PABLO ISRAEL ALVAREZ ROMERO

MICROBIOME ANALYSIS OF ANDEAN SOILS AND HIGH-THROUGHPUT MOLECULAR APPROACHES FOR TRANSLATIONAL EPIDEMIOLOGY OF EARLY BLIGHT

Thesis submitted to the Universidade Federal de Viçosa, as part of the requirements of the Graduate Program in Plant Pathology, to obtain the title of Doctor Scientiae.

Advisor: Eduardo Seiti Gomide Mizubuti

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ABSTRACT

ALVAREZ, Pablo Israel Romero, D.Sc., Universidade Federal de Viçosa, August, 2019. **Microbiome Analysis of Andean Soils and High-throughput Molecular Aproaches for Translational Epidemiology of Early Blight.** Advisor: Eduardo Seiti Gomide Mizubuti.

Early blight (EB) is one of the most devastating foliar diseases of solanaceous crops. As chemical control is the main strategy used to control EB, the active microbiota of the soil has the potential to help to managing plant diseases. It was demonstrated that the microbiota associated with Andean soils has an important role in potato diseases suppressiveness. Based in this hypothesis we used high-throughput sequencing (HTS) and metabarcoding to investigate the composition, structure, and diversity of microbiota associated with the rhizosphere of native (landraces) and improved potatoes (commercial) from the Ecuadorian Andes as a quest for potential biological control agents of early blight. The main fungi phyla found were Ascomycota (51.96%), Mortierellomycota (28.39%) and the Basidiomycota (5.56%), while bacterial community was dominated by the Proteobacteria (39.17%), Acidobacteria (17.89%), and Bacteroidetes (14.10%) phyla. Edaphic factors affected the composition, diversity, and structure of the microbiota across the different places sampled. Higher microbial diversity was found in the rhizosphere from landrace cultivars and it constitutes a new source of candidate microorganisms for the use in biological control. Moreover, we used an ecological approach involving reciprocal soil transplant to test the effect of putative suppressive Andean soils to potato and tomato EB epidemics. The direct transfer of soil had an effect only on the epidemics on potato EB. The rhizosphere microbiome from the plants was structured by soil and crop type. Highest levels of fungal diversity were found in potato rhizosphere associated with Ecuadorian soils. Also in the same study, we used HTS and metabarcoding to assess the temporal variation of the fungal and bacterial leaf-microbiota associated with potatoes and tomatoes plants growth in Brazilian and Ecuadorian soils and infected by different species of Alternaria. The leaf profile of microbiota composition was affected by the soil transfer. Thirteen families of fungi belonging to the Ascomycota phylum and 15 families belonging to the Basidiomycota were found in potatoes associated with Brazilian soils, in contrast, eight families of fungi belonging to Ascomycota phylum and 17 families belonging to the Basidiomycota phylum were found in potatoes associated with Ecuadorian soils. Also, 15 families of Fungi belonging to Ascomycota and 10 families belonging to Basidiomycota were found in tomatoes grown in Brazilian soil while,

respectively, 14 and 16 families were recorded in plants grown in Ecuadorian soil. Finally, to differentiate closely related species of Alternaria associated with EB in potato and tomato crops, a single round polymerase chain reaction coupled with high-resolution melting curve (PCR-HRM) using primers for the cadmodulin gene were develop and validated. The HRM-assay was a useful diagnostic tool. All techniques explored in this thesis may enhance management strategies for early blight epidemics.

Keywords: Metabarcoding. Sequencing. Soil Suppressiveness. Alternaria. PCR. Population biology.

RESUMO

ALVAREZ, Pablo Israel Romero, D.Sc., Universidade Federal de Viçosa, agosto de 2019. **Análise de Microbioma de Solos Andinos e Abordagens Moleculares de Alto Rendimento para Epidemiologia Translacional de Pinta Preta.** Orientador: Eduardo Seiti Gomide Mizubuti.

A pinta preta (PP) é uma das doenças mais devastadoras das solanáceas. A pesar de a aplicação de fungicidas ser a principal estratégia para o controle da PP, a microbiota ativa do solo tem o potencial de contribuir para o manejo de doenças de plantas. Foi demonstrado que a microbiota associada aos solos andinos tem papel importante na supressividade das doenças da batata. Com base nessa hipótese, utilizamos o sequenciamento de alto rendimento (SAR) e metabarcoding para investigar a composição, estrutura e diversidade da microbiota associada à rizosfera de batatas nativas e melhoradas dos Andes equatorianos para uma busca de potenciais agentes de controle biológico da PP. Os fungos encontrados foram predominantemente representado por indivíduos dos filos Ascomycota (51,96%), Mortierellomycota (28,39%) e Basidiomycota (5,56%), enquanto a comunidade bacteriana foi dominada pelos filos Proteobacteria (39,17%), Acidobacteria (17,89%) e Bacteroidetes (14,10%). Fatores edáficos afetaram a composição, diversidade e estrutura da microbiota nos diferentes locais amostrados. Maior diversidade microbiana foi encontrada na rizosfera dos genótipos nativos que constituem uma nova fonte de microrganismos candidatos para uso no controle biológico. Além disso, utilizou-se uma abordagem ecológica envolvendo o transplante de solo recíproco para testar o efeito de potenciais solos supressivos andinos nas epidemias de PP em batata e tomate. A transferência direta de solo teve efeito apenas sobre as epidemias de PP em batata. O microbioma da rizosfera das plantas foi estruturado por solo e tipo de cultura. Os maiores níveis de diversidade fúngica foram encontrados na rizosfera de batatas associada aos solos equatorianos. Também no mesmo estudo, utilizou-se SAR e metabarcoding para acessar a variação temporal da microbiota fúngica e bacteriana associadas às plantas de batata e tomate infectadas por diferentes espécies de Alternaria e crescidas em solos brasileiros e equatorianos. O perfil foliar da composição da microbiota foi afetado pela transferência de solo. Treze famílias de fungos pertencentes ao filo Ascomycota e 15 famílias pertencentes ao filo Basidiomycota foram observadas em batatas associadas a solos brasileiros. Em contrapartida, oito famílias de fungos pertencentes ao filo Ascomycota e 17 famílias pertencentes ao filo Basidiomycota foram observadas em batatas associadas a solos equatorianos. Além disso, 15 famílias de fungos

pertencentes a Ascomycota e 10 famílias pertencentes a Basidiomycota foram observadas em tomates cultivados em solo brasileiro, enquanto em plantas cultivadas em solo equatoriano foram registradas 14 e 16 famílias de fungos pertencentes a Ascomycota e Basidiomycota, respectivamente. Finalmente, para diferenciar as espécies de Alternaria associadas à PP em plantações de batata e tomate, uma reação de polimerase em cadeia única acoplada à curva de dissociação de alta resolução (PCR-HRM) foi desenvolvida e validada utilizando primers desenhados com base nas diferenças de nucleotídeos da região calmodulina. O ensaio HRM foi uma ferramenta de diagnóstico útil para melhorar as estratégias de manejo para epidemias de PP.

Palavras-chave: Metabarcoding. Sequenciamento. Supressividade de solo. Alternaria. PCR. Biologia de populações.

SUMMARY

CHAPTER 3

GENERAL INTRODUCTION

The quest for gathering data of high precision, resolution and accuracy is a continuous defiance for plant pathology studies (Bulman et al., 2018). The main barrier for many procedures in biological sciences has been the time-consuming and demanding process of classifying and identifying organisms. In this regard, the rapid development of DNA metabarcoding provides a new potential solution. The high-throughput capacity, cost-effectiveness, comprehensive taxonomic classification, and greater sample processing capacity are key features of these methods or approaches (Bush et al., 2019).

Early blight (EB) is one of the most destructive fungal foliar diseases of solanaceous crops worldwide (Grigolli et al., 2011; Bessadat et al., 2017). The causal agent of EB was initially considered to be Alternaria solani Sorauer, an Ascomycete. Then, two additional morphological species of Alternaria were shown to be associated with potato, and tomato: A. grandis and A. linariae (syn. A. tomatophila), respectively (Simmons, 2000). The development of rapid and reliable molecular identification technique is crucial for fast and accurate identification of Alternaria spp. associated with EB. High resolution melting (HRM) analysis has been developed and utilized for DNA genotyping (Ganopoulos et al., 2012; Tucker & Huynh, 2014). This approach was already used to efficiently discriminate other species of Alternaria (Zambounis et al., 2015; Garganese et al., 2018). Increasing the number of genomic regions useful for taxonomic purposes may improve the detection and classification of closely related fungal species. Currently, four genomic regions have been more commonly used to sort Alternaria spp. However, resolution and accuracy could be much improved with more informative regions.

Microbial communities in soils promise new functions to improve crop yields, and there is a growing interest in understanding these communities to enhance

sustainability (Xue et al., 2018). The active microbiota of the soil offers a potentially useful resource to manage plant diseases (Cook & Baker, 1983; Mazzola, 2004). Soils with high antagonistic potential have higher chances to suppress pathogens. In a suppressive soil, pathogens do not establish, persist or cause significant damages (Cook & Baker, 1983). Suppressive soils have already been described for many pathogens (Weller et al., 2002). The suppressiveness of soils to plant diseases is a subject that has recently gained attention due to the current availability of high-throughput sequencing tools.

The suppression mechanisms are still not fully understood, but the biotic factors, i.e. a diverse soil microbiota, are commonly associated with this phenomenom (Weller et al., 2002). Soil microbial diversity and structure depend on the type of soil, plant and management and the interaction of microorganisms with those other factors can lead to disease suppressivess (Garbeva et al., 2004).

Ecuador is located in a region considered to be the center of origin of several crops of the Solanaceae family. Recently, it was demonstrated that the microbiota associated with Andean soils has an important role in the suppressiveness of some potato diseases (Orquera-Tornakian et al., 2018). The Ecuadorian soils have a wide range of physicochemical characteristics across the different provinces due to the different origins of the soils (Espinosa et al., 2018), and these characteristics could strongly influence the microbial diversity that turns Ecuadorian soils to be suppressive. EB is not a yield constraint in Andean countries and therefore does not cause significant damage as in other countries where its presence was reported. In this perspective, it is possible that the soil associated-microbiome from the Andes can be suppressive to EB epidemics.

Thus, the aims of this study were: i. To investigate the composition, structure, and diversity of microbiota associated with the rhizosphere of native (landraces) and improved potatoes (commercial) from the Ecuadorian Andes as a quest for potential biological control agents of early blight; ii: To study the effect of the microbiome transfer on the natural leaf microbiome of potato and tomato plants inoculated with A. grandis, A. solani and A. linariae; and iii: To develop and validate a HRM-assay for sorting different species of Alternaria associated with EB in potato and tomato crops.

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CHAPTER 1

Assessment of the potato soil microbiome: A quest for potential biological control agents of early blight

Abstract

The rescue of soil microbiome of indigenous plants can be a new approach to improve crop production and plant health by establishing competitive environments that can be deleterious for plant pathogens. To assess this potential, we used high-throughput sequencing (HTS) and metabarcoding to investigate the composition, structure, and diversity of fungal and bacterial communities associated with the rhizosphere of native (landraces) and improved potatoes (commercial) from the Ecuadorian Andes. The HTS generated a total of 9,419 OTUs for fungi and 36,744 OTUs for bacteria. The relative abundance of species varied across the provinces, and was dominated by the Ascomycota (51.96%), Mortierellomycota (28.39%) and the Basidiomycota (5.56%) phyla, while bacterial community was dominated by the Proteobacteria (39.17%), Acidobacteria (17.89%), and Bacteroidetes (14.10%) phyla. Edaphic characteristics affected the composition, diversity, and structure of fungal and bacterial communities across the different places sampled. The rhizosphere from landrace cultivars showed a higher microbial diversity and it constitutes a new source of candidate microorganisms for the use in sustainable agriculture.

Keywords: metabarcoding, HST, suppressiveness, landraces.

INTRODUCTION

The soil microbiome has a crucial role in plant health, growth and other important plant functions and its manipulation may be a potential instrument to improve crop production (Lareen et al., 2016; Pérez-Jaramillo et al., 2016). Plants possess different allowance abilities to biotic or abiotic stresses and, in most cases, the microbiome plays a key role allowing them to thrive under such conditions (Santoyo et al., 2017). Unfortunately, studies evaluating the composition, diversity, structure, and effect of the microbiota in economically important crops are still uncommon (de Souza et al., 2016) and understanding the interactions in the microbiome is crucial for manipulating the assembly and functioning of the plant-microbiome to reduce plant diseases or improve crop yields (Gdanetz & Trail, 2017).

The active microbiota of the soil has the potential to contribute to crop production as sustainable tools to manage plant diseases (Cook & Baker, 1983; Mazzola, 2004). The suppressiveness of soils to plant diseases has been a subject of interest for many years. However, this has recently gained attention probably due to the current availability of high-throughput sequencing and computational tools. A suppressive soil is one in which disease does not develop even when the pathogen and the root system of susceptible plants share the same (Srour et al., 2017). The advancements in sequencing technologies and computational resources allowed the exploration in more details the biotic component of naturally suppressive soils, including both cultivated and non-cultivated microbes that may be associated with this process. The Ecuadorian Andes soils are naturally suppressive to pathogens that cause important diseases in potato crops, suchs as late blight. In Ecuador, despite a strong impact of this disease on the foliage, tuber blight is rare (Orquera-Tornakian et al., 2018). A recent study showed that bacteria naturally present in suppressive soils from four potato-growing regions of Ecuador had the

potential to inhibit tuber infection by Phytophthora infestans and also the growth of the pathogen in vitro (Orquera-Tornakian et al., 2018).

Crop varieties when grown under agricultural conditions away from their centers of origin and diversity, necessarily assemble their associated microbiota from ex situ microbiomes. This community assemblage probably contrasts with the in situ microbiomes with which their wild relatives co-evolved (Hale et al., 2014). The Andean region is the most important center of potato diversity and more than 4,000 varieties and landraces were discovered in this area. In such a diverse environment, local farmers have an important function in the conservation of approximately 3,000 landrace potatoes (Spooner et al., 2014).

Ecuador is one of the centers of origin and diversity of cultivated and wild potatoes and more than 400 landraces of native potatoes have been registered in this country (Cuesta et al., 2005) including 23 wild species and 3 cultivated taxa (Solanum tuberosum subsp. andigenum and Solanum phureja) (Spooner & van den Berg, 1992; Spooner et al., 2007). In the Andes, microcenters of diversity of native and cultivated potato are not randomly dispersed (Harlan, 1951). In Ecuador, the Chimborazo and Carchi provinces are recognized as important microcenters of potato diversity (CIP, 2018).

The study of the associated microbiota of native potatoes has been largely neglected. Many relevant gaps need to be fulfilled, but the investigation of the microbial community composition that interacts with in situ crop wild relatives using a microbiome approach is a powerful option to search and discover effective biological control agents. Ultimately, these agents can help improve tolerance of potato crops to biotic or abiotic stresses.

Here we adopted a Vavilovian approach using high-throughput sequencing (HTS) and metabarcoding to generate a comprehensive inventory of the composition, structure,

and diversity of fungal and bacterial communities associated with the rhizosphere of native (landraces) and improved potatoes (commercial) collected in the Ecuadorian Andes. We hypothesize that the rhizosphere of potato landraces has higher microbial diversity than the rhizosphere of commercial cultivars and that the recruitment of specific fungal and bacterial taxa within their root vicinity is influenced by spatial, climatic and soil conditions. The study aimed to yield a solid base for researching the potential ecological role of microbes associated with potato landraces, especially with respect to plant protection.

MATERIALS AND METHODS

Sample collection and storage conditions.

Soil samples were taken from the rhizosphere of 14 commercial and 22 landrace potato crops from 18 fields across 10 provinces in the highlands of Ecuador (Figure 1). Field sites were characterized at the time of sample collection (Table 1). Three planting rows were selected for sampling, with two subsamples per planting row. Each subsample consisted of ~1.5 L of soil collected in row, between plants, and at a 15 to 30 cm depth. A composite sample was made for each field site and used for soil chemical analyses (Table 2) and DNA extraction. Soil samples were placed in sterile plastic bags and kept in a cooler during transportation to the laboratory where they were maintained a (4-8 °C) prior to processing.

Soil DNA extraction and processing.

We extracted DNA from 0.25 g sub-sample of soil using the PowerSoil® DNA Isolation Kit (MoBio Laboratories Inc., Carlsbad, CA, USA), following modifications outlined by the Earth Microbiome Project (EMP; www.earthmicrobiome.org/emp-standard-protocols) (Caporaso et al., 2012). We quantified the extracted DNA using NanoDrop® 2000 spectrophotometer (Thermo Fisher Scientific), and quality of DNA was checked on a 1 % agarose gel with GelRed (Biotium, Fermont, CA). The final DNA concentration was adjusted to 20 ng/µL, and samples were stored at -20 °C until sequencing.

Fungal and bacterial amplification and sequencing.

A total of 72 genomic DNA samples (including duplicates) were lyophilized and sent to Argonne National Laboratory Next Generation Sequencing (ANL-NGS) core facility. All downstream processes, including library preparation, amplification, and

sequencing were conducted at ANL-NGS. Samples were processed for sequencing using the Illumina Miseq® reagent kit v2 following a modified manufacturer´s protocol.

Fungal communities were accessed by amplifying the internal transcribed spacer (ITS) region of the rRNA, with the ITS1f and ITS2 primers using the protocol described at ANL-NGS (https://bit.ly/307E5L3).

The PCR reactions of both libraries were carried out in 25 µL volumes, including 1 µL DNA template, 0.5 µL of each primer at 10 µM, 13 µL of PCR-grade water (Sigma), 10 µL 2x of Platinum™ Hot Start PCR master mix (Thermo Fisher Scientific). The PCR conditions for bacterial analysis were: initial denaturing at 94 °C for 3 min, followed by 35 cycles of 94 °C for 45 s, 50 °C for 60 s, 72 °C for 90 s and a final extension of 10 min at 72 °C. For fungal amplifications, the conditions were 94 °C for 1 min, followed by 35 cycles of 94 °C for 30 s, 52 °C for 30 s, 68 °C for 30 and a final extension of 7 min at 68 °C.

Bacterial DNA was amplified with the universal 16S rRNA gene primer set 515F and 806R, targeting the V4 region of the 16S SSU rRNA combined with the sequencer adapter used in the Illumina flowcell (Caporaso et al., 2011, 2012). The reverse amplification primer also contains a 12 bp barcode sequence that supports pooling of up to 2,167 different samples in each lane. To avoid the plant host plastid and mitochondrial 16S contamination, PNA clamp sequences were included in the amplification (Lundberg et al., 2013). The 16S protocol followed the proposed by the Earth microbiome project (http://www.earthmicrobiome.org).

Sequence clean-up.

All sequences were demultiplexed using the software CASAVA (Consensus Assessment of Sequence and Variation) version 1.8.2 (Eren et al., 2013). Further sequence processing was performed using Mothur version 1.39.5 (Schloss et al., 2009)

following an adapted protocol (Kozich et al., 2013). Briefly, paired-end sequences were combined into contigs and poor quality sequences were removed. Bacterial sequences were aligned against the SILVA 16 S rRNA gene reference alignment database (Pruesse et al., 2007) and screened for alignment to the correct region. Fungal sequences were not aligned to a database due to the frequency of insertions and deletions in ITS, and de novo alignment within the dataset was performed instead (Misof & Misof, 2009). Sequences were pre-clustered to decrease sequencing error and chimera detection and removal were performed. Finally, bacterial sequences were classified using the GreenGenes database (DeSantis et al., 2006) and fungal sequences were classified using the UNITE-ITS database (Kõljalg et al., 2013).

Sequence analysis and statistics.

All sequences were grouped into 97% operational taxonomic units (OTUs) by uncorrected pairwise distances and furthest neighbor clustering (Schloss et al., 2009). Coverage was assessed by Good's coverage calculated in Mothur. Fungal and bacterial communities were normalized to equal sequence counts, and normalized OTU tables were used in all further analyses. All statistical analyses were performed in R v3.4.3 (R Development Core Team, 2017) using packages vegan v2.4-1 (Noetzli et al., 2007), phyloseq v1.19.1 (McMurdie & Holmes, 2013), ggplot2 v2.2.1 (Bodenhofer et al., 2011), and iNEXT v2.0.12 (Hsieh et al., 2016). The Hills numbers (q=0, species richness, q=1, Shanon diversity and q=2, Simpson diversity) were used to estimate the species diversity and construct the extrapolation/interpolation rarefaction curves. Species richness, Shannon diversity, and Simpson diversity were used to assess the fungal and bacterial diversity in the samples collected. Evenness (Shannon diversity divided by the natural logarithm of species richness) was also calculated by province, place, and cultivar. The α-diversity was summarized by province, place, and cultivar and these measures were

evaluated for correlation with spatial and edaphic conditions using Spearman's rank correlation.

In order to study community structure, the Bray-Curtis distance metric was calculated to determine the β-diversity and the resulting dissimilarity matrices were used to assess clustering of the communities by province, and to evaluate communities by province and by cultivar using ordination analysis as principal coordinate analysis (PCoA).

Climatic parameters of the sampled fields were acquired based on geographic information system (GIS) coordinates. Air temperature (maximum and minimum), precipitation (rain) and radiation (radn) data for different places were obtained from the NASA-POWER package of R. Chemical analysis was performed on rhizosphere soils from the fields surveyed. The different spatial, climatic and edaphic factors were studied for diversity and community structure association by using vector fitting in conjunction with the ordination analysis. The estimates of the effects of spatial, climatic and soil factors were also checked with the Mantel test (Ramette, 2007). The ecological importance of the fungal phylotypes was established using FUNGuild (Nguyen et al., 2016) and the bacterial functional prediction was done using the Functional Annotation of Prokaryotic Taxa (FAPROTAX) (Louca et al., 2016).

RESULTS

Composition of soil microbial communities at the province level.

A total of 1,958,956 high-quality sequences using ITS primers were obtained and the average number of sequences per sample was 27,207.22 (range: 7,315 to 58,588). A total of 9,419 operational taxonomic units (OTUs) were obtained for fungi, with 97% sequence similarity. For the 16S analysis, 1,339,976 high-quality sequences were generated and the average number of sequences per sample was 18,610.78 (range 6,187 to 30,761). A total of 36,744 OTUs were obtained for bacteria, with 97% sequence similarity. For all amplicons, Good´s Coverage of each sample was greater than 0.98.

The most common fungal phylum in the communities was the Ascomycota (51.96%). The second and third most abundant phyla were the Mortierellomycota (28.39%) and the Basidiomycota (5.56%). After the analyses, 13.83% of the sequences were not classified at the phylum level. The remaining phyla had relative abundances lower than 1% each.

Focusing on the three most abundant phyla, the abundance of the 50 most common taxa was evaluated summarizing by family. Thirty-nine families were represented in the Ascomycota and the most important genera were Giberella (16.48%), Fusarium (9.5%), Illyonectria (2.12%), Cylindrocarpon (1.59%), Cladosporium (1.01%), Alternaria (0.86%), Chaetomium (0.81%), Exophiala (0.8%), Fusicola (0.76%), Humicola (0.6%), Peziza (0.57%), Ulocladium (0.55%), Clonostachys (0.53%), Sclerotinia (0.51%), Lecanicillium (0.43%), Metarhizium (0.42%), Truncatella (0.41%), Tetracladium (0.36%), Preussia (0.28%), Microdochium (0.25%), Schizothecium (0.12%) and Pochonia (0.04%). For the Mortierellomycota, two families were identified in the top 50 taxa. Mortierella was the dominant genus within the phylum Mortierellomycota. Finally, the phylum Basidiomycota was represented by eight families and the most common

genera were Panaeollus (2.14%), Scleroderma (0.92%), Minimedusa (0.5%), Solicoccozyma (0.45%), Coprinellus (0.35%) and Tanatephorus (0.06%).

The abundances of Ascomycota and Mortierellomycota shifted across provinces. The phylum Ascomycota was more abundant in the provinces of Cotopaxi, Loja, Chimborazo, Tungurahua, and Bolívar, while Mortierellomycota was more abundant in the Cañar and Carchi provinces. The unclassified fungi were more frequently found in the Azuay and Imbabura provinces. The phylum Basidiomycota had low abundance in almost all provinces (Figure 2A).

For the bacterial community composition, the most abundant phyla were Proteobacteria (39.17%), Acidobacteria (17.89%), Bacteroidetes (14.10%), Verrumicrobia (7.07%), Actinobacteria (6.36%), Gemmatinomadetes (4.80%), Planctomycetes (2.74%), Chlorofexi (2.38%) and Nitrospirae (1.27%). Regarding the three most abundant phyla, the 50 most common taxa were studied and 20 families were represented in the Proteobacteria. In this phylum, the most abundant families were: Sphingomonadaceae (7.43%), Comamonadaceae (3.33%), Xanthomonadaceae (3.13%), Syntrophobacteraceae (1.69%), Hyphomicrobiaceae (2.05%), Pseudomonadaceae (1.53%), Oxalobacteraceae (1.33%), Rodospirillaceae (1.03%), Haliangiaceae (0.71%), Bradyrhizobiaceae (0.76%), Polyargiaceae (0.55%) and Caulobacteraceae (0.59%). In the Acidobacteria phylum, eight families were represented in the top 50 taxa and the most important were Ellin6075 (3.48%) and Koribacteraceae (1.43%). With respect to the Bacteroidetes phylum, five families were identified in the top 50 taxa, and the dominant were: Chitinophagaceae (7.28%), Cytophagaceae (2.54%), Sphingobactereriaceae (0.90%), and Saprospiraceae (0.53%).

In terms of bacterial community composition, the phylum Proteobacteria had a higher abundance in the provinces of Bolivar, Pichincha, Imbabura, Azuay, and Cañar.

The phylum Acidobacteria was more abundant in Loja, Carchi and Azuay provinces. The phylum Bacteroidetes was more frequently found in the Carchi, Imbabura and Pichincha provinces (Figure 2B).

Composition of soil microbial communities in the microcenter of potato diversity.

In the microcenter of potato diversity located in the Chimborazo province, 60.2% of the fungal OTUs were classified as Ascomycota, 20.9% as Mortierellomycota, and 5.2% as Basidiomycota. The unidentified OTUs were 13.7%. In the Laime community, 39.7% of the OTUs could be classified. The highest abundance values for the phylum Ascomycota were recorded in the San Francisco de Telán (79.2%), San Vicente de Nanzag (67.7%) and Achullay (62.8%) communities. The phylum Mortierellomycota was the dominant phylum in the Candelaria community (42.7%) and the phylum Basidiomycota had low abundance in all locations.

When stratifying according to the types of plants, a higher number of OTUs of the Ascomycota and Mortierellomycota phyla were found associated with the landraces cultivars in Laime community than with the commercial cultivars. The number of Basidiomycota and unclassified OTUs was higher in the commercial cultivars than in the landrances. On the other hand, in the Pull Quishuar community, the abundance of the OTUs of the phylum Ascomycota in the commercial cultivars was higher than that of landraces. The unclassified OTUs, phylum Mortierellomycota and Basidiomycota were more frequently found in landraces cultivars.

The majority of bacterial OTUs found in the Chimborazo province were from the phyla Proteobacteria (31.5%), Acidobacteria (19.4), Bacteroidetes (11.4%), Verrumicrobia (11.6%), Actinobacteria (8.0%), Gemmatimonadetes (4.7%), Planctomycetes (3.5%). Chlorofexi (2.45%) and Nitrospirae (2.04%). The Phylum

Proteobacteria had approximately the same distribution across the different places studied in Chimborazo. In the Laime and Pull Quishuar communities there were no clear differences between OTUs of the phyla Proteobacteria, Acidobacteria, Bacteroidetes found in commercial and landrace cultivars. The phylum Acidobacteria was more abundant in the Candelaria community. The phylum Bacteroidetes was found in high frequency in San Vicente de Nanzag.

Diversity of soil microbial communities per field across provinces.

The α-diversity was calculated by province using the Species richness, Shannon diversity and Simpson diversity based on Hill´s number. For fungi, the Bolivar province had the highest microbial diversity in the potato soil microbiome. The average species richness was 634.7, while Imbabura, Tungurahua, and Pichincha had the lowest levels of species richness (Table 3). On the other hand, highest bacteria diversity was found in Pichincha, Chimborazo, Cotopaxi and Carchi. The lowest level of bacterial species richness was found in the Azuay Province (Table 4).

The Shannon diversity calculated by province showed that diversity ranged from 10.4 to 33.8 for fungi and from 502.7 to 1076.9 for bacteria. The Simpson diversity, which is more sensitive to the dominant or common microbes in the community, calculated for each province ranged from 3.4 to 13.5 for the fungi and from 120.2 to 376.3, for the bacteria. Evenness within the samples, ranged from 2.0 to 5.8 for the fungi and from 69.5 to 135.1 for the bacteria.

To assess the β-diversity the Bray-Curtis dissimilarity index was subjected to cluster analysis and plotted as a dendrogram to evaluate inter-province diversity. Geographically adjacent provinces had similar community structure and as such grouped together (Figure 3A and 3B). The fungal and bacterial PCoA analysis did not show clear distance separation of samples by province or type of cultivar (landrace or commercial)

and the percent of variance explained by the first coordinate axes was low for both groups of microbes (Figure 4A and 4B).

Diversity of soil microbial communities in the microcenter of potato diversity.

The microbial diversity in Chimborazo estimated with the Hill´s numbers indicated that the highest species richness values for fungi were found in the landraces cultivars sampled in the Pull Quishuar community (Figure 5). For bacteria, highest species richness was found in landraces cultivars in the Laime community (Figure 6). The species richness values for fungi ranged from 110.0 to 358.2 and for bacteria from 3002.1 to 3548.0. Shannon and Simpson diversity had the same trend with higher values in landrace cultivars in the Pull Quishuar community for fungi (Figure 5) and for bacteria in the Laime community (Figure 6). The Shannon diversity values ranged from 43.7 to 3.4 for the fungi and from 584.3 to 1111.5 for the bacteria. The Simpson diversity values ranged from 1.7 to 16.9 for fungi and from 71.6 to 198.7 for bacteria. Notably, the samples with the lowest levels of values of species richness, Shannon and Simpson diversities, and evenness for fungi were found in commercial potato cultivars from the Pull Quishuar community (Table 5). Regarding the cultivars sampled in Chimborazo, the landraces cultivars Turca morango showed the highest values of species richness for fungi (Table 7) and bacteria (Table 8). The lowest values were found in the landraces cultivars Uvilla Blanca for fungi and Puña Negra for bacteria.

Spatial and edaphic effects on microbial community diversity and composition.

Using diversity per province, the effect of latitude, longitude and altitude as spatial factors; and pH and organic matter as edaphic factors were investigated for a potential role on microbial community diversity. Significant positive correlations were observed for fungi between latitude and longitude with species richness (Figure 7A and 7B), and

Shannon and Simpson diversities with pH (Figure 7C and 7D). Greater species richness was found at higher latitudes and longitudes and high values of Shannon and Simpson diversities at higher pH values. Moreover, significant negative correlations were observed for bacteria between latitude and longitude with Shannon and Simpson diversities (Figure 8A, 8B, 8D and 8E), and pH with Shannon diversity (Figure 8C). High values of Shannon and Simpson diversities at low latitudes, longitudes and pH values.

Using the similarity (anosim) permutation test, fields were grouped by latitude, longitude or altitude and evaluated for microbial community similarity. There were differences between fungal community composition at different latitudes ($R = 0.03$, $P = 0.017$) and more similar composition at similar latitudes. For bacterial community composition, the trend was similar ($R = 0.05$, $P = 0.008$). Low differentiation of the bacterial communities was found within provinces, suggesting a similar community composition ($R = 0.42$, $P = 0.01$), but there was significant differentiation among provinces when the province was used as a group. In addition, longitude, altitude, and cultivar were also evaluated to determine the contribution of each factor to differences observed across communities at the field level. Using longitude, altitude, and type of cultivar as grouping factors there was no differentiation between fungal and bacterial communities. In order to corroborate the anosim results and determine the contribution to the variance of the different factors using fungal and bacterial community composition as response, a permanova (adonis) test was performed that resulted in significant differences for province and type of cultivar sampled (Table 9 and Table 10). The province grouping explained 28.6% of the fungal community and 15.6% of the bacterial community structure variability.

Spatial, climatic and edaphic effects on community structure. Further research of the β-diversity (among-group diversity) between provinces and type of cultivars was

studied using a PCoA and their correlation with spatial, climatic and edaphic factors. A total of 18 different factors were studied. Four factors for fungi (Table 11 and Figure 4C) and nine for bacteria correlated with the ordination (Table 12 and Figure 4D). Soil variables were the main factors associated with bacterial community structure: pH, organic matter, nitrogen (N), phosphorus (P), calcium (Ca), magnesium (Mg), iron (Fe), and zinc (Zn). Additionally, altitude was also associated with community structure. The elements Fe, Mg, Ca and minimal temperature were the factors associated with fungal community structure. Latitude, longitude, maximal temperature, precipitation and radiation did not correlate with fungal or bacterial community structure.

Factors significant for envit analysis (fitting environmental vectors) were also examined using the Mantel test. Most factors for bacteria were significant in both tests. Mg was not significant for both fungi and bacteria, (Table 11 and Table 12). The correlations for significant parameters were lower than 0.13 for fungi. For bacteria the correlation ranged from 0.12 to 0.76. Some factors were found to be correlated and to contribute to the bacterial community composition. Contrary to what was found for bacteria, only Fe, Mg, Ca and minimal temperature were found to be correlated and contributed to the fungal community composition. Soil variables, pH, organic matter, N, P, Ca, Fe, Zn and spatial factors as altitude were the most correlated factors for bacterial community structure and Fe was the factor correlated for fungal community structure according to both tests. These correlations were also studied using the correlated PCoA axis with the respective linked factor.

Additionally, samples were visualized with color by latitude. For fungi, Fe was negatively correlated, indicating that fungal community composition similarity was higher at locations with low content of iron (Figure 9A and 9B). For bacteria, soil pH was negatively correlated (Figure 10A and 10B) with relatedness among communities.

Therefore, soils with lower pH had communities less similar to each other than communities in high pH soils. Organic matter and N were positively correlated with community similarity (Figure 10 and 11). Bacterial community composition similarity was lower at places with low organic matter and N soil content.

Functional prediction.

Functional prediction using ecological guild indicated 9 possible trophic modes (Figure 14) and 69 guilds (Figure 12) assigned into the fungal community found in potato rhizosphere. The dominant trophic modes were Saprotroph, Saprotroph-Symbiotroph, and Pathotroph-Saprotroph-Symbiotroph. The more common guilds were Endophyte-Soil Saprotroph -Undefined Saprotroph, Undefined Saprotroph, Plant Pathogen, and Plant Pathogen-Soil Saprotroph-Wood Saprotroph.

The analysis of functional prediction revealed 68 functions associated with bacteria communities in potato rhizosphere (Figure 13). The more often assigned functions were chemoheterotrophy (1151 records), aerobic chemoheterotrophy (968 records), intracellular parasites (861 records), phototrophy (234 records), photoautotrophy (222 records), nitrate reduction (172 records), predatory or exoparasitic (130 records), cyanobacteria (127 records), oxygen photoautotrophy (127 records), fermentation (118 records), nitrogen respiration (102 records), photoheterotrophy (102 records), nitrate respiration (101 records), nitrification (100 records), anoxygenic photoautotrophy (95 records), nitrite denitrification (93 records), nitrate denitrification (91 records), nitrous oxide denitrification (91 records), denitrification (91 records) and anoxygenic photoautotrophy S oxidizing (91 records).

Relative abundance and distribution of the potential biological control agents.

The abundance of fungi of the genera Clonostachys, Trichoderma, Pochonia, Lecanicillium, Metarhizium and Mortierella as potential biological control agents were examined by provinces and cultivars. Species varied across provinces (Figure 15) and cultivars (Figure 16). In the rhizosphere associated with landraces potatoes from the Chimborazo province there were greater diversity of potential biocontrol agents. One hundred and two OTUs of the genus Clonostachys, 18 of Trichoderma, 13 of Pochonia, 17 of Lecanicillium, and 43 of Metarhizium were found across different provinces, and cultivars sampled. Additionally, 1101 OTUs belonged to different species of Mortierella were found associated to potato rhizosphere.

Three species of Clonostachys were found: C. rosea, C. miodochialis and C. divergens. Three species of Trichoderma were recorded: T. harzianum, T. rossicum, T. spinolosum. Two species of Pochonia were detected: P. bulbillosa and P. cordycepisociata. Two species of Metarhizium, M. cameum and M. marquandii and two of Lecanicillium, L. primulinum, and L. psaliotae were found. Finally, 12 species of Mortierella were found, M. alpina, M. gamsii, M. minutissima, M. rishikesha, M. polygonia, M. elongata, M. sarnyensis, M. exigua, M.antarctica, M.humilis, M. falshederae and M. olygospora.

DISCUSSION

This is one of the first studies using the NGS approach that was conducted to investigate fungal and bacterial microbial communities in potato ecosystems of Ecuadorian Highlands, one of the centers of origin and diversity of potato. The effects of climatic, spatial and edaphic variables on the composition and structure of microbial communities have been examined (Kaiser et al., 2016; Rojas et al., 2017), however, studies have rarely addressed these factors together with the role of native potato plants. The microbial community associated with the rhizosphere of 22 landraces (natives) and 14 commercial (improved) potato cultivars was characterized, and the combined effects of different soil types, climatic conditions and cultivars in the potato rhizobiome were explored.

Culture-independent studies of the potato microbiome have primarily been addressed on bacterial community composition (Peiffer et al., 2013) and to our knowledge, this is the first report of culture-independent analyses of the fungal communities associated with potato rhizosphere in one of the centers of origin and diversity of potato. Fungi have a leading role in the plant microbiome and they are a key determinant of plant health (Shoresh et al., 2010; Pieterse et al., 2014). Unraveling their biological functions could lead to new approaches for plant disease management. For example, fungi produce a diversity of structurally distinctive compounds with antimicrobial properties (Porras-Alfaro & Bayman, 2011; Aldrich et al., 2015). Considering the limited options of fungicides with new modes of actions that meet the safety and efficacy requirements, prospecting molecules of biological origin may be an interesting alternative to increase the portfolio of fungicides to be used in crop protection.

Ascomycota was the most abundant phylum associated with Ecuadorian potato rhizosphere. Similar to our findings, several studies reported that Ascomycota was the

most relatively abundant fungal phylum in soil (Kazeeroni & Al-Sadi, 2016). Gibberella and Fusarium were the most frequently found genera of the Ascomycota. Members of these genera occur frequently in the rhizosphere of plants, while known to cause plant diseases, they may frequently inhabit the soils as nonpathogenic saprophytes, suppressing plant pathogens via competition for space and nutrients (Srour et al., 2017). Moreover, in some cases, several species of Fusarium have been reported to trigger ISR-based defenses in plants (Fravel et al., 2003).

Mortierellomycota was the second most predominant phylum identified in this study, and members of the Mortierella genus are filamentous fungi commonly found in soil (Yadav et al., 2015). Mortierella was more frequently associated with landraces than to commercial potato soils in Laime and Pull Quishuar. These two sampling sites are located in Chimborazo, one of the Ecuadorian microcenters of potato diversity. This finding may indicate a possible mechanism used by landrace potatoes to recruit these microorganisms, for some specific function. For example, many filamentous fungi belonging to the genus Mortierella are promising candidates for use as producers of arachidonic acid and other polyunsaturated fatty acids (PUFAs) (Kendrick & Ratledge, 1992; Botha et al., 1999). Additionally, several isolates have been researched as potential antagonistic agents against many plant pathogens (Tagawa et al., 2010). Mortierella alpina enhanced tolerance to corm rot disease in Crocus sativus by releasing arachidonic acid which acts as conserved defense signal and induces jasmonic acid production. This fungus also improved many morphological and physiological traits of this medicinal crop (Wani et al., 2017). Furthermore, fungi belonging to this genus were commonly found associated with Fusarium wilt suppressive soils in vanilla crops (Xiong et al., 2017) and related with the suppressiveness of the infection of Arabidopsis by Alternaria brassicae (Johnson et al., 2018).

Proteobacteria was the most abundant taxonomic group of bacteria identified in the potato soils in the Ecuadorian Highlands. Representatives of these phylum have previously been found in potato soils (Pfeiffer et al., 2017). Within the Proteobacteria, the families Sphingomonadaceae, Comamonadaceae, Xanthomonadaceae, Syntrophobacteraceae, Hyphomicrobiaceae, Pseudomonadaceae and Oxalobactereraceae were in significantly higher numbers in Ecuadorian potato rhizosphere. Species of Spingomonadaceae have been found in the rhizosphere of tobacco plants grown in suppressive soils to Ralstonia solanacearum (Liu et al., 2016). Additionally, some strains from this family are closely associated with nitrogen fixation (Adhikari et al., 2001). The families Comamonadaceae, Pseudomonadaceae, and Oxalobacteraceae were associated in previous works with disease-suppressive soils (Rosenzweig et al., 2011; Chapelle et al., 2015).

After the Proteobacteria, the other abundant taxonomic group identified in this study was Acidobacteria. This phylum was discovered in some soil types from North and South America (Jones et al., 2009; Lauber et al., 2009). The soils were cultivated with wheat (Yin et al., 2010) and associated with later stages of the take-all decline disease (Sanguin et al., 2009), or the rhizosphere of chestnut trees (Lee et al., 2008). At the phylum level, the relative abundance of sequences assigned to Acidobacteria was high and present in all the Ecuadorian potato rhizospheres sampled, markedly in Loja, Carchi and Azuay provinces. Members of this phylum are known to inhabit acidic soils, suggesting an adaptation to this condition (Kaiser et al., 2016). Acidobacteria also have been described as possessing growth-promoting effects associated to auxin production (Kielak et al., 2016), and are hence linked to the regulation of seed germination, increase in shoots and roots biomass as well as morphological alterations in root system (Shu et al., 2016). Proteobacteria, Acidobacteria and Bacteroidetes were the main phyla found.

Our results differ from similar studies that were conducted in Peru (Pfeiffer et al., 2017), where Proteobacteria, Firmicutes and Actinobacteria were the dominant phyla discovered in the potato soils from Peruvian highlands Andes.

Some fields across the different provinces in the Ecuadorian highlands had greater fungal and bacterial diversities. In the same way, the diversity of soil associated to landrace potatoes had higher microbial diversity than soils from commercial potatoes. Different plant genotypes of the same species may differ in their rhizosphere microbiome composition (Pérez-Jaramillo et al., 2016). For example, the portion of OTUs observed in three different potato cultivars was cultivar-specific (Weinert et al., 2011). Similar cultivar-dependent effects were observed for the rhizobacterial communities in the rhizosphere of potato plants (İnceoğlu et al., 2011). Peiffer et al. (2013) observed in a study with 27 modern maize lines, grown in field environments that OTU richness was influenced by maize genotypes.

This great diversity found can be associated with a global increase in soil microbial biomass and it is a greater opportunity to study how to develop and establish competitive environments that can be detrimental for the plant pathogens (Lareen et al., 2016). Thus, fields that had great diversity of microbial communities are hot spots to be explored as candidate suppressive soils. The value of diversity may display new microorganisms and multiple distinct mechanisms that can be used to manage different plant diseases. Presumably, the diversity is well-established and this allows functional stability across a wide range of ecological communities. A diversified pathogen-suppressive population will yield more stable function (disease suppression) than a limited group of antagonist (Schlatter et al., 2017a).

Studying soil microbiome diversity modifications are important to understand the impact of spatial and edaphic factors on the autochthonous microbial communities. Here

we analyzed the impact of these parameters on fungal and bacterial communities under field conditions. We found significant correlations between fungal and bacterial diversity and spatial and edaphic variables. Latitude, longitude and pH were the principal factors analyzed that had significant effect on the microbial diversity. The microbial communities associated with plant hosts are probably assembled by a wide variety of environmental and host-related factors, including geographic location, plant genotype and soil chemistry (Müller et al., 2016).

The diversity of fungal and bacterial communities varied importantly across the provinces and across the different places and cultivars sampled. This study determined that the biogeography and soil chemistry played an important role in the variation of fungal and bacterial microbiome diversity. Studies in several different experimental systems have similarly reported that fungal communities have different composition by geographic distance (Peay et al., 2007; Shakya et al., 2013; Meiser et al., 2014), which suggests that fungal endemism may be a community-modeling force operating at multiple scales and in multiple habitats. On the other hand, the same studies suggest that the geographic effect in the bacterial communities is less important than for fungi.

The soil is an important resource of an agricultural ecosystem (Wang et al., 2016) and depicts a dynamic and complex environment. Microbial communities in this ecosystem are guided by a host of different biotic or abiotic factors (Kaiser et al., 2016). The Ecuadorian soils have a wide range of physicochemical characteristics across the different provinces due to the different origins of the soils, and these characteristics can strongly influence the microbial diversity. Ecuadorian Highlands have three different regions according to the soil origin: The northern highlands, that received contributions of volcanic projections (ash and lapilli) over which diverse fertile and generally deep Andisols have developed. The central highlands that do not have recent pyroclastic

projections and soil parent material consists of old volcanic and metamorphic rocks in a monotonous relief. There are different types of soils depending on the altitude. Finally, the southern highlands that are characterized by a more rugged relief over granites, metamorphic formations, tertiary sediments, and glacis of colluvial deposits (Espinosa et al., 2018).

Soil pH has a fundamental function on the composition and the diversity of soil microbial communities. Our results highlighted the overriding effect of pH over other soil abiotic variables in assembling fungal and bacterial microbiome in the Ecuadorian potato rhizosphere. Soils with high pH values presented high fungal diversity values. Conversely, soils with low pH values showed high bacterial diversity values. This corroborates the relationship between bacterial diversity and pH observed in, for example, Lauber et al. (2009). Authors observed that the overall bacterial community composition in different soils from across North and South America was mainly associated with soil pH, supporting that this pattern is constant across different soil types (Lauber et al., 2009). Soil pH is often referred to as a critical variable due to its considerable effect on biotic and abiotic processes. Soil pH is also considered as one of the principal components influencing the soil microbiome assembling (Lauber et al., 2009; Andrew et al., 2012; Zhalnina et al., 2015), through impacting pH homeostasis of the microbial cell or regulating the availability of soil nutrients. Extremes of soil pH present strong selection pressures, particularly relating to nutrient availabilities. Soils with pH <5.5 cause Al, Fe, Mn and H toxicities. Concurrently, low pH soils tend to show P, N and base cation deficiencies, resulting in inhibition of root growth, and poor yields. Neutral to alkaline soils are limited in Fe, Mn and P availability (George et al., 2012). Soil pH also affects the structure of soil microbial communities by affecting nitrogen availability (Cederlund

et al., 2014), soil organic carbon content (Sul et al., 2013), and redox status (Pett-Ridge & Firestone, 2005).

This study suggest that soil characteristics influenced the potato rhizosphere microbiome establishment more than spatial and climatic factors, and they importantly affected the fungal and bacterial microbiome structure. We analyzed the effects of the soil variables including Mg, Fe, Ca, pH, Mn, K, Zn, Cu, P, N and Organic matter on the fungal and bacterial communities in the potato rhizosphere, and found that Fe for fungal and pH, organic matter, N, P, Fe, Zn and Ca for bacterial communities, substantially contributed to the variation in the microbial structure. Maize studies demonstrated that the rhizosphere microbiome structure and diversity were also driven by soil characteristics (Castellanos et al., 2009). In other studies, place- and management-specific soil properties were associated to model the structure of the rhizosphere bacterial microbiome (Aira et al., 2010; Peiffer et al., 2013; Gannibal et al., 2014). The present study corroborates studies on other crop-species highlighting the deterministic roles of soil characteristics in structuring the rhizosphere microbiome (Schreiter et al., 2014; Edwards et al., 2015; Yao et al., 2016).

The abundance of several classical and new candidate fungal biocontrol agents were also observed. Reports highlighting the benefits of these fungi in terms of antagonistic effects on plant pathogens and pests and recommending the organisms in agriculture are continuing to emerge as per the recent research publications (Yao et al., 2016; Iqbal et al., 2017; Wani et al., 2017; Krell et al., 2018; Johnson et al., 2018). Great diversity of these fungi in the microcenter of potato diversity was observed with emphasis in landraces cultivars. However, studies do not mention about the monitoring of these fungi with culture-independent approach. This could be a new tool in prospecting new and promising beneficial microorganisms.

Describing microbial communities in the potato rhizosphere correlated with candidate disease-suppressive soil is a pivotal first step toward understanding the potential of microbiota to protect crops against plant pathogens. The potato diversity has been recognized as critical to improving global food security and this study in the Ecuadorian Andes supplies additional informational to considering the conservation of native potatoes and encourage invisible biodiversity they hide.

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FIGURE LEGENDS

Figure 1. Geographical location of potato fields sampled across the different provinces of Ecuador. The provinces and places sampled are color-coded according to the legend from top to bottom.

Figure 2. Relative abundance of OTUs summarized by Phyla and province for fungi (A) and for bacteria (B). The Phyla are color-coded according to the legend from top to bottom.

Figure 3. Cluster analysis based on Bray-Curtis distance by province surveyed. Dendrogram was constructed using hierarchical clustering with complete linkage. A) Dendrogram for fungi. B) Dendrogram for bacteria.

Figure 4 Principal coordinate analysis (PCoA) of microbial communities collected from potato rhizosphere in the Ecuador Highlands based on a Bray-Curtis distance. A) PCoA for fungi. B) PCoA for bacteria. C) PCoA with envit analysis for fungi. D) PCoA with *envit* analysis for bacteria. Spatial, climatic and edaphic factors are plotted as vectors based on correlations with community distance. Only vectors with significance correlations are represented in the figures.

Figure 5 Alpha fungal diversity different places in the potato microcenter of diversity using Hill's numbers (effective number of species). A) Species richness (q = 0), B) Shannon diversity (q = 1) and C) Simpson diversity (q = 2).

Figure 6. Alpha bacterial diversity different places in the potato microcenter of diversity using Hill's numbers (effective number of species). A) Species richness (q = 0), B) Shannon diversity (q = 1) and C) Simpson diversity (q = 2).

Figure 7. Diversity of fungal communities. A) Diversity expressed as species richness across the latitudes of the fields sampled. B) Diversity expressed as species richness across the longitudes of the fields sampled. C) Diversity expressed as Shannon diversity across the different soil pHs. D) Diversity expressed as Simpson diversity across the different soil pHs. Spearman correlation values (rho) and *P-value* are presented for each comparison.

Figure 8. Diversity of bacterial communities. A) Diversity expressed as Shannon diversity across the latitudes and B) across the longitudes of the fields sampled. C) Diversity expressed as Shannon diversity across the different pHs. D) Diversity expressed as Simpson diversity across the differents latitudes and E) longitudes of the fields sampled. Spearman correlation values (rho) and *P-value* are presented for each comparison.

Figure 9. Representation of the significant correlations of among fungal community similarity (PCoA ordination axis) and the different spatial, climatic and edaphic factors. A) and B) significant correlations with Fe. C)and D) significant correlations with Ca. E) and F) significant correlations with Mg. G) and H) correlations with minimal temperature (mint).

Figure 10. Representation of the significant correlations of among bacterial community similarity (PCoA ordination axis) and the different spatial, climatic and edaphic factors. A) and B) significant correlations with soil pH. C)and D) significant correlations with organic matter E) and F) significant correlations with altitude (m.s.a.l.). G) and H) correlations with minimal temperature (mint).

Figure 11. Representation of the significant correlations among bacterial community similarity (PCoA ordination axis) and the different spatial, climatic and edaphic factors. A) and B) significant correlations with P. C) and D) significant correlations with Ca. E) and F) significant correlations with N. G) and H) correlations with Mg.

Figure 12. Frequency of guilds associated to fungal communities from potato rhizosphere.

Figure 13. Frequency of the functions associated to bacterial communities from potato rhizosphere.

Figure 14. Frequency of the trophic modes associated to fungal communities from potato rhizosphere.

Figure 15. Heatmap depicts the relative abundance percentage of potential beneficial fungi abundance assessed in the different provinces.

Figure 16. Heatmap depicts the relative abundance percentage of potential beneficial fungi abundance assessed in the different places from the microcenter of diversity.

Figure 17. Heatmap depicts the relative abundance percentage of potential beneficial fungi abundance assessed in the different cultivars sampled from the microcenter of diversity.

FIGURES AND TABLES

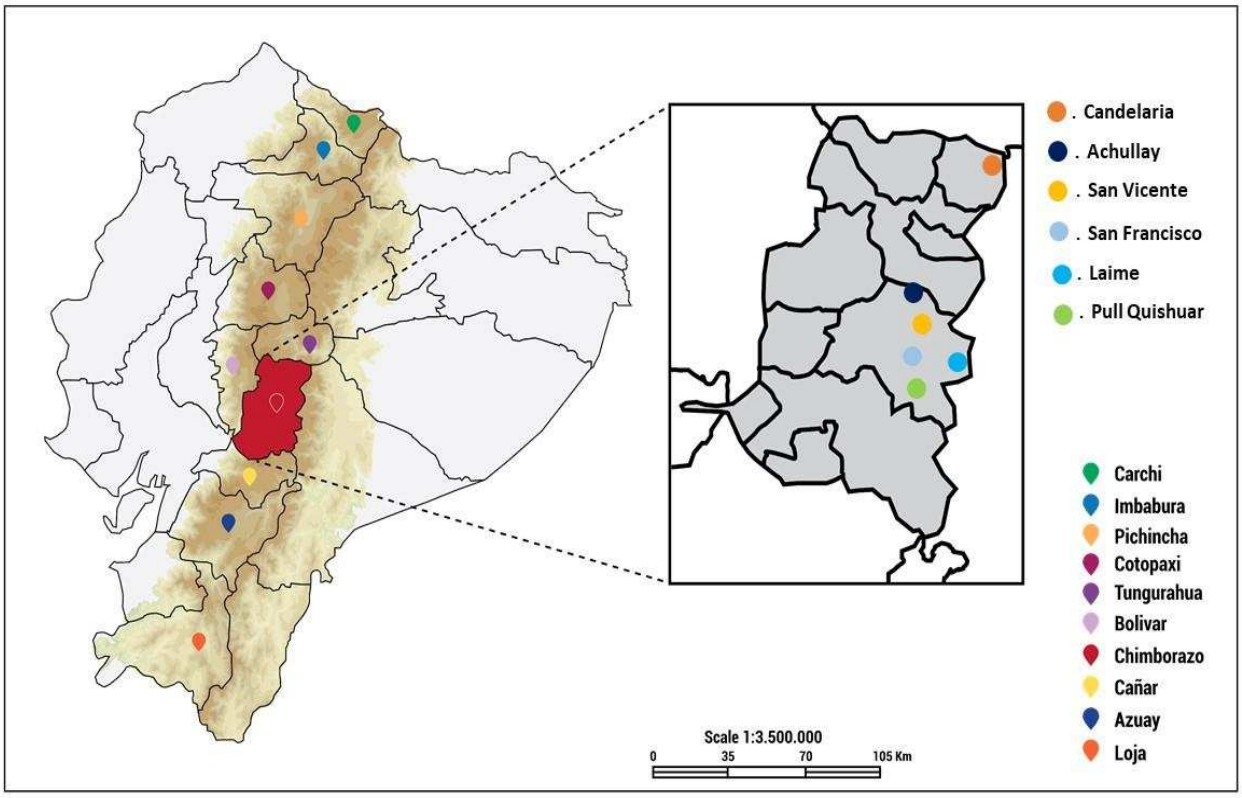
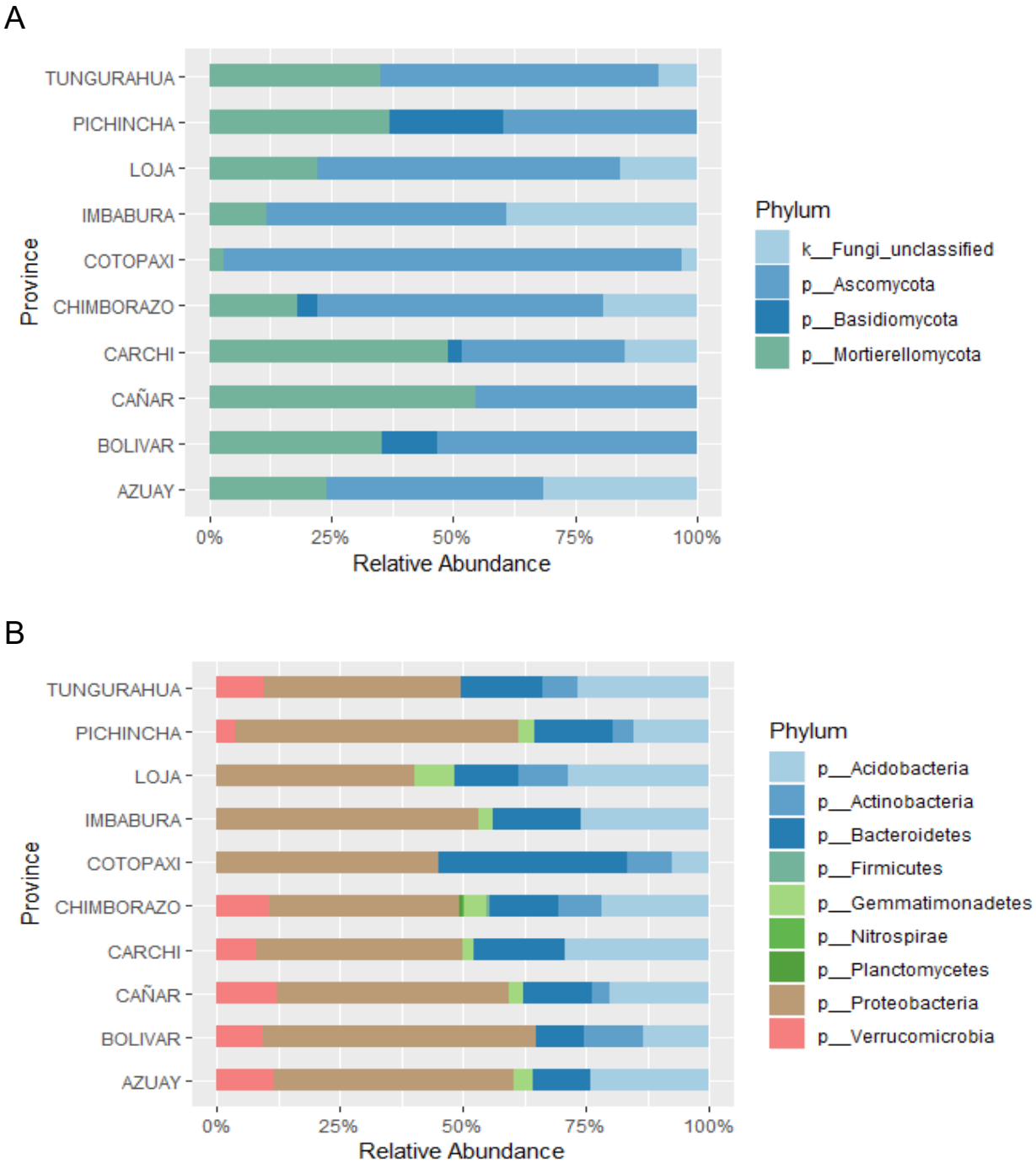


Figure 1. Álvarez-Romero et al. 2019.



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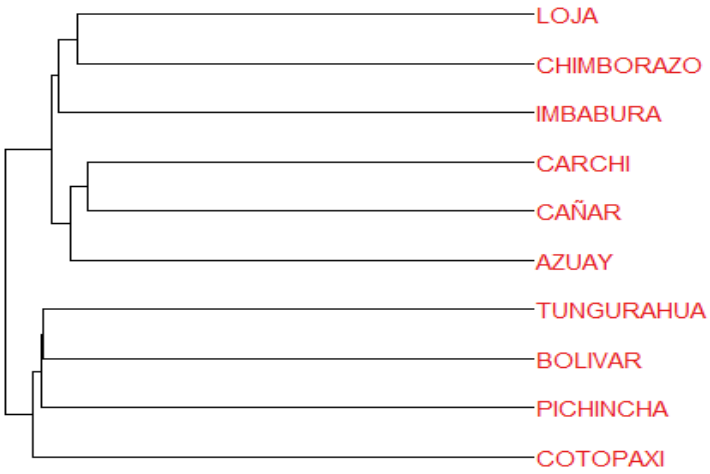

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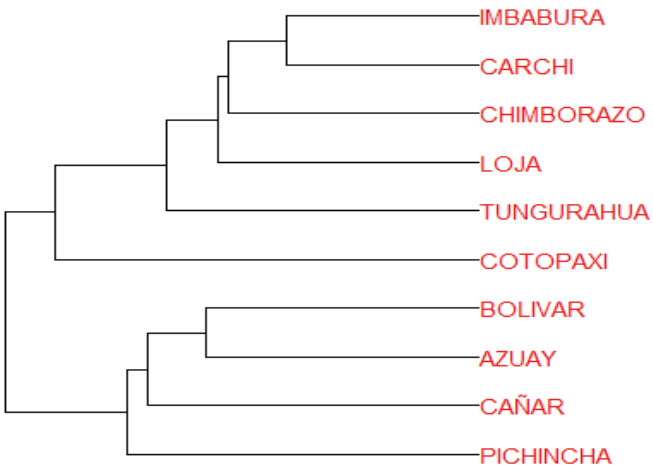

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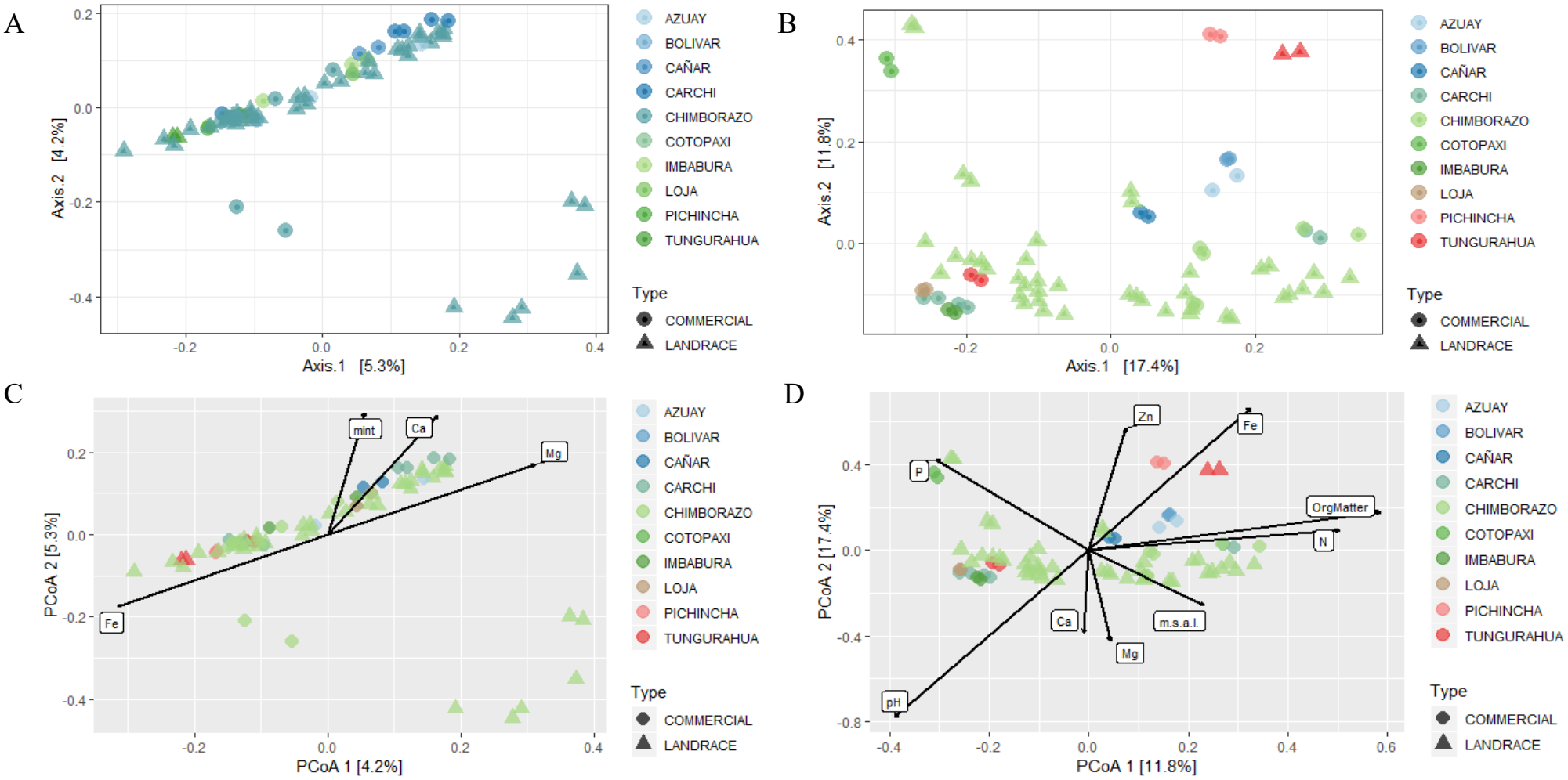

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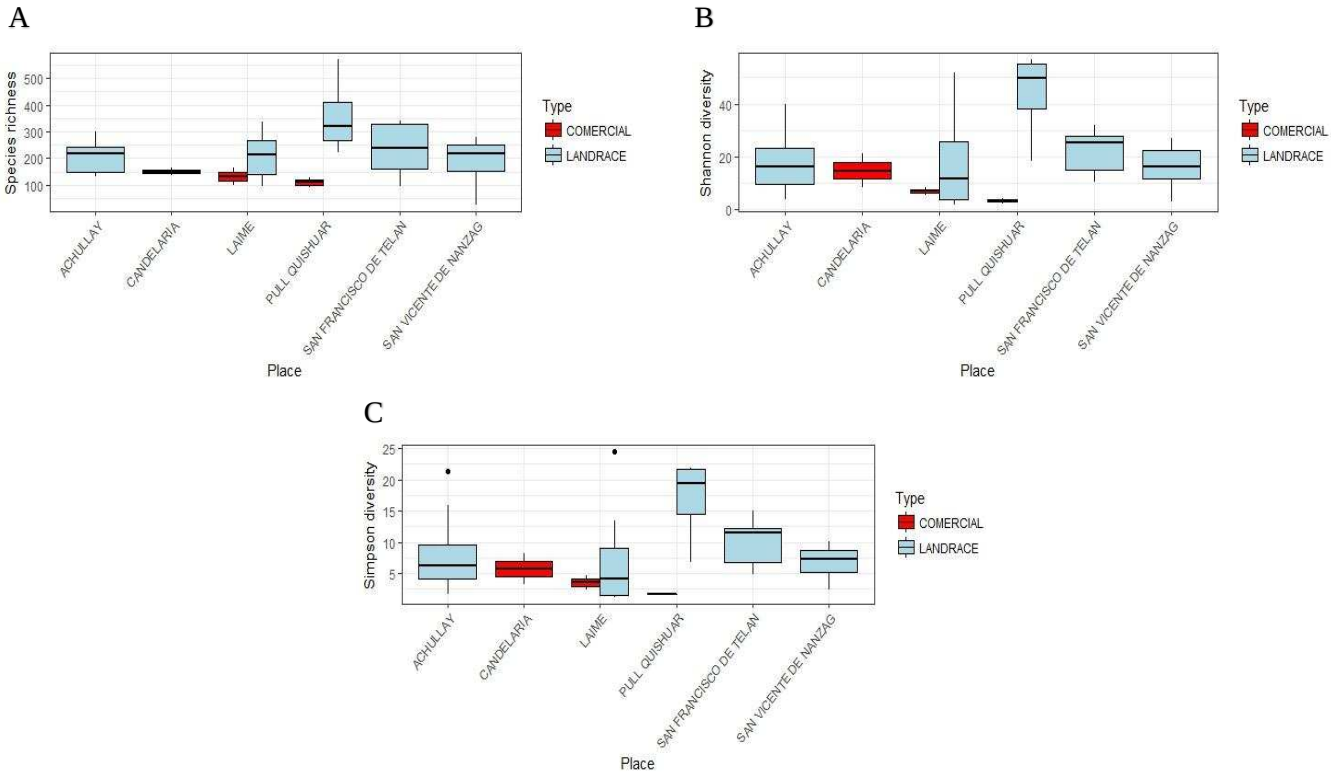

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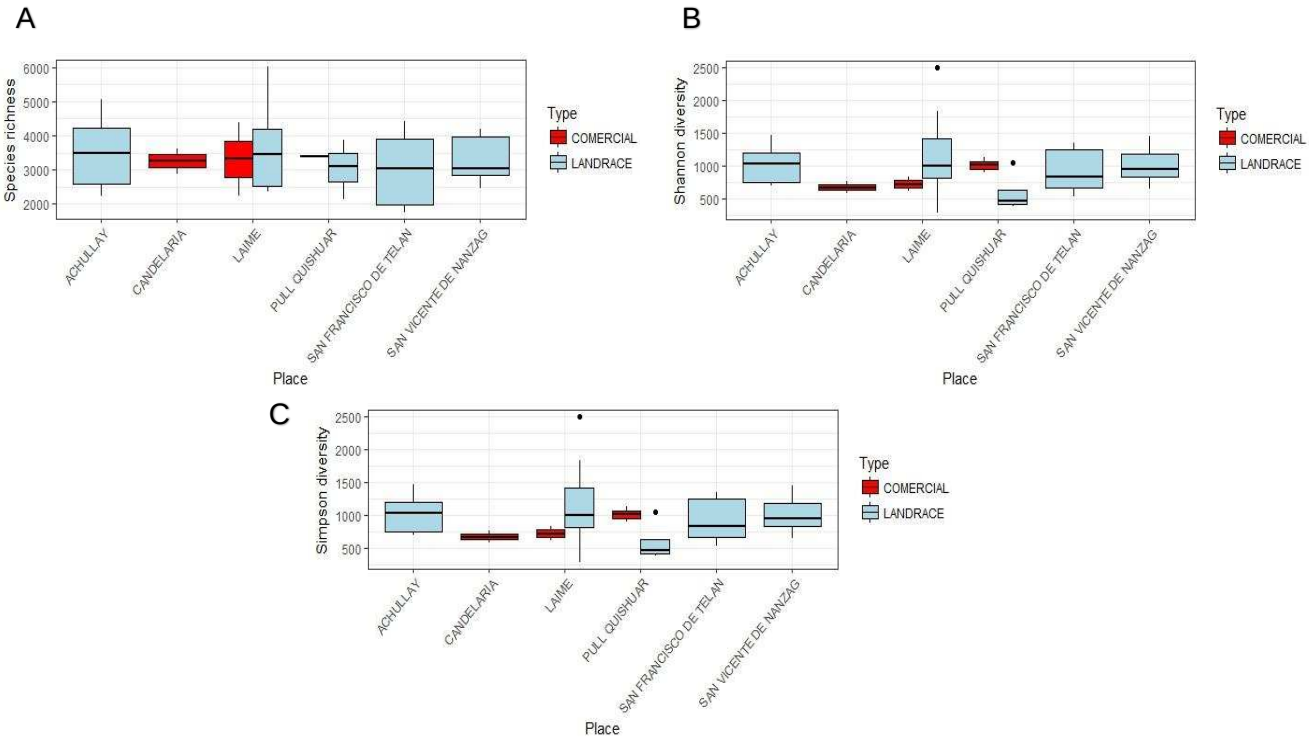

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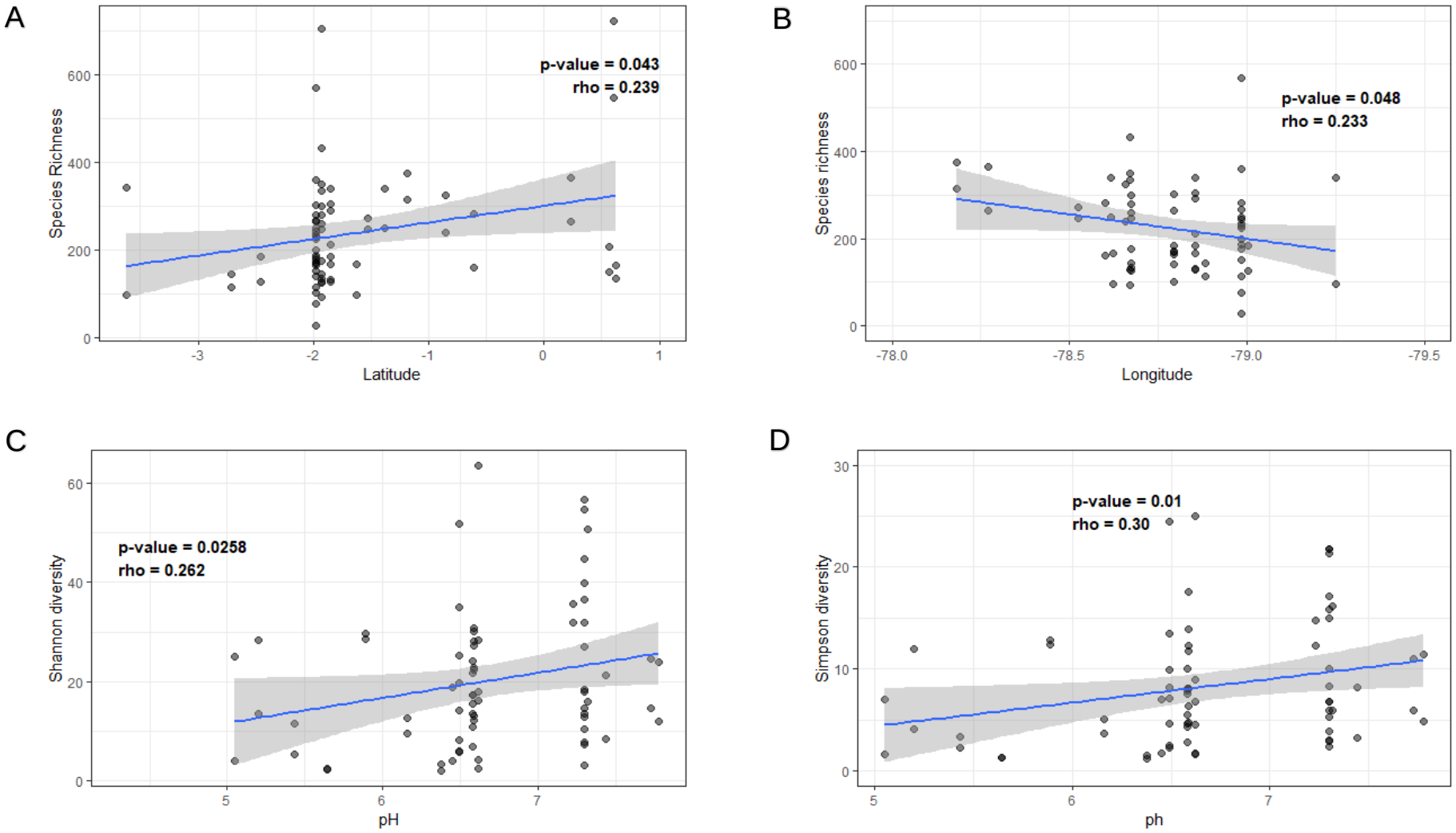

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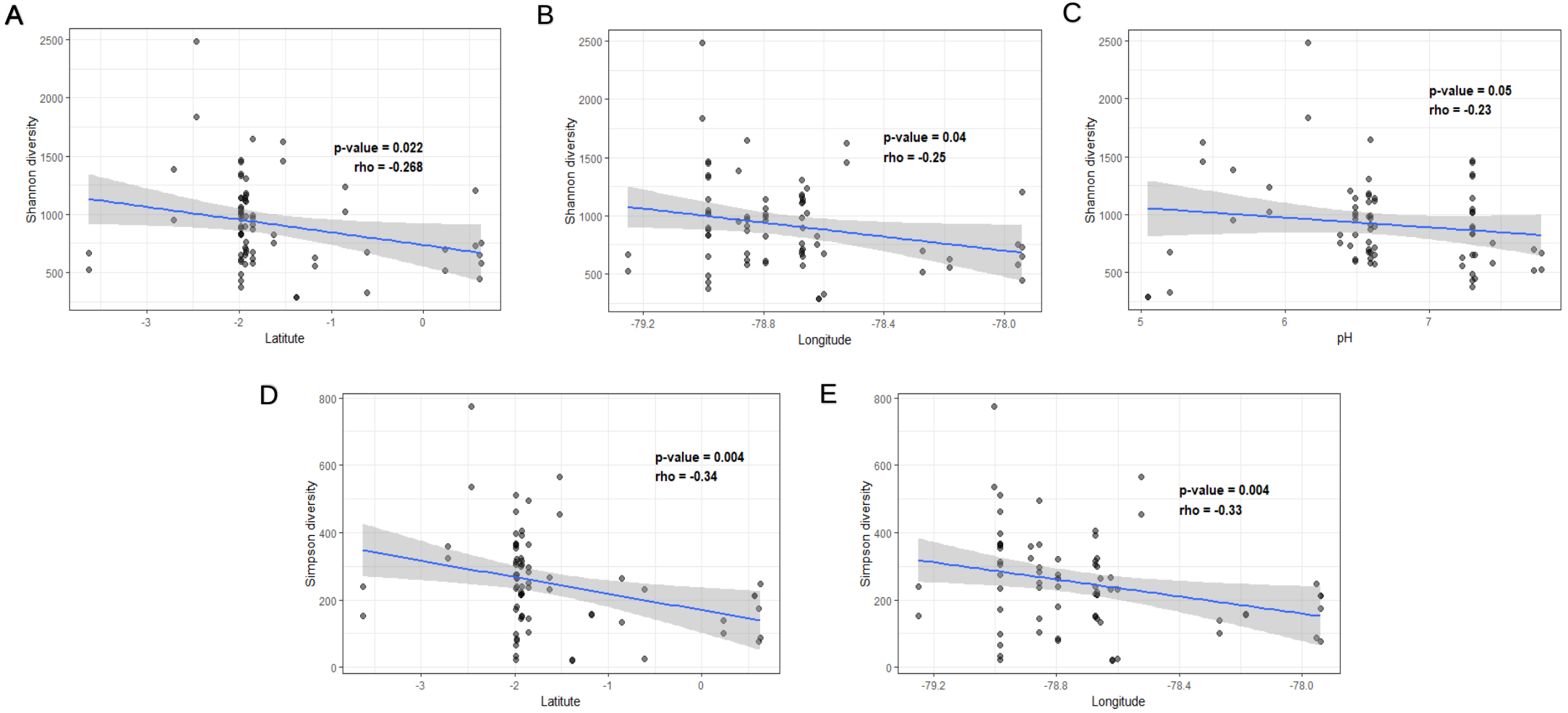

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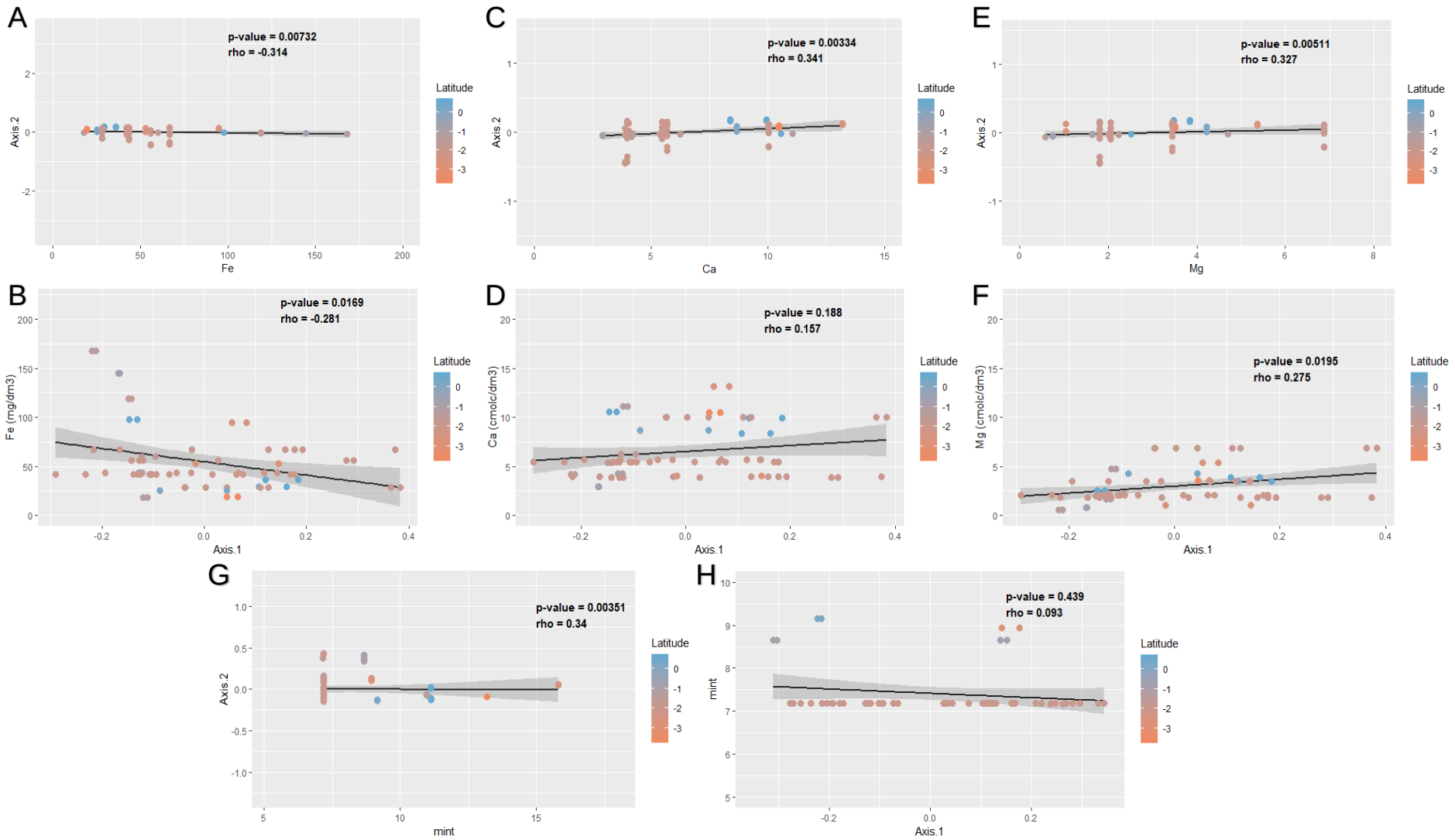

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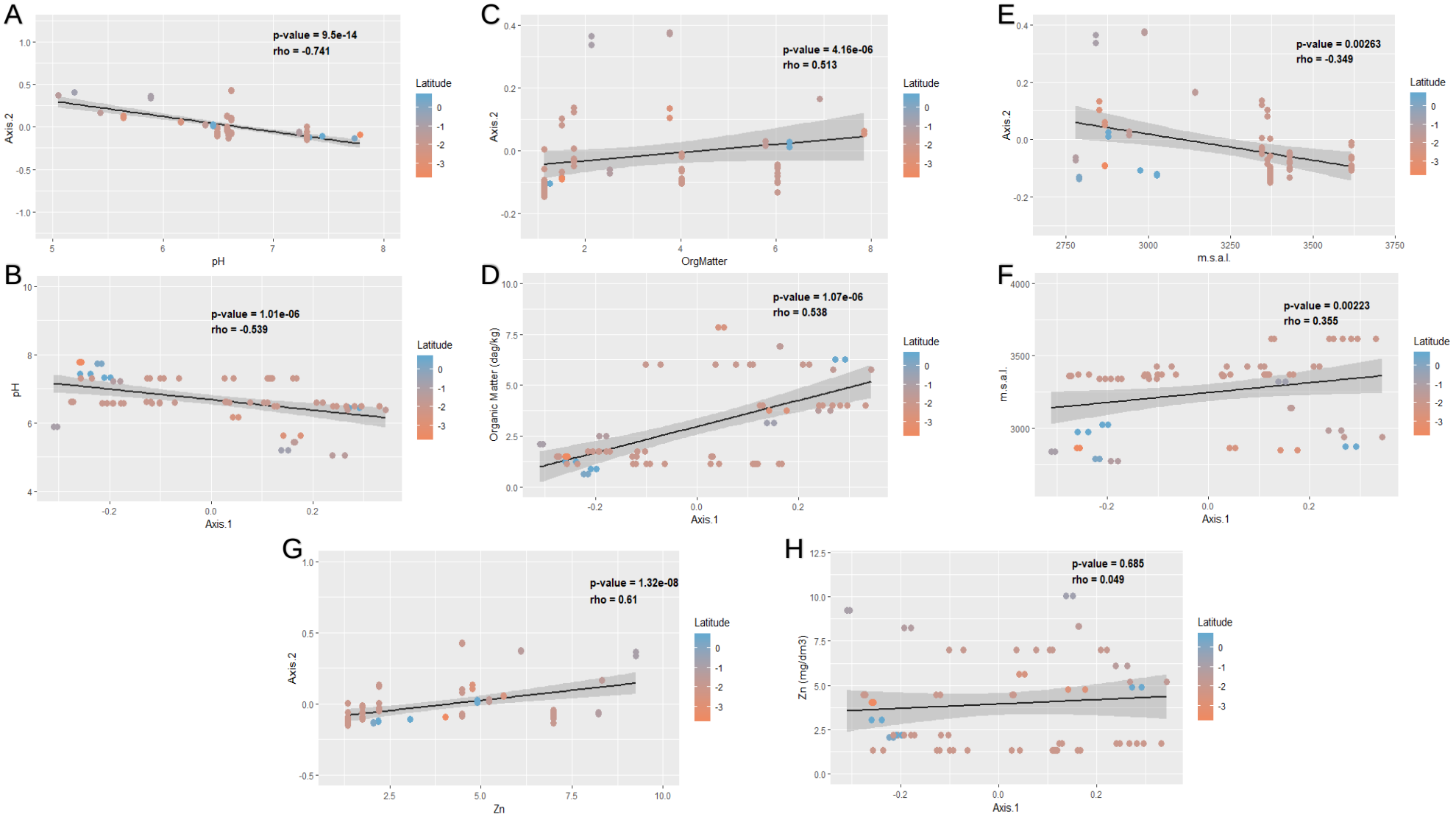

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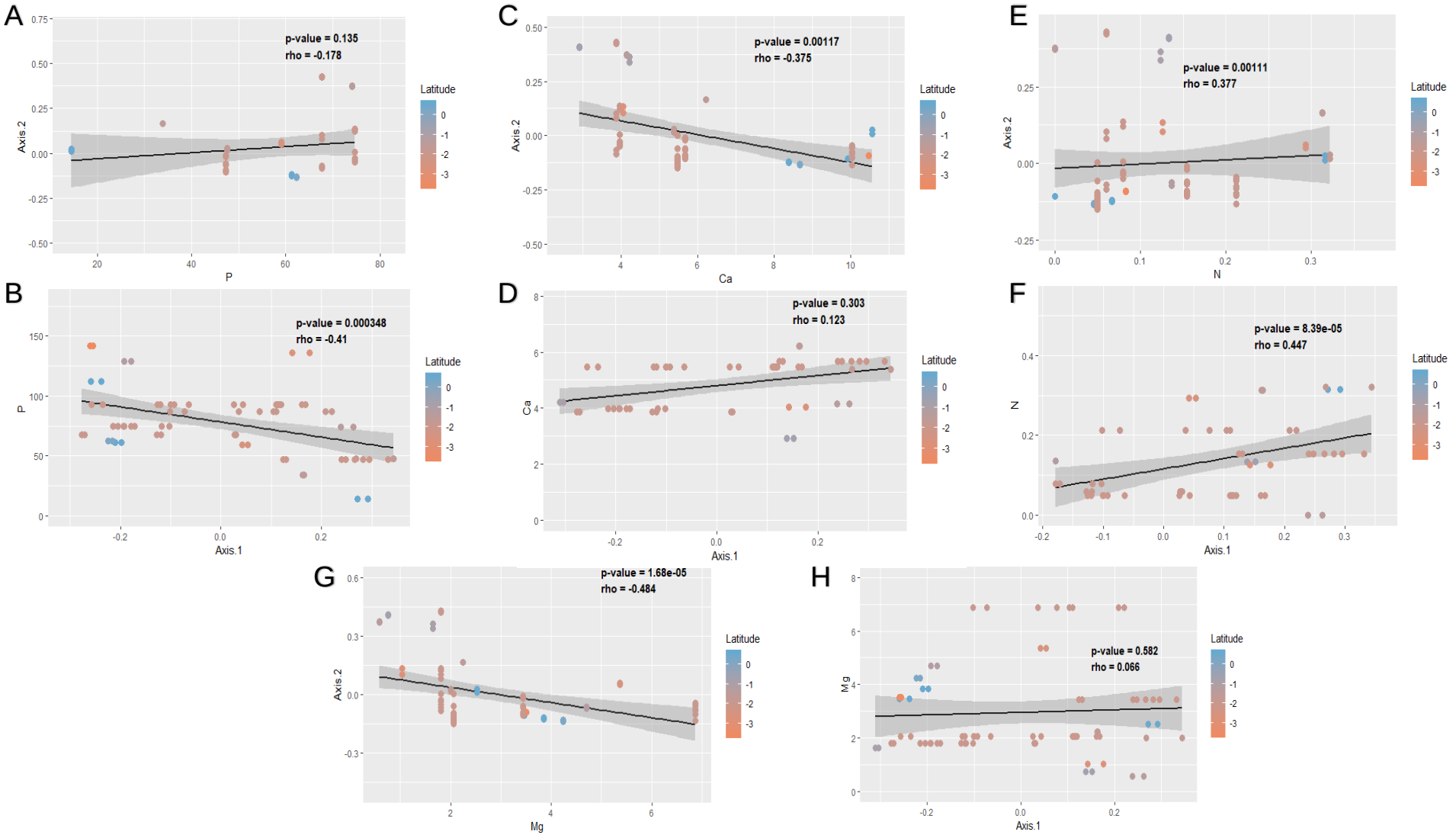

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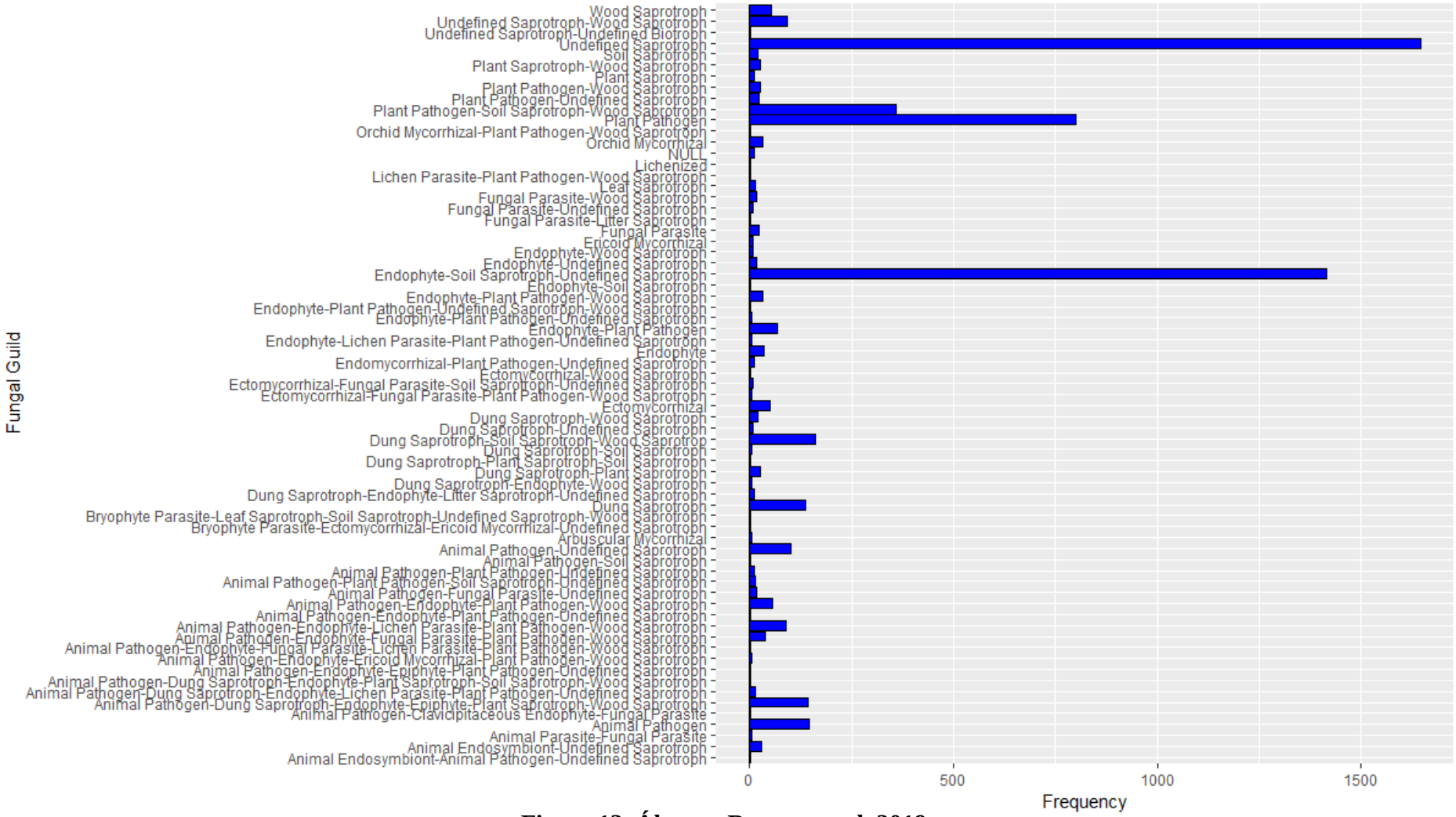

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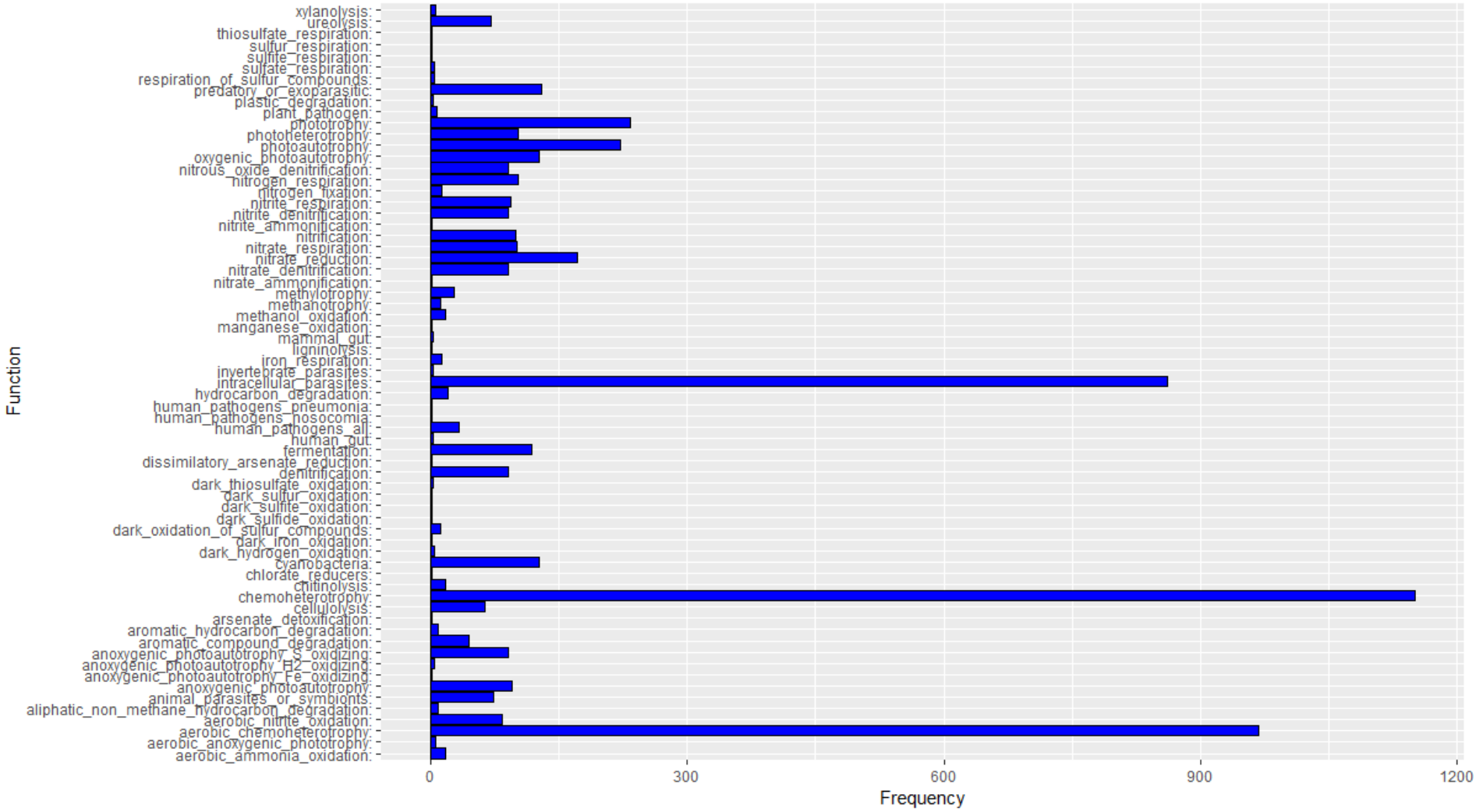

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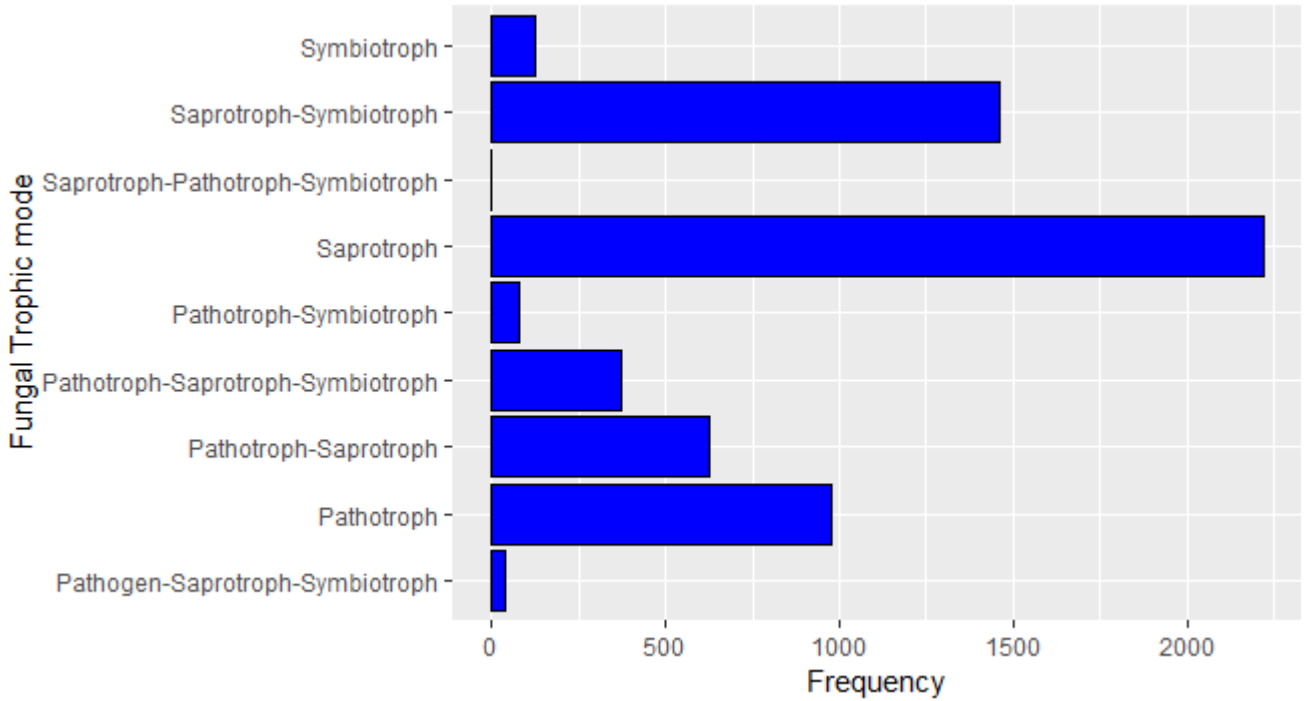

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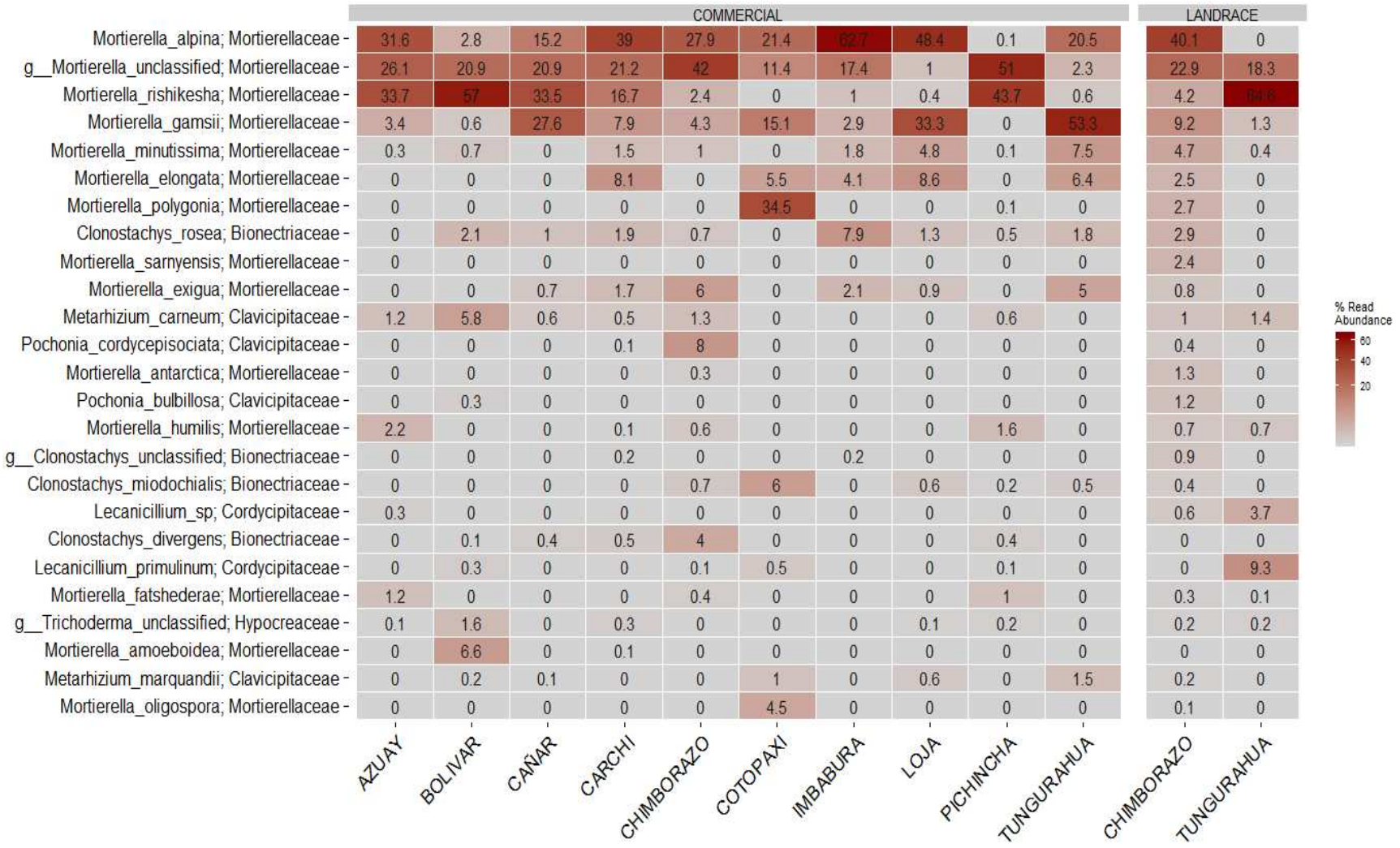

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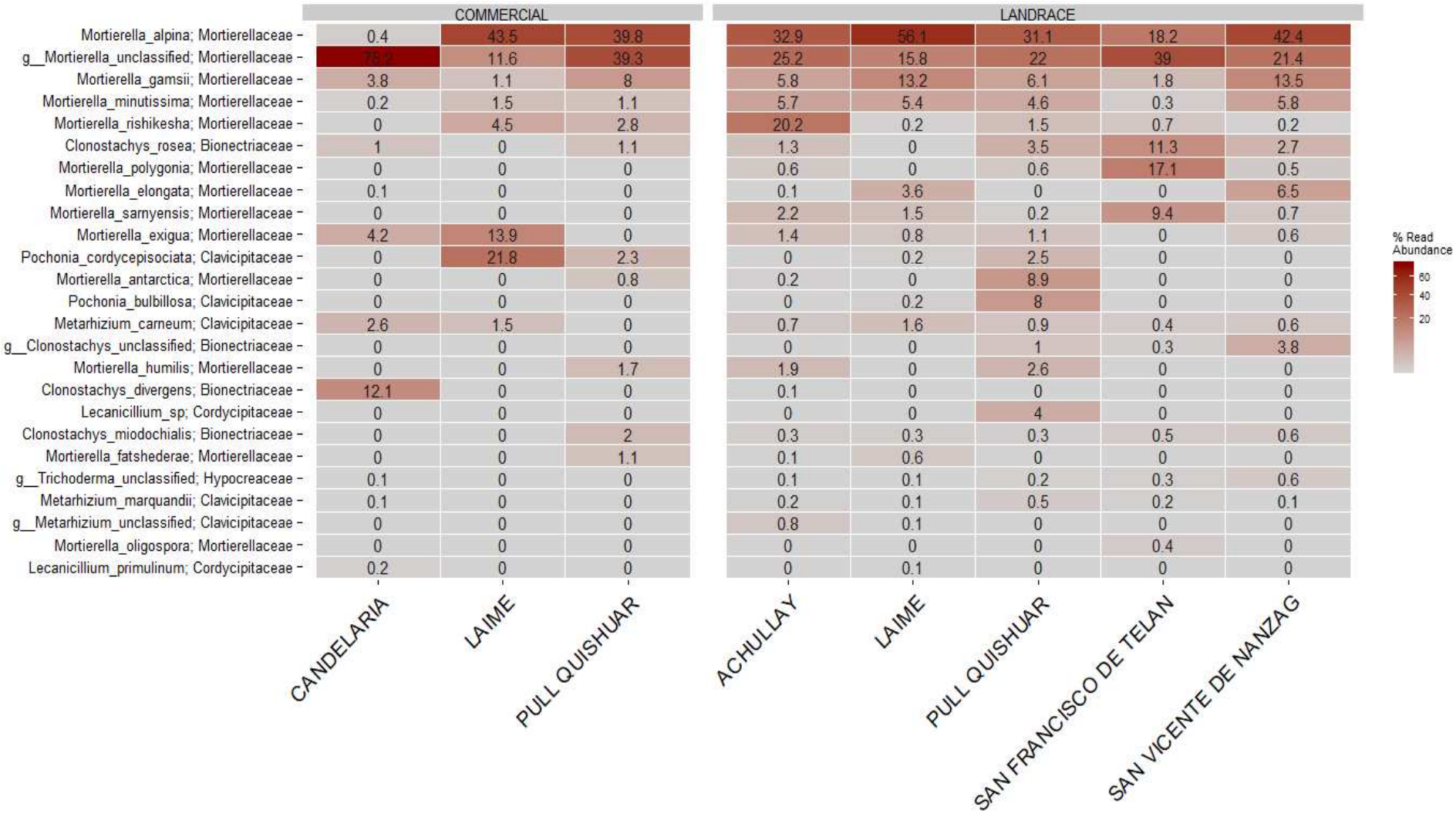

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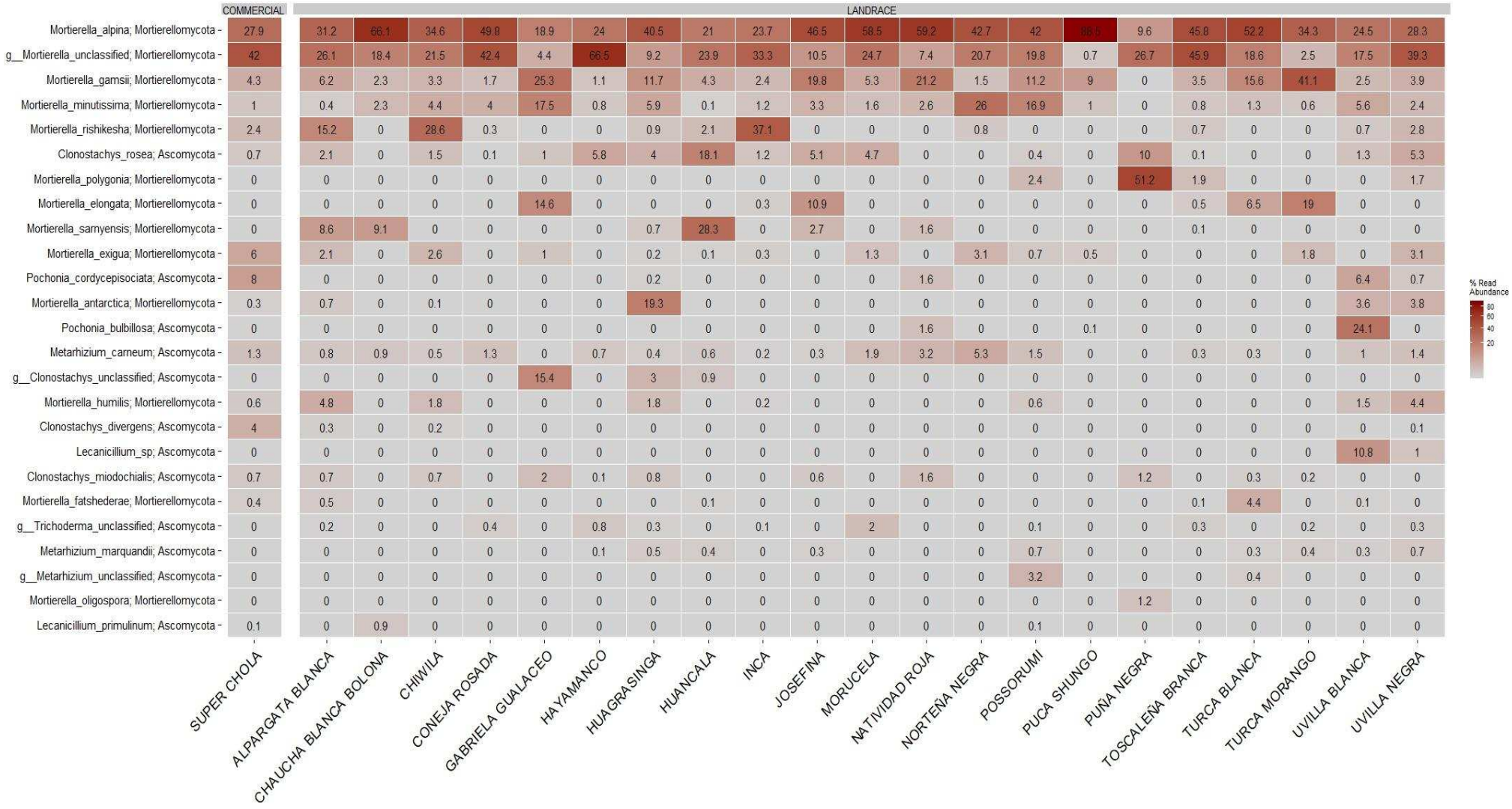

Figure 17. Álvarez-Romero et al. 2019.

Table 1. Characteristics of fields sites from which soil samples were collected in January 2017.

Number of samples	Field sites		Crop characteristics			Geographic parameters		
	Province	County Community	System	Potato varieties	Crop age	Latitude	Longitude	Altitude (m.a.s.l)
1	Carchi	El Angel S.F	Conventional	improved (Commercial)	3 months	0.623	-77.954	2975
1	Carchi	Montufar D. B.	Conventional	improved (Commercial)	3 months	0.565	-77.942	2877
1	Carchi	El Angel I.	Conventional	improved (Commercial)	4 months	0.605	-77.942	3025
1	Imbabura	S. J. Ibarra	Conventional	improved (Commercial)	3 months	0.231	-78.271	2790
1	Pichincha	Machachi	Conventional	improved (Commercial)	3 months	-0.613	-78.600	3321
1	Cotopaxi	Saquisilí	Conventional	improved (Commercial)	3 months	-0.851	-78.657	2841
1	Tungurahua	Pillaro	Conventional	improved (Commercial)	4 months	-1.183	-78.183	2778
1	Tungurahua	Quero	Conventional	native (landrace)	4 months	-1.383	-78.617	2987
1	Bolívar	Guanujo	Conventional	improved (Commercial)	3 months	-1.524	-78.524	3142
4	Chimborazo	Achullay	Organic	native (landrace)	3 months	-1.860	-78.860	3142
1	Chimborazo	Candelaria	Conventional	improved (Commercial)	3 months	-0.162	-78.623	2941
4	Chimborazo	S. Vicente N.	Landrace	native (landrace)	3 months	-1.930	-78.670	3344
3	Chimborazo	S. Francisco T.	Landrace	native (landrace)	3 months	-1.930	-78.670	3362
8(N); 1(I)	Chimborazo	Laime	Landrace	native (N); improved (I)	3 months	-1.983	-78.983	3371
2(N); 1(I)	Chimborazo	P. Quishuar	Organic	native (N); improved (I)	3 months	-1.980	-78.795	3616
1	Cañar	Zhud	Conventional	improved (Commercial)	3 months	-2.460	-79.003	2868
1	Azuay	Biblian	Conventional	improved (Commercial)	3 months	-2.713	-78.883	2851
1	Loja	Saraguro	Conventional	improved (Commercial)	3 months	-3.625	-79.249	2868

Table 2. Physicochemical properties of potato soils fields in Ecuador.

Province	County	Soil nutrients								pH (H2O)	Organic Matter (dag/kg)
		N (dag/kg)	P (mg/dm^3)	Ca2+ ($cmolc/dm^3$)	Mg2+ ($cmolc/dm^3$)	Fe (mg/dm^2)	Cu (mg/dm^3)	Zn (mg/dm^3)	Mn (mg/dm^3)		
Chimborazo	Achullay	0.212	87.2	10.04	6.87	28	2.84	7	20.5	6.59	6.03
Biblian	Azuay	0.126	136.1	4.05	1.04	52.9	2.83	4.77	16.7	5.64	3.77
Zhud	Cañar	0.293	59	13.19	5.37	94.7	4.26	5.63	98.6	6.16	7.85
Chimborazo	Candelaria	0.322	47.6	5.38	2	118.8	2.84	5.22	32.1	6.38	5.78
Carchi	El Angel Ishpingo	0.066	61.3	8.38	3.85	29.2	1.74	2.18	34.2	7.32	0.88
Carchi	El Angel San Francisco	Nd.	112.1	9.93	3.47	35.9	1.94	3.05	52	7.44	1.26
Bolívar	Guanujo	0.313	33.9	6.23	2.24	60.1	1.23	8.32	31.9	5.43	6.91
Chimborazo	Laime	0.049	92.6	5.47	2.06	42	13.24	1.35	13.6	7.3	1.13
Saraguro	Loja	0.083	142.1	10.48	3.51	19.2	4.25	4.02	24.1	7.78	1.51
Pichincha	Machachi	0.133	191.9	2.91	0.75	144.6	3.76	10.06	31.1	5.2	3.14
Carchi	Montufar La Delicia Bajo	0.316	14.5	10.57	2.52	97.6	2.38	4.9	25	6.45	6.28
Tungurahua	Pillaro	0.136	129.2	11.7	4.7	17.8	2.98	8.23	44.1	7.23	2.51
Chimborazo	Pull Quishuar	0.154	47.2	5.67	3.44	43.3	6.01	1.74	16.7	6.49	4.02
Tungurahua	Quero	Nd.	74	4.15	0.58	168.2	4.37	6.1	35.1	5.05	3.77
Chimborazo	San Francisco de Telán	0.06	67.6	3.88	1.81	56	11.33	4.48	12.8	6.62	1.51
Imbabura	San Juan Ibarra	0.045	62.3	8.68	4.23	25	1.83	2.06	25.5	7.73	0.63
Chimborazo	San Vicente de Nanzag	0.079	74.7	3.97	1.8	66.8	8.55	2.2	19.7	6.58	1.76
Cotopaxi	Saquisilí	0.123	651.2	4.22	1.64	43.4	1.74	9.26	22.8	5.89	2.13

Table 3. Fungal community diversity and evenness by province. Data represent province average and standard deviation of potato rhizosphere sampled by field.

Province	Fields Sampled	Cultivars Sampled	Species Richness [a]		Shannon Diversity [d]		Simpson Diversity [e]		Evenness[f]
			Mean [b]	SD [c]	Mean	SD	Mean	SD	
Carchi	3	1	381.88	± 180.11	27.59	± 18.48	10.39	± 7.38	4.64
Imbabura	1	1	161.62	± 30.42	12.89	± 9.72	5.25	± 4.19	2.53
Pichincha	1	1	171.81	± 57.34	17.95	± 6.75	7.19	± 1.29	3.49
Cotopaxi	1	1	190.68	± 107.83	10.39	± 4.38	3.41	± 0.71	1.98
Tungurahua	2	2	163.21	± 25.96	22.96	± 8.85	11.01	± 5.91	4.51
Bolívar	1	1	634.66	± 123.79	33.29	± 24.59	11.06	± 7.28	5.16
Chimborazo	6	24	211.42	± 96.36	18.65	± 14.42	7.82	± 6.13	3.48
Cañar	1	1	314.26	± 70.01	19.56	± 7.09	8.46	± 3.57	3.40
Azuay	1	1	220.97	± 85.19	20.96	± 10.49	8.05	± 5.52	3.88
Loja	1	1	344.79	± 42.92	33.79	± 2.77	13.52	± 1.76	5.78

[a] Hill´s number q=0
[b] Mean across fields sampled for the corresponding province
[c] Standard deviation for fields sampled for the corresponding province
[d] Hill´s number q=1
[e] Hill´s number q=2
[f] Pielou´s evenness: Shannon diversity divided by the natural logarithm of species richness in a sample

Table 4. Bacterial community diversity and evenness by province. Data represent province average and standard deviation of potato rhizosphere sampled by field.

Province	Fields Sampled	Cultivars Sampled	Species Richness [a]			Shannon Diversity [d]			Simpson Diversity [e]			Evenness[f]
			Mean [b]		SD [c]	Mean		SD	Mean		SD	
Carchi	3	1	3269.1	±	1092.49	858.34	±	240.06	229.69	±	52.3	106.07
Imbabura	1	1	3343.48	±	954.06	791.62	±	280.95	174.82	±	126.3	97.55
Pichincha	1	1	3393.03	±	524.71	774.52	±	272.07	170.87	±	94.29	95.27
Cotopaxi	1	1	3283.31	±	602.37	1017.02	±	9.33	374.42	±	30.67	125.61
Tungurahua	2	2	2900.69	±	1487.7	1076.86	±	420.27	376.3	±	106.67	135.07
Bolívar	1	1	2636.26	±	547.67	547.73	±	141.13	125.86	±	67.74	69.53
Chimborazo	6	24	3361.22	±	979.54	971.16	±	415.54	262.41	±	152.52	119.6
Cañar	1	1	2819.65	±	971.41	604.75	±	125.85	120.23	±	28.28	76.12
Azuay	1	1	2113.74	±	298.24	502.68	±	245.71	128.1	±	144.96	65.66
Loja	1	1	2621.29	±	734.01	590.66	±	49.61	156.63	±	0.74	75.04

[a] Hill´s number q=0
[b] Mean across fields sampled for the corresponding province
[c] Standard deviation for fields sampled for the corresponding province
[d] Hill´s number q=1
[e] Hill´s number q=2
[f] Pielou´s evenness: Shannon diversity divided by the natural logarithm of speciess richness in a sample

Table 5. Fungal community diversity and evenness by place in the microcenter of potato diversity. Data represent place average and standard deviation of potato rhizosphere sampled by field.

Place	Fields Sampled	Cultivars Sampled	System	Type	Species Richness [a]		Shannon Diversity [d]		Simpson Diversity [e]		Evenness[f]
					Mean [b]	SD [c]	Mean	SD	Mean	SD	
Achullay	1	4	Organic	Landrace	206.73 ±	59.12	18.71 ±	13.09	8.31 ±	6.79	3.51
Candelaria	1	1	Convencional	Commercial	149.73 ±	20.90	14.82 ±	9.12	5.74 ±	3.54	2.96
Laime	1	1	Organic	Commercial	132.43 ±	44.42	7.02 ±	1.84	3.54 ±	1.52	1.44
Laime	1	8	Organic	Landrace	210.67 ±	79.23	16.12 ±	14.57	6.52 ±	6.42	3.01
Pull Quishuar	1	1	Organic	Commercial	110.02 ±	25.11	3.36 ±	1.25	1.7 ±	0.11	0.71
Pull Quishuar	1	2	Organic	Landrace	358.18 ±	151.23	43.67 ±	17.59	16.87 ±	7.07	7.43
San Francisco	1	3	Organic	Landrace	234.18 ±	103.47	22.28 ±	8.92	10.1 ±	4.1	4.08
San Vicente	1	4	Organic	Landrace	187.70 ±	91.02	16.15 ±	8.22	6.82 ±	2.89	3.09

[a] Hill´s number q=0
[b] Mean across fields sampled for the corresponding place
[c] Standard deviation for fields sampled for the corresponding place
[d] Hill´s number q=1
[e] Hill´s number q=2
[f] Pielou´s evenness: Shannon diversity divided by the natural logarithm of speciess richness in a sample

Table 6. Bacterial community diversity and evenness by place in the microcenter of potato diversity. Data represent province average and standard deviation of potato rhizosphere sampled by field.

Place	Fields Sampled	Cultivars Sampled	System	Type	Species Richness [a]			Shannon Diversity [d]			Simpson Diversity[e]			Evenness[f]
					Mean [b]		SD [c]	Mean		SD	Mean		SD	
Achullay	1	4	Organic	Landrace	3507.75	±	1056.82	1011.21	±	281.18	258.82	±	117.5	123.88
Candelaria	1	1	Convencional	Commercial	3254.21	±	527.86	665.63	±	125.11	112.52	±	112.52	82.30
Laime	1	1	Organic	Commercial	3309.02	±	1535.27	716.29	±	153.85	71.83	±	71.83	88.38
Laime	1	8	Organic	Landrace	3548.99	±	1172.56	1111.51	±	565.82	198.7	±	198.69	135.97
Pull Quishuar	1	1	Organic	Commercial	3387.52	±	0.12	1007.41	±	159.87	71.62	±	71.62	123.94
Pull Quishuar	1	2	Organic	Landrace	3047.45	±	737.42	584.29	±	311.03	118.49	±	118.49	72.83
San Francisco	1	3	Organic	Landrace	3002.13	±	1186.95	921.72	±	355.27	80.54	±	80.54	115.11
San Vicente	1	4	Organic	Landrace	3298.55	±	694.78	1011.93	±	267.21	119.57	±	119.57	124.91

[a] Hill´s number q=0
[b] Mean across fields sampled for the corresponding place
[c] Standard deviation for fields sampled for the corresponding place
[d] Hill´s number q=1
[e] Hill´s number q=2
[f] Pielou´s evenness: Shannon diversity divided by the natural logarithm of species richness in a sample

Table 7. Fungal community diversity and evenness by cultivar in the microcenter of potato diversity. Data represent cultivar average and standard deviation of potato rhizosphere sampled by cultivar.

Cultivar	Place	System	Type	Species Richness[a]		Shannon Diversity[d]		Simpson Diversity[e]		Evenness[f]
				Mean[b]	SD[c]	Mean	SD	Mean	SD	
Alpargata Blanca	Achullay	Organic	Landrace	185.94 ±	46.47	8.83 ±	2.8	3.75 ±	1.31	1.69
Chaucha blana B	Laime	Organic	Landrace	341.06 ±	26.37	2.27 ±	0.18	1.27 ±	0.04	0.39
Chiwila	Achullay	Organic	Landrace	211.58 ±	0.01	11.43 ±	10.53	4.38 ±	3.71	2.13
Coneja rosada	Laime	Organic	Landrace	298.08 ±	33.89	43.37 ±	11.93	18.97 ±	7.71	7.61
Gabriela Gualaceo	San Vicente	Organic	Landrace	316.47 ±	205.49	8.83 ±	8.13	5.35 ±	4.17	1.53
Hayamanco	San Francisco	Organic	Landrace	266.92 ±	24.69	27.68 ±	0.59	12.02 ±	0.36	4.95
Huagrasinga	Pull Quishuar	Organic	Landrace	44.71 ±	30.48	31.64 ±	18.65	11.96 ±	7.3	8.33
Huancala	San Francisco	Organic	Landrace	363.94 ±	3.18	21.19 ±	15.14	10.15 ±	6.89	3.59
Inca	Achullay	Organic	Landrace	227.69 ±	109.33	16.35 ±	1.3	6.5 ±	1.47	3.01
Josefina	San Vicente	Organic	Landrace	231.56 ±	187.01	15.44 ±	3.49	6.37 ±	0.64	2.83
Morucela	San Vicente	Organic	Landrace	273.39 ±	56.88	17.39 ±	13.72	6.56 ±	4.96	3.09
Natividad Roja	Laime	Organic	Landrace	508.79 ±	78.37	8.49 ±	4.31	2.85 ±	0.75	1.36
Norteña negra	Laime	Organic	Landrace	248.1 ±	25.09	2.71 ±	0.99	1.37 ±	0.18	0.49
Possorumi	Achullay	Organic	Landrace	408.07 ±	144.77	38.21 ±	2.43	18.61 ±	3.86	6.36
Puca shungo	Laime	Organic	Landrace	221.1 ±	107.03	17.36 ±	7.21	6.42 ±	2.31	3.22
Puña negra	San Francisco	Organic	Landrace	196.97 ±	60.76	17.97 ±	8.39	8.14 ±	4.63	3.40
Super chola	Candelaria	Conventional	Commercial	166.97 ±	112.52	14.81 ±	9.12	5.74 ±	3.54	2.89
Super chola	Laime	Organic	Commercial	130.08 ±	71.83	7.02 ±	1.84	3.54 ±	1.52	1.44
Super chola	Pull Quishuar	Organic	Commercial	273.17 ±	71.62	3.33 ±	1.25	1.7 ±	0.11	0.59
Toscaleña blanca	San Vicente	Organic	Landrace	353.78 ±	53.27	22.95 ±	1.58	8.98 ±	1.51	3.91

Turca blanca	Laime	Organic	Landrace	198.38	±	92.83	29.19	±	0.71	12.58	±	0.28	5.52
Turca morango	Laime	Organic	Landrace	655.14	±	169.63	11.11	±	2.28	4.35	±	0.96	1.71
Uvilla blanca	Laime	Organic	Landrace	21.13	±	2.15	14.44	±	14.88	4.31	±	3.83	4.73
Uvilla negra	Pull Quishuar	Organic	Landrace	153.8	±	170.52	55.69	±	1.32	21.78	±	0.06	11.06

[a] Hill´s number q=0
[b] Mean across fields sampled for the corresponding cultivar
[c] Standard deviation for fields sampled for the corresponding cultivar
[d] Hill´s number q=1
[e] Hill´s number q=2
[f] Pielou´s evenness: Shannon diversity divided by the natural logarithm of species richness in a sample

Table 8. Bacterial community diversity and evenness by cultivar in the microcenter of potato diversity. Data represent cultivar average and standard deviation of potato rhizosphere sampled by cultivar.

Cultivar	Place	System	Type	Species Richness[a]			Shannon Diversity[d]			Simpson Diversity[e]			Evenness[f]
				Mean[b]		SD[c]	Mean		SD	Mean		SD	
Alpargata Blanca	Achullay	Organic	Landrace	3413.69	±	1657.13	941.05	±	340.31	185.94	±	46.47	115.67
Chaucha blana B	Laime	Organic	Landrace	3426.92	±	1513.94	1169.65	±	310.87	341.06	±	26.34	143.70
Chiwila	Achullay	Organic	Landrace	3782.13	±	1805.12	966.98	±	338.34	211.58	±	0.01	117.37
Coneja rosada	Laime	Organic	Landrace	3952.86	±	203.66	1049.76	±	126.74	298.08	±	33.88	126.74
Gabriela Gualaceo	San Vicente	Organic	Landrace	3559.99	±	913.47	1140.89	±	435.11	316.47	±	205.49	139.51
Hayamanco	San Francisco	Organic	Landrace	2827.44	±	1509.27	831.21	±	224.37	266.92	±	24.69	104.59
Huagrasinga	Pull Quishuar	Organic	Landrace	2746.06	±	867.09	429.66	±	76.45	44.71	±	30.48	54.26
Huancala	San Francisco	Organic	Landrace	4150.75	±	368.43	1336.41	±	11.71	363.94	±	3.18	160.41
Inca	Achullay	Organic	Landrace	3481.44	±	651.89	962.6	±	283.69	227.69	±	109.33	118.03
Josefina	San Vicente	Organic	Landrace	3525.57	±	879.85	897.36	±	345.74	231.56	±	187	109.86
Morucela	San Vicente	Organic	Landrace	2556.94	±	151.96	865.93	±	43.1	273.39	±	56.88	110.35
Natividad Roja	Laime	Organic	Landrace	3700.06	±	218.07	1541.17	±	119.14	508.79	±	78.37	187.57
Norteña negra	Laime	Organic	Landrace	2509.89	±	42.16	790.59	±	49.37	248.11	±	25.09	100.99
Possorumi	Achullay	Organic	Landrace	3353.75	±	1082.38	1174.19	±	413.58	408.07	±	144.77	144.64
Puca shungo	Laime	Organic	Landrace	2560.27	±	19.85	765.69	±	206.15	221.1	±	107.03	97.56
Puña negra	San Francisco	Organic	Landrace	2028.2	±	183.93	597.53	±	100.44	196.97	±	112.52	78.46
Super chola	Candelaria	Conventional	Commercial	3254.2	±	527.86	665.63	±	125.11	166.97	±	112.52	82.30
Super chola	Laime	Organic	Commercial	3309.02	±	1535.27	716.29	±	153.85	130.08	±	71.83	88.38
Super chola	Pull Quishuar	Organic	Commercial	3387.52	±	0.11	1007.41	±	159.87	273.17	±	71.62	123.94
Toscaleña blanca	San Vicente	Organic	Landrace	3551.7	±	528.63	1143.52	±	228.68	353.78	±	53.27	139.87
Turca blanca	Laime	Organic	Landrace	4243.63	±	1231.59	1127.35	±	149.45	198.38	±	92.83	134.96

Turca morango	Laime	Organic	Landrace	5581.33	±	610.51	2161.34	±	461.69	655.14	± 169.63	250.52
Uvilla blanca	Laime	Organic	Landrace	2406.96	±	69.31	286.48	±	1.31	21.13	± 2.15	36.79
Uvilla negra	Pull Quishuar	Organic	Landrace	3348.84	±	718.46	738.93	±	4343.42	154.8	± 170.52	91.04

[a] Hill´s number q=0
[b] Mean across fields sampled for the corresponding cultivar
[c] Standard deviation for fields sampled for the corresponding cultivar
[d] Hill´s number q=1
[e] Hill´s number q=2
[f] Pielou´s evenness: Shannon diversity divided by the natural logarithm of species richness in a sample.

Table 9. Evaluation of Fungal Community structure (β diversity) differences across provinces, type of cultivars and province-type of cultivar interaction based on Bray-Curtis distance using ADONIS.

Adonis on Bray-Curtis distance	Df[a]	F Statistic	R^{2b}	Pr(>F)[b]
Province	9	1.293	0.156	0.001
Type of cultivar (landrace or commercial)	1	1.553	0.021	0.001
Province: Type of cultivar	1	1.468	0.020	0.003
Residuals	60			
Total	71			

[a] Degrees of freedom

[b] R-squared and P-value based on 999 permutations

Table 10. Evaluation of Bacterial Community structure (β diversity) differences across provinces, type of cultivars and province-type of cultivar interaction based on Bray-Curtis distance using ADONIS.

Adonis on Bray-Curtis distance	Df[a]	F Statistic	R^{2b}	Pr(>F)[b]
Province	9	2.981	0.289	0.001
Type of cultivar (landrace or commercial)	1	1.779	0.019	0.029
Province: Type of cultivar	1	4.291	0.046	0.001
Residuals	60			
Total	71			

[a] Degrees of freedom

[b] R-squared and P-value based on 999 permutations

Table 11. Significance of spatial, climatic and edaphic factors affecting fungal communities associated with rhizosphere potato, based on using envfit function from vegan and Mantel test.

			envit test		Mantel test	
En.var	Axis.1[a]	Axis.2[b]	R^2	P. value	Statistic	P. value
Mg	0,312	0,171	0,126	0,009	-0,076	0,869
Fe	-0,316	-0,175	0,130	0,011	0,339	0,001
Ca	0,166	0,288	0,111	0,019	0,003	0,475
mint	0,056	0,295	0,090	0,040	-0,007	0,486
maxt	0,053	0,265	0,073	0,064	0,021	0,388
radn	-0,113	-0,251	0,076	0,070	0,001	0,482
pH	0,172	0,206	0,072	0,078	0,199	0,005
m.s.a.l.	0,143	-0,179	0,053	0,152	0,041	0,233
Mn	0,016	0,222	0,049	0,162	0,129	0,042
K	0,176	-0,125	0,047	0,202	0,047	0,223
Zn	-0,114	-0,122	0,028	0,373	0,174	0,002
Cu	-0,043	-0,144	0,023	0,466	0,090	0,007
Latitude	-0,081	0,125	0,022	0,477	0,091	0,137
P	-0,131	0,007	0,017	0,496	0,197	0,012
rain	-0,004	-0,068	0,005	0,835	-0,041	0,674
Longitude	-0,030	0,047	0,003	0,909	0,064	0,174
N	-0,037	-0,004	0,001	0,952	0,158	0,007
OrgMatter	0,009	0,001	0,000	0,998	0,070	0,104

[a] Axis 1 explains 5.3% of the variability between communities
[b] Axis 2 explains 4.2% of the variability between comunities

Table 12. Significance of spatial, climatic and edaphic factors affecting bacterial communities associated with rhizosphere potato, based on using envfit function from vegan and Mantel test.

			envit test		Mantel test	
En.var	Axis.1 [a]	Axis.2 [b]	R^2	P.value	Statistic	P. value
pH	-0,390	-0,781	0,762	0,001	0,456	0,001
OrgMatter	0,585	0,179	0,374	0,001	0,218	0,001
N	0,503	0,093	0,261	0,001	0,244	0,001
P	-0,305	0,422	0,271	0,001	0,407	0,001
Fe	0,324	0,666	0,549	0,001	0,420	0,001
Zn	0,075	0,570	0,330	0,001	0,256	0,001
Mg	0,044	-0,421	0,179	0,002	0,017	0,369
Ca	-0,011	-0,385	0,148	0,004	0,123	0,025
m.s.a.l.	0,232	-0,256	0,120	0,009	0,237	0,001
Cu	-0,128	-0,229	0,069	0,094	0,058	0,032
mint	-0,209	-0,009	0,044	0,209	0,235	0,007
maxt	-0,190	0,062	0,040	0,245	0,274	0,001
Longitude	-0,117	0,137	0,032	0,338	0,198	0,002
Mn	-0,017	0,144	0,021	0,435	0,264	0,001
K	-0,132	-0,080	0,024	0,439	0,043	0,218
Latitude	-0,096	0,086	0,017	0,562	0,304	0,001
radn	0,090	-0,068	0,013	0,650	0,215	0,008
rain	-0,076	0,012	0,006	0,821	0,186	0,029

[a] Axis 1 explains 11.8% of the variability between communities,

[b] Axis 2 explains 17.4% of the variability between comunities

CHAPTER 2

Comparative Early Blight Pathobiome and Plant Microbiome Engineering.

Abstract

Early blight (EB) epidemics can lead to total defoliation and yield losses of economically important solanaceous crops. The Andean soils have an important role in the suppressiveness of some potato diseases, therefore an ecological approach involving reciprocal transplant was used in order to test the hypothesis of the possible effect of the microbiota associated with Andean soils to EB epidemics. Additionally, temporal changes in the microbial populations associated with specific Alternaria species in potato and tomato was assessed to identify possible microorganisms associated with disease suppression. The metabarcoding approach was used to compare the microbiota associated with potato and tomato inoculated with different species of Alternaria. There were temporal changes in the microbial communities induced by the presence of a different species of Alternaria. The approach developed here can be viewed as a model for studying interactions in other pathosystems involving necrotrophic pathogens.

Keywords: metabarcoding, Alternaria, microbiota, epidemiology

INTRODUCTION

Early blight (EB) is one of the most destructive fungal foliar diseases of potato (Solanum tuberosum L.) and tomato (Solanum lycopersium L.) crops worldwide. Yield losses can reach up to 80 % (Grigolli et al., 2011; Bessadat et al., 2017). Common symptoms on leaves are dark necrotic lesions with concentric rings (Van Der Waals et al., 2003). Severe EB epidemics can lead to total defoliation in short periods of time (Chaerani & Voorrips, 2006) and the causal agent of the disease was initially considered to be Alternaria solani Sorauer, an Ascomycete. Currently, different Alternaria spp. have been related to EB epidemics in potato and tomato crops (Rodrigues et al., 2010a; Gannibal et al., 2014; Bessadat et al., 2017). In the late 1990's two morphological species of Alternaria were shown to be associated with potato and tomato EB: A. grandis and A. linariae (syn. A. tomatophila), respectively (Simmons, 2000). These species have been confirmed by inoculation studies, molecular and metabolite profiling analyses (Andersen et al., 2008; Rodrigues et al., 2010a; Lawrence et al., 2013; Woudenberg et al., 2013; Gannibal et al., 2014; Woudenberg et al., 2014; Ozkilinc et al., 2018). In Brazil, A. grandis is the species most commonly found on potato crops and A. linariae can be found on tomato fields (Rodrigues et al., 2010a).

Plants can host a complex micro-ecosystem supporting different microbial communities on various different organs (Bulgarelli et al., 2013). The leaf associated microbiota is composed of a broad range of filamentous fungi, bacteria, and yeasts (Müller & Ruppel, 2014). The dynamics of the microbiota have been studied during different stages of the plant cycle (Kerdraon et al., 2019). Fungal and bacterial communities are the two major groups of the plant microbiome, and their interactions have an important role in shaping the environmental microbial communities and in

influencing fitness, colonization, or pathogenesis of the interacting partners (Chen et al., 2018).

Studies of plant, animal, and human epidemiology attempt to characterize the changes in the microbial populations associated with specific disease stages, and the temporal dynamics of the microbial population during disease progress (Cho & Blaser, 2012; Jakuschkin et al., 2016; Lebreton et al., 2019). In plant pathology, disease development has classically been considered as the result of the relationships among the pathogen, the plant, and the environment. This dogma, nevertheless, does not take into account the communities of commensal, mutualistic microorganisms and other pathogens that colonize the host plant along with the pathogen itself, and their potential effects on epidemics (Sapkota et al., 2017).

The microbiota associated to pathogens is known as the "pathobiome" (Vayssier-Taussat et al., 2014), which may have a fundamental role in pathogen persistence, transmission, and evolution. However, the complexity of the interactions and the limitations imposed by the technology available to properly understand the extant relationships hampered advancements in this area. Metabarcoding and high-throughput sequencing technologies progress enabled more detailed insights about pathogens during the epidemics (Kerdraon et al., 2019). Moreover, microbial communities change during the course of plant disease epidemics, probably modifying interactions between microorganisms over time (Kerdraon et al., 2019). The pathobiome role in early blight epidemics was long neglected. There are no studies focusing on the characterization and the temporal evolution of the microbiota interacting with the different species of Alternaria associated with potato and tomato EB. This knowledge could be and alternative for controlling EB in a more sustainable way, particularly for the strategic use of biological control measures.

Putative interactions between microorganisms were studied, explored and analyzed using different approaches. For instance, significant changes in leaf associated fungal and bacterial communities were detected following the infection of pedunculate oak with Erysiphe alphitoides (Jakuschkin et al., 2016), and potential candidate antagonists of toxigenic Fusarium spp. species present in maize residues were identified (Cobo-Díaz et al., 2019). In the case of the different species of Alternaria associated with EB, there is no information on the impact of these species on the microbiota of potato and tomato leaves, nor on the possible interactions that may exist.

Ecuador is located in a region considered to be the center of origin of potato, where there is high plant genotype diversity. Recently, it was demonstrated that the microbiota associated with Andean soils has an important role in the suppressiveness of some potato diseases (Orquera-Tornakian et al., 2018). For instance, EB is neither of widespread occurrence nor such a destructive disease to potato and tomato crops in the Andean countries. One hypothesis is: the soil associated-microbiome from the Andes suppresses EB epidemics. To test this hypothesis an ecological approach involving reciprocal soil transplant was used. The goal is to transfer the whole microbiome and to reproduce its functions and interactions in the new "environment" (Howard et al., 2017).

Various microbiome transfer approaches have been applied in agricultural systems, including direct soil transfer and soil washes (Howard et al., 2017). However, this approach has been intuitively used to study soil suppressiveness for more than half a century (Schlatter et al., 2017b). Most experiments, whether "traditional" or "modern" ones using NGS techniques, have shown the impact of microorganisms on disease intensity (Wagner et al., 2014; Yergeau et al., 2015; Panke-Buisse et al., 2015). Direct transfer of soil microbiome could potentially influence the composition, diversity, structure and functional properties of the leaf-associated microbiome. Thence, the goal of

this study was to identify the fungal and bacterial pathobiome interacting with A. grandis, A. linariae and A. solani during EB epidemics on potato and tomato crops. We were also interested in studying the impact of the soil microbiome transplant on the associated leaf microbiome of potato and tomato plants. Finally, we intended to assess the effects of microbiome transference on EB epidemics development caused by different Alternaria species. We hypothesized that (i) the soil microbiome transfer has an important effect on potato and tomato EB epidemics; (ii) the soil microbiome transfer affects the composition, diversity, structure and interactions of the associated leaf microbiome of potato and tomato plants; and (iii) potential antagonists of plant pathogens are enriched in native soils compared with commercial soils and, thus, may play a role in EB reduction.

MATERIAL AND METHODS

Soil sampling collection

Soil samples from three locations were collected. One sample was obtained from a landrace potato field in Ecuador (Lat 0° 0’ 54’’ S, Long 7° 29’ 19.4" W) on January 5th, 2018. A second sample was taken from a commercial potato field in Brazil (Lat 20° 45’ 37’’ S, Long 42° 52’ 04’’ W) on September 3rd, 2017. The third sample was taken from a tomato field in Brazil (Lat 42° 47’ 48’’W, Long 42° 47’ 48’’ W) on May 6th, 2018. All samples were taken using shovels, gathered at approximately 30 cm-deep, transported to the laboratory, and stored at 5°C until use on August 3rd, 2018. Samples were mixed, homogenized and chemical analyses were conducted at the Department of Soil Science of the Universidade Federal de Viçosa (Table 1).

Planting and reciprocal soil transfer experiment

Potato sprouts (c.v. Asterix) and tomato seeds (cv. Santa Clara) susceptible to EB were used in the experiments. Potato sprouts and tomato seeds were sown into the three different soils. Sterilized 0.1 L-plastic pots were used in the experiments. The pots were watered daily with sterile distilled water (SDW) to avoid the influence of the external microbiota in the experiments. Then, four-week-old seedlings were transplanted into 2L-pots containing a solarized soil-substrate mixture (3:1). Before planting, three biological replicates of the soil from the rhizosphere of potato and tomato plants were selected for DNA extraction and Illumina Miseq sequencing in order to evalute the initial potato and tomato rhizosphere microbiome that was already established.

Potato and tomato greenhouse experiment and different Alternaria species inoculation

The greenhouse experiments were set up as a randomized complete block design with two factors (soil type and Alternaria spp.). The first factor of each experiment had two levels (Brazilian soil and Ecuadorian soil) and the second factor had three levels (inoculation with A. solani, with A. grandis and water-control for the potato experiment and A. solani, A. linariae and water-control for the tomato experiment). Each treatment had three replicates and the potato and tomato experiments were carried out independently. Three isolates of Alternaria spp. were used in the experiments. One isolate of A. grandis (AS604) and one isolate of A. linariae (AS1000), these isolates were previously characterized by molecular tools. The Ex-type isolate of A. solani (CBS 109157) was included as a treatment in the potato and tomato experiments. Additional information about the isolates used in this study is presented in Table 2. All monosporic isolates are kept in the Culture Collection of the Populations Biology Laboratory at the Universidade Federal de Viçosa.

For inoculum production, sporulation was induced according to previously described procedures (Rodrigues et al., 2010b). Five-day-old colonies of Alternaria species were grown in V8 $CaCO_3$ agar (175 mL of V8 juice, 3g $CaCO_3$, 20 g agar, 1 L) at 25 ± 2°C, at 12 h-photoperiod. Superficial mycelium was removed using 10 mL of SDW and a clean paintbrush. The suspension was discarded, then the Petri plates were kept uncovered at 23 ± 2 °C, under near-UV light for 12 h and 12 h in the dark to induce sporulation. After 48 h, conidia were removed as above and a suspension was adjusted for 10^3 conidia/mL. The 45 days old-tomato and potato plants were sprayed until runoff with the inoculum suspension of each Alternaria species. After inoculation, plants were

kept for 12 h in a moist chamber at 23 °C, 12 h-photoperiod. After this time, the potato and tomato plants were transferred to greenhouse conditions.

Early blight severity assessments and leaf sampling

Early blight severity was assessed using a descriptive standard area diagram (SAD) (Duarte et al., 2013). Disease severity was assessed at every two days, beginning with the appearance of the first symptoms. Ten severity assessments were performed in each experiment.

For the potato experiment, two middle leaves were collected for each replicate from each treatment, at 24, 48, 96 and 144 h after inoculation. For the tomato experiment, two middle leaves were sampled from each replicate, from each treatment, at the same sampling times.

Sample processing and DNA extraction

Leaves from potato and tomato plants subjected to each treatment were sampled and submitted to surface disinfestation using 70% ethanol for 30s, 2% sodium hypochlorite for 5 min, and rinsed three times in SDW. The efficiency of the disinfestation process was checked by plating aliquots (0.1 mL) of the last water used during the disinfestation on Petri dishes with PDA medium (dextrose 20g, potato 200g, and agar 20 gL^{-1}) and incubated at 25 °C for 7 days. After confirming that epiphytes that could grow in culture medium were not present on tissues, 500 mg of leaf were used to DNA extraction. The leaves were lysed using steel beads on a TissueLyser LT (Quiagen, UK) for 3 minutes and placed in 20 mL of 1x PBS solution (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4) for 12 h under stirring. The liquid phase obtained was centrifuged at 4000 x g for 5 min at 10 °C to separate the remaining tissue fragments. The supernatant was centrifuged at 18000 x g for 30 minutes at 10°C to concentrate the pellet of the

microbial cells. The pellet obtained was used for DNA extraction using PowerSoil® DNA Isolation Kit (MoBio Laboratories Inc., Carlsbad, CA, USA), according to the manufacturer's recommendations. For the soil reciprocal transplant experiment, the DNA was extracted from a 0.25 g sub-sample soil using the same kit mentioned above. We quantified extracted DNA using NanoDrop® 2000 spectrophotometer (Thermo Fisher Scientific), and quality of DNA was checked on a 1% agarose gel with GelRed (Biotium, Fermont, CA). The DNA samples were stored at -80 °C until sequencing.

ITS rRNA gene and 16S RNA gene V4 amplification and sequencing.

Six genomic DNA samples from the reciprocal soil transplantation, 72 DNA samples from the potato experiment and 58 genomic DNA samples from the tomato experiment were lyophilized and sent to Environmental Sample Preparation and Sequencing Facility (ESPSF) of the Argonne National Laboratory (ANL) (Lemont, Illinois, USA), for paired-end amplicon sequencing using Illumina Miseq platform. All downstream processes (library preparation, amplification, and sequencing) were performed at ANL. All samples were processed employing the Illumina Miseq reagent kit v2 following the manufacturer's protocol.

To assess the fungal microbiota an amplification was performed using the primers ITS1f and ITS2 from the (ITS) region of the rRNA. The full protocol used is detailed at (http://press.igsb.anl.gov/earthmicrobiome/protocols-and-standards/its/). To assess the bacterial microbiota an amplification was performed using the primers 515F and 806R from the V4 region of the 16S rRNA combined with the sequencer adapter used in the Illumina flow cell (Caporaso et al., 2011, 2012). The16S protocol used is detailed elsewhere (http://press.igsb.anl.gov/earthmiCcrobiome/protocols-and-standards/16S/).

For both libraries, the PCR reactions were performed in 25 μL volumes, including 1 μL DNA template, 0.5 μL of each primer at 10 μM, 13 μL of PCR-grade water (Sigma),

10 μL 2x of Platinum^{TM} Hot Start PCR master mix (Thermo Fisher Scientific). For fungal amplifications, the conditions used were initial denaturation at 94 °C for 1 min, followed by 35 cycles of 94 °C for 30 s, 52 °C for 30 s, 68 °C for 30 s and a final extension of 7 min at 68 °C. The PCR conditions for bacteria were initial denaturation at 94 °C for 3 min, followed by 35 cycles of 94 °C for 45 s, 50 °C for 60 s, 72 ºC for 90 s and a final extension of 10 min at 72 °C.

Sequence processing

The sequences obtained from the Miseq runs were demultiplexed using the software CASAVA (Consensus Assessment of Sequence and Variation) version 1.8.2 (Eren et al., 2013). Further processing of sequences was carried out using the software Mothur version 1.39.5 (Schloss et al., 2009), following the standard protocol proposed by Kozich et al.(2013). The sequences were overlapped to form contiguous reads using Mothur's command make.contigs (Schloss et al., 2009). Reads containing any ambiguous bases and poor quality were then discarded. Bacterial sequences were aligned against the SILVA 16S r RNA gene reference alignment database (Pruesse et al., 2007) and then screened for the correct region. For the fungal sequences, it was not possible to align to a database due to the frequency of insertions and deletions. Thus, de novo alignment within the dataset was carried out (Misof & Misof, 2009). Sequences were pre-clustered and chimera detection and removal were performed. UNITE-ITS (Kõljalg et al., 2013) and GreenGenes (DeSantis et al., 2006) databases were used for the classification of the fungal and bacterial sequences. All OTUs identified as belonging to chloroplast and mitochondria were removed from the data set.

Data analysis and statistics

The sequences were then clustered into operational taxonomic units (OTUs) by uncorrected pairwise distances and neighbor clustering was performed. All further analyses were conducted in R v3.6.0 (R Development Core Team, 2017) with the packages vegan v2.4.4 (Oksanen, 2017) , phyloseq v1.19.1 (McMurdie & Holmes, 2013) , ggplot v2.2.1 (Wickham, 2011), AmpVis v2.0, and iNEXT v2.0.1.2 (Hsieh et al., 2016). For the α-diversity the exponents of Shannon and Simpson indices were estimated by Hill's numbers using the iNEXT package. From now on these exponents will be referred to as richness, Shannon and Simpson's indices. The divergence of microbial communities between samples was assessed by calculating the Bray-Curtis dissimilarity matrix, and then illustrated by PCoA. The Richness, Shannon and Simpson indices were used to assess the effect of each set of conditions of fungal and bacterial diversity.

The area under the disease progress curve (AUDPC) was correlated with α diversity. To know the role of the foliar associated microbiome from potato and tomato crops assessed by NGS on the different early blight epidemics, we investigated by correlating the values of AUDPC using the Spearman's rank correlation method, before doing that the different indices calculated in four sampling time were transformed using the same AUDPC approach, to check whether there was any temporal variation of diversity and, if positive, whether there was any relation with the temporal variation of EB intensity.

For characterization of the interactions within the different treatments we used ecological interaction networks calculated with SPIEC-EASI (Kurtz et al., 2015) for each dataset. We used the neighborhood selection as graphical inference model (Meinshausen and Bühlmann MB method). Networks were then analyzed with the igraph package

v.1.2.2 (Csardi & Nepusz, 2006). The dataset with Brazilian fungi belonging to two species of Alternaria (A.grandis and A. solani), the former commonly associated with potato and the latter species (A. linariae) frequently found on tomato were chosen and used as models for the network analysis. Basic features of the networks such as number of nodes, edges, modularity, clustering coefficient were analyzed and compared between the different models. Additionally, the network's degree of distribution was analyzed to determine if the network was small-word or scale-free type. Finally, the small-wordness score was checked to determine if the network was of the small-word type.

RESULTS

Composition, structure, and diversity of the rhizosphere microbiome of potato and tomato plants grown in soils from Brazil and Ecuador.

Using the internal transcribed spacer (ITS) region of rRNA it was possible to obtain 408,885 high-quality sequences clustered in 1643 operational taxonomic units (OTUs). The V4 region of the 16 SSU rRNA gene was used for bacteria and generated 262,732 high-quality sequences clustered in 287 OTUs.

Brazilian-soil grown potato and tomato contained in their rhizosphere significantly higher proportions of Ascomycota, represented primarily by Nectriaceae (25.3% and 10.8 % for potato and tomato rhizosphere, respectively) and Chaetomiaceae families (17.8 % and 5.8 % for potato and tomato rhizosphere, respectively) (Fig. 1). In contrast, the Ascomycota present in the microbiome of the rhizosphere of potato plants grown in Ecuador was represented primarily by the Pyrenomataceae (52.7 %) and the Mortierellomycota was represented by the Moriellaceae (11.2 %). Moreover, tomato plants grown in Ecuadorian soil contained higher proportions of Ascomycota and Mortierellomycota represented by the Nectriaceae (33%) and the Mortierellaceae (32 %) in their rhizosphere.

There were high percentages of unclassified bacteria at the kingdom level (43.4 % and 45.8 % for potato and tomato rhizosphere, respectively) and most Proteobacteria (23.5% and 35.1 % for potato and tomato rhizosphere, respectively), Actinobacteria (4.9% for potato) and Alphaproteobacteria (8.7% and 3.6 % for potato and tomato rhizosphere, respectively) could only be classified at the phylum level in the rhizosphere of plants grown in Brazilian soils. The same way Ecuadorian rhizosphere had highest levels of bacteria unclassified at the kingdom level (27.60% and 41.0 % for potato and

tomato rhizosphere, respectively), Proteobacteria unclassified at the phylum level (21.3% and 17% for potato and tomato rhizosphere, respectively) and Actinobacteria at the phylum level (6% and 11.6% for potato and tomato rhizosphere, respectively) (Fig.4).

The PCoA on Bray-Curtis (considering abundance) indices produced similar clusters with Brazilian rhizosphere fungal communities associated with positive axis two score, whereas Ecuadorian rhizosphere communities were more related with negative axis two scores. Furthermore, in each of the soil clusters, two subgroups can be detected, one for the potato rhizosphere and the other for the tomato rhizosphere. For the fungal communities and their dispersion, the first and second axes explained 41.4 % of the variance (Fig. 2). The PCoA analysis for bacteria (Fig. 5) showed that four groups were formed according to the soils and crops. The axes explained 41.2% of the variance. In order to determine the contribution of the factors (soil and crop) to the total variance, a permanova (adonis test) was performed and the effects of both factors were significant ($P<0.05$). The soil factor explained 19.1 % of the variance associated with fungal community and 22.5 % of the bacteria community structure. The crop factor explained 17.1 % of the variance associated with fungal community and 17.8 % of the bacterial community.

The fungal α-diversity for the Ecuadorian differed from the Brazilian soil samples according to the diversity indices. Fungal microbiota associated with potato plants grown in Ecuadorian soil had significantly higher richness (331.97 ± 27.22), Shannon (32.83 ± 6.44) and Simpson indices (11.28 ±1.36) compared to potato plants grown in Brazilian soil (Fig. 3). On the other hand, only the Simpson index was higher for the fungal communities (15.23±1.36) in the Ecuadorian tomato rhizosphere. Conversely, there was greater diversity for bacterial communities associated with tomato plants when they were grown in Brazilian soil compared to tomato grown in Ecuadorian soil.

Microbiota community composition, structure and diversity associated with different soil types, solanaceous crops and Alternaria species.

The 872,018 fungal sequences for the potato experiment were clustered in 1133 OTUs. Thirteen families of the Ascomycota phylum, and 15 families of Basidiomycota from potato leaves associated with Brazilian soils were identified. In contrast, eight families of the Ascomycota and 17 of the Basidiomycota associated with Ecuadorian soils were found (Figs. 8 and 9).

The V4 region of the 16 SSU rRNA gene was used for assessing bacteria diversity and the 1,049,904 sequences clustered in 68 OTUs. The bacterial taxonomy composition for all potato treatments included members from two phyla, Proteobacteria and Firmicutes. Four families, Moraxellaceae, Pseudomonadaceae, and Bacillaceae were identified (Fig. 10). The potato composition profile of the fungal and bacterial communities varied according to soil, Alternaria species and sampling time (Fig. 7 and 10).

To estimate β-diversity among samples from potato leaves we used a Bray-curtis distance matrix. Most of the variation in the total dataset from the Brazilian and Ecuadorian samples could be attributed to Alternaria species and sampling time (Fig. 19). The permanova analysis revealed significant differences ($P<0.01$) for Alternaria species and sampling time, each factor accounting for low, but significant, percentages of the total variance, 7.1 % and 2.8 % respectively. The permanova was carried out for bacteria associated to potatoes, but neither Alternaria spp. nor soil types were significant. Thus, no PCoAs were performed.

Soil, Alternaria species and sampling time had significant effects on the diversity of potato fungal communities (Fig. 21). Plants associated with Brazilian soils and inoculated with A. solani showed highest values of species richness (107 ± 22.5) at 24 h after inoculation (a.i) decreasing to 48.8 ± 16.88 at 144 h. On the other hand, plants not inoculated had the lowest species richness values (39.1 ± 14.89) at 24 h a.i. increasing to 54.8±7.98 at 144 h a.i. Non-inoculated potato plants grown in Ecuadorian soils had the highest values of richness (60.8 ± 46.64) at 24 h a.i. and, as observed with the situation in Brazilian soils, the values of the indices also decreased at 144 h a.i. (26.5 ± 4.95). The lowest species diversity was presented by plants inoculated with A. grandis (32.3 ± 9.9) at 24 h a.i.

The same parameters also had important effects on bacterial potato communities diversity (Fig. 22). The values of microbial richness in potato plants associated with Brazilian soil and inoculated with A. grandis varied from 1 at 24 h a.i. to 4.17 ± 2.93 at 144 h a.i., from 2.5 ± 1.80 at 24 h a.i. to 5.75 ± 3.89 at 48 h a.i. when the plants were inoculated with A. solani and from 3.81 ± 1.59 at 24 h a.i. to 14.50 ± 17.68 for non-inoculated plants. On the other hand, the plants grown in Ecuadorian soil and inoculated with Alternaria species had the lowest values of richness at 24 h a.i. and higher values of richness at 96h.

For the tomato experiment, the 483,001 fungal sequences were clustered in 491 OTUs. Fifteen families of the Ascomycota phylum, and 10 families of Basidiomycota from tomato leaves associated with Brazilian soils were identified. On the other hand, 14 families of Ascomycota and 16 of Basidiomycota associated with Ecuadorian soils were found in tomato leaves (Figs. 14 and 15).

The V4 region of the 16 SSU rRNA generated 879,200 sequences clustered in 60 OTUs. The bacterial communities composition for plants grown in Brazilian soils

included members of the Cyanobacteria and Proteobacteria phyla and three families, Pseudomonadaceae, Moraxellaceae, and Xanthomonadaceae. The microbiota associated with plants grown in Ecuadorian soils were composed by Pseudomonadaceae, Moraxellaceae, Xanthomonadaceae, and Exiguobacteraceae, all of the Proteobacteria phylum and another unclassified family belonging to the Cyanobacteria phylum. Soil type, Alternaria species and sampling time affected the composition of the microbiota of tomato leaves (Figs.16, 17 and 18).

In both, soils of Brazil and Ecuador, a possible structuring of the fungal communities was found and the effect of inoculation with different species of Alternaria (permanova <0.05) was evidenced by the PCoA. The total variance explained using the PCoA was 24.6% for plants grown in Brazilian soils and 35.7% for plants grown in Ecuadorian soil (Fig. 20). Similarly, there was no contribution (permanova >0.05) of soil or species of Alternaria in the total variance of the diversity of bacteria communities associated with potato plants grown in Ecuadorian soil and no PCoA was performed.

The fungal α-diversity differed between tomatoes grown in different soils and markedly between plants inoculated with different species of Alternaria. The plants from Brazilian soils and non-inoculated showed highest values of species richness (22.7 ± 10.66) at 24 h after inoculation (a.i.) increasing to 23.56 ± 6.03 at 96 h a.i., but the plants inoculated with A. linariae presented the highest values of diversity at 96 h a.i. In contrast, highest values of species richness (25.33 ± 5.88) were recorded for communities associated with plants grown in Ecuadorian soil inoculated with A. solani at 24 h. The lowest value of richness was recorded for communities associated with non-inoculated plants (Fig. 23). The values of bacterial richness in non-inoculated tomato plants grown in Brazilian soil were highest at 24 h a.i. For the plants inoculated with A. solani, the highest species richness was recorded at 24 and 96 h a.i. (Fig. 24).

Correlation of fungal and bacterial diversity with early blight epidemics

The different species of Alternaria had an effect on the intensity of early blight epidemics on potato and tomato ($P<0.01$) (Figs. 25 and 26). The direct transfer of soil had an effect only on the epidemics on potato. A. solani had the lowest values of severity in potato and tomato crops (Fig. 25).

There was no correlation between the fungal microbiota associated with potato leaves and diversity. In contrast, the richness of bacterial microbiota from potato leaves was negatively correlated with AUDPC (rho = -0.81; $P=0.049$), but there was no correlation for Shannon and Simpson indices with AUDPC. The Shannon diversity index for the fungal microbiota from tomato leaves was negatively correlated with AUDPC (rho= -0.89, $P = 0.014$), but there was no correlation between AUDPC and species richness and Simpson's index. There was no correlation between species richness, Shannon and Simpson's diversity indices and AUDPC of bacterial microbiota from tomato leaves.

Ecological interactions networks.

The networks obtained are shown in Figs. 27 to 30. The A. grandis network presented higher number of nodes (395), edges (3063) and modularity (0.67) than the A. solani network (nodes = 358, edges = 2548, modularity = 0.07), suggesting more connections and interrelations between fungal communities. The clustering coefficient was similar between the two networks (0.55 for A. grandis and 0.53 for A. solani). In contrast, the A. linariae network presented 129 nodes, 400 edges, modularity = 0.73, and the clustering coefficient was 0.57. Based on the power law and the smallwordness score the networks for A. grandis ($P<0.01$ and smallwordness score =3.97) and A. solani

(P<0.01 and smallwordness score=3.69) can be considered as scale-free networks. Conversely, the network for A. linariae can be considered as small word (P>0.01 and smallwordness score = 0.86).

DISCUSSION

Soil amendments and direct transfer soil have been shown to have a markedly effect on the microbiome of the rhizosphere, but beneficial effects were mostly assessed for the management of soil-borne diseases (Inderbitzin et al., 2017; Howard et al., 2017). Here we describe leaf associated fungal and bacterial community composition, structure and diversity from potato and tomato and the potential effects of direct soil transfer inferred by high-throughput DNA sequencing to early blight epidemics caused by different species of Alternaria.

The approach used for soil microbiome transfer had a remarkable effect on the microbiome of potato and tomato rhizosphere. After four weeks of incubation the transplanted microbiomes differed to a great extent. This is consistent with previous findings in which different profiles of composition were observed when this approach was used (Howard et al., 2017). Other studies using molecular and cultivation-based methods have assessed the fungal and bacterial communities present in different agricultural systems (van Geel et al., 2015; Al-Sadi et al., 2015) and have shown that fungal communities vary with the soil physicochemical properties and cropping systems (Thomson et al., 2015; Huang et al., 2015).

The role of the host in structuring the rhizosphere microbiome of potato and tomato plants was evident. Both host plants are from the same family, Solanaceae, but the microbiota of potato differed from that of tomato even when these plants were grown in the same soil. This fact was demonstrated in other studies where specific plant genotypes and conditional environmental conditions contribute to selecting and structuring specific microbiota from the soil according to their own needs (Bodenhausen et al., 2014; Edwards et al., 2015; Pérez-Jaramillo et al., 2016).

Additionally, high fungal diversity was found in potato associated with Ecuadorian soils. This may be related with the lower EB severity on potato plants in Ecuadorian soil compared to plants grown in Brazilian soils. Root microbiomes are prominent diverse (Engelbrektson et al., 2012; Bulgarelli et al., 2012) and some of the diversity may arise from particular microbes being of benefit only to special hosts under particular conditions or stresses, such as pathogens (Gaiero et al., 2013). Native root-associated bacteria in Nicotiana attenuata reduced damage to sudden-wilt disease caused by a Fusarium-Alternaria complex in a field plot after consecutive planting of N. attenuata (Santhanam et al., 2015). Our research supports the link between diversity and disease protection. Albeit there exists a theoretical relationship between diversity and the strength of a microbiome's disease-resistance effect, there is little empirical evidence to support or disprove this (Morella et al., 2019). The results reported here are more related to support the effects of diversity in reducing disease intensity.

To date, there is almost no research on temporal variation of microbiota associated with aerial plant pathogens. The few studies available are on microbiota associated with root pathogens. In our study, the profile of abundances of fungal and bacterial communities associated with potato and tomato and diversity were influenced by Alternaria species inoculation and sampling time. Similar results were described when using microbial inoculants containing plant growth-promoting rhizobacteria. The supply of microbes affected the diversity, evenness, richness and bacterial profile composition in sprouting broccoli roots (Gadhave et al., 2018).

Members of the Cladosporiaceae family can be saprotrophs and strong fungal competitors in necrotic tissues. They can be involved in complex symbiotic relationships with plants, and they have the potential to be used as biological agents. For instance, Cladosporium cladosporioides and C. pseudocladosporioides were studied as antagonists

of Puccinia horiana, biotrophic fungi causal agent of chrysanthemum white rust (Torres et al., 2017). For the potato experiment, the highest abundances of fungi from the Cladosporiaceae family was found in the inoculated and non-inoculated plants with different Alternaria species. The levels of abundance of the Cladosporiaceae between the plants inoculated with Alternaria and not inoculated in Brazilian and Ecuadorian soils were different. The inoculated plants had high number of members of the Cladosporiaceae in the early stages of the infection, while in non-inoculated plants the number of individuals of this fungal family remained in equilibrium during the evaluation period. On the other hand, for the tomato experiment, both Brazilian and Ecuadorian soils, the highest levels of Cladosporiaceae was detected in non-inoculated plants at 96 h.

The use saprophytic fungi as competitors has also been tested for necrotrophic pathogenic fungi in perennial systems. The control of some plant diseases by reducing initial inoculum produced on necrotic leaves is possible. The use of Ulloclaudium atrum to control gray mold in strawberry crops was tested under low disease pressure and gray mold intensity was reduced in four of seven experiments (Boff et al., 2002). The clear role of the Cladosporiaceae is still unknown, but in natural conditions, without perturbations of other competitors, they may inhabit potato tissues without causing damage.

The high number of families of the Basidiomycota found in both potato and tomato leaves associated with Ecuadorian soils may indicate an underexplored group of fungi to be used as biological control agents. This group of fungi can be a rich source of natural antibiotics and other products. The secondary metabolites produced by them possess antimicrobial properties that have been tested against pathogenic fungi with good results (Sivanandhan et al., 2017).

Many members of the Bacillaceae family are known to suppress the growth of several fungal pathogens by producing antifungal compounds (Berg, 2009). It is worth mentioning that in the potato plants grown in Brazilian soil, the average bacterial profiles of Bacillaceae were maintained at higher averages (25%) in non-inoculated plants than in inoculated ones (16.9 for A. grandis and 15.08% for A. solani). We hypothesize that inoculation with a necrotrophic pathogen causes an imbalance in the bacterial communities of the Bacillaceae which reduces the defense response of plants. This can be related with the negative correlation (-0.81) between richness index and AUDPC.

In the potato and tomato experiments there was greater virulence of A. grandis in relation to A. solani and of A. linariae in relation to A. solani, respectively. This was already reported (Rodrigues et al., 2010b; Wolters et al., 2019). Interestingly, the abundance profiles of fungi of the Pleosporaceae, related to Alternaria, changed in each of the treatments. In the case of potato associated with Brazilian soil and inoculated with A. grandis the lowest values were recorded in the first hours of the infection, and the highest percentages of relative abundance at 144 h after inoculation. In contrast, the percentage of Pleosporaceae members in plants inoculated with A. solani was highest in the first hours of inoculation and maintained high values during the evaluation period. We hypothesized that A. solani rapidly colonizes and infects the internal tissues of potato plants, leading to relative high abundance, what does not happen with A. grandis that apparently during the first hours of the process of colonization and infection grows more slowly. In contrast, the relative abundance of Pleosporaceae associated with A. solani was highest at early stages of the disease, but not efficient. This could be due to the triggering of an early response of defense by the plant. Conversely, in the potato plants associated with Ecuadorian soils the two-species had highest levels in the early stages of

the infection, but decreasing during the epidemic. In this case, there may exist a suppressive mechanism associated to soil diversity not deciphered yet.

In tomato, the abundance of Pleosporaceae in plants grown in Brazilian soil and inoculated with A. linariae and A. solani were highest at 24 and 96h. For the tomatoes grown in Ecuadorian soil, the values of the relative abundance of the Pleosporaceae were highest for the two Alternaria species at 24 h and A. linariae had a small reduction at 96h. Therefore, there was no clear pattern of association. However, the microbiome specificity could have a role in the non-reduction of the disease and the abundance of the Pleosporaceae, since specific hosts shape their microbiome according to their needs and the soil microbiome came from potato. Probably more complex microbiome interrelationships are involved.

In the case of the two pathogens associated with potato EB (A. grandis and A. solani), the modularity was higher in A. grandis' network indicating that this network had dense connections within certain group of nodes and sparse connections between these groups. A. linariae had higher modularity in relation to A. solani, showing that the interactions in this case are also dense. The high virulence of these two species in their specific hosts in comparison to A. solani the classical causal agent of EB can help explain the results. Higher numbers of nodes and edges in the A. grandis network indicate higher microbiome members interacting in that network and higher number of interactions. Thus, this demonstrates the greatest complexity present in this epidemic. Additional studies will be developed using co-occurrence analyses to try to elucidate the role and putative interactions of all members involved in the networks.

The direct transfer soil had an effect on EB potato epidemic and influenced the composition and diversity of the associated leaf microbiome of potato and tomato. No classical antagonists were found but the role of fungi belonging to the Basidiomycota

phylum found frequently needs to be disentangled. There was no correlation between diversity indices and disease score. This suggests that different pathobiomes may be associated with the different species of Alternaria. This research evidences the complexity of fungal and bacterial communities relationships and provides new insights into the possible interactions between natural microbiota of potato and tomato with emphasis on pathogens.

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FIGURE LEGENDS

Figure 1. Heatmap depicts the relative abundance percentage of fungal families associated with the rhizosphere from potato and tomato plants grown in different soils from Brazil or Ecuador.

Figure 2. Beta diversity estimates using Principal Coordinates Analysis (PCoA) with Bray Curtis distances calculated from relative OTU abundances for fungal communities associated with the rhizosphere of potato and tomato plants grown from Brazil and Ecuador.

Figure 3. Alpha diversity metrics. A) Species richness, B) Shannon, and C) Simpson indices of fungal communities associated with the rhizosphere of potato and tomato plants grown in Brazil and Ecuador. Center line in rectangles represents the median value. The upper end lower lines of the boxes represent the third and first quartiles, respectively. Whiskers represent ± 1.5 times the interquartile range. The black dots represent outliers.

Figure 4. Heatmap depicts the relative abundance percentage of bacteria families associated with the rhizosphere from potato and tomato plants grown in different soils from Brazil or Ecuador.

Figure 5. Beta diversity estimates using Principal Coordinates Analysis (PCoA) with Bray Curtis distances calculated from relative OTU abundances for bacterial communities associated with the rhizosphere of potato plants grown in Brazil and Ecuador .

Figure 6. Alpha diversity metrics. A) Species richness, B) Shannon, and C) Simpson indices of bacterial communities associated with the rhizosphere of potato and tomato plants grown in Brazil and Ecuador. Center line in rectangles represents the median value. The upper end lower lines of the boxes represent the third and first quartiles, respectively. Whiskers represent ± 1.5 times the interquartile range. The black dots represent outliers.

Figure 7. Temporal Relative abundance of leaf fungal communities of potato inoculated with different species of Alternaria (A. grandis, A. solani and non-inoculated (without). (A) Plants grown in Brazilian soil (B) Plants grown in Ecuadorian soil.

Figure 8. Heatmap depicts the temporal relative abundance of leaf fungal communities of potato growth in Brazilian soil and inoculated with different species of Alternaria (A. grandis, A.solani and non-inoculated (without)).

Figure 9. Heatmap depicts the temporal relative abundance of leaf fungal communities of potato growth in Ecuadorian soil and inoculated with different species of Alternaria (A. grandis, A. solani and non-inoculated (without)).

Figure 10. Temporal relative abundance of leaf bacterial communities of potato inoculated with different species of Alternaria (A. grandis, A. solani and non-inoculated (without)). (A) Plants grown in Brazilian soil (B) Plants grown in Ecuadorian soil

Figure 11. Heatmap depicts the temporal relative abundance of leaf bacterial communities of potato growth in Brazilian soil and inoculated with different species of Alternaria (A. grandis, A. solani and non-inoculated (without)).

Figure 12. Heatmap depicts the temporal relative abundance of leaf bacterial communities of potato growth in Ecuadorian soil and inoculated with different species of Alternaria (A. linariae, A. solani and non-inoculated (without)).

Figure 13. Temporal Relative abundance of leaf fungal communities of tomato inoculated with different species of Alternaria (A. linariae, A. solani and non-inoculated (without)). (A) Plants grown in Brazilian soil (B) Plants grown in Ecuadorian soil.

Figure 14. Heatmap depicts the temporal relative abundance of leaf fungal communities of tomato growth in Brazilian soil and inoculated with different species of Alternaria (A.linariae, A.solani and non-inoculated (without)).

Figure 15. Heatmap depicts the temporal relative abundance of leaf fungal communities of tomato growth in Ecuadorian soil and inoculated with different species of Alternaria (A.linariae, A.solani and non-inoculated (without)).

Figure 16. Temporal relative abundance of leaf bacterial communites of tomato inoculated with different species of Alternaria (A. linariae, A. solani and non-inoculated (without)). (A) Plants grown in Brazilian soil (B) Plants grown in Ecuadorian soil.

Figure 17. Heatmap depicts the temporal relative abundance of leaf bacterial communities of tomato growth in Brazilian soil and inoculated with different species of Alternaria (A.linariae, A. solani and non-inoculated (without)).

Figure 18. Heatmap depicts the temporal relative abundance of leaf bacterial communities of tomato growth in Ecuadorian soil and inoculated with different species of Alternaria (A. linariae, A.solani, non-inoculated (without)).

Figure 19. Beta diversity estimates using Principal Coordinates Analysis (PCoA) with Bray Curtis distances calculated from relative OTU abundances for fungal communities of potato growth in A) Brazilian and B) Ecuadorian soil and inoculated with different species of Alternaria (A .grandis, A.solani, non-inoculated (without)).

Figure 20. Beta diversity estimates using Principal Coordinates Analysis (PCoA) with Bray Curtis distances calculated from relative OTU abundances for fungal communities of tomato growth in A) Brazilian and B) Ecuadorian soil and inoculated with different species of Alternaria (A. linariae, A. solani and non-inoculated (without).

Figure 21. Alpha diversity metrics. A) Species richness, B) Shannon, and C) Simpson indices of leaf fungal communities of potato incoculated with different species of Alternaria (A.grandis, A .solani and non-inoculated (without). Center line in rectangles represents the median value. The upper end lower lines of the boxes represent the third and first quartiles, respectively. Whiskers represent ± 1.5 times the interquartile range. The black dots represent outliers.

Figure 22. Alpha diversity metrics. A) Species richness, B) Shannon, and C) Simpson indices of leaf bacterial communities of potato incoculated with different species of Alternaria. (A. grandis, A. solani and non-inoculated (without)) Center line in rectangles represents the median value. The upper end lower lines of the boxes represent the third and first quartiles, respectively. Whiskers represent ± 1.5 times the interquartile range. The black dots represent outliers.

Figure 23. Alpha diversity metrics. A) Species richness, B) Shannon, and C) Simpson indices of leaf fungal communities of tomato inoculated with different species of Alternaria (A. linariae, A.solani and non-inoculated (without)). Center line in rectangles represents the median value. The upper end lower lines of the boxes represent the third and first quartiles, respectively. Whiskers represent ± 1.5 times the interquartile range. The black dots represent outliers.

Figure 24. Alpha diversity metrics. A) Species richness, B) Shannon, and C) Simpson indices of leaf bacterial communities of tomato inoculated with different species of Alternaria (A. linariae, A. solani and non-inoculated (without)). Center line in rectangles represents the median value. The upper end lower lines of the boxes represent the third and first quartiles, respectively. Whiskers represent ± 1.5 times the interquartile range. The black dots represent outliers.

Figure 25. Soil transfer and Alternaria species effect on the EB epidemics. Disease progress curve of A. grandis and A. solani in potato plants growth in A) Brazilian soil, B) Ecuadorian soil. C) Boxplots showing the area under the disease progress curve (AUDPC) of potato grown in Brazilian and Ecuadorian soils and inoculate with A. grandis and A.solani.

Figure 26. Soil transfer and Alternaria species effect on the EB epidemics. Disease progress curve of A. linariae and A. solani in tomato plants growth in A) Brazilian soil, B) Ecuadorian soil. C) Boxplots showing the area under the disease progress curve (AUDPC) of tomato grown in Brazilian and Ecuadorian soils and inoculate with A. grandis and A.solani.

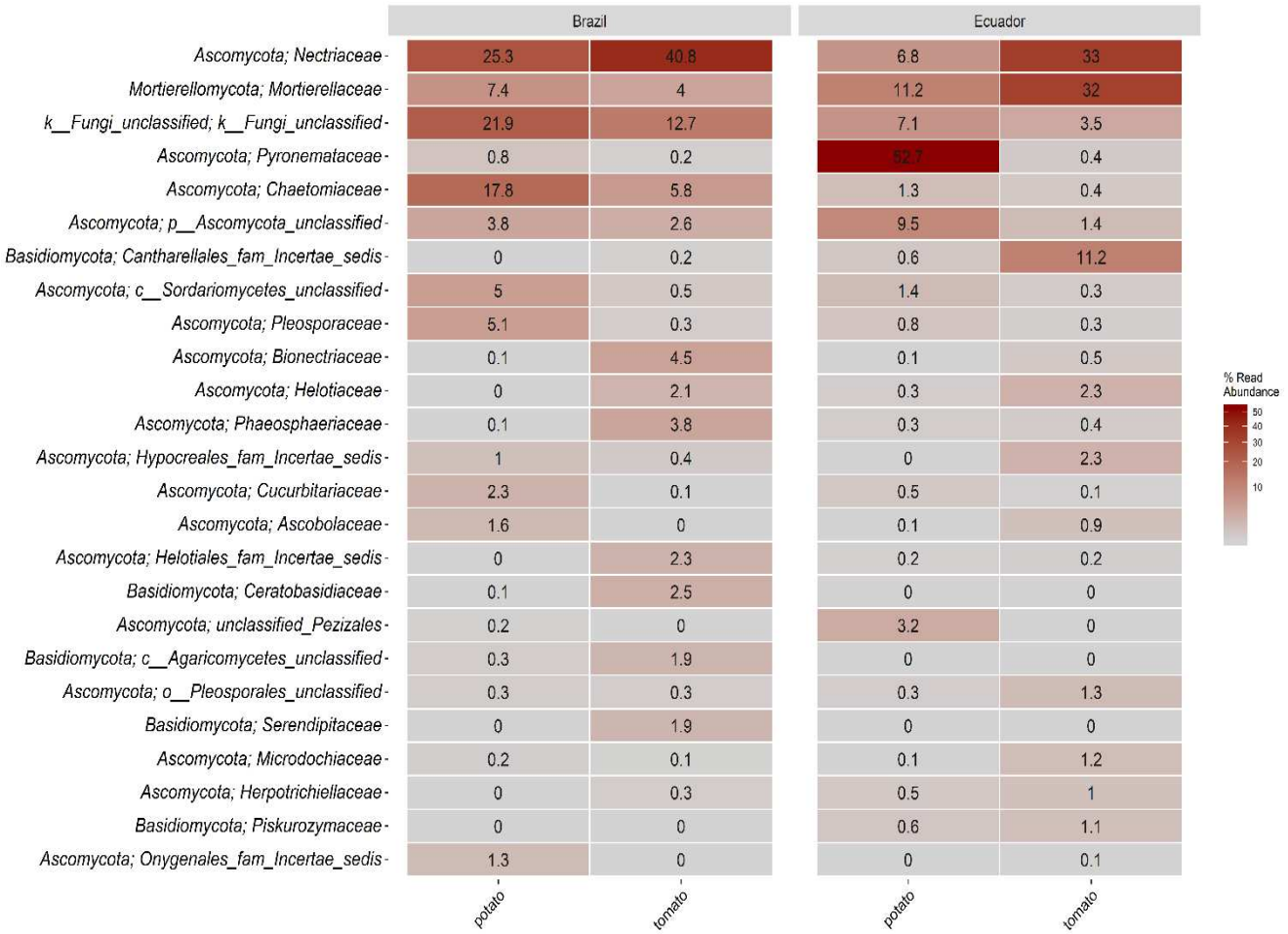
Figure 27. Microbiome associated-network . A) Alternaria grandis, B) Alternaria solani, C) Alternaria linariae.

Figure 28. Alternaria grandis microbiome associated-network

Figure 29. Alternaria solani microbiome associated-network

Figure 30. Alternaria linariae microbiome associated-network

FIGURES AND TABLES



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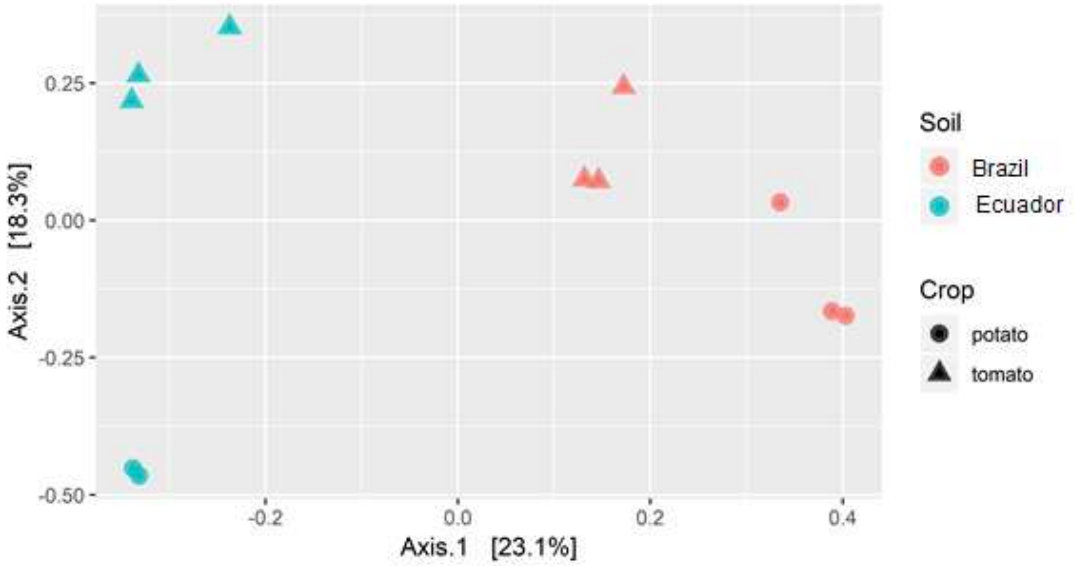

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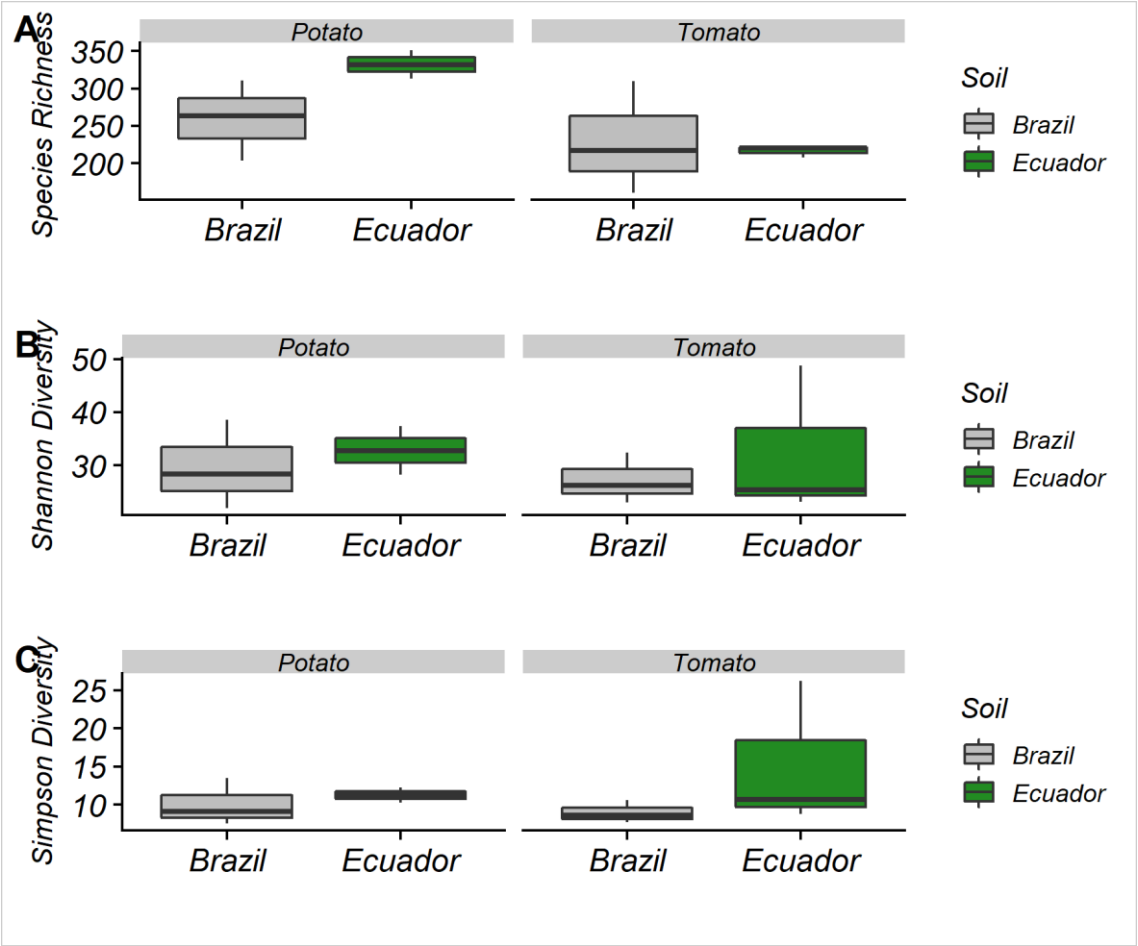

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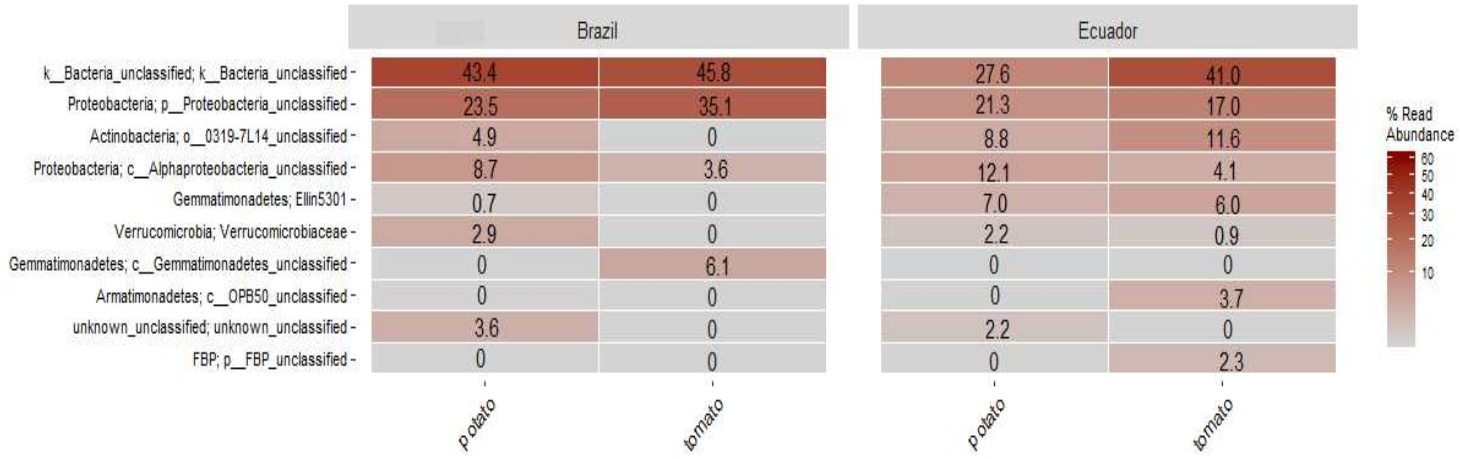

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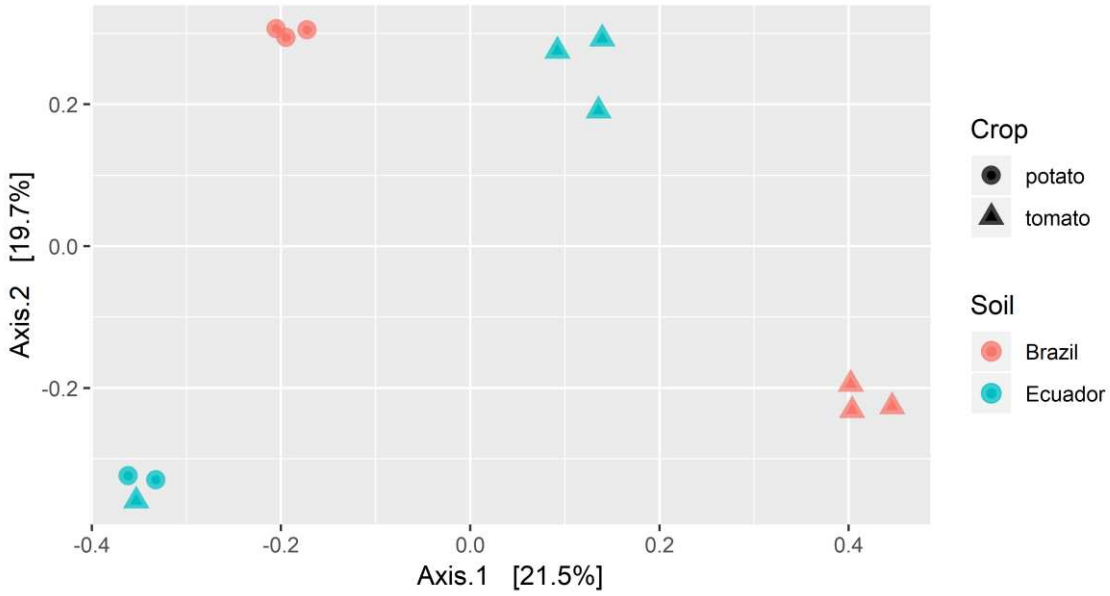

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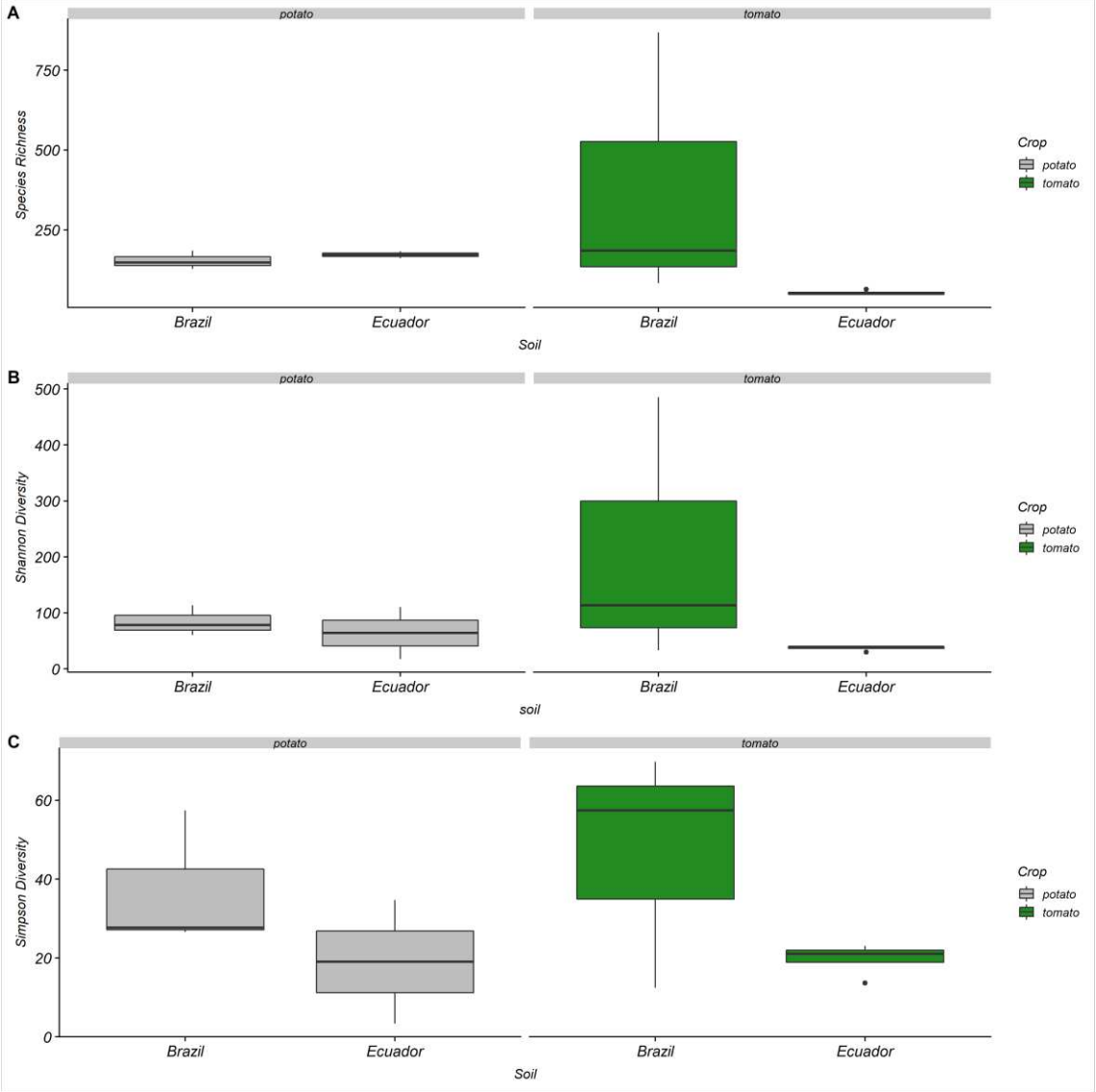

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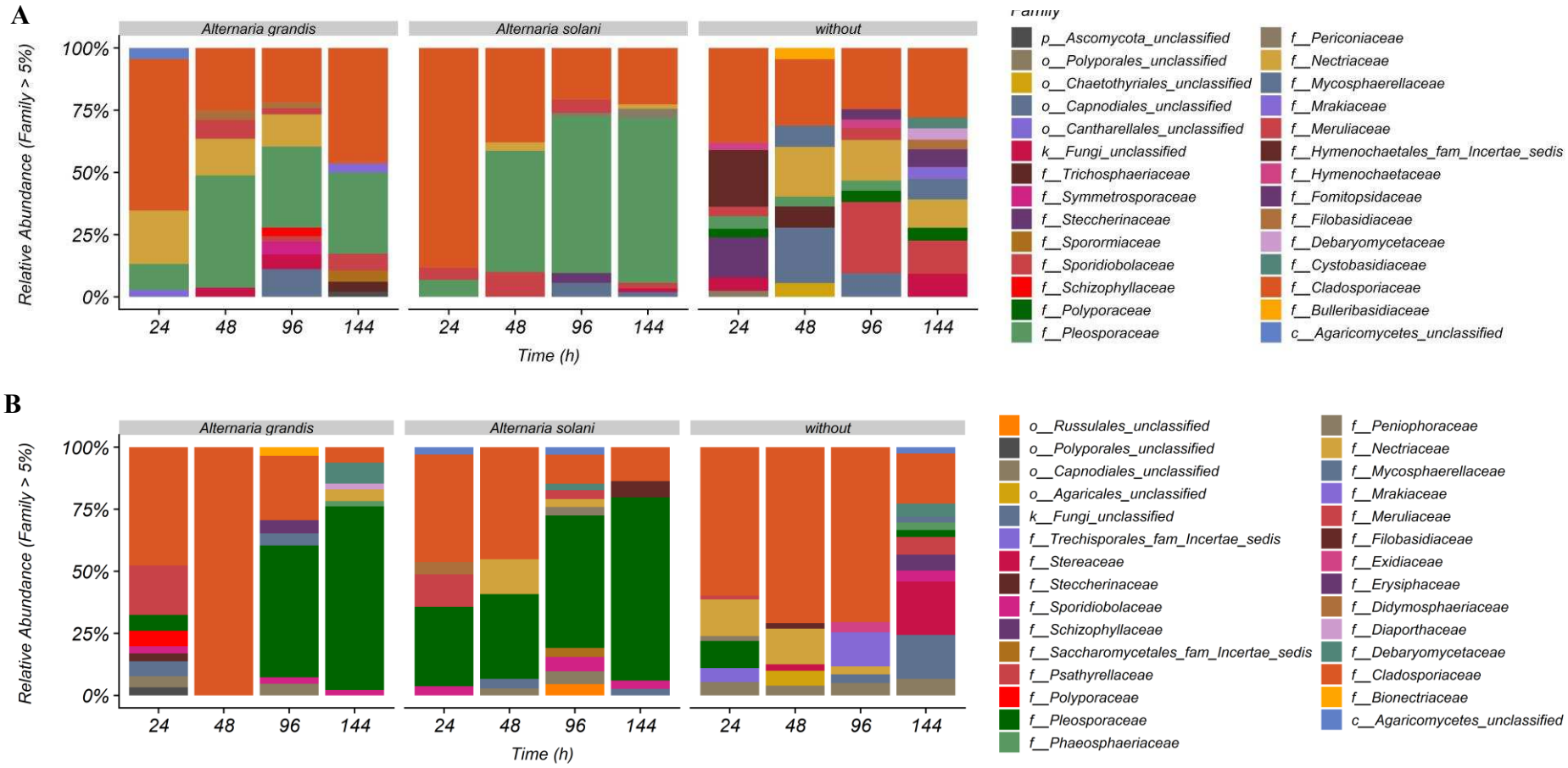

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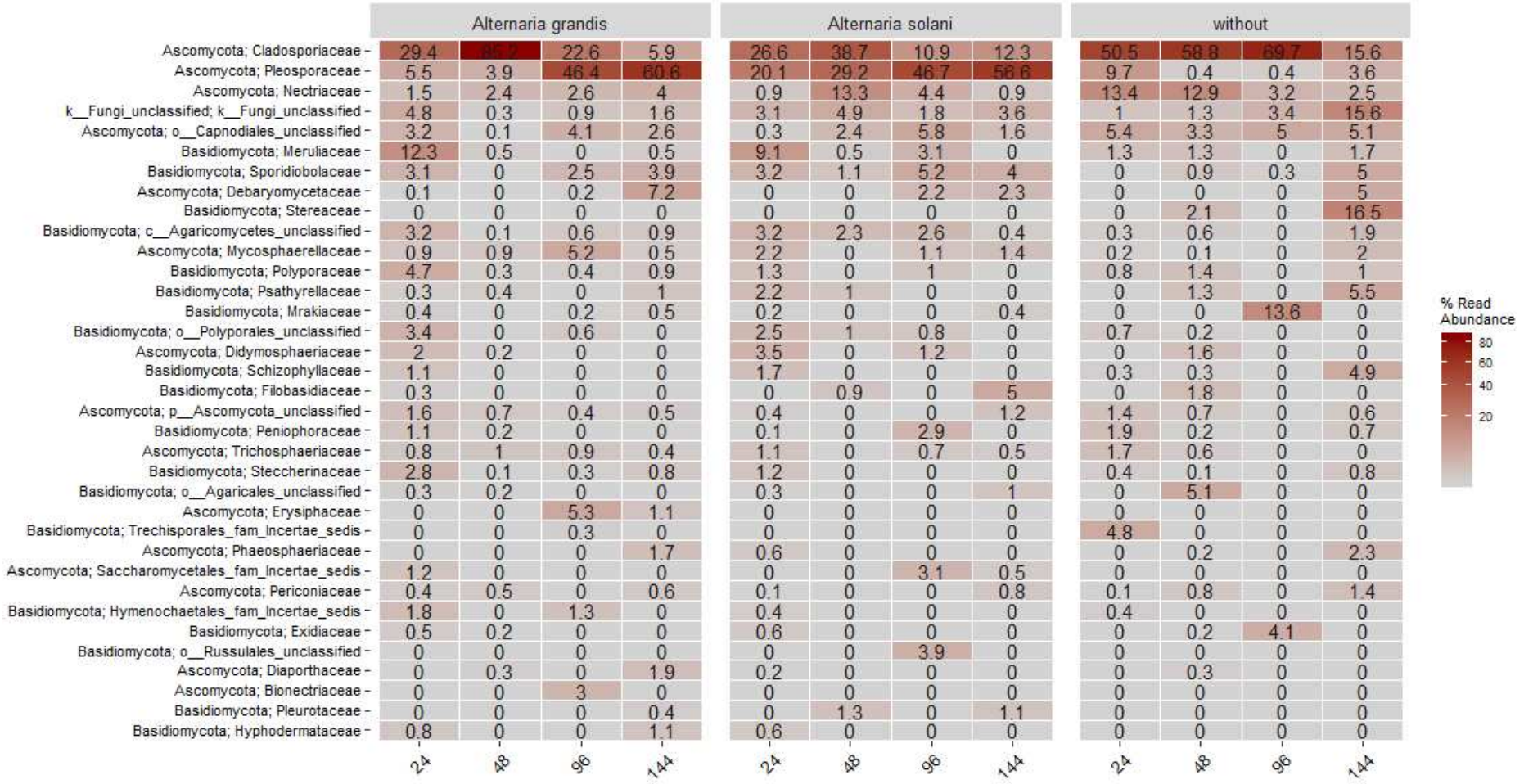

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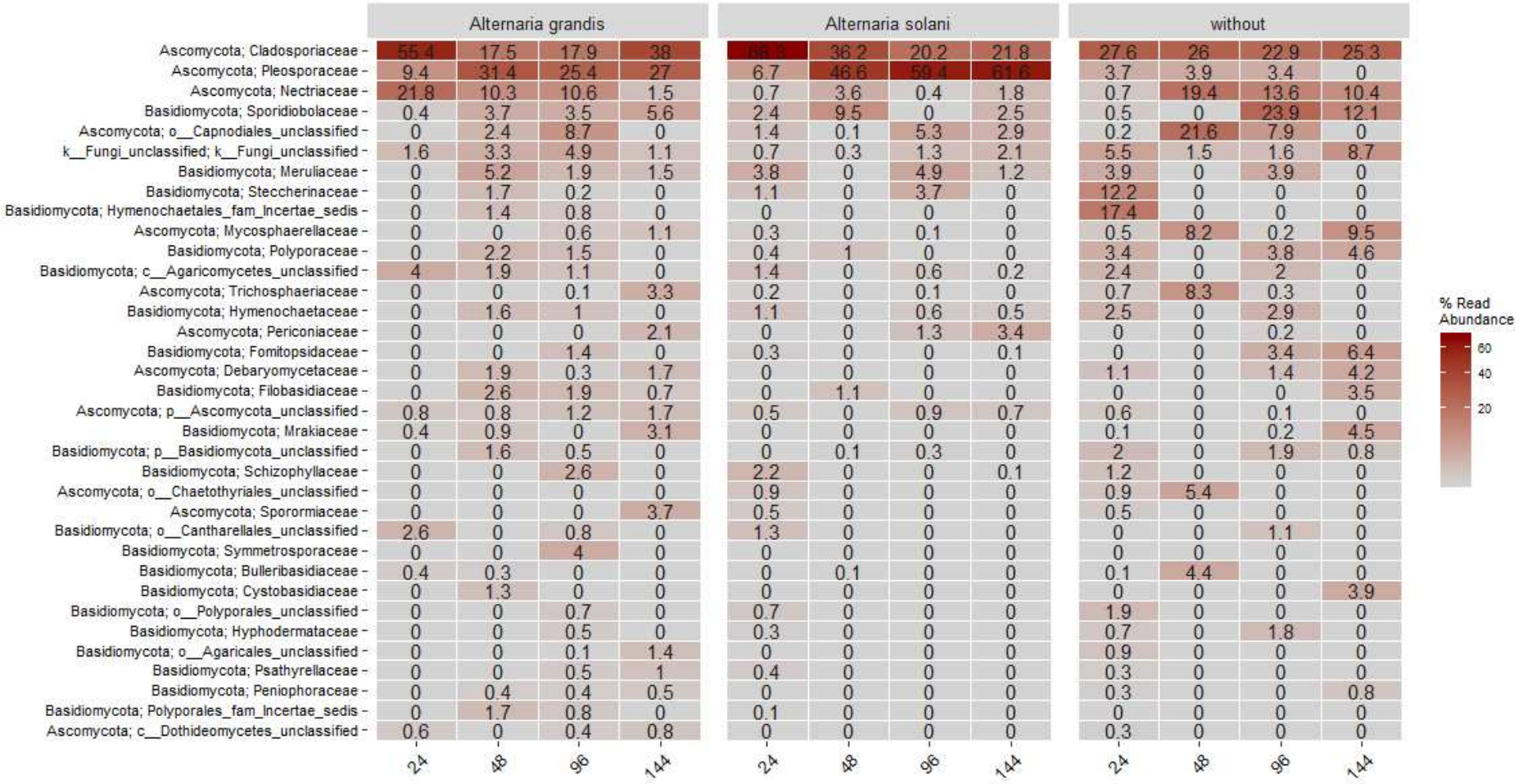

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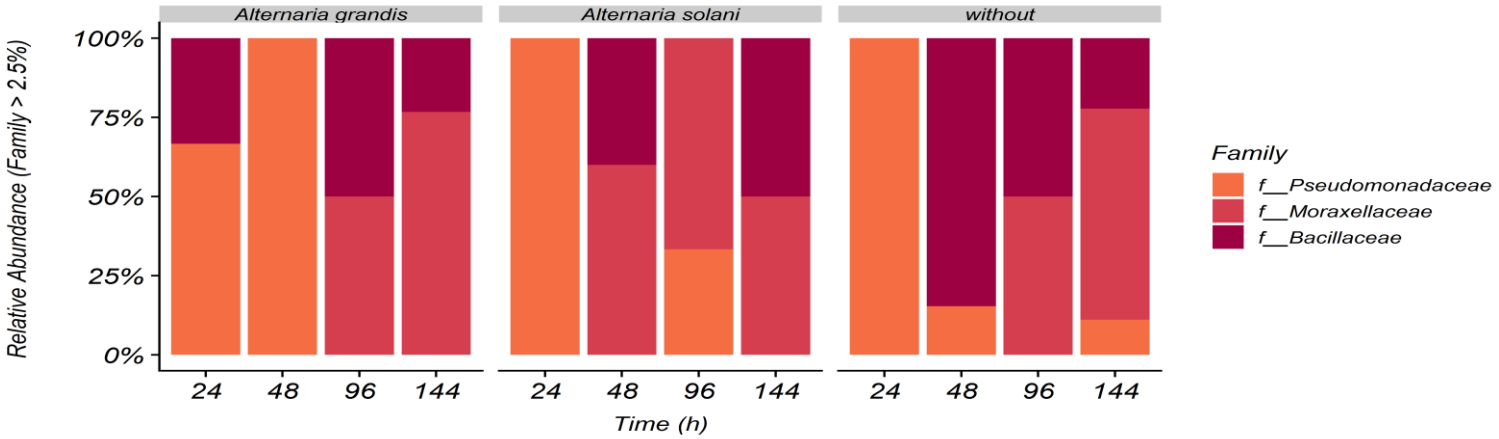
A



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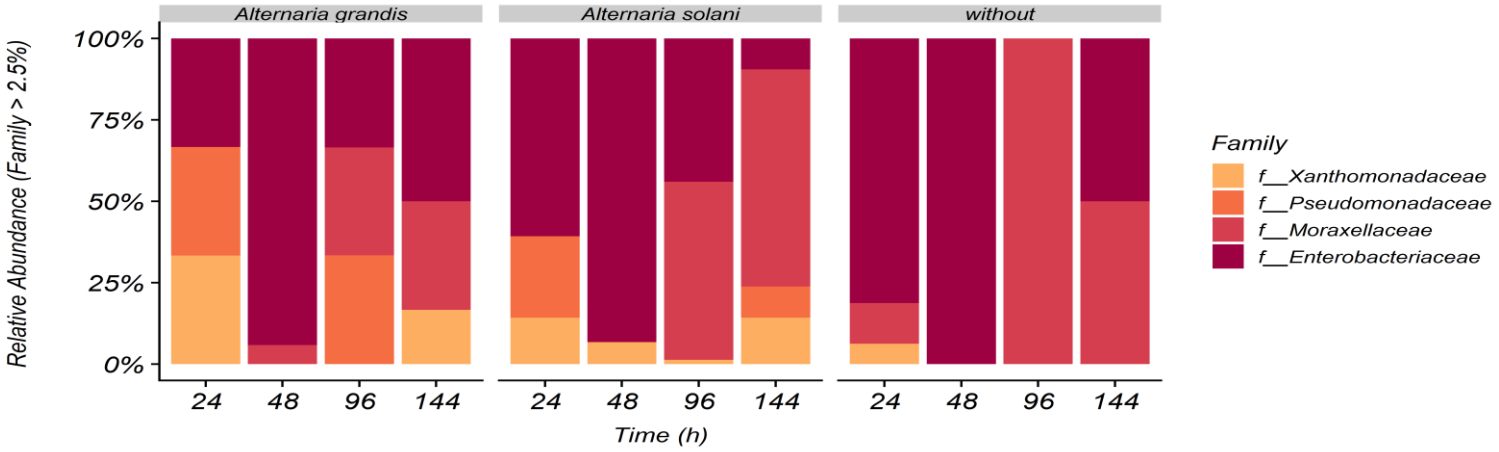

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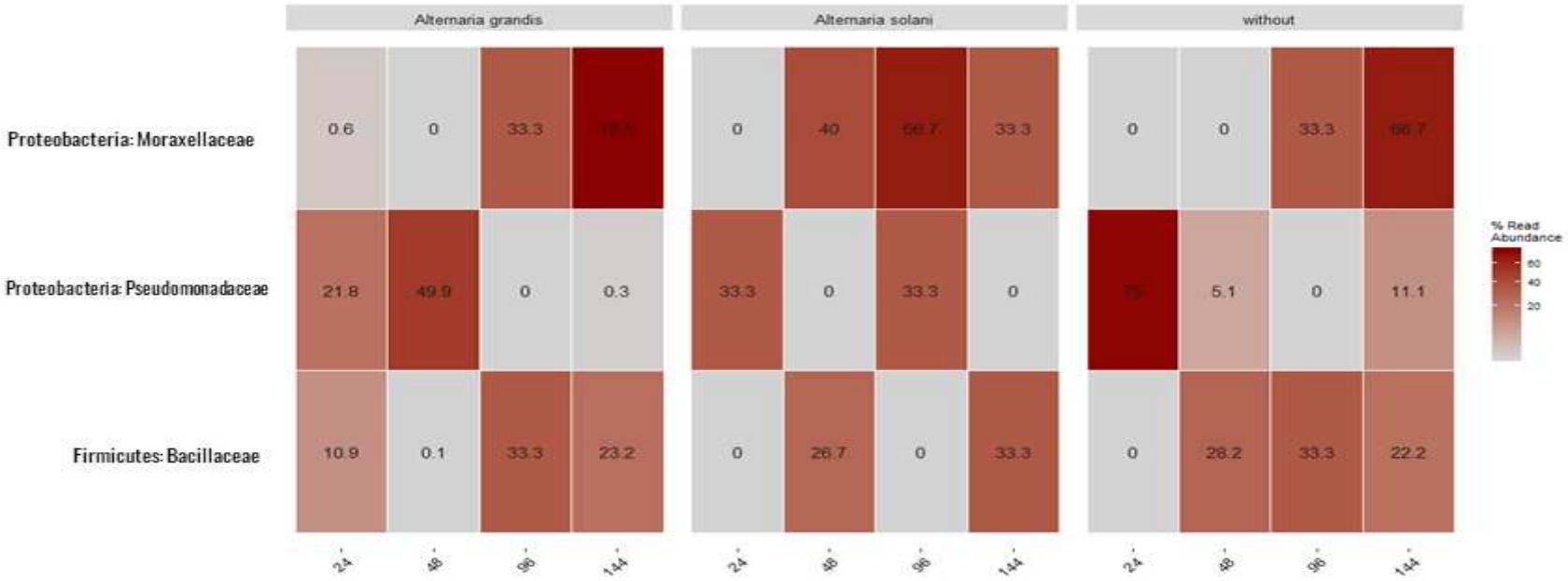

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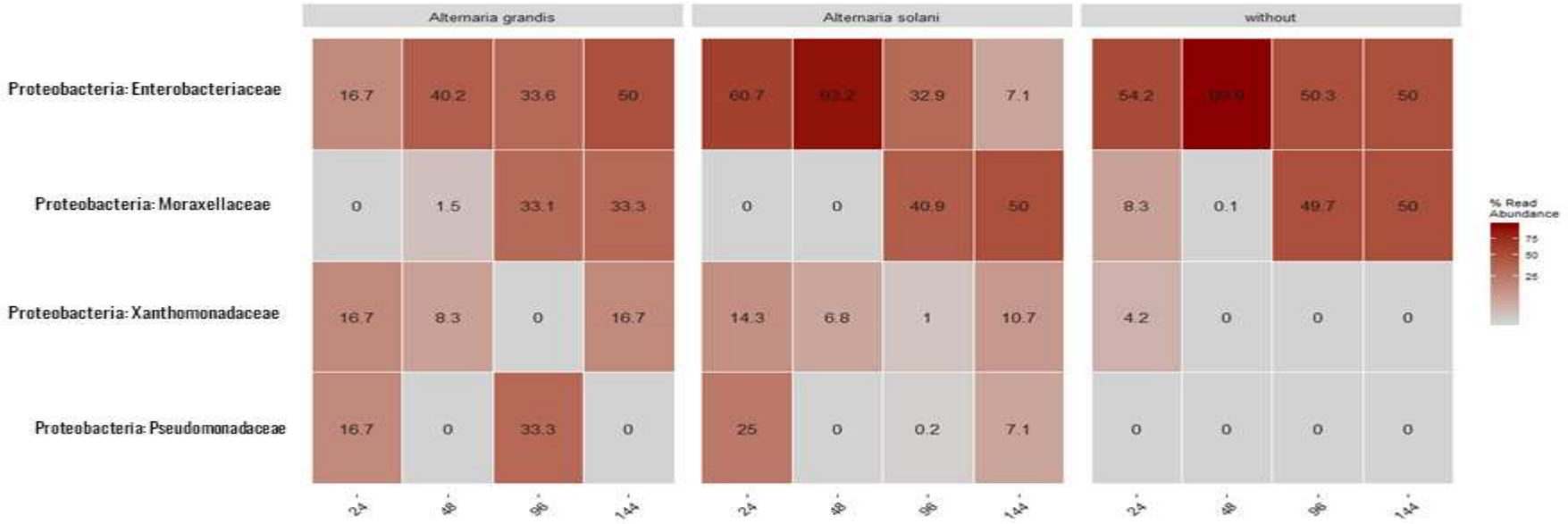

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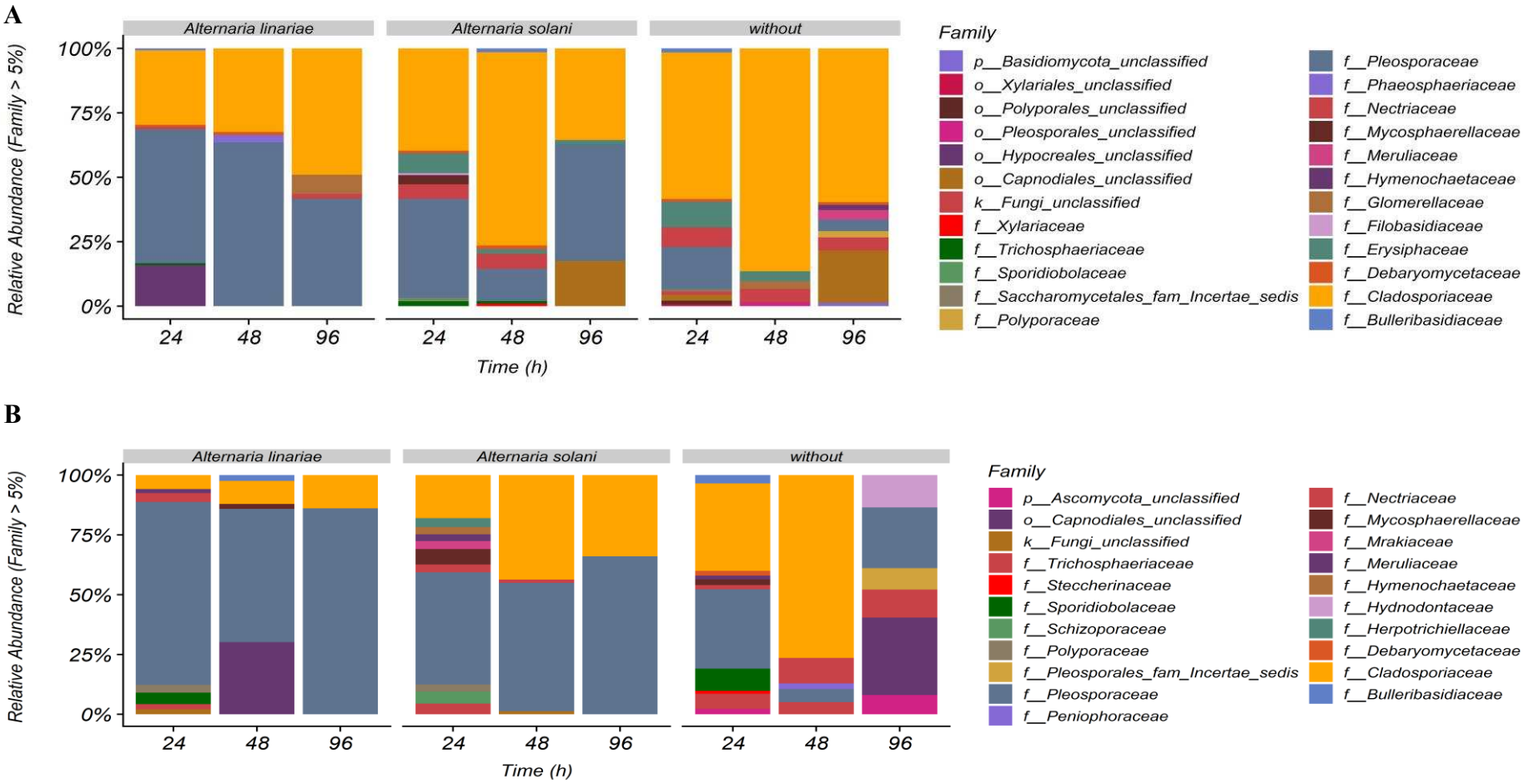
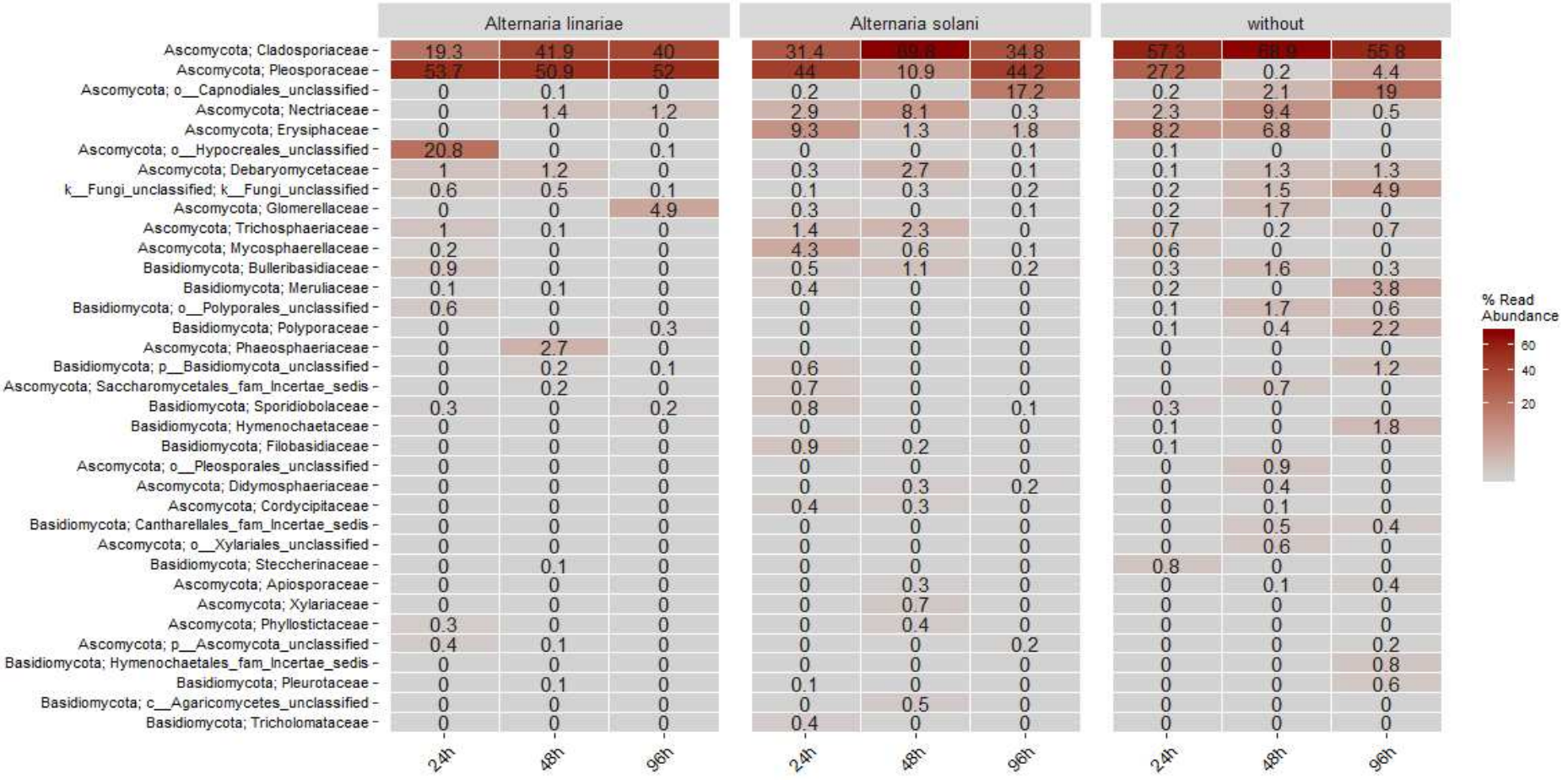


Figure 13. Alvarez et al. 2019



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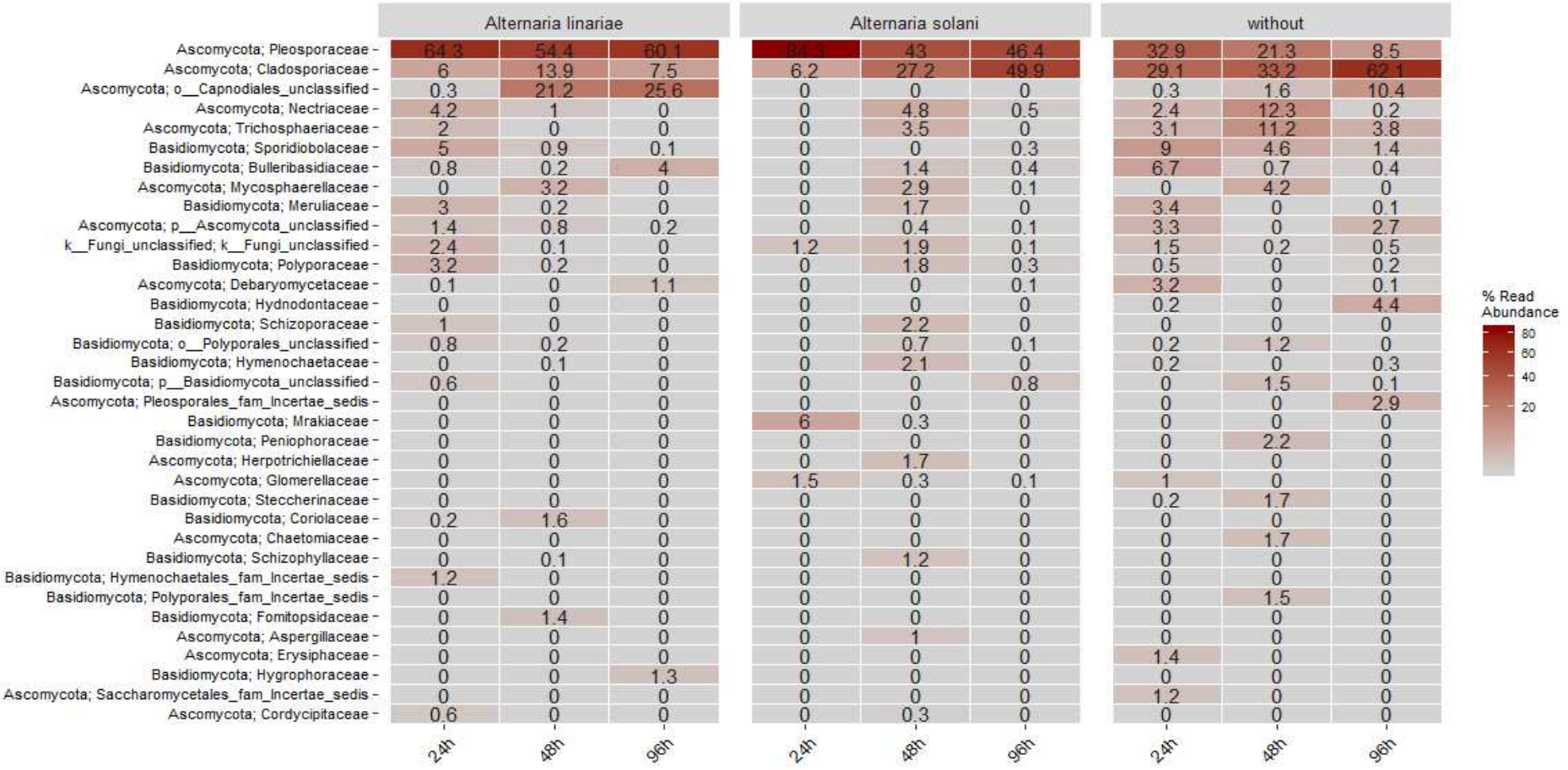

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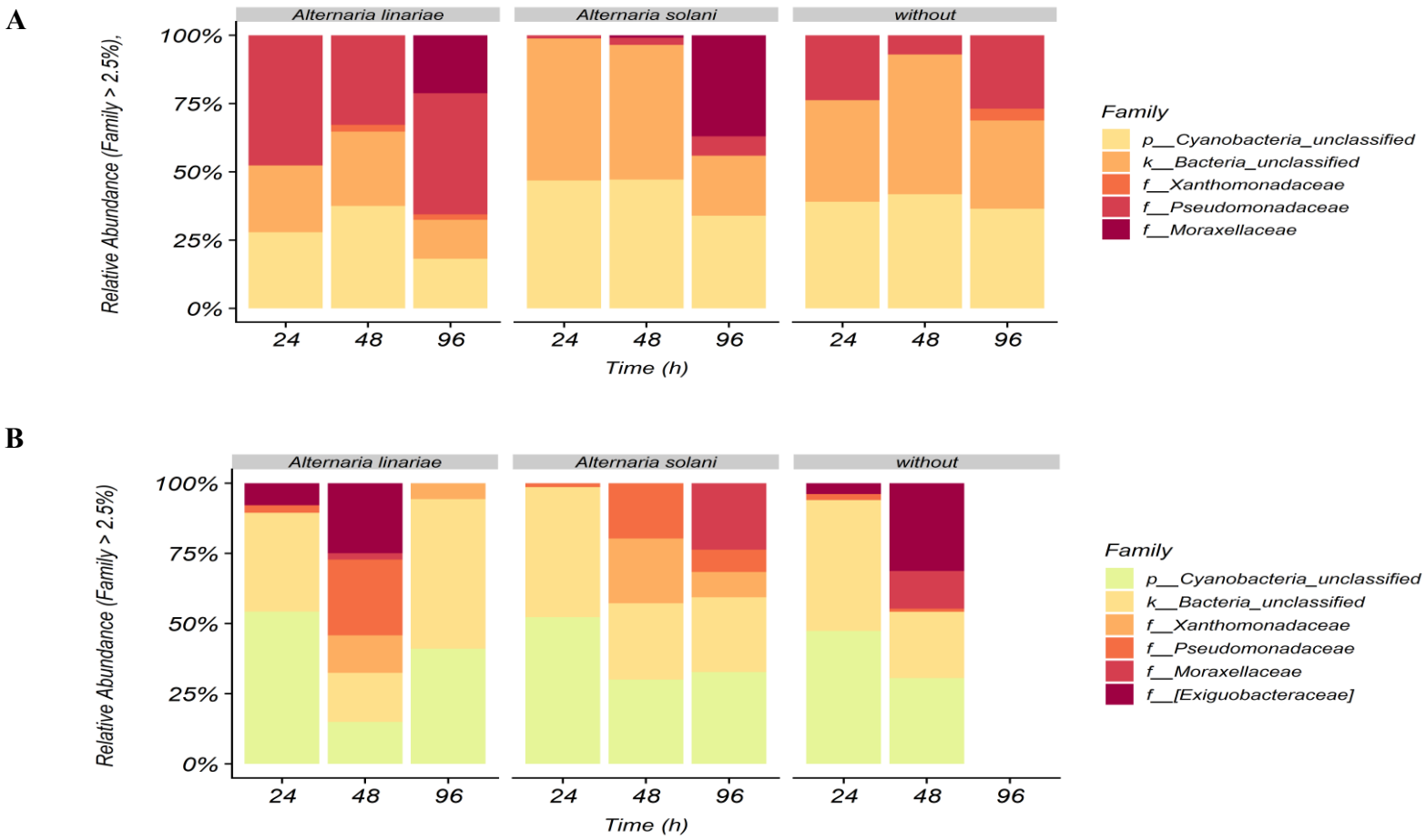

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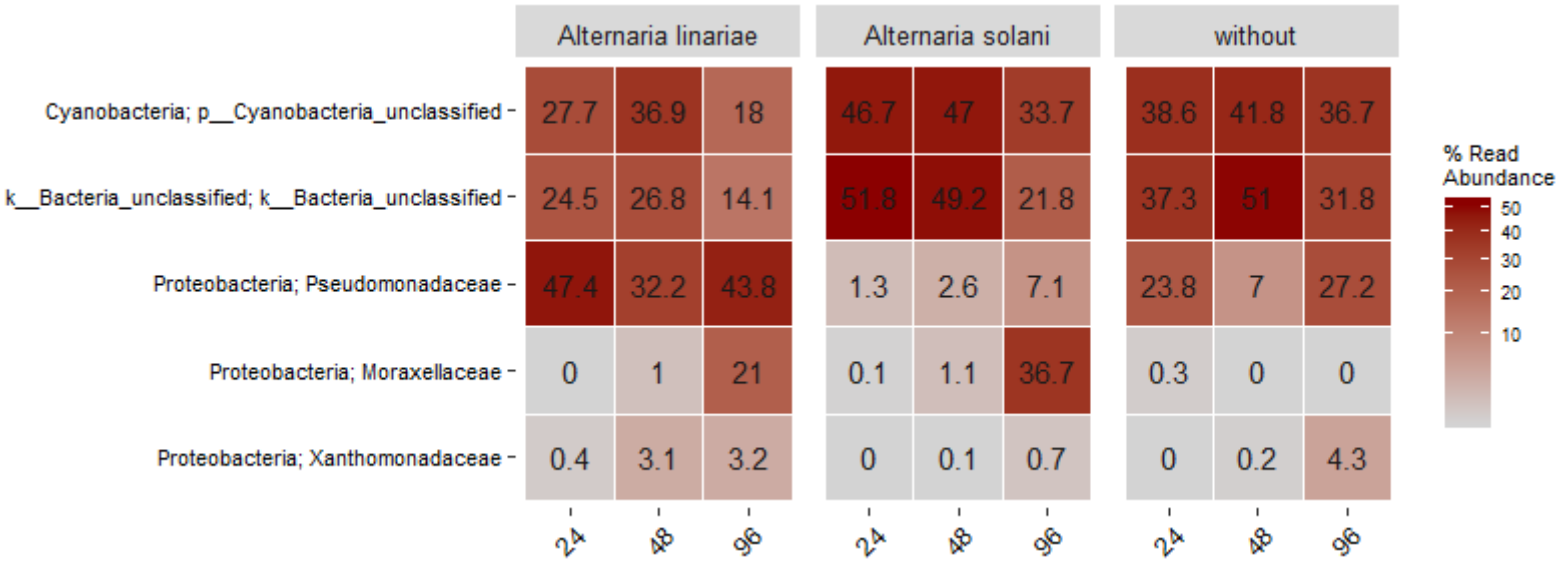

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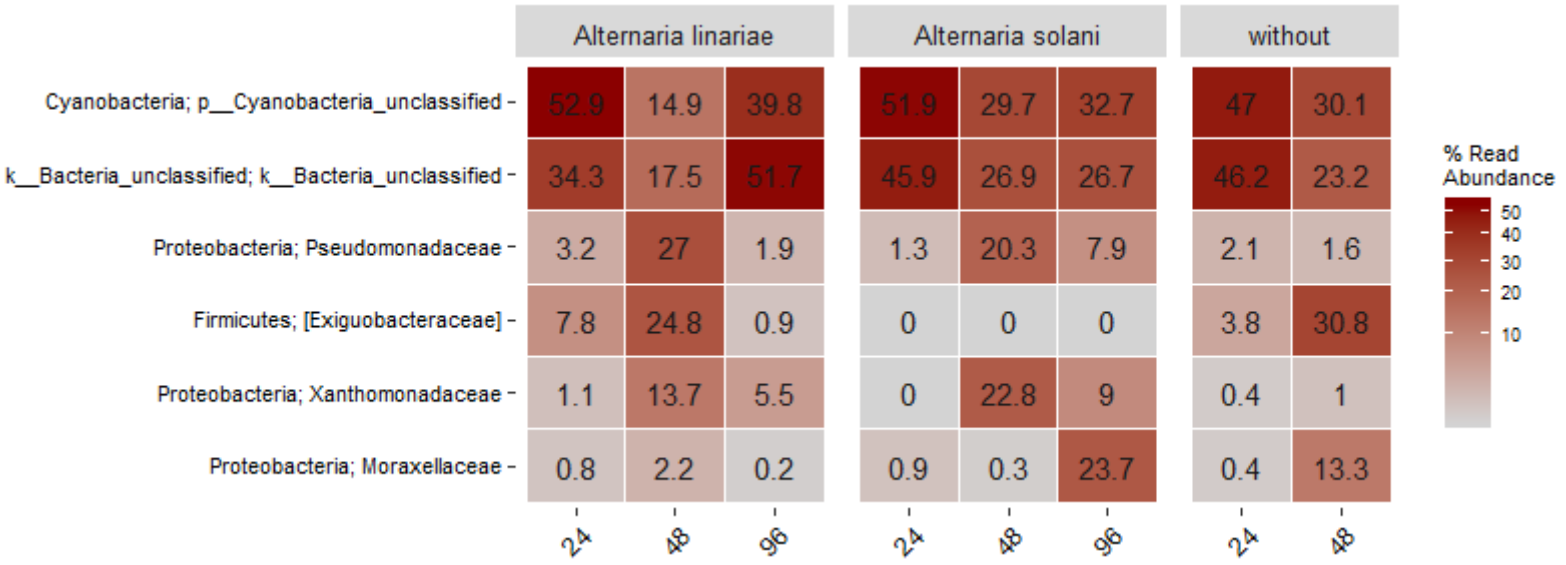

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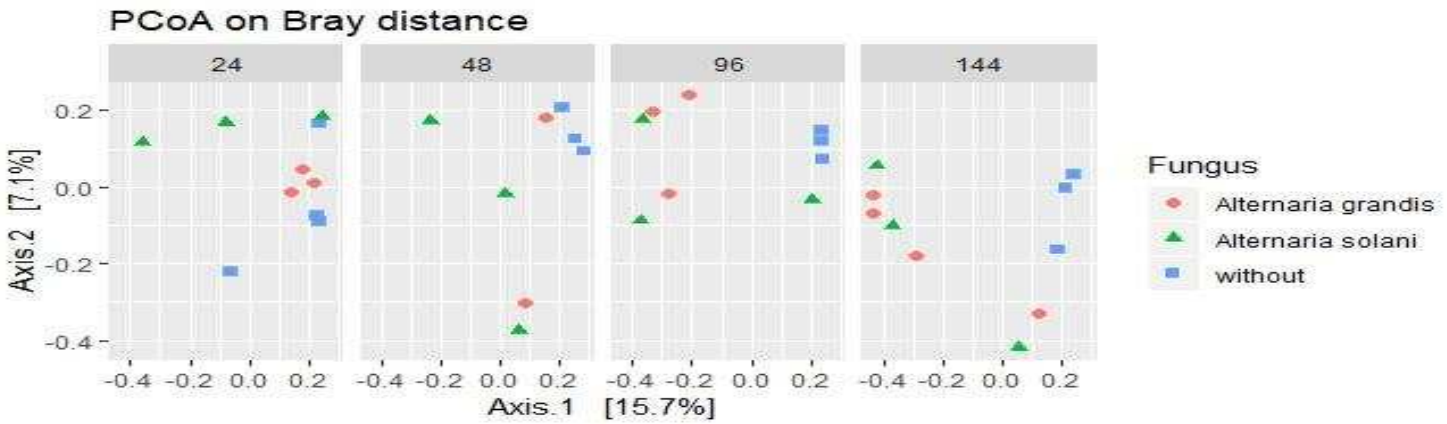
A



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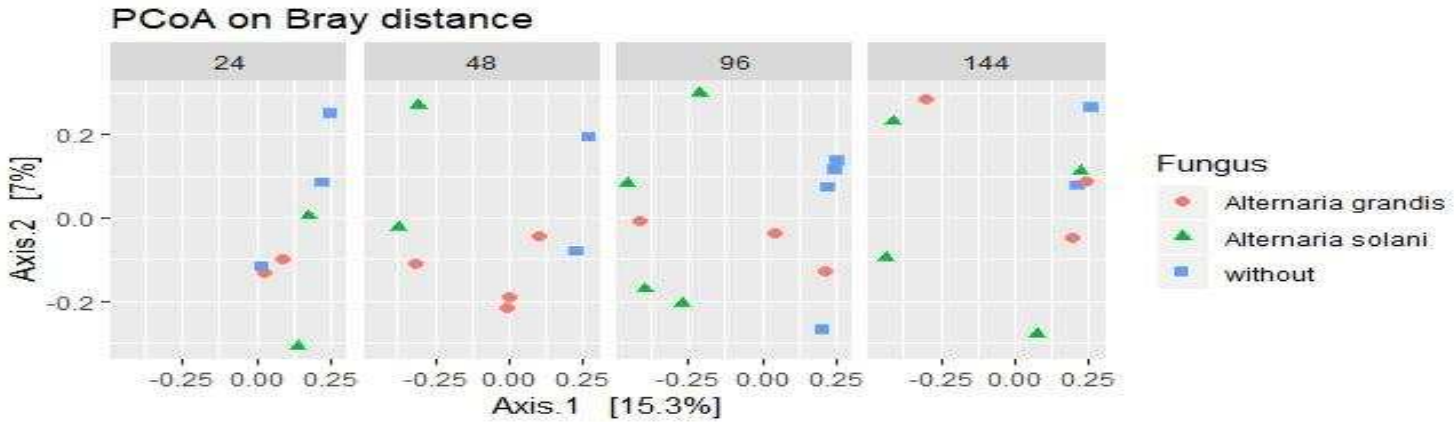

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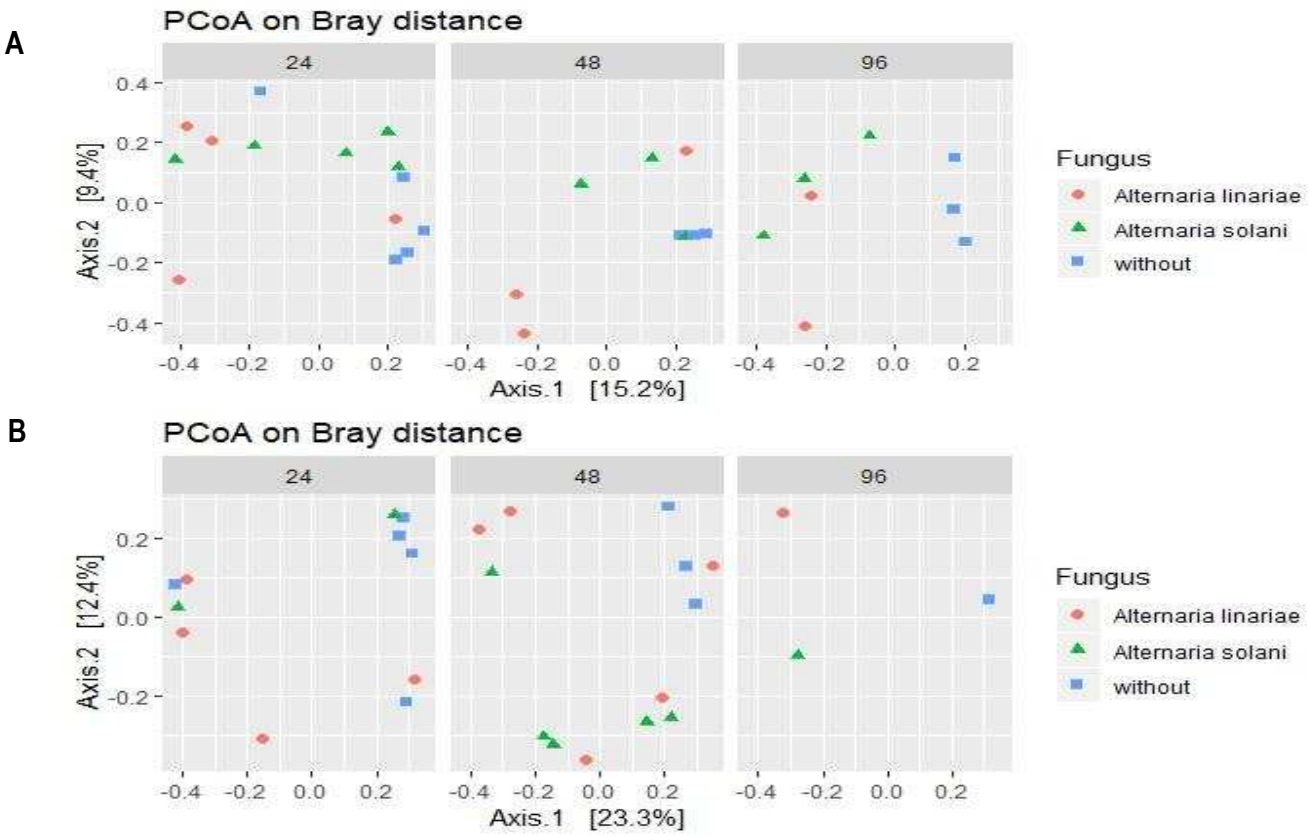

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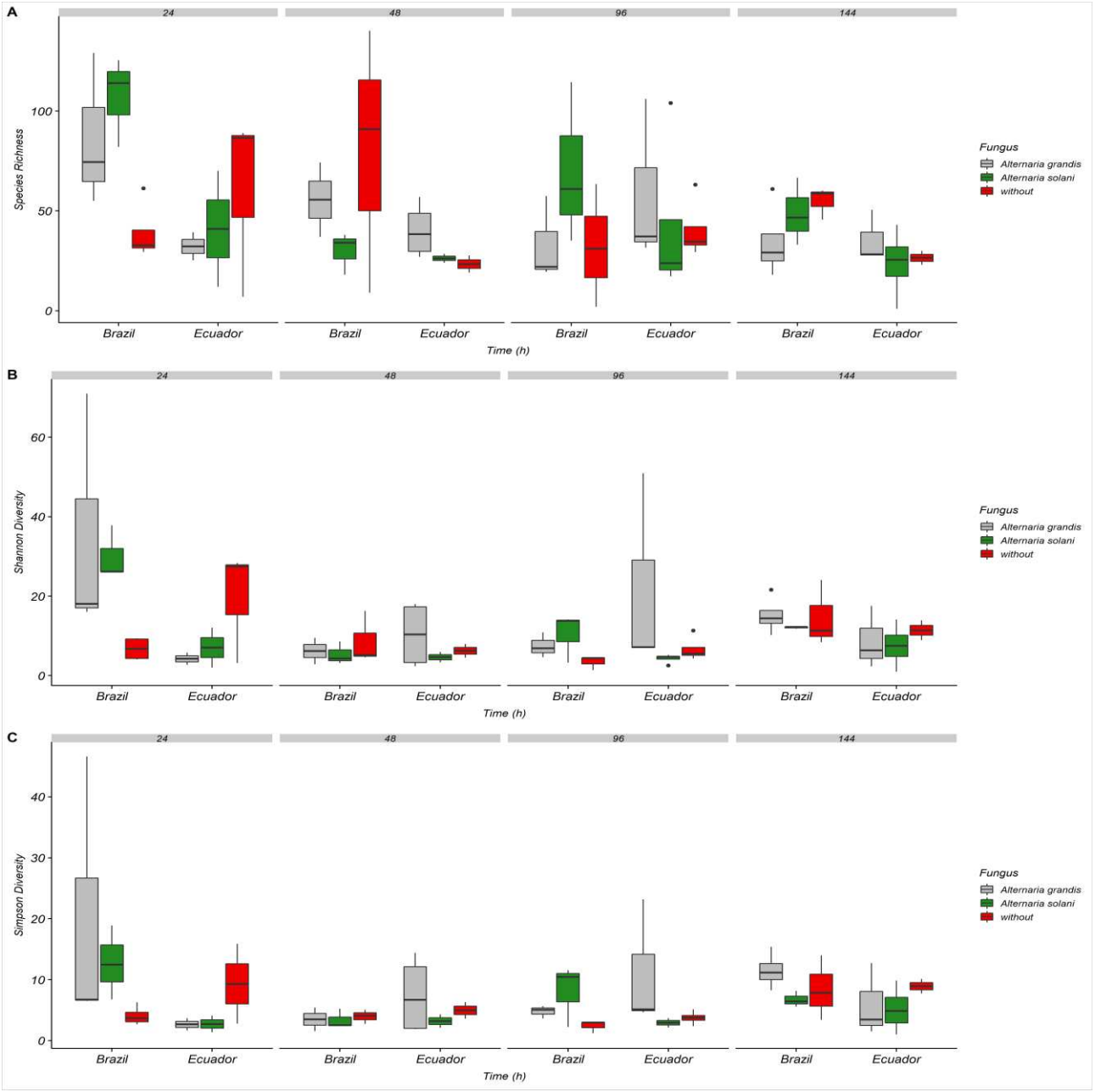

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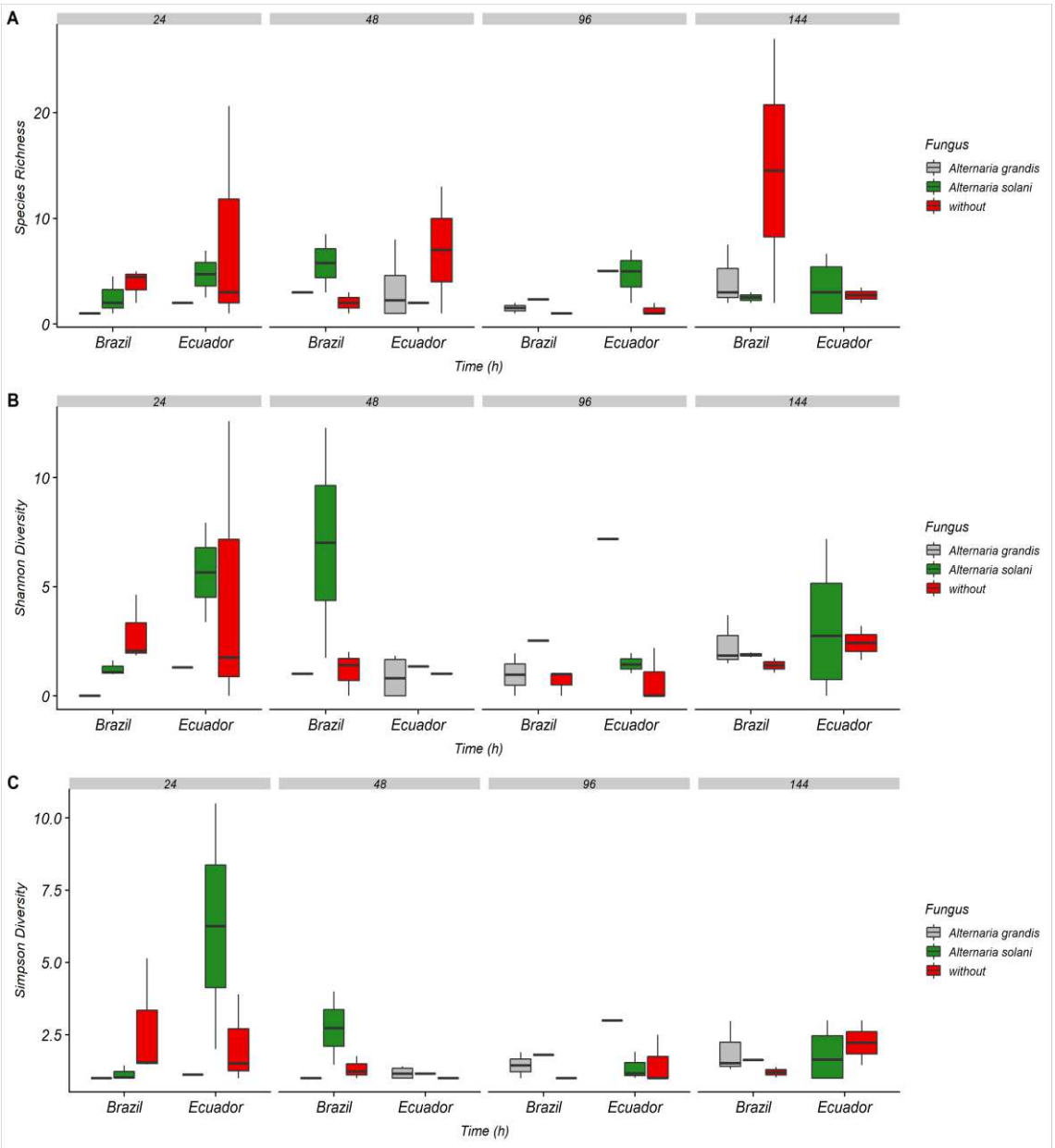

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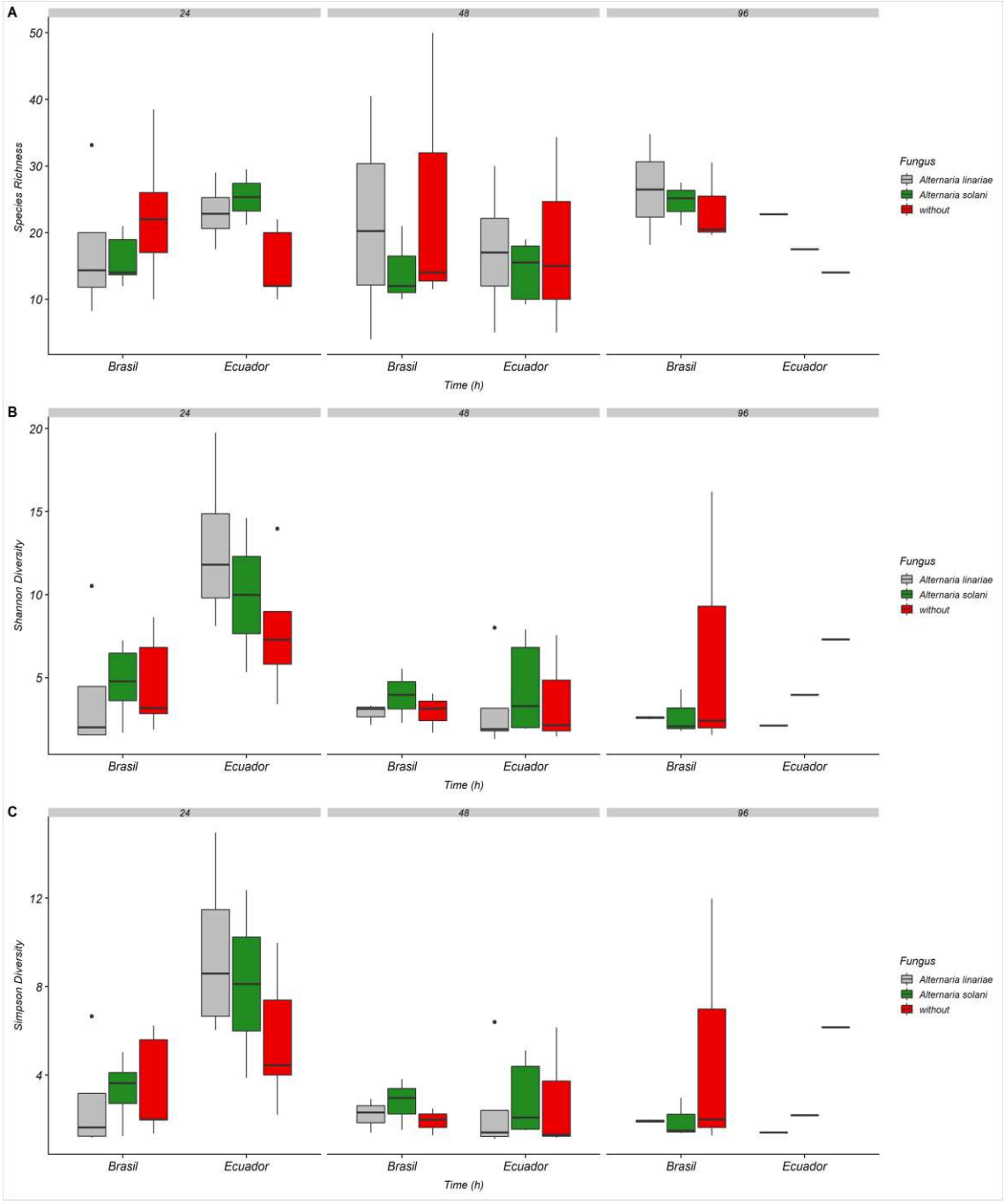

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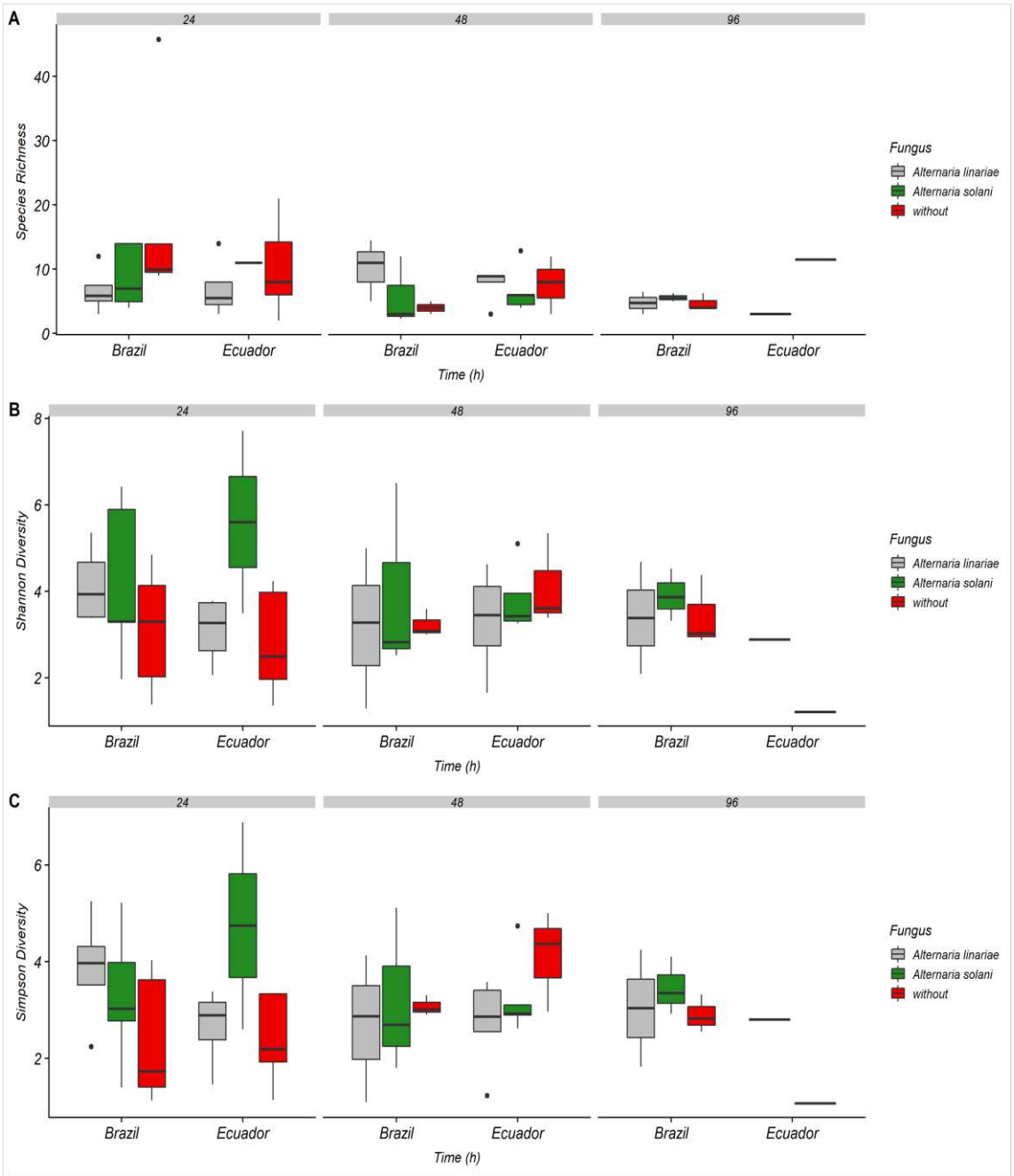

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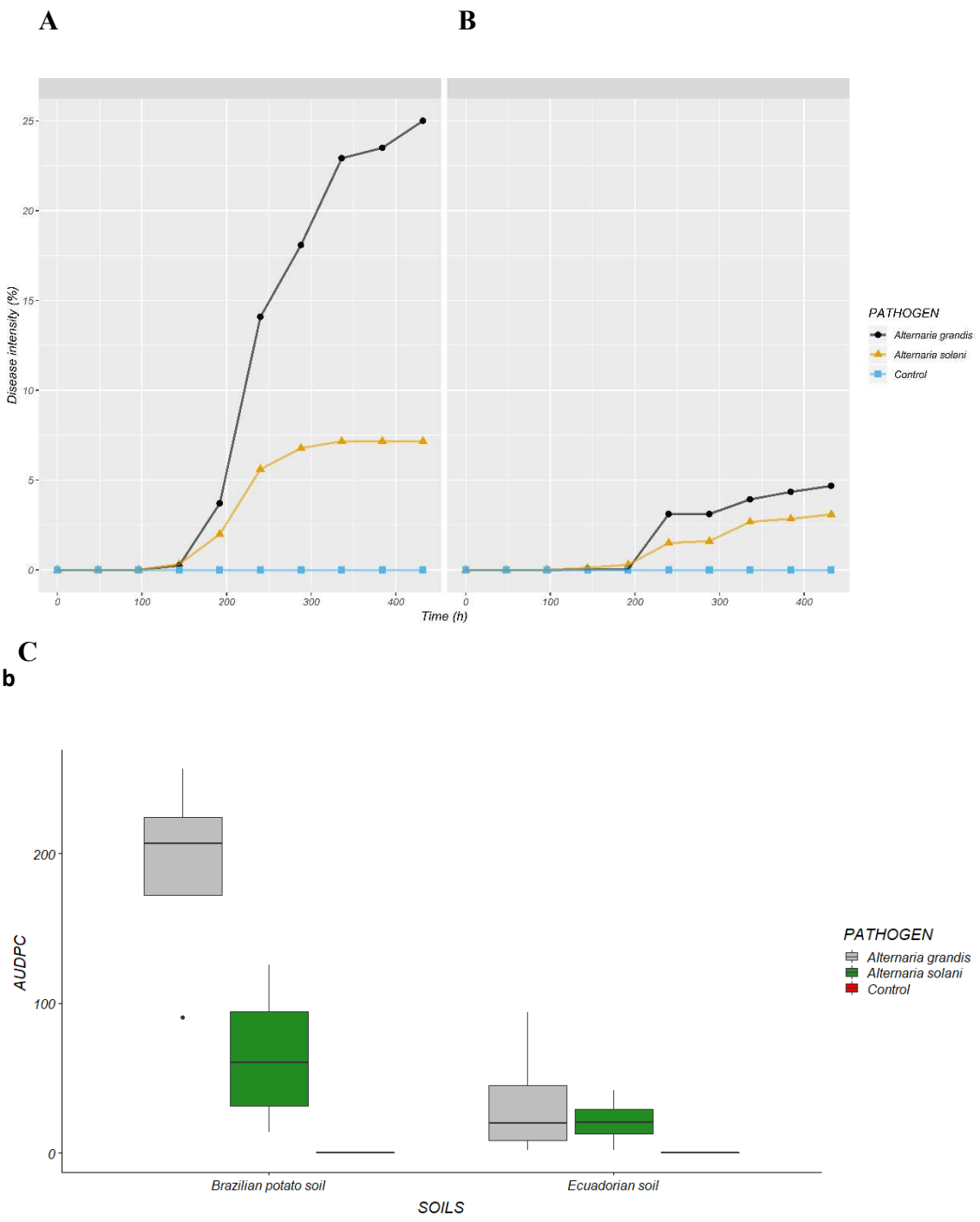

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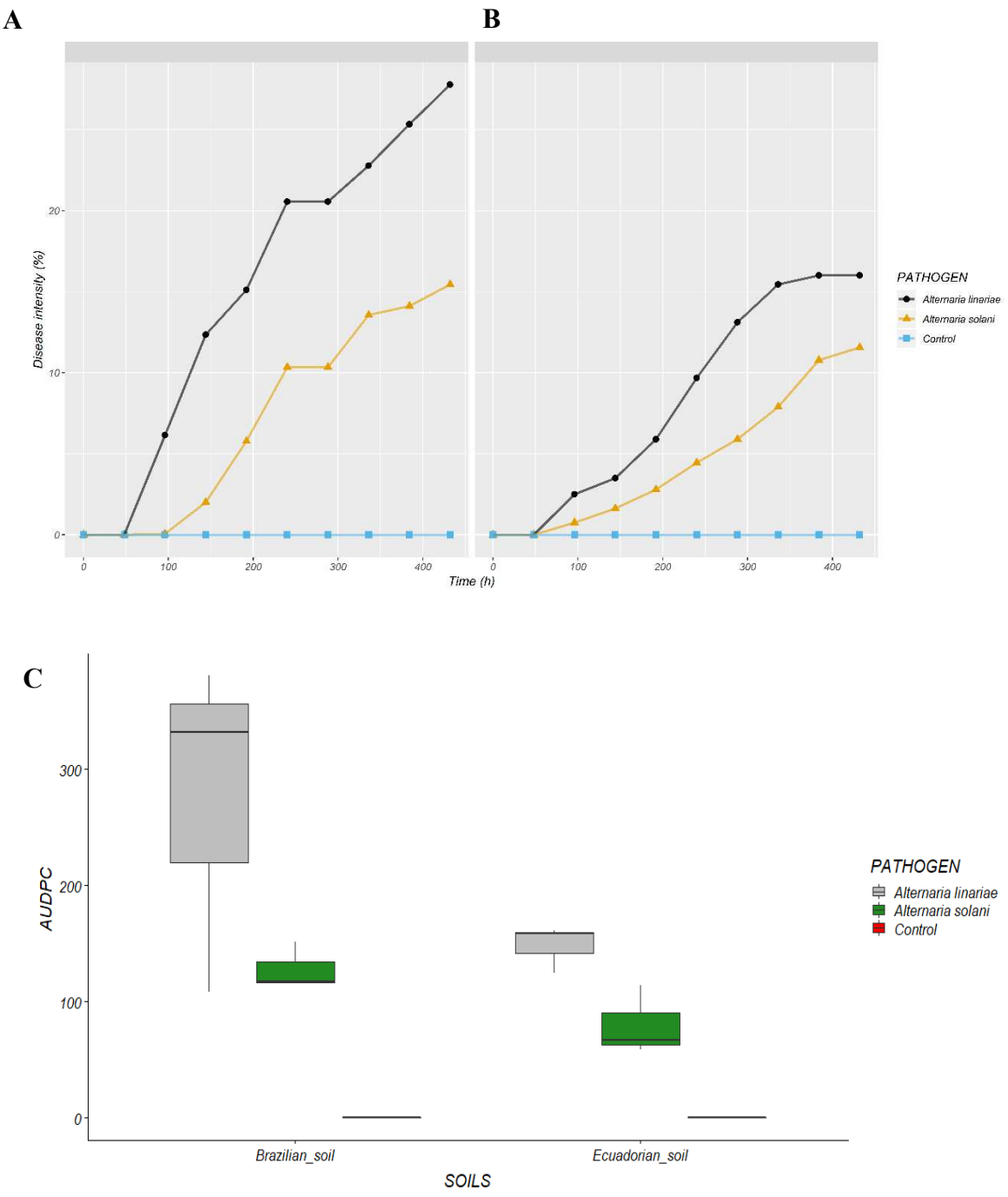

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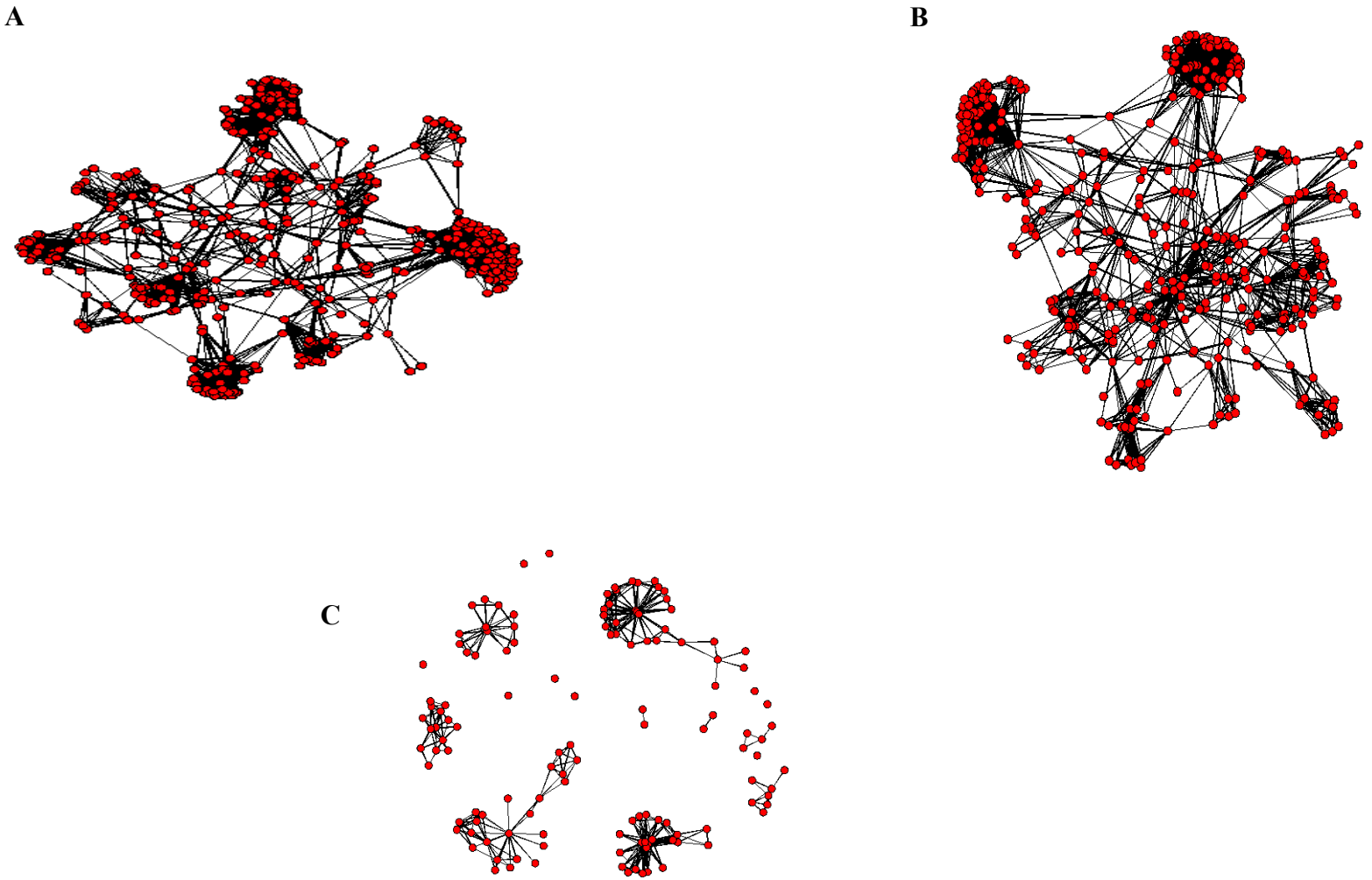

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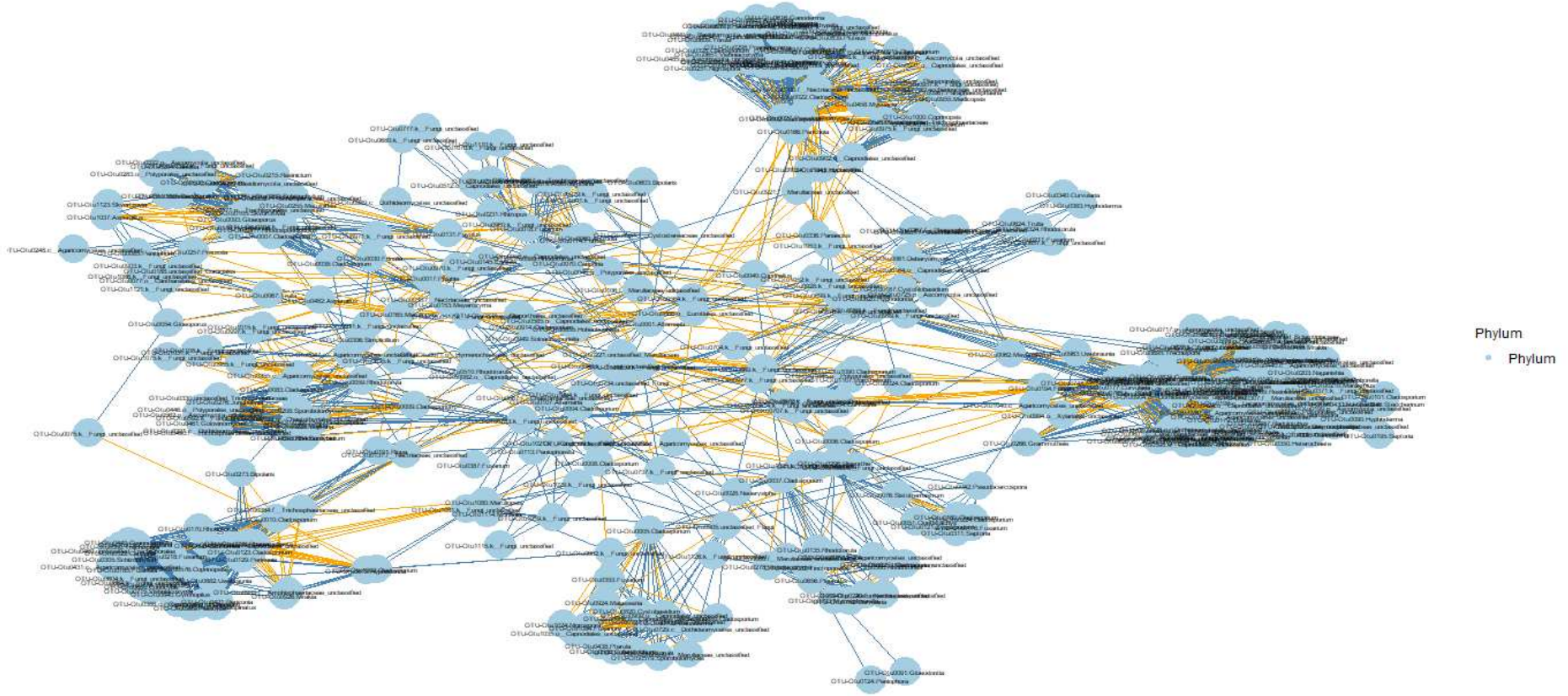

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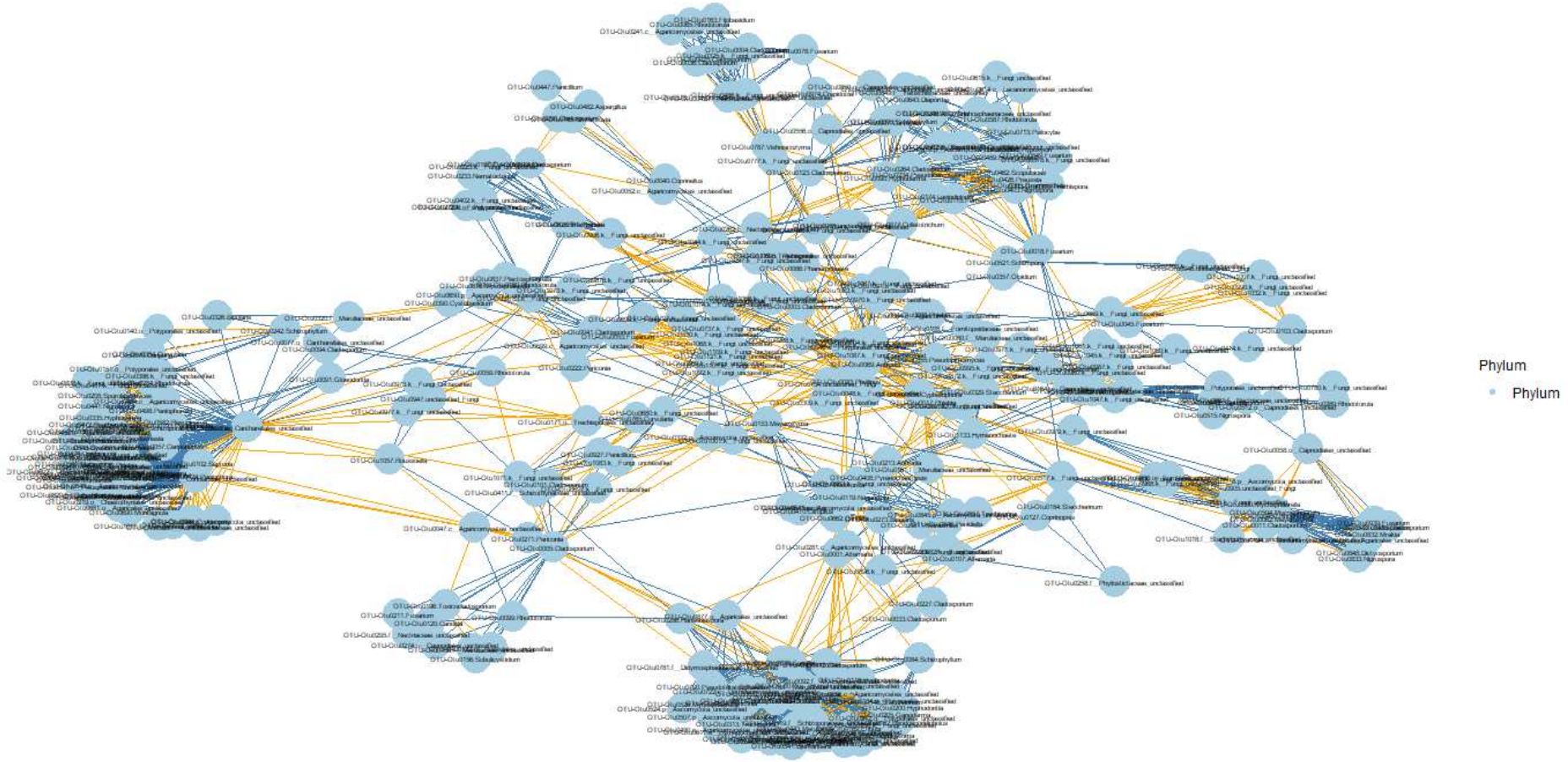

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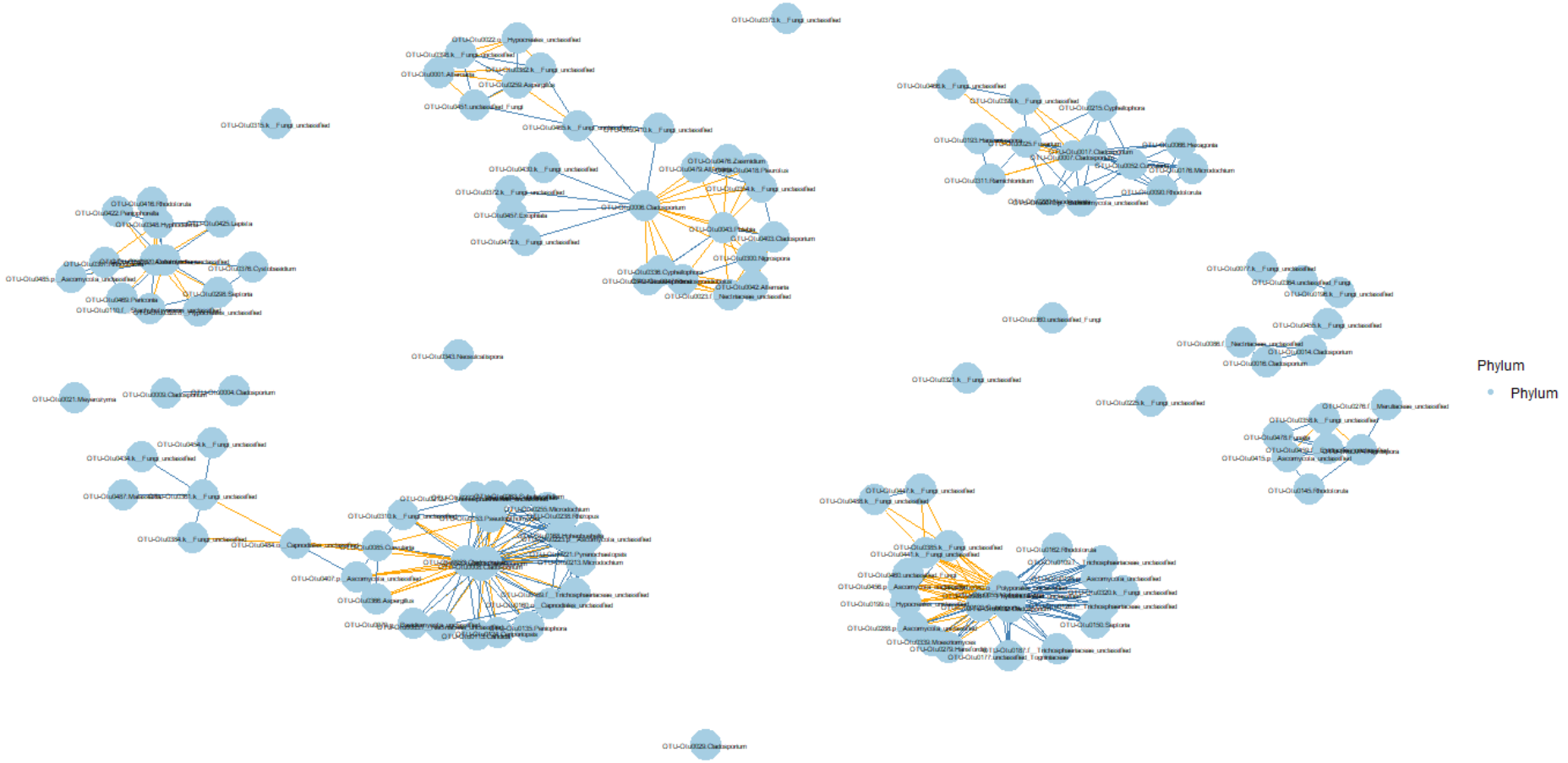

Figure 30. Alvarez et al. 2019

Table 1. Physicochemical properties of soils used in this study

Crop	Place	Soil nutrients									pH (H_2O)	Organic Matter (dag/kg)
		N (dag/kg)	P (mg/dm^3)	K (mg/dm^3)	Ca^{2+} ($cmolc/dm^3$)	Mg^{2+} ($cmolc/dm^3$)	Fe (mg/dm^2)	Cu (mg/dm^3)	Zn (mg/dm^3)	Mn (mg/dm^3)		
Potato	**Brazil** (Rio Paranaiba, MG)	0.3	192.0	1137.0	9.4	2.6	33.2	0.9	21.2	49.1	7.1	7.4
Tomato	**Brazil** (Cajuri, MG)	0.2	526.3	170.0	4.5	0.8	56.5	3.2	8.5	65.9	5.7	4.0
Potato	**Ecuador** (Achullay, Chimborazo).	0.2	87.2	145.0	10.0	6.9	28.0	2.8	7.0	20.5	6.6	6.0

Table 2. Isolates used in the experiments.

Isolate-Source	Species	Origin of isolates	Genotyping method
AS1000	A. Linariae	Cajuri, MG	RPB2-sequencing
CBS 109157	A. Solani	USA, California	Ex -Type
AS624	A. grandis	Rio Paranaiba, MG	HRM-calmodulin

CHAPTER 3

Sorting Alternaria Species Associated with Early Blight in Potato and Tomato Crops using High-Resolution Melting Analysis.

Abstract

Early blight caused by A. solani, A. grandis, and A. linariae, is a serious constraint to solanaceous crops in Brazil. A reliable method for rapid and accurate identification of these species is the first step when studying EB epidemics and for establishing suitable control measures. A single-round polymerase chain reaction coupled with high-resolution melting curve analysis (PCR-HRM) using primers designed based on the calmodulin gene was developed and validated to differentiate closely related A. species. The HRM assay based on the calmodulin region proved to be an accurate technique for the separation of the three Alternaria species and can be a powerful diagnostic tool for enhancing management strategies for early blight epidemics.

Keywords: Solanaceous, Plant Disease Diagnosis, PCR, Rapid detection, Epidemiology.

INTRODUCTION

Different species of large-spored Alternaria, namely A. solani, A. grandis and A. linariae, all classified in the section Porri, are related as causal agents of potato (Solanum tuberosum L.), and tomato (Solanum lycopersicum) early blight (EB) (Rodrigues et al., 2010a; Gannibal et al., 2014). Regardless of the large-spored Alternaria species, the main symptom of EB in leaf, stem, and tuber or fruit are dark, sunken, necrotic lesions (Palm & Rotem, 1997; Duarte et al., 2014). In leaves lesions are generally circular and concentric rings are commonly present (Bessadat et al., 2017). Correct diagnosis of the causal agent can be confusing. Epidemiological knowledge about these "new" large-spored Alternaria species is scarce, but it is likely that species might differ in the way they respond to the environment and to host genotypes. Thus, the accurate identification of the pathogen is an important component for EB management purposes.

The identification of Alternaria spp. has been traditionally based on morphological characteristics such as color, size, shape of conidia and sporulation patterns (Simmons, 2000). Nevertheless, these morphological characters are not necessarily markedly distinct among the species and can vary depending on the temperature and substrate used during the culture growth (Simmons & Roberts, 1993), making the use of this type of markers unreliable. Currently, molecular approaches based on sequence dataset of the glyceraldehyde-3-phosphate dehydrogenase (gpd), plasma membrane ATPase, Alternaria allergen a 1 (Alt a1), actin and calmodulin gene fragments have been broadly used to study Alternaria species associated with early blight worldwide (Rodrigues et al., 2010a; Lawrence et al., 2013; Gannibal et al., 2014; Bessadat et al., 2017; Ozkilinc et al., 2018).

The development of rapid and reliable molecular identification technique is crucial for fast and accurate identification of Alternaria spp. associated with EB. High-

resolution melting (HRM) analysis has been developed and utilized for DNA genotyping (Ganopoulos et al., 2012; Tucker & Huynh, 2014). HRM analysis is a post-PCR method for identifying genetic variation in DNA sequences by quantifying changes in fluorescence level of the melting DNA amplicon (Erali et al., 2008; Erali & Wittwer, 2010; Lu et al., 2017). Briefly, before HRM analysis, the target region is amplified using PCR in the presence of a double-stranded (ds) DNA binding dye that does not affect the reaction and fluoresces brightly when bound to DNA or fluoresces at a low level in the unbound state (Tucker & Huynh, 2014). Following the PCR amplification, the amplicon is slowly heated until the dsDNA denatures into two single strands, releasing the binding dye which is sensed as a reduction in the fluorescence level. A melting profile is obtained for each genotype and the profile is analyzed by a software (Ganopoulos et al., 2012).

The HRM data are assessed using two methods: melting point (T_M) determination and difference curve analysis (Hrncirova et al., 2010; Gago et al., 2011; Alnuaimi et al., 2014). T_M is frequently used to differentiate species of fungi because each species has a specific T_M for its DNA constitution (Ganopoulos et al., 2012). Difference curves are constructed by subtracting a test curve from a standard curve after normalization shifting of the raw melt curve (Lu et al., 2017). Multiple studies have used the rDNA internal transcribed spacer (ITS) region as the target amplicon to sort fungal species. Dunyach et al. (2008) employed primers to amplify the ITS1-5.8S-ITS2 region and determined that the use of the HRM based on this region could discriminate five species of Candida. Similarly, the HRM-curves for the ITS2 region of Aspergillus carbonarius, A. tubigensis, A. niger, A. ibericus and A. japonicus were used to distinguish these species (Xanthopoulou et al., 2019). Other studies have examined other amplicons. Garganese et al. (2018) analyzed the HRM-curves of the OPA1-3 region of Alternaria species associated with potato brown spot and successfully separated two small-spored ones: A.

alternata and A. arborescens. Additionally, an HRM real-time PCR assay targeting the endopolygalacturonase (EndoPG) was used to identify and genotype A. alternata, A.tenuissima and A.arborescens, all members of section Alternaria and commonly associated with fruit rot of apple and pomegranate (Zambounis et al., 2015).

Despite the importance of early blight in potato and tomato crops, little work on new molecular tools for diagnosis have been developed; therefore, the aim of this study was to develop and validate a specific HRM method for identification of large-spored Alternaria spp. associated to early blight based on differences in melting curve characteristics via the calmodulin region.

MATERIAL AND METHODS

Primer design

Primers were designed to amplify a portion of the calmodulin gene of three large-spored Alternaria spp., section Porri, that includes both highly conserved and variable regions (Fig. 1). The partial sequence of the calmodulin gene of A. solani, A. grandis, A. linariae were obtained from GenBank (NCBI, Bethesda MD, USA) (Table 1) and alignment was carried out using Muscle® v.3.6 software (Edgar, 2004) implemented in Mega 7.0 (Kumar et al., 2016). Once the specific and conserved regions for each Alternaria spp. were identified, a pair of common primers was designed for the HRM assay using Primer 3 v.4.0 (http://simgene.com/Primer3). In silico analysis using BLAST (NCBI) was performed on the primer pair to evaluate its specificity. The folding characteristics of these primers and amplicons were evaluated using OligoAnalyzer 3.1 (http://sg.idtdna.com/calc/analyzer) (Integrated DNA Technologies, Coralville, Iowa, USA). The primer pair HRMCAL300F (5' ACAACGGCACCATTTGACTTC 3') and HRMCAL300R (5' TGATGCAAGCAGGTGCAA 3') were designed for amplification of the calmodulin region for subsequent HRM analysis.

Fungal isolates and DNA extraction

Eight previously characterized isolates of Alternaria species: one of A. solani, three of A. grandis, three of A. linariae and one of A. alternata were selected for the development and validation of the method. The Ex-Type of A. solani (CBS109157), A. grandis (CBS109158), and A. linariae (CBS109156) were used in this study (Table 2). Later, 46 uncharacterized Alternaria isolates from potato and tomato plants were used for evaluation of HRM analysis. Single-conidium isolations were performed and cultures were maintained as single-spore colonies stored using Castellani's method at 10 °C.

All Alternaria isolates were cultured on PDA medium at 24 °C for 7 days. Two discs of 5 mm diameter were cut from the mycelial margin, transferred to 100 mL of synthetic media and incubated for 5 days at 27 °C. The mycelia were recovered, washed twice with distilled water and dried on sterile filter paper for 5-10 min under a laminar air-flow hood. DNA was extracted with Wizard Genomic DNA Kit (Promega) following the manufacturer's instructions and the DNA was eluted in 50 µl of TE buffer. Quality and quantity of the extracted DNA were checked using agarose gel electrophoresis and spectrophotometer (NanoDrop 2000).

HRM analysis

The HRM assay was performed using an HRM-equipped real-time rotary analyzer (Rotor Gene Q). The PCR reactions were performed with the Type-it HRM PCR Kit according to the manufacturer protocol (Qiagen). Each reaction was performed in a total volume of 10 µL mixture containing 1x of HRM Master mix (Qiagen), 0.35 µM of each primer and 10 ng of each DNA sample. The thermal profile comprised initialization step at 95 °C for 5 min, followed by 40 cycles of amplification which included denaturation of double-stranded DNA at 95 °C for 15 s and annealing of DNA strands at 54 °C for 1 min. The PCR products were then subjected to a melt program: denaturation of double-stranded DNA at 95 °C for 10 s, annealing of double-stranded DNA at 60 °C for 15 s. Finally, melting curve plots were plotted and analyzed using the Rotor-Gene Q associated software v.2.0.2.4 to establish average melting temperature (Tm) for each Alternaria spp. associated to EB in potato and tomato.

Analytical sensitivity evaluation.

The analytical sensitivity or detection limit of HRM assay was assessed by using 6-fold serial dilutions with 20, 2, 0.2, 0.02, 0.002, and 0.0002 ng of DNA of A. solani, isolate CBS 109157 (Ex-Type). The procedure was repeated twice to ensure

reproducibility. The PCR product was run on a 1.0% agarose gel to confirm the product amplification and the band size.

RESULTS

The HRM data were interpreted by means of conventional derivative genotype plots with each of the three large-spored Alternaria species (Table 3) being assigned to a unique genotype profile (Fig. 2). The three large-spored and the small-spored Alternaria genotypes were represented by three different melting curves (Tm) peaks profiles (Table 3, Fig. 2). Each of the genotypes showing similar melting peak values was differentiated from the others. The range of the first peak was from 84.86 ± 0.11 °C (A. alternata) to 86.14 ± 0.03 °C (A. solani). The second peak varied from 85.88 ± 0.08 °C (A. alternata) to 86.85 ± 0.02 °C (A. solani). Finally, the third peak varied from 86.43 ± 0.04 °C (A. alternata) to 87.33 ± 0.002 °C (A. solani). (Table 3).

Although Alternaria species melting profiles were very similar, they could be discerned by the plot of the temperature-shifted fluorescence differences and the normalized melting curves (Fig. 2). All 50 samples tested were identified and differentiated with a level of confidence of 81.68- 99.49% (Table 5).

The reproducibility of the results was confirmed, and the lowest detectable limit of the HRM assay was of 0.02 ng, the 6-fold serial diluted of A. solani DNA is shown in the standard curve and melting curve (Fig. 3).

DISCUSSION

Early blight has been an increasing yield reducing factor for potato and tomato crops. Fast and reliable identification of Alternaria species are prerequisites for the disease management. This aspect is particularly important when considering the many Alternaria outbreaks that have recently emerged, particularly in potato crops, associated with previously undescribed species (Simmons, 2000; Rodrigues et al., 2010a).

In the present study, we developed and validated an innovative HRM method based on the differences in the melting curves of specific amplicons of the calmodulin gene for the rapid differentiation of three Alternaria species. The calmodulin gene was previously shown as efficient to delineate closely related and unrelated Alternaria species (Gannibal et al., 2014; Ozkilinc et al., 2018). The different species tested generated distinct HRM profiles, thus permitting an accurate identification and discrimination of Alternaria species. The potential discriminative efficiency of this technique is higher than that of conventional melting curve approaches. In HRM analysis, melting curves can be efficiently differentiated even when they show the same Tm, because of the heterozygosity-derived composite melting curve profiles (Ganopoulos et al., 2012). The HRM assay allows to discriminate the different Alternaria species in a single round assay with accurate and reliable results. The genomic region chosen to develop the HRM assay was reliable enough to sort closely related Alternaria species and the short length of the amplicons enabled good optimization of the analyses. Additionally, the inclusion of one characterized isolate of A. alternata (section Alternaria) formed a group different from the other Alternaria species section. There was clear separation of the two sections.

Although our genotyping method is a promising approach to assign new unclassified Alternaria species to a specific taxon, this should be carefully assessed because of the potential occurrence of encrypted genetic variation among Alternaria

species. The HRM analysis is not to substitute the traditional morphological and molecular tools but can be a powerful complement to the identification of species, especially because of its high throughput screening capacity. The data presented in this study, therefore, support the usefulness of HRM technique in differentiating closely related Alternaria species. In conclusion, this is the first study employing the HRM analysis for the accurate identification and differentiation of Alternaria species associated with EB belonging to Porri section.

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FIGURE LEGENDS

Figure 1. A) Alignment of consensus sequences of three Alternaria species showed the SNPs used for species separations in HRM analyses. B) Calmodulin region used for HMR-analysis of Alternaria species.

Figure 2. Melting curve variance of the four Alternaria spp. in A) aligned (normalized melting curves), B) derivative plot with melting peaks and C) difference plot analysis.

Figure 3. Analytical sensitivity evaluation. A) Derivative melting curves, B) 6-fold serial diluted A. solani target sequence.

Figure 4. Agarose (1%) gel electrophoresis of calmodulin gene PCR product. Lane M: molecular marker (100 base pair DNA ladder), Lane D1: PCR product with 20 ng/µL de DNA, D2: 2 ng/µL, D3: 0.2 ng/µL, D4: 0.02 ng/µL, D5:0.02 ng/µL,D6: 0.002 ng/µL and water.

FIGURES AND TABLES

A

B

5'GTCCGCGCGGCTGCCTGGTAGCCCCCATAGCCCTGCGCCGTCACGAACACGCAACTGACCAGCTCCCTCGTGCTCTAGGACAAGGATGGCGATGGTCAGTACT
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TCATGCGCTCGCTCGGCCAAAATCCCAGCGAGTCTGAGCTCCAGGACATGATCAACGAGGTCGATGCCGACAACAACGGCACCATTGACTTCCCAGGTGCGCCTC
ATCCGCTCCAGCA**CCAAGTACCACAGCTAACCCGTCCAGAATTCCTCACCATGATGGCCCGCAAGATGAAGGACACCGACTCCGAGGAAGAGATCCGGGAAG**
CCTTCAAGGTCTTCGACCGCGACAACAATGGCTTCATCTCCGCCGCCGAACTGCGCCACGTCATGACTTCTATTGGCGAGAAATTGACCGATGACGAGGTCGACG
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Figure 1. Alvarez et al., 2019.

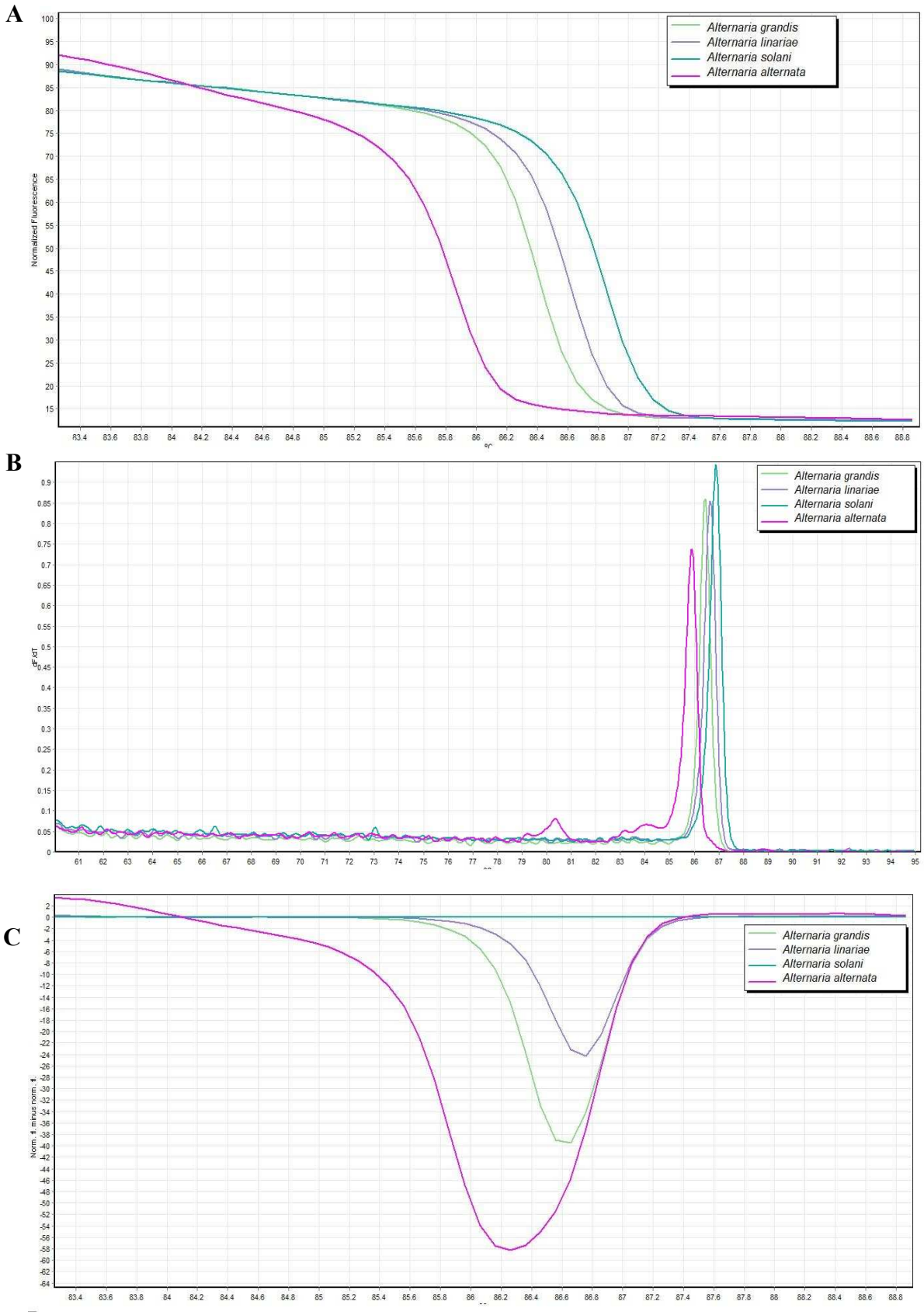
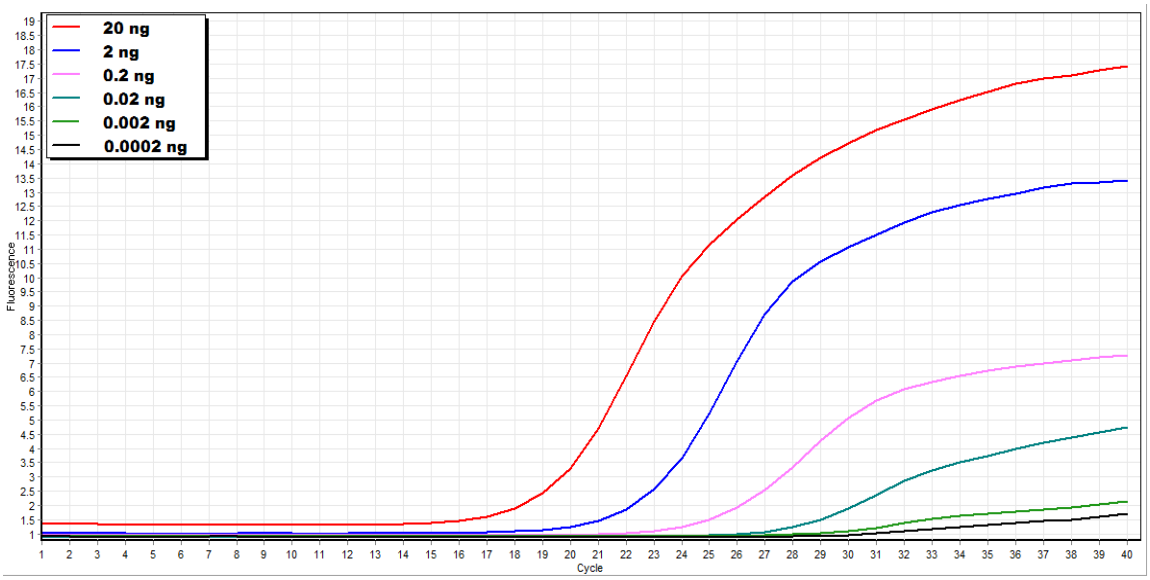


Figure 2. Alvarez et al., 2019.

A



B

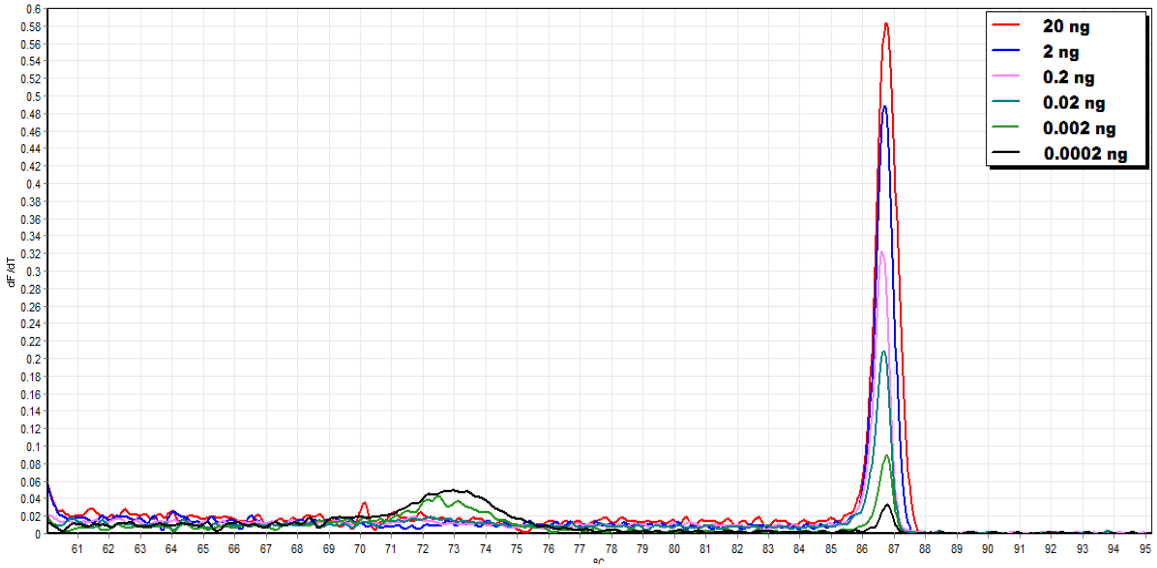


Figure 3. Alvarez et al., 2019.

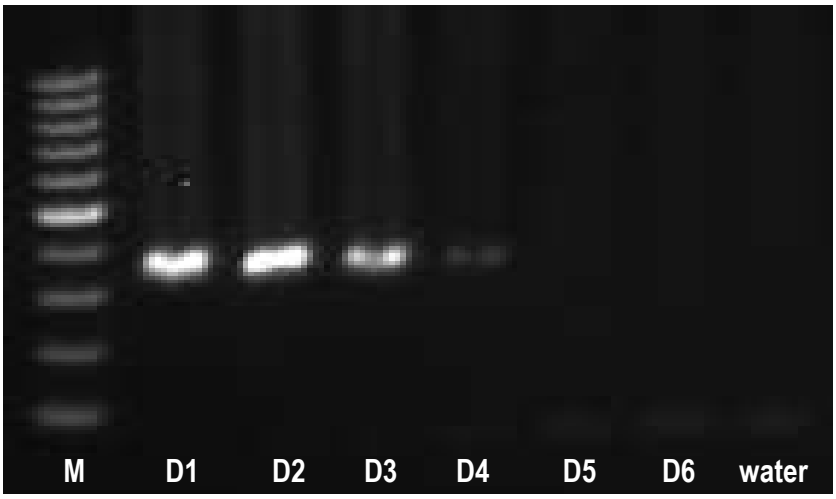


Figure 4. Alvarez et al., 2019.

Table 1. Information of the Calmodulin sequences of Alternaria ssp. that were analyzed to design primers.

Isolate-Source	Species	Location	GenBank accession
CBS 109158	A. grandis	USA, Pennsylvania	JQ646249.1
ATCC 58177	A. solani	USA, California	JQ646232.1
CBS 109156	A. linariae	Indiana	JQ646257.1

Table 2. Isolates used for the development and validation of the method.

Isolate-Source	Species	Location	Description
CBS 109158	A. grandis	USA, Pennsylvania	Ex-Type
CBS 109157	A. solani	USA, California	Ex-Type
CBS 109156	A. linariae	Indiana	Ex-Type

Table 3. Melting analysis of Alternaria species with three melting peaks distribution.

Alternaria species	Peak 1 (°C)	Peak 2 (°C)	Peak 3 (°C)
A. solani	86.14 ± 0.03	86.85 ± 0.02	87.33 ± 0.02
A. grandis	85.70 ± 0.09	86.48 ± 0.06	87.00 ± 0.02
A. linariae	85.91 ± 0.08	86.60 ± 0.02	87.09 ± 0.11
A. alternata	84.86 ± 0.11	85.88 ± 0.08	86.43 ± 0.04

Table 4. Information of the isolates of Alternaria spp. used for HRM analysis.

Isolate-Source	Host of origin	Species	Location	Year	HRM genotyping	Confidence threshold (%)
AS626	Potato cv. Agata	Undefined	Rio Paranaiba, MG	2017	Alternaria grandis	97.85 ± 0.12
AS633	Unknown Potato	Undefined	Rio Paranaiba, MG	2017	Alternaria grandis	95.36 ± 0.16
AS800	Unknown Potato	Undefined	Rio Paranaiba, MG	2017	Alternaria grandis	93.18 ± 0.11
AS801	Unknown Potato	Undefined	Rio Paranaiba, MG	2017	Alternaria grandis	81.68 ± 16.42
AS802	Unknown Potato	Undefined	Rio Paranaiba, MG	2017	Alternaria grandis	96.90 ± 1.84
AS803	Unknown Potato	Undefined	Rio Paranaiba, MG	2017	Alternaria grandis	95.32 ± 0.86
AS804	Unknown Potato	Undefined	Rio Paranaiba, MG	2017	Alternaria grandis	90.76 ± 1.63
AS805	Unknown Potato	Undefined	Rio Paranaiba, MG	2017	Alternaria grandis	90.41 ± 0.39
AS806	Unknown Potato	Undefined	Rio Paranaiba, MG	2017	Alternaria grandis	97.83 ± 0.60
AS807	Unknown Potato	Undefined	Rio Paranaiba, MG	2017	Alternaria grandis	91.70 ± 3.37
AS631	Unknown Potato	Undefined	Rio Paranaiba, MG	2017	Alternaria grandis	94.73 ± 0.07
AS808	Unknown Potato	Undefined	Rio Paranaiba, MG	2017	Alternaria grandis	92.06 ± 0.08
AS809	Unknown Potato	Undefined	Rio Paranaiba, MG	2017	Alternaria grandis	91.12 ± 0.50
AS810	Unknown Potato	Undefined	Rio Paranaiba, MG	2017	Alternaria grandis	94.05 ± 0.15
AS811	Unknown Potato	Undefined	Rio Paranaiba, MG	2017	Alternaria grandis	84.75 ± 7.82
AS812	Unknown Potato	Undefined	Rio Paranaiba, MG	2017	Alternaria grandis	97.41 ± 0.26

AS613	Unknown Potato	Undefined	Rio Paranaiba, MG	2017	Alternaria grandis	91.54 ± 3.05
AS814	Unknown Potato	Undefined	Rio Paranaiba, MG	2017	Alternaria grandis	81.92 ± 3.05
AS830	Unknown Potato	Undefined	Rio Paranaiba, MG	2017	Alternaria grandis	95.82 ± 1.06
AS185*	**Potato cv Asterix**	**Alter grandis**	**Bom Repouso, MG**	**2005**	**Alternaria grandis**	**97.12 ± 1.89**
AS260	**Potato cv. Agata**	**Alternaria grandis**	**Cristalina, GO**	**2005**	**Alternaria grandis**	**92.50± 0.21**
AS232	**Tomato cv. Miss Brazil**	**Alternaria linariae**	**Conselheiro L., MG**	**2005**	**Alternaria linariae**	**99.49± 0.05**
AS94	Unknown Tomato	Alternaria linariae	Rio Paranaiba, MG	2005	Alternaria linariae	91.20± 0.22
AS1010	Unknown Tomato	Undefined	Cajuri, MG	2018	Alternaria linariae	90.20± 0.35
AS1000	**Unknown Tomato**	**Alternaria linariae**	**Cajuri, MG**	**2018**	**Alternaria linariae**	**96.93±1.06**
AS636	Unknown Potato	Undefined	Rio Paranaiba, MG	2017	Alternaria grandis	91.20± 0.11
AS623	Unknown Potato	Undefined	Rio Paranaiba, MG	2017	Alternaria grandis	90.40± 0.17
AS815	Unknown Potato	Undefined	Rio Paranaiba, MG	2017	Alternaria grandis	94.31± 0.34
AS620	Unknown Potato	Undefined	Rio Paranaiba, MG	2017	Alternaria grandis	95.42± 0.21
AS612	Unknown Potato	Undefined	Rio Paranaiba, MG	2017	Alternaria grandis	92.14± 0.22
AS627	Unknown Potato	Undefined	Rio Paranaiba, MG	2017	Alternaria grandis	91.21± 0.12
AS816	Unknown Potato	Undefined	Rio Paranaiba, MG	2017	Alternaria grandis	90.21± 0.10
AS220	Unknown Potato	Undenifed	Rio Paranaiba, MG	2005	Alternaria grandis	94.17± 0.13
AS594	Unknown Potato	Undefined	Rio Paranaiba, MG	2016	Alternaria grandis	91.23± 0.12
AS817	Unknown Potato	Undefined	Rio Paranaiba, MG	2017	Alternaria grandis	96.16 ±2.47

AS632	Unknown Potato	Undefined	Rio Paranaiba, MG	2017	Alternaria grandis	90.22 ± 6.86
AS609	Potato c.v. Markies	Undefined	Rio Paranaiba, MG	2017	Alternaria grandis	90.31 ± 2.93
AS621	Potato c.v. Agata	Undefined	Rio Paranaiba, MG	2017	Alternaria grandis	98.38 ± 1.01
AS604	Potato c.v. Agata	Undefined	Rio Paranaiba, MG	2017	Alternaria grandis	92.59 ± 0.78
AS618	Potato c.v. Agata	Undefined	Rio Paranaiba, MG	2017	Alternaria grandis	94.26 ± 0.71
AS606	Potato c.v. Markis	Undefined	Rio Paranaiba, MG	2017	Alternaria grandis	90.31 ± 4.08
AS634	Potato c.v. F100-08-03	Undefined	Rio Paranaiba, MG	2017	Alternaria grandis	96.81 ± 0.29
AS818	Unknown Potato	Undefined	Rio Paranaiba, MG	2017	Alternaria grandis	90.54 ± 8.64
AS88	Unknown Potato	Undefined	Rio Paranaiba, MG	2005	Alternaria grandis	91.92 ± 5.85
AS819	Unknown Potato	Undefined	Rio Paranaiba, MG	2017	Alternaria grandis	94.88 ± 1.77
AS630	Unknown Potato	Undefined	Rio Paranaiba, MG	2017	Alternaria grandis	98.38 ± 0.96
AS605	Potato c.v. Agata	Undefined	Rio Paranaiba, MG	2017	Alternaria grandis	95.03 ± 4.49
AS820	Unknown Potato	Undefined	São Paulo	2019	Alternaria grandis	92.45± 2.1
AS821	Unknown Potato	Undefined	São Paulo	2019	Alternaria grandis	91.36± 3.2
AS607	Unknown Potato	Undefined	Rio Paranaiba, MG	2017	Alternaria alternata	91.23± 0.15
AS628	**Potato c.v. Agata**	**Alternaria alternata**	**Rio Paranaiba, MG**	**2017**	**Alternaria alternata**	--------

***Isolates used for the validation of the method**

GENERAL CONCLUSIONS

1. The HST and metabarcoding allowed to reveal and assess the hidden diversity of the Ecuadorian landraces soil. Edaphic factors are the main forces that shape the structure of the potato rhizosphere microbiome in the Ecuadorian Andes.

2. The microbiome structure was strongly influenced by soil and host characteristics in the soil reciprocal transplant experiment.

3. The potato microbiome transfer had a remarkable effect on the epidemics of early blight on potato.

4. The infection by different species of Alternaria impacted the composition profile of the potato and tomato leaf microbiome. Complex relationships are present between different species of Alternaria spp. related to early blight and potato and tomato microbiota.

5. The HRM-calmodulin assay allowed to discriminate to different species of large-spored Alternaria classified in the section Porri (A.solani, A.grandis and A. linariae) with accurate and reliable results.