Universidad de Oviedo
Universidá d'Uviéu
University of Oviedo

Diverse arbuscular mycorrhizal fungal species colonize roots of important agricultural crops (Pequin pepper, soybean and orange) in the northeast Mexico as revealed by Illumina Mi-Seq sequencing

MASTER IN BIOTECNOLOGY OF ENVIRONMENT AND HEALTH

SALVADOR GIMÉNEZ BRU

2017/2018

JULIO 2018

Diverse arbuscular mycorrhizal fungal species colonize roots of important agricultural crops (Pequin pepper, soybean and orange) in the northeast Mexico as revealed by Illumina Mi-Seq sequencing

by

Salvador Giménez Bru

Abstract

Arbuscular mycorrhizal fungi (AMF) are obligate symbionts in 80% of land plants. Their influence on plant development and yield has made them important players in sustainable agricultural practices. However, AMF efficiency may differ depending on their host and environmental conditions. Pequin pepper, soybean and orange are important crops in the northeast Mexico that grow in arid areas exposed to drought conditions. Hence, AMF community characterization is relevant for their production by sustainable management practices. In this study, AMF species that colonized the roots of these crops were phylogenetically characterized using a 450 bp region of the large ribosomal gene that was sequenced with the MiSeq-Illumina platform followed by taxonomic affiliation based on an evolutionary placement algorithm (EPA). Twenty species from 13 different genera were found with this approach. AMF community composition, based on read relative abundance of the AMF species, was different in each crop. In Pequin pepper roots, several *Rhizophagus* species represented the majority of the community, being *Rhizophagus clarus* the most abundant. Meanwhile, soybean AMF community was dominated by *Rhizophaus irregularis* and *Funneliformis mosseae,* and orange community by species of *Dominikia,* which represented a set of species only found in this crop. Interestingly, sampling time and stage of plant development did not show a significant effect in AMF community composition. On the contrary, plant host-specificity was shown as a significant factor shaping AMF communities. The newly proposed ribosomal region and sequencing strategy coupled with the EPA phylogenetic affiliation permitted the study of AMF communities at the species level in economically important crops in Mexico. Unraveling AMF host preferences by affordable and reliable sequencing methods such as Illumina is important to study AMF communities and for the development of effective crop-specific biofertilizers that could improve sustainable agricultural practices.

Keywords: Arbuscular mycorrhizal fungi – Metagenomics – MiSeq sequencing – Evolutionary placement algorithm – Mexico – Host preference – Plant-fungal associations – Mycorrhizal ecology

Resumen

Los hongos micorrícicos arbusculares (HMA) son simbiontes obligados en el 80% de las plantas terrestres. Su influencia en el desarrollo y rendimiento de la planta los ha convertido en actores importantes de las prácticas agrícolas sostenibles. Sin embargo, la eficacia de AMF puede diferir dependiendo de su anfitrión y las condiciones ambientales. El pimiento piquín, la soja y la naranja son cultivos importantes en el noreste de México que crecen en áreas áridas expuestas a condiciones de sequía. Por lo tanto, la caracterización de las comunidades de HMA es relevante para su producción mediante prácticas de agricultura sostenible. En este estudio, las especies de HMA que colonizaron las raíces de estos cultivos se caracterizaron filogenéticamente utilizando una región de 450 pb del gen ribosomal grande que se secuenció con la plataforma MiSeq-Illumina seguida de una asociación taxonómica basada en el algoritmo "Evolutionary Placement Algorithm" (EPA). Veinte especies de 13 géneros diferentes se encontraron con este enfoque. La composición de la comunidad de HMA, basada en la abundancia relativa de las especies, fue diferente en cada cultivo. En las raíces de pimiento piquín, varias especies de *Rhizophagus* representaban la mayoría de la comunidad, siendo *Rhizophagus clarus* la más abundante. Mientras tanto, la comunidad de HMA de soja estuvo dominada por *Rhizophaus irregularis* y *Funneliformis mosseae*, y la comunidad naranja por especie de *Dominikia*, que representaba un conjunto de especies que solo se encuentran en este cultivo. Curiosamente, el tiempo de muestreo y la etapa de desarrollo de la planta no mostraron un efecto significativo en la composición de la comunidad de HMA. Por el contrario, la especificidad del huésped de la planta se demostró como un factor importante que dio forma a las comunidades de HMA. La nueva región ribosomal propuesta y la estrategia de secuenciación con la filiación filogenética de EPA permitieron el estudio de las comunidades de HMA a nivel de especie en cultivos económicamente importantes en México. Desentrañar las preferencias de huésped de los HMA para métodos de secuenciación asequibles y confiables como Illumina es importante para estudiar las comunidades de HMA y para el desarrollo de biofertilizantes efectivos específicos de cultivos que podrían mejorar las prácticas agrícolas sostenibles.

Universidad de Oviedo
Universidá d'Uviéu
University of Oviedo

MASTER IN BIOTECNOLOGY OF ENVIRONMENT AND HEALTH

La Dra. Adriana Pacheco Moscoa, Profesor Investigador del Tecnológico de Monterrey, Campus Monterrey, México,

CERTIFICA:

Que el presente trabajo titulado "Diverse arbuscular mycorrhizal fungal species colonize roots of important agricultural crops (Pequin pepper, soybean and orange) in the northeast Mexico as revealed by Illumina Mi-Seq sequencing" ha sido realizado por la Graduada/o en Biotecnología D./Dña. Salvador Giménez Bru en el Tecnológico de Monterrey bajo su dirección, constituyendo el Trabajo Fin de Máster del/la interesado/a, cuya presentación se autoriza.

Oviedo, a 9 de Julio 2018

Fdo: Dra. Adriana Pacheco Moscoa

Fdo: Dra. Ana Isabel Peláez de Andrés

List of Figures

List of Tables

Abbreviations and acronyms

Abbreviations.

	Description
AMF	Arbuscular Mycorrhizal Fungi
ITS	Internal Transcribed Spacer
LSU	Long Subunit
SSU	Short Subunit
rDNA	Ribosomal DNA
PCR	Polymerase Chain Reaction
RS	Representative Sequence
NCBI	National Center for Biotechnology Information
ML	Maximum Likelihood
EPA	Evolutionary Placement Algorithm
PerMANOVA	Permutational Multivariate Analysis of Variance
ANOSIM	Analysis of Similarity
CAP	Canonical Analysis of Principal coordinates

Content

1. Introduction

One of the most important challenges for humanity today is to ensure food production for an increasing global population. One promising sustainable approach is to improve crop productivity by using biofertilizers (Cordell et al., 2009). Nutrients are key elements associated with plant productivity. For example, phosphorous (P) is an essential element naturally limited in soils that cannot be substituted by another nutrient (Steen, 1998). Moreover, as a non-renewable resource, it is estimated that phosphate reserves would be depleted by 2050-2090 (Runge-Metzger, 1995), placing global food security at risk (Cordell et al., 2009). In this scenario, new fertilizers are taking into account soil microorganisms in their formulations because of their proved benefits to plants (van Loon, 2007; van der Heijden et al., 2008).

Among important microorganisms for plant development, arbuscular mycorrhizal fungi (AMF) have received great attention because they form symbiotic relations with the most relevant food crops for human consumption (Brundrett, 2009; FAO, 2012). AMF are obligate symbionts of 80% of land plants, providing them with a wide range of benefits such as nutrient and water transport, soil adhesion, protection against pathogens and carbon recycling (Rillig et al., 2002; Smith and Read, 2008; Gianinazzi et al., 2010; Wehner et al., 2011; Zhao et al., 2015). However, one of the most significant roles of AMF is phosphorous (P) transport from the soil to the plant, increasing the efficiency of fertilizers (Tawaraya et al., 2012). Because of the benefits that AMF offer to plants, their use in agricultural practices is promising. Colinization of AMF used as inoculants has been found to be effective under laboratory or greenhouse conditions but is less reliable when applied in the field (Berruti et al., 2016). Until recent years, it has been shown that species specificity or differences in adaptation to particular environmental conditions should be considered when selecting AMF species to formulate a biofertilizer (Antunes et al., 2011; Zoppellari et al., 2014). These specific biofertilizers might represent a sustainable solution to improve crop yield in harsh environmental conditions as in dry desert-like areas or poor soils of countries like Mexico. For example, orange trees and peppers are crops vulnerable to droughts that represent two of the 10 most produced cultivars in Mexico. Another

susceptible important crop is soybean for which Mexico is in the top 20 world producers (FAO, 2016).

Furthermore, agricultural practices as well as environmental conditions of the site could impact AMF capability to improve crop yield and health (Gosling et al. 2006). AMF community composition can be affected by soil pH (Meadow and Zabinski, 2012), soil tillage practices (Jansa

et al., 2002; Avio et al., 2013), nutrient availability (Daleo et al., 2008, Johnson et al., 2008; Collins and Foster, 2009), land use (Antoninka et al., 2011), management practices (Castillo et al., 2006; Alguacil et al., 2014) and soil type (Oehl et al., 2010). Moreover, preferential associations with plant host species or cultivars and competition with indigenous AMF communities are other factors that have shown an influence on AMF inoculum establishment (Verbruggen et al. 2013). Undeniably, the host plant would influence the AMF community composition. Some studies even suggest that host-AMF species preferential associations exist (Vandenkoornhuyse et al., 2002; Sanders, 2003; Martínez-García and Pugnaire, 2011; Torrecillas et al., 2012), whereas other studies disagree (Hart and Reader, 2002; Santos et al., 2006; Schechter and Bruns, 2013). Indeed, unraveling AMF specificity towards the host plant requires further research.

AMF communities in host plants have been characterized by using molecular markers and DNA sequencing. In the past 10 years, the use of high-throughput technologies has been the preferred method for molecular ecological studies. Most studies relied on the use of partial sequences of the small-subunit (SSU) ribosomal gene (Öpik et al., 2013; García de León et al., 2018), which has a phylogenetic resolution intermediate between genus and species (Stockinger et al., 2009). Recently, Pacific BioSciences (PacBio) Single-Molecule Real-Time (SMRT) sequencing was successfully used to characterize AMF species (Schlaeppi et al., 2016). The strategy consisted on sequencing a 1.5 kb fragment that contemplated a partial sequence of the SSU, the full ribosomal internal transcribed spacer (ITS) and a partial sequence of the large-subunit (LSU) ribosomal gene using AMF specific primers (Krüger et al., 2009). This region has been defined as the AMF barcode (Krüger et al., 2010) and can resolve closely related species. However, SMRT sequencing is not the most used high-

throughput sequencing technology due to its high error rate dominated by insertions and deletions, and the special format of the obtained throughput data (Schadt et al., 2010) and, most importantly, it is not as easily accessible, as for example the Illumina sequencing platform.

Another molecular strategy to achieve species level phylogenetic resolution of AMF microorganisms was proposed by Senés-Guerrero and Schüßler (2016), using 454 GS-FLX+ high-throughput sequencing and an evolutionary placement algorithm (EPA). This approach proved to have the sufficient phylogenetic resolution to characterize AMF species; however, 454 pyrosequencing was phased out around mid-2016 and bypassed by other next generation sequencers, mainly Illumina. Currently, there is no strategy to characterize AMF species using Illumina sequencing. Therefore, in this study we adapted the approach of Senés-Guerrero and Schüßler (2016) and generated a similar strategy for the MiSeq-Illumina platform. For this, a reference phylogenetic tree consisting of 1.5 kb AMF sequences was used to affiliate 450 bp sequences of the LSU-D2 rDNA region of the samples.

This new AMF sequencing strategy will be used to accurately identify AMF species from environmental samples. Therefore, as a second objective of this study we characterized the AMF communities of three important crops of the northeast of Mexico; Pequin pepper, soybean and orange. In addition, we described specific host-AMF species preferences and the effect of sampling and stage of development on crops' AMF community composition. By unravelling AMF-host specificity in economically important crops we can improve our knowledge on AMF community behavior and facilitate sustainable agriculture implementation, especially in drought-vulnerable regions such as northeast Mexico. The establishment of a new sequencing strategy and robust phylogenetic pipeline to identify AMF species is crucial for the development of effective crop-specific biofertilizers.

1.1. Objectives of the study

The general objective of that study is:

- To develop, validate and apply a high-throughput sequencing method and phylogenetic annotation to identify AMF species associated with agricultural crops of relevance in northeastern Mexico, in order to generate inocula with potential as biofertilizers for use in sustainable agriculture.

To reach that goal, the study aims to achieve these specific objectives:

- To develop specific primers of AMF species with sufficient resolution for sequencing on the Illumina MiSeq platform and a bioinformatic analysis protocol that can be used worldwide.
- To adapt the Illumina sequencing data to the "evolutionary placement algorithm" (EPA) to determine the phylogeny of the AMF communities at the species level.
- To characterize AMF communities of relevant agricultural crops from northeastern Mexico and describe preferential plant-microorganism associations and stage of crop development.

2. Materials and Methods

2.1. Field site and sampling

Sampled crops were Pequin pepper (*Capsicum annuum* var. *glabriusculum*), soybean (*Glycine max*) and orange trees (*Citrus sinensis*) from the Tecnologico de Monterrey experimental field site in Hualahuises, Nuevo Leon, Mexico (24°88.06' N, 99°62.39' W). In the state (Nuevo Leon, México), around 612.2 mm of precipitation are expected annually and the climate around the field site is classified as semi-warm arid with an average annual temperature for 2016 of 30, 23 and 16 °C as maximum, average and minimum, respectively (SIAP, 2017). During the sampling period (May 19 to September 6, 2017), the experimental field experienced 153 mm of rain (Fig. 1). In the field, the area planted with soybeans and peppers was closer to each other (10 m apart), while the orange trees were planted around 50 m from these crops (Fig. 2a). Soil analyses from composite samples of a 0-30 cm depth were performed in two sites of the field (Fig. 2a) by the ISO certificated company Fertilab (Guanajuato, Mexico) (Fig. 2a, 2c). No differences in soil parameters were found between the two sampling sites. Soils possess a clay-loam medium texture, an alkaline pH of 8.48, low organic matter (2.75%), moderately low water conductivity, moderately high carbonate content (19.5%), low salinity (0.65 ds/m) and, of all nutrients, deficiency in phosphorous (9.01 ppm), sulfur (2.87 ppm), iron (8.04 ppm), zinc (0.43 ppm), manganese (2.00 ppm) and boron (0.58 ppm) (Fig. 2c).

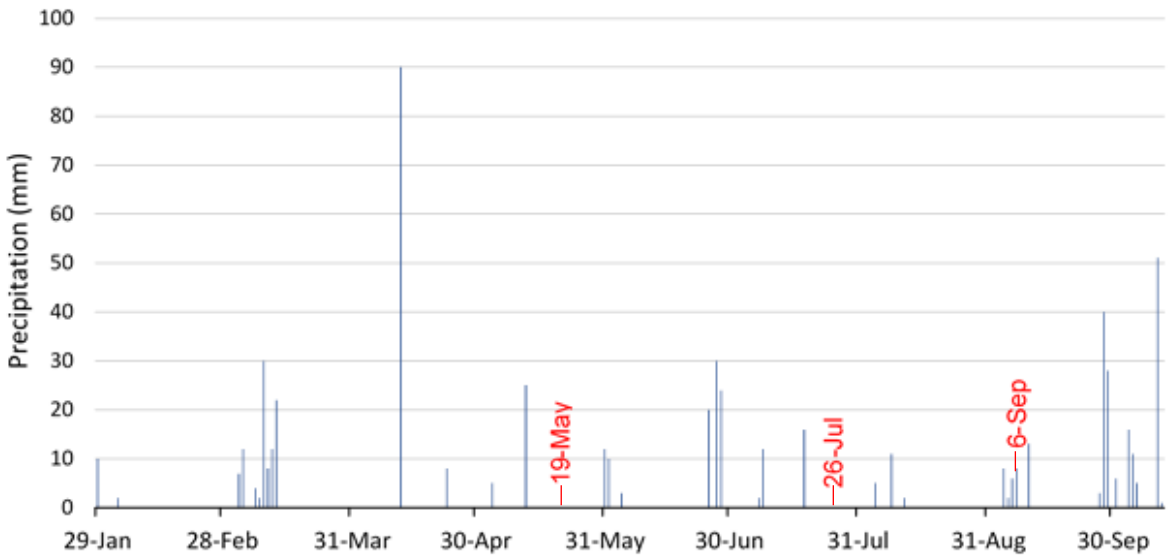
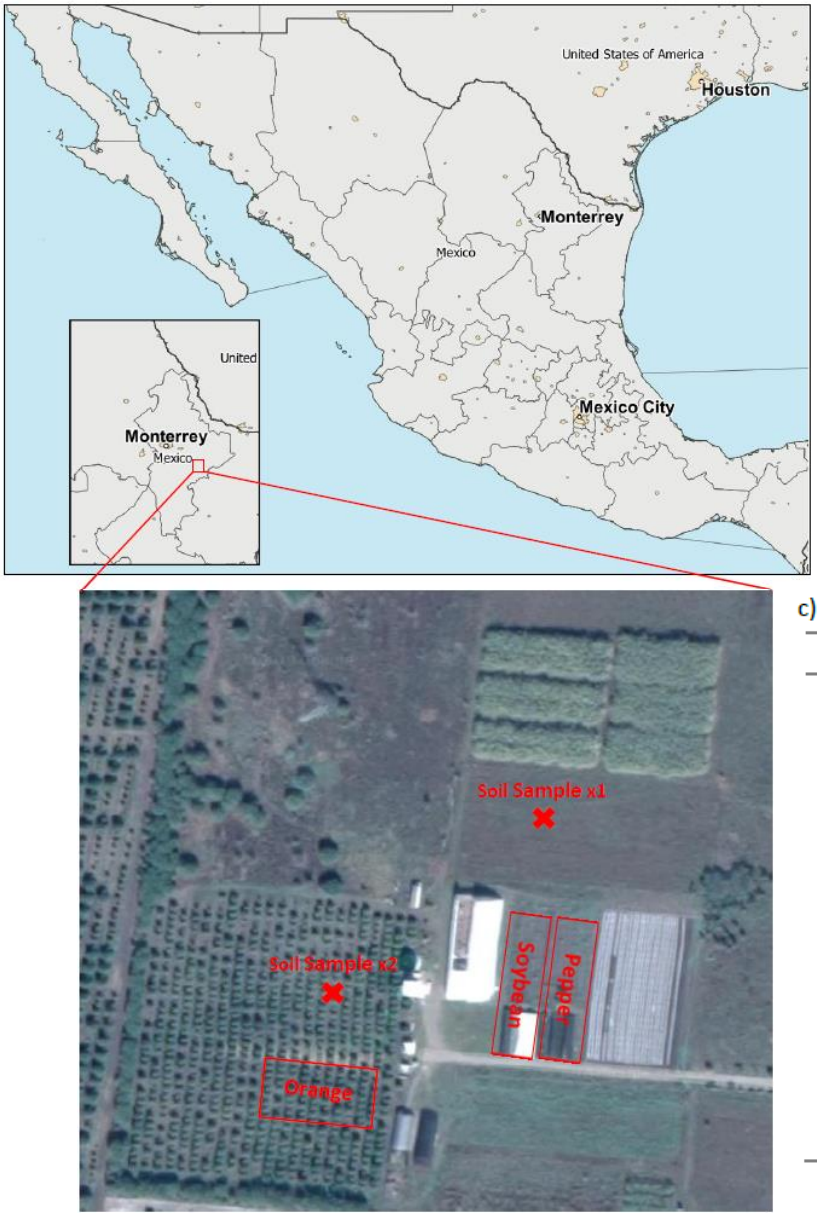


Figure 1. Precipitation in the experimental field site (Nuevo Leon, Mexico) during the study. Sampling times are indicated with the specific date of sampling during 2017.

Root samples from the three studied crops were collected in May, July and September 2017 with an interval of approximately two months among each sampling. Four independent replicates were collected from different plants of soybean and Pequin pepper. The orange trees being mature plants (12-year-old trees) were sampled in triplicate. Secondary roots were collected in sterile Whirl-Pak bags from a soil depth of 0-10 cm using a sterile wooden stick and avoiding roots of invasive plants. Three plant stages (young, mature and senescent) were determined for pepper and soybean, which corresponded to each sampling time (Fig. 2b). A young stage was defined as flowering plants, mature stage when the plants had green fruits and, finally, a senescent plant when fruits where ripe or already harvest. All samples were conserved at 4 °C until laboratory arrival, where they were stored at -20 °C.

a)



b)

Sampling	Crop	Age	Stage
May 19			
	Pequin Pepper	12 months	Young
	Soybean	2 months	Young
	Orange	12 years	Mature
July 26			
	Pequin Pepper	14 months	Mature (fruits)
	Soybean	4 months	Mature (fruits)
	Orange	12.2 years	Mature
September 6			
	Pequin Pepper	15 months	Senescent
	Soybean	5 months	Senescent
	Orange	12.4 years	Mature

c)

Soil fertility	
MO	2.75%
N-NO_3	11.9 ppm
P	9.01 ppm
K	342 ppm
Ca	6677 ppm
Mg	360 ppm
Na	63.3 ppm
Fe	8.04 ppm
Zn	0.43 ppm
Mn	2.00 ppm
Cu	0.96 ppm
B	0.58 ppm
S	2.87 ppm

Soil properties	
Texture	Clay loam
Saturation point	21.20%
Field capability	27.30%
Hidraulic cond.	2.60 cm/hr
Aparent density	1.12 g/cm^3
pH (1:2 water)	8.48
Total carbonates	19.50%
Salinity	0.65 ds/m

Figure 2. Location of the experimental field site in Nuevo León, México (a), crop sampling times and developmental stages (b) and physicochemical soil analysis (c).

2.2. Sample preparation and DNA extraction

All 33 samples were processed between two days of arrival and storage at -20 °C. First, roots were selected, washed with tap water and conserved in 96 % ethanol in 50 mL tubes at -20 °C. Lateral roots were cut into 1 cm segments under sterile conditions to obtain 20 pieces of roots per sample and conserved in 96% ethanol in 2 mL tubes at -20 °C until DNA extraction. Before DNA extraction,

ethanol was removed with a pipette and samples were dried at 60 °C in a sterile environment until all the ethanol had evaporated. Then, 200 µl of molecular grade water (DNase-free) were added to the roots for rehydration. DNA was extracted with the FastDNA Spin Kit for soil (MP Biomedicals, Santa Ana, CA, USA) following manufacturer's instructions with the lysing matrix type A and one extra ¼ inch ceramic sphere to assure complete rupture of the roots. DNA was conserved at -20 °C until sequencing library preparation.

2.3. MiSeq sequencing

AMF amplification was conducted with a nested PCR approach. For the first PCR, the primer mixture SSUmAf and LSUmAr described by Krüger et al. (2009) were used. These AMF-specific primers amplify approximately a 1.8 kb fragment covering a segment of the SSU rDNA gene, full ITS region (ITS1-5.8S-ITS2) and a segment of the LSU rDNA gene. Each sample was amplified with a 15 µl PCR mixture containing 0.5 µl of DNA, 0.75 µl of each primer at 10 µM and 7.5 µl of the 2x Phusion High-Fidelity PCR Mastermix (New England Biolabs, Ipswich, MA, USA). Cycling conditions included initial denaturation at 99 °C for 5 min, followed by 35 cycles of denaturation at 99 °C for 10 s, annealing at 60 °C for 30 s and extension at 72 °C for 1 min; a final extension phase was conducted at 72 °C for 10 min (Krüger et al., 2009). These PCR products were used as template for a nested PCR to amplify a ~450 bp fragment of the LSU-D2 rDNA region. The forward primer LSUD2mod (5'-TGAAATTGTTRAWARGGAAACG-3') was designed for this study and the reverse primers (LSUmBr) were previously published (Krüger et al., 2009). Nested PCR was performed in a volume of 20 µl using 10 µl of the 2x Phusion High-Fidelity PCR Mastermix, 1 µl of each primer at 10 µM and 0.5 µl of the first PCR amplicon. The cycling conditions were 99 °C for 2 min, followed by 30 cycles of 99 °C for 10 s, 63 °C for 30 s, 72 °C for 20 s, a final extension step was conducted at 72 °C for 5 min. After each PCR reaction, correct amplification was visualized in a 2 % agarose gel. Every reaction was done in triplicate and once correct amplification of each replicate was determined by agarose visualization, triplicates were pooled together and used for library preparation.

Pooled PCR products were purified using AMPure XP beads (Qiagen, Hilden, Germany) and visualized in a 2 % agarose gels. Nextera v2 indexes (Illumina, San Diego, CA, USA) were added by PCR at the edges of the amplicon to mark each one in a unique way. For this, PCR cycling conditions were identical to the nested PCR reaction but using 8 cycles of amplification. As before, PCR clean-up was performed using AMPure XP beads and indexed PCR products were observed in a 2 % agarose gel, the expected amplicon size was 630 bp.

Library quantification was done with the Qubit® dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) in a Qubit 2.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA). Paired-end sequencing (2 x 300 bp) was performed with a MiSeq Reagent Kit v3 (Illumina, San Diego, CA, USA) at a final loading concentration of 6.3 pM in a MiSeq System (Illumina, San Diego, CA, USA). PhiX sequencing control represented 30 % of the pooled library at 6.3 pM.

The sequencing run has been stored at the Sequence Read Archive at the NCBI with accession number PRJNA472054.

2.4. Bioinformatic sequencing data analysis

Sequence raw reads were analyzed using QIIME v1.9.1. (Caporaso et al., 2010) and the fungi Illumina pipeline from Balint et al. (2014). First, paired ends were joined with a minimum 10 bp overlap, allowing an error rate of 25 %. In the next step, sequences that did not have a quality score of minimum 25 and 70 % of consecutive high-quality base calls were discarded. The resulting sequences were clustered into representative sequences (RS) using UCLUST (Edgar, 2010) with a similarity threshold of 98 %. An open-reference approach was performed using published sequences (Krüger et al., 2012) as database to reduce the time and resources needed for this clustering. After clustering, singletons were removed. The remaining RS were analyzed with USEARCH v10.0.240 (Edgar, 2010) to find and discard chimeric sequences. Afterwards, non-AMF sequences were removed from the remaining RS by BLAST against the NCBI Nucleotide database using the CLC Genomics Workbench (Qiagen, Hilden, Germany). The remaining RS, after quality filtering, chimera and non-AMF sequence removal were used for downstream analyses.

2.5. Species delimitation

For AMF species delimitation, a reference phylogenetic tree was computed using published reference sequences (Krüger et al., 2012) and 1.5 kb sequences from newly described AMF species available in public repositories. This served as a backbone for the placement of the approximately 450 bp LSU-D2 representative sequences (RS). Sequences for the reference phylogenetic tree were aligned with MAFFT v7.311 (Katoh et al., 2002) and manually revised in CLC Genomics Workbench (Qiagen, Hilden, Germany). A maximum-likelihood phylogenetic tree was calculated with RAxML-HPC v.8 on XSEDE at the CIPRES Science Gateway (http://www.phylo.org/portal2/) using the GTRGAMMA model and 1,000 bootstraps (Stamatakis, 2015).

As a second step, every RS was aligned to the previous 1.5 kb sequence alignment with MAFFT. Hence, the resulting alignment contains 1.5 kb sequences from described AMF which compose the phylogenetic backbone and ~450 bp LSU-D2 representative sequences. This alignment file is used as an input at the CIPRES Science Gateway using RAxML-HPC v.8 on XSEDE. Here, there is an option to perform the RAxML evolutionary placement algorithm (EPA) with the GTRGAMMA model (Berger et al., 2011; Berger and Stamatakis, 2011). For each RS, EPA assigns a place in the phylogenetic tree used as a backbone. EPA was shown to be useful for the placement of short AMF reads (~780 bp) with high accuracy (Senés-Guerrero and Schüßler, 2016). Sequences that had a maximum placement likelihood weight of >0.5 were considered to have an uncertain placement and were discarded (Zhang et al., 2013; Berger et al., 2011). The resulting phylogenetic tree with the placement of the RS was visualized in Archaeopteryx Treeviewer v0.9920 (Han and Zmasek 2009). RS were manually annotated to a taxonomic level corresponding to the branch where they were affiliated in the phylogenetic backbone tree.

2.6. Validation of species annotation by evolutionary placement algorithm (EPA)

To validate EPA affiliation of the ~450 bp LSU-D2 sequences, eight *Rhizophagus* species from 138 reference sequences were used to generate a maximum-likelihood reference phylogenetic tree, which was used as a backbone for short

sequence affiliation. Two sequences of *Claroideoglomus claroideum* were used as tree root. To obtain reliable and described 450 bp LSU-D2 sequences to conduct a test, five sequences of *Rhizophagus intraradices* and 16 sequences of *Rhizophagus irregularis* which are well characterized and published in public databases were used as query sequences by shortening them to the same ~450 bp LSU-D2 rDNA fragment amplified in the nested PCR. These short sequences were aligned and affiliated to the reference phylogenetic tree as previously described. Their correct taxonomic affiliation was manually evaluated.

2.7. Statistical analysis

All statistical analyses were performed in R 3.4.4 (R Core Team, 2018) using the vegan v2.4-6 (Oksanen et al. 2013) and BiodiversityR v2.9-2 (Kindt, 2016) packages, unless stated otherwise. Rarefaction curves for each crop and sampling time were calculated using the rarefaction function of Vegan package. The read count matrix was square root normalized and analyzed by canonical analysis of principal coordinates (CAP) (Anderson et al., 2003) using the BiodiversityR function CAPdiscrim, which is based on Bray-Curtis dissimilarities and 999 permutations. Sample clustering shown in CAP was done with the ordiellipse function, which highlights groups with a 95% of confidence. Beta-diversity among samples was calculated using the Bray-Curtis dissimilarity with the vegdist function of vegan. Beta-diversity changes among samples was tested by permutational MANOVA (PerMANOVA) using the adonis function of vegan and 999 permutations. To validate the obtained P values, the statistical power of the PerMANOVA test was corroborated using G*Power v3.1.9.2 (Faul et al., 2007) with an effect size of 0.6666. The effect size was calculated by taking into account the 33 samples, 6 groups (Pequin pepper, soybean, orange and three samplings), 2 predictors (crop and sampling) and 1 response variable (species abundance). Results were further validated by an ANOSIM carried out using the vegan function anosim and the same parameters of PerMANOVA.

3. Results

3.1. Validation of phylogenetic affiliation

A phylogenetic backbone tree was constructed using 138 sequence variants of eight reference *Rhizophagus* species, all sequences were 1.5 kb and correspond to the AMF barcode. Variants of the species *Rhizophagus intraradices* and *Rhizophagus irregularis* were used as query sequences by shortening them to 450 bp in the LSU-D2 rDNA region, as our proposed amplicon. All 21 query sequences were correctly affiliated to the tree branches associated to the species showing a maximum placement likelihood weight <0.5 (Table 1), suggesting that the phylogenetic affiliation of this fragment is a robust and accurate method to characterize AMF species.

Species	Accesion number [1]	Tree Branch [2]
Rhizophagus intraradices	JF439108	I48
Rhizophagus intraradices	FM865566	I62
Rhizophagus intraradices	FM865573	I39
Rhizophagus intraradices	FM865575	I39
Rhizophagus intraradices	FM865603	I39
Rhizophagus irregularis	FM992377	I210
Rhizophagus irregularis	FM992383	I183
Rhizophagus irregularis	FM992386	I182
Rhizophagus irregularis	AJ854584	I218
Rhizophagus irregularis	AJ854585	I218
Rhizophagus irregularis	AJ854586	I153
Rhizophagus irregularis	AJ854587	I102
Rhizophagus irregularis	AJ854590	I218
Rhizophagus irregularis	AJ854593	I218
Rhizophagus irregularis	AJ854595	I102
Rhizophagus irregularis	AJ854596	I102
Rhizophagus irregularis	AJ854597	I102
Rhizophagus irregularis	AJ854611	I153

Table 1. Tree branch affiliation of published sequences of *Rhizophagus* used to validate method of taxonomic affiliation of AMF species. [1]Accession number according to the NCBI database. [2]Branch of the reference tree to which the sequence was affiliated.

3.2. Library generation and sequencing data analysis

A total of 33 samples were processed for library generation, which contemplates crop (n=3), sampling time (n=3) and replicate (n=3-4). All PCR amplicon replicates of each of these samples resulted in measurable products that were pooled together and sequenced. After sequencing, paired-end reads were joined and filtered for quality. Sequences were clustered using a 98% similarity threshold into 55,337 no-singletons references sequences (RS). Then, 5,769 chimeras and 3,575 non-AMF sequences were removed, leaving 45,993 RS to be affiliated into the phylogenetic backbone tree. These RS contained 1,315,642 reads with lengths between 357 and 592 bp. Using RAxML-EPA, RS were annotated into 20 AMF species from 13 genera. All sample libraries exceeded 7,000 reads affiliated at the genus level; hence, none of them were dismissed from the analysis. In addition, rarefaction curves of the samples showed that the analyzed libraries were close or reached AMF species saturation, suggesting coverage of AMF diversity in all samples (Fig. 3).

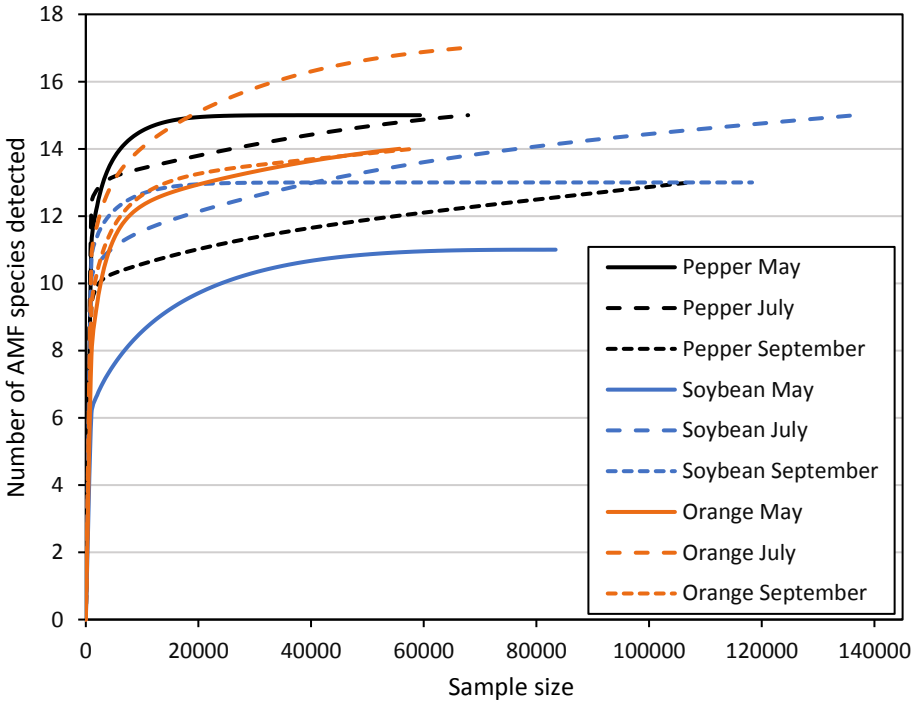


Figure 3. Rarefaction curves of the number of arbuscular mycorrhizal fungal (AMF) species detected per crop and sampling time.

3.3. AMF species diversity on crops

The AMF community of each crop was evaluated by determining their relative read abundance at the species and genus level (Fig. 4). Generalist species like *Funneliformis mosseae* and *Rhizophagus irregularis* were observed in all crops, with a higher combined relative read abundance of these two species in soybean (71%) followed by pepper (31%) and, lastly, orange (28%) (Fig. 4a). Pepper and soybean shared most AMF species identified in the samples but in different proportions. Soybean also showed a considerable presence of *Claroideoglomus hanlinii* (5%), that was not represented in the other crops (<1%), and *Kamienskia perpusilla* (6%). Pepper AMF community presented a high abundance of *Sclerocystis sinuosa* (22%), *Rhizophagus clarus* (22%), *Rhizophagus arabicus* (8%) and *Rhizophagus fasciculatus* (8%). In contrast, orange AMF community was considerably unique, dominated by *Dominikia* spp. (52%), while in pepper (7%) and soybean (3%) this species was in low abundance. Also, orange showed *Rhizophagus intraradices* (5%) and *Rhizophagus diaphanus* (10%) not present in high proportion (<1%) in the other crops.

Along sampling period of the pepper plants, from first to third sampling, relative read abundance of *R. irregularis* (39%, 18% and 3%) and *F. mosseae* (18%, 33%, 1%) significant decrease, whereas the opposite was shown for *R. clarus* (3%, 22%, 33%), *S. sinuosa* (<1%, 2%, 47%) and *R. fasciculatus* (< 1%, 33%, 13%) (Fig. 4b). In soybean, generalist species like *R. irregularis* (28%, 55% and 41%) and *F. mosseae* (64%, 14% and 16%) were highly abundant throughout sampling, while the relative read abundance of other species such as *S. sinuosa*, *C. hanlinii*, *R. clarus*, *R. diaphanus* and *R. fasciculatus* increased with time (Fig. 4b). For orange, a clear domination of non-determined species of the genus *Dominikia* (82%, 24%, 56%) is shown regardless of sampling time, whereas generalists like *R. irregularis* increased in their abundance (<1%, 25%, 12%) or showed irregular patterns like *F. mosseae* (< 1%, 35%, 3%) and *R. intraradices* (16%, < 1% for the second and third sampling times) (Fig. 4b).

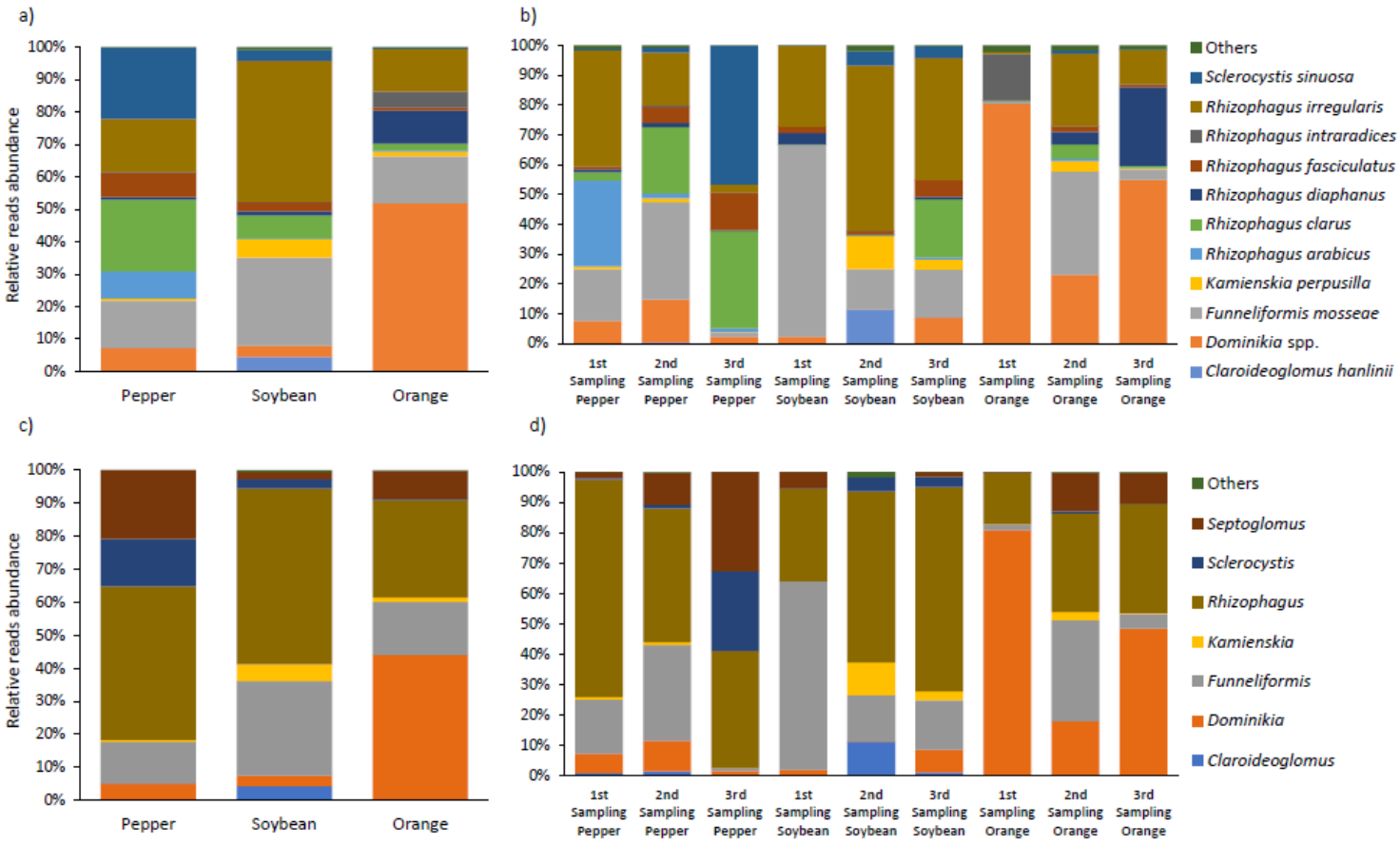


Figure 4. Relative abundance of AMF species and genera for crop type (a and c, respectively) and sampling time (b and d, respectively). "Others" represent those species and genera that did not exceed 1 % in samples. *Dominikia* spp. represents undetermined species of this genus with a highly probable affiliation.

At the genus level, pepper and soybean were dominated by *Rhizophagus* (>47%), while orange by *Dominikia* (44%) followed by *Rhizophagus* (30%) (Fig. 4c). In pepper, *Septoglomus* (21%) and *Sclerocystis* (14%) contributed more than the generalist *Funneliformis* (13%). However, in soybean, *Funneliformis* was a major genus (29%) and the genera *Claroideoglomus* (4%) and *Kamienskia* (5%) were in a higher proportion than in the other crops (<1%) (Fig. 4c). Accordingly to sampling time, *Septoglomus* and *Sclerocystis* in pepper drastically increased with time from 2 to 33% and <1 to 26%, respectively. *Dominikia* and *Funneliformis* showed high relative abundances in the first two samplings (6-10 and 18-31%, respectively) but declined to around 1% in the third sampling. Although *Rhizophagus* was highly abundant in the second (44%) and third (38%) sampling, in the first sampling represented 72% of the AMF community (Fig. 4d). In

soybean, *Rhizophagus* maintained high relative read abundances through sampling time (31, 56 and 67%), while *Funneliformis* reached its maximum abundance in the first sampling (62%) and decreased to 15-16% in the other two samplings. Other genera found with abundances >10% were *Claroideoglomus* and *Kamienskia* (Fig. 4d). As mentioned above, orange trees were highly colonized by *Dominikia* and other genera such as *Septoglomus*, *Rhizophagus* and *Funneliformis* showed relative read abundances >10% at some sampling time (Fig. 4d).

The presence or absence of AMF species among all replicates within each crop was evaluated to determine if certain species were specifically related to one crop than another. Figure 5 shows all AMF species identified in this study and the number of replicates where they were observed. As previously mentioned, generalist species as *Funneliformis mosseae* and *Rhizophagus irregularis* were present in all replicates of all crops. In contrast, the orange crop presented a unique set of five AMF species; three species of the genus *Dominikia (D. disticha, D. irania and D. achara)*, *Kamienskia bistrata* and *Dentiscutata savannicola* (Fig. 5c). Also, *Rhizophagus proliferus* was only observed in pepper (Fig. 5a). Although soybean did not exhibit any particular AMF species, *Diversispora omaniana* and *Claroideoglomus claroideum* were observed in a higher number of replicates compared to pepper and orange (Fig. 5b). *Acaulospora scrobiculata*, *Rhizopagus aggregatum* and *R. arabicus* were also more common in pepper than in the other crops.

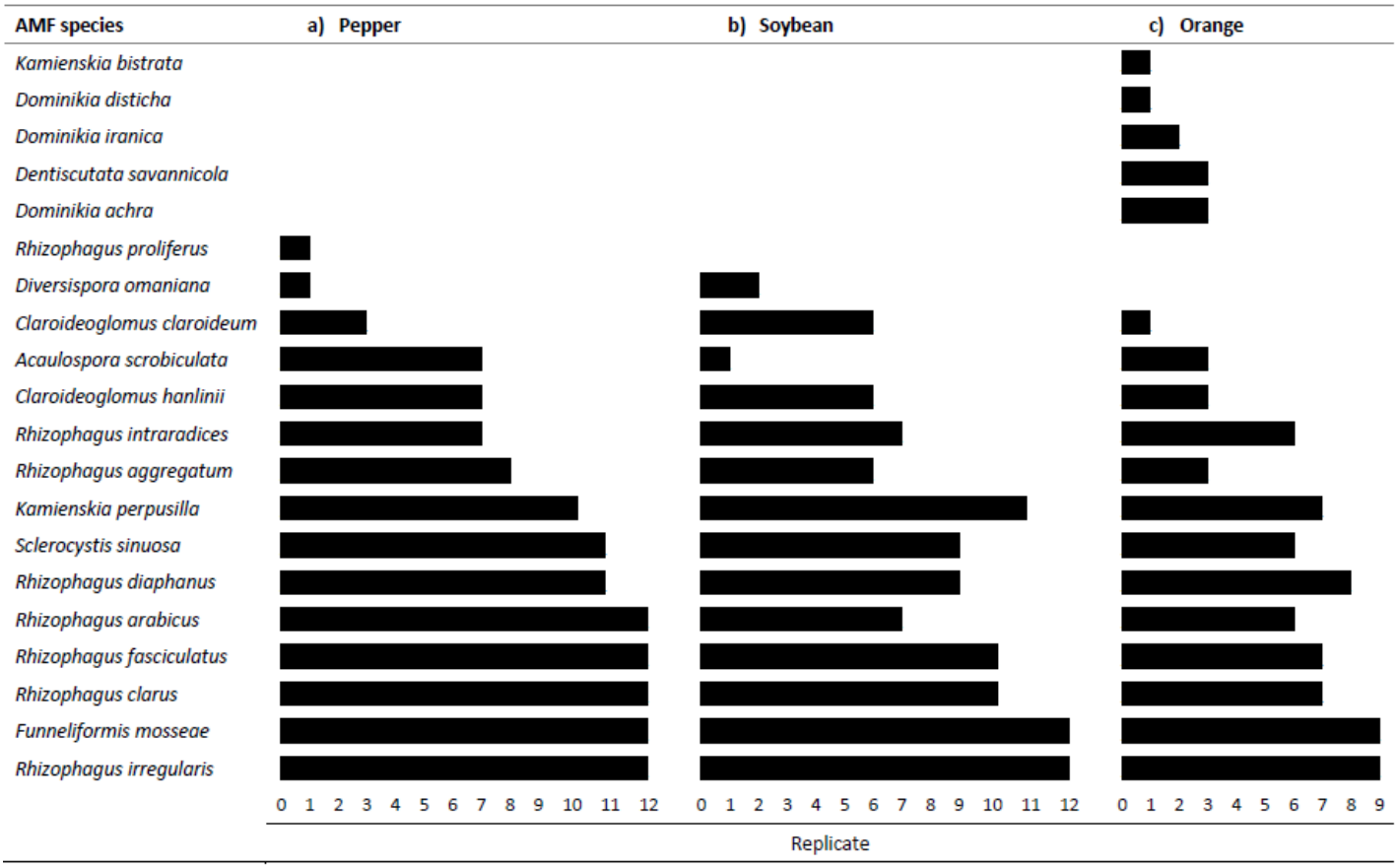


Figure 5. Presence or absence of AMF species in all replicates of the study for Pekin pepper (a), soybean (b) and orange (c).

3.4. Influence of crop, sampling time and developmental stage on AMF community composition

CAP analysis of the effect of crop species on AMF community composition resulted in a two-dimensional solution with a 72.73% of correct allocations, which strongly separated three clusters that corresponded to crop species (Fig. 6a). Furthermore, PerMANOVA and ANOSIM analyses indicated that crop species had a significant effect on AMF community composition (P=0.001; Table 2). Contrary, the effect of sampling time for all crops, developmental stage of pepper and developmental stage of soybean generated for each effect a two-dimensional solution with 48.48, 63.67 and 58.33 % of correct allocations, respectively, where clusters of these three variables did not separated from each other (Fig. 6b-d). The two-way PerMANOVA and ANOSIM analysis of each effect showed no significant influence of these variables on AMF community composition (Table 2).

However, the soybean developmental stage ANOSIM test displayed a significance of 0.033 contradictory to the results of the PerMANOVA (P=0.119; Table 2).

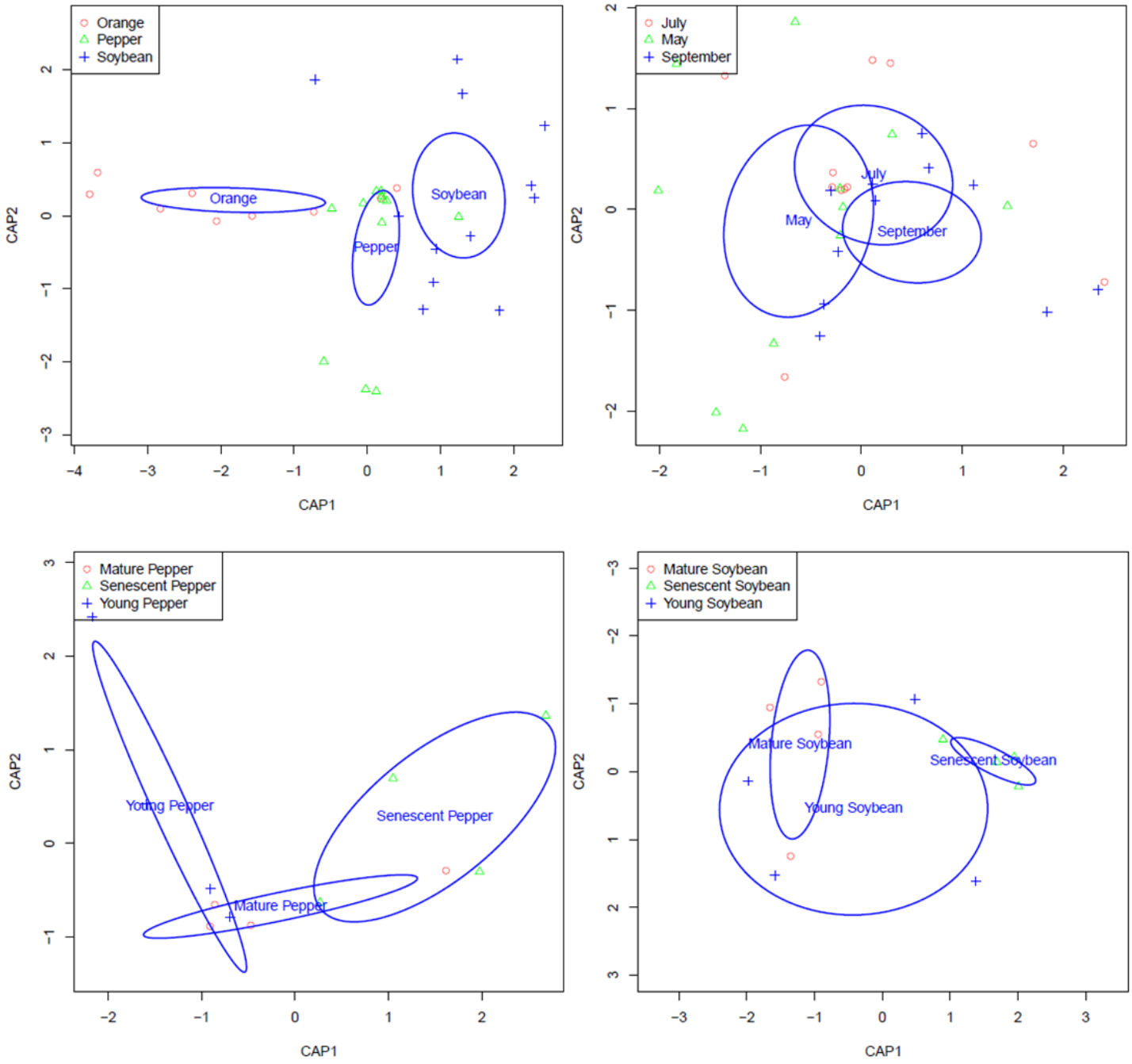


Figure 6. Canonical analysis of principal coordinates (CAP) of the AMF community among crop type (a), sampling time (b) and developmental stage of pepper (c) and soybean (d). Weight-calculated ellipses highlights groups with a 95 % confidence level.

	PerMANOVA			ANOSIM	
Variable	**F value**	**R^2**	***p* value**	**R**	**Significance**
Crop	4.3246	0.22379	0.001	0.235	0.001
Sampling	1.3370	0.08184	0.182	0.031	0.163
Pepper stage	1.5627	0.25775	0.099	0.148	0.101
Soybean stage	1.8105	0.28690	0.119	0.292	0.033

Table 2. PerMANOVA and ANOSIM test parameters of the influence of crop type, sampling time and developmental stage in the AMF community composition.

4. Discussion

In this study, we aimed to characterize AMF communities associated with economically important crops in the northeast Mexico. To accomplish this goal, we first developed and validated a molecular strategy using the sequencing platform of MiSeq-Illumina and a high-throughput phylogenetic annotation to identify AMF microorganisms at the species level. These results are crucial for future applications and the development of effective crop-specific AMF-based biofertilizers. Particularly, these microorganisms might be adapted to harsh environmental conditions such as high temperature and drought, typical of the northeast Mexico.

4.1. AMF species delimitation method

By using a strategy based on a two-step PCR followed by MiSeq-Illumina sequencing and a phylogenetic pipeline that contemplated a reference phylogenetic tree and the evolutionary placement algorithm (EPA), we were able to annotate to the species level members of the studied AMF communities. The selection of the genetic region was crucial to obtain sufficient phylogenetic resolution when characterizing sequences to the species level. The LSU-D2 rDNA region was shown to be a good candidate for species identification from roots and soils (Krüger et al., 2009; Stockinger et al., 2009); therefore, we focused on this region to conduct in silico tests to define a set of primers that targeted all described species publicly available (Krüger et al., 2012). In addition, the amplified fragment was limited to a maximum size of 550 bp, as recommended by Illumina when running a 2x300 bp paired-end sequencing read.

As there was no reported bioinformatic pipeline for the Illumina sequencing method and amplified region used, we adapted related protocols to process and annotate the generated sequences (Caporaso et al., 2010; Balint et al., 2014; Senés-Guerrero & Schüßler, 2016). After raw sequence assembly and quality filtering, a relevant step is to determine the similarity threshold for sequence clustering, as it is important to avoid that single clusters contain different species. For threshold selection, the length and variability of the sequenced region should be taken into account. Although many studies have used 97% similarity to cluster

AMF sequences (Haugh et al., 2013; Bell et al., 2014; Lekberg et al. (2014), this threshold depends on the level of interpretation desired. Due to the short length of our reads and the region used, which possesses an intraspecific rDNA sequence variation of 0-15.7% (Stockinger et al., 2010), we decided to use a similarity threshold of 98%, as other authors have suggested (Schechter and Bruns, 2013; Senés-Guerrero and Schüßler, 2016).

EPA has been shown to be an accurate method to affiliate short sequences into a robust phylogenetic tree (Berger and Stamatakis, 2011). Here, we constructed a maximum-likelihood phylogenetic tree using published AMF reference sequences and affiliated ~450 bp LSU-D2 rDNA sequences. All query sequences are affiliated into tree branches; however, it has been shown that maximum placement likelihood weights (PLW) > 0.5 could be artifacts (Zhang et al., 2013; Berger et al., 2011). Therefore, we used BLAST to compare the affiliated RS sequences with a PLW > 0.5 to their closest species in the phylogenetic backbone tree. Indeed, sequences above the threshold previously mentioned were not similar to their closest phylogenetic species and were discarded (conserved sequences represented 99.25% of affiliated RS).

To further test EPA affiliation using the LSU-D2 rDNA region and to validate whether it is capable of distinguishing closely related species, we affiliated ~450 bp characterized sequences of *R. irregularis* and *R. intraradices*. EPA placed all sequences correctly, suggesting that our selected region can be used for the molecular characterization of communities of AMF species by a high-throughput sequencing method such as MiSeq-Illumina.

4.2. AMF communities associated with crops

The studied crops were analyzed with sufficient depth to characterize all AMF species diversity, as observed in the rarefaction curves. In terms of species richness, orange trees showed a more complex community with 18 AMF species identified compared to Pequin pepper (15 species) and soybean (14 species). Five orange-specific AMF species were found (*Dominikia achra*, *D. iranica*, *D. disticha, Kamienskia bistrata* and *Diversispora savannicola*), while the unique difference between pepper and soybean was R. proliferus, only present in pepper.

Rhizophagus arabicus, Rhizophagus clarus, Rhizophagus fasciculatus and *Sclerocystis sinuosa* were found in high abundances in Pequin pepper compared to soybean or orange. In relation to the beneficial effects of these AMF species to the host plant, *R. fasciculatus* has been reported as an important species in chlorophyll and carotenoid composition and quality of pepper fruits in drought conditions (Mena-Violante et al., 2006). Other reported species associated with pepper roots are *Rhizophagus intraradices* (Cekic et al., 2012; Beltrano et al., 2013), which was not a major component in this study. It was suggested that *R. intraradices* colonization is related to salinity stress (Beltrano et al., 2013) and the studied soil was low in salinity. In contrast, *F. mosseae*, also related with salinity stress alleviation in pepper (Latef and Chaoxing, 2014), was found in high proportion and in all sampling replicates. Spore-based studies in pepper agreed with our results, Carballar-Hernández et al. (2017) found *R. fasciculatus, F. mosseae* and *S. sinuosa* in relative high proportions in different agro-ecosystems and Sánchez-Sánchez et al. (2018) reported as *F. mosseae* and *R. intraradices* as abundant species. The latter species was not as abundant in this study as compared to *R. irregularis*, however *R. intraradices* could be difficult to differentiate from *R. irregularis* using spores morphology because these two species are very closely related (Stockinger et al., 2009).

Soybean samples showed almost the same AMF species than pepper but in different proportions. *R. irregularis* and *F. mosseae* represented most of the AMF community of soybean through time. It has been reported that after soybean cultivation, AMF species in the soil are reduced, affecting low abundance species more than generalists (Higo et al., 2013). Furthermore*, R. irregularis* (Peyret-Guzzon et al., 2016) and *F. mosseae* (Sýkorová et al., 2007; Lekberg et al., 2012) are early-stage colonizers and adapted species to disturbed systems, like agriculture fields. These results could suggest that soybean AMF community is more sensitive than others to soil disturbance. Besides generalist AMF species, *Claroideoglomus hanlinii* and *Kamienskia perpusilla* were particularly abundant in soybean. These AMF species were recently described and there are no sufficient studies to explain their role. However, Jansa et al (2008) demonstrated that members of the genera *Rhizophagus* and *Claroideoglomus* had a synergetic effect and increased the phosphorous uptake when they were inoculated

together. Overall, soybean AMF community has not been described before, so the community presented here should be compared with new studies to elucidate differences among important agricultural parameters.

Orange AMF community was dominated by different species of the genus *Dominikia*. Moreover, a particular set of AMF species only found in a few replicates of orange was composed of *D. achra, D. iranica, D. disticha, K. bistrata* and *D. savannicola*. These species of *Dominikia* have been described in a different ecosystem, in maritime dunes, and although it has been suggested as an important microorganism for establishing, shaping and functioning of plants with high colonization power, its specific role has not been elucidated (Błaszkowski et al., 2018). Other abundant AMF species in orange were *F. mosseae, R. irregularis, R. diaphanous* and *R. intraradices*, the latter two *Rhizophagus* species were in low abundance (<1%) in pepper and soybean. Moreover, among all described functions of *R. intraradices* it plays an important role in drought tolerance (Li et al., 2014; Ortiz et al., 2015), suggesting that the orange crop possesses a high-water requirement in the semi-warm arid climate of the studied field site. Citrus crops have been widely studied and reported to possess a high AMF dependence especially in drought conditions (Peyronel, 1922; Wu et al., 2013). The AMF community found in this study is similar to those characterized by Youpensuk et al. (2008) and Chakraborty et al. (2011). These studies were carried out with *Citrus reticulata* using spores but showed the presence of AMF species highly represented in our study like *F. mosseae, R. intraradices* and *R. fasciculatus*. Furthermore, Camprubí and Calvet (1996) and Zhang (2010) also found these species along with *R. diaphanous* in citrus crops.

4.3. Influence of crop, sampling time and developmental stage on AMF community composition

AMF community composition can be influenced by many factors such as geography, temperature, soil type, tillage, etc. (Antoninka et al., 2011; Meadow and Zabinski, 2012; Avio et al., 2013; Alguacil et al., 2014). In this study, we sampled three different crops within a small experimental field site; therefore, we can assure similar environmental and soil conditions along with agronomic practices for all samples. Then, the variables that could have an effect on AMF

community composition were host specificity, sampling time and developmental stage. To understand the effect of these variables, we evaluated their significance in AMF beta-diversity changes among the studied crops by using them as pre-established factors and conducting a CAP analysis with Bray-Curtis dissimilarities among groups. Only the variable crop showed a significant effect on the AMF community composition. This result was supported by PerMANOVA analysis using Bray-Curtis dissimilarities. Neither sampling time nor developmental stage was a determinant factor shaping AMF species communities.

Some studies have reported an effect of host plant and deny changes in the AMF community due to season (Liu et al., 2009; Davison et al., 2011). On the contrary, a seasonal study from Drumbell et al. (2011) showed a clear difference in AMF communities between winter and summer. In addition, Santos-Gonzalez et al. (2007) and Rosendahl and Stukenbrock (2004) indicated that composition and diversity of AMF assemblages was maintained throughout warmer months, a phenomenon that agreed with our results.

Although not statically significantly, slight differences among Pequin pepper developmental stages were observed in the CAP plot and relative read abundances, being the mature stage an intermediate state between young and senescent plants. This result agrees with studies that suggest that early plants are colonized mainly by AMF generalists, like *R. irregularis* and *F. mosseae*, and then there are progressive changes in AMF species through plant development (Sýkorová et al., 2007; Werner and Kiers, 2014). For soybean, the senescent stage showed more differences in AMF abundance while young plants seemed similar to the mature stage. Schreiner and Mihara (2009) found different AMF phylotypes depending on vine age. In the same way, Muleta et al. (2008) suggested that the age of the plant affected the AMF community associated with coffee plants. However, these studies characterized perennial plants, in which the stage of development might be more relevant than in these short-cycle crops as pepper and soybean. Interestingly, plants with shorter cycles more similar to pepper and soybean life cycles, like *Alliaria petiolata*, showed more impact in the determination of AMF communities in early stages than older, possibly because of an AMF growth-promotion role (Lankau, 2011). Accordingly, the stages of development evaluated in this study could have presented no differences on the

AMF community because it was determined in earlier stages and remain without significant change through the cycle.

5. Conclusions

Overall, we studied in depth the AMF species community composition of three important crops growing in the same field site, which allowed control of climatic and soil biochemical variables and focused the analysis in host specificity and sampling time. Sampling time and stage of plant development were not significant factors influencing AMF communities. On the contrary, host-specificity was shown as a significant factor shaping AMF communities, which is in agreement with several published studies (Vandenkoornhuyse et al., 2002; Sanders, 2003; Martínez-García and Pugnaire, 2011; Torrecillas et al., 2012) and suggests a strong host preference of AMF species.

The development of a high-throughput annotation strategy based on EPA phylogenetic affiliation permitted the study of AMF communities at the species level in economically important crops in Mexico. Unraveling AMF host preferences by affordable and reliable sequencing methods such as the Illumina platform is important to study AMF communities and their intricate dynamics, helping in the future goal of selecting specific AMF species for the development of biofertilizers that could improve sustainable agricultural practices.

6. References

Abdel Latef A, Chaoxing H (2014) Does inoculation with *Glomus mosseae* improve salt tolerance in pepper plants? Journal of Plant Growth Regulation 33:644-653

Alguacil MM, Torrecillas E, García-Orenes F, Roldán A (2014) Changes in the composition and diversity of AMF communities mediated by management practices in a Mediterranean soil are related with increases in soil biological activity. Soil Biology and Biochemistry 76:34-44

Anderson M, Willis T (2003) Canonical analysis of principal coordinates: a useful method of constrained ordination for ecology. Ecology 84(2):511-525

Antoninka A, Reich PB, Johnson NC (2011) Seven years of carbon dioxide enrichment, nitrogen fertilization and plant diversity influence arbuscular mycorrhizal fungi in a grassland ecosystem. New Phytologist 192:200–214.

Antunes PM, Koch AM, Morton JB, Rillig MC, Klironomos JN (2011) Evidence for functional divergence in arbuscular mycorrhizal fungi from contrasting climatic origins. New Phytologist 189:507–514

Avio L, Castaldini M, Fabiani A, Bedini S, Sbrana C, Turrini A, Giovannetty M (2013) Impact of nitrogen fertilization and soil tillage on arbuscular mycorrhizal fungal communities in a Mediterranean agroecosystem. Soil Biology and Biochemistry 67:285-294

Bálint M, Schmidt PA, Sharma R, Thines M, Schmitt I (2014) An Illumina metabarcoding pipeline for fungi. Ecology and Evolution 4(13):2642-2653

Bell T, Hassan S, Lauron-Moreau A, Al-Otaibi F, Hijri M, Yergeau E, St-Arnaud M (2014) Linkage between bacterial and fungal rhizosphere communities in hydrocarbon-contaminated soils is related to plant phylogeny. The ISME Journal 8:331-343

Beltrano J, Ruscitti M, Arango M, Ronco M (2013) Effects of arbuscular mycorrhiza inoculation on plant growth, biological and physiological

parameters and mineral nutrition in pepper grown under different salinity and P levels. Journal of soil science and plant nutrition, 13(1): 123-141.

Berger SA, Krompass D, Stamatakis A (2011) Performance, accuracy, and web server for evolutionary placement of short sequence reads under maximum-likelihood. Systematic Biology 60:291–302

Berger SA, Stamatakis A (2011) Aligning short reads to reference alignments and trees. Bioinformatics 27:2068–2075

Berruti A, Lumini E, Balestrini R and Bianciotto V (2016) Arbuscular Mycorrhizal Fungi as Natural Biofertilizers: Let's Benefit from Past Successes. Frontiers in Microbiology 6:1559 doi: 10.3389/fmicb.2015.01559

Błaszkowski J, Ryszka P, Kozłowska A (2018) *Dominikia litorea*, a new species in the Glomeromycotina, and biogeographic distribution of *Dominikia*. Phytotaxa 338(3):241-254

Brundrett MC (2009) Mycorrhizal associations and other means of nutrition of vascular plants: understanding the global diversity of host plants by resolving conflicting information and developing reliable means of diagnosis. Plant Soil 320:37–77

Camprubí A, Calvet C (1996) Isolation and screening of mycorrhizal fungi from citrus nurseries and orchards and inoculation studies. HortScience 31:366–369

Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Pena AG, Goodrich JK, Gordon JI et al (2010) QIIME allows analysis of high-throughput community sequencing data. Nature Methods 7:335–336

Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Huntley J, Fierer N, Owens SM, Betley J, Fraser L, Bauer M, Gormley N, Gilbert JA, Smith G, Knight R (2012) Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. The ISME Journal 6:1621-1624

Carballar-Hernández S, Hernández-Cuevas L, Montaño N, Larsen J, Ferrera-Cerrato R, Taboada-Gaytán O, Montiel-González A, Alarcón A (2017) Native communities of arbuscular mycorrhizal fungi associated with *Capsicum annuum* L. respond to soil properties and agronomic management under field conditions. Agriculture, Ecosystems and Environment 245:43-51

Castillo GC, Rubio R, Rouanet JL, Borie F (2006) Early effects of tillage and crop rotation on arbuscular mycorrhizal fungal propagules in an Ultisol. Biology and Fertility of Soils 43(1):83-92

Cekic F, Unyayar S, Ortas I (2012) Effects of arbuscular mycorrhizal inoculation on biochemical parameters in *Capsicum annuum* grown under long term salt stress. Turkish Journal of Botany 36: 63-72.

Chakraborty U, Chakraborty B, Allay S, De U, Chakraborty A (2011) Dual application of *Bacillus pumilus* and *Glomus mosseae* for improvement of health status of mandarin plants. Acta Horticulturae 892:215–230.

Collins CD and Foster BL (2009) Community-level consequences of mycorrhizae depend on phosphorus availability. Ecology 90(9):2567-2576

Cordell D, Drangert J, White S (2009) The story of phosphorus: Global food security and food for thought. Global Environmental Change 19(2):292-305

Daleo P, Alberti A, Canepuccia M, Escapa E, Fanjul B, Silliman M, Bertness D, Iribarne O (2008) Mycorrhizal fungi determine salt-marsh plant zonation depending on nutrient supply. Journal of Ecology 96:431–437

Davison J, Öpik M, Daniell T, Moora M, Zobel M (2011) Arbuscular mycorrhizal fungal communities in plant roots are not random assemblages. FEMS Microbiology Ecology 78:103-115

Degola F, Fattorini L, Bona E, Sprimuto C, Argese E, Berta G, di Toppi L (2015) The symbiosis between *Nicotiana tabacum* and the endomycorrhizal fungus *Funneliformis mosseae* increases the plant glutathione level and decreases leaf cadmium and root arsenic contents. Plant Physiology and Biochemistry 92:11-18

Drumbell A, Ashton P, Aziz N, Feng G, Nelson M, Dytham C, Fitter A, Helgason T (2011) Distinct seasonal assemblages of arbuscular mycorrhizal fungi revealed by massively parallel pyrosequencing. New Phytologist 190(3):794-804

Edgar RC (2010) Search and clustering orders of magnitude faster than BLAST. Bioinformatics 26:2460–24

FAO (2012) Food and Agriculture Organization of the United Nations, Land Resources. FAOSTATS-Crops. Available at http://faostat.fao.org/site/567/default.aspx#ancor [Accessed 10 November 2017].

FAO (2016) Food and Agriculture Organization of the United Nations, Land Resources. FAOSTATS-Crops. Available at http://www.fao.org/faostat/es/#rankings/ [Accessed 13 May 2018].

Faul F, Erdfelder E, Lang AG, Buchner A (2007) G*Power 3: A flexible statistical power analysis program for the social, behavioral, and biomedical sciences. Behavior Research Methods 39:175-191.

García de León D, Cantero J, Moora M, Öpik M, Davison J, Vasar M, Jairus T, Zobel M (2018) Soybean cultivation supports a diverse arbuscular mycorrhizal fungal community in central Argentina. Applied Soil Ecology 124:289-297

Gianinazzi S, Gollote A, Binet M, van Tuinen D, Redecker D, Wipf D (2010) Agroecology: the key role of arbuscular mycorrhizas in ecosystem services. Mycorrhiza 20(8):519-530

Gosling P, Hodge A, Goodlass G, Bending DG (2003) Arbuscular mycorrhizal fungi and organic farming. Agriculture, Ecosystems & Environment 113:17-35

Hall T (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series 41:95-98

Han M, Zmasek C (2009) phyloXML: XML for evolutionary biology and comparative genomics. BMC Bioinformatics 10:356

Hart MM and Reader RJ (2002) Host plant benefit from association with arbuscular mycorrhizal fungi: variation due to differences in size of mycelium. Biology and Fertility of Soils 36(5):357-366

Haug I, Setaro S, Suárez J (2013) Reforestation Sites Show Similar and Nested AMF Communities to an Adjacent Pristine Forest in a Tropical Mountain Area of South Ecuador. PLos ONE 8(5): e63524. https://doi.org/10.1371/journal.pone.0063524

Higo M, Isobe K, Yamaguchi M, Drijber R, Jeske E, Ishii R (2013) Diversity and vertical distribution of indigenous arbuscular mycorrhizal fungi under two soybean rotational systems. Biology and Fertility of Soils 49(8):1085-1096

Illumina (2013). 16S Metagenomic Sequencing Library Preparation: Preparing 16S ribosomal RNA gene amplicons for the Illumina MiSeq System. Part # 15044223 Rev. B. Illumina, San Diego, CA, USA. pp. 28.

Jansa J, Mozafar A, Anken T, Ruh R, Sanders I, Frossard E (2002) Diversity and structure of AMF communities as affected by tillage in a temperate soil. Mycorrhiza 12(5):225-234.

Jansa J, Smith SA, Smith SE (2008) Are there benefits of simultaneous root colonization by different arbuscular mycorrhizal fungi? New Phytologist 177(5):779-789

Jiang Q, Tan S, Zhuo F, Yang D, Ye Z, Jing Y (2016) Effect of *Funneliformis mosseae* on the growth, cadmium accumulation and antioxidant activities of Solanum nigrum. Applied Soil Ecology 98:112-120

Johnson NC, Rowland DL, Corkidi L, Allen EB (2008) Plant winners and losers during grassland N-eutrophication differ in biomass allocation and mycorrhizas. Ecology 89:2868–2878

Katoh K,Misawa K, Kuma K,Miyata T (2002) MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. Nucleic Acids Research 30:3059–3066

Kindt R (2016) BiodiversityR: Package for Community Ecology and Suitability Analysis. R package version 1.8-8. http://CRAN.R-project.org/package=BiodiversityR

Krüger M, Stockinger H, Krüger C, Schüßler A (2009) DNA-based species level detection of *Glomeromycota*: one PCR primer set for all arbuscular mycorrhizal fungi. New Phytologist 183:212–223

Krüger C (2013) Arbuscular mycorrhizal fungi for reforestation of native tropical trees in the Andes of South Ecuador. PhD, Ludwig Maximilian University of Munich, Munich, Germany

Lankau R (2011) Intraspecific variation in allelochemistry determines an invasive species' impact on soil microbial communities. Oecologia 165(2):453-463

Lekberg Y, Schnoor T, Kjøller R, Gibbons SM, Hansen LH, Al-Soud WA, Sørensen SJ, Rosendahl S (2012) 454-sequencing reveals stochastic local reassembly and high disturbance tolerance within arbuscular mycorrhizal fungal communities. Journal of Ecology 100(1):151-160

Li T, Lin G, Zhang X, Chen Y, Zhang S, Chen B (2014) Relative importance of an arbuscular mycorrhizal fungus (*Rhizophagus intraradices*) and root hairs in plant drought tolerance. Mycorrhiza 24(8):595-602

Liu Y, He L, An L, Helgason T, Feng H (2009) Arbuscular mycorrhizal dynamics in a chronosequence of *Caragana korshinskii* plantations. FEMS Microbiology Ecology 67:81–92.

Loman NJ, Misra RV, Dallman TJ, Constantinidou C, Gharbia SE, Wain J, Pallen MJ (2012) Performance comparison of benchtop high-throughput sequencing platforms. Nature Biotechnology 30:434-439

Martínez-García LB and Pugnaire FI (2011) Arbuscular mycorrhizal fungi host preference and site effects in two plant species in a semiarid environment. Applied Soil Ecology 48(3):313-317

Meadow JF and Zabinski CA (2012) Linking symbiont community structures in a model arbuscular mycorrhizal system. New Phytologist 194:800–809.

Mena-Violante H, Ocampo-Jimenez O, Dendooven L, Martínez-Soto G, González-Castañeda J, Davies F, Olalde-Portugal V (2006) Arbuscular mycorrhizal fungi enhance fruit growth and quality of chile ancho (*Capsicum annuum* L. cv San Luis) plants exposed to drought. Mycorrhiza 16:261-267

Muleta D, Assefa F, Nemomissa N, Granhall U (2008) Distribution of arbuscular mycorrhizal fungi spores in soils of smallholder agroforestry and monocultural coffee systems in southwestern Ethiopia. Biology and Fertility of Soils 44(4):653-659

Oksanen J, Guillaume-Blanchet F, Kindt R, Legendre P, Minchin PR, O'Hara RB, Simpson GL, Solymos P, Henry M, Stevens H, Wagner H (2013) vegan: Community ecology package. R package version 2.0-10. http://CRAN.R-project.org/package=vegan

Öpik M, Zobel M, Cantero J, Davison J, Facelli J, Hiiesalu I, Jairus T, Kalwij J, Koorem K, Leal M et al (2013) Global sampling of plant roots expands the described molecular diversity of arbuscular mycorrhizal fungi. Mycorrhiza 23:411–430

Ortiz N, Armada E, Duque E, Roldán A, Azcón R (2015) Contribution of arbuscular mycorrhizal fungi and/or bacteria to enhancing plant drought tolerance under natural soil conditions: Effectiveness of autochthonous or allochthonous strains. Journal of Plant Physiology 174:87-96

Peyret-Guzzon M, Stockinger H, Bouffaud ML, Farcy P, Wipf D, Redecker D (2016) Arbuscular mycorrhizal fungal communities and *Rhizophagus irregularis* populations shift in response to short-term ploughing and fertilisation in a buffer strip. Mycorrhiza 26(1):33-46

Peyronel, B (1922) Sulla presenza di micorize nel grano e in alter piante cultivate spontance. Bolletino della Regia Stazione Patologia Vegetate 3:43–50

R Core Team (2018). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org/

Redecker D (2000) Specific PCR primers to identify arbuscular mycorrhizal fungi within colonized roots. Mycorrhiza 10:73–80

Rillig M, Wright S, Eviner V (2002) The role of arbuscular mycorrhizal fungi and glomalin in soil aggregation: comparing effects of five plant species. Pant and Soil 238(2):325-333

Rosendahl S, Stukenbrock E (2004) Community structure of arbuscular mycorrhizal fungi in undisturbed vegetation revealed by analyses of LSU rDNA sequences. Molecular Ecology 13: 3179–3186.

Runge-Metzger A (1995). Closing the cycle: obstacles to efficient P management for improved global security. In: TiessenH, ed. Phosphorus in the global environment. Chichester, UK: John Wiley and Sons Ltd, 27–42

Sánchez-Sánchez A, Salcedo-Martínez S, Mendoza-Villarreal R, Pinedo-Espinoza J, Moreno-Limón S (2018) Aislamiento e Identificación de Micorrizas Arbúsculares (MA) Asociadas a la Rizósfera del chile piquín (*Capsicum annuum* var. *aviculare* L.). Investigación y Desarrollo en Ciencia y Tecnología de Alimentos 3:86-91

Sanders IR (2003) Preference, specificity and cheating in the arbuscular mycorrhizal symbiosis. Trends in Plant Science 8(4):143-145

Santos JC, Finlay RD, Tehler A (2006) Molecular analysis of arbuscular mycorrhizal fungi colonizing a semi-natural grassland along a fertilization gradient. New Phytologist 172(1):159-168

Santos-Gonzalez J, Finlay R, Tehler A (2007) Seasonal dynamics of arbuscular mycorrhizal fungal communities in roots in a seminatural grassland. Applied Environmental Microbiology 73: 5613–5623

Schadt E, Turner S, Kasarskis A (2010) A window into third-generation sequencing. Human Molecular Genetics 19:227-240

Schechter SP, Bruns TD (2013) A Common Garden Test of Host-Symbiont Specificity Supports a Dominant Role for Soil Type in Determining AMF Assemblage Structure in *Collinsia sparsiflora*. PLoS ONE 8(2): e55507. https://doi.org/10.1371/journal.pone.0055507

Schlaeppi K, Bender F, Mascher F, Russo G, Patrignani A, Camenzind T, Hempel S, Rillig M, van der Heijden M (2016) High-resolution community profiling of arbuscular mycorrhizal fungi. New Phytologist 212(3):780-791

Schreiner P and Mihara K (2009) The diversity of arbuscular mycorrhizal fungi amplified from grapevine roots (*Vitis vinifera* L.) in Oregon vineyards is seasonally stable and influenced by soil and vine age. Mycologia 101(5):599-611

Senés-Guerrero C and Schüßler A (2016) DNA-Based Characterization and Identification of Arbuscular Mycorrhizal Fungi Species. In: Martin F and Uroz S. (eds) Microbial Environmental Genomics (MEG). Methods in Molecular Biology, vol 1399. Humana Press, New York, NY

SIAP-Servicio de Información Agroalimentaria y Pesquera (2017) Nuevo León: Infografía agroalimentaria 2017. SAGARPA, CDMX. pp 52.

Smith SE, Read DJ (2008) Mycorrhizal symbiosis 3rd edn. Academic, London.

Stamatakis, A (2015) Using RAxML to infer phylogenies. Current Protocols in Bioinformatics 51:6.14.1-6.14.14.

Steen I (1998) Phosphorus availability in the 21st Century: management of a non-renewable resource Phosphorus and Potassium. Phosphorus Potassium 217:25-31

Stockinger H, Walker C, Schüßler A (2009) '*Glomus intraradices* DAOM197198', a model fungus in arbuscular mycorrhiza research, is not *Glomus intraradices*. New Phytologist 183:1176–1187

Stockinger H, Krüger M, Schüßler A (2010) DNA barcoding of arbuscular mycorrhizal fungi. New Phytologist 187:461–474

Sýkorová Z, Ineichen K, Wiemken A, Redecker D (2007) The cultivation bias: different communities of arbuscular mycorrhizal fungi detected in roots from the field, from bait plants transplanted to the field, and from a greenhouse trap experiment. Mycorrhiza 18:1-14

Tamayo E, Gómez-Gallego T, Azcón-Aguilar C, Ferrol N (2014) Genome-wide analysis of copper, iron and zinc transporters in the arbuscular mycorrhizal fungus *Rhizophagus irregularis*. Frontiers in Plat Sciences 5:547-560

Tawaraya K, Hirose R, Wagatsuma T (2012) Inoculation of arbuscular mycorrhizal fungi can substantially reduce phosphate fertilizer application to *Allium fistulosum* L. and achieve marketable yield under field condition. Biology and Fertility of Soils 48: 839–843

Torrecillas E, Alguacil MM, Roldán A (2012) Host Preferences of arbuscular mycorrhizal fungi colonizing annual herbaceous plant species in semiarid mediterranean prairies. Applied Environmental Microbiology 78:6180–6186

van der Heijden MG, Richard DB, van Straalen NM (2008) The unseen majority: soil microbes as drivers of plant diversity and productivity in terrestrial ecosystems. Ecology Letters 11:296–310

van Loon LC (2007) Plant responses to plant growth-promoting rhizobacteria. European Journal of Plant Pathology 119:243–254

Vandenkoornhuyse P, Husband R, Daniell TJ, Watson IJ, Duck JM, Fitter AH, Young JPW (2002) Arbuscular mycorrhizal community composition associated with two plant species in a grassland ecosystem. Molecular Ecology 11(8):1555-1564

Verbruggen E, van der Heijden M, Rillig M, Kiers T (2013) Mycorrhizal fungal establishment in agricultural soils: factors determining inoculation success. New Phytologist 197(4):1104-1009

Wang L, Huang X, Ma F, Ho S, Wu J, Zhu S (2017) Role of *Rhizophagus irregularis* in alleviating cadmium toxicity via improving the growth, micro- and macroelements uptake in *Phragmites australis*. Environmental Science and Pollution Research 24(4):3593-3607

Wehner J, Antunes PM, Powell JR, Caruso T, Rillig MC (2011) Indigenous Arbuscular Mycorrhizal Fungal Assemblages Protect Grassland Host Plants from Pathogens. PLoS ONE 6(11): e27381. https://doi.org/10.1371/journal.pone.0027381

Werner G and Kiers T (2014) Order of arrival structures arbuscular mycorrhizal colonization of plants. New Phytologist 205(4):1515-1524

Wu Q, Srivastava A, Zou Y (2013) AMF-induced tolerance to drought stress in citrus: A review. Scientia Horticulturae 164:77-87

Youpensuk S, Lordkaew S, Rerkasem B (2008) Arbuscular mycorrhizal fungi associated with tangerine (*Citrus reticulata*) in Chiang Mai province, northern Thailand, and their effects on the host plant. Science Asia 34:259–264

Zhao R, Guo W, Bi N, Guo J, Wang L, Zhao L, Zhang J (2015) Arbuscular mycorrhizal fungi affect the growth, nutrient uptake and water status of maize (*Zea mays* L.) grown in two types of coal mine spoils under drought stress. Applied Soil Ecology 88:41-49

Zhang J (2010) The diversity of arbuscular mycorrhizal fungi in yellow-brown soil citrus orchards. Huazhong Agricultural University, Wuhan, China, MD Dissertation (in Chinese with English abstract).

Zhang J, Kapli P, Pavlidis P, Stamatakis A (2013) A general species delimitation method with applications to phylogenetic placements. Bioinformatics 29(22):2869-2876

Zoppellari F, Malusà E, Chitarra W, Lovisolo C, Spanna F, Bardi L (2014) Improvement of drought tolerance in maize (Zea mays L.) by selected rhizospheric microorganisms. Italian Journal of Agrometeorology 18(1):5–18