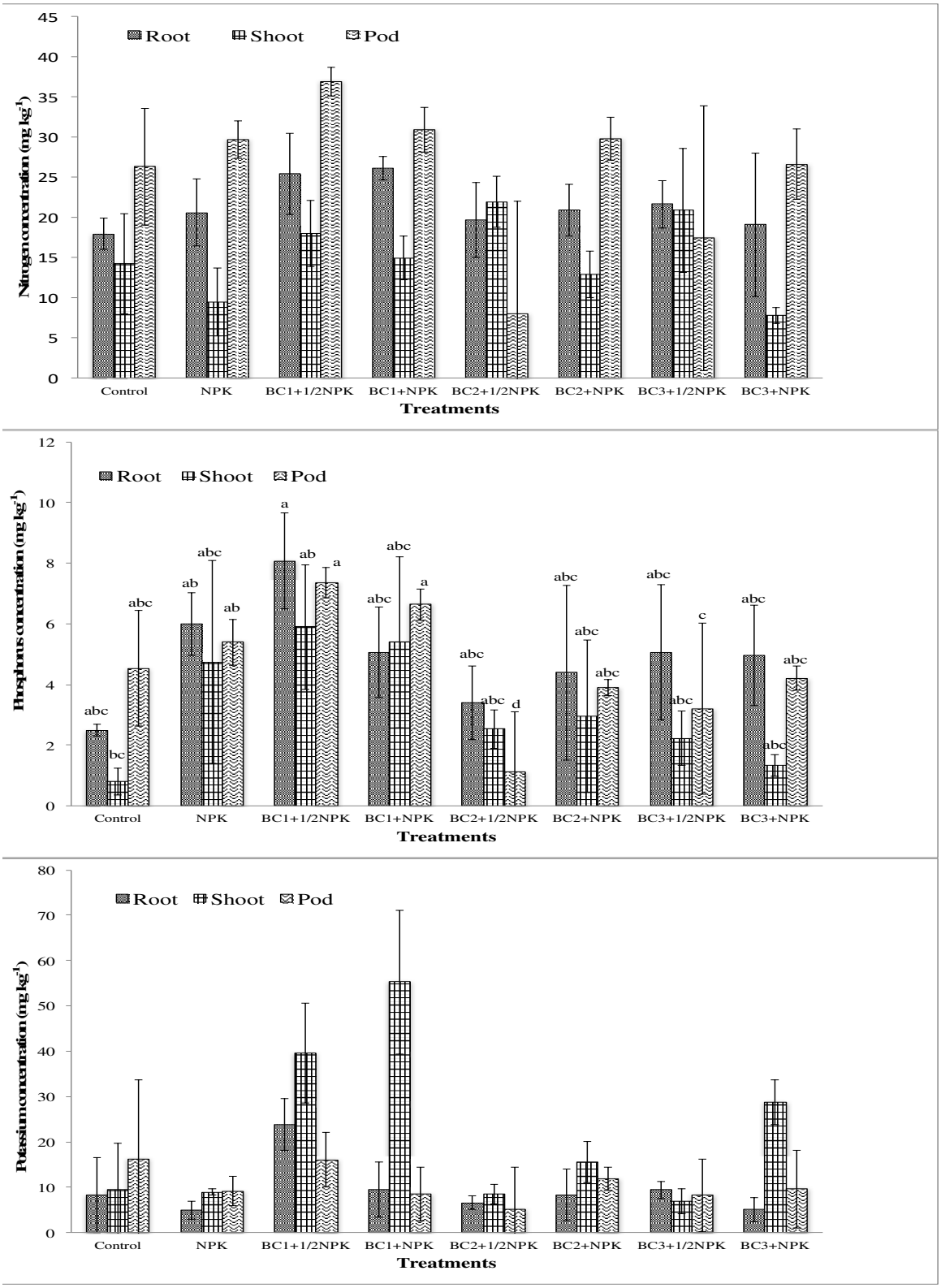

Fig. 2 N, P and K uptake (mg kg^{-1}) by cowpea after the pot experiment with acid Arenosol of Mozambique. Means followed by different lowercase letters differed significantly among treatments at $P < 0.05$ by Duncan multiple mean comparison test. The whiskers indicate the standard deviation (n=3).

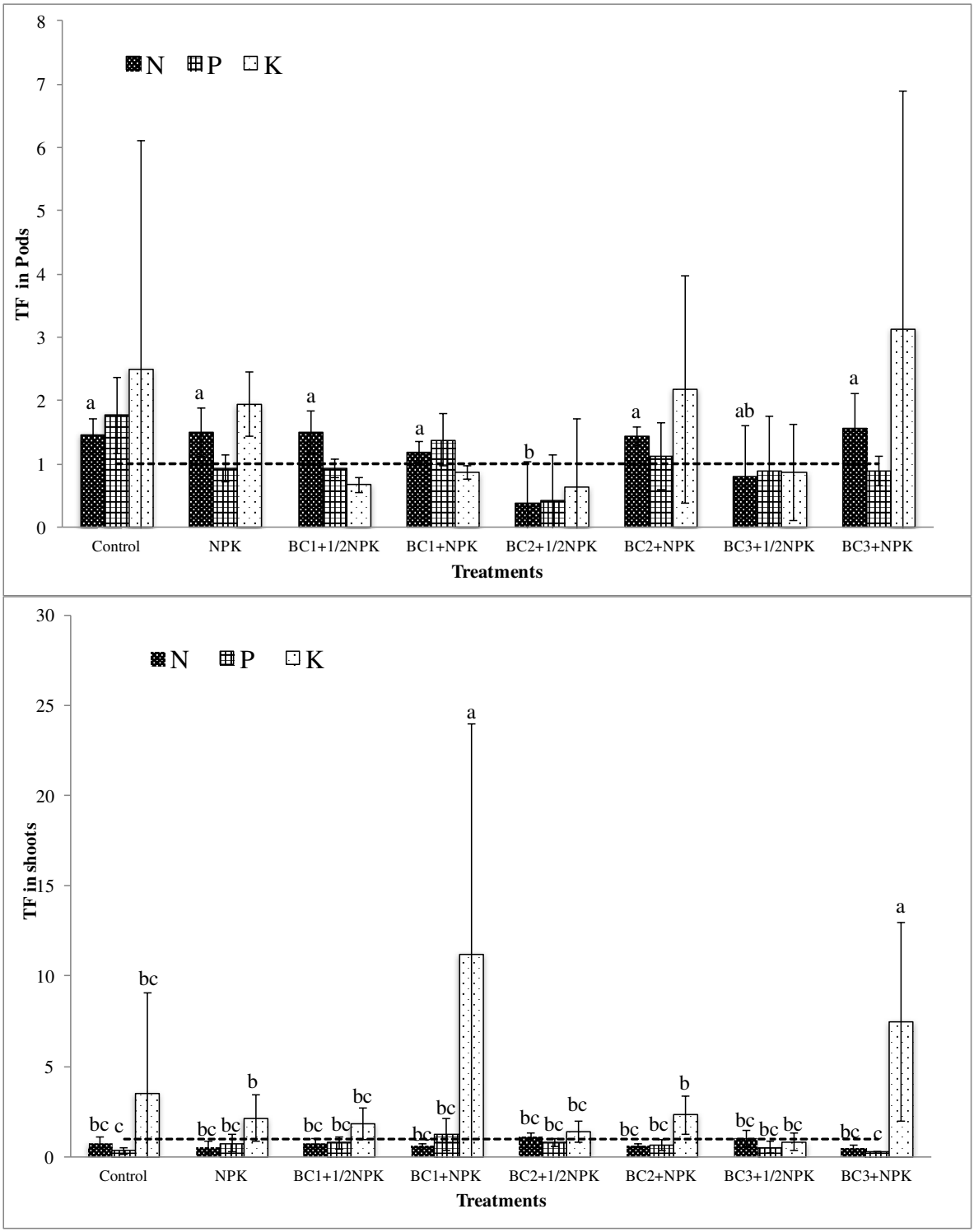

Fig. 3 N, P, and K translocation factor by cowpea in pod and shoot (stem + leaves) after the pot experiment with acid Arenosol of Mozambique. Means followed by different lowercase letters differed significantly among treatments at $P < 0.05$ by Duncan multiple mean comparison test. The whiskers indicate the standard deviation (n=3).

The higher agronomic and physiological efficiency were ascribed to the better nutrient uptake and efficient utilization for production growth and yield attributes (Sanginga et al., 2000; Singh et al., 2003; Randal et al., 2006; Choudhary and Kumar, 2014). Higher N, P, and K uptake by cowpea in these treatments implies that the biochar treated soil maintained higher concentration of these nutrients in soil solution (Muthukumar and Udaiyan, 2002; Agegnehu et al., 2015; Agegnehu et al., 2016a, b). Similarly, Steiner et al. (2007) and van Zwieten et al. (2010) observed a high availability of soil nutrients in treatments with biochars. High nutrient uptake efficiency is expected in acid soil amended with biochar because of the increased CEC, together with more favourable root environmental conditions as the acidity and available Al are reduced (Chan et al., 2007; van Zwieten et al., 2010).

4.6. Rhizosphere processes influencing cowpea growth

4.6.1. Rhizosphere effect on soil chemical properties

The cowpea rhizosphere was not significant for pH (both in H_2O and KCl) and available P, while it was significant (P<0.05) for CEC and exchangeable Ca and Mg (Fig. 5). This was a rather unexpected result as, generally, plant species belonging to the families of *Fabaceae* and *Poaceae* have a strong rhizosphere effect on available P and biological properties (Dotaniya and Meena, 2015). This is mainly due to the fact that N source fertilizers affect rhizosphere pH through H^+ or OH^- displacement in reply to the absorption of NH_4^+ or NO_3^-, respectively, and to denitrification/nitrification reactions (Marschner et al., 2005; Bleam, 2012; Dotaniya and Meena, 2015; Murphy et al., 2017). Thus, changes of the rhizosphere pH may promote nutrient conversion from non-available to available forms, so modifying biochemical activities too.

In our case, the absence of a rhizosphere effect for pH and available P was attributed to the addition of biochar. Houben and Sonnet (2015) asserted that biochars have potential for reducing rhizosphere acidification, and this is probably due to the reduction of H^+ activity. Further, the higher values of CEC and exchangeable Ca and Mg in the bulk soil relative to the rhizosphere were attributed to the constraint of plant charge balancing (e.g. van Beusichem et al., 1988; Bleam, 2012). Because of this, during the growing season plant roots are forced to uptake more cations (Ca, Mg, K) than anions because of the self-production of anions, and this absorption is balanced by releasing H^+. However, the effect of H^+ release on acidification and availability of nutrients like P is made null by biochar application (Inal et al., 2007; Marschner et al., 2011).

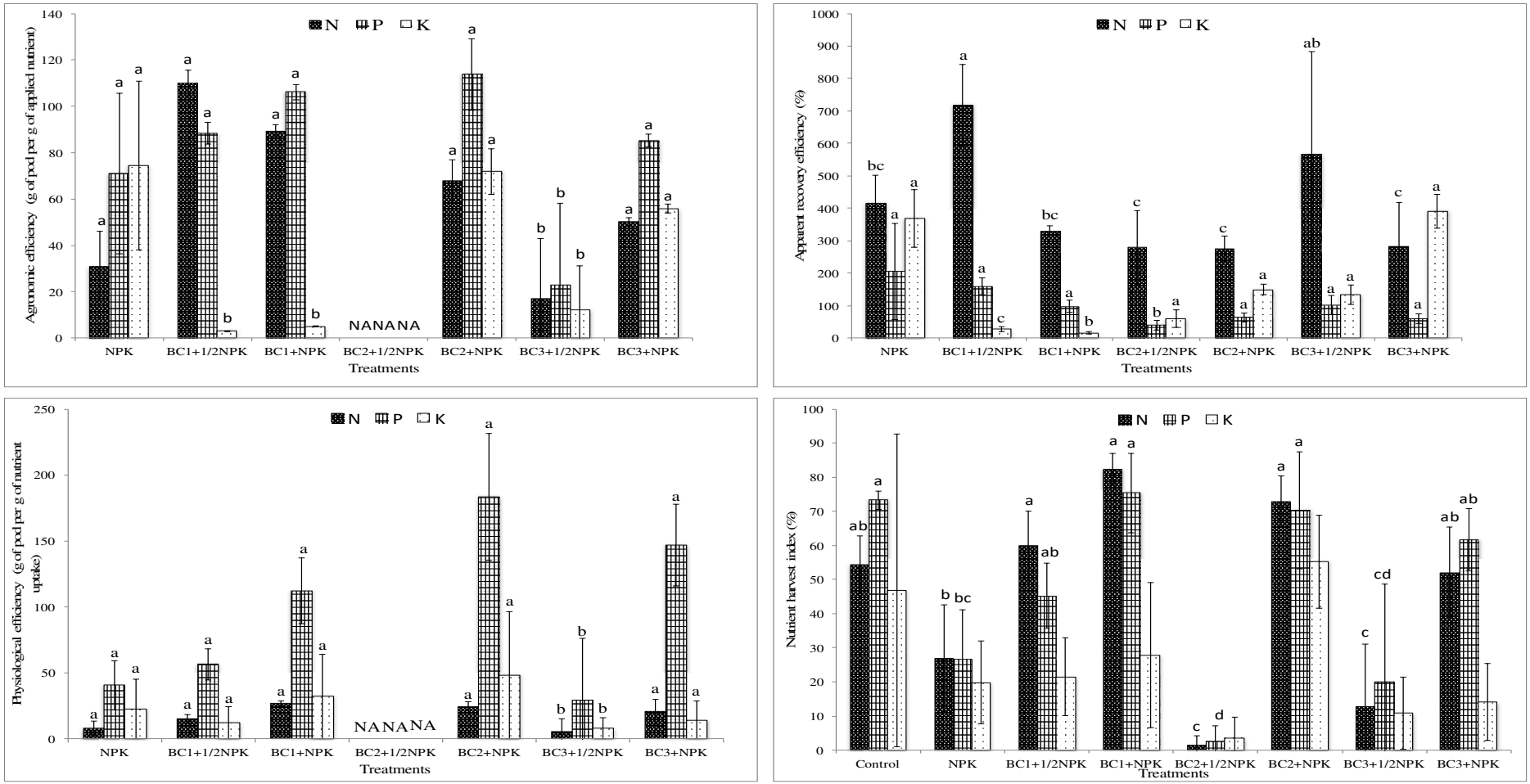

Fig. 4 N, P, and K agronomic (AE, g g^{-1}) and physiological efficiency (PE, g g^{-1}), apparent nutrient recovery (ARE, %), and harvest index (HI, %) by cowpea after the pot experiment with acid Arenosol of Mozambique. AE, PE, ARE, and HI are significant at $P = 0.001$, except HI for K. Means followed by different lowercase letters differed significantly among treatments at $P < 0.05$ by Duncan multiple mean comparison test. The whiskers indicate the standard deviation (n=3).

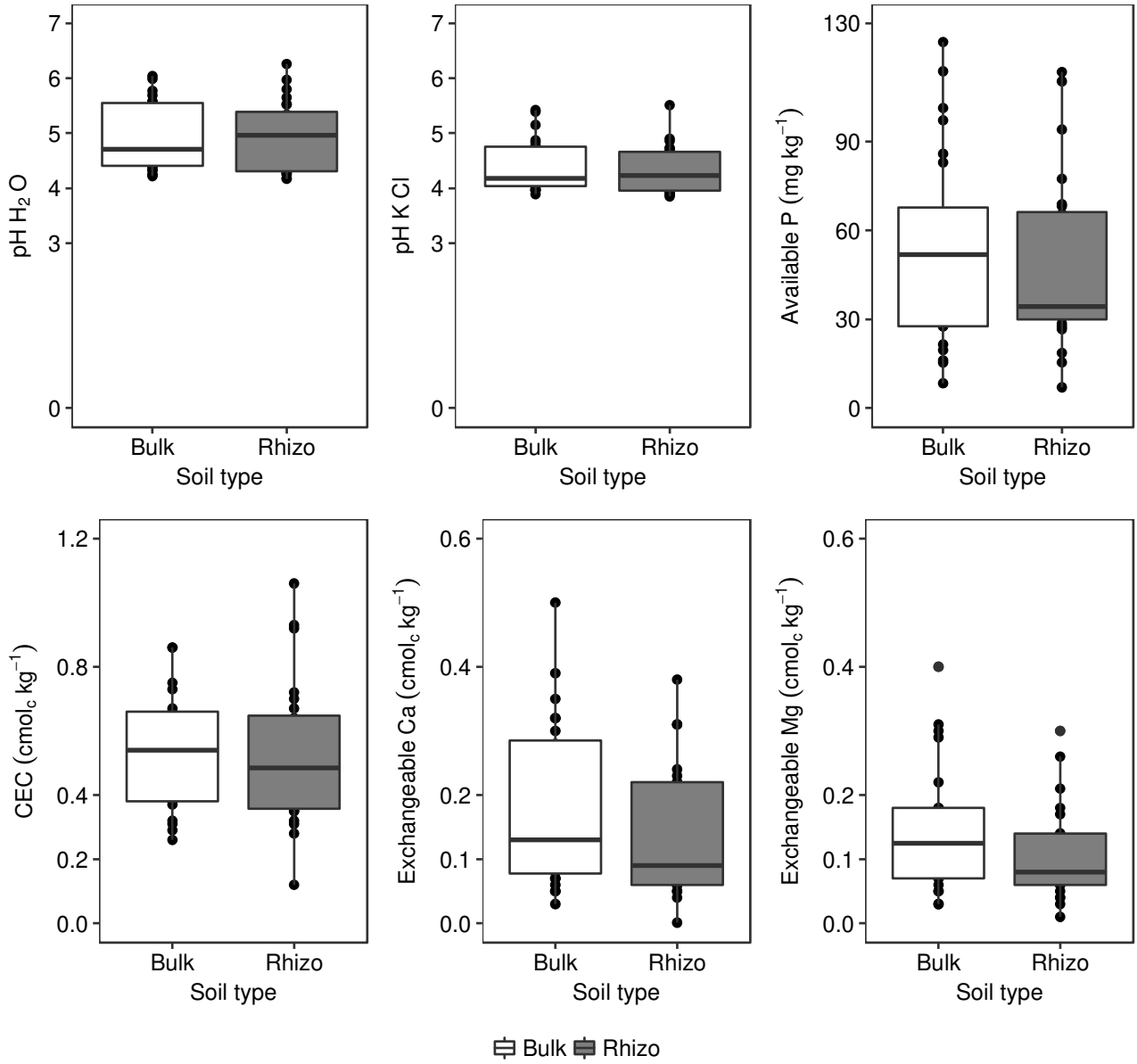

Fig. 5 Cowpea rhizosphre effect on pH-H_2O (not significant) and pH-KCl (not significant), available P (mg kg^{-1}) (not significant), cation exchange capacity (CEC, $cmol_c$ kg^{-1}) (*P = 0.05*), exchangeable Ca ($cmol_c$ kg^{-1}) (*P = 0.01*), and exchangeable Mg ($cmol_c$ kg^{-1}) (*P = 0.01*) after pot experiment with acid Arenosol of Mozambique (Bulk – Bulk soil; Rhizo – Rhizospheric soil)[7].

[7]Features of the boxes: upper whisker represents the maximum value excluding outliers; upper part of the box represents the upper quartile (25% of data is greater than this value); horizonal whisker represents the median (50% of data is greater than this value); lower part of the box represents the lower quartile (25% of data is less than this value); lower whisker represents the minimum value, excluding outliers; points located out of the upper and lower whisker are outliers.

Principal component analysis (PCA) was run assessing the variation of soil chemical properties as affected by the rhizosphere, which contributed to ~ 70% of the variation. PC1 and PC2 were responsible for 51.3 and 16.8% of the variation, respectively (Fig. 6a). The PCA biplot showed variations of the soil properties between bulk and rhizosphere soils. Soil parameters such as pH (in H_2O and KCl), exchangeable Al, base saturation, and Al saturation showed positive correlation with PC1 contributing ~ 57% of the variability (Table S2 of Supplementary materials). Instead, parameters such as available P, exchangeable Ca, Mg and K, and CEC showed positive correlation with PC2 contributing ~ 70% of the variability. The cowpea rhizosphere usually showed higher content of exchangeable Al and Al saturation, and lower pH (both in H_2O and KCl), EOOC, TOC, available P, exchangeable Ca, Mg, and CEC compared with the bulk soils. With respect to the bulk soil, Ruan et al. (2004) reported lower pH and higher extractable Al values in the rhizosphere of tea plant after litter incorporation and N fertilization. Therefore, under acidification a decrease of alkaline nutrients (such as Ca and Mg) and available P contents were expected in the rhizosphere (Dotaniya and Meena, 2015). In our experiment, the cowpea rhizosphere influenced nutrient availability by increasing nutrient uptake from biochar and NPK fertilizer, and led to higher nutrient efficiency of the treatments where a half dose of NPK fertilizer was applied.

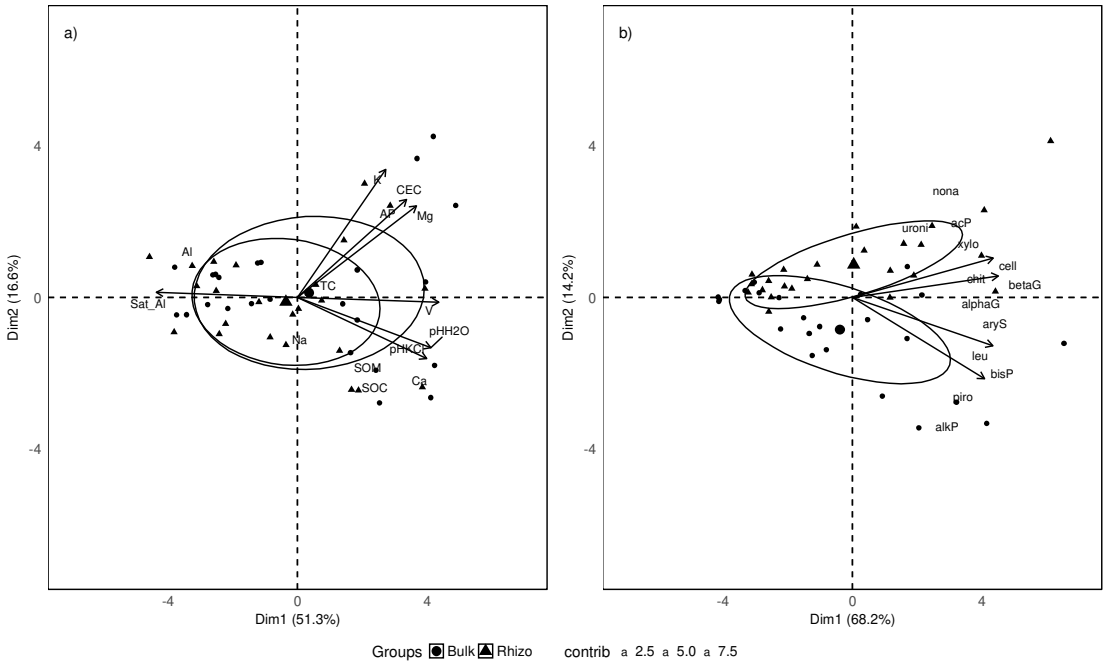

Fig. 6 Principal component analysis of soil chemical parameters (a) and enzyme activities (b) as affected by the rhizosphere of Cowpea after the pot experiment with acid Arenosol of Mozambique (contrib. = contribution (%) of variables to the principal components (Dim1 - PC1 and Dim2 - PC2)).

a) graph of individuals (symbols) and variables (arrows) for chemical parameters; b) graph of individuals (symbols) and variables (arrows) for enzyme activities. Individuals close to each other have similar properties, whereas individuals far apart differ strongly. Symbols with bigger size represent the main effect of the group (Bulk or Rhizo). The grey circle surrounds bulk samples, the light grey circle surrounds rhizosphere soil samples; Contrib.: Intensity of arrows (variables) in scale from 2-8%, in which arrows with color tending to light grey has less contribution to PCA, whereas arrows with color tending to black has higher contribution to PCA.

4.6.2. Rhizosphere effect on soil potential enzyme activities

Significant differences between bulk and rhizosphere soils were found only for the activities of alkaline phosphomonoesterase, leucine aminopeptidase, α-glucosidase, nonanoate esterase, and glucuronidase. For alkaline phosphomonoesterase and leucine aminopeptidase there was a significant interaction between the soil component (bulk or rhizosphere) and the treatments (Fig. S1 of Supplementary materials). Bulk soil of the BC1+1/2NPK, BC2+1/2NPK, and BC2+NPK treatments showed higher activities of alkaline phosphomonoesterase than the rhizosphere soil; treatments with the higher soil pH. Chhabra et al. (2013) stated that soil pH can affect the production and the persistence of alkaline phosphomonoesterase during plant decomposition, and the addition of biochar could have promoted a pH increase able to trigger the decomposition of the fine organic remnants present in the soil. For leucine aminopeptidase, only the bulk soil of the BC2+NPK treatment showed higher activities than the rhizosphere soil, as also reported by Bell et al. (2015).

This enzyme is related to the N cycle, and this result suggests that soil N resource availability was different between the rhizosphere and bulk soil. The increase of both these potential enzyme activities was related to soil chemical changes promoted by the BC1+1/2NPK, BC2+1/2NPK, and BC2+NPK treatments, and was ascribed to the higher pH and nutrient availability able to support a greater microbial biomass (Trabelsi et al., 2017). This did not affect all the potential enzyme activities as the activity of some enzymes are higher in strongly acid conditions. Further, soil pH and moisture (higher in biochar treatments) have been identified by Bell et al. (2015) as the key factors affecting the plant rhizosphere bacterial community structure and functions.

No significant interaction between soil component (bulk or rhizosphere) and treatment was found for glucuronidase, nonanoate esterase, and α-glucosidase. However, rhizosphere soil presented higher activities of these three enzymes compared with the bulk soil (Fig. S2 of Supplementary materials). A PCA on enzyme data as affected by rhizosphere soil, which explained 82.4% of the variations, subdivided into 68.2% for PC1 and 14.2% for PC2 (Fig. 6b). We found negative and strong

correlation between nonanoate esterase and alkaline phosphomonoesterase, while positive and strong correlation was found between nonanoate esterase and acid phosphomonoesterase. This result gave an indication that nonanoate esterase activity in soil depends on soil pH; preferably acid pH.

There was a clear difference between rhizosphere and bulk soil in terms of potential enzyme activities. Rhizosphere soil showed higher activities of nonanoate esterase and acid phosphomonoesterase, while bulk soil presented higher activity of alkaline phosphomonoesterase, leucine aminopeptidase, pyrophosphate-phosphodiesterase, and phosphodiesterase. These results demonstrated the occurrence of a rhizosphere effect on plant uptake since the form in which a nutrient is absorbed by plants largely determines the rhizosphere acidification or alkalinisation (Yong-liang et al., 2002). This rhizosphere effect depends on plant species, but most plants and soil microorganisms are able to release H^+ ions, organic anions (e.g. citrate, malate and oxalate), and enzymes (phosphatases, peptidases, and others) to increase nutrient uptake (Marschner et al., 2011). Zhou et al. (2009) and Maltais-Landry (2015), reported that *Fabaceae* species had a greater rhizosphere effect, with lower pH, higher organic acid release, and major phosphatase activity than cereals. In our case, since the sandy soil used had low pH buffering capacity, the effect of the released H^+ ions, organic anions and enzymes by cowpea roots in soil was evident.

5. Conclusions

This study showed that the application of biochars in combination with NPK fertilizer in acid Arenosol improved soil chemical properties and potential enzyme activities. These changes in soil properties were accompanied by higher cowpea N, P, and K uptake and efficiency from the treatments containing biochars, and therefore higher cowpea growth and yield were obtained. Cowpea rhizosphere had a major effect on potential activities of enzymes related with P and N cycles. Application of biochar resulted in higher activity of alkaline phosphomonoesterase and leucine aminopeptidase in bulk soil, while rhizosphere soil always promoted the activities of glucuronidase, nonanoate esterase, and α-glucosidase.

Among the biochars, baby corn peel biochar (BC1) followed by the mango tree branches biochar (BC2) were more influential on soil properties and cowpea yield than rice husk biochar (BC3). Higher pH, available P and K, and CEC of BC1 were the dominant characteristics affecting the soil. Conversely, higher pH, Ca, and CEC of BC2 contributed to the main changes in soil properties. Rice husk biochar (BC3) was less effective in improving soil properties and cowpea yield. Application of

biochars increased the N, P, and K use efficiency by cowpea. Under cowpea cultivation, soil properties and yield parameters were generally equal with a full or half dose of NPK for each type of biochar, indicating that poor farmers can reduce fertilizer application, and consequently the cost of food production, by increasing biochar in soil. Further investigation under field condition is needed to validate these results and quantify the long-term benefit of biochars.

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Supplementary Materials

Table S1
Contributions of the variables to the principal components (PC1 and PC2).

Variables[a)]	PC1	PC2
	%	
pH_{H2O}	**4.27**	2.21
pH_{KCl}	**3.73**	1.64
TOC	**1.75**	1.02
EOOC	1.00	1.90
AP	**2.12**	3.88
Exchangeable Ca	**3.03**	0.77
Exchangeable Mg	1.87	6.44
Exchangeable K	0.87	4.66
Exchangeable Na	0.19	2.29
Exchangeable Al	**4.05**	1.57
CEC	1.57	**5.31**
Base saturation	**4.35**	3.17
AryS	**5.30**	1.29
α-G	**4.41**	1.84
β-G	3.69	**5.41**
Cell	2.85	**5.62**
Xylo	2.82	**6.16**
Uroni	**4.83**	0.07
Chit	3.68	**3.85**
Leu	**4.95**	2.18
AcP	0.41	**10.69**
Pyro	**4.12**	2.50
BisP	**5.68**	1.44
AlkP	**5.64**	0.02
Nona	0.79	**7.74**
dsDNA	0.51	**3.31**
Number nodules	**3.26**	0.68
Dry biomass	**3.96**	1.74
Dry root	0.57	0.03
Dry shoot	0.78	0.22
Dry leaves	0.70	0.10
Dry pods	1.96	2.12
Stem diameter	2.31	3.67
Plant height	**2.63**	0.18
Root depth	0.02	1.34
N in root	**1.93**	0.38
P in root	**1.21**	0.58
K in root	1.19	1.95

[a)]Numbers of variables in bold contribute most to PC1 or PC2. TOC: total organic carbon; EOOC: easily oxidizable organic carbon; AP: available phosphorus; CEC: cation exchange capacity; AryS: arylsulfatase; α-G: α-glucosidase; β-G: β-glucosidase; Cell: cellulase; Xylo: xyloxidase; Uroni: glucuronidase; Chit: chitinase; Leu: leucine aminopeptidase; AcP: acid phosphomonoesterase; Pyro: pyrophosphate phosphodiesterase; BisP: phosphodiesterase; AlkP: alkaline phosphomonoesterase; Nona: nonanoate esterase; dsDNA: double-strand DNA.

Table S2
Contributions of the variables to the principal components (PC1 and PC2).

Variables[a)]	PC1	PC2
	%	
pH_{H2O}	**11.28**	4.46
pH_{KCl}	**10.60**	6.93
TOC	0.49	2.58
EOOC	3.97	2.26
AP	6.69	**11.75**
Exchangeable Ca	8.13	**12.06**
Exchangeable Mg	9.47	**12.66**
Exchangeable K	5.57	**22.21**
Exchangeable Na	0.01	9.26
Exchangeable Al	**9.16**	3.00
CEC	8.28	**11.41**
Base saturation	**13.17**	0.71
Al saturation	**13.17**	0.71

[a)]Numbers of variables in bold contribute most to PC1 or PC2. TOC: total organic carbon; EOOC: easily oxidizable organic carbon; AP: available phosphorus; CEC: cation exchange capacity.

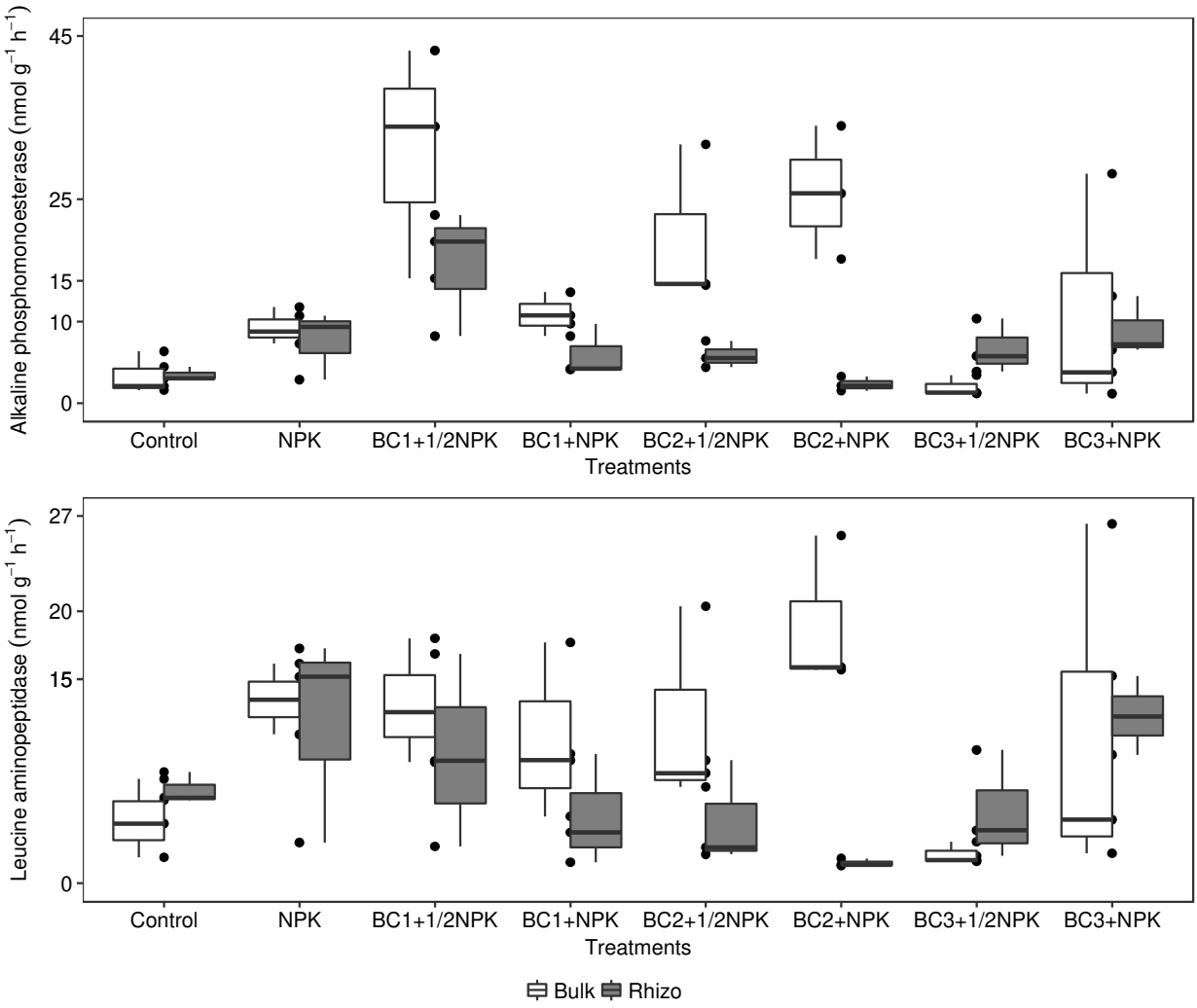

Fig. S1 Rhizosphere effect on soil alkaline phosphomonoesterase (P = 0.001) and leucine aminopeptidase (P = 0.01) activities after pot experiment with acid Arenosol of Mozambique. Features of the boxes: upper whisker represents the maximum value excluding outliers; upper part of the box represents the upper quartile (25% of data is greater than this value); horizonal whisker represents the median (50% of data is greater than this value); lower part of the box represents the lower quartile (25% of data is less than this value); lower whisker represents the minimum value, excluding outliers; points located out of the upper and lower whisker are outliers.

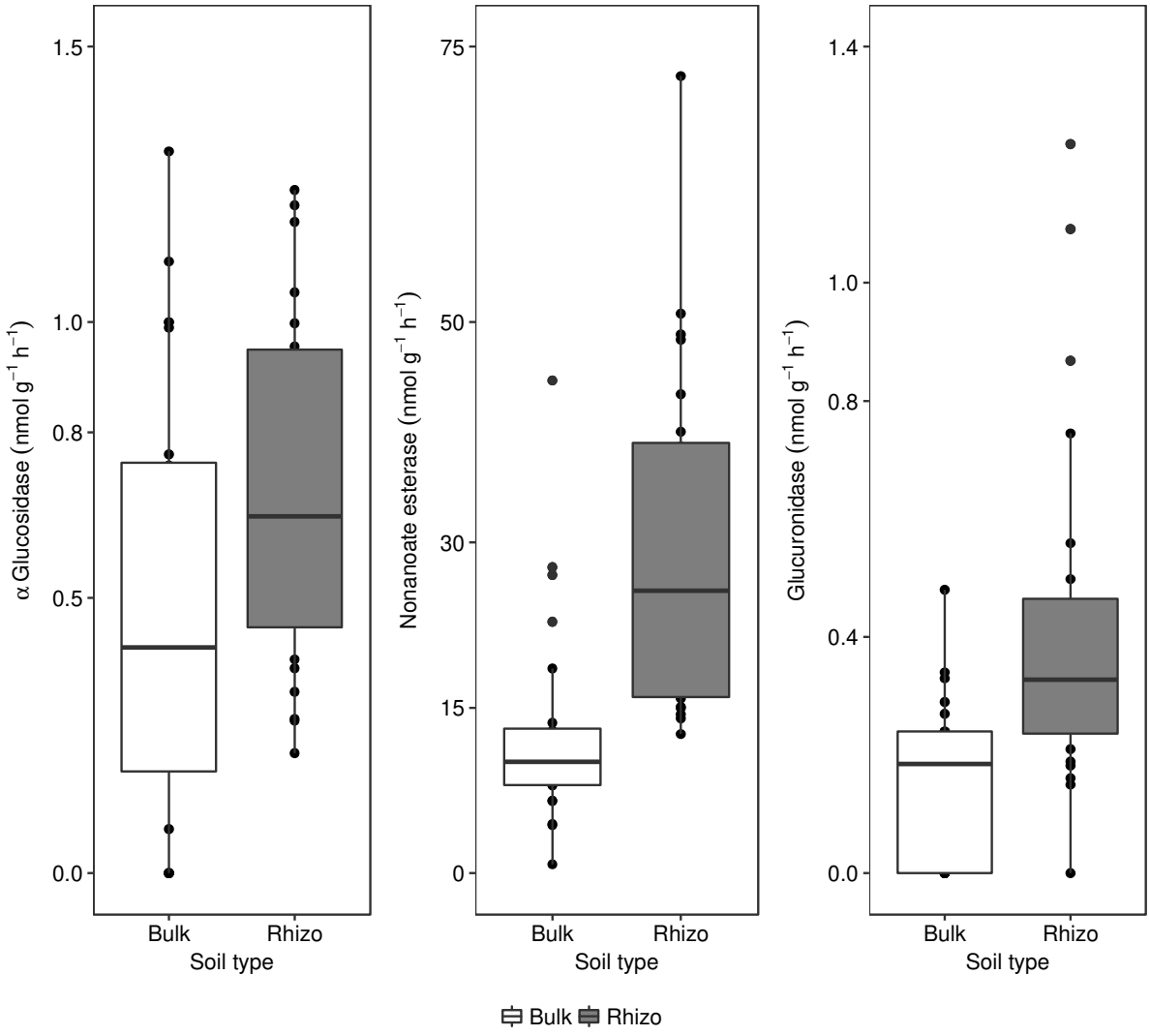

Fig. S2 Rhizosphere effect on soil α-glucosidase (P =0.05), nonanoate esterase (P =0.001), and glucuronidase (P = 0.001) activities after pot experiment with acid Arenosol of Mozambique. Features of the boxes: upper whisker represents the maximum value excluding outliers; upper part of the box represents the upper quartile (25% of data is greater than this value); horizonal whisker represents the median (50% of data is greater than this value); lower part of the box represents the lower quartile (25% of data is less than this value); lower whisker represents the minimum value, excluding outliers; points located out of the upper and lower whisker are outliers.

CHAPTER 6 - DISCUSSION AND CONCLUSIONS

DISCUSSION AND CONCLUSIONS

Phosphate rock, dolostone and biochar are available resources in Mozambique. This opens an opportunity to use these local resources as soil amendment for improving soil fertility, mainly in acid poor soils. In this doctoral research, several aspects regarding characterization and agronomic effectiveness of these resources were evaluated as preliminary study in this subject in Mozambique before field experiments for widespread recommendation for the farmers. In addition, plant-soil relationship response to application of these inputs was assessed. All the experiments were run in controlled conditions, pot trials established in greenhouse with an acid Arenosol, very deficient in nutrients, which is one of the representative soils types from the Southern region of Mozambique. Therefore, the novelty found in this research can be resumed as follows:

1. Local phosphate rock and dolostone can be used as slow release fertilizer and amendment in strongly acid soils. These rocks are composed respectively, by fluoroapatite and dolomite, both less soluble minerals if compared with other minerals from their family (e.g. hydroxyapatite or chlorapatite in the case of phosphate rock and calcite for calcareous rock). These rocks are potential sources of P, Ca, and Mg with the additional benefit of liming. The release of nutrients by these rock fertilizers is controlled by surface reactions and affected by particle size, acidity, and time.

2. Single application of phosphate or dolostone to acid Arenosol fails to improve soil fertility, but tremendous improvement of soil fertility is achieved in combination with biochar. Baby corn peel biochar can be used as P and K source, with the additional benefit of liming. Simultaneous application of phosphate rock and biochar can be a suitable combination to improve soil fertility and crop yields in acid soils. Biochar can ensure short-term availability of P, while the phosphate rock assure the mid to long-term. Application of high rate of phosphate rock did not interfere negatively in soil microbial biomass. These findings are paramount because higher rate of phosphate rock is needed to reach similar results as those found with water soluble P fertilizer.

3. Root-induced change was enzyme and crop-specific, and was affected by application of phosphate rock and biochar. Cowpea root-induced acidification was higher than corn, which means that cowpea is more efficient absorving nutrients from phosphate rock. Rhizosphere soils where phosphate rock was applied alone increased acid phosphomonoesterase and xylosidase activities, P and C cycle enzymes, respectively, while the higher soil pH (bulk and rhizosphere) in phosphate rock and biochar treatments increased the activities of C (β-glucosidase, cellulase), N (leucine aminopeptidase), and P (alkaline phosphomonoesterase, phosphodiesterase, and pyrophosphatase-

phosphodiesterase) cycle enzymes. Rhizosphere is an active environment which regulate nutrient availability through the activation of extracellular enzymes. Therefore, root induced changes contributed to the faster mineralization of soil organic matter and possible N_2 fixation in cowpea as verified by root nodulation.

4. Biochar properties vary with feedstock. Application of baby corn peel, tree branch, and rice husk biochars improved soil quality. Baby corn peel and tree branch biochar are relatively more recalcitrant, have liming effect in soil, higher CEC than rice husk biochar, and are more suitable for application to acid soils. Under cowpea cultivation, soil properties and yield parameters were generally equal with a full or half dose of commercial NPK fertilizer for each type of biochar, indicating that poor farmers can reduce fertilizer application, and consequently the cost of food production, by increasing biochar in soil.

5. In general, both corn and cowpea yields dramatically increased with simultaneous application of phosphate rock or dolostone and biochar, or biochar in combination with inorganic fertilizer with respect to the Control due soil fertility improvement. The yield increase reflect increase of profit and food security, however further evaluation under field conditions assessing both agronomic effectiveness and economic benefit is necessary to validate these results. For phosphate rock, strategies to increase its reactivity must be applied. Practically it can be done by: i) using staple crops that can solubilize phosphate through root induced acidification; ii) Processing techniques (e.g. phosphor-composting, inoculation with mycorrhiza fungi) before application should be considered. A mixture of finer (0.063 – 0.25 mm) and medium (0.25 – 0.5 mm) particle sizes represent a good balance to apply in soil so the cost of grinding can be reduced. Biochar has paramount importance as soil amendment and to increase crop yield. However, 1.1 % (w:w) biochar in 1 hectare (for a soil depth of 20 cm with a soil bulk density of 1.5 kg dm^{-3}) would require approximately 545 ton ha^{-1} of fresh biomass (assuming that dry weight biomass correspond to ~ 20 % of fresh biomass weight, and biochar production efficiency is ~ 30% of feedstock dry weight). Therefore, biochar production demands a huge amount of biomass making it impossible to be applied in larger areas due to the lack of availability of enough biomass, unless wastes are used as raw materials for biochar production, and also to the operational cost inherent to its application. Evaluation of biomass value chain is mandatory before making a recommendation regarding biochar in larger areas. In this study a waste residue was used as feedstock, which would be a potential source of feedstock biomass. Further research regarding biochar should focus on identifying appropriate farm systems it could be coupled.