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Evaluating the impact of the biocontrol agent *Trichoderma harzianum* ITEM 3636 on indigenous microbial communities from field soils

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Running headline: biocontrol agent impact on soil microorganisms

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Abstract

Aim: to investigate the impact of inoculating peanut seeds with the biocontrol agent *Trichoderma harzianum* ITEM 3636 on the structure of bacterial and fungal communities from agricultural soils.

Methods and Results: PCR-Denaturing gradient gel electrophoresis (PCR-DGGE) and next-generation sequencing (NGS) of amplicons (or marker gene amplification metagenomics) were performed to investigate potential changes in the structure of microbial communities from fields located in a peanut-producing area in the province of Córdoba, Argentina. Fields had history of peanut smut (caused by *Thecaphora frezii*) incidence. The Shannon indexes (H'), which estimate diversity, obtained from the PCR-DGGE assays did not show significant

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differences neither for bacterial nor for fungal communities between control and inoculation treatments. On the other hand, the number of Operational Taxonomic Units (OTUs) obtained after NGS was similar between all the analyzed samples. Moreover, results of alpha and beta diversity showed that there were no significant variations between the relative abundances of the most representative bacterial and fungal phyla and genera, in both fields.

Conclusions: *T. harzianum* ITEM 3636 decreases the incidence and severity of agriculturally relevant diseases without causing significant changes in the microbial communities of agricultural soils.

Significance and Impact of the Study: our investigations provide information on the structure of bacterial and fungal communities in peanut-producing fields after inoculation of seeds with a biocontrol agent.

Keywords: biocontrol; DGGE (denaturing gradient gel electrophoresis); microbial structure; metagenomics; soil.

Introduction

In Argentina, between 350 and 400 thousand hectares are planted annually with peanuts (*Arachis hypogaea* L.), approximately 90% of which are sown in the province of Córdoba. The average yield varies between 3.3 and 3.5 tons of peanuts per hectare, which represents around one million tons per year. Argentina is the main country exporting high quality peanuts or confectionery quality peanuts, although its production represents less than 2% of the world's production (Pedelini, 2014). Peanut plants are susceptible to a large number of diseases that develop due to numerous factors such as the susceptibility of the cultivar to diseases, type of pathogen (fungi, bacteria or viruses), a favorable environment and biotic factors (including the microbiota associated to the plant). Such factors are part of the tetrahedron of the disease described by Brader et al. (2017). Within a favorable environment, not only climate is included, but also agricultural practices, which have a great influence on the dissemination of plant pathologies (Marinelli and March, 2005). Some of the pathogens that affect peanut cultivation in Argentina and cause great economic losses are: *Fusarium solani*, *Botrytis cinerea*, *Rhizoctonia solani*, *Sclerotinia minor*, *Sclerotinia sclerotiorum*, *Sclerotium rolfsii*, *Aspergillus niger*, *Cercospora arachidicola*, *Cercosporidium personatum*, *Sphaceloma arachidis* and *Thecaphora Frezzi* (Andrés et al., 2016). *T. frezii* was reported for the first time as a wild peanut pathogen (*Arachis* sp.) in Brazil. Thus, this fungus was identified as the causal agent of peanut smut. The symptoms are observed in the fruits, which present hypertrophy and deformations (pods and seeds). Within the pods, the seeds have small sori (small deformations or dark-colored blisters, formed by teliospores of the pathogen) on their surfaces. The seeds may have only one affected region or they may be completely affected by a carbonaceous mass of spores (Marinelli and March, 2005).

In previous studies conducted in commercial fields with peanut smut history, the protective effect of a bioformulation containing *Trichoderma harzianum* ITEM 3636 was evaluated. The inoculation of the peanut seeds caused significant reductions in the incidence and severity of peanut smut, also causing significant increases in the weight of grains/plant (Ganuja et al., 2018). *Trichoderma* spp. are highly studied fungi and are among the most widely used microbial agents for biological control in agriculture. Currently, they are marketed as biopesticides, biofertilizers, growth promoters and stimulants of natural resistance. The effectiveness of these fungi can be attributed to their ability to protect plants, increase vegetative growth, contain pathogen populations under different agricultural conditions and act as amendments/inoculants for increasing nutrient availability,

decomposition and biodegradation. Fungal spores are incorporated in several formulations, both traditional and novel, to be applied to seeds or by foliar spray, incorporated into the soil during sowing or transplantation, or by irrigation. Therefore, *Trichoderma*-based preparations are marketed worldwide and are used to protect crops against numerous plant pathogens or to increase plant growth and productivity in multiple environments (Woo et al., 2014).

The microbial diversity in the ecosystems of the soils is massive. In fact, it is estimated that one gram of soil can contain up to 10 million microorganisms from thousands of different species (Torsvik and Øvreås, 2002). Since soil microorganisms play an essential role in the decomposition of organic matter and in the release of mineral nutrients, they affect nutrient cycles by regulating their content and physical-chemical properties and, consequently, primary productivity (Rutigliano et al., 2004). Microbial diversity is fundamental for soil quality (Liu et al., 2006). Heterogeneity in soils is composed of species diversity, genetic diversity and biodiversity of ecosystems (Solbrig et al., 1991). The diversity of species is based on their richness, which represents the total number of species present and their distribution (Ovreas, 2000). However, the classical definition of species used for, for example, higher plants can't be easily applied neither to prokaryotes (Godfray and Lawton, 2001) nor to asexual organisms. Microbial diversity is usually considered as the number of individual sequences assigned to different taxa and their distribution among taxa. Consequently, microorganisms are referred to as functional groups or as communities (Liu et al., 2006).

The study of the survival of bacterial and fungal strains inoculated in fields and the effect of their release on the structure of microbial communities present in these soils has been important since the development of the practical use of microorganisms. The inoculation of seeds could have as a consequence changes in the structure of the microbial communities present in the soils, which could generate concerns regarding the safety of the introduction of formulations containing microorganisms in the environment. It is known that the inoculation of microorganisms can influence, even temporarily, the native microbial communities (Trabelsi and Mhamdi, 2013).

In the present study, PCR-denaturing gradient gel electrophoresis (PCR-DGGE) and next-generation sequencing (NGS) of amplicons (or marker gene amplification metagenomics) were carried out to investigate the impact of inoculation with *Trichoderma harzianum* ITEM 3636 on soil bacterial and fungal communities.

Materials and methods

Fungal strain

Trichoderma harzianum strain ITEM 3636, originally isolated from peanut cropped soil by researchers of the Plant Pathology Department, UNRC, was deposited at the Istituto Tossine e Micotossine da Parassiti Vegetali, Italy (Rojo et al., 2007). The isolate was kept in 15% glycerol and frozen at -80°C. The identity of the strain was confirmed using sequence analyses of the 1 α (TEF1) and calmodulin (CAL) genes (Ganuja et al., 2018). These DNA sequences were uploaded to GenBank (accession numbers KY595072 and KY595073). The inocula of *T. harzianum* ITEM 3636 were obtained from 7-day-old cultures on Petri dishes containing malt extract agar (MEA) at 28°C under a 12 h light/dark cycle. The suspensions of conidia were harvested by covering each of 10 plates with 10 ml of sterile distilled water and scraping the surfaces of the cultures with a sterile glass spatula. The inoculum density was adjusted to 5×10^6 conidia/ml by adding sterile distilled water.

Field assays

Experimental design and sample processing

In a previous work, we reported on the evaluation of a bioformulation containing *T. harzianum* strain ITEM 3636, an effective biocontrol agent against the peanut pathogen *Fusarium solani*, for control of peanut smut, an emergent disease caused by *Thecaphora frezii*. Field assays, from November 2014 to April 2015 and from November 2015 to April 2016, were conducted in commercial fields located in the province of Córdoba, Argentina. Both fields had a long history of peanut smut incidence (Ganuza et al., 2018) and were previously sown with maize. *T. harzianum* ITEM 3636 was applied on seeds previously coated with the Options Advance (NOVA) chemical fungicide. The treatments were: Pelleting Control (C), where each kg of seeds was pelleted with 100 ml of 2% carboxymethyl cellulose (CMC) and 100 ml of sterile distilled water; *T. harzianum* ITEM 3636 inoculated on seeds by pelleting (Th), where each kg of seeds was pelleted with 100 ml of 2% CMC and 100 ml of the suspension of *T. harzianum* ITEM 3636; Furrow control (CF), where seeds were placed into furrows and sprayed with sterile distilled water before covering them with soil, and *T. harzianum* ITEM 3636 inoculated into furrows (ThF), where seeds were placed into furrows and sprayed with the suspension of *T. harzianum* ITEM 3636 before covering them with soil. The sowing was performed during November. A one-furrow hand sowing machine (Ferreti, model MSE301) was used for sowing. Randomized complete block designs, with four replicates, were used. The plots consisted of four rows, each of 10 m long, with an area of 28 m² (2.8 m × 10 m). The spacing between rows was ~0.7 m, with 0.08 m between plants. The seed rate was of ~120 kg/ha, with a depth of 5 cm. Pesticides were applied at recommended doses to avoid the detrimental effects of weeds and pests on plant growth and crop yield.

From these fields, soil samples were collected for the present study, at three different times: before sowing (initial condition or pre-sowing), 75 days after sowing (Plants in R2-R3 stage; gynophore formation) and during drying in the field (120 days after sowing). Samples, consisting of approximately 300 g of soil and collected from the upper 10 cm of soil, were placed in polyethylene bags and stored at 4°C. Samples obtained before sowing were collected considering the entire sowing surface. For this, two transects were contemplated from the vertices of the mentioned surface and samples were collected at intervals. Later, three sub-samples were combined into one sample. Samples collected at 75 days after sowing were taken from each of the sites from which a peanut plant was extracted for the analysis of growth parameters. Sub-samples were combined to obtain a final sample from each plot. Samples obtained at 120 days, during drying in the field, were collected from under peanut plants that were already digged. Three sub-samples were collected within each plot and combined to obtain a final sample.

Based on the results obtained in the PCR-DGGE, we decided to use samples obtained before sowing and from the plots corresponding to the C and Th treatments at 120 days after sowing, during drying in the field, for the study of next-generation sequencing of amplicons.

DNA extraction from each of these samples was carried out from 250 mg of soil, using the commercial kit Powersoil DNA isolation Kit (Mo Bio) and following the manufacturer's recommendations.

Soil parameters

Soil samples were subjected to granulometry using a laser diffraction particle analyzer. The pH of the samples was recorded using the 1/2.5 potentiometric technique. Measurements of organic matter content and total nitrogen content were carried out by the method described by Walkley and Black (1934) and the method described by Kjeldahl (1883), respectively. The electrical conductivity of soils was assessed with a conductivity meter that was placed in a dilution of soil and water (at 25°C) and the density of soils was measured through the method of the metallic cylinder, where a fix volume of soil was taken without disturbance and weighed once dry (oven at 105°C for 24 hours).

Fingerprinting of bacterial and fungal communities by PCR-DGGE

PCR and DGGE conditions

A region of the 16S rRNA gene was amplified using a nested polymerase chain reaction (PCR) approach. In the first amplification reactions, the 27F and 534R primer set was used, at 0.2 $\mu\text{mol l}^{-1}$ each primer (see table 1). PCR reaction mixture (final volume of 25 μl) included 1X *Taq* polymerase buffer (Promega) with 1.5 mmol l^{-1} MgCl_2 , 1.5 U of *Go Taq* enzyme (Promega), 5% (v v^{-1}) dimethylsulfoxide (DMSO), 0.2 mmol l^{-1} dNTPs (Promega), 0.2 $\mu\text{g } \mu\text{l}^{-1}$ bovine serum albumin (Fermentas), 0.5 to 2 ng of each DNA sample and nuclease-free water. The amplification was carried out as follows: initial denaturation at 94°C for 5 min, followed by 35 cycles of 30 s of denaturation at 94°C, 45 s of annealing at 55°C and 45 s of extension at 72°C, with one final extension step of 10 min at 72°C. For the second PCR, 341F-GC and 534R primers (see table 1) were used, at 0.2 $\mu\text{mol l}^{-1}$. PCR reaction mixture (final volume of 40 μl) included 0.5 μl of product obtained in the first PCR, 1X *Taq* polymerase buffer (Promega) with 1.5 mmol l^{-1} MgCl_2 , 2.4 U of *Go Taq* enzyme (Promega), 5% (v v^{-1}) DMSO, 0.2 mmol l^{-1} dNTPs (Promega), 0.2 $\mu\text{g } \mu\text{l}^{-1}$ bovine serum albumin (Fermentas) and nuclease-free water. The conditions for the amplification program were the same as for the reaction mentioned above.

The ITS region was amplified using a nested polymerase chain reaction (PCR) approach. In the first amplification reactions, the forward primer ITS1 and reverse primer ITS4 were used, at 0.2 $\mu\text{mol l}^{-1}$ each (see table 1). PCR reaction mixture (final volume of 25 μl) included 1X *Taq* polymerase buffer (Promega) with 1.5 mmol l^{-1} MgCl_2 , 1.25U of *Go Taq* enzyme (Promega), 5% (v v^{-1}) DMSO, 0.2 mmol l^{-1} dNTPs (Promega), 0.2 $\mu\text{g } \mu\text{l}^{-1}$ bovine serum albumin (Fermentas), 0.5 to 2 ng of each DNA sample and nuclease-free water. The amplification conditions consisted of an initial denaturation of 5 min at 94°C, followed by 31 cycles of 45 s of denaturation at 94°C, 45 s of annealing at 58°C and 45 s of extension at 72°C, with one final extension step of 7 min at 72°C. For the second PCR, ITS1F-GC and ITS2 primers (see table 1) were used, at 0.2 $\mu\text{mol l}^{-1}$. PCR reaction mixture (final volume of 40 μl) included 2 μl of product obtained in the first PCR, 1X *Taq* polymerase buffer (Promega) with 1.5 mmol l^{-1} MgCl_2 , 2U of *Go Taq* enzyme (Promega), 5% (v v^{-1}) DMSO, 0.2 mmol l^{-1} dNTPs (Promega), 0.2 $\mu\text{g } \mu\text{l}^{-1}$ bovine serum albumin (Fermentas) and nuclease-free water. The amplification conditions consisted of an initial denaturation of 5 min at 94°C, followed by 10 cycles of 30 s of denaturation at 94°C, the annealing temperature began at 65°C for 30 s and decreased by 1°C over the course of the 10 cycles, and 30 s of extension at 72°C. In addition, 25 cycles of 30 s of denaturation at 94°C, 30 s of annealing at 50°C and 30 s of extension at 72°C were performed, with a final extension step of 7 min at 72°C.

The PCR reactions were conducted in a MULTIGENE GRADIENT TC 9600 thermal cycler (Labnet). Reactions included a negative and positive control. Aliquots (1-2 μl) of PCR products were analyzed by electrophoresis in 1% (w v⁻¹) agarose gel stained with 2 μl GelRed Nucleic Acid Stain 10000X (Biotium) and visualized with TM 26 transilluminator (Labnet).

DGGE conditions: the gels were made using a SG15 gradient generator (Amersham Biosciences) and a DYNAMAX RP1 peristaltic pump (Rainin). PCR products (42 μl) were loaded on an 8% acrylamide gel with a denaturing gradient ranging from 40-70% and 30-60% for total bacteria and fungi, respectively, where 100% denaturant corresponded to 7 mol l⁻¹ urea (Promega) and 40% (v v⁻¹) formamide (Sigma-Aldrich, Co., St. Louis, MO). DGGE was performed within TAE 1 \times buffer at 60 °C applying 60 V (between 23 to 25 mA) during 17 hours, using a PowerPac Basic power source (BIO-RAD). Gels were stained in 100 ml of 1X TAE buffer with 20 μl of GelRed Nucleic Acid Stain 10000X (Biotium) for 45 min, washed in distilled water for 10 min and visualized in a LABNET UV transilluminator (Biodynamics).

The DGGE profiles were digitized and analyzed with the GELCOMPARE II software V4.602 (Applied Maths). The position and intensity of the bands (previously normalized) were included in a band matrix that was used as starting material for the analysis of clustering (or conglomerates) and for the calculation of sample diversity. In this work, we evaluated the similarity in the structure of total bacterial and fungal communities between the different treatments, considering the bands profiles from each sample in the DGGE.

Determination of diversity

To estimate the diversity of the community from the band intensity matrices of the DGGE, each band in the gel was considered as a specific phylotype or species in the community and the intensity of each band was taken as a reflection of the abundance of that sequence (phylotype) in the community (Escalante, 2007; Zheng et al., 2008). Each band matrix was analyzed with the PAST program V2.17b (Hammer et al., 2001) for the calculation of the Shannon-Weaver index (H') (Shannon and Weaver, 1949).

Statistical analyses of data

The analyses of the mean values for the Shannon indexes from the treatments were performed with ANOVA. The differences between the means were calculated by means of the Fisher's Least Significant Difference (LSD) test ($P < 0.05$) using the program INFOSTAT (Di Rienzo et al., 2014).

Analysis of next-generation sequencing (NGS) of amplicons

Sequencing and processing of sequenced data

Soil DNA samples were lyophilized and sent to CD Genomics (USA) for PCR amplification, purification, construction of DNA libraries, sequencing and analyses of sequences. To study the diversity of bacteria, the 338F and 806R primers set were used (see table 1). These primers amplify the hypervariable regions V3 and V4 from the 16S rRNA gene. To study the diversity of fungi, the ITS1F and ITS2 primers set was used (see table 1). These primers amplify the ITS1 region between the 18S and 5.8S rRNA genes.

The sequencing was performed with the HiSeq2500 (Illumina) equipment to generate raw paired end readings. Raw paired end readings were assigned to each sample based on their specific barcodes, which were then removed along with the sequences of the primers. The readings of paired ends were merged using the FLASH V1.2.7 program and the spliced sequences obtained were called raw labels. The trimming of the raw labels was performed using the Trimmomatic V0.33 software, which carries out a quality control process. After trimming, high quality, clean labels were obtained. Next, the labels were compared with the reference Gold database using the UCHIME algorithm to detect the chimeric sequences, which were eliminated. Thus, the process generated effective labels. The Operational Taxonomic Units (OTU) are groups of similar sequences of the 16S rRNA or ITS marker genes. Each of these groups represents a taxonomic unit of a species or genus of bacteria or fungi, depending on the threshold of sequence similarity. The OTU groups are defined by an identity threshold of 97%. From each OTU group, a unique sequence was selected as a representative sequence. The representative sequence was annotated using a classification method and all the sequences within that OTU inherited that same classification. UCLUST (Caporaso et al., 2010) was used in QIIME (Edgar, 2010) V1.8.0 to group the labels with 97% similarity and the obtained OTUs were then annotated based on Silva's taxonomic database (bacteria) and UNITE (fungi). The OTUs that were annotated as mitochondria, chloroplast or unknown were eliminated. The abundance information in each taxonomic level was generated using QIIME, and the microbial community structure, heatmaps, UPGMA and PCoA graphs were made with R version 3.5.0. Principal coordinate analysis (PCoA) was performed using the "ape" package. Pearson correlation coefficient and visualization by heatmap were performed by R using the "corrplot" package.

Statistical analyses

The significant difference analyses included the LEfSe Analysis between groups and the METASTAT Analysis. Line Discriminant Analysis (LDA) Effect Size (LEfSe) (Segata et al., 2011) is a statistical method that relies on the Wilcoxon rank-sum test, with an additional step to estimate the effect size, for high-dimensional biomarker discovery and explanation that identifies genomic features (genes, pathways, or taxa) and characterizes the differences between two or more biological conditions (or classes), the screening criteria for biomarkers is LDA score > 4. METASTAT (White et al., 2009) is the method that uses t-tests with sample permutation for detecting differentially abundant features in a metagenome, which is a non-parametric T-test that determines whether there are taxa that are differentially represented between the samples. On the other hand, the Multivariate Statistical Analysis included an ANOVA Analysis and a Wilcoxon Test. ANOVA was used to determine whether there were any statistically significant differences between the means of the groups. ANOVA analyses in diverse taxonomy levels were performed. The Wilcoxon Test is a non-parametric statistical hypothesis test used when comparing two related samples to evaluate whether their mean population ranges differ. Differences between the mean values of alpha diversity indexes and were assessed by means of the Holm-Sidak method ($P < 0.05$).

Accession numbers

Data were deposited in the NCBI (National Center for Biotechnology Information Search database) Sequence Read Archive (SRA) database with accession number SRP146071, and will be accessible after the release date.

Results

Soil parameters

Textural data of samples showed that the field from the 2014/2015 season had loam soil (clay 8.1%, silt 18.3% and sand 73.6%) while the field from the 2015/2016 season had a sandy-loam soil (clay 13.9%, silt 37.8% and sand 48.3%). Table 2 includes other soil properties from both fields. Table 3 shows that inoculation with *T. harzianum* ITEM 3636 increased the organic matter content of soil in 8% at 120 days, as compared to the control treatment, during the 2014/2015 season. However, the difference was not significantly different ($P \leq 0.05$). Furthermore, the organic matter content of the soil during this season was higher after harvest (in both treatments) than before sowing. On the other hand, the organic matter content of the soil during the 2015/2016 season remained stable during the whole assay. Table 3 also shows that the soil pH did not vary neither with time nor treatment during both seasons. Also, the presence of peanut plants caused a slight increase in soil pH during the 2015/2016 season. Finally, table 3 shows that the presence of peanut plants increased the content of total nitrogen in the soil during the 2014/2015 season. No statistically significant differences ($P \leq 0.05$) were observed between control and inoculation treatment soils during plant growth and after harvest. By comparison, the content of total nitrogen in the soil did not vary with time or treatment during the 2015/2016 season.

PCR-Denaturing gradient gel electrophoresis (DGGE) assays

DGGE allows observing the diversity of the most predominant phylotypes. Table 4 shows that there were no statistically significant differences in the diversity of bacteria and fungi, measured through their respective Shannon indexes (H'), for the different treatments and during both seasons. Therefore, the application of *T. harzianum* ITEM 3636 did not cause a significant disturbance within the predominant communities of the studied agricultural soils. Regarding the methodology of application of the biocontrol agent, by seed pelleting or by inoculation into furrows by irrigation, no statistically significant differences were observed. We only observed significant differences when comparing the diversity of the bacterial communities at 75 and at 120 days, during both seasons. Table 4 also includes the indexes for bacteria and fungi from the initial condition (before sowing or pre-sowing) as a reference for the initial diversity of soils. The fingerprinting profiles obtained can be observed in supplementary Figures S1 and S2.

Analyses of next-generation sequencing (NGS) of amplicons

Bacterial communities: next-generation sequencing of amplicons from the 16S ribosomal RNA gene provided an average of 80,000 readings per sample with an average size of ~420 bp. After the cleaning of the paired end readings, approximately 69,000 effective sequences remained per sample. These sequences were used to continue with the diversity study. The observed GC percentage of the sequences was similar in all the samples

(~57%). Between 2000 and 2150 Operational taxonomic units (OTUs) were obtained from each of our 12 samples. Each OTU is a group of sequence variants which are similar to the sequence of the 16S rRNA marker gene. Each of these groups is intended to represent a taxonomic unit of a bacterial species or genus, depending on the threshold of sequence similarity. The number of OTUs obtained was highly similar between the samples obtained before sowing and the samples corresponding to 120 days after sowing.

The relative abundances (%) of bacterial phyla and genera present in the soils from the different treatments are shown in Fig. 1. Only the relative abundances of the 10 most representative phyla are shown. Notably, these representative phyla were present in all the samples, being *Proteobacteria* the most abundant (its relative abundance ranged between 33 and 37% among the samples). Then, following a descending order in relative abundances, *Actinobacteria* was found to range between 19 and 30%. *Acidobacteria* ranged from 10 to 16% of relative abundance, and was lower in samples obtained from inoculated soil, as compared to samples obtained before sowing, during the 2014/2015 season. The *Gemmatimonadetes* phylum did not show a notorious variation, and ranged between 9.87 and 12% among all the samples. The *Chloroflexi* phylum was also present at similar relative abundances among the samples (from 4 to 5.34%). The relative abundance of this phylum was observed to be lower in samples obtained from inoculated soil, as compared to control samples, during the 2014/2015 season. Also, the *Bacteroidetes* phylum showed a significantly lower relative abundance in samples from the control treatment during the 2014/2015 season, and a significantly higher relative abundance in the inoculation treatment, as compared to pre-sowing samples. On the other hand, during the 2015/2016 season, the relative abundance of this phylum was lower in samples from the control treatment, as compared to the pre-sowing treatment. With regard to the *Nitrospirae* phylum, its relative abundance was observed to be lower in samples from inoculated soil during the 2015/2016 season, as compared to pre-sowing samples. The relative abundance for the *Firmicutes* phylum did not significantly vary between treatments during both years of experimentation. The *Verrucomicrobia* phylum showed a variation between 0.88 and 1.7%. No significant differences were detected. The relative abundances of the *Latescibacteria* phylum did not significantly vary between treatments during both years of experimentation. Finally, the group named as Others (including *Euryarchaeota*, *Armatimonadetes*, *Chlamydiae*, *Chlorobi*, *Cyanobacteria*, *Elusimicrobia*, *Fibrobacteres*, *Ignavibacteriae*, *Parcubacteria*, *Planctomycetes*, *Zixibacteria*, *Saccharibacteria*, *Spirochaetae* and *Tectomicrobia*) showed relative abundances values between 0.9% and 1.77% during both years, with significant differences between phyla such as *Spirochaetae* and *Ignavibacteriae*.

Only the relative abundances of the 10 most representative genera are shown in Fig. 1.B. The relative abundances of bacterium (~50%), Others (~27%) and Unknown (~6%) were the highest throughout all the samples. Bacterium comprises uncultured bacteria while the taxonomies with abundance ranks of less than 1 were grouped into "Others". The rest of the dominant genera across all samples were RB41 (~2.5%), H16 (~2.2%), which was significantly lower in the control treatment during the 2015/2016 season, *Sphingomonas* (~2.1%), *Candidatus_Solibacter* (~2.1%), *Gemmatimonas* (~1.7%), *Bacillus* (~1.4%), *Nitrospira* (~1.2%), which was significantly higher in the inoculation treatment during the 2015/2016 season, *Bradyrhizobium* (~1.1%) and *Rhizomicrobium* (~1%). Notably, most of the relative abundances of the 10 most representative bacterial genera did not significantly vary across the different samples from both seasons (pre-sowing, control and inoculated with *T. harzianum* ITEM 3636). In addition, the heatmap and dendrogram (Figure S3 in supplementary material) depict the relative abundances of the different genera within each treatment.

Rarefaction Curves are used to assess whether the number of generated OTUs is large enough to represent the communities under study. Fig. 2.A. shows the rarefaction curves obtained for bacteria with the samples from both fields. The curves become flatter to the right, meaning that a reasonable number of individual samples were collected. Thus, the number of obtained OTUs for each of the samples was sufficient to continue the studies of microbial diversity and was sufficiently representative of the bacterial communities of those soils. In addition,

Table 5 shows, similarly to results from PCR-DGGE, that application of *T. harzianum* ITEM 3636 did not significantly affect the diversity of bacterial communities. Specifically, no significant differences were observed between the H' indexes of control and inoculation treatments. On the other hand, significant differences were observed between the indexes for bacterial communities over the different sampling times, i.e. among samples obtained before sowing (initial condition) and samples obtained at 120 days after sowing (during drying in the field), in both seasons. Finally, the ACE, Chao1 and Simpson indexes (Table S.1. in supplementary material) were obtained from the alpha diversity study. In general, no significant differences were observed, except for the ACE and Chao1 indexes for bacterial communities from the 2015-2016 season (between the pre-sowing samples and samples obtained at 120 days post sowing). However, it must be highlighted that there were no statistically significant differences, for any of the calculated indexes, between the control and inoculation treatments.

Beta diversity represents the explicit comparison of microbial communities based on their composition. Beta diversity from both field assays, analyzed as principal coordinates (PCoA) is shown in Fig. 3.A. First, samples corresponding to the 2014-2015 season were separated by time of sampling, i.e., they were separated into samples taken before sowing and samples taken during drying in the field. In the same way, samples corresponding to the 2015-2016 season were separated by time of sampling. Interestingly, both the inoculation and control treatments are grouped within the same quadrant. Such grouping shows that, on the whole, there were no significant variations between the bacterial communities from Control and Inoculation treatments. Furthermore, Fig. S4A shows the integration of UPGMA clustering trees and genus-level abundance histogram graph for bacteria. In each of these clusters, a separation between sampling times (initial condition and 120 days after sowing) is observed, similarly to results shown in Fig. 3.A.

From the LEfSe analysis, we observed that there were no statistically significant differences between samples (Figure S5A in supplementary material), since a biomarker with LDA score > 4 was not found. On the other hand, no significant differences were found between the control and inoculation treatments at the phylum and genus level, in both seasons, with multivariate statistical analyses. That is, ANOVA and Wilcoxon Test (data not shown).

Fungal communities: An average of 80,000 readings per sample, with an average size of ~240 bp, was obtained. After the cleaning of the paired end readings, approximately 71,000 effective sequences remained per sample. These sequences were used to continue with the diversity study. The GC percentage of the sequences was highly similar in all the samples (~44%). In addition, between 330 and 610 OTUs were obtained for each of the 12 samples. Each OTU is a group of sequence variants which are similar to the sequence of the ITS marker gene that is intended to represent a taxonomic unit of a fungal species or genus depending on the sequence similarity threshold. A higher number of OTUs was obtained from the samples of the 2014/2015 season. This may have been due to the fact that, during this season, the assayed field had a higher fungal diversity or that there were environmental conditions that favored a greater fungal diversity.

The relative abundance (%) of fungal phyla and genera present in the soils of the different treatments are shown in Fig. 4.A. and B. The most abundant phylum in the samples was shown to be *Ascomycota* and its abundance ranged between 39.5% (sample Th5601) and 68% (samples C4502 and Th4501). In this case, samples from the 2015/2016 season showed lower values than those from the 2014/2015 season. Nevertheless, differences were not statistically significant. Then, following a descending order in relative abundances, the phylum *Zygomycota* showed significantly higher values in samples from inoculated soil during the first year, as compared to pre-sowing soil. The relative abundance values of the *Basidiomycota* phylum were higher in control samples during

the 2014/2015 season. Also, samples from the inoculation treatment showed the highest relative abundance percentages for this phylum in the 2015/2016 season. With respect to the *Chytridiomycota* phylum, values ranged between 0.2% (sample C5601) and 5.7% (sample I5602). The relative abundance of *Glomeromycota* was higher in control samples than in pre-sowing samples during the first year and significantly higher in samples from the inoculation treatment during the second year, compared to pre-sowing samples. Finally, in the group named as “Unknown” the variation of the relative abundances was not significant. However, it should be highlighted that there was a greater number of unknown OTUs (they could not be assigned to a certain phylum) in samples corresponding to the 2015/2016 season, as compared to the previous season.

The dominant fungal genera in all the samples were *Mortierella*, *Gibberella* and *Fusarium*. However, there were significant differences in the relative abundances of some genera. A total of 130 genera were detected. During the 2014/2015 season, inoculation varied the abundances of 9 genera such as *Thanatephorus*, *Clonostachys*, *Delfinachytrium*, *Aspergillus* and *Periconia* (whose relative abundances increased) and *Pseudogymnoascus*, *Spizellomyces*, *Humicola* and *Mortierella* (whose relative abundances decreased), compared with the pre-sowing treatment. When comparing with the control treatment, we observed that inoculation reduced the relative abundances of genera such as *Colletotrichum*, *Ochrocladosporium*, *Podospora* and *Purpureocillium* while it increased those of *Thanatephorus*, *Clonostachys*, *Cyphellophora* and *Flagelloscypha*. During the 2015/2016 season, the relative abundances of the genera *Gibberella* and *Trichoderma* increased in the control plots while those of *Oidiodendron*, *Sarocladium*, *Microdochium* and *Pseudaleuria* decreased compared to the pre-sowing soil. In addition, we observed a decrease in the relative abundances of the genera *Clitopilus*, *Oidiodendron*, *Erythrobasidium*, *Pseudaleuria* and *Phialemonium*, while increasing those of *Trichoderma*, *Fusarium*, *Phaeoacremonium*, *Scytalidium* and *Gibberella*, in the inoculation treatment, compared to the pre-sowing soil. Finally, the inoculation increased the relative abundances of *Phaeoacremonium*, *Scytalidium* and *Trichoderma*, compared to the control. The heatmap (Figure S6 in supplementary material) depicts the relative abundances of the different fungal genera within each treatment.

The obtained OTUs for the samples are sufficient to perform further studies of microbial diversity and they represent the fungal communities of the soil (Fig. 2.B.), since the rarefaction curves become flatter to the right. Furthermore, table 5 shows, as results from PCR-DGGE, that there were no significant differences between the *H'* indexes of control and inoculation treatments. On the other hand, there were no significant differences between the indexes for fungal communities over the different sampling times, i.e. among samples obtained before sowing (Initial condition) and samples obtained at 120 days after sowing (during drying in the field), in both seasons. Finally, the ACE, Chao1 and Simpson indexes (Table S1 in supplementary material) did not show statistically significant differences in none of the cases.

The principal coordinates analysis (PCoA) for fungal beta diversity shows that samples within seasons (2014-2015 and 2015-2016) are separated by sampling time. That is, pre-sowing samples on one side, and samples obtained during drying in the field on the other (Fig. 3.B.). Crucially, no variations in distribution were observed between the control and inoculation by pelleting treatments in these analyses. Thus, these findings also suggest that application of *T. harzianum* ITEM 3636 would not be affecting the diversity of fungal communities. Figure S4B shows the integration of UPGMA clustering trees and genus-level abundance histogram graph for fungi. This figure shows the same pattern as that observed in Fig. 3.B., i.e., samples diverge according to the different sampling times (initial condition and 120 days after sowing), for both seasons.

Similarly to the results obtained for bacteria when performing the LEfSe analysis, we observed that there were no statistically significant differences between samples (Figure S5B in supplementary material), since a biomarker with LDA score > 4 was not found. In addition, no significant differences were found between the control and inoculation treatments at the fungal phylum and genus level, in both seasons, with multivariate statistical analyses. That is, ANOVA and Wilcoxon Test (data not shown).

Discussion

In studies carried out in commercial fields with history of peanut smut, we evaluated the protective effect of a bioformulation containing strain ITEM 3636 of *T. harzianum*. Inoculation of peanut seeds caused significant reductions in the incidence and severity of peanut smut, and has the potential to exert beneficial effects on peanut plants, increasing crop yield. Another soil-borne disease that causes severe economic losses is brown root rot, caused by *Fusarium solani*. Both phytopathogens, *T. frezii* and *F. solani*, may be present in the soil and, depending on different environmental conditions, cause disease. *T. harzianum* ITEM 3636 is a microbial agent with high potential for controlling both diseases, thus the application of a single bioformulation could protect the health of peanut plants against two high impact pathogens (Rojo et al., 2007; Ganuza et al., 2018).

The aim of the present work was to study the impact of the introduction of *T. harzianum* ITEM 3636 on the native soil bacterial and fungal communities of fields used for peanut production. Sustainability in agriculture implicates an optimization in the handling of our agricultural resources in order to meet human food needs while simultaneously preserving environmental health and protecting natural resources. Enhancements in the sustainability of agricultural systems need an appropriate exploitation of soil fertility and of its physicochemical properties. Both depend on biological processes derived from soil biodiversity (Singh et al., 2011). Soil microbiota is responsible for indispensable contributions to the sustainability of ecosystems, since its components are involved in nutrient cycling, modulation of the dynamics of organic matter and soil carbon sequestration, which modify the soils' physical structure and water regimes, thus increasing the effectiveness of nutrient acquisition by plants and their health. Such assistances are essential to the operation of ecosystems as well as to the sustainable management of agricultural ecosystems (Singh et al., 2011). For this, before the release of bacterial or fungal strains (that exert beneficial effects on plants) into soils, researchers must estimate their effects on the populations of resident microorganisms to determine their potential impact on the functioning of the inoculated ecosystems. Reduction of native microbial species from the soil environment is unwanted as there is a possibility of hampering their structure and/or activity, which are important for the functioning of ecosystems (Sharma et al., 2012). The diversity of soil microbial communities could be the major determinant without which the realization of different objectives for sustainable development would not be achievable (Singh, 2015).

With the advent of advanced technologies such as NGS, researchers are able to combine microbial community studies with classic biocontrol approaches. This integration could answer new biocontrol hypotheses (Massart et al., 2015).

In the present study, we first used PCR-DGGE to investigate the impact of inoculation on soil microbial communities. This molecular fingerprinting technique allows the detection of the most abundant microbial populations, culturable and non-culturable, in the rhizosphere (Roesti et al., 2006). We observed that *T. harzianum* ITEM 3636 did not significantly alter the structure of such communities. The effect of greater

disturbance, according to our results, could have been due to time and, possibly, to the presence of the crop. By comparison, Roesti et al. (2006) evaluated the effects of bio-inoculations on the bacterial community structure and wheat growth during a growing season in 3 Indian rain-fed wheat fields. Those fields differed in fertilizer application. They found that inoculation with arbuscular mycorrhizal fungi and plant growth promoting rhizobacteria induced modifications in the structure of bacterial communities and claimed that a substantial part of the bacterial populations present in rhizosphere soil was affected by factors other than growth stage, fertilizer application or type of bio-inoculant since their experiments were performed under field conditions, meaning a higher heterogeneity between the samples compared to samples that would have been obtained from more controlled conditions. Even though our results were promising, considering that they were as expected, we performed further studies to deepen in the understanding of microbial diversity in agricultural soils after applying *T. harzianum* ITEM 3636. To this end, we used an amplicon next-generation sequencing (NGS) approach.

Sequences of 16S rRNA genes consistent with a total of 27 phyla were identified in the bacterial communities from pre-sowing soil during the first year of sampling as well as 25 phyla during the second year. Halder et al. (2011) used a combination of procedures, including molecular typing based on 16S rDNA, to characterize the bacterial community in soil from an agricultural field destined for commercial production of peanut. The two important bacterial phyla that they detected from bulk soil were *Proteobacteria* (41%) and *Bacteroidetes* (24%). Also, they only detected the genera *Agrobacterium* and *Bacillus* in bulk soil. In this study, we observed that only the relative abundance of the bacterial phylum *Spirochaetae* was significantly higher in samples from the inoculation treatment, as compared to pre-sowing samples, during the 2014/2015 season. Members of this phylum have been associated with an effective degradation of ciprofloxacin, one of most used quinolone antibiotics detected frequently in agricultural soils (Huang et al., 2017). Also, only the relative abundances of the phyla *Acidobacteria*, *Planctomycetes* and *Bacteroidetes* were lower in samples from the inoculation treatment, as compared to pre-sowing samples. During the 2015/2016 season, we observed that only the relative abundances of the bacterial phyla *Euryarchaeota*, *Nitrospirae*, and *Ignavibacteriae* were lower in samples from the inoculation treatment, as compared to pre-sowing samples, while only the relative abundance of the phylum *Ignavibacteriae* was lower compared to the control. Members of *Euryarchaeota* are involved in the production of methane, which accounts for ~ 74% of the emitted atmospheric methane on Earth (Liu and Whitman, 2008). Additionally, Rodrigues et al. (2015) studied bacterial taxa that cycle nitrogen in soils and concluded that they tend to increase in abundance in association with plant invasion. They observed that ammonia-oxidizing microorganisms were among the bacteria with highest abundance in the invaded root-zone soils. Root exudation is important for maintaining root–soil contact in the rhizosphere by reshaping the biochemical and physical properties of the rhizosphere and promoting plant growth. Soil microorganisms are attracted to the roots by a known mechanism, which involves cross signaling with roots (Bais et al., 2004). Thus, inoculation would not have impacted on such taxa in our study since their abundance seems to depend on the presence of plant roots and the compounds that they excrete to the rhizosphere during plant development. Notably, there were no variations in the relative abundances of the phylum *Actinobacteria*. Cuesta et al. (2012), among others, reported that the presence of *Actinobacteria* is typical of suppressive substrates, since they have potential to act as biocontrol agents. On the other hand, *Ascomycota* and *Zygomycota* were the most prominent fungal phyla identified in all the samples from our study, followed by *Basidiomycota*, *Chitridiomycota*, *Glomeromycota* and *Rozellomycota*. Interestingly, the relative abundance of the phylum *Glomeromycota* was significantly higher in samples from the inoculation treatment, as compared to pre-sowing samples, during the 2015/2016 season. Arbuscular mycorrhizal fungi (AMF; phylum *Glomeromycota*) are among the soil microorganisms with the highest positive influence on plant nutrition since they establish interdependence with their hosts. These fungi colonize the plant root cortex and expand their hyphae into the surrounding soil where they search for low mobility nutrients like phosphorus, which they translocate to plants (Goetten et al., 2016). By comparison, Zhang et al. (2018) studied the consequences of organic or *Trichoderma* biofertilizer applications on grassland soil. They found that biofertilization with *Trichoderma* (used at 9,000 kg ha⁻¹) increased the relative abundances of *Archaeorhizomyces* and *Trichoderma* while decreasing that of *Ophiosphaerella*. On the other hand, the phylum *Cercozoa* (Kingdom *Rhizaria*) included a high number of the

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sequences detected in this study. However, it is common for databases of fungi to include non-fungal sequences in order to provide references that allow differentiation between fungal and non-fungal sequences, at least at the phylum level (Detheridge et al., 2016). The study of NGS-based amplicon sequencing is a tool that allows determining, quite precisely, if there are alterations in microbial communities. *T. harzianum* ITEM 3636 did not significantly alter the populations of fungi and bacteria at the phylum and genera levels, as shown here. The effect of greater disturbance of the analyzed populations, according to the presented results, was probably due to, for instance, the sampling time. Take the case of the beta diversity analysis to illustrate such assumption, since we did not observe variations in the indexes of alpha diversity. As mentioned throughout this work, there are a number of factors that may have introduced variation between sampling times. For instance, climatic conditions, agronomic management practices and fauna, among others. The analyzed results from PCR-DGGE and NGS-based amplicon sequencing indicate that there were no variations in the bacterial and fungal phyla when applying the bioproduct. Thus, the two methods used in this study provided complementary results and allowed concluding that inoculation with *T. harzianum* ITEM 3636 did not alter the structure of microbial communities in agricultural soils. In a similar study, Cordier and Alabouvette (2009) carried out experiments to detect any modification in the structure of fungal and bacterial communities from two soils after inoculation with the biocontrol strain I-1237 of *T. atroviride*, which showed biocontrol capacities against soil-borne pathogens. They observed that introduction of I-1237 in the soil resulted in a significant modification of the fungal community structure 3 days post-inoculation. Such significant difference between inoculated and control soils persisted for 3 months after inoculation. However, at the end of their experiment, the fungal community structures of the inoculated and control soils were not different from each other. Also, introduction of strain I-1237 did not significantly affect the structure of bacterial communities.

To summarize, the structures of fungal and bacterial communities from inoculated peanut fields were successfully analyzed using PCR-DGGE and NGS-based amplicon sequencing. Our results from the PCR-DGGE method were highly correlated with the results obtained using next-generation sequencing (NGS) of amplicons. Thus, our findings highlight the potential of *T. harzianum* ITEM 3636 as a fungal biocontrol agent for sustainable management of peanut diseases, since it decreases the incidence and severity of agriculturally relevant diseases without causing significant changes in the microbial communities of agricultural soils.

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Conflict of interest:

No conflict of interest to declare.

References

- Anderson, I.C., Campbell, C.D. and Prosser, J.I. (2003a) Diversity of fungi in organic soils under a moorland - Scots pine (*Pinus sylvestris* L.) gradient. *Environ Microbiol* **5**, 1121–1132.
- Anderson, I.C., Campbell, C.D. and Prosser, J.I. (2003b) Potential bias of fungal 18S rDNA and internal transcribed spacer polymerase chain reaction primers for estimating fungal biodiversity in soil. *Environ Microbiol* **5**, 36–47.
- Andrés, J.A., Pastor, N.A., Ganuza, M., Rovera, M., Reynoso, M.M. and Torres, A. (2016) Biopesticides: An Eco-Friendly Approach for the Control of Soilborne Pathogens in Peanut. In *Microbial Inoculants in Sustainable Agricultural Productivity: Vol. 1: Research Perspectives* eds. Singh, D.P., Singh, H.B. and Prabha, R. pp.161–179. New Delhi: Springer India.
- Bais, H.P., Park, S.-W., Weir, T.L., Callaway, R.M. and Vivanco, J.M. (2004) How plants communicate using the underground information superhighway. *Trends Plant Sci* **9**, 26–32.
- Brader, G., Compant, S., Vescio, K., Mitter, B., Trognitz, F., Ma, L.-J. and Sessitsch, A. (2017) Ecology and Genomic Insights into Plant-Pathogenic and Plant-Nonpathogenic Endophytes. *Annu Rev Phytopathol* **55**, 61–83.
- Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Peña, A.G., Goodrich, J.K., Gordon, J.I., Huttley, G.A., Kelley, S.T., Knights, D., Koenig, J.E., Ley, R.E., Lozupone, C.A., McDonald, D., Muegge, B.D., Pirrung, M., Reeder, J., Sevinsky, J.R., Turnbaugh, P.J., Walters, W.A., Widmann, J., Yatsunencko, T., Zaneveld, J. and Knight, R. (2010) QIIME allows analysis of high-throughput community sequencing data. *Nat Methods* **7**, 335.
- Caporaso, J.G., Lauber, C.L., Walters, W.A., Berg-Lyons, D., Lozupone, C.A., Turnbaugh, P.J., Fierer, N. and Knight, R. (2011) Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *PNAS* **108**, 4516–4522.
- Cordier, C. and Alabouvette, C. (2009) Effects of the introduction of a biocontrol strain of *Trichoderma atroviride* on non-target soil micro-organisms. *Eur J Soil Biol* **45**, 267–274.
- Cuesta, G., García-de-la-Fuente, R., Abad, M. and Fornes, F. (2012) Isolation and identification of actinomycetes from a compost-amended soil with potential as biocontrol agents. *J Environ Manage* **95**, S280–S284.
- Detheridge, A.P., Brand, G., Fychan, R., Crotty, F.V., Sanderson, R., Griffith, G.W. and Marley, C.L. (2016) The legacy effect of cover crops on soil fungal populations in a cereal rotation. *Agr Ecosyst Environ* **228**, 49–61.
- Di Rienzo, J.A., Casanoves, F., Balzarini, M.G., Gonzalez, L., Tablada, M. and Robledo, C.W. (2014) *InfoStat versión 2014*. Grupo InfoStat, FCA, Universidad Nacional de Córdoba, Argentina.
- Edgar, R.C. (2010) Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* **26**, 2460–2461.
- Escalante, A. (2007) La ecología molecular en el estudio de las comunidades bacterianas. In *Ecología molecular* ed. Eguiarte, L.E., Souza, V. and Aguirre, X. pp.393–425.
- Ganuza, M., Pastor, N., Erazo, J., Andrés, J., Reynoso, M.M., Rovera, M. and Torres, A.M. (2018) Efficacy of the biocontrol agent *Trichoderma harzianum* ITEM 3636 against peanut smut, an emergent disease caused by *Thecaphora frezii*. *Eur J Plant Pathol* **151**, 257–262.
- Gardes, M. and Bruns, T.D. (1993) ITS primers with enhanced specificity for basidiomycetes--application to the identification of mycorrhizae and rusts. *Mol Ecol* **2**, 113–118.
- Godfray, H.C.J. and Lawton, J.H. (2001) Scale and species numbers. *Trends Ecol Evol* **16**, 400–404.

- Goetten, L.C., Moretto, G. and Stürmer, S.L. (2016) Influence of arbuscular mycorrhizal fungi inoculum produced on-farm and phosphorus on growth and nutrition of native woody plant species from Brazil. *Acta Bot Bras* **30**, 9–16.
- Grifoni, A., Bazzicalupo, M., Di Serio, C., Fancelli, S. and Fani, renato (1995) Identification of *Azospirillum* strains by restriction fragment length polymorphism of the 16S rDNA and of the histidine operon. *FEMS Microbiol Lett* **127**, 85–91.
- Haldar, S., Roy Choudhury, S. and Sengupta, S. (2011) Genetic and functional diversities of bacterial communities in the rhizosphere of *Arachis hypogaea*. *A van Leeuw* **100**, 161–170.
- Hammer, O., Harper, D.A.T. and Ryan, P.D. (2001) PAST Paleontological Statistics software package for education and data analysis. *Paleontol Electron* **4**, 9 pp.
- Huang, X.-P., Mo, C.-H., Yu, J., Zhao, H.-M., Meng, C., Li, Y.-W., Li, H., Cai, Q.-Y. and Wong, M.-H. (2017) Variations in microbial community and ciprofloxacin removal in rhizospheric soils between two cultivars of *Brassica parachinensis* L. *Sci Total Environ* **603–604**, 66–76.
- Huse, S.M., Dethlefsen, L., Huber, J.A., Welch, D.M., Relman, D.A. and Sogin, M.L. (2008) Exploring Microbial Diversity and Taxonomy Using SSU rRNA Hypervariable Tag Sequencing. *PLOS Genet* **4**, e1000255.
- Kjeldahl, J. (1883) A new method for the determination of nitrogen in organic matter. *Z Anal Chem* **22**, 366–382.
- LIU, B.-R., JIA, G.-M., CHEN, J. and WANG, G. (2006) A Review of Methods for Studying Microbial Diversity in Soils1 IProject supported by the National Key Basic Research and Development Program of China (No. 2002CB111505). *Pedosphere* **16**, 18–24.
- Liu Yuchen and Whitman William B. (2008) Metabolic, Phylogenetic, and Ecological Diversity of the Methanogenic *Archaea*. *Ann N Y Acad Sci* **1125**, 171–189.
- Marinelli, A.D. and March, G.J. (2005) El carbón del maní. In *Enfermedades del maní en la Argentina* eds. March, G.J. and Marinelli, A.D. pp.88–89. Biglia Impresores.
- Massart, S., Martinez-Medina, M. and Jijakli, M.H. (2015) Biological control in the microbiome era: Challenges and opportunities. *Biol Control* **89**, 98–108.
- Muyzer, G., de Waal, E.C. and Uitterlinden, A.G. (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl Environ Microb* **59**, 695–700.
- Ovreas, L. (2000) Population and community level approaches for analysing microbial diversity in natural environments. *Ecol Lett* **3**, 236–251.
- Pedelini, R. (2014) Maní. Guía práctica para su cultivo. Instituto Nacional de Tecnología Agropecuaria.
- Pereira, V.J., Fernandes, D., Carvalho, G., Benoliel, M.J., San Romão, M.V. and Barreto Crespo, M.T. (2010) Assessment of the presence and dynamics of fungi in drinking water sources using cultural and molecular methods. *Water Res* **44**, 4850–4859.
- Rodrigues, R.R., Pineda, R.P., Barney, J.N., Nilsen, E.T., Barrett, J.E. and Williams, M.A. (2015) Plant Invasions Associated with Change in Root-Zone Microbial Community Structure and Diversity. *PLOS ONE*. **10**, e0141424.
- Roesti, D., Gaur, R., Johri, B.N., Imfeld, G., Sharma, S., Kawaljeet, K. and Aragno, M. (2006) Plant growth stage, fertiliser management and bio-inoculation of arbuscular mycorrhizal fungi and plant growth promoting rhizobacteria affect the rhizobacterial community structure in rain-fed wheat fields. *Soil Biol Biochem* **38**, 1111-1120.
- Rojó, F.G., Reynoso, M.M., Ferez, M., Chulze, S.N. and Torres, A.M. (2007) Biological control by *Trichoderma* species of *Fusarium solani* causing peanut brown root rot under field conditions. *Crop*

Prot **26**, 549–555.

- Rutigliano, F., D'Ascoli, R. and Virzo De Santo, A. (2004) Soil microbial metabolism and nutrient status in a Mediterranean area as affected by plant cover. *Soil Biol Biochem* **36**, 1719–1729.
- Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W.S. and Huttenhower, C. (2011) Metagenomic biomarker discovery and explanation. *Genome Biol* **12**, R60–R60.
- Shannon, C.E. and Weaver, W. (1949) *The mathematical theory of communication*. The mathematical theory of communication. Champaign, IL, US: University of Illinois Press.
- Sharma, S., Gupta, R., Dugar, G. and Srivastava, A.K. (2012) Impact of Application of Biofertilizers on Soil Structure and Resident Microbial Community Structure and Function. In *Bacteria in Agrobiolgy: Plant Probiotics* ed. Maheshwari, D.K. pp.65–77. Berlin, Heidelberg: Springer Berlin Heidelberg.
- Singh, J.S. (2015) Microbes: The chief ecological engineers in reinstating equilibrium in degraded ecosystems. *Agriculture, ecosystems & environment*. v. **203**, 80–82.
- Singh, J.S., Pandey, V.C. and Singh, D.P. (2011) Efficient soil microorganisms: A new dimension for sustainable agriculture and environmental development. *Agr Ecosyst Environ* **140**, 339–353.
- Solbrig, O.T. (1991) From genes to ecosystems: a research agenda for biodiversity : report of a IUBS-SCOPE-UNESCO workshop, Harvard Forest, Petersham, Ma., USA, June 27-July 1, 1991.
- Torsvik, V. and Øvreås, L. (2002) Microbial diversity and function in soil: from genes to ecosystems. *Curr Opin Microbiol* **5**, 240–245.
- Trabelsi, D. and Mhamdi, R. (2013) Microbial Inoculants and Their Impact on Soil Microbial Communities: A Review. *BioMed Res Int* doi: 10.1155/2013/863240.
- Walkley, A. and Black, I.A. (1934) An examination of the Degtjareff method for determining soil organic matter, and a proposed modification of the chromic acid titration method. *Soil Sci* **37**, 29-38.
- White, J.R., Nagarajan, N. and Pop, M. (2009) Statistical Methods for Detecting Differentially Abundant Features in Clinical Metagenomic Samples. *PLoS Comput Biol* **5**(4): e1000352. <https://doi.org/10.1371/journal.pcbi.1000352>.
- White, T., Bruns, T., Lee, S. and Taylor, J. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR Protocols: A Guide to Methods and Applications* eds. Innis, M., Gelfand, D., Shinsky, J. and White, T. pp.315–322. New York: Academic Press.
- Woo, S.L., Ruocco, M., Vinale, F., Nigro, M., Marra, R., Lombardi, N., Pascale, A., Lanzuise, S., Manganiello, G. and Lorito, M. (2014) *Trichoderma*-based Products and their Widespread Use in Agriculture. *Open Mycol J* **8**, 71–126.
- Zhang, F., Huo, Y., Cobb, A.B., Luo, G., Zhou, J., Yang, G., Wilson, G.W.T. and Zhang, Y. (2018) *Trichoderma* Biofertilizer Links to Altered Soil Chemistry, Altered Microbial Communities, and Improved Grassland Biomass. *Front Microbiol* **9**,848. doi: 10.3389/fmicb.2018.00848.
- Zheng, Y., Zhang, L.-M., Zheng, Y.-M., Di, H. and He, J.-Z. (2008) Abundance and community composition of methanotrophs in a Chinese paddy soil under long-term fertilization practices. *J Soil Sediment* **8**, 406–414.

Table 1. Primers used for PCR amplifications of 16S rRNA and ITS regions.

Name	Sequence (5' to 3')	Reference
27F	GAGAGTTTGATCCTGGCTCAG	Grifoni et al. (1995)
341F-GC	CGCCCGCCGCGCGCGGGCGGGGCGGGGGCAC GGGGGCCTACGGGAGGCAGCAG	Muyzer et al. (1993)
534R	ATTACCGCGGCTGCTGG	Muyzer et al. (1993)
ITS1-F	CTTGGTCATTTAGAGGAAGTAA	Gardes and Bruns (1993); Pereira et al. (2010)
ITS1F-GC	CGCCCGCCGCGCGCGGGCGGGGCGGGGG CACGGGGGGCT TGG TCA TTT AGA GGA AGT AA	White et al. (1990), Anderson et al. (2003a, b)
ITS2	GCTGCGTTCTTCATCGATGC	White et al. (1990), Anderson et al. (2003a, b)
ITS4	TCCTCCGCTTATTGATATGC	White et al. (1990)
338F	ACTCCTACGGGAGGCAGCA	Huse et al. (2008)
806R	GGACTACHVGGGTWTCTAAT	Caporaso et al. (2011)

Table 2. Soil properties during both seasons.

Field in General Cabrera, season 2014-2015			Field in General Cabrera, season 2015-2016		
Soil salinity	Electrical conductivity (dS/m or mmhos/cm) ^a	Bulk density (g/cm ³) ^b	Soil salinity	Electrical conductivity (dS/m or mmhos/cm) ^a	Bulk density ^b
Non-saline	< 2	1.4	Non-saline	< 2	1.4

^a Conductimeter for direct measurements on soils and solutions.

^b Cylinder method.

Table 3. Percentage of organic matter, pH and nitrogen content of field soils at 75 and 120 days after sowing during the 2014/2015 and 2015/2016 peanut growing seasons.

Treatment	2014/2015 season	2015/2016 season	2014/2015 season	2015/2016 season	2014/2015 season	2015/2016 season
	Organic matter in soil (%)	Organic matter in soil (%)	Soil pH	Soil pH	N content in soil (%)	N content in soil (%)
Initial condition	1.784 ± 0.008b	1.430 ± 0.001a	5.885 ± 0.007a	5.880 ± 0.020a	0.096 ± 0.015b	0.075 ± 0.001a
Pelleting control after 75 days	2.668 ± 0.087a	1.555 ± 0.239a	6.035 ± 0.021a	6.100 ± 0.065a	0.191 ± 0.004a	0.082 ± 0.002a
Inoculation by pelleting after 75 days	2.792 ± 0.322a	1.282 ± 0.041a	5.865 ± 0.063a	6.030 ± 0.075a	0.169 ± 0.006a	0.079 ± 0.010a
Pelleting control after 120 days	2.812 ± 0.117a	1.316 ± 0.040a	5.870 ± 0.014a	6.155 ± 0.100a	0.172 ± 0.020a	0.074 ± 0.007a
Inoculation by pelleting after 120 days	3.059 ± 0.524a	1.199 ± 0.083a	5.910 ± 0.070a	6.100 ± 0.105a	0.171 ± 0.017a	0.080 ± 0.004a
Furrow control after 75 days	TNI	1.555 ± 0.000a	TNI	6.505 ± 0.180a	TNI	0.091 ± 0.001a
Inoculation into furrows after 75 days	TNI	1.365 ± 0.041a	TNI	6.385 ± 0.045a	TNI	0.087 ± 0.008a
Furrow control after 120 days	TNI	1.396 ± 0.120a	TNI	6.470 ± 0.050a	TNI	0.074 ± 0.002a
Inoculation into furrows after 120 days	TNI	1.180 ± 0.180a	TNI	6.335 ± 0.001a	TNI	0.092 ± 0.019a

Each value represents the mean from 2 replicates ± S.D.

TNI: treatment not included.

Different letters in each column indicate statistically significant differences between means according to the Holm-Sidak method ($P \leq 0.05$).

Table 4. Shannon indexes (H') obtained in the PCR-DGGE assays.

Field in General Cabrera, season 2014-2015					Field in General Cabrera, season 2015-2016				
Treatment	Shannon indexes (H') for bacteria		Shannon indexes (H') for fungi		Treatment	Shannon indexes (H') for bacteria		Shannon indexes (H') for fungi	
	75 DAS	120 DAS	75 DAS	120 DAS		75 DAS	120 DAS	75 DAS	120 DAS
C	2.65±0.15a	2.84±0.14b	2.84±0.14a	2.76±0.13a	C	4.01 ± 0.01a	4.08 ± 0.01b	2.79 ± 0.05a	2.85 ± 0.12a
Th	2.61±0.12a	2.96±0.06b	2.96±0.06a	2.95±0.08a	Th	4.03 ± 0.07a	4.11 ± 0.01b	2.79 ± 0.13a	2.63 ± 0.11a
---	---	---	---	---	CF	4.04 ± 0.02a	4.12 ± 0.01b	2.76 ± 0.09a	2.82 ± 0.08a
---	---	---	---	---	ThF	4.01 ± 0.01a	4.15 ± 0.04b	2.88 ± 0.08a	2.73 ± 0.05a
Initial condition	2.55 ± 0.16		2.96 ± 0.03		Initial condition	3.83 ± 0.04		2.70 ± 0.09	

Mean values followed by the same letter are not significantly different according to the Holm-Sidak method ($P<0.05$). The numbers after "±" correspond to standard deviations. DAS: days after sowing. C: pelleting control; Th: inoculation by pelleting; CF: control for irrigation into furrows; ThF: inoculation by irrigation into furrows.

Table 5. Shannon indexes (H') obtained in the analyses of NGS-based amplicon sequencing

Field in General Cabrera, season 2014-2015			Field in General Cabrera, season 2015-2016		
Treatment	Shannon indexes (H') for bacteria	Shannon indexes (H') for fungi	Treatment	Shannon indexes (H') for bacteria	Shannon indexes (H') for fungi
Initial condition	6.53 ± 0.02a	4.45 ± 0.03a	Initial condition	6.57 ± 0.02a	4.12 ± 0.35a
C	6.59 ± 0.03ab	4.25 ± 0.05a	C	6.66 ± 0.01b	3.98 ± 0.04a
Th	6.64 ± 0.01b	4.34 ± 0.06a	Th	6.67 ± 0.01b	4.25 ± 0.15a

Values from the same column followed by the same letter are not significantly different according to the Holm-Sidak test ($P < 0.05$). The numbers after "±" correspond to standard deviations. C: control; Th: inoculation with *Trichoderma harzianum* ITEM 3636.

The level of alpha diversity was calculated using Mothur (Version 1.30).

Fig. 1. Relative abundance (%) of bacterial phyla (A) and genera (B) present in the soils of the different treatments.

Phyla: Proteobacteria; Actinobacteria; Acidobacteria; Gemmatimonadetes; Chloroflexi; Bacteroidetes; Nitrospirae; Firmicutes; Verrucomicrobia; Latescibacteria; Others.

Genera: bacterium; RB41; H16; Sphingomonas; Gemmatimonas; Candidatus_Solibacter; Bacillus; Nitrospira; Bradyrhizobium; Rhizomicrobium; others; Unknown. The length of each color block indicates the proportion of the relative abundance of the taxonomy. I4501 and I4502: Initial condition samples obtained during the 2014-2015 season; C4501 and C4502: samples obtained 120 days after sowing from the control treatment during the 2014-2015 season; Th4501 and Th4502: samples obtained 120 days after sowing from the inoculation treatment (*T. harzianum* ITEM 3636) during the 2014-2015 season; I5601 and I5602: Initial condition samples obtained during the 2015-2016 season; C5601 and C5602: samples obtained 120 days after sowing from the control treatment during the 2015-2016 season; Th5601 and Th5602: samples obtained 120 days after sowing from the inoculation treatment (*T. harzianum* ITEM 3636) during the 2015-2016 season.

Fig. 2. Rarefaction curves obtained with the bacterial (A) and fungal (B) sequenced readings from all the samples.

C4501; C4502; C5601; C5602; I4501; I4502; I5601; I5602; Th4501; Th4502; Th5601; Th5602

I4501 and I4502: Initial condition samples obtained during the 2014-2015 season; C4501 and C4502: samples obtained 120 days after sowing from the control treatment during the 2014-2015 season; Th4501 and Th4502: samples obtained 120 days after sowing from the inoculation treatment (*T. harzianum* ITEM 3636) during the 2014-2015 season; I5601 and I5602: Initial condition samples obtained during the 2015-2016 season; C5601 and C5602: samples obtained 120 days after sowing from the control treatment during the 2015-2016 season; Th5601 and Th5602: samples obtained 120 days after sowing from the inoculation treatment (*T. harzianum* ITEM 3636) during the 2015-2016 season.

Fig. 3. PCoA analysis based on the weighted Unifrac algorithm for bacteria (A) and bray Curtis for fungi (B). +

I1415; ○ C1415; ◇ Th1415; × I1516; △ C1516; ▽ Th1516. Each colored dot represents a sample, traced by a main coordinate on the X axis and other principal coordinate on the Y axis. The percentage on each axis indicates the value of the contribution to the discrepancy between the samples. I4501 and I4502: Initial condition samples obtained during the 2014-2015 season; C4501 and C4502: samples obtained 120 days after sowing from the control treatment during the 2014-2015 season; Th4501 and Th4502: samples obtained 120 days after sowing from the inoculation treatment (*T. harzianum* ITEM 3636) during the 2014-2015 season; I5601 and I5602: Initial condition samples obtained during the 2015-2016 season; C5601 and C5602: samples obtained 120 days after sowing from the control treatment during the 2015-2016 season; Th5601 and Th5602: samples obtained 120 days after sowing from the inoculation treatment (*T. harzianum* ITEM 3636) during the 2015-2016 season.

Fig. 4. Relative abundance (%) of fungal phyla (A) and genera (B) present in the soils of the different treatments. Phyla:

Ascomycota; Zygomycota; Basidiomycota; Cercozoa; Chytridiomycota; Glomeromycota; Rozellomycota; Unkown.

Genera: Mortierella; Fusarium; Gibberella; Neurospora; Penicillium; Aspergillus; Periconia; Unkown.

Podospora; Chrysosporium; Alternaria; Others; Unkown. The length of each color block indicates the proportion of the relative abundance of the taxonomy. I4501 and I4502: Initial condition samples obtained during the 2014-2015 season; C4501 and C4502: samples obtained 120 days after sowing from the control treatment during the 2014-2015 season; Th4501 and Th4502: samples obtained 120 days after sowing from the inoculation treatment (*T. harzianum* ITEM 3636) during the 2014-2015 season; I5601 and I5602: Initial condition samples obtained during the 2015-2016 season; C5601 and C5602: samples obtained 120 days after sowing from the control treatment during the 2015-2016 season; Th5601 and Th5602: samples obtained 120 days after sowing from the inoculation treatment (*T. harzianum* ITEM 3636) during the 2015-2016 season.







