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ORIGINAL ARTICLE

Influence of resistance breeding in common bean on rhizosphere microbiome composition and function

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The rhizosphere microbiome has a key role in plant growth and health, providing a first line of defense against root infections by soil-borne pathogens. Here, we investigated the composition and metabolic potential of the rhizobacterial community of different common bean (*Phaseolus vulgaris*) cultivars with variable levels of resistance to the fungal root pathogen *Fusarium oxysporum* (*Fox*). For the different bean cultivars grown in two soils with contrasting physicochemical properties and microbial diversity, rhizobacterial abundance was positively correlated with *Fox* resistance. Pseudomonadaceae, bacillaceae, solibacteraceae and cytophagaceae were more abundant in the rhizosphere of the *Fox*-resistant cultivar. Network analyses showed a modular topology of the rhizosphere microbiome of the *Fox*-resistant cultivar, suggesting a more complex and highly connected bacterial community than in the rhizosphere of the *Fox*-susceptible cultivar. Metagenome analyses further revealed that specific functional traits such as protein secretion systems and biosynthesis genes of antifungal phenazines and rhamnolipids were more abundant in the rhizobacterial community of the *Fox*-resistant cultivar. Our findings suggest that breeding for *Fox* resistance in common bean may have co-selected for other unknown plant traits that support a higher abundance of specific beneficial bacterial families in the rhizosphere with functional traits that reinforce the first line of defense.
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Introduction

The rhizosphere microbiome, that is, the totality of microorganisms, their genomes and interactions in the rhizosphere, has been the focus of numerous studies to resolve its role in plant growth and health (Mendes *et al.*, 2013; Philippot *et al.*, 2013; Berg *et al.*, 2014). Microorganisms inhabiting the rhizosphere may provide a range of beneficial functions for the host plant, in particular nutrient acquisition, stress tolerance and protection against soil-borne pathogens (Mendes *et al.*, 2011, 2014; Pérez-Jaramillo *et al.*, 2015). The bulk soil is the main source of microbial species colonizing the rhizosphere, and the plant genotype drives, in part, the selection of the microorganisms by depositing specific exudates in the soil-root interface (Jones *et al.*, 2009; Mendes *et al.*, 2013). Hence, plant species, cultivars and soil type are key drivers of rhizosphere microbiome composition and functioning (Marschner *et al.*, 2001; Berg and Smalla, 2009; Bulgarelli *et al.*, 2012, 2015; Inceoglu *et al.*, 2012).

Over the past decades, plant breeders have exploited plant genetic traits to improve plant growth and tolerance to biotic and abiotic stresses (Pérez-Jaramillo *et al.*, 2015). Despite the increasing knowledge about the beneficial role of the rhizosphere microbiome for plants, there are only few examples of breeding programs that have considered rhizosphere-related traits (Smith and Goodman, 1999; Smith *et al.*, 1999; Wissuwa *et al.*, 2009). In this context, Bakker *et al.* (2012) stated that future breeding programs should evaluate plant lines for their broad interaction with soil microorganisms. Previous studies on the microbial basis of disease suppressive soils already showed that plants rely, at least in part, on the rhizosphere microbiome as a first line of defense against pathogen invasion (Mendes *et al.*, 2011; Chapelle *et al.*, 2015; Raaijmakers and Mazzola, 2016). A recent study has shown that the phytohormone salicylic acid modulates colonization of *Arabidopsis* roots by specific bacterial taxa, illustrating how plant defense pathways impact on the rhizosphere microbiome composition (Lebeis *et al.*, 2015). This study provided, for the first time,

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evidence of a connection between the plant immune system and the rhizosphere microbiome composition. Yao and Wu (2010) previously showed that the rhizobacterial community composition of a cucumber cultivar resistant to *Fusarium oxysporum* (*Fox*) differed from that of susceptible cultivars. Whether disease resistance also affects rhizosphere microbiome assembly of other crop species and, more importantly, the functional potential of the rhizosphere microbiome is yet unknown.

Here, we investigated how breeding for resistance of common bean (*Phaseolus vulgaris*) to the root pathogen *Fox* influences rhizosphere microbiome assembly and the abundance of specific functional traits associated with the first line of defense provided by the rhizosphere microbiome. Genetic resistance in common bean to *Fox* is based on a pool of genes with epistasis (Mukankusi *et al*., 2011) and nine quantitative trait loci were found significantly associated with *Fox* resistance (Román-Avilés and Kelly, 2005). The resistance is quantitatively inherited and influenced by environmental conditions (Baggett *et al*., 1965; Schneider and Kelly, 2000). Combining 16S ribosomal RNA (rRNA) amplicon sequencing and shotgun metagenomics, we assessed the taxonomical and functional profile of the rhizosphere microbiome associated with four common bean cultivars, with different levels of *Fox* resistance, growing in two soils with contrasting physicochemical properties and microbial diversity. This approach allowed us to investigate the effects of soil type and host genotype on rhizosphere microbiome composition, and to identify potential beneficial microbial groups and functional traits affected by resistance breeding.

Materials and methods

Soil sampling and physicochemical parameters

In order to test the effect of soil properties and diversity on the rhizosphere microbiome we conducted mesocosm experiments with two contrasting soil types specially related to microbial diversity, that is, Amazon Dark Earth (ADE) and an agricultural soil (AGR). The ADE soil was collected at the Hatahara site, located within the Amazon basin near Iranduba-Manaus, Brazil (03°16'S and 60°12'W). The ADE soils are anthropogenic horizons built-up by the Pre-Colombian Indians between 500 and 8700 years ago, and they are characterized by their high fertility and high microbial diversity (Brossi *et al*., 2014). The AGR was collected in an experimental area of the 'Luiz de Queiroz' College of Agriculture (ESALQ/USP, Piracicaba, Brazil), located at Anhembi municipality, Sao Paulo, Brazil (22°40'S and 48°10'W). The AGR soil is characterized as oxisol and this area has been used for agricultural experiments with common bean and corn. Soil physical and chemical properties were determined in triplicate for ADE and AGR based on 400 g of soil, performed at the Laboratory of Soil Analysis at ESALQ/USP according to the methodology described by Camargo *et al*. (2009). In brief, soil pH was measured in a 1:2.5 soil/water suspension. Exchangeable Al, Ca and Mg were extracted with KCl 1 M. Calcium and Magnesium were determined by atomic absorption spectrometry and Al by acid–base titration. Phosphorus and K were extracted by ion-exchange resin. Potential acidity (H+Al) was calculated based on the pH determined in SMP buffer solution (pH SMP). Mehlic 1 was used to extract the available micronutrients (Fe, Cu, Mn and Zn) being determined by atomic absorption spectrometry. Hot water was used to extract boron, being further determined by spectrophotometry with azomethine-H at 420 nm. The results of macro and micronutrients allowed the calculation of exchangeable bases (SB), the sum of Ca, Mg, and K; cation exchange capacity, the sum of Ca, Mg, K, Al and H; base saturation (V), the percentage relation between SB and cation exchange capacity; and Al saturation (m%), the percentage relation between exchangeable Al and cation exchange capacity. The texture of the soil samples was determined using Bouyoucos densimeter after shaking the soil vigorously with NaOH 1 M as dispersant. Total nitrogen was determined by Kieldahl method and NH_4+ and NO_3- by Raney/Kieldahl.

Plant material, experimental design and sampling

Soil samples previously collected at the field were used to grow common bean plants in a mesocosm experiment conducted in greenhouse at CENA-University of Sao Paulo (USP), Piracicaba, Brazil. Four common bean cultivars were used in the mesocosms. The choice of the common bean cultivars was based on levels of genetic resistance to *Fox* (Supplementary Table 1), and the seeds were provided by the Agronomic Institute of Campinas (IAC, Campinas, Sao Paulo). The mesocosms were assembled in ceramic pots (30 cm high × 20 cm diameter) with a stone layer of 5 cm on the bottom. Approximately 8 kg of soil were used to fill the pots, and four seeds were sowed in each pot. Each cultivar was grown in three independent pots per soil type, that is, four common bean cultivars × 2 soil types × 3 pots (replicates), resulting in 24 samples (independent pots). The plants germinated at 28/19 °C (day/night) with 12 h photoperiod. The moisture and temperature were regularly adjusted for optimal growth conditions for the plants. Plants were collected at R1 stage (early flower) and the roots with attached soil were removed from the pots and transported on ice to the laboratory. The roots were shaken to remove the loosely adhering soil. The soil attached firmly to the roots was collected with sterile brushes and considered to be the rhizosphere soil. Soil samples collected prior to the bean cultivation, without the effect of common bean roots, were considered as initial soil community and called here

as 'soil' treatment. For each pot we obtained a single DNA sample, which was used for both shotgun metagenome and 16S rRNA sequencing.

DNA extraction

Total DNA was extracted from 250 mg of soil (soil and rhizosphere) using the PowerLyzer PowerSoil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA), according to the manufacturer's protocol. Measurements of DNA quality and quantity were performed by 1% sodium boric acid (Brody and Kern, 2004) agarose gel electrophoresis and NanoDrop 1000 spectrophotometry (Thermo Scientific, Waltham, MA, EUA). The same DNA sample was used for DNA shotgun sequencing and 16S rRNA sequencing.

Quantitative PCR analysis

Quantitative PCR analysis (qPCR) was performed to quantify the number of bacterial gene copies in soil and rhizosphere samples of the four bean cultivars. The amplification reactions were run with universal primers for Bacteria U968F (5'-AACGCGAAGAACC TTAC-3') and R1387 (5'-CGGTGTGTACAAGGCCC GGGAACG-3') (Heuer *et al*., 1997), which generated amplicons size of 419 bp. The reactions were performed in a final volume of 10 µl containing 5 µl of SYBR Green Rox qPCR Kit (Fermentas, Brazil), 2.5 µM of each primer and 10 ng of DNA template. DNA amplification was performed with an initial denaturation temperature at 94 °C for 10 min, followed by 40 cycles of 94 °C for 30 s, 56 °C for 30 s and 72 °C for 40 s with a final melting curve of 95 °C for 15 s, 56 °C for 1 min and temperature increasing to 95 °C for 15 s, with data reading at each 0.7 °C. Reactions were carried out on the equipment StepOnePlus (Applied Biosystems, Foster City, CA, USA). The Cts values (cycle threshold) were used as standards for determining the amount of DNA template in each sample. Standard curves were produced for the 16S rRNA using cloned fragments of *Pseudomonas fluorescens* DSM 8369. Gene fragments were quantified in a spectrophotometer (190 and 840 nm–NanoDrop ND-1000) and diluted (10^7–10^3 genes µl) to generate the standard curve. The gene copy number in different samples was expressed as log copy numbers of gene per gram of soil. Statistical data analyses were performed using One-way analysis of variance and Tukey's test to determine the significance of differences between all soil samples, using the PAST 3 software (Hammer *et al*., 2001).

16S rRNA gene sequencing, data processing and taxonomical affiliation

For taxonomical profiling of the bacterial communities, triplicate PCR reactions of a total of 30 samples ((4 bean cultivars+soil) × 3 replicates × 2 soils) targeting the V3-V4 region of the 16S rRNA gene were performed. The amplification was conducted with the primers S-D-Bact-0341-b-17 (5'-CCTACGGGNGGCWGCAG-3') and S-D-Bact-0785-a-A-21 (5'-GACTACHVGGGTATCTAATCC-3'), with an amplicon size of 464 bp (Klindworth *et al*., 2013). An overhang adapter sequence was added to the locus-specific primers as recommended by Illumina, San Diego, CA, USA. For amplification, the 20-µl reaction mixtures contained 2 µl of reaction buffer × 10 (Invitrogen, Carlsbad, CA, USA), 1 µl of $MgCl_2$ (50 mM), 1 µl of the primer set (5 pmol each), 1 µl of deoxyribonucleotide triphosphate mixture (2.5 mM each), 0.25 µl of Bovine Serum Albumin (1 ng ml^{-1}), 0.2 µl (5 U) of Platinum Taq DNA Polymerase (Invitrogen), 1 µl of DNA template (20 ng) and 12.55 µl of sterilized ultrapure water. PCR amplification was performed using a GeneAmp PCR System 9700 Thermal Cycler (Applied Biosystems) with the following amplification cycles: 95 °C for 5 min, followed by 25 cycles of 95 °C for 40 s, 56 °C for 1.5 min, and 72 °C for 1 min with a final extension step at 72 °C for 7 min. Negative and positive controls were run in all amplifications. Products were purified with Agencourt AMPure XP reagents (Beckman Coulter, Brea, CA, USA), on a magnetic rack, as manufacturer's instructions. The purified amplicons were used for the insertion of barcoding indexes with the Nextera XT Index Kit (Illumina). A combination of 5 × 6 indexes was used totaling 30 samples per sequencing run. For each PCR, the 50-µl reaction mixtures contained 5 µl of reaction buffer × 10 (Invitrogen), 1.5 µl of $MgCl_2$ (50 mM), 5 µl of both forward and reverse indexes, 2 µl of deoxyribonucleotide triphosphate mixture (2.5 mM each), 0.3 µl (5 U) of Platinum Taq DNA Polymerase (Invitrogen), 20 µl of purified PCR products and 11.2 µl of sterilized ultrapure water. PCR amplification was performed using a GeneAmp PCR System 9700 Thermal Cycler (Applied Biosystems) with the following amplification cycles: 72 °C for 3 min, 95 °C for 30 s, followed by 12 cycles of 95 °C for 10 s, 55 °C for 30 s, and 72 °C for 30 s with a final extension step at 72 °C for 5 min. The PCR products were pooled in one single sample and gel purified with GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Prior to sequencing, the pooled purified sample was quantified with the KAPA SYBR FAST Universal qPCR Kit (Kapa Biosystems, Woburn, MA, USA) in a StepOnePlus thermocycler (Thermo Fisher Scientific, Waltham, MA, USA) according to manufacturer's protocol. The paired-end sequencing was performed with Miseq Reagent Kit v3 (600 cycles; Illumina) in a Miseq Personal Sequencing System (Illumina).

The data obtained by the sequencing was analyzed with bioinformatics tools as follow. Initially, primer sequences were removed from the per sample FASTQ files using Flexbar version 2.5 (Dodt *et al*., 2012). All reads were trimmed to a minimum length

of 150 bp and at least a Phred score of 25 by using fastq-mcf (https://expressionanalysis.github.io/ea-utils/). The remaining sequences were converted to FASTA format and concatenated into a single file. All reads were clustered into OTUs using the UPARSE strategy by dereplication, sorting by abundance with at least two sequences and clustering using the UCLUST smallmem algorithm (Edgar, 2010). These steps were performed with VSEARCH version 1.0.10 (Rognes *et al.*, 2015), which is an open-source and 64-bit multithreaded compatible alternative to USEARCH. Next, chimeric sequences were detected using the UCHIME algorithm (Edgar *et al.*, 2011) implemented in VSEARCH. All reads were mapped before the dereplication to OTUs using the usearch_global method implemented in VSEARCH to create an OTU table and converted to BIOM-Format 1.3.1 (McDonald *et al.*, 2012). Finally, taxonomic information for each OTU was added to the BIOM file by using the RDP Classifier version 2.10 (Cole *et al.*, 2014). All steps were implemented in a Snakemake workflow (Köster and Rahmann, 2012) as shown in Supplementary Figure 1. Singletons and doubletons, mitochondrion, chloroplast and eukaryotic sequences were removed and the BIOM file generated was used for statistical analyses. The 16S rRNA data are available at EBI (PRJEB14409).

Shotgun metagenomics, data processing and functional annotation

In total, 30 DNA sample libraries ((4 bean cultivars +soil) × 3 replicates × 2 soils) were prepared using the Miseq Reagent Kit v2 (500 cycles; Illumina), according to the manufacturer's protocol for shotgun metagenomic sequencing in a Miseq Personal Sequencing System (Illumina). The paired-end sequences obtained were merged using FLASH version 1.2.5 (Magoč and Salzberg, 2011) and low-quality bases (quality score lower than 20) from merged and unmerged sequences were trimmed from both ends using the Phred algorithm with SeqyClean script (https://github.com/ibest/seqyclean). Unassembled DNA sequences were annotated with Metagenomics Rapid Annotation (MG-RAST) pipeline version 3.5 (Meyer *et al.*, 2008). The functional profile was generated using the normalized abundance of sequence matches to the SEED database (Aziz *et al.*, 2008). For each metagenome, a table of the frequency of subsystems hits was generated and normalized by dividing by the total number of hits to remove bias indifference in sequencing efforts and read length. To identify hits, BlastX was used with minimum alignment length of 15 bp, minimal identity of 60%, and an *E*-value cutoff of $E < 1 \times 10^{-5}$. The data matrices generated were used for statistical analyses. The shotgun metagenome data are available at MG-RAST under the project 'Common Bean Rhizosphere Microbiome' (ID 12156) (more information on Supplementary Table 2).

Data analysis

Redundancy analysis (RDA) was used to visualize the community structure and determine its correlation with environmental parameters. First, the matrices were analyzed using Detrended Correspondence analysis to evaluate the gradient size of the species distribution, which indicated linearly distributed data (length of gradient < 3), suggesting the RDA as the best-fit mathematical model for the data. Forward selection and the Monte Carlo permutation test were applied with 1000 random permutations to verify the significance of environmental parameters upon the microbial community structure. RDA plots were generated using Canoco 4.5 software (Biometrics, Wageningen, The Netherlands). We used permutational multivariate analysis of variance (PERMANOVA) (Anderson, 2001) to test whether sample categories harbored significantly different metagenome or microbial community structures. Alpha diversity was calculated from a matrix of richness using Shannon's index. PERMANOVA and alpha diversity indexes were calculated with the software PAST 3 (Hammer *et al.*, 2001). Phylogenetic diversity was calculated as Faith's PD (Faith, 1992) by using Qiime (Caporaso *et al.*, 2010). Venn diagrams were also constructed to verify the proportion of groups exclusive and shared between samples using the webtool Venny 2.0.2 (Oliveiros, 2007). To determine the statistical differences between the treatments, the Statistical Analysis of Metagenomics Profile v2.1.3 (STAMP) software was used (Parks *et al.*, 2014). For 16S profiling, the BIOM file was used as input. For functional profiling a table with hits frequency of the functional subsystems (SEED database) for each metagenome was generated from MG-RAST and used as input. *P*-values were calculated using the two-sided Fisher's exact test (Fisher, 1958), confidence intervals were calculated using the Newcombe–Wilson method (Newcombe, 1998) and correction was made using Benjamini–Hochberg false discovery rate (Benjamini and Hochberg, 1995).

In addition, network analyses were performed to assess the complexity of the interactions among microbial taxa. Non-random co-occurrence analyses were performed using SparCC, a tool capable of estimating correlation values from compositional data (Friedman and Alm, 2012). For this, quality reads were clustered at 97% identity and an OTU table affiliated at genus level was used for analysis. For each network, *P*-values were obtained by 99 permutations of random selections of the data table, subjected to the same analytical pipeline. Statistically significant ($P < 0.01$) SparCC correlations with a magnitude of > 0.7 or < -0.7 were included into the network analyses. The nodes in the reconstructed network represent taxa at genus level, whereas the edges represent significantly positive or negative

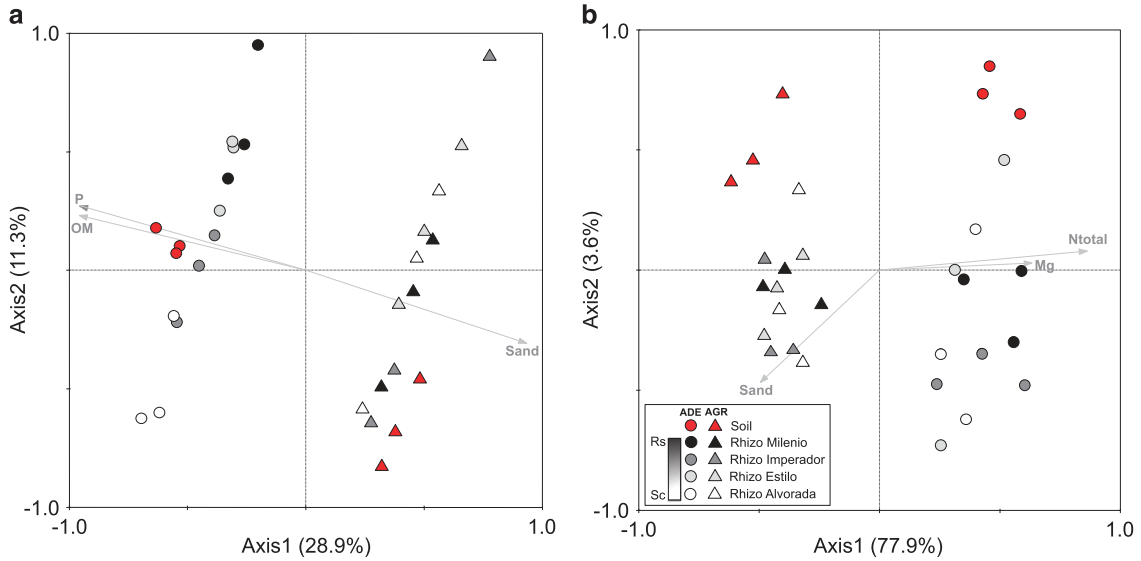

Figure 1 Redundancy analysis (RDA) performed on (**a**) taxonomic (16S rRNA) and (**b**) functional (metagenome) profiles and environmental characteristics for two types of soils and rhizosphere of four common bean cultivars. Arrows indicate correlation between environmental parameters and microbial structure. The shown environmental parameters were chosen based on significant correlation evaluated via the Monte Carlo permutation test ($P<0.05$); Rhizo = rhizosphere soil; Rs = resistant; Sc = susceptible; Mg = Magnesium; Ntotal = Total Nitrogen; OM = Organic matter; P = Phosphorus.

correlations between nodes. The network graphs were made based on a set of measures, as number of nodes, number of edges, modularity, number of communities, average node connectivity, average path length, diameter and cumulative degree distribution. Co-occurrence analyses were carried out using the Python module 'SparCC' and networks visualization and properties measurements were calculated with the interactive platform Gephi (Bastian and Jacomy, 2009).

Results and discussion

Rhizosphere effect on soil properties and microbial recruitment

The soil is considered as the prime source of microbial species richness and diversity in the plant rhizosphere. Hence, many studies have demonstrated the influence of soil type on the composition and structure of microbial communities in the rhizosphere (Ridder-Duine *et al.*, 2005; Berg and Smalla, 2009; Bulgarelli *et al.*, 2012; Inceoglu *et al.*, 2012; Lundberg *et al.*, 2012; Bakker *et al.*, 2015). The two soil types used in our experiment, Amazon Dark Earth (ADE) and AGR, differed significantly in physicochemical properties (Supplementary Table 3). This contrast was confirmed by molecular analyses, which revealed higher amounts of DNA retrieved and higher bacterial abundance in ADE than in AGR soil (Supplementary Figure 2). For both ADE and AGR soils, cultivation of all four bean cultivars had substantial effects on the physical and chemical properties of the rhizosphere soil, including significant declines in K and H+Al as compared with the soil ($P<0.05$). However, bean cultivation increased base saturation index as well as Ca and Fe contents ($P<0.05$). Overall, the rhizosphere effect was stronger in the AGR soil (Supplementary Tables 4 and 5). These changes are most likely due to several processes occurring at the root-soil interface during plant development, including the release of exudates, uptake of water and nutrients, and root respiration (Philippot *et al.*, 2013). In addition, the nutrient availability in rhizosphere is controlled by interactions among soil properties, plant characteristics, and microbial activity. Although a decrease of iron and calcium in the rhizosphere is common, accumulation owing to exudates and microbial solubilization may occur at some plant developmental stage (Moore *et al.*, 1965; Calba *et al.*, 2004; Rengel, 2015).

In addition to these chemical alterations, the taxonomic composition and functional profile of the bacterial community in the rhizosphere of all bean cultivars was different from that of the bulk soil, exemplifying the well-known rhizosphere effect. RDA of the taxonomic composition resulted in samples clustering firstly according to the soil type (Figure 1a) as confirmed by PERMANOVA ($F=10.35$, $P=0.0001$), followed by a separation by sample type, that is, soil or rhizosphere (PERMANOVA–ADE $F=4.937$, $P=0.0038$; AGR $F=3.035$, $P=0.0026$) (Supplementary Table 6; for taxonomic affiliation see Supplementary Figure 3). The taxonomic differences between the common bean cultivars were more evident in ADE soil, where the structure of the rhizosphere communities was significantly different among the cultivars (PERMANOVA $F=2.469$, $P=0.0007$).

For the functional annotation of genes, the samples were first clustered according to the soil type, which

was explained by the variation of the first axis of the plot (77.9%) and confirmed by one-way PERMANOVA ($F=87.81$, $P=0.0001$) (Figure 1b). Second, the samples were clustered in the second axis of the RDA based on sample type (PERMANOVA $F=1.903$, $P=0.0365$). However, it was not possible to discriminate the functional profiles based on the four different cultivars. The forward selection, followed by Monte Carlo permutation test, showed that the structure of the rhizobacterial communities correlates with physicochemical properties such as phosphorus content, organic matter, Cu and total nitrogen (Figure 1). These parameters were found to be distinctly different between ADE and AGR ($P<0.0005$).

When the OTUs of each cultivar were compared with those of the bulk soils, we found on average 8.6 and 9.4% of the OTUs exclusively present in the rhizosphere of beans grown in ADE or AGR, respectively. The core rhizosphere microbiome, that is, the bacterial OTUs shared between all four cultivars, accounted for ~73% of all OTUs (Supplementary Figure 4A). When the OTUs of the core microbiome were compared with the bulk soil, we found an average of 8.6% of OTUs present in the rhizosphere of all cultivars that were not found at detectable numbers in the soil (Supplementary Figure 4B). When rhizosphere OTUs were compared among the cultivars, an average of 2.5% were cultivar-specific. This cultivar specificity was more prominent in the AGR soil, where the *Fox*-resistant cultivar harbored 4.5% unique OTUs. The OTUs exclusively present in the rhizosphere of the *Fox*-resistant cultivar were affiliated to 13 phyla, dominated by Actinobacteria (21.2%), Bacteroidetes (15.8%), Verrucomicrobia (15.5%) and Proteobacteria (11.9), with some variation between ADE and AGR soils (Supplementary Figure 5).

Based on the results of RDA and PERMANOVA, we grouped the samples of rhizosphere and compared the phyla abundance to that in soil. Considering both soil types, five phyla were more abundant in the rhizosphere, namely Armatimonadetes, Chlamydiae, Cyanobacteria, Candidatus Saccharibacteria and Deinococcus-Thermus (Supplementary Figure 6). Some members of these groups have an important role in nutrient cycling. In a previous study, we showed that soybean plants are able to recruit microbial taxa based on the functional cores related to benefits for the plant, such as growth promotion and nutrition (Mendes *et al.*, 2014). Analyzing the functional profile (Supplementary Figure 7A), we detected a high abundance of sequences in the rhizosphere-related to motility and chemotaxis. Flagellar motility, along with quorum sensing, allows the microbial populations to actively access available resources and avoid feeding by predators (Mallon *et al.*, 2015). We also found a high abundance of sequences related to membrane transport. Some membrane transport systems can contribute to the competitiveness of a microbial community, such as Type VI secretion systems (T6SS), whose function is to mediate extracellular export of virulence factors and their translocation into target eukaryotic cells (Leiman *et al.*, 2009). Some of these functions may also hamper pathogen invasion, contributing to the function of the rhizosphere microbiome as a first line of defense. Interestingly, when we analyzed these two functions for each cultivar we found that *Fox*-resistant cultivars showed a higher abundance of membrane transport genes than *Fox*-susceptible cultivars in ADE soil and also a higher abundance of motility and chemotaxis genes in AGR soil (Supplementary Figure 7B).

Modulation of the rhizosphere microbiome by the Fox-resistant bean cultivar

In order to explore possible correlations between pathogen resistance in plants and the rhizosphere microbiome composition, we selected the most resistant (IAC Milenio) and most susceptible (IAC Alvorada) bean cultivars for detailed analysis. The resistant cultivar IAC Milenio used in our experiments is derived from a cross between two cultivars, one being a sibling line of the susceptible cultivar IAC Alvorada (Carbonell *et al.*, 2014). Although they are genetically related, we hypothesized that even small changes in the genome of the *Fox*-resistant cultivar may affect rhizosphere community assembly. In the *Fox*-resistant bean cultivar, the colonization of the fungus between adjacent xylem vessels is restricted by chemical and structural alterations, including vascular occlusion by gel plugs, tyloses, deposition of additional cell wall layers and infusion of these structures with phenols and other metabolites (Mace *et al.*, 1981; Pereira *et al.*, 2013). In a recent study, Beckers *et al.* (2016) demonstrated that variation in lignin biosynthesis modulates the composition of the plant endosphere microbiome. Considering that plants rely, at least in part, on the rhizosphere microbiome for functions and traits related to growth, development and health (Mendes *et al.*, 2013), we postulated that breeding for *Fox* resistance in common bean may have unintentionally co-selected for plant traits affecting the recruitment of beneficial cultivar-specific microbiota.

According to richness and both diversity indices used, that is, Shannon and Faith's PD, the *Fox*-resistant cultivar presented higher bacterial community diversity when compared with the bulk soil (Figures 2a–c). Interestingly, the bacterial abundance was higher in the rhizosphere of the *Fox*-resistant cultivar grown in ADE soil (Figure 2d), and this abundance decreased in the rhizosphere of bean cultivars with increasing susceptibility to the pathogen. For diversity, we could observe a similar trend for both soil types, however, not statistically significant ($P>0.05$). Higher bacterial abundance found in the rhizosphere of the *Fox*-resistant cultivar may result in a higher competition for resource

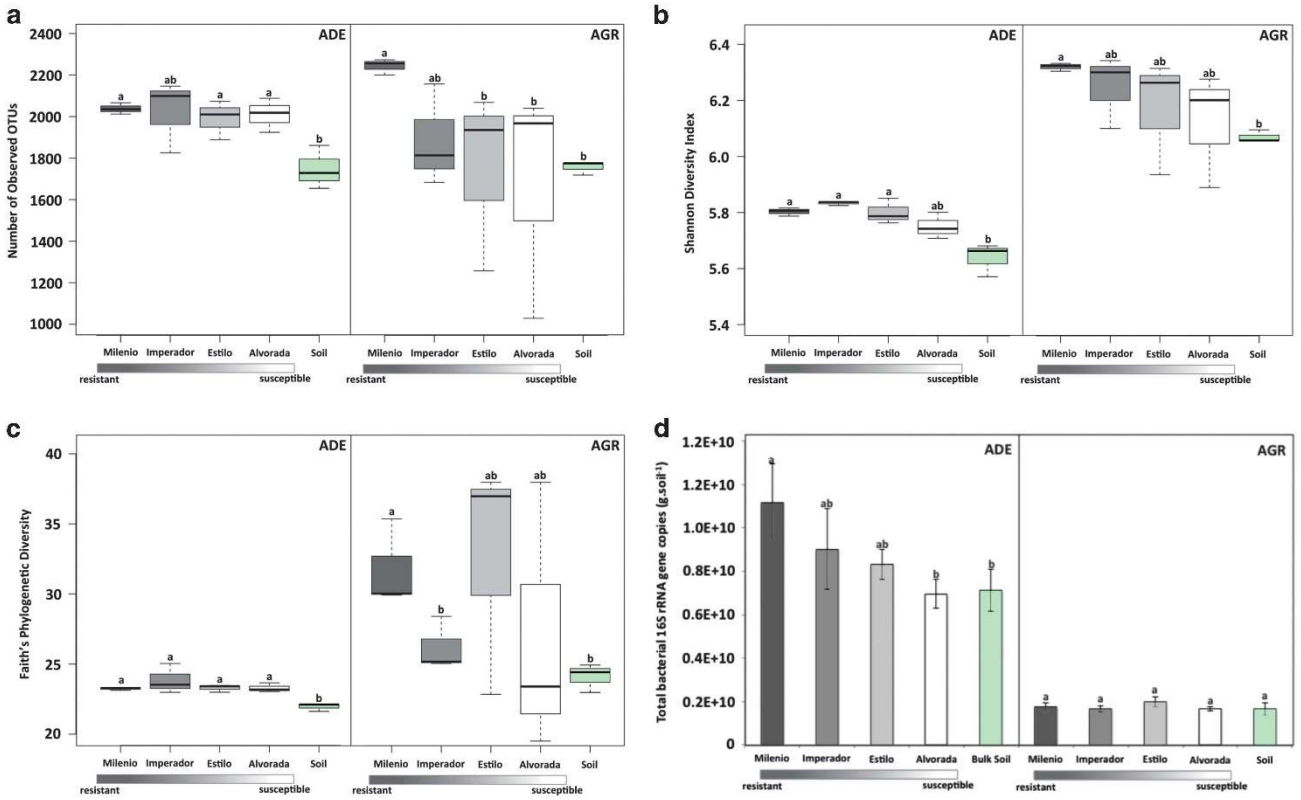

Figure 2 Diversity measurements based of 16S rRNA of soil and rhizosphere microbial communities for Amazon Dark Earth and agricultural soil. (**a**) Richness based on the number of observed OTUs; (**b**) phylogenetic diversity calculated as Faith's PD based on 97% similarity; (**c**) diversity based on Shannon's index; and (**d**) abundance of bacterial 16S rRNA gene. Error bars represent the standard deviation of four independent replicates. Different lower case letters refer to significant differences between treatments based on Tukey's test ($P<0.05$).

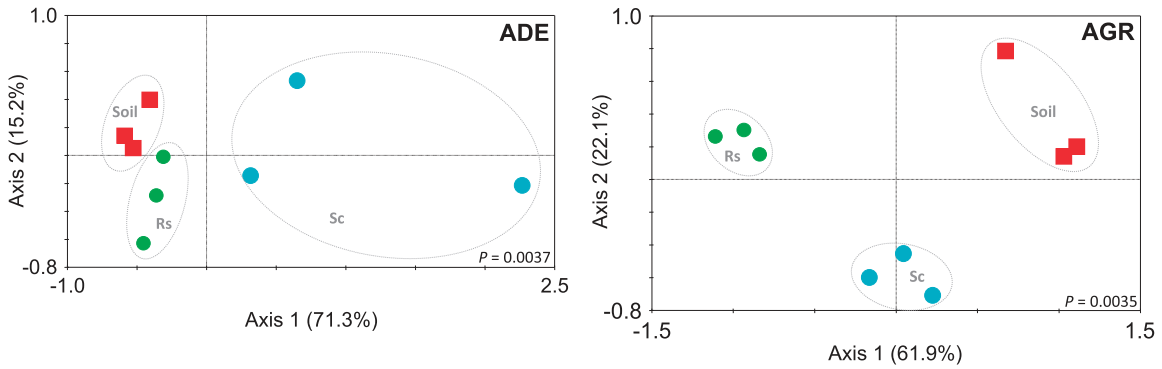

Figure 3 Principal component analysis (PCA) performed on taxonomic profile (OTU level for 16S rRNA data set) for two types of soils and rhizosphere of two common bean cultivars with contrasting levels of resistance to the pathogen *Fusarium oxysporum*. *P*-value evaluated via one-way PERMANOVA test. Rs = rhizosphere of the resistant cultivar Milenio; Sc = rhizosphere of the susceptible cultivar Alvorada; Soil = initial soil community; ADE = Amazon Dark Earth; AGR = agricultural soil.

contributing to protection against fungal infection. Resource competition is enhanced in highly abundant and more diverse communities and has been proposed as a key factor for the success or failure of pathogen invasion (Wei *et al.*, 2015).

Comparative analysis of the rhizosphere microbiome between the *Fox*-resistant and susceptible bean cultivars revealed a distinct community structure and differential abundance of specific family groups (Figures 3 and 4). These results extend the results of Yao and Wu (2010), who demonstrated that the rhizospheric community structure of a cucumber cultivar resistant to *F. oxysporum* differed in comparison to susceptible cultivars. Our 16S rRNA and metagenome sequence data for both soil types further pointed to a higher abundance of the families Pseudomonadaceae, Bacillaceae, Cytophagaceae and Solibacteraceae in the rhizosphere of the *Fox*-resistant cultivar (Figures 4a-d). At a lower taxonomical level, we found higher abundance of the

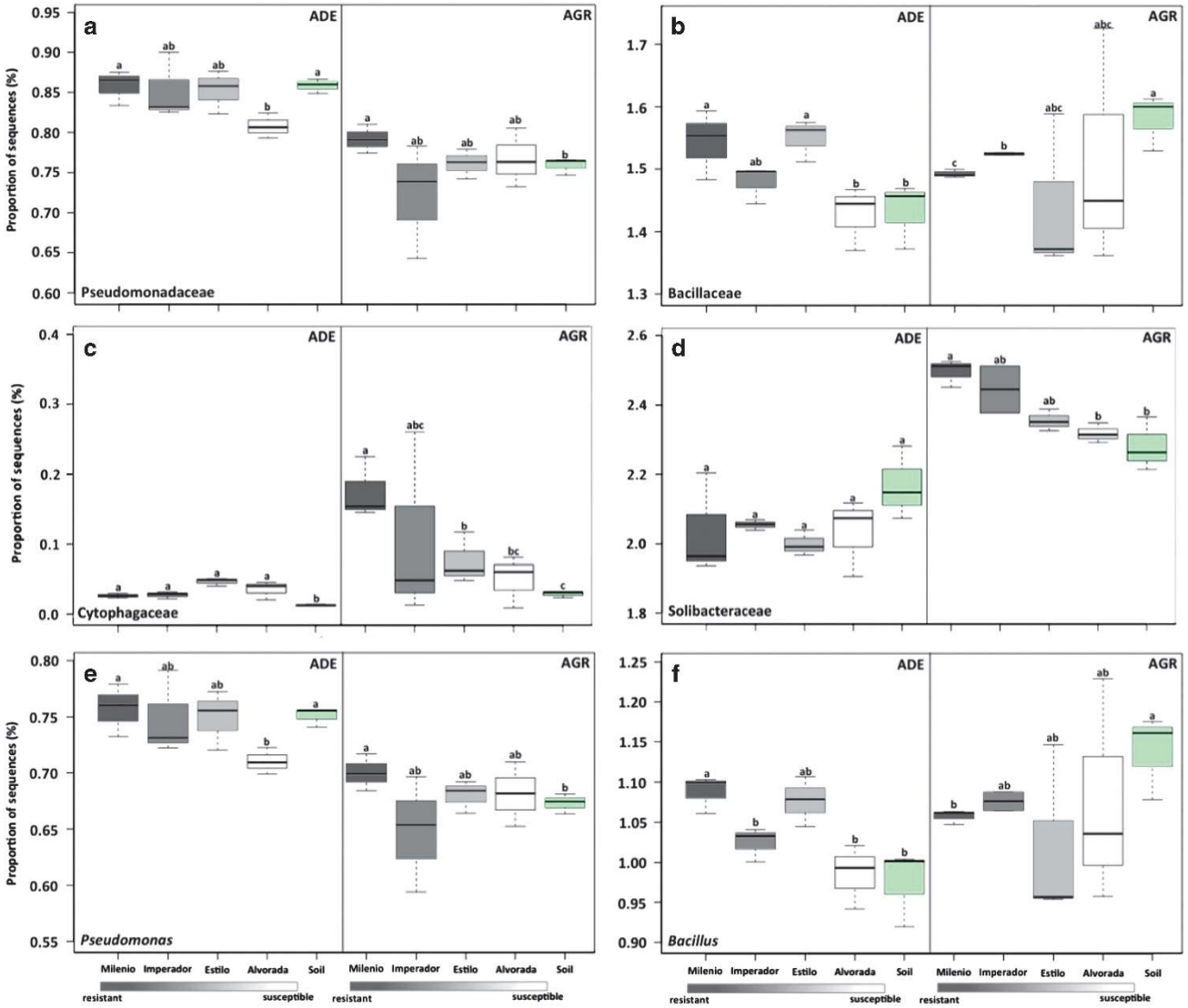

Figure 4 Distribution of the most differential bacterial groups ((**a–d**) family level and (**e–f**) genera level, based on 16S rRNA dataset) in the rhizosphere of different common bean cultivars in Amazon Dark Earth (ADE) and agricultural soil (AGR). Boxes indicate IQR (75th to 25th of the data). The median value is shown as a line within the box and outliers are represented by dots. Different lower case letters refer to significant differences between treatments within each soil type based on Tukey's test ($P<0.05$).

genera *Pseudomonas* and *Bacillus* in the rhizosphere of the *Fox*-resistant cultivar growing in ADE soil (Figures 4e and f). These two rhizobacterial genera are well-known for biofertilization, stimulation of root growth, rhizo-remediation, control of abiotic stress and plant diseases (Mendes *et al.*, 2013). In a study with common bean, Gilard *et al.* (2008) showed that *Pseudomonas chlororaphis* exhibited biological control against fusarium root rot. Also, member of the genus *Bacillus* are well-known for their ability to protect plants against root pathogens (Cavaglieri *et al.*, 2005). For common bean, Kumar *et al.* (2012) isolated *Bacillus* strains from the rhizosphere and showed plant growth promotion and antagonistic activity against several phytopathogens, including *Fusarium oxysporum*. Based on our metagenome data, sequences assigned to *Pseudomonas* and *Bacillus* were affiliated to nutrient-related metabolism and several antagonistic traits such as phenazine and chitinase biosynthesis, stress response, secondary metabolism, motility, chemotaxis, dormancy and sporulation (Supplementary Table 7). Further exploration of the metagenome data revealed a higher abundance of sequences associated with the biosynthesis of phenazines and rhamnolipids in the rhizosphere of the *Fox*-resistant cultivar IAC Milenio grown in ADE soil (Figures 5a and b). Phenazine antibiotics have a key role in suppression of *F. oxysporum* on diverse crops (Anjaiah *et al.*, 1998; Chin-a-Woeng *et al.*, 2000). Furthermore, Mazurier *et al.* (2009) reported that phenazine antibiotics produced by *Pseudomonas* species contribute to natural soil suppressiveness to fusarium wilt. Rhamnolipids, also produced by *Pseudomonas*,

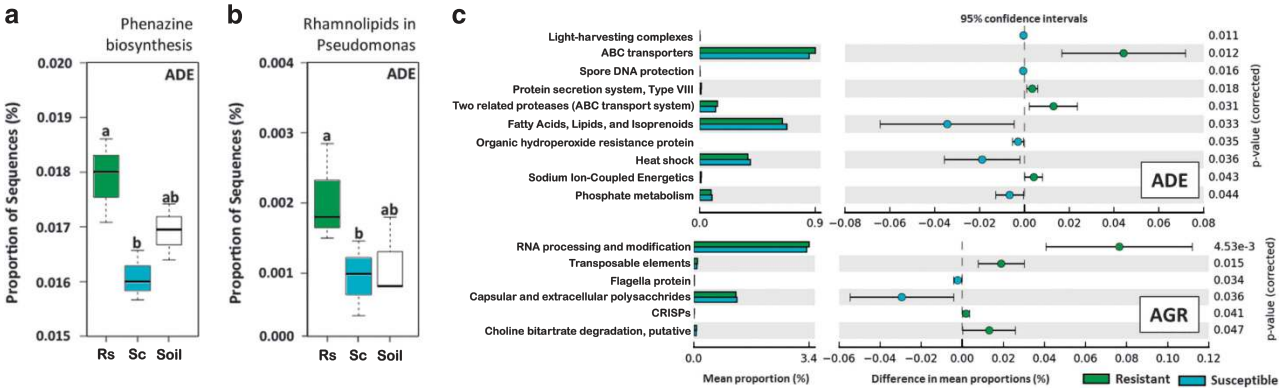

Figure 5 Differential abundance of sequences affiliated to bacterial functions (based on metagenome data set) in the rhizosphere microbiome of resistant and susceptible common bean cultivars grown in two different soils. The sequences were affiliated to functions based on SEED databank (**a** and **b**) level 3 and (**c**) level 2. Resistant = rhizosphere of the resistant cultivar Milenio; Susceptible = rhizosphere of the susceptible cultivar Alvorada; ADE = Amazon Dark Earth; AGR = Agricultural soil.

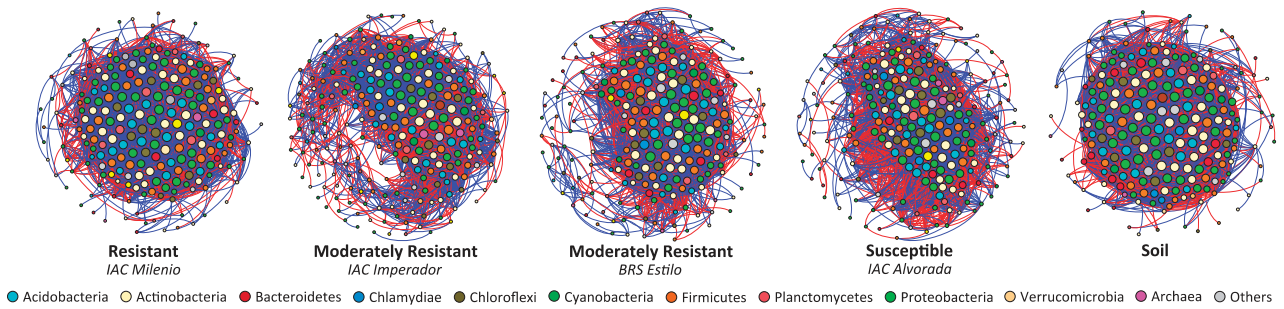

Figure 6 Network co-occurrence analysis of microbial communities of rhizosphere and soil samples. A connection stands for SparCC correlation with magnitude > 0.7 (positive correlation–blue edges) or < −0.7 (negative correlation–red edges) and statistically significant ($P < 0.01$). Each node represents taxa affiliated at genus level (based on 16S rRNA), and the size of node is proportional to the number of connections (that is, degree). Each node was labeled at phylum level.

have antimicrobial properties and also act against several fungi including *Fusarium* (Haba *et al.*, 2003). We also found a higher abundance of sequences affiliated to ABC transporters and protein secretion system in the rhizobacterial community of the *Fox*-resistant cultivar (Figure 5c). Some of these functions are related to the export of various virulence factors, such as antibiotics, bacteriocins, toxins, that may increase competition within a community hindering pathogen invasion. Therefore, based on our findings we hypothesize that the *Fox*-resistant cultivar is better able to recruit beneficial microbial groups that, in addition to the plant genetic traits, may complement the defense against pathogen infection.

Network structure of the rhizosphere microbiome

We then used co-occurrence network analysis to explore the complexity of connections within the rhizosphere microbiomes of the different common bean cultivars. For this, we calculated SparCC correlations between microbial taxa at genus level based on 16S rRNA data. Following this, we calculated the topological properties of the obtained networks to identify differences between the samples. The *Fox*-resistant cultivar showed the highest level of complexity and modular structure, whereas the *Fox*-susceptible cultivar presented a less complex (Figure 6) and less modular network (Table 1). It has been assumed that highly diverse bacterial communities are often more resistant to pathogen invasion (Latz *et al.*, 2012; Mallon *et al.*, 2015). This hypothesis is based on the assumption that diverse communities exhibit a higher number of species interactions and intensified competition for niche space (Kennedy *et al.*, 2002; van Elsas *et al.*, 2012). Although we did not find significant differences in the rhizobacterial diversity between the *Fox*-resistant and the susceptible bean cultivars, we found a higher complexity in the network of the *Fox*-resistant cultivar. Wei *et al.* (2015) suggested that the relationships of diversity-invasion resistance could be mechanistically explained by the underlying interaction network architecture. The network of the *Fox*-resistant cultivar presented the highest number of connections per node (average degree = 84.59), and a lower average path length (1.66) and diameter (6) in comparison with the other cultivars, indicating a highly connected community (Table 1). Average path length is defined as the average number of steps along the shortest paths between each node, being a measure of efficiency on a network (Zhou

Table 1 Correlations and topological properties of common bean rhizosphere microbiome networks

Network properties	*Resistant IAC Milenio*	*Mod. resistant IAC Imperador*	*Mod. resistant BRS Estilo*	*Susceptible IAC Alvorada*	*Soil*
Number of nodes[a]	230	253	239	241	222
Number of edges[b]	9728	5243	6474	5883	9457
Positive edges[c]	5049	3124	3342	3239	4831
Negative edges[d]	4679	2119	3132	2644	4626
Modularity[e]	12.27	2.45	14.46	4.57	18.08
Number of communities[f]	9	17	19	17	9
Network diameter[g]	6	9	7	7	6
Average path length[h]	1.66	2.32	1.88	2.03	1.63
Average degree[i]	84.59	41.44	54.17	48.82	85.19
Average clustering coefficient[j]	0.342	0.277	0.300	0.29	0.350

[a]Microbial taxon (at genus level) with at least one significant ($P<0.01$) and strong (SparCC>0.7 or<-0.7) correlation.
[b]Number of connections/correlations obtained by SparCC analysis.
[c]SparCC-positive correlation (>0.7 with $P<0.01$).
[d]SparCC-negative correlation (<-0.7 with $P<0.01$).
[e]The capability of the nodes to form highly connected communities, that is, a structure with high density of between nodes connections (inferred by Gephi).
[f]A community is defined as a group of nodes densely connected internally (Gephi).
[g]The longest distance between nodes in the network, measured in number of edges (Gephi).
[h]Average network distance between all pair of nodes or the average length off all edges in the network (Gephi).
[i]The average number of connections per node in the network, that is, the node connectivity (Gephi).
[j]How nodes are embedded in their neighborhood and the degree to which they tend to cluster together (Gephi).

et al., 2010). Also, the network of the *Fox*-resistant cultivar presented a more modular structure (Table 1), which is characterized by the presence of different groups of nodes with high numbers of interconnections within, with some degree of independencies between groups (Newman, 2006). A modular structure suggests diversity in species roles and functionality, increasing niche overlap (Poudel *et al*., 2016). In this sense, a highly connected and modular rhizosphere microbiome could decrease pathogen invasion success if it leads to more efficient consumption for resources (van Elsas *et al*., 2012; Wei *et al*., 2015). Also, modularity combined with a short average path length may imply a more prompt response of the microbial community to environmental perturbations (Faust and Raes, 2012).

Based on the network properties, we identified three bacterial groups with more betweenness centrality, which is defined as the number of times a node acts as a bridge along the shortest path between two other nodes (Poudel *et al*., 2016). In network analysis, the centrality indicates the most important nodes, which may be interpreted as key taxa inside a connected community (Borgatti, 2005). The top three nodes with more centrality were (1) the genus *Dyadobacter* (phylum Bacteroidetes), (2) a member of the family Comamonadaceae (phylum Proteobacteria) and (3) one taxon affiliated to *Acidobacteria* Gp16. Interestingly, the genus *Dyadobacter* was suggested as a potential taxon involved in the suppression of fusarium wilt in the rhizosphere of banana (Fu *et al*., 2017), whereas the family Comamonadaceae was identified as *Fox*-suppressive bacterial taxon associated with cotton plants (Li *et al*., 2015). Also, all three key bacterial groups are interconnected in the network of the *Fox*-resistant cultivar. Key taxa represent nodes that are associated with many others, and a removal of this node may have a large impact on the community structure (Steele *et al*., 2011). It is important to note that a pathogen invader may manage to displace the key taxa and collapse the network structure (Albrecht *et al*., 2014).

Conclusions

The importance of the rhizosphere microbiome in the plant ecosystem functioning has been widely recognized, but traditional approaches of plant breeding do not take the plant microbiome into account. Here, we presented an in-depth analysis of the rhizosphere microbiomes of common bean cultivars with different degrees of *Fox* resistance. Although *Fox* resistance is based on plant genetic traits, our data support the hypothesis that breeding for resistance may have unintentionally altered the rhizosphere microbiome composition, altering the frequency of beneficial microorganisms and traits that may contribute to plant growth or assist in protection against the pathogen. Considering that *Fox* resistance is based on genetic and chemical alterations in the plant, our findings suggest that the observed changes in rhizosphere microbiome may enforce the first line of defense, limiting pathogen invasion by means of a higher abundance of specific microbial groups and functions, high microbial diversity, abundance and a more complex network structure. We also showed that the recruitment of the rhizosphere microbiome is highly dependent on the soil type, where the Amazonian Dark Earth soil provided a richer source of potentially beneficial microorganisms. Future studies will require experimental validation of the putative beneficial effects of

the identified bacterial taxa. Our results reinforce the importance of understanding the genetic and (bio) chemical mechanisms involved in the interplay between soil type, plant genotype, rhizosphere microbiome, plant growth and plant health. Hence, future plant breeding programs should be directed toward unraveling the molecular basis of interaction between plants and beneficial members of the rhizosphere microbiome. The use of next-generation sequencing together with culture-dependent approaches are essential to identify and functionally validate the importance of key microbial groups and traits in pathogen suppression. Unraveling the link between specific regions in the plant genome and microbial recruitment is essential for identifying molecular markers in plants that can be used in future breeding programs.

Conflict of Interest

The authors declare no conflict of interest.

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