**University of São Paulo
“Luiz de Queiroz” College of Agriculture**

**The rhizosphere microbiome of common bean (*Phaseolus vulgaris* L.) and
the effects on phosphorus uptake**

Josiane Barros Chiaramonte

Thesis presented to obtain the degree of Doctor in Science.
Area: Agricultural Microbiology

**Piracicaba
2018**

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Licensed in Biological Sciences

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DEDICATION

In memoriam of my beloved grandma, who taught me the good ways and gave me strength
to go on no matter what.

My endless love to you, Olga Trevisan Barros (★19/02/1919 - † 30/07/2017).

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EPIGRAPH

*“If I have the gift of prophecy
and know all mysteries and
all knowledge; and if I have
all faith, so as to remove
mountains, but do not have
love, I am nothing.”
(Cor 13,2).*

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RESUMO

O microbioma da rizosfera de feijão comum (*Phaseolus vulgaris* L.) e os efeitos na absorção de fósforo

O atual aumento populacional irá demandar uma maior produção agrícola para completar a necessidade de alimento. Para suprir essa necessidade e preservar o meio ambiente, muitos recursos serão aplicados para promover a agricultura sustentável. A depleção de fósforo é um dos principais fatores que limita a produção agrícola em solos tropicais, onde o pH e o conteúdo de argila fixam rapidamente esse nutriente. Os melhoristas de plantas visam solucionar esse problema alterando a necessidade de fósforo das plantas e adaptando-as as baixas disponibilidade de fósforo. No entanto, com essas estratégias a demanda por fertilizantes fosfatados irá continuar assim como a exploração das reservas naturais de fósforo. Nesse estudo foi proposto que as plantas contrastantes em relação a eficiência na absorção de fósforo, i.e. P-eficiente e P-ineficiente, recrutariam um microbioma rizosférico distinto em relação a mobilização de fósforo. Essa hipótese foi testada cultivando plantas em um gradiente usando duas fontes distintas de P, triplo fosfato ou fosfato de rocha Bayovar. O microbioma da rizosfera de feijão foi então avaliado por técnicas dependentes e independentes de cultivo, análise enzimática, predição metagenômica e análises de *network*. Um enriquecimento diferencial de várias OTUs observado na rizosfera do genótipo de feijão P-ineficiente, e o enriquecimento de funções de quimiotaxia bacteriana e envolvidas na mobilização de fósforo sugerem que esse genótipo tem uma maior comunicação com o microbioma rizosférico e é altamente dependente deste para a mobilização de fósforo. Como prova de conceito, o genótipo P-eficiente foi plantado em solo previamente cultivado com o genótipo P-ineficiente. Os resultados mostraram que o genótipo P-eficiente responde positivamente à rizosfera modificada nos estádios iniciais de crescimento, ou seja, o microbioma selecionado e enriquecido pelo genótipo P-ineficiente melhorou a absorção de fósforo no genótipo cultivado posteriormente no mesmo solo. Coletivamente, esses resultados sugerem que as plantas dependem parcialmente do microbioma da rizosfera para a absorção de P e que a exploração destas interações durante o melhoramento vegetal permitiria a seleção de genótipos muito mais eficientes, conduzindo à uma agricultura sustentável explorando o fósforo residual do solo.

Palavras-chave: Microbioma; Fósforo; Nutrição vegetal; Ecologia microbiana; Sustentabilidade

ABSTRACT

The rhizosphere microbiome of common bean (*Phaseolus vulgaris* L) and the effects on phosphorus uptake

The current population growth will demand a higher productive agriculture to full the food requirement. To supply this need and preserve the environment, many resources are applied to promote sustainable agriculture. Phosphorus depletion is the main factor that limits crops yields in tropical soils, where the pH and clay content rapid fixate this nutrient. Plant breeders aim to solve this issue by changing the plant requirements for phosphorus and adapting them to low P availability. However, with these approaches the demand for phosphorus fertilizers will continue and so the depletion of the natural deposits. In this study is proposed that plants with contrasting phosphorus uptake efficiency, i.e. P-efficient and P-inefficient, recruits distinct rhizosphere microbiome specialized in phosphorus mobilization. This hypothesis was tested growing plants in a gradient of two sources of P, triple superphosphate or rock phosphate Bayovar. Thebean rhizosphere microbiome was assessed with culture dependent and independent approaches, enzymatic assays, predictive metagenomics and networks analysis. A differential enrichment of several OTUs in the rhizosphere of the P-inefficient common bean genotype, and the enrichment of bacterial chemotaxis functions and functions involved in phosphorus mobilization suggest that this genotype has superior communication with the rhizosphere microbiome and is highly dependent on it for phosphorus mobilization. As a proof of concept, the P-efficient genotype was sown in soil previously cultivated with P-inefficient genotype. The results showed that P-efficient genotype positively responded to the modified rhizosphere in early stages, that is, the microbiome selected and enriched by the P-inefficient genotype improved the P uptake in the genotype cultivated afterwards in the same soil. Taken collectively, these results suggest that plants partly rely on the rhizosphere microbiome for P uptake and that the exploration of these interactions during plant breeding would allow the selection of even more efficient genotypes, leading to a sustainable agriculture by exploring soil residual P.

Keywords: Microbiome; Phosphorus; Vegetal nutrition; Microbial ecology; Sustainability

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1. INTRODUCTION

The current population growth rates will lead to a higher demand of resources mainly for energy and food production. The development of renewable forms of energy will cause a management in the priorities focusing in the sustainable agriculture, primarily concerning to phosphorus (WELLMER; SCHOLZ, 2017). Acid soils rapidly retain phosphorus fertilizers applied and consequently shows a low availability of this nutrient to plants (VALLADARES et al., 2003). Several breeders focused in the development of genotypes able to grow and produce good yield under P limiting conditions (WISSUWA et al., 2015). However, plant genotypes also influence the composition of rhizosphere community (BULGARELLI et al., 2015; LUNDBERG et al., 2012), and these changes in the rhizosphere microbial community was erroneously unconsidered during the plant breeding (BAKKER; SHEFLIN; WEIR, 2012).

Rhizosphere microbial community has an important role in nutrient supply to plants, either facilitating the acquisition or mobilizing them in the soil. The solubilization of inorganic phosphate and mineralization of organic compounds by rhizobacteria action are responsible for converting stable phosphorus fractions in the soil into phosphate ions that are uptaken by plants (RODRÍGUEZ; FRAGA, 1999; SINGH; PANDEY; SINGH, 2011). In addition, the microbial community enable the application of insoluble sources of phosphate in agriculture (KHAN et al., 2009), without compromising the yield (CABELLO et al., 2005; GOENADI DIDIEK H, SISWANTO, SUGIARTO, 2000). This is an important and sustainable strategy to decrease phosphate fertilizers fixation, consequently reducing the demand for phosphorus applications and economical losses.

Without considering the rhizosphere microbiome in plant breeding programs, its role in plant phenotype is also neglected. Considering that the plant at least in part depends on its rhizosphere to accomplish several functions (LEMANCEAU et al., 2017; MENDES; GARBEVA; RAAIJMAKERS, 2013), is important to access how much of the plant phenotype is explained by its rhizosphere microbiome.

In this study was hypothesized that genotypes inefficient in phosphorus uptake, when exposed to less soluble sources of phosphate and phosphorus limiting conditions in the soil, would rely in the rhizosphere microbiome by enriching the rhizosphere with phosphorus mobilizing microorganisms. This hypothesis was tested by performing a total randomised experiment, comparing the rhizosphere of two common bean genotypes contrasting in phosphate uptake efficiency, growing in a gradient of Triple Superphosphate (TSP) and Rock Phosphate Bayovar (RPB). Our results demonstrate how the host plant depends, at least in part,

on its bacterial community for P uptake. A prediction of this observation is that our work will pave the way to alternative strategies conjugating sustainable crop production with reduced application of P-fertilizers.

2. LITERATURE REVIEW

2.1. The phosphorus scenario

Available phosphates constitute a small fraction of the greater amount of phosphorus found in soils. The weathering of rocks and mineralization of organic compounds is responsible for turning phosphates available to plants, and then it can be recycled. Once in the soil, soluble phosphates can be lost by lixiviation to superficial waterbodies causing environmental unbalances, which can lead to eutrophication. The phosphates can also become precipitated in soil particles and metallic ions. In this sense, the only way to be reutilized is through the deposition in ocean sediments; however, this process takes eras to occur, and during this time phosphorus is inaccessible to agricultural and industrial purposes, what makes phosphorus a finite resource (FILIPPELLI, 2011).

The efforts of preserving phosphorus rely on the fact that it is a basic element of the periodic table and cannot be replaced for another nutrient. Furthermore, it is required for every living organism as one of the main constituents of cell membrane and having an important role in energy transference. For this reason, about 90% of global demand for phosphorus currently is applied to food production (SMIL, 2000).

Along with the increase of the population, an increase in food production will arise, and ratios of phosphorus input in the soil will enhance even more. The rates of exploration in phosphate rock reserves reached 44.5 million tons in 2016 (US GEOLOGICAL SURVEY; JASINSKI, 2017). An enhance of 5 million tons is expected in the next 5 years (US GEOLOGICAL SURVEY; JASINSKI, 2017), the estimative is that 40–60% of the current resource will be extracted by 2100 (VAN VUUREN; BOUWMAN; BEUSEN, 2010), if these projections come true, the agriculture will be severely affected in the incoming years (CORDELL; WHITE, 2015; LEGHARI et al., 2016). These facts justify the need of developing sustainable system for the use of phosphorus in agriculture.

Several strategies are being considered in plant breeding to achieve phosphate efficient plants. Approaches such as selection of high development of roots in plants to enhance scavenging area improving nutrient uptake, and the reduction in phosphorus translocation to the grains, allows better phosphate use efficiency. These strategies are highly dependent on plant species, and is time consuming to achieve considerable results. Furthermore, these

strategies will not significantly reduce the need of chemical phosphate fertilization if the phosphorus continues to be fixed in tropical soils.

Brazil is one of the largest words suppliers of agricultura products and since 1960 the application of phosphate fertilizers in soil largely exceeds plant demands, this has caused a significant cumulative effect (WITHERS et al., 2018). Phosphorus is the least mobile macronutrient when compared to the other nutrients required in great quantities by the plants. The slow mobility of inorganic phosphate in the soil occurs due to its strong retention. About 30% of the world soils show a high capacity of fixing phosphates (KOCHIAN, 2012; VAN DE WIEL; VAN DER LINDEN; SCHOLTEN, 2016). This is a concern mainly to tropical and subtropical regions of the world, where the soil is highly weathered and acid, causing rapid phosphate precipitations in metallic ions such as Fe^{3+} and Al^{3+} (KHAN et al., 2014), what cause great economical losses due to the rapid precipitation of fertilizers. SATTARI et al., (2012) showed that residual phosphorus in Europe caused by the extensive fertilization can support the agricultural production for a considerable period. In Brazil, was also recently demonstrated that residual phosphorus can consist in a suitable strategy to promote agricultural sustainability (WITHERS et al., 2018).

The need to decrease the chemical phosphate fertilization inputs in agriculture can be approached in three ways i) enhancing the availability of the phosphorus in soil solution, ii) improving plant efficiency in acquiring phosphorus and iii) enhancing plant efficiency in phosphorus utilization (Figure 1). In this context, the rhizosphere microbiome can directly influence in the availability and acquisition of soluble phosphate in soils, which can be explored as strategy towards a more sustainable agriculture.

Figure 1. Strategies to decrease phosphorus applications in soil by increasing availability, acquisition, and utilization of phosphorus by the host plant.

2.2. Phosphorus mobilization

Organic matter is the most labile source of phosphate to plants. There is a great diversity of organic P compounds with different rates of mineralization. Phytate (myo-inositol hexakisphosphate) is the most abundant organic P compound in the soil, and due to its difficult mineralization, its deposition reaches about 80% of organic P (TURNER et al., 2002). The phytate cycle in soil is still poorly understood, it becomes available through deposition of plant and animal's residues and is adsorbed to soil components (TURNER et al., 2002). The utilization of phytate by plants is limited due to their capacity to hydrolyse it (HAYES; SIMPSON; RICHARDSON, 2000). Mineralization by enzymes produced by microorganisms are responsible for the availability of phytate and other sources of organic P, such as, nucleotides, proteins and phospholipids (KHAN et al., 2014).

Phytases are enzymes produced by almost all living organisms and are responsible for the mineralization of the main organic phosphorus compound in soils into smaller phosphates such as mioinositol. There are different classes of phytases responsible for the mineralization of phytate (CHU et al., 2004; MULLANEY; ULLAH, 2003). However, its mobilization in the soil is limited due to its solubility (TANG et al., 2006), but once leached, they are readily

hydrolysed in aquatic environments (SUZUMURA; KAMATANI, 1995). In the soil, some microorganisms such as *Bacillus sp.*, *Aspergillus niger*, *Penicillium rubrum* are known to produce phytases (GULATI; CHADHA; SAINI, 2007; YADAV; TARAFDAR, 2003). YADAV; TARAFDAR, (2004) showed that the phytase activity in rhizosphere can enhance 1 - 3 mg Pi released g⁻¹ dry soil h⁻¹.

Acid or alkaline phosphatases are also responsible by the hydrolysis of several types of organic phosphorus available in the soil into phosphate ions. Up to 60% of total organic phosphorus is hydrolysed by phosphatases, which can be originated from plants or microorganisms (BÜNEMANN, 2008). Plants do not release alkaline phosphatase, so the activity of this enzyme in soil is predominantly from microorganisms in the soil (RICHARDSON; SIMPSON, 2011; YADAV; TARAFDAR, 2003). Despite of evidences that microorganism-origin phosphatases are more efficient in releasing orthophosphates (TARAFDAR; YADAV; MEENA, 2001), acid phosphatase is widely released in acid soils, then its activity many times overcomes microbial phosphatase.

The production of phosphatases by microorganisms is controlled by the regulon PHO-box, expressed when phosphorus become unavailable (RODRÍGUEZ et al., 2006). In the rhizosphere of several crops was already reported higher phosphatase activity in low P availability. Soybean cultivars efficient in P uptake showed an enhance in the exudation of acid phosphatase in the rhizosphere under low phosphate availability (ZHOU et al., 2016), the same trend was observed in several studies for different crops such as tomato (GOLDSTEIN; BAERTLEIN; MCDANIEL, 1988), lupin and rice (TADANO et al., 1993). Even in forestall environments is observed correlation between the levels of phosphate in the soil and the amount of phosphatase released by different trees species (CABUGAO et al., 2017).

There is a limited knowledge on understanding the contribution of microorganism or plant origin phosphatases in phosphorus mobilization. Some studies stated that microbial derived phosphatase responded more to soil phosphate contents than plant-originated phosphatase, being promoted in low phosphorus availability (CABUGAO et al., 2017; EDER et al., 1996; WANNER, 1993). A better understanding of the microorganism's role in releasing phosphatases in P-depleted soils were shown by BERGKEMPER et al., (2016); using metagenomic approaches, they also observed that in phosphorus depleted forest soils, functions involved in the mineralization of phosphates were enriched compared to soils where this nutrient was highly available.

Inorganic phosphate solubilization and mineralization of organic P by the action of rhizobacteria (RODRÍGUEZ; FRAGA, 1999; SINGH; PANDEY; SINGH, 2011) are responsible for converting fractions of phosphorus of the soil in phosphate ions, that are absorbed by the plants. The potential of production and secretion of low weight organic acids by some bacteria genera is the most studied mechanism of inorganic phosphate solubilization (KHAN et al., 2014; MARRA et al., 2015). Organic acids act like chelators of metallic ions releasing phosphates that become available to the plants.

Besides the production and secretion of organic acids, microorganisms can solubilize phosphorus through different mechanisms. It has been demonstrated that, despite of the ability of the fungi *Trichoderma harzianum* T-22 in solubilizing phosphorus, it did not produce organic acids in vitro (ALTAMORE et al., 1999), the same was observed for several actinobacteria lineages (*Streptomyces sp* e *Micromonospora sp*) isolated from a phosphate mine in Morocco (HAMDALI et al., 2008), in both cases the solubilization was attributed to chelator activities. In this case the solubilization of phosphate may be a secondary effect due to the absorption of iron by the microorganisms.

Microorganisms able to solubilize phosphate are being targeted in many studies that aims to enhance the availability of this nutrient to the soil improving agriculture production (LUCY; REED; GLICK, 2004). Great part of these microorganisms solubilize phosphates bounded to calcium (Ca) while some of them are able to solubilize more complex compounds of rock phosphate, and phosphates bounded to iron (Fe) and aluminium (Al) (GYANESHWAR et al., 2002).

Recycling waste products is an important strategy to prolong the duration of the phosphate reserves (CORDELL; DRANGERT; WHITE, 2009). Although waste products application is limited due to the risk of diseases that might be transmitted (BEKCHANOV, 2017; CASE et al., 2017), other organic compounds can be used as source of phosphorus, such as food and garden waste, bone meal, ash and algae (BEKCHANOV, 2017). After application in the soil, these products also require the action of microorganisms and plant enzymes to release soluble phosphates.

Sugarcane crops is responsible for generating a large amount of waste, ESTRADA-BONILLA et al., (2017) studied the advantage of using phosphate mobilizing microorganisms in sugarcane compost, in order to improve the liberation of phosphate and other nutrients. They observed that PSM could be used in the compost, the positive responses included the solubilization of phosphate bound to calcium. Phosphate mobilizing bacteria can also be used

to enhance the soluble P availability of biochar, the presence of *Penicillium aculeatum* liberate citric acid that was shown to have positive effects in the release of phosphates and consequently in plant growth promotion potential (EFTHYMIU et al., 2018).

Microbial action might enable the application of rock phosphate directly to agriculture production (KHAN et al., 2009), that allied to solubilizing bacteria can enhance field production in about 70% (CABELLO et al., 2005; GOENADI DIDIEK H, SISWANTO, SUGIARTO, 2000) and reduce losses associated to precipitation and fixation of phosphorus in the soil.

Besides the strategies related to the source of phosphorus and association with microorganism in the process of solubilization of P to plants, approaches enrolling the genetic improvement of plant has also contributed significantly to agricultural production. Plants can mineralize organic phosphates by the production and secretion of phosphatases (VANCE; UHDE-STONE; ALLAN, 2003), and solubilize phosphorus through the exudation of organic acids such as citrate, malate and oxalate (YAN et al., 2001).

The impact of the plant in the mobilization of phosphate in the soil, can also be attributed to differences in exudations patterns. Considering that phosphorus is the main constituent of biological membranes, its deficiency might lead to a higher permeability of the cell membrane, that would lead to a high or modified exudation pattern (TAWARAYA et al., 2014a). Once the rhizosphere microbial community structure is determined mainly through the rhizodeposition of substrates from the roots, allied to the plant species and soil conditions (BULGARELLI et al., 2012), phosphorus deficiency consequently changes the rhizosphere microbial composition.

It has already been demonstrated that bean genotypes that are more efficient in phosphorus uptake produces significantly more acid exudates than inefficient genotypes (YAN et al., 2001). Thus, plant roots can promote acidification of the rhizosphere and exudate organic acids and enzymes to change the solution Pi availability (HINSINGER, 2015). However, plant release soil region with less availability of phosphorus and its diffusion through the soil is limited (RICHARDSON et al., 2011), then organic acids might be rapidly absorbed in acid soils or depredated in calcareous soils (WANG; SHEN; LIAO, 2010).

2.3. Efficiency in P Acquisition

After going through the first obstacle that is the availability of soluble phosphates in soil, the second step is the acquisition of this nutrient. Despite of being soluble, phosphorus transportation in the soil occurs through diffusion rather than mass flow, due to the strong reactions with soil components (HINSINGER, 2015). The rapidly uptake by the roots results in a depletion zone at the root surface and to acquire phosphorus out of this zone, plants should evolve either mutualistic association with mycorrhizal fungi or evolve adaptations of its root systems.

The acquisition of phosphorus can be promoted by the enhance of root: shoot ratio and root structural modifications. In common bean, genotypic adaptations are responsible for the changes in root structure that facilitate the acquisition of phosphorus in topsoil (LYNCH; BROWN, 2012). The allocation of more carbon to roots results in increased root growth, density of lateral root, that allow higher topsoil scavenging, and the number of basal roots and whorls (LYNCH; BROWN, 2012). It is also observed the increased length and number of root hairs (LYNCH; BROWN, 2001), and enhanced expression of P transporters (JIANZHONG et al., 2001). The increase of the root systems besides enhancing the scavenging area can also promote niche availability for a higher diversity of microorganisms. ROBERTSON et al., (2017), comparing the rhizosphere of wild barley genotypes with mutant's genotypes exhibiting limited root hair growth, observed that, in part, the root structure promoted a recruitment of a less diverse microbiota in the mutants compared to the wild types.

Some plant species do not respond to phosphorus deficiency altering the structure of the roots (BROWN et al., 2013). In these scenarios; the most important strategy to acquire phosphorus under limiting conditions is the association with arbuscular mycorrhizal fungi (AMF). BROWN et al., (2013), observed that barley mutants with no development of root hairs under P deficient conditions, showed higher AMF colonization. From their observation a very traditional and accepted concept from BAYLIS (1970) is recovered, stating that the absence of root hairs and AMF the plants might need a higher input of phosphate fertilizers.

Based on morphological traits of spores, about 240 species of AMF are known (SCHÜSSLER; WALKER, 2010), with the development and popularization of molecular techniques, is expected that a higher number of species exists (KRÜGER et al., 2012). Once, most of AMF are able to establish an effective symbiosis with a large number of host plants, it is considered that this fungal group is nonspecific.

It is already known that arbuscular mycorrhizal fungi are found in a large variety of soils, from arid grasslands (CAVAGNARO et al., 2017), forests (BENNETT et al., 2017; FERNANDES et al., 2016), to agricultural crops (FERNANDES et al., 2016); where the management and land use can drastically impact community diversity (FERNANDES et al., 2016) or select less efficient lineages (CONVERSA et al., 2013). All the functions that these fungi exert in these environments are far from being completely understood. But the role in plant nutrient uptake, mainly the participation in the phosphorus nourishment and exchange of carbon derived molecules between the symbionts is well recognized (VAN DER HEIJDEN et al., 1998). Recently, AMF was described to impact the formation of soil aggregate (LEIFHEIT et al., 2014); respiration (LANGLEY; JOHNSON; KOCH, 2005; WAMBERG et al., 2003) and even in the protection of the host against stressful situations (BOTHE, 2012).

In low phosphorus soil, AMF can partially supply the amount of phosphate required (CONVERSA et al., 2013). A recent study suggested that phosphatase enzymes and increased root colonization levels by AM fungi enhance the capacity of legumes to acquire soil P and prosper in tropical forest (NASTO et al., 2014). The interactions of *Rhizobium* and AMF was previously described by LARIMER; CLAY; BEVER, (2014), however it was VAN DER HEIJDEN et al., (2016), that reinforced and highlighted the importance of considering not only the AMF community but the interaction of different members of the root microbiome; in his studies he accessed the influence of the association of *Rhizobium* and AMF and observed a promotion in plant diversity and nutrient supply in depleted soils.

In the phosphorus nourishment context, it is known that mycorrhizal associated bacteria might act in the mobilization of phosphorus, facilitating the transport to the plants performed by the fungi. Dual inoculation tests of AMF with the phosphorus mobilizing bacteria of genera *Streptomyces* (BATTINI et al., 2017), *Bacillus* (JANGANDI et al., 2017), *Enterobacter* (KIM; JORDAN; MCDONALD, 1998), *Pseudomonas* (GHORCHIANI; ETESAMI; ALIKHANI, 2018), *Burkholderia* (SAXENA; MINAXI; JHA, 2014); frequently the results show that the plant performance is better with both microorganisms than with each of them separately. Besides the increase in the soluble phosphorus content, in several studies an increased colonization by AMF in dual inoculation is observed. This fact is known by synergistic effect and is attributed to changes in the root morphology caused by the presence of a bacterial inoculum (SAXENA; MINAXI; JHA, 2014).

2.4. Efficiency in P utilization

Phosphorus utilization efficiency is defined as the ability to produce biomass or yield using the acquired P (WANG; SHEN; LIAO, 2010) or as shoot biomass produced per unit of P in shoots (ROSE; WISSUWA, 2012). The utilization efficiency is related to the translocation and re-use of stored phosphorus in plants. When growing in P deficient environments plants evolved several adaptive responses to improve internal use of phosphorus by changing metabolism (VANCE; UHDE-STONE; ALLAN, 2003). Plants usually store secondary metabolites such as flavonoids and indole alkaloids (ZHANG; LIAO; LUCAS, 2014), and phenolic compounds (AE et al., 1990), that acts as chelators and promote the release of inorganic phosphorus. Another metabolic response of phosphorus starvation is the synthesis of intra and extra phosphatases. These enzymes are responsible for mineralizing phosphorus and promote seed germination (BRINCH-PEDERSEN; SØRENSEN; HOLM, 2002); and internal remobilization of P (BALDWIN, 2001). The vacuoles of plants also store phosphorus that is released under P deficient conditions to maintain Pi homeostasis in plants (AKHTAR; OKI; ADACHI, 2008).

Recently plant breeding is aiming for phosphorus use efficiency. Gene engineering that lead to high utilization efficiency depends on recovering genes involved in phosphorus use efficiency and utilization. WISSUWA et al., (2015) reported for the first time a locus for PUE that have the capacity to enhance biomass production per unit of P uptake and therefore have utility in plant breeding. GAMUYAO et al., (2012) also showed that the overexpression of PSTOL1 gene in rice varieties significantly enhances grain yield in phosphorus-deficient soil.

The high P removal rate in harvested grains drives the need to replace soil P by fertilizer application or leads to P mining where fertilizer application rates are low. One of the potential approaches of plant breeding target the quantity and quality of the phosphorus that are transferred to the grain, aiming to short the exported phosphorus from the field during the harvest (ROSE; LIU; WISSUWA, 2013). The effects of the substantial reduction of phosphorus in the grains still not clear. While some studies showed the effect of low phosphorus on seeds caused strength and germination commitment (ZHU; SMITH, 2001); experiments using seeds differing in P concentration that were not obtained from plants grown under severe P deficiency found no differences in yields when plants were grown in P replete or P deficient soil (ROSE; WISSUWA, 2012). Another worry is the impact of lowering the grain phosphate content for human and animal feed.

Despite of the fact, that recently endophyte bacteria are being investigated for its phosphorus mobilizing potential and plant growth promotion (OTEINO et al., 2015), the mechanisms of phosphorus solubilization is being attributed to organic acid production and there are no evidences that these strains are involved in the mobilization of phosphorus inside the plant. As previously stated in this strategy to promote phosphorus efficiency, the participation of microorganisms is still not properly addressed and for this reason it will not be deeper explored.

2.5. Changes in rhizosphere microbial community due to phosphorus fertilization

Once explored how the rhizosphere bacterial community can influence the plant phosphorus efficiency, it is important to consider that the availability of phosphorus in the soil and its management can also impact the bacteria rhizospheric community.

In the past five years, several studies targeted the influence of organic and inorganic phosphorus addition in crops and how it changed the rhizosphere microbial community. TAN et al., (2013) observed a predominance of Acidobacteria in the control treatments, while Firmicutes and Proteobacteria, mainly Alphaproteobacteria, abundances increased with P fertilization. They also demonstrated that P fertilization caused a decrease in bacterial groups able to efficiently mineralize phosphorus in the soil and therefore, caused a decrease in alkaline phosphatase capacity with higher P amendments.

Long term fertilization was described to have a significant impact in the rhizosphere composition. LI et al., (2014) have explored the impacts of nitrogen and phosphorus long term addition on microbial community composition in secondary tropical forest in China. They demonstrated that phosphorus addition always had positive effect on the microbial community, possibly it is the most limiting nutrient in the soil. They also described that the fungal community was more sensitive to phosphate fertilization, showing an increase in the ratio between F/B.

The bacterial community also showed limited changes in long term P, N and manure application soils. In the study conducted by WANG; JI; GAO, 2016, the changes in bacterial community after phosphorus addition were observed only in the lower taxonomic levels; it showed a significant increase of the family Micrococcaceae in the addition of P with N and manure.

Different levels of phosphorus fertilization affect differently the bacterial community. HUANG et al., (2016) found that different P addition had significant influences on soil microbial biomass but attributed these effects mainly due to indirect alterations of alterations in pH and dissolved organic carbon.

However, if these changes in the rhizosphere microbial community are beneficial or not to plant physiology and development is not yet elucidate, except for the study of BERGKEMPER et al., (2016), which suggested that the microbial community is assembled according to phosphorus depleted conditions, and that it may actually enhance the levels of phosphorus in forest soil historically depleted in this nutrient.

2.6. Perspective: rhizosphere microbiome manipulation for a more sustainable agriculture

The missing piece of this line of research perhaps is to access and explore how the manipulation of the rhizosphere microbiome towards to a more sustainable agriculture. According to SINGH; PANDEY; SINGH, (2011), agents with potential of promoting the growth of plants has been progressively applied in agriculture in order to reduce the application of phosphate fertilizer of high cost and dangerous to the environment. Besides, seed inoculation allied or not to application of phosphate fertilizers is being pointed to promote grain production, and the use of different species of bacteria combined is even more promising (KHAN et al., 2009). EGAMBERDIYEVA, (2007) related that the efficiency of rhizobacteria inoculants is affected directly by the nutritional condition of the soil, and the bacterial stimulants shows higher effect in plant growth when in depleted soils than in rich ones.

However, the studies targeting the inoculation of a given microorganisms to verify its potential to promote plant growth, disregard the associations of the entirely rhizosphere community. The inoculation of a selected bacteria or fungi consider that it will be able to find an available niche and succeed in an already established rhizospheric community. This is one of the reasons why the field studies do not show consistent results. Besides, the large amount of different conditions observed in soils must also be considered when expecting an inoculum to thrive in the new environment (EIDA; HIRT; SAAD, 2017).

The rhizosphere area is extremely influenced by the plant roots, and its physical and chemical characteristics is also changed according to plant genotype (HINSINGER et al., 2005;

MENDES et al., 2018). Several studies have already described that the changes in the exudations profiles of the plants under P stressful conditions, might as well affect the rhizosphere microbial communities. CARVALHAIS et al., (2011) showed that in P limiting conditions the release of c-aminobutyric acid and carbohydrates were stimulated in wheat. In soybean was observed an enhance in metabolites, amino acids and tricarboxylic acid cycle intermediates (TAWARAYA et al., 2014a, 2014b), and in common bean up to 10 % of primary and secondary metabolites exudation is promoted in P depleted conditions (TAWARAYA et al., 2014b, 2014a). The secretion of L-asparagine by soybean plants in P deficient conditions was already related to changes microbial community respiration (PENNANEN et al., 2004). Some other metabolites were investigated regarding its role in assembling bacterial community structure, Asparagine, glutamic acid and tryptophan, for example, were reported to have chemotactic to plant-associated bacteria, of genus *Rhizobium* sp., *Bacillus* sp. and *Pseudomonas* sp (GUPTA SOOD, 2003). However, there are still much to understand about the exudation patterns and its correlation with the rhizosphere community structure.

Modern cultivars usually are inefficient in phosphorus acquisition and utilization of phosphorus under limiting conditions. This is observed due to the fact that they were raised with optimal supply that selected against these characteristics, commonly present in Landrace genotypes (WISSUWA; MAZZOLA; PICARD, 2008). It is possible that plant breeding aiming cultivars more efficient in phosphorus uptake leaded to a less specialized microbiome in phosphorus provision to the plant. Until recently the plant breeding programs did not consider the rhizosphere microbiome (BAKKER; SHEFLIN; WEIR, 2012). Soon, breeding approaching microbial community to enhance the mobilization of phosphorus might become possible once identified the genes expressed that are involved in the recruitment of a given bacteria or a community of interest.

One of the major challenges to be addressed is correlating the microbiome to its function in the plant phenotypes. The identification of a functional microbiome starts with the acknowledgement of a microbial core directly related to the host phenotype. This can be achieved throughout the integration of taxonomic and metagenomic analysis and tested through microbiome manipulation (LEMANCEAU et al., 2017). However, this manipulation will only be able if the rhizosphere complexity of the rhizosphere is also considered (MUELLER; SACHS, 2015). Then, a specifically microorganisms do not need to be abundant, but the interactions between them and the environment can lead to an adapted community able to develop the desired function. To do this, is also important the identification of key stone species

that have determinant role in structuring the microbiome, whose addition or exclusion from the community can change all its functionality (YAM et al., 2017).

One of the ways of being successful in the microbiome manipulation is the enrichment of the rhizosphere microbial communities by repetitive cultivation. This approach, which considers the association between the plant and the rhizosphere microbial community is advantage by allowing the inclusion of non-culturable microorganisms, but of a well-structured community. This allows the microbiome to be projected to optimize a desired function. SWENSON; WILSON; ELIAS (2000), first applied this method to select the higher biomass of the model plant *Arabidopsis thaliana*. And recently, PANKE-BUISSE et al., (2015), showed the effect of the rhizosphere microbial community in the time of flowering of the same species.

2.7. CONCLUSIONS

Phosphorus sustainability is one of the main concerns to food security. Several strategies are evaluating the role of the rhizosphere microbiome in releasing soil fixed phosphates to plants. The use of microorganisms in agriculture cannot be limited to the approach of using single (or few) microorganisms as inoculants but the strategy need to be expanded to searching for a functional rhizosphere microbial community as a whole. In the future, linking this community to plant genes involved in its recruitment could be a suitable strategy for plant breeding in generating more efficient plants, considering also the rhizosphere microbiome.

3. MATERIAL E METHODS

3.1. Quantification of phosphate fixation in the soil

To establish the phosphorus gradient to be used during the bioassay, we set up an experiment to quantify the phosphate fixation in the soil. This analysis allowed to predict the amount of phosphate that should be added to ensure the constitution and conservation of the desired phosphate gradient until the end of the experiment.

To do this, five levels of phosphate: 15, 30, 60, 120, and 240 kg ha⁻¹ of P₂O₅, from Triple Superphosphate (TSP) or Rock Phosphate Bayovar (RPB), were added to 200 ml pots containing soil and kept under greenhouse conditions (~27,4°C, 24% moisture) for 10 days with constant moisture (70%). The sources of P₂O₅ were Triple Superphosphate (TSP, 46% P₂O₅) ground in ball mill for 1 min; and phosphate rock Bayovar (31% P₂O₅, 4.42% SiO₂, 0.96% Al₂O₃, 0.87% Fe₂O₃, 46% CaO, 0.53% MgO, 1.98% Na₂O, 0.3% K₂O, 0.01% MnO), ground for 10 min. By the end of the test, each pot was analysed to quantify the available P, by colorimetric method extracted with ion exchange resin at the Laboratório de solos, Embrapa Meio Ambiente, Jaguariúna, SP.

3.2. Experimental Design and Bioassay

The experiment was conducted according to a factorial scheme (2x2x4) in completely randomized design with 5 replicates. The studied factors were: 2 common bean genotypes (IAC-Imperador and Dor-364), 2 sources of phosphate (Triple Superphosphate and Rock Phosphate Bayovar) and 4 P-levels (0, 30, 60 and 240 kg ha⁻¹). The soil used in the experiment was obtained from the experimental field of Embrapa Meio Ambiente, Jaguariúna, SP, and was characterized as slightly acid and with high quantity of clay (Table 1).

Table 1. Physical and chemical properties of the soil used in the experiment

Total Sand	Clay	Silt	pH	MO	P _{resin}	H+Al	K	Ca	Mg	SB	CTC	V%
----- g kg ⁻¹ -----			(CaCl ₂)	(g dm ⁻³)	(mg dm ⁻³)	-----mmole dm ⁻³ -----						
429	528	43	4.6	21	4	38	1	17	10	28.0	66.0	42

The common bean genotypes selected, IAC-Imperador and Dor-364, were previously characterized in hydroponics studies as efficient and non-efficient in phosphorus uptake (SILVA et al., 2014). The sources of phosphate differ about their solubility, being TSP a readily available source, while RPB demand some time and relies mainly of microorganism and plant exudates to become available.

The experiment was conducted under greenhouse conditions, in 5-liter pots (147.4 ± 2.0 g), containing 4.5 kg of soil, kept under field capacity varying from 60 to 70% (total height 5.2 to 5.3 kg). The moisture was reset once or twice every day. To correct the base saturation and supply nitrogen, irrigations with nutrient solution containing $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ (0.2 mM) and $\text{Mg}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ (0.03 mM) were performed, 10 days after germination and repeated every 10 days until flowering state (R6) when sampling was performed. Total supply of nitrogen in each pot by the end of the experiment was 900 mg (720 mg via $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ and 180 mg via $\text{Mg}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$). The average temperature along the experiment varied between 17-29°C, photoperiod of 10h/14h (light/dark).

3.3. Phenotypic characterization of common bean

To verify phenotypic differences between the genotypes, were performed analysis of relative chlorophyll index (RChI) by non-destructive methods (SPAD-502Plus – Konica Minolta), height (H), and shoot diameter (ShDi) during stage V3 (First trifoliate leaf fully expanded), i.e. before the appearance of P depletion deficiency symptoms. The same parameters were analysed when the first symptoms began to appear during the end of the vegetative stages (V4-R5). During the flowering stage (R6), besides RChI, H and ShDi; the number of Nodes per plant (NPP) was also verified, and after the sampling of the rhizosphere the roots were storage in 20% ethylic alcohol and further analysed. Root structure analysis considered total length (TRL); superficial area (RSA), total volume (RTV) and average diameter (RoDi). The images of each root were obtained in scanner LA2400 (EPSON) and the characteristics were calculated using software WinRHIZO® (Regent Instruments Inc., Quebec, Canada), after the structural analysis they were air dried in room temperature until constant weigh to determine dried biomass (RDB). Right after the sampling, leaves were digitalized in Multifunctional Officejet 4400 (HP), and total leaf area (TLA) was accessed through a software that through the images of the scanned leaves, makes the segmentation by thresholding and counting of the pixels belonging to the leaf. For this, the calibration of the area of one pixel in the scanning process

was carried out by means of comparison with a figure of known area. The leaves were also dried in air forced oven (50°C) until constant weight to determine the shoot dried biomass (SDB). The evaluation of phosphorus content in the leaves were carried out through nitric perchloric digestion.

3.4. Isolation of phosphate solubilizing bacteria

Subsamples of 1g of rhizospheric soil was used to perform serial dilutions in 9 ml of sterile saline solution (NaCl 0.85%). To ensure the liberation of microbial cells from soil particles the first dilution was homogenized for 10 min followed by 1 min of ultrasound. Three dilutions (10^{-3} , 10^{-4} and 10^{-5}) were inoculated in Petri dishes containing Tryptic Soy Agar (TSA) 10% to access total bacteria; and phosphate media NBRIP (National Botanical Research Institute's Phosphate) containing 10 g de $C_6H_{12}O_6$, 5 g de $MgCl_2 \cdot 6H_2O$, 0.25 g de $MgSO_4 \cdot 7H_2O$, 0.2 g de KCl, 0.1 g $(NH_4)_2SO_4$, 5 g de $Ca_3(PO_4)$ and 15 g de agar in 1000 ml distilled water (NAUTIYAL, 1999), to identify and quantify culturable phosphate solubilizing bacteria (PSB).

After the quantification, colonies showing good potential of phosphate solubilization (identified by the size of the formed halo) were selected and isolated. The isolates were purified in NBRIP media and evaluated about the potential to solubilize phosphate rock Bayovar and mineralize calcium phytate ($C_6H_6Ca_6O_{24}P_6$). To evaluate the potential to solubilize phosphate rock Bayovar, a modified NBRIP media containing 20 g $C_6H_{12}O_6$, 5g $MgCl_2 \cdot 6H_2O$, 0.25g $MgSO_4 \cdot 7H_2O$, 0.2 g KCl, 0.1g $(NH_4)_2SO_4$, 5 g rock phosphate bayovar, 5 ml Bromo cresol green solution 0,5% and 15 g agar (GADAGI; SA, 2015) was used; to evaluate mineralization potential was applied a Phytate Specific Media (PSM), containing 15 g $C_6H_{12}O_6$, 5 g $(NH_4)_2SO_4$, 0.1 g de NaCl, 0.5 g KCl, 0.01g $FeSO_4$, 0.1 g de $MgSO_4 \cdot 7H_2O$, 0.01 g $MnSO_4$, 5 g calcium phytate and 15 g agar (SINGH; MALIK; SINGH, 2013).

The quantification of the amount of phosphate solubilized was performed according to (MARRA et al., 2011) with some modifications. The bacterial isolates were cultivated in Luria Bertani (10 casein, 5 g yeast extract, 10 g NaCl, 15 g agar) solid media for 2 days, the colonies were diluted from the Petri dishes culture in saline sterile solution (NaCl 0.85%) and the optical density was adjusted to $OD_{560}=0.5$, corresponding to 10^8 cells per ml. Erlenmeyer flasks containing 30 ml of Liquid NBRIP media received 1 ml of this inoculum and cultivated under constant 130 rpm agitation at 25°C for 10 days. Aliquots of 1 ml were taken and

centrifuged at 10,000 g for 5 min, the supernatant was used to quantify the solubilized phosphate with phosphorus-molibdate solution (Murphy e Riley, 1962).

A total of 11 isolates that showed higher potential to mineralize and solubilize phosphorus from different sources were selected to be identified through the sequencing of the 16S rRNA at the Centro de Pesquisas sobre o Genoma Humano e Células-Tronco, Instituto de Biociências, USP. The potential to produce organic acids was also evaluated in high performance liquid chromatography (HPLC). The organisms were grown in NBRIP media as previously described, after 10 days aliquots were taken, centrifuged at 10,000 g for 5 min and filtered in Millipore® membranes 0.2 µm. The extract was applied at column Bio-Red aminex HPX-87H, with mobile phase of acetonitrile 10.8 % in 0.0035M H₂SO₄, and constant efflux 0.5 ml min⁻¹, 35°C, UV (210nm) for 15 min.

3.5. Bacterial community assessment by 16S rRNA amplicon sequencing

The metagenomic DNA was extracted from 250 mg of rhizosphere using Power Soil DNA Extraction TM kit® (MoBio) following the manufacturer's instructions. Two replicates of each sample had the DNA extracted and then they were pooled together for higher representativity. DNA quality was accessed using NanoDrop™ 2000/2000c Spectrophotometer and 0.8% Agarose gel. Quantification was performed on Qubit® 2.0 Fluorometer (Invitrogen, USA), using Qubit® ds DNA HS Assay kit according to manufacturer's instructions. A total of 100 ng of DNA (20 µl at 5 ng µl⁻¹) was amplified using primers 515F-926R and the 16S rRNA amplicons were sequenced at Argonne National Laboratory (USA).

3.6. Taxonomical annotation and data analysis

The sequences obtained from the 16S rRNA sequencing were pre-processed using QIIME v.1.9 (CAPORASO et al., 2010). The forward and reverse reads generated were joint using fastq-join method (ARONESTY, 2011), followed by demultiplexing, removal of barcode and primer sequences. Quality filtering of the sequences was performed, and the sequences were truncate considering the Phred Quality threshold higher than Q20. After identification and removal of chimera using Usearch Database (Edgar, 2010), de novo Operational Taxonomic

Unit (OTU) picking was performed by assigning similar sequences to OTUs, selecting the representative OTUs, PyNAST alignment (CAPORASO et al., 2010); and taxonomy classification was attributed considering Silva's database (Quast et al., 2013). After the removal of chloroplast and mitochondria-related sequences, the pipeline followed to multivariate analysis. A PERMANOVA (Permutational Multivariate Analysis of Variance) was performed to access the effect of each factor in the rhizosphere microbial community using function ADONIS in Vegan Package (ANDERSON, 2001; DIXON, 2003). The constrained ordination analysis was performed with function Ordinate in R software Phyloseq Package (MCMURDIE; HOLMES, 2013).

After verifying the existence of the rhizosphere effect, the bulk soil samples were removed to visualize the effect of the genotypes tested. Further to verify the differences between the genotypes, IAC Imperador [P-efficientefficient] and DOR-364 [P-inefficientinefficient] were compared separately in each level and source of phosphate (Figure 3). Differently abundant OTUs between the genotypes in each condition were assessed through DESeq2 Package (LOVE; ANDERS; HUBER, 2014), in R environment (R Development Core Team, 2008). In this analysis the dispersion of the OTU counts were estimated by empirical Bayes shrinkage, that fits a generalized linear model (GLM) to OTU abundances with the treatments as explanatory variables.

The input data consisted in a matrix containing raw counts of sequencing of reads (LOVE; ANDERS; HUBER, 2014; MCMURDIE; HOLMES, 2014), after removing OTUs with less than 15 reads in each treatment. The visualization of differentially enriched OTUs was performed on iTOL (LETUNIC; BORK, 2016). To identify direct and indirect interactions among community members and the nature of this association in response to phosphorus levels, was performed a Network analysis with SPARCC (FRIEDMAN; ALM, 2012), considering only significant correlations ($p < 0,01$) that were higher than 0.9. The visualization of the network was performed in Gephi (BASTIAN; HEYMANN; JACOMY, 2009).

3.7. Functional data analysis

The hypothesis proposes that the less efficient genotypes regarding phosphate uptake would promote a rhizosphere enriched with phosphate mobilizing bacteria. To investigate whether differences in taxonomic composition mirrored different functions provided by the rhizosphere microbiota to their host plants a predictive metagenomics approach was

implemented. This strategy allows inferring the function putatively encoded by a given community based on their taxonomic composition. The prediction of the functions based on 16S rRNA sequencing was performed with function Tax4Fun (AßHAUER et al., 2015) available at R Environment (R Development Core Team, 2008) in the OTU Table containing taxonomic information according to Silva's database (Quast et al., 2013). The differentially enriched functions (KEGG orthology, KO) were accessed using a non-parametrical T-Test performed on QIIME (CAPORASO et al., 2010). A list of 46 KEGG functions (Table) involved in phosphorus metabolism was selected to analyse separately in a more specifically level with a non-parametrical T-test.

3.8. Enzymatic assays

3.8.1. Phosphatases analysis

To evaluate phosphatase activity, to 1 g of rhizospheric soil was added 0.2 ml of toluene, 4 ml modified universal buffer (MUB; pH 6.5 to access acid phosphatase and pH 11 to alkaline phosphatase) and 1 ml of p-nitrophenyl sodium phosphate as substrate. After incubating the flasks for 1 h, 37°C; 1 ml of CaCl₂ (0.5M) and 4 ml of NaOH (0.5M) were added to stop the enzymatic activity. The suspension was filtered in Whatman n° 2 paper filters, and the colour intensity was measured at 420 nm (TABATABAI; BREMNER, 1969).

3.8.2. Fluorescein Diacetate Hydrolysis (FDA) analysis

The analysis of fluorescein diacetate hydrolysis allows to evaluate the microorganisms activity in soil samples, once FDA is hydrolysed by several enzymes like proteases, lipases and esterase produced by living microorganisms (ADAM; DUNCAN, 2001). In glass recipients of 250 g, were added 5g of rhizospheric soil and 20 ml of potassium phosphate buffer and 200 µL of FDA solution (20 mg de FDA, 10 ml de acetone at 2 mg ml⁻¹). The samples were placed at shaker at 160 rpm in 25°C during 20 min. After the incubation 20 ml of acetone were added to recipients to stop the enzymatic activity. Each sample was filtered in Whatman ® n° 1 membranes, and the absorbance were measured at spectrophotometer 490 nm (FERNANDEZ; PITELLI; CADENAZZI, 2009).

3.8.3. Beta Glucosidase analysis

Beta glucosidase activity was measured from 0.5g of rhizospheric soil incubated with 0.1ml of toluene for 10 min at room temperature. After incubation 0.9 ml of distilled water, 1.5 ml of buffer and 0.6 ml of p-Nitrophenol-D-glucosidase were added and incubated for another 1 h. Ethanol was added to stop enzymatic reaction and 2 ml of Tris 2M was added to the solution. Enzymatic activity was measured through spectrophotometer at 400nm (HOFFMANN; DEDEKEN, 1995).

3.9. Arbuscular Mycorrhizal Fungi quantification

Quantitative Polymerase Chain Reaction (qPCR) was applied to quantify arbuscular mycorrhizal fungi gene. To perform the quantification, the primers proposed by LEE; LEE; YOUNG (2008) that generate 800 bp amplicons were modified to be applied on qPCR. Twenty four sequences of SSU rRNA belonging to arbuscular mycorrhizal fungi (1 to 4 genera of each of the 8 families belonging to Glomeromycota) were selected at GenBank (CLARK et al., 2016). Sequences were aligned using ClustalW Multiple Alignment on software BioEdit Sequence Alignment Editor (HALL, 1999). The primer AML-1 proposed by (LEE; LEE; YOUNG, 2008) was identified in the alignment and kept to ensure specificity to the selected mycorrhizal fungi genera. A second conserved region was identified in the alignment (AMF01), that in association to AML-1, generates a 100 bp amplicon. This primer was selected using the tool *Primer Blast* available at NCBI (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>), considering i) the size of the generated amplicon (100-250 bp), ii) melting temperature 56 - 64°C iii) primer size about 20 pb, iv) 50% GC content, v) 3 bases maximum complementarity. The primers were tested *in silico* using PrimerStat (available at http://www.bioinformatics.org/sms2/pcr_primer_stats.html). Non-mycorrhizal fungal SSU rRNA sequences were obtained at Genbank as previously reported. *In silico* evaluations showed that the primers were highly specific to AMF (Table 2).

A nested PCR was first performed in Thermocycler Veriti (*Applied Biosystems, USA*) using the primers NS1 - NS4 to 18S rRNA gene in the according conditions: denaturation 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, 40°C for 1 min and 72°C for 1 min and final extension of 72°C for 10 min. After that the PCR using AMF primers new pair of primers: Forward AML1 (LEE; LEE; YOUNG, 2008) and Reverse AMF01 according the conditions:

denaturation at 94°C for 3 min, followed by 35 cycles at 94°C for 1 min, 50°C for 1 min and 72°C for 30 s and a final extension at 72°C for 4 min (LEE; LEE; YOUNG, 2008). The integrity of the PCR amplicons was evaluated in 2% agarose gel in TAE buffer 1X. The purified amplicons were cloned using P-Gem Easy Vector[®], according to manufacturer's instructions. Copies of the gene were obtained by transforming *Escherichia coli* DH5- α . Viable clones were purified in alkaline hydrolyses. After the quantification Qubit[®] Fluorometer (Invitrogen, USA), five dilutions were performed to construct the calibration curve. The quantification was performed in *Applied Biosystems StepOne™ Instrument*, using only the arbuscular mycorrhizal fungi primers (AML1 e AMF01) following the conditions described previously. Each 10 μ l reaction consisted of buffer 1 X, MgCl₂ 2.5 mM, 0.25 pMol each primer, 5 μ l de SYBR Green and 1 μ l of metagenomic DNA.

Table 2. Sequences analysed to design the primer to be used in qPCR

Target	GenB ank c ode	Forward primer position		Reverse primer position	
		307	330	378	397
Arbusc ular Myc orrhizal Fungi					
<i>Glomus claroidium</i>	AJ276075	A T C A A C T T T C G A T G G T A G G A T A G A		G G A G A G G G G A G C C T G A G A A A C	
<i>Archaeospora leptoticha</i>	AJ301861	.	.	.	.
<i>Scutellospora cerradensis</i>	AB041344	.	.	.	.
<i>Archaeospora leptoticha</i>	AJ301861	.	.	.	.
<i>Paraglomus occultum</i>	AJ276081	.	.	.	.
<i>Diversispora spurca</i>	AJ276077	.	.	.	.
<i>Scutellospora projecturata</i>	AJ242729	.	.	.	.
<i>Glomus sinuosum</i>	AJ133706	.	T	.	.
<i>Glomus mossae</i>	AJ306438	.	.	.	.
<i>Paraglomus brasilianum</i>	AJ301862	.	.	.	.
<i>Glomus luteum</i>	AJ276089	.	.	.	.
<i>Diversispora epigae</i>	AJ276088	.	.	.	.
<i>Gigaspora rosea</i>	X58726	.	.	.	.
<i>Gigaspora intraradices</i>	X58725	.	.	.	.
<i>Ambispora fennica</i>	AM268196	.	.	.	.
<i>Pacispora scintillans</i>	AJ619947	.	.	.	.
<i>Pacispora scintillans</i>	AJ619946	.	C	.	.
<i>Glomus Manihotis</i>	Y17648	.	.	.	.
<i>Diversispora sp.</i>	Y17644	.	.	.	.
<i>Glomus caledonium</i>	Y17635	.	.	.	.
<i>Archaeospora trapei</i>	Y17634	.	.	T	.
<i>Acaulospora laevis</i>	Y17633	.	.	.	.
<i>Gigaspora albida</i>	Z14009	.	.	.	.
<i>Entrophospora colombiana</i>	Z14006	.	.	.	.
<i>Acaulospora spinosa</i>	Z14004	.	.	.	T
Non-myc orrhizal Fungi					
<i>Leveillula taurica</i>	AB033479	.	T	T G	.
<i>Microidium phyllanti</i>	AB120753	.	T	T G	.
<i>Chaetomium globosum</i>	AB048285	.	C	CT G C T T	A
<i>Cordyceps takaomontana</i>	AB044631	.	T T	G T T	A
<i>Fusarium oxysporum f.</i>	AF219124	.	T T	G T T	A
<i>Keissleriella cladophila</i>	AF164360	.	A	G T T	.
<i>Fusarium equiseti</i>	AF141949	.	T T	G T T	A
<i>Medicago truncatula</i>	AF093506	.	.	T	.
<i>Myrothecium sp.</i>	AJ301998	.	T T	G T T	A
<i>Dichostereum pallescens</i>	AF334912	.	.	T	.
<i>Lewia infectoria</i>	U43465	.	A	G T T	C
<i>Trichoderma sp.</i>	DQ401107	.	A	T G G T G	A A G A
<i>Trichoderma viride</i>	AY489694	.	T T	G T T	A
<i>Nectria lugdunensis</i>	AY357278	.	T T	G T T	A
Plants					
<i>Soybean (Glycine max)</i>	X02623	.	.	T C	.
<i>Trifolium repen</i>	AF071069	.	.	T C	.
<i>Lilium superbum</i>	AF206952	.	.	G C	.
<i>Sorghum bicolor</i>	AH001770	.	K	G C	N N W
<i>Melanoma Sanguinarum</i>	AF242265	.	A G	T T C	A

3.10. Rhizosphere microbiome manipulation of bean genotypes to improve phosphorus uptake

Thirty plants of each common bean genotype (IAC Imperador and Dor-364) were sown in 5-liter pots (147.4 ± 2.0 g), containing 4.5 kg of soil, kept under field capacity varying from 60 to 70% (total height 5.2 to 5.3 kg). Base saturation and supply of nitrogen were corrected with nutrient irrigations as previously described ($\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ (0.2 mM) and $\text{Mg}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ (0.03 mM)). The first cycle of the experiment last from January 29th until March 23rd, 2018, and it was conducted under greenhouse conditions. The first cycle of cultivation the different genotypes showed very distinct characteristics as expected (Figure 2). During the flowering stage, the plants were carefully removed, and the rhizosphere collected as previously described.

Figure 2. Illustration of genotypes differences in the first cultivation cycle. Showing IAC Imperador with higher structure with small and few leaves and number of nodes per plant; and Dor-364 with smaller structure, more leaves and higher number of nodes per plant.

The rhizosphere of 30 plants of each genotype was pooled together and homogenized to be transplanted, the same process was performed for bulk soil samples. The rhizosphere pool of IAC Imperador provided 703.60 g of material while Dor-364 rhizosphere pool was represented by 559.65 g of material. From the bulk soil 600 g of material was randomly collected across the pots to consist the inoculum. Equal portions (50 g) of rhizosphere was added to new pots containing the same soil (10 replicates each genotype). In addition, 10 control

pots received the homogenised bulk soil samples. The new pots that received the inoculum were previously prepared with 4,500 g of soil around a 50 ml falcon tube placed in the centre of the pot (Figure 3). After humidification, the falcon tubes were removed, and the inoculum was carefully placed inside the role left in the pots.

Figure 3. Experimental procedure illustrations showing a) the inoculum example, b) the pots containing the falcon tube in the centre and c) the removal of the falcon tubes leaving the role where the inoculum was carefully placed.

IAC Imperador [P-efficient] was sown in all the 30 pots that received rhizosphere or bulk soil inoculum. Bulk soil pots without cultivation continued to be kept during this cycle of growth. To evaluate if the rhizosphere from DOR-364 is able to change the phenotype of the IAC Imperador, periodically measurements of relative chlorophyll index, height, shoot diameter were taken in the V3 and V4 stages. At flowering stage (R6) besides RChI, H and ShDi. The leaves were also dried in air forced oven (50°C) until constant weight to determine the shoot dried biomass (SDB). The evaluation of phosphorus content in the leaves were carried out through nitric perchloric digestion. To calculate the effect of each genotype rhizosphere in the plant phenotype, the plants that received bulk soil inoculum were treated as negative control and the effect was calculate as described in Equation 1.

Equation 1.
$$Effect (\%) = \frac{T - (C-)}{C-} \times 100$$

Where *T* is the average value of height in the rhizosphere treatments and *C-* is the average value of height in plants that received the bulk soil inoculum.

4. RESULTS

4.1. Establishment of a phosphorus gradient in the soil

To verify the existence of a linear relationship between the phosphate added and the available phosphorus, a simple linear regression was performed (Figure 4). The available P remained in soil solution after the experiment time was about 10 times less than the phosphate added (Figure 5). Four levels of phosphorus were selected to continue the experiment: L0) with no phosphorus addition, L50) 30 kg ha⁻¹, which is half of the recommended amount, L100) 60 kg ha⁻¹, which corresponds to the indicated level of phosphorus to be applied in the soil to obtain a high yield of common bean (Instituto Agronômico, 1996), and L200) 240 kg ha⁻¹ of P₂O₅, which is the double of the recommended amount (Table 3).

Figure 4. Phosphorus fixation potential in the soil used in the experiment a) linear regression of the amount of triple superphosphate added in the soil and the remaining available phosphorus after 10 days incubated in greenhouse conditions ($P_{disp} = 0.0806P_{add} + 5.467$; $R^2 = 0.97$); b) linear regression of the amount of phosphate rock Bayovar added in the soil and the remaining available phosphorus after 10 days incubated in greenhouse conditions ($P_{disp} = 0.0583P_{add} + 5.2194$; $R^2 = 0.95$). X-axis shows the amount of phosphorus added (Padd) in the soil and Y-axis shows the amount of phosphorus available after incubation.

Figure 5. Phosphorus gradient selection a) levels of remaining available phosphorus after 10 days of incubation of triple superphosphate in the soil; b) levels of remaining available phosphorus after 10 days of incubation of phosphate rock Bayovar in the soil. Different letters indicate significant differences according to Tukey test considering $p < 0.05$. Arrows indicate the selected phosphorus levels to be used in the experiment.

Table 3. Levels of available phosphate selected to initiate the assay.

Natural	Triple superphosphate				Phosphate Rock Bayovar		
L0	L50	L100	L200		L50	L100	L200
-----mg ml ⁻¹ -----							
4	6	10	21		7	9	23

4.2. Phenotypic characterization of the common bean

During the early stage V3, differences between the two genotypes were clear (Figure 6). The constrained PCoA distinguished the genotypes in the first axis, explaining 55.3% of the data (ANOVA: $F=19.03$; $p=0.001$). The genotype Dor-364 showed smaller height and RChI compared to IAC Imperador, shoot diameter did not differ significantly between the cultivars (Table 4).

Figure 6 Constrained principal coordinates analysis showing differences between IAC Imperador and Dor-364 genotypes considering the plant features in Stage V3 (ANOVA: $F=19.03$; $p=0.001$)

Table 4 Differences between the genotype features during the early stage V3

	RChI ¹			H ²			ShDi ³		
	Mean±SD	F	p	Mean±SD	F	p	Mean±SD	F	p
IAC Imperador	34.28±1.32 ^{a*}	99.305	<0.01	12.34±1.98 ^a	40.06	<0.01	3.41±0.3	1.20	0.27
Dor-364	30.29±1.59 ^b			9.96±1.02 ^b			3.33±0.25		

¹RChI, Relative chlorophyll index

²H, Height (cm)

³ShDi, shoot diameter (mm)

*Different letters indicate significant differences accord Tukey test considering $p<0.05$.

During the stages V4 and beginning of R5 (40-44 days after planting), differences between the genotypes became more discrete and differences between the levels of phosphate applied become more evident (Figure 7). The only feature that continued to distinguish the two genotypes was height (Table 5)

Figure 7. Constrained principal coordinates analysis showing differences between IAC Imperador and Dor-364 genotypes considering the plant features in Stage V4/R5 ($F=34.86$, $p=0.001$)

Table 5. Differences between the genotypes features during the early stage V4-R5

	RChI ¹			H ²			ShDi ³		
	Mean±SD	F	p	Mean±SD	F	p	Mean±SD	F	p
IAC Imperador	27.69±2.98 ^{a*}	58.85	<0.01	17.39±5.96	2.18	0.14	4.15±0.68	0.026	0.87
Dor-364	21.46±3.77 ^b			15.59±4.05			4.12±0.55		

¹RChI, Relative chlorophyll index

²H, Height (cm)

³ShDi, shoot diameter (mm)

*Different letters indicate significant differences accord Tukey test considering $p<0.05$.

The phenotypical characterization was also performed at stage R6, just before the sampling. The levels of phosphate caused a visible effect on plant height and number of nodes (Figure 8). The levels of phosphate caused significant differences when considering all the previously features and the root structure analysis in the constrained analysis of principal coordinates (Figure 9).

Figure 8. Illustration of the genotypes along the phosphate gradient a) IAC Imperador growing in TSP gradient b) IAC Imperador growing in RPB gradient, c) Dor-364 growing in TSP gradient and d) Dor-364 growing in RPB gradient

Figure 9 Constrained principal coordinates analysis showing the differences in the plant features during R6 stage. a) IAC Imperador growing in TSP gradient (ANOVA, $F=16.582$, $p=0.001$), b) IAC Imperador growing in RPB gradient (ANOVA, $F=17.254$, $p=0.001$), c) DOR-364 growing in TSP gradient (ANOVA, $F=38.865$, $p=0.001$) and d) DOR_364 growing in RPB gradient (ANOVA, $F=48.625$, $p=0.001$)

The root structure showed to be more developed in IAC Imperador genotype compared to the Dor-364, furthermore, there was a negative effect of the levels of phosphate in the root related to the shoot development (Table 6).

Table 6. Root architectural analysis

Genotype	Source	Level	Length ¹	Area (cm ²)	Volume (cm ³)	Diameter (mm)	Biomass (g)
IAC-Imperador	L0	L0	4894.53±1215	470.10±119.53	3.60±0.96	0.31±0.01	0.19±0.06
		L50	5888.11±397	553.35±32.53	4.14±0.31	0.30±0.01	0.27±0.05
	TSP	L100	6067.24±678	573.28±75.56	4.31±0.66	0.30±0.01	0.28±0.06
		L200	4788.96±657	491.23±72.60	4.02±0.72	0.33±0.02	0.35±0.07
	RPB	L50	5123.47±324	476.88±25.75	3.54±0.24	0.30±0.01	0.25±0.02
		L100	5426.49±592	484.98±119.46	4.04±0.43	0.31±0.01	0.26±0.04
		L200	3474.05±449	368.77±48.13	3.12±0.42	0.34±0.01	0.17±0.05
		L200	3446.53±252	336.31±21.89	2.61±0.17	0.31±0.01	0.22±0.05
Dor-364	L0	L50	4862.57±931	482.94±77.23	3.82±0.51	0.32±0.01	0.26±0.09
		L100	5221.83±793	503.32±42.14	3.87±0.41	0.31±0.01	0.32±0.09
	TSP	L200	4529.31±417	470.26±48.66	3.89±0.41	0.33±0.01	0.34±0.06
		L50	3839.05±817	366.13±69.82	2.78±0.49	0.31±0.01	0.26±0.11
	RPB	L100	5871.88±1552	560.61±72.67	4.26±0.56	0.30±0.01	0.30±0.08
		L200	2114.82±1498	226.12±37.14	1.94±0.38	0.34±0.03	0.19±0.03
		L200					
		L200					

¹Mean±Standart deviation

Analysis of available phosphorus by resin colorimetric method were conducted before and after the experiment to confirm the existence of a phosphorus gradient from the beginning of the experiment until sampling. Shoot P were accessed to confirm the deficiency of phosphorus in plants and their response to phosphate additions. During the experiment, the amount of TSP and RPB fixed were minimum (Figure 10a and d), the IAC Imperador [P-efficient] responded more clearly to RPB addition than to TSP (Figure 10b and e), and in both sources of phosphate IAC Imperador showed to be more responsive to phosphorus addition than DOR-364 [P-inefficient] (Figure 10c and f).

The differences in shoot content of phosphorus between the treatments show, as well as the phenotypic features of plants, that the conditions achieved during the experiment were robust to access and evaluate the rhizosphere microbiome of common bean genotypes contrasting in phosphorus uptake efficiency growing in distinct phosphates sources gradient. The plants were carefully removed from the pots, loosing soil particles were removed from the roots and the plant was placed in a sterile plastic bag. A vigorous shaking promoted the release of the closely attached soil from the root that consisted in the rhizosphere samples.

Figure 10. Establishment of phosphate gradient in the beginning and gradient of available phosphorus in the end of the experiment and total content of phosphorus in the leaves of common bean genotypes, a) Bulk soil with TSP Additions, b) IAC Imperador with TSP additions, c) DOR-364 with TSP additions, d) Bulk soil with RPB additions, e) IAC Imperador with RPB additions and f) DOR-364 with RPB additions. Grey lines represent triple superphosphate additions and brown lines represents rock phosphate bayovar additions. Dashed line shows the concentration of available P in the beginning of the experiment, full lines show the concentration of available phosphorus in the end of the experiment and green lines show P content in shoot of both genotypes.

4.3. Isolation and quantification of phosphate solubilizing bacteria

The cultivation of microorganisms showed differences in counting of CFUs between IAC Imperador and Dor-364 only in L100 of TSP (Figure 11a) and L50 of RPB (Figure 11b), being higher in IAC Imperador with TSP addition and on Dor-364 with RPB addition. No significant differences were observed along TSP (Figure 11a) or RPB levels (Figure 11b)

Figure 11. Total of colony forming units count in TSA media observed in a) triple superphosphate gradient and b) rock phosphate bayovar

Considering that the quantification of CFUs is limited as only a small portion of the rhizosphere bacteria can be cultured, these results were confirmed with the quantification of

16S rRNA genes through qPCR. IAC Imperador showed higher abundance of total bacteria in L0, when compared to RPB additions (Figure 12b), the same trend was observed in Dor-364 growing in TSP (Figure 12a) and RPB (Figure 12b) gradient, but not in IAC Imperador growing in TSP (Figure 12a), in this case, L100 showed higher numbers of copies of 16S rRNA genes.

Figure 12. Bacterial quantification of 16S rRNA genes through qPCR in IAC Imperador and Dor-364 genotypes observed in a) triple superphosphate gradient and b) rock phosphate bayovar. Different letter represent significant differences in Tukey test considering $p < 0.05$

The number of phosphate solubilizing bacteria, identified by halo formation in NBRIP medium, was higher in Dor-364 rhizosphere along the TSP gradient (Figure 13a). No genotype effect was observed in RPB additions, and the frequency of phosphorus mobilizing bacteria in both genotypes (Figure 13b) decreased as the level of both sources of phosphate increased in IAC Imperador and in Dor-364 rhizosphere (Figure 13b), being more evident in Dor-364.

Figure 13. Phosphorus mobilizing bacteria observed by the formation of halo in NBRIP media in a) triple superphosphate gradient and b) rock phosphate bayovar. Different letter represent significant differences in Tukey test considering $p < 0.05$.

To confirm these results and remove the bias caused by the limitation of the culture dependent approach, the quantification of *gcd* gene, responsible for the production and release of organic acids able to chelate phosphate ions and promote solubilization was quantified through qPCR. Higher levels of *gcd* gene were observed under phosphorus limited conditions and in L200 of TSP, being higher in Dor-364 compared to IAC Imperador (Figure 14a). RPB additions caused an increase in *gcd* gene abundance in both genotypes until L100 but a drastically decrease was observed in L200 (Figure 14b). Despite of great variability between the abundance of *gcd* gene between the genotypes, no significant differences were observed.

Figure 14. Bacterial quantification of *gcd* genes through qPCR in IAC Imperador and Dor-364 genotypes observed in a) triple superphosphate gradient and b) rock phosphate bayovar. Different letter represent significant differences in Tukey test considering $p < 0.05$

From culture dependent approach 94 bacteria were isolated able to solubilize calcium phosphate. These isolated were also evaluated for the ability to mobilize other sources of phosphorus, where 27 were able to solubilize RPB and 62 besides solubilizing inorganic P, could also mineralize calcium phytate (Table 7).

Table 7. Isolates with potential to mobilize phosphate *in vitro* conditions

Isolate Id	Origin of the Isolate		Mobilization potential		
	Level/Source of P	Genotype	Ca ₃ PO ₄	RPB	Phytate
1	L200/TSP	IAC Imperador	*1	+	+
2			**	+	+
3			*	+	+
4			**	-	+
5			***	-	+
6			***	+	+
7			*	+	+
8			*	-	+
9			*	-	+
10			*	-	+
14	L200/TSP	Dor-364	*	-	+
16			*	-	+
18			*	+	+
19	L200/RPB	Dor-364	***	-	+
20			***	-	+
22	L200/RPB	IAC Imperador	***	-	+
23			*	+	+
25			*	+	+
26			***	+	+
27	L0	IAC Imperador	*	+	+
29			*	-	+
30			*	-	+
31			*	-	+
32			**	-	+
37	L50/TSP	IAC Imperador	*	-	-
38			*	-	-
41			*	-	-
42			*	-	+
43			*	-	+
46	L50/TSP	Dor-364	*	+	+
49			*	+	+
50			**	+	+
52			**	-	+
53	L100/TSP	Dor-364	*	+	+
54			**	+	+
55	L100/TSP	IAC Imperador	*	-	+
56			*	-	+
58			*	+	+
59			*	-	+
60			***	+	+
61			*	-	+
62			*	-	+
63			*	-	+
64	L50/RPB	IAC Imperador	*	-	+
65			*	-	+
66			*	-	+
67			*	-	+
68			*	-	-
69			*	-	-

70			*	-	-
71			*	-	+
72	L100/RPB	IAC Imperador	*	-	+
73			*	-	-
74			*	+	+
75	L100/RPB	Dor-364	***	+	+
76			*	+	+
77			*	+	+
79			*	-	+
80			***	+	+
81			*	-	-
82	L0	Dor-364	*	-	+
85			*	-	+
86			*	-	+
87			*	+	+
88			***	-	+
89			*	+	-
91			**	+	+
92	L50/RPB	Dor-364	*	-	+
93			*	-	+
94			*	+	+

¹. Quantification of phosphate liberated from Ca_3CO_4 , solubilization halo diameter, *=0.5 cm>1.5 cm, **=1.5 cm>3.0 cm, ***=>3 cm, + or - shows positive and negative potential to mobilize RPB or Phytate, these assays were not quantified.

Isolates highlighted in black were the selected to be identified through partial 16S rRNA sequencing

The phosphate liberation was quantitatively evaluated in liquid media in all bacterial isolates. Most of the isolates released less than 3 mg l⁻¹ of PO₄ from Ca₃(PO₄)₂, after 10 days of incubation at 25°C; only three isolates released more than 3,1 mg ml⁻¹ (Table 7). After the screening of the isolates, 11 bacteria were selected based on the ability of releasing more phosphate from calcium compounds and solubilize rock phosphate Bayovar on liquid media. These selected isolates were submitted to partial 16S rRNA sequencing to identification. Also, the release of organic acids was also evaluated.

Three species of the genera *Burkholderia*, two species of *Paraburkholderia* and two Actinobacteria were identified as showing the better phosphorus mobilization activity (Table 8). The species of *Burkholderia* (*B. multivorans*, *B. contaminans*, *B. territori*) produced citric acid, while *Paraburkholderia* (*P. carebensis* and *P. caledonica*) produced gluconic acid, and the Actinobacteria *Leucobacter aridicollis* produced gluconic acid and *Curtobacterium oceanosedimentum* produced oxalic acid (Figure 15).

Table 8. Partial 16S rRNA sequencing identification of the isolates with high potential to mobilize phosphorus

Taxonomical Classification					Isolate identification/ number		Similarity (%)	Completeness (%)	Top-hit strain
Phylum	Class	Order	Family	Genus	Specie	Nº			
Proteobacteria	Betaproteobacteria	Burkholderiales	Burkholderiaceae	<i>Burkholderia</i>	<i>B. contaminans</i>	5	99.24	100	^T LMG 23361
					<i>B. territorii</i>	26	99.73	58.0	^T LMG 28158
					<i>B. multivorans</i>	60	99.65	100	^T ATCC BAA-247
					<i>B. multivorans</i>	80	100	100	^T ATCC BAA-247
					<i>B. contaminans</i>	88	99.72	100	^T LMG 23361
				<i>Paraburkholderia</i>	<i>P. caribensis</i>	6	99.86	100	^T MWAP64
					<i>P. caribensis</i>	7	99.86	100	^T MWAP64
					<i>P. caledonica</i>	19	99.65	100	^T NBRC 102488
					<i>P. caribensis</i>	20	99.27	76.6	^T MWAP64
					<i>Leucobacter</i>	22	99.65	100	^T CIP 108388
Actinobacteria	Actinobacteria	Micrococcales	Microbacteriaceae	<i>Curtobacterium</i>	<i>C. oceanosedimentum</i>	75	99.17	100	^T ATCC 31317

Figure 15. Chromatograms showing a) negative control, b) organic acid standards, c) citric acid production from *Burkholderia* sp, d) gluconic acid production from *Paraburkholderia* sp, e) gluconic acid production from *Leucobacter aridicollis* and f) oxalic acid from *Curtobacterium oceanosedimentum*.

4.4. 16S rRNA Sequencing

From the sequencing results, 10,656 OTUs remained after pre-processing and filtering mitochondria and chloroplast from raw data. IAC Imperador [P-efficient] samples showed smaller number of OTUs along the treatments (Figure 16a), however no significant differences were observed; in the same way, higher richness was observed in the bulk soil compared to the rhizosphere of IAC Imperador and Dor-364 (Figure 16b). Shannon and Simpson indexes showed higher diversity in bulk soil samples, however, significant differences were observed only in Shannon values (Figure 16c), but not for Simpson (Figure 16d). Shannon diversity did not differ between the genotypes, but was lower in L0 and L200, and higher in intermediary levels of phosphors from both sources tested.

Figure 16. Alpha diversity indexes between the treatments a) number of observed OTUs b) richness index Chao1, c) Shannon diversity index and d) Simpson diversity index. Letters shows significance by Tukey test considering $p < 0.05$, Bulk soil, and rhizosphere of IAC Imperador and DOR-364 are identified by upper bars. Different colours show sources of phosphate (white= L0, grey=triple superphosphate source (TSP) and brown= Rock phosphate Bayovar (RPB))

The constrained ordination clearly separated bulk soil microbial community from the IAC Imperador and Dor-364 rhizospheres (Figure 17a) and showed increase in many phyla with known ecological importance, such as Actinobacteria, Proteobacteria and Bacteroidetes (Figure 17b).

Figure 17. The rhizosphere effect is demonstrated by a) constrained principal coordinate analysis (CAP) showing the differences between the genotypes (shapes) and levels of phosphate (colours); and b) Phylogenetic tree showing the differentially enriched OTU in the rhizosphere of both genotypes compared to the bulk soil.

4.5. Genotype effect on rhizosphere taxonomical and functional assembly in phosphate depleted conditions

A permutational multivariate analysis (PERMANOVA) showed significant ($p < 0.05$) influence of the genotype and the level among the samples, despite of that, the correlation (R^2) of these two factors and the rhizosphere microbial community was very low (Table 9)

Table 9. Permutational Multivariate Analysis of Variance showing the contribution (R^2) and significance (p) of each factor accessed in this study in the rhizosphere community assembly.

	Df	SumsOfSqs	MeanSqs	F.Model	R^2	p
Genotype	1	0.164	0.164	2.29	0.029	0.032
Level	1	0.254	0.254	3.55	0.046	0.006
Phosphate Source	2	0.181	0.090	1.26	0.032	0.192
Genotype*Level	1	0.111	0.111	1.56	0.020	0.119
Genotype*Source	2	0.357	0.178	2.49	0.064	0.006
Level*Source	1	0.111	0.111	1.56	0.020	0.123
Genotype*Level*Source	1	0.109	0.109	1.52	0.020	0.131
Residuals	60	4.293	0.071		0.769	
Total	69	5.580			1.000	

There are significant differences between the genotypes in phosphate deficient conditions (L0). Constrained analysis of principal coordinates significant separated Dor-364 from IAC Imperador rhizosphere microbial community in the first axis, explaining 18.2% of data variance (ANOVA, $F=2.33$, $p=0.001$) (Figure 18a). Predominantly there is a significant improvement of OTUs in Dor-364 compared to IAC Imperador. An enrichment of several OTU belonging to the phylum Actinobacteria, Bacteroidetes, Proteobacteria (mainly Alphaproteobacteria) was observed in the Dor-364 [P-inefficient] (Figure 18b). No OTUs were significantly enriched in IAC Imperador compared to Dor-364.

Figure 18. Differences between the common bean genotypes under phosphorus limiting conditions showed by a) Constrained principal coordinate analysis highlighting the differences between the genotypes and b) phylogenetic tree showing the differently enriched OTU between the genotypes. Red bars represent differential enrichment of OTU in Dor-364 rhizosphere. No significant differentially enriched OTU was observed in IAC Imperador rhizosphere under phosphorus depleted conditions.

Network analysis showed higher number of connections (edges) in Dor-364 rhizosphere, compared to IAC Imperador and bulk soil. Dor-364 network showed 354 nodes and 1432 interactions (Figure 19c), while IAC Imperador showed 110 nodes and 96 edges (Figure 19b) in this condition (L0). Higher degree, diameter and density was observed in Dor-364 rhizosphere network, however, IAC Imperador network showed higher modularity (Table 10). The analysis showed higher degree, diameter and average path length in Dor-364 network compared to IAC Imperador (Table 10). Bulk soil network also showed to be loose with lower modularity and high average path length and diameter.

Figure 19. Network visualization of rhizosphere communities in phosphorus deficient conditions of a) bulk soil community b) IAC Imperador rhizosphere and c) Dor-364 rhizosphere. Blue connections show positive correlations ($R^2 > 0.9$; $p < 0.01$), red connections show negative correlations ($R^2 < -0.9$, $p < 0.01$). The networks were constructed using five replicates for bulk soil and for each genotype. The size of the node is proportional to the number of connections it makes (degree). The colours represent different phylum.

In the IAC Imperador rhizosphere community, higher betweenness centrality (BC) was observed for an OTUs representing Alphaproteobacteria (*Sphingomonas*) and Actinobacteria (Conexibacteraceae). In Dor-364 rhizosphere the higher betweenness centrality was observed OTUs representing Chloroflexi (Ktedonobacterales) and Actinobacteria (*Actinoallomurus*).

Table 10. Network statistics of communities of Bulk soil, IAC Imperador and Dor-364 rhizosphere growing in P limiting conditions (L0)

	Bulk Soil	IAC Imperador	Dor-364
Number of nodes	269	110	354
Number of edges	255	96	1432
Positive Edges	151	79	853
Negative Edges	124	17	579
Modularity	0.86	0.87	0.37
Number of communities	61	27	35
Network Diameter	5	4	8
Average Path Length	1.67	1.47	2.77
Average Degree	2.04	1.75	8.09
Clustering Coefficient	0.039	0.019	0.083

The predictive metagenomics approach showed a significant enrichment of the general KO functions involved in Sugars and Glutathione metabolism and pentose phosphate pathway in IAC Imperador [P-efficient] rhizosphere; while in the rhizosphere of Dor-364 [P-inefficient],

Lysine degradation, biotin, propanoate and galactose metabolism and Fatty acid biosynthesis functions were enriched (Figure 20).

Figure 20. Differentially enriched functions accessed by the predictive metagenomics on 16S rRNA sequencing of IAC Imperador and Dor-364 rhizosphere under P limiting conditions.

Under phosphorus depleted conditions in IAC Imperador [P-efficient] rhizosphere, 6 predicted functions were differentially enriched, including quinoprotein glucose dehydrogenase. While 13 functions were enriched in Dor-364 rhizosphere consisting mainly in functions involved in phosphorus binding and transport (Figure 21; Table 11).

Figure 21. Differentially enrichment of functions involved in phosphorus mobilization accessed by the predictive metagenomics on 16S rRNA sequencing of IAC Imperador and Dor-364 rhizosphere under P limiting conditions.

Table 11. Non parametrical t test evaluating differential enrichment of KEGG functions involved in phosphorus mobilization between IAC Imperador and Dor-364 rhizosphere under phosphorus limiting conditions

KEGG Functions involved in P mobilization	T-Test	p^1	Dor-364 ²	IAC Imperador ²
Quinoprotein glucose dehydrogenase (K00117)	-4.37	0.03	8.84E-04	1.15E-03
Alkaline phosphatase (K01077)	3.16	0.05	3.43E-04	2.88E-04
Acid phosphatase (K01078)	0.85	0.44	2.20E-05	2.14E-05
Phosphoserine phosphatase (K01079)	-0.18	0.90	2.32E-04	2.33E-04
3-Phytase (K01083)	5.15	0.05	4.17E-05	3.18E-05
Glucose-1-Phosphatase (K01085)	5.37	0.03	2.43E-06	1.67E-06
Trehalose 6-phosphate phosphatase (K01087)	2.63	0.03	2.03E-04	1.90E-04
Imidazoleglycerol-phosphate dehydratase / histidinol-phosphatase (K01089)	3.27	0.03	5.23E-05	2.83E-05
Protein phosphatase (K01090)	-4.60	0.03	5.96E-04	6.82E-04
Phosphoglycolate phosphatase (K01091)	-0.41	0.82	5.38E-04	5.41E-04
Myo-inositol-1(or 4)-monophosphatase (K01092)	1.65	0.15	7.45E-04	7.28E-04
4-Phytase / acid phosphatase (K01093)	-2.25	0.08	3.44E-05	3.94E-05
4-Nitrophenyl phosphatase (K01101)	0.33	0.84	1.78E-05	1.73E-05
Alkaline phosphatase D (K01113)	-1.25	0.31	4.33E-04	4.56E-04
Phospholipase C (K01114)	-3.57	0.04	7.48E-04	9.01E-04
Phospholipase D (K01115)	1.24	0.34	2.01E-05	1.89E-05
Glycerophosphoryl diester phosphodiesterase (K01126)	1.95	0.14	7.20E-04	7.04E-04
Phosphate transport system ATP-binding protein (K02036)	-0.23	0.84	4.79E-04	4.82E-04
Phosphate transport system permease protein (K02037)	0.90	0.44	5.30E-04	5.22E-04
Phosphate transport system permease protein (K02038)	0.72	0.57	4.32E-04	4.28E-04
Phosphate transport system protein (K02039)	-2.75	0.03	3.25E-04	3.46E-04
Phosphate transport system substrate-binding protein (K02044)	-2.20	0.03	6.46E-04	6.67E-04
Phosphonate transport system ATP-binding protein (K02041)	3.71	0.03	1.06E-04	8.92E-05
Phosphonate transport system permease protein (K02042)	2.95	0.03	1.62E-04	1.36E-04
Phosphonate transport system substrate-binding protein (K02044)	4.24	0.03	1.49E-04	1.31E-04
Inorganic phosphate transporter, PiT family (K03306)	-3.21	0.06	5.40E-04	5.63E-04
Phosphonoacetaldehyde hydrolase (K05306)	-2.56	0.03	2.93E-05	3.48E-05

Putative phosphonate transport system ATP-binding protein (K05780)	2.17	0.03	3.96E-05	3.32E-05
Putative phosphonate transport system ATP-binding protein (K05781)	2.45	0.03	4.98E-05	4.16E-05
Sn-glycerol 3-phosphate transport system substrate-binding protein (K05813)	2.16	0.04	1.78E-04	1.47E-04
Sn-glycerol 3-phosphate transport system permease protein (K05814)	2.80	0.03	1.03E-04	8.91E-05
Sn-glycerol 3-phosphate transport system permease protein (K05815)	2.57	0.05	8.81E-05	7.57E-05
Sn-glycerol 3-phosphate transport system ATP-binding protein (K05816)	3.51	0.03	1.28E-04	1.12E-04
PhnM protein (K06162)	1.83	0.11	1.23E-04	1.03E-04
PhnJ protein (K06163)	2.08	0.04	4.53E-05	3.71E-05
PhnI protein (K06164)	1.98	0.06	5.74E-05	4.78E-05
PhnH protein (K06165)	2.01	0.06	2.78E-05	2.23E-05
PhnG protein (K06166)	1.79	0.11	2.24E-05	1.89E-05
PhnP protein (K06167)	-1.72	0.20	2.62E-04	2.80E-04
Phosphotriesterase-related protein (K07048)	0.39	0.75	1.62E-04	1.55E-04
Two-component system, OmpR family, phosphate regulon sensor histidine kinase PhoR (K07636)	0.09	0.90	7.60E-04	7.58E-04
Two-component system, OmpR family, sensor histidine kinase PhoQ (K07637)	3.00	0.03	2.81E-05	2.39E-05
Two-component system, OmpR family, phosphate regulon response regulator PhoB (K07657)	-1.48	0.24	2.93E-04	3.03E-04
Two-component system, OmpR family, alkaline phosphatase synthesis response regulator PhoP (K07658)	3.19	0.06	1.58E-04	1.39E-04
Acid phosphatase (class A) (K09474)	1.39	0.24	2.83E-05	2.64E-05
Outer membrane pore protein E (K11929)	2.55	0.05	1.50E-07	6.66E-08

¹ *p* values corrected for False Discovery Rate (FDR, Benjamini-Hochberg), values of *p*<0.05 are highlighted in black.

² Values of functions frequencies in each genotype standardized to the number of reads in the original file

4.6. Genotype effect on rhizosphere assembly in phosphate gradient of Triple Superphosphate and Rock Phosphate Bayovar

Genotypes contrasting in P uptake efficiency showed different assembly of the rhizosphere microbial community under phosphate depleted condition. To verify if these differences were consistent, these analyses were also performed in different levels and sources of phosphates. Along the levels of TSP applied to the soil, the genotype effect was still observed comparing IAC Imperador and DOR-364 (Figure 22a), constrained ordination of principal coordinates, significantly separated Dor-364 rhizosphere from IAC Imperador in the first axis explaining 11.2% the data (ANOVA, $F=2.84$, $p=0.001$) while RPB additions decreased the differences between the genotypes (ANOVA, $F=1.81$, $p=0.05$) (Figure 22b).

Figure 22. Constrained principal coordinate analysis showing the genotype effect along a) TSP additions and b) RPB additions. Different colours represent the different genotypes and different shapes represent different levels of phosphorus applied to the soil.

As previously described, there was a significant improvement of OTUs in Dor-364 [P-inefficient] rhizosphere compared to IAC Imperador [P-efficient] in phosphorus limiting conditions (L0, Figure 18). Along the TSP additions, the rhizosphere of Dor-364 [P-inefficient] still show a higher number of enriched OTUs compared to IAC Imperador [P-efficient] rhizosphere, this trend was observed until L100 (optimal level of P), followed by a decrease in genotype effect in L200 (that largely exceeds plants nutritional requirements). Dor-364 [P-inefficient] showed a markedly enhance in OTUs belonging to Bacteroidetes in L50, L100 and L200; while IAC Imperador [P-efficient] showed enriched OTUs mainly from Actinobacteria and Acidobacteria only in L100 (Figure 23).

Figure 23. Phylogenetic trees showing the differently enriched OTUs observed between IAC Imperador (blue bars) and Dor-364 (red bars) in each level of TSP applied to the soil.

Opposite to what is observed in TSP additions, the differences between the genotypes was not very clear when RPB was added to soil. Different enriched OTUs were observed only in L0 and L50 (Figure 24). where RPB addition results in significant enrichment of OTUs belonging to Actinobacteria in Dor-364 [P-inefficient] rhizosphere in L50 (similar to the observed in L0 conditions). Following, the increase of rock phosphate levels, decreased the genotype effect.

Figure 24. Phylogenetic trees showing the differently enriched OTUs observed between IAC Imperador (blue bars) and Dor-364 (red bars) in each level of RPB applied to the soil. No differentially enriched OTUs were observed in higher levels (L100 and L200) of RPB applied to the soil.

A small supply of TSP (L50), promoted the increase in the number of differentially enriched OTUs reflected the differences in the functional assembly. According to the predictive metagenome, Dor-364 [P-inefficient] rhizosphere was enriched with Bacterial Chemotaxis and Flagellar Assembly, suggesting a higher potential to recruit beneficial bacteria. Aminoacids and nitrogen metabolism were also enriched in Dor-364 [P-inefficient] rhizosphere compared to IAC Imperador [P-efficient] rhizosphere (Figure 25). However, significant enrichment of KEGG functions involved in phosphorus mobilization was not observed in Dor-364 [P-inefficient] rhizosphere compared to IAC Imperador [P-efficient] rhizosphere (Table 12).

Figure 25. Differentially enriched functions accessed by the predictive metagenomics on 16S rRNA sequencing of IAC Imperador and DOR-364 rhizosphere with L50 of TSP applied to the soil.

Table 12. Non parametrical t test evaluating differential enrichment of KEGG functions involved in phosphorus mobilization between IAC Imperador and Dor-364 rhizosphere with L50 of TSP applied to the soil.

KEGG Functions involved in P mobilization	T-Test	<i>p</i>¹	Dor-364²	IAC Imperador²
Quinoprotein glucose dehydrogenase (K00117)	1.56	0.42	1.15E-03	1.08E-03
Alkaline phosphatase (K01077)	-0.95	0.62	2.49E-04	2.71E-04
Acid phosphatase (K01078)	5.59	0.14	2.36E-05	2.11E-05
Phosphoserine phosphatase (K01079)	1.20	0.57	2.43E-04	2.34E-04
3-Phytase (K01083)	0.66	0.74	2.86E-05	2.71E-05
Glucose-1-Phosphatase (K01085)	-0.44	0.86	1.44E-06	1.49E-06
Trehalose 6-phosphate phosphatase (K01087)	-1.64	0.42	1.75E-04	1.85E-04
Imidazoleglycerol-phosphate dehydratase / histidinol-phosphatase (K01089)	0.89	0.64	2.63E-05	2.21E-05
Protein phosphatase (K01090)	1.41	0.42	7.70E-04	7.34E-04
Phosphoglycolate phosphatase (K01091)	1.88	0.34	5.35E-04	5.27E-04
Myo-inositol-1(or 4) -monophosphatase (K01092)	-3.28	0.14	7.02E-04	7.14E-04
4-Phytase / acid phosphatase (K01093)	0.24	0.86	4.02E-05	3.95E-05
4-Nitrophenyl phosphatase (K01101)	-0.35	0.86	1.56E-05	1.61E-05
Alkaline phosphatase D (K01113)	2.74	0.14	5.01E-04	4.66E-04
Phospholipase C (K01114)	0.44	0.85	1.04E-03	9.91E-04
Phospholipase D (K01115)	-0.31	0.86	1.66E-05	1.69E-05
Glycerophosphoryl diester phosphodiesterase (K01126)	1,75	0.64	6.97E-04	7.03E-04
Phosphate transport system ATP-binding protein (K02036)	-0.91	0.62	4.82E-04	4.91E-04
Phosphate transport system permease protein (K02037)	-1.03	0.58	5.13E-04	5.22E-04
Phosphate transport system permease protein (K02038)	1.60	0.42	4.34E-04	4.27E-04
Phosphate transport system protein (K02039)	0.06	0.99	3.60E-04	3.60E-04
Phosphate transport system substrate-binding protein (K02044)	0.91	0.64	7.00E-04	6.89E-04
Phosphonate transport system ATP-binding protein (K02041)	-0.40	0.85	7.72E-05	7.88E-05
Phosphonate transport system permease protein (K02042)	-0.21	0.86	1.18E-04	1.19E-04
Phosphonate transport system substrate-binding protein (K02044)	1.55	0.32	1.24E-04	1.17E-04
Inorganic phosphate transporter, PiT family (K03306)	0.58	0.81	5.74E-04	5.67E-04
Phosphonoacetaldehyde hydrolase (K05306)	0.50	0.82	4.03E-05	3.91E-05

Putative phosphonate transport system ATP-binding protein (K05780)	-2.38	0.28	2.83E-05	3.02E-05
Putative phosphonate transport system ATP-binding protein (K05781)	-2.32	0.31	3.41E-05	3.80E-05
sn-glycerol 3-phosphate transport system substrate-binding protein (K05813)	-1.06	0.56	1.28E-04	1.32E-04
sn-glycerol 3-phosphate transport system permease protein (K05814)	-1.38	0.43	7.68E-05	7.99E-05
sn-glycerol 3-phosphate transport system permease protein (K05815)	-0.67	0.68	6.55E-05	6.69E-05
Sn-glycerol 3-phosphate transport system ATP-binding protein (K05816)	-1.96	0.32	9.50E-05	1.03E-04
PhnM protein (K06162)	-2.43	0.29	8.61E-05	9.13E-05
PhnJ protein (K06163)	-1.32	0.46	3.13E-05	3.27E-05
PhnI protein (K06164)	-1.27	0.50	4.06E-05	4.22E-05
PhnH protein (K06165)	-1.59	0.42	1.87E-05	1.97E-05
PhnG protein (K06166)	-1.38	0.42	1.64E-05	1.72E-05
PhnP protein (K06167)	0.58	0.81	2.96E-04	2.86E-04
Phosphotriesterase-related protein (K07048)	-1.85	0.34	1.19E-04	1.58E-04
Two-component system, OmpR family, phosphate regulon sensor histidine kinase PhoR (K07636)	3.10	0.18	8.07E-04	7.53E-04
Two-component system, OmpR family, sensor histidine kinase PhoQ (K07637)	2.98	0.14	2.43E-05	2.13E-05
Two-component system, OmpR family, phosphate regulon response regulator PhoB (K07657)	3.55	0.14	3.21E-04	3.03E-04
Two-component system, OmpR family, alkaline phosphatase synthesis response regulator PhoP (K07658)	0.81	0.66	1.38E-04	1.33E-04
Acid phosphatase (class A) (K09474)	-0.52	0.85	2.41E-05	2.49E-05
Outer membrane pore protein E (K11929)	-1.24	0.31	2.97E-08	8.80E-08

¹ *p* values corrected for False Discovery Rate (FDR, Benjamini-Hochberg) , values of *p*<0.05 are highlighted in black.

² Values of functions frequencies in each genotype standardized to the number of reads in the original file

Optimal level of TSP (L100) increased the number of predicted functions differentially enriched between the genotypes. Flagellar assembly, bacteria chemotaxis, bacterial secretion system and two component system; as well as aminoacids and nitrogen metabolism continued to be enriched in DOR-364 [P-inefficient] rhizosphere. While ABC transport systems, some aminoacids metabolism and carbon pathway were increased in the rhizosphere of IAC Imperador [P-efficient] rhizosphere (Figure 26).

Figure 26. Differentially enriched functions accessed by the predictive metagenomics on 16S rRNA sequencing of IAC Imperador and DOR-364 rhizosphere with L100 of TSP applied to the soil.

Dor-364 [P-inefficient] rhizosphere showed a higher number of differentially enriched KEGG predicted functions involved in the phosphorus mobilization, compared to IAC Imperador [P-efficient] rhizosphere. These functions consist in P mineralizing enzymes and phosphatases regulons (Figure 27; Table 13).

Figure 27. Differentially enrichment of functions involved in phosphorus mobilization accessed by the predictive metagenomics on 16S rRNA sequencing of IAC Imperador and DOR-364 rhizosphere with L100 of TSP addition to the soil.

Table 13. Non parametrical t test evaluating differential enrichment of functions involved in phosphorus mobilization in between IAC Imperador and Dor-364 rhizosphere with L100 of TSP applied to the soil

KEGG Functions involved in P mobilization	T-Test	<i>p</i> ¹	Dor-364 ²	IAC Imperador ²
Quinoprotein glucose dehydrogenase (K00117)	-3.56	0.04	1.14E-03	9.44E-04
Alkaline phosphatase (K01077)	5.28	0.03	2.56E-04	3.21E-04
Acid phosphatase (K01078)	-3.50	0.07	2.27E-05	2.01E-05
Phosphoserine phosphatase (K01079)	-1.99	0.12	2.38E-04	2.29E-04
3-Phytase (K01083)	-1.93	0.12	2.97E-05	2.56E-05
Glucose-1-Phosphatase (K01085)	-2.85	0.05	1.70E-06	1.40E-06
Trehalose 6-phosphate phosphatase (K01087)	3.16	0.04	1.80E-04	1.98E-04
Imidazoleglycerol-phosphate dehydratase / histidinol-phosphatase (K01089)	-3.64	0.03	2.87E-05	1.66E-05
Protein phosphatase (K01090)	-4.82	0.03	7.57E-04	6.79E-04
Phosphoglycolate phosphatase (K01091)	-3.35	0.04	5.24E-04	5.11E-04
Myo-inositol-1(or 4)-monophosphatase (K01092)	4.18	0.03	7.03E-04	7.19E-04
4-Phytase / acid phosphatase (K01093)	-3.95	0.03	3.90E-05	3.17E-05
4-Nitrophenyl phosphatase (K01101)	3.36	0.05	1.70E-05	2.13E-05
Alkaline phosphatase D (K01113)	-7.09	0.03	5.01E-04	4.03E-04
Phospholipase C (K01114)	-3.61	0.03	1.01E-03	7.92E-04
Phospholipase D (K01115)	2.17	0.10	1.68E-05	1.82E-05
Glycerophosphoryl diester phosphodiesterase (K01126)	0.06	0.98	7.11E-04	7.11E-04
Phosphate transport system ATP-binding protein (K02036)	7.34	0.03	4.82E-04	5.37E-04
Phosphate transport system permease protein (K02037)	8.70	0.03	5.11E-04	5.68E-04
Phosphate transport system permease protein (K02038)	5.28	0.03	4.27E-04	4.46E-04
Phosphate transport system protein (K02039)	2.02	0.12	3.54E-04	3.63E-04
Phosphate transport system substrate-binding protein (K02044)	0.85	0.48	6.90E-04	6.96E-04
Phosphonate transport system ATP-binding protein (K02041)	2.48	0.09	8.09E-05	8.88E-05
Phosphonate transport system permease protein (K02042)	1.31	0.33	1.23E-04	1.30E-04
Phosphonate transport system substrate-binding protein (K02044)	0.30	0.77	1.25E-04	1.27E-04
Inorganic phosphate transporter, PiT family (K03306)	-5.37	0.04	5.78E-04	5.38E-04
Phosphonoacetaldehyde hydrolase (K05306)	-1.36	0.27	3.84E-05	3.64E-05

Putative phosphonate transport system ATP-binding protein (K05780)	0.63	0.62	2.86E-05	2.94E-05
Putative phosphonate transport system ATP-binding protein (K05781)	2.81	0.07	3.54E-05	4.02E-05
Sn-glycerol 3-phosphate transport system substrate-binding protein (K05813)	-0.29	0.84	1.28E-04	1.26E-04
Sn-glycerol 3-phosphate transport system permease protein (K05814)	1.77	0.14	7.47E-05	7.92E-05
Sn-glycerol 3-phosphate transport system permease protein (K05815)	0.24	0.85	6.28E-05	6.34E-05
Sn-glycerol 3-phosphate transport system ATP-binding protein (K05816)	4.77	0.03	9.47E-05	1.10E-04
PhnM protein (K06162)	-0.53	0.70	8.59E-05	8.41E-05
PhnJ protein (K06163)	-0.98	0.43	3.15E-05	2.99E-05
PhnI protein (K06164)	-1.18	0.36	4.08E-05	3.86E-05
PhnH protein (K06165)	-1.04	0.44	1.90E-05	1.79E-05
PhnG protein (K06166)	-1.66	0.17	1.66E-05	1.54E-05
PhnP protein (K06167)	-5.51	0.03	2.88E-04	2.40E-04
Phosphotriesterase-related protein (K07048)	5.71	0.03	1.34E-04	2.28E-04
Two-component system, OmpR family, phosphate regulon sensor histidine kinase PhoR (K07636)	-6.71	0.03	7.89E-04	6.60E-04
Two-component system, OmpR family, sensor histidine kinase PhoQ (K07637)	-2.45	0.10	2.38E-05	2.04E-05
Two-component system, OmpR family, phosphate regulon response regulator PhoB (K07657)	-8.33	0.03	3.13E-04	2.73E-04
Two-component system, OmpR family, alkaline phosphatase synthesis response regulator PhoP (K07658)	-2.68	0.07	1.40E-04	1.34E-04
Acid phosphatase (class A) (K09474)	-2.53	0.09	2.42E-05	2.14E-05
Outer membrane pore protein E (K11929)	-1.35	0.34	6.35E-08	4.53E-08

¹ *p* values corrected for False Discovery Rate (FDR, Benjamini-Hochberg) , values of *p*<0.05 are highlighted in black.

² Values of functions frequencies in each genotype standardized to the number of reads in the original file

Following the trend of the taxonomical assembly, with L200 TSP addition, the number of differentially enriched KO predicted functions between the genotypes decreased. The genotype effect was lower in this level of TSP, that is significantly higher than the plant requirement (Figure 28). Despite of that, flagellar assembly, bacteria chemotaxis, bacterial secretion system and two component system; as well as aminoacids and nitrogen metabolism are enriched in Dor-364 [P-inefficient]. While, ABC transport systems and valine, leucine and isoleucine metabolism were increased in the rhizosphere of IAC Imperador [P-efficient] (Figure 28). Considering the predicted KEGG functions involved in phosphorus mobilization, no genotype effect was observed in this level of TSP (L200) (Table 14).

Figure 28. Differentially enriched functions accessed by the predictive metagenomics on 16S rRNA sequencing of IAC Imperador and DOR-364 rhizosphere with L200 of TSP applied to the soil.

Table 14. Non parametrical t test evaluating differentially enrichment of functions involved in phosphorus mobilization between IAC Imperador and Dor-364 rhizosphere with L200 of TSP applied to the soil

KEGG Functions involved in P mobilization	T-Test	p^1	Dor-364 ²	IAC Imperador ²
Quinoprotein glucose dehydrogenase (K00117)	-1.03	0.58	1.00E-03	9.72E-04
Alkaline phosphatase (K01077)	3.79	0.10	2.79E-04	3.02E-04
Acid phosphatase (K01078)	-0.29	0.90	2.48E-05	2.45E-05
Phosphoserine phosphatase (K01079)	-3.96	0.06	2.48E-04	2.36E-04
3-Phytase (K01083)	1.59	0.36	3.16E-05	3.36E-05
Glucose-1-Phosphatase (K01085)	2.55	0.13	1.78E-06	2.21E-06
Trehalose 6-phosphate phosphatase (K01087)	4.63	0.06	1.74E-04	1.85E-04
Imidazoleglycerol-phosphate dehydratase / histidinol-phosphatase (K01089)	-0.82	0.70	3.89E-05	3.58E-05
Protein phosphatase (K01090)	-2.75	0.09	7.17E-04	6.82E-04
Phosphoglycolate phosphatase (K01091)	0.36	0.90	5.26E-04	5.28E-04
myo-inositol-1(or 4)-monophosphatase (K01092)	-1.60	0.36	7.17E-04	7.10E-04
4-Phytase / acid phosphatase (K01093)	-0.29	0.90	3.39E-05	3.37E-05
4-Nitrophenyl phosphatase (K01101)	1.53	0.36	1.91E-05	2.12E-05
Alkaline phosphatase D (K01113)	-1.92	0.33	4.80E-04	4.53E-04
Phospholipase C (K01114)	-1.16	0.54	9.10E-04	8.76E-04
Phospholipase D (K01115)	1.23	0.54	1.80E-05	1.85E-05
Glycerophosphoryl diester fosfodiesterase (K01126)	2.45	0.20	7.10E-04	7.22E-04
Phosphate transport system ATP-binding protein (K02036)	0.53	0.89	4.99E-04	5.02E-04
Phosphate transport system permease protein (K02037)	0.89	0.74	5.32E-04	5.36E-04
Phosphate transport system permease protein (K02038)	-1.96	0.28	4.51E-04	4.42E-04
Phosphate transport system protein (K02039)	-0.97	0.70	3.52E-04	3.49E-04
Phosphate transport system substrate-binding protein (K02044)	-3.13	0.05	7.03E-04	6.89E-04
Phosphonate transport system ATP-binding protein (K02041)	0.23	0.90	9.03E-05	9.10E-05
Phosphonate transport system permease protein (K02042)	-0.43	0.90	1.40E-04	1.38E-04
Phosphonate transport system substrate-binding protein (K02044)	-1.61	0.36	1.43E-04	1.37E-04
Inorganic phosphate transporter, PiT family (K03306)	-1.53	0.36	5.56E-04	5.51E-04
Phosphonoacetaldehyde hydrolase (K05306)	-0.44	0.89	3.94E-05	3.88E-05
Putative phosphonate transport system ATP-binding protein (K05780)	0.70	0.70	3.01E-05	3.10E-05

Putative phosphonate transport system ATP-binding protein (K05781)	1.77	0.36	3.71E-05	3.95E-05
sn-glycerol 3-phosphate transport system substrate-binding protein (K05813)	0.40	0.90	1.37E-04	1.40E-04
sn-glycerol 3-phosphate transport system permease protein (K05814)	1.21	0.54	8.02E-05	8.36E-05
sn-glycerol 3-phosphate transport system permease protein (K05815)	0.58	0.85	6.85E-05	7.01E-05
Sn-glycerol 3-phosphate transport system ATP-binding protein (K05816)	3.02	0.13	1.01E-04	1.09E-04
PhnM protein (K06162)	-0.16	0.90	9.12E-05	9.06E-05
PhnJ protein (K06163)	0.21	0.90	3.38E-05	3.42E-05
PhnI protein (K06164)	0.17	0.90	4.34E-05	4.38E-05
PhnH protein (K06165)	0.11	0.92	2.04E-05	2.05E-05
PhnG protein (K06166)	-0.02	1.00	1.73E-05	1.73E-05
PhnP protein (K06167)	-3.61	0.06	2.78E-04	2.62E-04
Phosphotriesterase-related protein (K07048)	4.52	0.06	1.31E-04	1.66E-04
Two-component system, OmpR family, phosphate regulon sensor histidine kinase PhoR (K07636)	-4.16	0.06	7.94E-04	7.51E-04
Two-component system, OmpR family, sensor histidine kinase PhoQ (K07637)	-0.20	0.90	2.64E-05	2.61E-05
Two-component system, OmpR family, phosphate regulon response regulator PhoB (K07657)	-4.02	0.06	3.12E-04	2.99E-04
Two-component system, OmpR family, alkaline phosphatase synthesis response regulator PhoP (K07658)	-0.54	0.89	1.53E-04	1.51E-04
Acid phosphatase (class A) (K09474)	0.95	0.69	2.25E-05	2.32E-05
Outer membrane pore protein E (K11929)	0.36	0.90	5.17E-08	5.82E-08

¹ *p* values corrected for False Discovery Rate (FDR, Benjamini-Hochberg), values of *p*<0.05 are highlighted in black.

² Values of functions frequencies in each genotype standardized to the number of reads in the original file

Rock phosphate additions, opposite to what is observed in TSP additions decreased the differences in taxonomical assembly of the two common bean genotypes (genotype effect). In the same way, neither KO general nor KEGG functions involved in phosphorus mobilization showed significant differential enrichment between genotypes along the RPB gradient.

4.7. Enzymatic analysis of the rhizosphere

Triple superphosphate gradient caused an increase in FDA activity in the rhizosphere of both genotypes, only in L50 FDA activity was significantly higher in Dor-364 compared to IAC Imperador (Figure 29a). Beta glucosidase was significantly higher in IAC Imperador in L100 (Figure 29b). Acid phosphatase activity was higher in Dor-364 rhizosphere than in IAC Imperador under P depleted conditions (Figure 29c). Along the triple superphosphate additions, there was a higher activity of acid phosphatase in IAC Imperador than in DOR-364 rhizosphere, being higher in L200. In the other hand, alkaline phosphatase activity responded directly to triple superphosphate additions being higher in DOR-364 rhizosphere than in IAC Imperador in L0, L50 and L100, but no significant differences between the rhizosphere of the two genotypes were observed at L200 (Figure 29d). This enzyme also responded to TSP levels being higher at P depleted conditions.

Figure 29. Enzymatic activity of a) FDA, b) Beta glucosidase, c) acid phosphatase and d) alkaline phosphatase in IAC Imperador and Dor-364 rhizosphere with triple superphosphate gradient (compared with Tukey test, * means $p \leq 0.1$, ** means $p \leq 0.05$, and *** means $p \leq 0.001$).

With rock phosphate additions, the enzymes involved in carbon metabolism (FDA and Betaglucosidase) responded positively along the RPB additions, however no significant differences were observed between the genotypes in the rhizospheric activity of FDA (Figure 30) and beta glucosidase (Figure 30b). With this source of phosphorus, acid phosphatase activity in Dor-364 rhizosphere was high in L0, L50 and L200, but not on L100 (optimal level of phosphorus); significant differences between the genotypes were observed only in L0 and L50, being higher in Dor-364 [P-inefficient] rhizosphere (Figure 30c). Alkaline phosphatase responded negatively to phosphate rock additions, being higher in P depleted conditions and lower in higher levels of P. Alkaline activity was significantly higher in Dor-364 [P-inefficient] rhizosphere in L0, L100 and L200, but not on L50 (Figure 30d)

Figure 30. Enzymatic activity of a) FDA, b) B-glucosidase, c) acid phosphatase and d) alkaline phosphatase in IAC Imperador and Dor-364 rhizosphere with rock phosphate bayovar (compared with Tukey test, * means $p \leq 0.1$, ** means $p \leq 0.05$, and *** means $p \leq 0.001$).

4.8. Level effect on rhizosphere taxonomical and functional assembly in phosphate gradient of Triple Superphosphate and Rock Phosphate Bayovar

Until now we focused the results in the differences between the genotypes. However, phosphate levels were also responsible for changes in microbial community structure as demonstrated previously in the permutational multivariate analysis (Table 9).

TSP additions caused the increase of diversity in the bulk soil; however, in both genotypes, the intermediary levels of phosphate were responsible for higher values of Shannon index, showing the effect of the level of phosphate in the rhizosphere community structure (Figure 16).

In IAC Imperador [P-efficient] low level of phosphate (L50) did not show any effect in the rhizosphere microbial community structure. Constrained analysis of principal coordinates, explained 16.6% of the data variance (ANOVA, $F=2.52$, $p=0.001$) and showed differences

between L200, L100 and grouped together L0 and L50 when analysing IAC Imperador rhizosphere microbial community along TSP additions (Figure 31a). Only in L200 there were differentially enriched OTUs compared to L0 (Figure 31b). While in Dor-364 [P-inefficient] growing in TSP additions, the constrained analysis of principal coordinates showed significant differences between the levels ($F=2.75$, $p=0.001$), where L50 was enough to cause a significant effect in the rhizosphere microbial community (Figure 31c), however the L100 had the community more distinct compared to the L0 (Figure 31d).

Figure 31. Level effect of triple superphosphate in the rhizosphere bacterial community structure a) Constrained principal coordinate analysis of IAC Imperador rhizosphere bacteria community structure b) number of differentially enriched OTUs along TSP gradient compared to P depleted conditions (L0) in IAC Imperador rhizosphere; c) Constrained principal coordinate analysis of DOR-364 rhizosphere bacteria community structure b) number of differentially enriched OTUs along TSP gradient compared to P depleted conditions (L0) in Dor-364 rhizosphere.

In the rhizosphere of IAC Imperador with the higher level of phosphate (L200) the phyla Verrucomicrobia, Planctomycetes, Actinobacteria and Proteobacteria were significantly

differentially enriched compared to P limiting conditions (Figure 32), where an enrichment of Bacteroidetes and Proteobacteria (mainly Alphaproteobacteria and Deltaproteobacteria) was observed. Comparing L200 with L0, no significant differentially enriched KO general predicted functions were observed. However, considering the KEGG functions involved in P mobilization, three were significantly affected by phosphate levels, including Phytase and Phosphonoacetaldehyde hydrolase that were enriched in L200 (Figure 33).

Figure 32. Level effect of triple superphosphate in IAC Imperador rhizosphere. Phylogenetic trees showing the differently enriched OTUs observed between L200 of triple superphosphate (light blue bars) and phosphorus depleted conditions (orange bars).

Figure 33. Level effect of triple superphosphate in IAC Imperador rhizosphere. Functions involved in phosphorus mobilization accessed by the predictive metagenomics on 16S rRNA sequencing differentially enriched between L200 of TSP (light blue bars) and phosphorus depleted conditions (orange bars) in IAC Imperador rhizosphere.

In Dor-364, the optimal level of TSP (L100) was responsible for showing a more evident difference compared to P limiting conditions (Figure 31d). In this level of TSP, rhizosphere

community showed enrichment of a great variety of microorganisms compared to L0. While P limiting conditions enriched OTUs belonging predominantly to Actinobacteria (Figure 34).

Figure 34. Level effect of triple superphosphate in Dor-364 rhizosphere. Phylogenetic trees showing the differently enriched OTUs observed between L100 of triple superphosphate (blue bars) and phosphorus depleted conditions (orange bars).

A significant differential enrichment of 28 KO general functions was observed between L0 and L100 (Figure 35). Where 11 were enriched in L0, including fatty acid biosynthesis and some amino acids metabolism pathways, and 16 were enriched in L100, including bacterial secretion systems, two component system and sugar metabolism pathways (Figure 35).

Figure 35. Level effect of triple superphosphate in Dor-364 rhizosphere. Differentially enriched functions between L100 TSP and phosphorus depleted conditions on Dor-364 rhizosphere accessed by the predictive metagenomics on 16S rRNA sequencing

Conversely, under phosphorus limiting conditions, the rhizosphere of Dor-364 [P-inefficient] showed significantly differential enrichment of KEGG predicted functions involved in phosphorus mobilization compared to L100 TSP (Figure 36). Twenty five functions involved in phosphorus transport and mineralization were enriched in L0 compared to L100, where only 11 functions were enriched (Figure 36).

Figure 36. Level effect of triple superphosphate in Dor-364 rhizosphere. Functions involved in phosphorus mobilization accessed by the predictive metagenomics on 16S rRNA sequencing differentially enriched between L100 of TSP and phosphorus depleted conditions in Dor-364 rhizosphere.

TSP additions caused a improvement in the structure of the community in both genotypes (Figure 37). In IAC Imperador, higher number of edges is observed in intermediary levels of TSP (Figure 37a), network diameter, average path length and average degree decreases as the levels of TSP increases (Table 15). Dor-364 showed a rhizosphere microbial community better structured than IAC Imperador under phosphorus depleted condition, as previously reported in this study (Figure 19c), however along additions of TSP, there is a decrease in the number of edges observed in the rhizosphere of this genotype (Figure 37b). Network diameter, average path length decreases in higher levels of TSP, however an increase in average degree and modularity is observed in higher levels of TSP (Table 15).

Figure 37. Level effect of triple superphosphate in co-occurrence network of a) the rhizosphere of IAC Imperador with different levels of triple super phosphate and b) the rhizosphere microbial community of DOR-364 in different levels of TSP. Blue connections show positive correlations ($r^2 > 0.9$; $p < 0.01$), red connections show negative correlations ($r^2 < -0.9$, $p < 0.01$). The size of the node is proportional to the number of connections it makes (degree). The colours represent different phylum.

In L50, higher betweenness centrality was observed in an OTU representing the Burkholderiales, Comamonadaceae in the rhizosphere of Dor-364. While in the rhizosphere of IAC Imperador, an OTU representing an Acidobacteria showed higher potential to be a keystone species in this condition.

In L100, the possible keystone species of Dor-364 rhizosphere is an OTU representing a Caulobacteraceae, while an OTU of Ktedonobacteria, showed higher betweenness centrality in IAC Imperador rhizosphere. In the higher level of TSP (L200), no potential keystone species was identified in IAC Imperador rhizosphere, while one OTU representing a Sphingobacteria, showed higher betweenness centrality in Dor-364 rhizosphere.

Table 15. Network statistics of rhizosphere communities of IAC Imperador and Dor-364 growing in TSP phosphate gradient

	IAC Imperador			Dor-364		
	L50	L100	L200	L50	L100	L200
Number of nodes	324	134	73	258	161	125
Number of edges	1598	122	52	241	171	108
Positive Edges	1020	97	33	162	104	79
Negative Edges	578	25	19	79	67	29
Modularity	0.35	0.86	0.92	0.80	0.88	0.88
Number of communities	27	34	24	24	48	30
Network Diameter	9	4	3	7	6	5
Average Path Length	2.65	1.48	1.22	2.162	1.84	1.62
Average Degree	9.86	1.82	1.42	2.12	1.87	7,73
Clustering Coefficient	0.097	0.027	0.026	0.049	0.022	0.046

4.9. Rock Phosphate Bayovar effect on rhizosphere microbial community

Rock phosphate Bayovar showed effect on rhizosphere microbial community of IAC Imperador even in the lower level applied. Constrained analysis of principal components, explained 14% of data and significantly (ANOVA, $F=2.04$, $p=0.001$) separated the rhizosphere microbial community of IAC Imperador between L0, L200, but clustered together L50 and L100 of RPB (Figure 38a). In the lowest level of RPB applied in the soil there was the highest number of differentially enriched OTUs in IAC Imperador rhizosphere compared to P depleted conditions (Figure 38b). The rhizosphere microbial community of Dor-364 also responded strongly to RPB additions (Figure 38c). The constrained analysis of variance significant (ANOVA, $F=2.47$, $p=0.001$) separated all levels of P, explaining 19.6% of the data variance. Higher number of differentially enriched OTU compared to phosphorus depleted condition was observed in L100 in Dor-364 rhizosphere (Figure 38d).

Figure 38. Level effect of rock phosphate Bayovar in the rhizosphere bacterial community structure a) Constrained principal coordinate analysis of IAC Imperador rhizosphere bacteria community structure b) number of differentially enriched OTUs along RPB gradient compared to phosphorus depleted conditions (L0) in IAC Imperador rhizosphere; c) Constrained principal coordinate analysis of DOR-364 rhizosphere bacteria community structure b) number of differentially enriched OTUs along RPB gradient compared to phosphorus depleted conditions (L0) in DOR-364 rhizosphere.

In IAC Imperador rhizosphere, significant differentially enriched OTUs in L50 belonged to phyla Bacteroidetes, Acidobacteria, Planctomycetes e Proteobacteria compared to phosphorus depleted conditions (Figure 39). No significant enriched predicted KO general functions were observed when comparing the L50 of RPB and P depleted conditions. However, considering the predicted KEGG functions involved in phosphorus mobilization, 15 were differentially enriched between L50 RPB and P depleted condition (Figure 40), most of them enriched under phosphorus depleted conditions, including functions involved in P transport (Figure 40).

Figure 39. Level effect of rock phosphate Bayovar in IAC Imperador rhizosphere. Phylogenetic tree showing the differently enriched OTUs observed between L50 of RPB (yellow bars) and phosphorus depleted conditions (orange bars).

Figure 40. Level effect of rock phosphate Bayovar in IAC Imperador rhizosphere. Functions involved in phosphorus mobilization accessed by the predictive metagenomics on 16S rRNA sequencing differentially enriched between L50 (yellow bars) of RPB and phosphorus depleted conditions (orange bars) in IAC Imperador rhizosphere.

In Dor-364 rhizosphere, a clear effect of the RPB level application was observed at L100 of RPB. In this condition, several phyla involved in the mobilization of phosphate in soil, such as Acidobacteria and Proteobacteria were differentially enriched compared to L0 (mainly class

Betaproteobacteria (Figure 41). P depleted conditions only showed differential enrichment of Actinobacteria compared to L100 of RPB in Dor-364 rhizosphere (Figure 41).

Figure 41. Level effect of rock phosphate Bayovar in Dor-364 rhizosphere. Phylogenetic tree showing the differently enriched OTUs observed between L100 of RPB (blue bars) and phosphorus depleted conditions (orange bars).

RPB additions caused a differential enrichment of 18 predicted KO general functions in L100 compared to P depleted conditions, where only 12 were differentially enriched in L0 (Figure 42). Functions involved in Alanine, aspartate and glutamate metabolism, Bacterial secretion system and flagellar assembly were enriched in L100 of RPB while in L0 functions involved in several amino acids metabolism (Valine, Leucine and Isoleucine, Glutathione, Lysine, Arginine and Proline) were significantly differentially enriched in L0 (Figure 42).

Figure 42. Level effect of rock phosphate Bayovar in Dor-364 rhizosphere. Differentially enriched functions between L100 RPB and phosphorus depleted conditions on Dor-364 rhizosphere accessed by the predictive metagenomics on 16S rRNA sequencing

Conversely, when considering the predicted functions involved in P mobilization, 40 functions were significantly differentially enriched, being 28 enriched in L0 conditions compared to L100 of RPB (Figure 43). Under P limiting conditions predicted functions like, acid and alkaline phosphatase enzymatic activity, phosphate transport systems and phytase activity are significantly differentially enriched; while in L100 RPB, besides of alkaline and acid phosphatase, the quinoprotein glucose dehydrogenase is also enriched (Figure 43).

Figure 43. Level effect of rock phosphate Bayovar in Dor-364 rhizosphere. Functions involved in phosphorus mobilization accessed by the predictive metagenomics on 16S rRNA sequencing differentially enriched between L100 of RPB and phosphorus depleted conditions in IAC Imperador rhizosphere.

Different from what was observed with TSP additions, RPB additions caused a neutral or negative response in the complexity of IAC Imperador [P-efficient] and Dor-364 [P-inefficient] rhizosphere network (Figure 44). Very low changes were observed in network attributes along RPB addition in the rhizosphere of IAC Imperador (Table 16), while RPB levels caused a decrease in Dor-364 rhizosphere complexity (Table 16). Despite of that, Dor-364 [P-inefficient] genotype show a higher complexity in the rhizosphere network compared to IAC Imperador [P-efficient], with this source of phosphate (Figure 44b).

Figure 44. Level effect of rock phosphate Bayovar in co-occurrence network of a) the rhizosphere of IAC Imperador with different levels of triple super phosphate and b) the rhizosphere microbial community of DOR-364 in different levels of rock phosphate. Blue connections show positive correlations ($r^2 > 0.9$; $p < 0.01$), red connections show negative correlations ($r^2 < -0.9$, $p < 0.01$). The size of the node is proportional to the number of connections it makes (degree). The colours represent different phylum.

Table 16. Network statistics of rhizosphere communities of IAC Imperador and Dor-364 growing in RPB phosphate gradient

	IAC Imperador			Dor-364		
	L50	L100	L200	L50	L100	L200
Number of nodes	177	151	144	271	209	169
Number of edges	162	135	152	328	211	151
Positive Edges	107	91	97	224	121	108
Negative Edges	54	44	55	104	90	43
Modularity	0.86	0.88	0.75	0.82	0.88	0.89
Number of communities	36	33	32	43	38	37
Network Diameter	4	4	3	5	4	5
Average Path Length	1.62	1.51	1.43	1.68	1.51	1.64
Average Degree	1.82	1.79	2.11	2.42	2.02	1.79
Clustering Coefficient	0.011	0.026	0.046	0.06	0.035	0.03

4.10. Arbuscular Mycorrhizal Fungi Quantification

Under phosphorus depleted conditions (L0), a higher abundance of AMF genes was observed in IAC Imperador [P-efficient] genotype compared to the Dor-364 rhizosphere (Figure 45a). In this condition (L0) the abundance of AMF was significantly higher than in the

rhizosphere treated with triple superphosphate ($p < 0.001$), however no significant differences were observed between the genotypes in each level of TSP.

The rock phosphate addition caused a significant decrease in AMF gene in L50 in both genotypes ($p < 0.01$). Phosphorus depleted conditions as well as higher levels of RPB applied in the soil showed higher abundance of AMF gene in rhizospheric soil. Despite of the trend showing higher abundance be observed in IAC Imperador genotype no significant genotype effect was observed (Figure 45b).

Figure 45. Quantification of arbuscular mycorrhizal fungi by qPCR a) in triple superphosphate gradient and b) in rock phosphate Bayovar gradient.

Taken together, the results suggest a significant influence of phosphorus uptake efficiency of the plants, levels and sources of phosphate in the rhizosphere microbial community assembly. The main results are summarized in (Table 17).

Table 17. Main finding of the high throughput 16S rRNA sequencing analysis using the proposed pipeline

Effect	Main findings	Results
Genotype effect under P-depleted conditions (L0)	Dor-364 [P-inefficient] showed several differentially enriched OTUs compared to IAC Imperador [P-efficient], a higher complex rhizosphere microbial community and higher number of functions involved in phosphorus mobilization, including Acid and Alkaline phosphatase activity in the rhizosphere.	Figure 18 Figure 19 Table 10 Figure 21 Figure 30c Figure 30d
Genotype effect in soil amended with a TSP gradient	Genotype effect continued clear along TSP additions, differences between the genotypes rhizosphere was more evident in L100. Despite there was a decrease in the number of functions involved in phosphorus mobilization differentially enriched between the genotypes.	Figure 22a Figure 23 Figure 27
Genotype effect in soil amended with a RPB gradient	Genotype effect was observed only in L50 of RPB. No significant differences between the genotypes rhizosphere were observed in higher levels.	Figure 22b Figure 24
TSP amendment effect on IAC Imperador	TSP additions slight changed the rhizosphere microbial community in IAC Imperador	Figure 31a Figure 31b
TSP amendment effect on Dor-364	L100 addition of TSP significantly changed the rhizosphere microbiome of Dor-364. L100 of TSP promoted the enrichment of several OTUs. However, higher number of functions involved in phosphorus mobilization was enriched in L0	Figure 31c Figure 31d Figure 34 Figure 36
RPB amendment effect on IAC Imperador	A small addition of RPB (L50) significantly changed the rhizosphere microbial community of IAC Imperador. This level of RPB promoted the enrichment of several OTUs, however, a higher number of functions involved in phosphorus mobilization was enriched in L0 compared to L50.	Figure 38a Figure 38b Figure 39 Figure 40
RPB smendment effect on Dor-364	L100 of RPB promoted the most significant differences in the rhizosphere microbial community of Dor-364, with a high number of differentially enriched OTU. However, higher number of differentially enriched functions were observed in L0 compared to L100.	Figure 38c Figure 38d Figure 41 Figure 43

4.1. Rhizosphere microbiome transplantation

The available phosphorus in the soil in the beginning of the experiment was 4 mg l⁻¹. After the first cycle of cultivation, bulk soil treatments remained with 4 mg l⁻¹ of phosphorus available. Rhizosphere of IAC Imperador [P-efficient] that was used as the inoculum in the second cultivation cycle showed about 7 mg l⁻¹ of available phosphorus, while in Dor-364, about 5 mg l⁻¹ was observed. Despite of that the phenotype of IAC Imperador plants growing with different rhizosphere microbiome was more evident in early stages (V3, Figure 46a), where the plants that received Dor-364 rhizosphere was about 7.45% higher and relative chlorophyll index

was about 3% superior (Table 18). In more advanced plant development stages, the effect of the rhizosphere microbiome in the phenotypic traits decreased. During the stage V4, the differences continued to be slight observed (Figure 46b), however the effect of the Dor-364 rhizosphere decreased during this stage, when plants that received Dor-364 microbiome were about 4% higher and relative chlorophyll index was about 2% superior (Table 18).

Figure 46. Differences between the IAC Imperador genotype growing with bulk soil (left), IAC Imperador rhizosphere microbiome (centre) and DOR-364 rhizosphere microbiome (right) in a) V3 stage, b) V4 stage and c) R5-R6 stage.

By the of the experiment, during R5-R stage the differences in average plant height growing with DOR-364 rhizosphere were about 3% superior (Figure 46c) while relative chlorophyll almost 10% lower than control plants (Table 18). Interestingly, IAC Imperador rhizosphere caused negatives effects during all stages compared to control plants. After the second cultivation cycle, the rhizosphere of IAC Imperador growing with BS, IAC Imperador and Dor-364 rhizosphere inoculum showed 4 mg l⁻¹ of available phosphorus.

Table 18: Effect of rhizosphere microbiome manipulation on IAC Imperador phenotype

	V3		V4		R5-R6	
	Height	IR Cl	Height	IR Cl	Height	IR Cl
Bulk soil	11.02±3.83	35.61±2.19	18.65±1.41	32.27±2.0	22.75±2.42	36.19±4.45
IAC Imperador	11.93±1.76	35.48±4.22	18.27±2.67	32.26±2.41	21.05±3.97	35.92±5.20
Dor-364	13.05±2.85	36.75±1.41	19.45±2.32	32.98±2.31	23.5±1.70	33.03±8.63
%Effect of IAC Imperador	-1.78	-0.35	-1.99	-0.01	-7.44	-0.7
%Effect of Dor-364	7.45	3.22	4.28	2.2	3.29	-8.72

5. DISCUSSION

5.1. Phosphorus crisis in agriculture and common bean culture

Since the 1960 when fertilizers applications became regular, Brazil soils are being supplied with more chemical and organic P fertilizers than the cultures can export from the fields (WITHERS et al., 2018). Brazil is the second greater agricultural producer in the world and continues to expanding its field boundaries (OECD/FAO, 2017). However, phosphorus consist in a finite resource, which extensive exploration might consume about 60% of the reserves in the next 100 years (US GEOLOGICAL SURVEY; JASINSKI, 2017). In this sense, to continue the agricultural production meeting the increasing demand for food, Brazil will have to find sustainable solutions to prevent economical losses and environmental impacts. In this scenario, a sustainable approach would be the exploration of the amount of phosphorus that exceed the plants requirement and are continuously storage in soils. Considering the Brazilian scenario 90-103 Tg of phosphorus is expected to remain in soils by 2050 (WITHERS et al., 2018).

Brazil in one of the main producers of common bean in the world, together with India, Myanmar, China, USA and Mexico. In 2016, was registered a total of 1,105 thousand hectares were cultivated with common bean in Brazil, an increase of about 13% compared to the previous year crop; and a yield of 1,388.6 thousand tons of grains, representing an increase of about 34% (CONAB, 2017). However, acid and high clay Brazilian soils are responsible for fixating great part of phosphorus fertilization, which is one of the main causes that still limits common bean cultivation in Brazil, once this culture is highly dependent on this nutrient (CUNHA; CASARIN; PROCHNOW, 2010; SILVA; VAHL, 2002).

Plants can show a great variety of adaptations to low phosphorus availability in the soil, including several mechanisms of uptake and phosphate mobilization (VANCE; UHDE-STONE; ALLAN, 2003). Once these characteristics are genetically defined, plant breeding traditionally search for the selection of cultivars more efficient on nutrient uptake and usage (FAGERIA, 1998). However, the relationship between the plants and its microbiome have been only partially considered in this process (BAKKER; SHEFLIN; WEIR, 2012). Once plants are usually selected under optimal nutritional conditions, functions related to plan nutrition might have been lost (PÉREZ-JARAMILLO; MENDES; RAAIJMAKERS, 2016; PHILIPPOT et al., 2013).

Some cultivars of common bean have already been classified about their efficiency in phosphorus uptake and usage. The genotypes IAPAR 81, Carioca Comum, IAC Carioca Tybatã, IAC Imperador and G2333 showed to be efficient and responsive to phosphorus fertilization, while the genotypes Dor-3645 and Jalo Precoco, were classified as inefficient and non-responsive in hydroponic studies (SILVA et al., 2014b). The genotype IAC Imperador showed better root length and superficial area development and also a higher yield compared to Dor-364 (SILVA et al., 2014b).

These two genotypes, despite of being classified as contrasting in phosphorus uptake efficiency in hydroponic studies, showed less contrasting differences when evaluated in soils. In eutrophic oxisol, root structure of IAC Imperador did not show significant differences compared to Dor-364, showing similar values of root length and volume; in addition, despite of Dor-364 yield be slight lower than IAC Imperador, no significant differences were observed (SILVA et al., 2016).

In this study, IAC Imperador also showed slight better structured root system compared to Dor-364. Higher values of root length, volume, average diameter and biomass were observed not only under phosphorus depleted conditions, but also along TSP and RPB additions. Although the yield was not evaluated in this study, the shoot P content showed that, Dor-364 had a higher content of phosphorus than IAC Imperador under phosphorus depleted conditions, but not on higher levels of P. This fact support that IAC Imperador is more sensitive to phosphorus additions than Dor-364 as previously reported.

5.2. Rhizosphere microbiome assembly in common bean genotype contrasting in P-uptake efficiency

The proposed hypothesis is that the common bean genotype Dor-364, inefficient in phosphorus uptake, would recruit a rhizosphere microbiome better structured and enriched in microorganisms able to mobilize and provide phosphorus for the plants. This hypothesis was accessed considering different levels and sources of phosphorus applied in the soil.

The rhizosphere bacterial community was very different from bulk soil community. Factors that lead to rhizosphere colonization were highly explored previously, being attributed mainly to the release of labile carbon from the roots. The exudation of chemical compounds through the roots confers to the rhizosphere labile carbon, together with primary metabolites like organic acids, amino acids, and secondary metabolites (alkaloids, terpenoids and phenolic compounds) that are potential drivers of the rhizosphere community assembly (VENTURI;

KEEL, 2016). The release of exudates from the plants might be related to phenological stages (CHAPARRO; BADRI; VIVANCO, 2014), but also by nutritional availability (CARVALHAIS et al., 2011; TAWARAYA et al., 2014b). Plant genotype also show a significant effect in the rhizosphere community assembly (BULGARELLI et al., 2015; WEINERT et al., 2011). But all these factors are delimited by the initial soil composition of microorganisms. Bulk soil diversity and richness are generally higher when compared to the rhizosphere microbial communities, due to the strong selection of microorganisms in this region.

The genotype effect on the rhizosphere community assembly was first analysed considering phosphorus deficient conditions (L0). Predominantly there is a significant improvement of OTUs in Dor-364 [P-inefficient] belonging to Actinobacteria, Bacteroidetes and class Alphaproteobacteria compared to IAC Imperador [P-efficient]. These taxa were already observed in the rhizosphere of several crops including common bean (TRABELSI et al., 2017), and their role in the soil are diverse once they exhibit different metabolisms and growth under several various conditions.

Considering that amplicon-based sequencing of 16S rRNA is widely applied for microbial ecology studies, the rhizosphere functional capabilities might be disregarded. Therefore, a tool for functional community profiling based on 16S rRNA data were applied to add some information about the rhizosphere community metabolism. The selected tool was Tax4Fun, which links 16S rRNA sequences with functional annotation with a nearest neighbour identification based on a minimum sequence similarity (AßHAUER et al., 2015). Despite of metagenomic prediction be imprecise due to its reliance in available sequenced genome, it is an important approach to provide insights about the community functional profile.

The inference of KEGG Orthology (KO) functional profiling of the rhizosphere of common bean genotypes under phosphorus limiting conditions, showed that biosynthesis functions continue to be enriched in the rhizosphere of Dor-364, compared to IAC Imperador rhizosphere. These results were supported with the enzymatic activity in the rhizosphere and the culture dependent approach. A higher activity of FDA, that represents microbial activity in the rhizosphere soil (ADAM; DUNCAN, 2001) and beta glucosidase enzymes and a higher number of CFU were observed in Dor-364 rhizosphere, showing a highly active microbial community.

Under phosphorus limiting conditions, bacteria might develop strategies to compete for the scarce resource. In this stressful condition, microorganisms that are able to adjusted cell physiology or replace the source of phosphorus by using a plentiful resource, use phosphorus

with different oxidation states, or that can regulate the synthesis of high-affinity transporters, and use sources from internal stores can over compete to those microorganisms that are not (CASEY et al., 2016; TAPIA-TORRES; RODRÍGUEZ-TORRES; OLMEDO-ÁLVAREZ, 2016). In the other hand, L200 might have increased the rhizosphere with dominant r-strategist microorganism, which significantly reduced the diversity and richness. This fact is in accordance to the intermediary disturbance hypothesis, that is observed in several communities in ecological studies (CONNELL; SERIES; MAR, 1978).

The analysis of KEGG predicted functions involved in phosphorus mobilization showed that, in Dor-364 [P-inefficient] rhizosphere under phosphorus limiting conditions, some phosphorus binding proteins, transport enzymes and some classes of phosphatases were more abundant than in IAC Imperador [P-efficient] rhizosphere. This suggests that, when phosphorus is limiting, Dor-364 rhizosphere bacteria community is specialized in mineralization of organic compounds. The shaping of a community higher specialized towards phosphorus cycling when phosphorus is limited was already described to forest soils (BERGKEMPER et al., 2016), many other studies related low phosphorus availability to changes in soil microorganisms (HUANG et al., 2016; ZENG et al., 2017). But no previously correlation between the rhizosphere microbial community and phosphorus uptake efficiency of the plants were accessed.

In IAC Imperador [P-efficient] was observed an increase in quinoprotein glucose dehydrogenase, an enzyme that facilitate the solubilization of inorganic phosphorus, promoting the efflux of ions during the oxidation of sugars (BERGKEMPER et al., 2016a). This fact suggest that its rhizosphere harbour a microbial community specialized in phosphorus solubilization from inorganic sources by releasing (keto)gluconic acid (GOLDSTEIN, 1995). When evaluating the phosphorus uptake efficiency of IAC Imperador and Dor-364 genotypes in previously studies (SILVA et al., 2014, 2016), only inorganic fertilizers were used. These findings might suggest that phosphorus uptake efficiency by the plants might correlate with the ability of the rhizosphere microbial to use inorganic P. It can also be proposed that Dor-364 is more efficient when using organic fertilizers; however, further studies are required to verify this statement. Furthermore, is still no possible to establish cause-effect relationships between the rhizosphere microbiome and phosphorus uptake efficiency.

Besides the rhizosphere community composition, and metabolic potential; accessing the interactions between the organisms might provide information about the available niches and strong positive/negative relationships between them. Visualising the rhizosphere community structure through network analysis allowed to observe that Dor-364 has higher number of organisms (nodes) making associations (edges) when than IAC Imperador. However, once the

average path length determines the average cohesion between nodes (ZHOU et al., 2010), and the diameter is the largest distance between a pair of node, IAC Imperador network is more robust than Dor-364, despite of the small number of nodes and correlations. Also, modularity and average path length that quantify the time of response of a community during a disturbance (FAUST, 2012), were higher in IAC Imperador, suggesting that this genotype has a rhizosphere microbial community more adapted to environmental disturbances.

The betweenness centrality of a node, is responsible for identification of possible keystone species in a given network (BORGATTI, 2005; POUDEL et al., 2016). Under phosphorus depleted conditions in IAC Imperador rhizosphere, the genus *Sphingomonas* showed to have an important role in the rhizosphere community structure. This genus was previously reported as potential plant growth promotion bacteria (YANG et al., 2014). In the rhizosphere of Dor-364, an OTU representing the order Ktedonobacteriales probably consist in a keystone species to structure the community. Very few information of this order is found due to the fact that only few representatives are cultivated (YABE et al., 2011), but it was already described as keystone species, being part of a core rhizosphere microbiome in sugarcane (HAMONTS et al., 2018).

Genotype effect continued to be clear along triple superphosphate additions, but not along RPB additions. Differences in the rhizosphere microbial community between the genotypes increased from phosphorus depleted conditions, until optimal application of TSP (L100), but in the higher level of TSP (L200) just few differences between the genotypes were observed. Once phosphorus is considered one of the main limiting factors in the soil, plants can exert a selective pressure by competing for it with microorganisms and select a rhizosphere microbiome able to thrive in depleted conditions (KUZYAKOV; XU, 2013). This selection can be driven by plant exudates, that are largely modified under different levels of phosphorus (TAWARAYA et al., 2014a, 2014b). For this reason, with L200 of triple superphosphate, which largely exceeds plant nutritional requirement, the selective pressure might have decreased and consequently, the genotype effect.

5.3. Rhizosphere microbial assembly in phosphorus gradient

The impact of triple superphosphate gradient was also observed in the KO predicted functional capacity. A small supply of TSP (L50) promoted functions involved in amino acids metabolism, flagellar assembly and bacterial chemotaxis in Dor-364 [P-inefficient]. These

findings suggest that in the level (L50) of TSP, the rhizosphere of the less efficient genotype in P uptake has a more intricate rhizosphere microbiome. Once, both flagellar assembly and chemotaxis can represent a community able to answer for chemical or physical characteristics (CELANI; VERGASSOLA, 2010; MITCHELL et al., 2009); and the amino acids metabolism can act like attractant agents (YANG et al., 2015). These results are in accordance with the network analysis in this condition (L50, TSP), where Dor-364 rhizosphere network structure showed higher modularity and lower diameter and average path length compared to the IAC Imperador rhizosphere in the same condition.

Despite of the lower number of correlated species (nodes), compared to L0 conditions, in this scenario, the rhizosphere microbiome of Dor-364 show higher number of co-occurrence (positive edges), than mutual exclusion or competition (negative edges) (FAUST et al., 2012). In this condition an OTU representing Comamonadaceae family, potentially involved in sulphur metabolism (SCHMALENBERGER et al., 2008) consisted in a possible keystone species of Dor-364 rhizosphere under phosphorus limiting conditions.

Although a significant enrichment of functions directly involved in phosphorus mobilization in the soil was not observed, nitrogen metabolism was promoted in Dor-364 rhizosphere compared to IAC Imperador. Possibly, under phosphorus depleted conditions, the environment was very aggressive, what also limited the richness and diversity of microorganisms in the rhizosphere, but a small addition of TSP exerted a priming effect in the rhizosphere of Dor-364 (HUO; LUO; CHENG, 2017; KUZUYAKOV, 2002), promoting higher diversity, metabolism (FDA enzymatic analysis) and a better structure community (network analysis).

With optimal supply of TSP (L100), more functions than those differentially enriched in L50 were also enriched in the rhizosphere of Dor-364. Bacterial secretion system, for example, is responsible for the transport of proteins through the membrane; these proteins can be from pathogenic bacteria to manipulate the host and allow the infection or help the bacteria to compete with nearby microorganisms and establish the association (GREEN; MECSAS, 2015). While, two component system functions involve the regulatory system of phosphatase production and secretion (GAO; STOCK, 2013; MORALEDA-MUÑOZ et al., 2003).

In all the levels of TSP added to the soil, rhizosphere microbiome showed the functions involved in Bacterial Secretions System, Flagellar assembly, chemotaxis enriched when compared to IAC Imperador rhizosphere. This strongly suggest that Dor-364 rhizosphere has an interaction much stronger with its rhizosphere microbiome than the genotype IAC Imperador.

Therefore, despite of not being responsive to phosphorus addition, Dor-364 show an improvement of several functions when compared to IAC Imperador along TSP gradient. Under L100 of triple superphosphate, Dor-364 also showed a higher number of functions involved in phosphorus mobilization enriched compared to IAC Imperador. Beyond, alkaline phosphatase regulons (two component systems), phytase, phospholipase, alkaline phosphatase D and quinoprotein glucose dehydrogenase were enriched in the P inefficient genotype compared to IAC Imperador. Probably the efficiency of IAC Imperador occurs due to physiological plant adaptations, like root development (CHIORATO et al., 2012; SILVA et al., 2014b, 2016; VANCE; UHDE-STONE; ALLAN, 2003). In this sense, the absence of these traits in Dor-364 genotype, possibly led to a higher reliance on its rhizosphere microbiome.

When RPB is added to the soil, genotype effect is observed only in L50 and is similar to the observed in L0. This result, followed by the absence of genotype effect in higher levels of RPB suggest that the diversity of microorganisms able to access phosphate rock is very limited.

The application of rock phosphate in the agriculture is advantageous mainly due to low cost, and for being more environmentally friendly than the application of soluble P. Rock phosphates consists in a natural source of fertilization, that requires a minimal or none manufacturing process (FAO, 2004). Furthermore, its chemical composition is extremely variable and complex, and they can be sources of many different nutrients beyond phosphorus, which is released slowly to the soil (FAO, 2012), reducing the fixation processes and therefore the need of repeated applications.

However, phosphate rock is less reactive than commercial fertilizers for direct application on the soil, consequently reducing the yield during few years following the application (ROSOLEM; MERLIN, 2014). Possibly, the results observed in this study reflects the difficult solubilization of the rock phosphate, that during the time of the experiment, did not selected a specialized rhizosphere microbial community.

The genotype effect along RPB levels did not show significant differences regarding the metabolic potential of the rhizosphere microbiome. However, the enzymatic activity in the rhizosphere showed a higher acid and alkaline phosphatase when RPB was added to the soil, compared to triple superphosphate additions. Several studies already reported that phosphatase activity is higher in phosphorus scarcity (BERGKEMPER et al., 2016b; CABUGAO et al., 2017; ZHOU et al., 2016), possibly with RPB additions the rhizosphere microbiome need to explore alternative sources of phosphorus like organic phosphates through enzymatic activity. In this sense, phosphate rock addition limited the access of phosphorus by the plant due to its

insoluble nature, and plant origin acid phosphatase activity was similar along RPB gradient. While the alkaline phosphatase activity, which is from microbial origin, slight decreased from phosphorus depleted conditions until higher level of RPB.

Despite of IAC Imperador be classified as responsive to phosphorus addition (SILVA et al., 2014b, 2016), its rhizosphere microbial community only responded to TSP additions in L100 and L200. Amendment of the soil with L200, which largely exceeds plants nutritional requirements, promoted on IAC Imperador the enrichment of members of many phyla such as Verrucomicrobia, Planctomycetes, Actinobacteria and Proteobacteria compared to phosphorus limiting conditions. Due to the high availability of phosphorus, possibly there was an increase in dominant species in higher levels of TSP, which also decreased the diversity of the rhizosphere bacterial community in this condition. For the same reason, a higher number of differentially enriched OTUs were observed in higher levels of TSP (L200) compared to phosphorus depleted conditions.

This finding suggests that the rhizosphere community did not respond to the P fertilizations as the plant phenotype does and that the most responsive genotype (IAC Imperador) is less dependent on the rhizosphere microbiome for P uptake. Reinforcing this statement, no differential enrichment was observed when comparing the general predicted functions of IAC Imperador rhizosphere under phosphorus depleted conditions and L200 of TSP. While, the functions involved in phosphorus mobilization were predominantly enriched in L200.

Conversely, in the rhizosphere of Dor-364 [P-inefficient], the smaller level of TSP (L50) applied to the soil was enough to cause a significant effect in the community structure. Dor-364 under L100 of TSP showed the most contrasting rhizosphere microbial community compared to L0 conditions. Despite of the largest number of differentially enriched OTU, and diversity in L100, phosphorus depleted conditions promoted the metabolic profile of Dor-364 rhizosphere with many functions including bacterial secretion system, two component systems, several amino acids metabolism and sugars metabolism compared to L100. Furthermore, phosphorus limiting conditions also showed an enrichment of several enzymes responsible for organic phosphorus hydrolysis like, phytase, phosphatases, P binding and transport proteins in Dor-364 rhizosphere.

Changes in rhizosphere community structure due to phosphate fertilization was reported in several previous studies. SILVA et al., (2017) showed a higher relative abundance of phyla Verrucomicrobia, Planctomycetes and Actinobacteria, but not Proteobacteria in maize rhizosphere added with 100 kg ha⁻¹ TSP, that corresponds to an inferior level of phosphate

compared to L200 (120 kg ha^{-1}). On the other hand, TRABELSI et al., (2017), showed a massive enrichment of common bean rhizosphere community structure with Proteobacteria supplemented with 50 kg ha^{-1} de TSP, a smaller addition compared to L100 applied in this study (60 kg ha^{-1}). However, these studies did not compare the application of phosphate in contrasting genotypes considering P efficiency.

Rock phosphate additions did not promoted differences between the genotypes (genotype effect); however, RPB additions caused an increase in diversity of IAC Imperador rhizosphere since low levels applications. The rhizosphere of IAC Imperador with L50 of RPB, showed an increase in several phyla, like Bacteroidetes, Acidobacteria, Planctomycetes and Proteobacteria compared to phosphorus depleted conditions. Changes in rhizosphere microbial community of maize was already described, showing a significant enrichment of Oxalobacteriaceae (Betaproteobacteria), Burkholderiaceae (Betaproteobacteria) and Bacillaceae (Firmicutes) was observed in maize rhizosphere (SILVA et al., 2017). However, considering the involvement of the rhizosphere microbiome in phosphorus mobilization a significant enrichment of binding and transport proteins is promoted under phosphorus limiting conditions in the IAC Imperador genotype. Conversely, Dor-364 rhizosphere show an enrichment of several OTUs belonging to Bacteroidetes, Proteobacteria and Verrucomicrobia in L100 of RPB, and this enrichment cause a potential promotion on several functions involved in bacterial recruitment like, flagellar assembly, bacterial secretion system and some amino acids metabolism. However, considering phosphorus mobilization an enrichment of enzymes and P binding a transport protein is still observed under phosphorus depleted condition.

In both genotype, RPB additions promote a higher diversity of microorganisms compared to TSP and no phosphorus additions. This was already observed in long term studies (SILVA et al., 2017). These findings, together with the previous observations regarding genotype effect, provide evidences to support the proposed hypothesis that there is a correlation between the plant efficiency in phosphorus uptake and its rhizosphere microbiome in phosphorus mobilization and that this recruitment is potentialized in phosphorus depleted conditions.

5.4. Effect of rhizosphere transplantation in plant phenotype

Assuming that, at least in part, plant microbiome influences the plant phenotype regarding nutrition. To determine to what extend the microbiome affects the plant nutrition, a proof of concept experiment was performed transplanting the rhizosphere microbiome of Dor-364 [donor] and IAC Imperador [receptor]. IAC Imperador plants growing with Dor-364

rhizosphere became higher than control plants (7.75%) and showed higher relative chlorophyll index (3.22%) in the early stages than IAC Imperador plants growing in their own rhizosphere.

The manipulation of the whole rhizosphere community is advantageous because it considers the rhizosphere complexity, interactions between the microorganisms, including non-cultivable ones and the metabolic potential of the community (MUELLER; SACHS, 2015). Repetitive plant cultivation is one of the approaches used to effectively enrich the rhizosphere microbiome as it considers the association between the plant and the rhizosphere microbial community. It was first applied by SWENSON; WILSON; ELIAS (2000) to obtain higher biomass of *Arabidopsis thaliana*. Flowering time was also changed by rhizosphere microbiome manipulation (PANKE-BUISSE et al., 2015). In both cases, the inoculum of the rhizosphere was composed by an enrichment of the rhizosphere of the plants with the desired characteristics.

The positive effect observed in the IAC Imperador phenotype growing in transplanted rhizosphere from Dor-364 was transient, i.e. limited to the initial development stages, and this can be explained by the fact that the plants were still under phosphorus depleted conditions or that more cultivation cycles would be needed to have a stronger and lasting impact on plant phenotype. In addition, another technical limitation could have impacted the phenotype outcome, which is the fact that after initial growth the root system explored soil out of the transplanted soil zone.

The effect of Dor-364 rhizosphere on IAC Imperador development reveals that despite of IAC Imperador be more efficient in P uptake, Dor-364 rhizosphere shows a higher dependency on its rhizosphere microbiome, enriching its rhizosphere with beneficial and phosphate mobilizing functions. These findings indicate that the efficiency of IAC Imperador occurs due to structural alterations in roots such as enhance of area and length; and reinforce the importance of properly consider the rhizosphere microbiome during plant breeding.

5.5. Considerations on data analyses approach

Considering the amount of data generated by high throughput sequencing, inference analysis is critical for interpretation of data. Some characteristics of the data such as the sample sizes, heteroskedasticity and multiple comparison tests performed can affect the inference if not correctly handled. Then, we proposed a pipeline that performs a strong inference analysis with methods to amend this issue and refining the inference.

Experiments involving several factors need to be partitioned to test multivariate hypotheses in ecology. The parametrical multifactorial analyses of variance are generally applied to ecological systems, and very often the assumptions are neglected. This fact can

potentially cause imprecise inferences and increase type I error. For this reason, there is a need for a robust analysis to be used in microbial ecology studies. To verify the effect of the factors in the structure of rhizosphere microbial community a PERMANOVA was applied (ANDERSON, 2001), which is advise for complex experimental designs and, allows partitioning of variability, considering actual Bray Curtis dissimilarity index.

The most commonly applied multivariate analysis, such as Principal Component Analysis (PCA), Correspondence Analysis (CA) and Non-Metric Multidimensional Scaling (NMDS) are considered Unconstrained Ordinations, and do not consider *a priori* hypothesis, just being applied to reduce dimensions of data (Anderson and Willis, 2003). We applied a Constrained Ordinations (CAP), which is able to uncover patterns masked in unconstrained ordinations, and application can provide important information about the ecological data (Anderson and Willis, 2003).

The differential analysis was performed through DESeq2 normalization (Weiss et al., 2017). The dispersion is estimated by empirical Bayes shrinkage, this function fits a generalized linear model (GLM) to OTU abundances with the treatments as explanatory variables; this approach is suitable when the limited number of samples in experiments might cause highly variable dispersion among the data. To avoid false positives, the shrinkage of log fold change (LFC) is fitted toward zero. With this approach, the shrinkage is stronger when OTU counts are low, have high dispersion and few degrees of freedom. The results of this analysis allow the identification of OTUs significantly enriched in each treatment and used as an input in a Wald Test pair-wise statistical comparison and the p values will be submitted to Benjamini-Holchberg correction.

The metagenomic prediction was performed using Tax4Fun which links 16S rRNA sequences with functional annotation with a nearest neighbour identification based on a minimum sequence similarity (AßHAUER et al., 2015). Despite of metagenomic prediction reliance on available sequenced genomes, that can cause a significant bias in the inference; Tax4Fun show a correlation of about 90% with metagenomic sequencing derived datasets from soil environment (AßHAUER et al., 2015), consisting in a strong tool to provide insights of the functional potential of the community.

Despite of advantage in applying DeSeq2 normalization to evaluate differentially enriched OTUs, the output of metagenomic prediction is standardized; making it inadequate to be analysed with DeSeq2. For this reason, a non-parametrical T-test was applied to evaluate differential enrichments in predicted metagenomics.

The multivariate ordinations can discriminate variable; however, they do not consider the interaction between populations. For this reason, we also propose to develop network analysis. This analysis can identify direct and indirect interactions among community members and the nature of this association in response to phosphorus levels (POUDEL et al., 2016).

To identify bacteria putatively relevant to specific treatments and investigate whether their abundances change in plant genotypes with contrasting P-efficiency uptake. A network analysis was performed using SPARCC correlations. This method minimizes the bias of compositional data (FRIEDMAN; ALM, 2012). Co-occurrence networks can identify keystone taxa in the rhizosphere (VAN DER HEIJDEN; HARTMANN, 2016). The calculation of the network using SPARCC infers associations in compositional data by estimating the linear Pearson's correlations between the log-transformed components (FRIEDMAN; ALM, 2012). To minimize the impact of rare OTUs, specific thresholds of frequency and abundance were included. A total of 99 correlations were performed to estimate the pairwise relation and the count data was permuted 100 times to generate randomized tables. The correlations obtained for the real data were compared to the shuffled data; and only pairwise correlations higher than 0.9 and p values below 0.001 were considered as significant interactions, suggesting strong evidence for association. Finally, the main findings of this study are summarized in Table 17.

6. CONCLUSION

The results show that the rhizosphere community of the genotypes responded differently, being DOR-364 [P-inefficient] a genotype that promote the recruitment of more OTUs when compared to IAC Imperador [P-efficient] in all levels of phosphate tested. Dor-364 [P-inefficient] also showed to have a higher communication with its rhizosphere microbiome and depends more on it for phosphorus mobilization. In despite of showing differences along the fertilizations with rock phosphate Bayovar, both genotypes showed similar rhizosphere microbial communities, mainly due to the limited number of organisms able to solubilize it.

Taken together, all the results, there is evidence that the less efficient genotype in P uptake is the main factor modulating the rhizosphere microbial community assembly compared to he different phosphate sources or levels. Therefore, we conclude that the less responsive to phosphorus fertilization, the more rhizosphere microbiome-dependent is a given genotype for P uptake. In addition, we demonstrated that this microbiome trait is transmissible using soil transplantation, as the rhizosphere microbiome of the less efficient genotype was able to promote growth of IAC Imperador [P-efficient] genotype in early stages of development. Finally, this study paves the way for plant breeding programs explore the rhizosphere microbiome and its functions related to phosphorus mobilization to promote sustainable agriculture through the exploration of residual phosphorus in the soil.

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