Microbial Community Analysis of Rhizosphere of Healthy and Wilted Pepper (*Capsicum Annuum* L.) in An Organic Farming System

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Research Article

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Abstract

Rhizosphere microorganisms play an important role in the growth and health of plants. Around the world, diverse soil-borne pathogens attack *Capsicum annuum* causing significant damage and economic losses; however, very little is known about how rhizosphere microbial communities are altered by infestation with root pathogens. This work aimed to determine whether the diversity and structure of microbial communities in the rhizosphere soil of *C. annuum* plants is significantly changed by wilt disease. We used 16S rRNA for bacteria and the internal transcribed spacer region for fungi, to characterize the rhizosphere microbiomes of healthy and wilted plants. The most abundant bacterial phyla were Proteobacteria and Gemmatimonadetes, while the most abundant fungal phyla were Ascomycota and Mucoromycota. The bacterial α -diversity did not show significant differences in richness and diversity, but did show a significant difference in evenness and dominance of species. Rare taxa were present in both healthy and wilted conditions with relative abundances < 1%. In the fungi, all evaluated estimators showed a significant reduction in the wilted condition. The β -diversity showed significant differences in the structure of bacterial and fungal communities, which were segregated according to plant health condition. The differential abundance analysis did not show significant results in the bacterial communities; however, in the fungal communities, *Rhizopus*, *Thanatephorus*, *Curvularia*, *Fusarium*, *Cladosporium*, and *Alternaria* were more abundant in the rhizosphere of wilted than healthy plants. Species from these genera have been previously reported as phytopathogens of several plants, including in reports on individual species as disease agents in *C. annuum*.

Introduction

The soil microbiota plays an essential role in decomposing organic matter, cycling nutrients and fertilizing the soil, as well as in interactions with plants to provide protection against pathogens [6], water stress [41], and the assimilation of minerals such as phosphorus [10]. The rhizosphere is a zone of high biological activity, with many interactions between plants and rhizobacteria that enhance plant growth and biological control activity [26]. This relationship provides the plants with protection against pathogens, by altering their microbiome to a beneficial community [6]. Beneficial organisms that have been found in the rhizosphere include plant growth-promoting rhizobacteria (PGPR), nitrogen-fixing bacteria, and mycorrhizal fungi [39, 44], as well as *Trichoderma* spp., *Metarhizium* spp., *Beauveria* spp. [23, 47], and others. Many genera of PGPR have been reported to interact with plants, including *Agrobacterium*, *Azotobacter*, *Azospirillum*, *Bacillus*, *Caulobacter*, *Chromobacterium*, *Erwinia*, *Flavobacterium*, *Micrococcus*, *Pseudomonas*, and *Serratia*, as well as nitrogen-fixing endophytic rhizobacterial genera, such as *Bradyrhizobium*, *Allorhizobium*, *Mesorhizobium*, and *Azorhizobium* [15, 25–27]. Actinomycetes are one of the major components of rhizobacteria that are beneficial to plants, and include *Streptomyces*, *Streptosporangium*, *Thermobifida*, and *Micromonospora*.

Chili pepper (*Capsicum annuum* L.) is one of the most important crops grown in Mexico. It can be used as a spice, a vegetable of high nutritional value, a food colorant, in addition to having pharmaceutical applications [3]. Diverse pathogens such as *Phytophthora capsici* [17], *Verticillium dahliae* [54, 69],

Fusarium oxysporum [68, 69], *Fusarium lateritium* [67], *Fusarium solani* [64], *Rhizoctonia solani*, *Pythium* spp. [69], *Sclerotinia sclerotiorum*, *Sclerotium rolfsii* [64], and *Macrophomina* sp. [67], trigger root-rot as well as wilting, stem-, leaf-, and fruit-blight, which cause significant damage to chili pepper crops, and thus, economic losses in production in Mexico [11, 22, 53, 57]. Previous studies have reported that beneficial microbes, such as PGPR and beneficial fungi [31, 32, 38], can be recruited by host plants to counteract pathogen infection [14].

For several years, the interactions between plants and pathogens have been studied under the concept of an individual plant–microorganism relationship, an approach that ignores the complexity of such interactions and the involvement of many other groups of microorganisms that affect the outcome of infection. Aside from these studies of diseasecausing agents and biocontrol microorganisms, to our knowledge few studies using Next-Generation Sequencing have been carried out to identify communities of microorganisms in the rhizosphere of *Capsicum annuum* L. Metagenomic analysis and comparison of plant-associated microbiomes have led to the successful identification of microorganisms that are either beneficial or pathogenic to plants. The objective of this study was to characterize and compare the diversity and structure of the rhizosphere microbiome in healthy and wilted *C. annuum* L. plants through 16S rRNA and ITS region amplicon sequencing by Illumina MiSeq. This study has deepened our understanding of the changes in microbial communities and the presence of beneficial and pathogenic members associated with the rhizosphere of *C. annuum* plants.

Methods

Site description and samples collection

We collected soil samples from organic farm plots under *C. annuum* cultivation, containing healthy and wilt-diseased plants, at a location in the municipality of Camargo, in Chihuahua State, Mexico (27°39'75"N, 105°07'84"W; 1240 masl) in June 2020. We took samples randomly with three replicates of soils from plants in each health condition (healthy or wilted). We selected rhizosphere soil samples from three plants at the fruiting stage (~40 cm tall) in each condition: first, we collected rhizosphere soils from wilt-diseased plants (wilted condition) 40 m apart from each other; then, we collected rhizosphere soil samples from healthy plants (healthy condition) at a distance of 100 m from the wilted plants, to complete a total of three replicates for each condition. We placed the rhizosphere soil samples individually in sterile bags and stored them on ice for transport to the laboratory, where they were then stored until processing.

Rhizosphere samples were numbered as follows: BJS1, BJS2, and BJS3, as well as HJS1, HJS2, and HJS3 were bacterial and fungal soil samples, respectively, from healthy *C. annuum* plants; and BJE1, BJE2, and BJE3, as well as HJE1, HJE2, and HJE3 were bacterial and fungal soil samples, respectively, from wilted *C. annuum* plants.

Metagenomic Dna Extraction, Library Preparation, And Sequencing

We extracted the metagenomic DNA individually from each soil sample using a ZymoBIOMICS™ DNA Miniprep Kit (Zymo Research, Irvine, CA, U.S.A.) following the manufacturer's instructions. We quantified the quality and integrity of the DNA using a NanoDrop 2000c spectrophotometer (Thermo Scientific, Wilmington, DE) based on its A260/280 ratio, and observed it in a 1.0% agarose gel electrophoresis. The 16S rRNA gene (V3-V4 region) and ITS region (ITS) libraries obtained from bacterial and fungal communities, respectively, were amplified and sequenced using paired-end 2 × 250 bp on an Illumina MiSeq sequencer (Illumina Inc., San Diego, CA, U.S.A.) at Novogene, Beijing, China.

Bioinformatic Analysis

We merged demultiplexed raw paired-end sequences in fastq format using FLASH v1.2.11 with default settings [40]. We quality-filtered, trimmed, and de-noised the merged sequences using DADA2 [8] in Quantitative Insights Into Microbial Ecology (QIIME2 v2020.2) [7] to obtain representative amplicon sequence variants (ASV). After completing the quality filtering step, we did multiple sequence alignment and phylogenetic reconstruction using MAFFT and FastTree, respectively, to generate a rooted phylogenetic tree [33, 51]. We extracted representative sequences and their abundances by feature-table, and did the taxonomy assignment with a pre-trained Naïve Bayes classifier using the Greengenes (v.13_8) database for bacteria, and the UNITE (v.8_99) database for fungi.

In order to analyze the α - and β -diversity of bacterial and fungal communities and conduct related statistical tests, we rarefied the samples at the depth of the library with the lowest number of reads, and calculated the metrics using the R package *vegan*. To explore α -diversity within these communities in both healthy and wilted conditions, we estimated species richness using Chao1, species diversity with the Shannon index, dominance with the Simpson index, and the species evenness index; using the Kruskal–Wallis test these were considered statistically significant at p values < 0.05.

To investigate differences in bacterial and fungal communities' composition between healthy and wilted conditions, we performed a principal coordinate analysis (PCoA) based on Bray–Curtis distances and unweighted UniFrac distances. We then used an analysis of similarities (ANOSIM) to test for significant differences among bacterial and fungal communities. Venn diagrams were plotted using the feature-table at the genus level with the R package *ggvenn*. Finally, we did a differential abundance test using ALDEx2 [20] to identify bacterial and fungal taxa that were significantly different across the rhizosphere samples from both healthy and wilted conditions at the genus level. We depicted the results as a volcano plot and bar plots using the R package *ggplot2*.

Results

Sequencing results

We obtained a total of 1,218,821 and 1,154,540 raw sequences from bacteria and fungi, respectively. After applying the quality control criteria, we retained a total of 857,743 and 1,040,617 high-quality sequences from bacteria and fungi, respectively. To conduct subsequent analyses, we rarefied the samples to the lowest number of reads per library; in the case of bacteria, we homogenized all samples to 134,116 reads (Supplementary Fig. 1a); and for fungi, we homogenized the samples to 162,089 reads (Supplementary Fig. 1b).

Microbial Community Composition

A total of 43 distinct phyla, 241 families, and 458 bacterial genera were identified. At the phylum level, Proteobacteria was the most abundant with > 36% of relative abundance, followed by Gemmatimonadetes (20.34%), Actinobacteria (11.51%), Bacteroidetes (9.71%), Acidobacteria (7.72%), Firmicutes (4.24%), Chloroflexi (4.11%), Nitrospirae (1.36%), and Verrucomicrobia (1.16%); the remaining 34 phyla had relative abundance < 1.00%. At the family level, Cytophagaceae was the most abundant (4%), followed by Sphingomonadaceae (3.73%), Xanthomonadaceae (2.83%), Rhodospirillaceae (2.61%), Hyphomicrobiaceae (1.66%), Bacillaceae (1.32%), Chitinophagaceae (1.15%), and Bradyrhizobiaceae (1.13%); the remaining families represented < 1.00%. At the genus level, a high number of bacteria were found (458 genera), the relative abundance distribution was very homogeneous; in fact, the most abundant genera were *Kaistobacter*, *Bacillus*, *Rubrobacter*, *Streptomyces*, *Balneimonas*, *Nitrospira*, and *Salinimicrobium*, with values ranging from 5.67–3.17% of relative abundance; 16 genera ranged from 2–1%, and the remaining 435 genera represented values < 1.00% of relative abundance (Fig. 1a shows the 20 most abundant bacterial genera).

Among the fungi, 13 distinct phyla, 132 families, and 257 genera were identified. The phylum Ascomycota was the most abundant taxonomic group, representing > 75% of the relative abundance, followed by Mucoromycota (14.88%), Mortierellomycota (2.84%), and Basidiomycota (1.2%); and the remaining 9 phyla were present at abundances < 1.00%. At the family level, the Aspergillaceae family represented > 28% of relative abundance, followed by Nectriaceae (25.43%), Rhizopodaceae (14.83%), Chaetomiaceae (5.97%), Hypocreales_fam_Incertae_sedis (3.2%), Mortierellaceae (2.83%), Sporormiaceae (1.29%), and Cladosporiaceae (1.25%); the remaining 124 families represented < 1.00%. At the genus level, the most abundant was *Aspergillus* with > 27% of relative abundance, followed by *Rhizopus* (14.83%), *Fusarium* (12.09%), *Acremonium* (3.09%), *Mortierella* (2.83%), *Chaetomium* (2.75%), *Cladosporium* (1.25%), *Acrophialophora* (1.19%), and *Preussia* (1.14%); the remaining 248 genera had relative abundances < 1.00% (Fig. 1b shows the 20 most abundant fungal genera).

Comparison of bacterial and fungal communities between plants in healthy and wilted condition

The Venn diagrams displayed the bacterial and fungal genera shared by plants in both healthy and wilted conditions, and those exclusive to plants in either condition. In the case of bacteria, out of a total of 458

genera found, 312 genera were shared by both conditions; and 82 and 64 genera were exclusively found in healthy and wilted conditions, respectively (detailed information on shared and exclusive bacterial genera is available in Supplementary Table 1). On the other hand, of the 257 fungal genera identified, 149 were shared by both conditions, while 75 were exclusive to the healthy condition, and 33 were unique to the wilted condition (detailed information on shared and exclusive fungal genera is available in Supplementary Table 2).

α - And β -diversity

The rarefaction curves of the bacterial and fungal samples all tended to approach the saturation plateau (Supplementary Fig. 1). The results of estimators of α -diversity did not show significant differences in the diversity and species richness of bacterial communities on the Shannon and Chao1 indexes ($p > 0.05$); however, the species equity and dominance measured with Evenness and Simpson index showed significant differences ($p < 0.05$) (Fig. 3a). For fungi, the Shannon, Chao1, Evenness, and Simpson indexes were significantly different, evidenced by higher values in the rhizospheres of healthy plants than those of wilted ones ($p < 0.05$) (Fig. 3b).

Using principal-coordinate analysis (PCoA) we examined the variation of bacteria between healthy and wilted conditions (β -diversity) based on weighted UniFract and Bray–Curtis distances, which explained 94.1% and 75.4% of the total observed variation, respectively, and revealed that rhizosphere bacterial communities were clustered by health conditions (ANOSIM; $p < 0.05$) (Fig. 4a). Similarly, in the fungal communities, PCoA showed clustering according to health conditions (ANOSIM; $p < 0.05$), which explained 99.6% (weighted UniFract) and 99.1% (Bray–Curtis) of the total observed variation (Fig. 4b).

Differential Abundance Analysis

Our analysis of the data with ALDEx2 helped us gain a better understanding of the significant changes in the abundance of microbial communities in both healthy and wilted conditions. In the bacteria, significant differences in abundance were not observed ($p > 0.05$) (Fig. 5a); however, in the fungi, a total of 17 genera had abundances that differed significantly between plant health conditions ($p < 0.05$) (Fig. 5b). The genera with significant abundance in the healthy condition were *Setophaeosphaeria*, *Pseudogymnoascus*, and *Mortierella*; while *Rhizopus*, *Conocybe*, *Thanatephorus*, *Trichophaeopsis*, *Myceliophthora*, *Curvularia*, *Fusarium*, *Podospora*, *Cladosporium*, *Chaetomium*, *Alternaria*, *Acrophialophora*, *Coprinopsis*, and *Neurospora* showed significant abundance in the wilted condition (Supplementary Fig. 2).

Discussion

The spread of *C. annuum* wilt and the high incidence of damage, together with the few studies of the associated microorganisms that inhabit the rhizosphere, result in great concern for all personnel involved

in the cultivation and management of this agriculturally important crop. In the present study, we analyzed the diversity and structure of bacterial and fungal communities through 16S rRNA and ITS region amplicon-based metagenomic sequencing in rhizosphere soil of healthy as well as wilt-diseased plants under organic farming conditions. From the results, we identified a diverse community of microorganisms, of which several bacterial and fungal members were either shared by, or exclusive to, the rhizospheres of *C. annuum* plants depending on their health condition.

The taxonomic assignment confirms that rhizospheric soil of *C. annuum* is composed mainly of the Proteobacteria, Gemmatimonadetes, Actinobacteria, Bacteroidetes, and Acidobacteria phyla, the first being the most abundant. The phylum Proteobacteria has been reported in many studies for its association with the rhizosphere of plants, particularly in some studies on soil growing *C. annuum* [4, 34, 60, 75]. In general, at the genus level, microbial diversity was very high and homogeneous, independent of the health conditions of the plants, although genera such as *Kaistobacter*, *Bacillus*, *Rubrobacter*, *Streptomyces*, *Balneimonas*, *Nitrospira*, and *Salinimicrobium* were slightly more abundant than others in the rhizosphere. These genera have been reported as the most frequent in soil [21, 71]. Little information is available about the genus *Kaistobacter*, however, some studies have reported species of this genus as being associated with active disease suppression in the rhizosphere of tobacco plants [21, 37]. Moreover, genera including *Bacillus*, *Rubrobacter*, and *Streptomyces* have been reported as suppressors of rhizosphere fungi that are pathogenic to other plant species, such as *Fusarium oxysporum*, *Rhizoctonia solani*, and *Verticillium dahliae* [9, 12, 30, 56, 73]. These species were also found in this study, so future analyses should be carried out to evaluate the biological control capabilities of these bacterial members.

The Ascomycota, Mucoromycota, Mortierellomycota, and Basidiomycota were the most abundant fungal phyla, of which the first had the highest abundance. Although it is reported as a widely found phylum in the rhizosphere, only few studies have described the fungal communities associated with *C. annuum* as well as other plants belonging to the Solanaceae family [46, 58]. The genus *Aspergillus*, the most abundant in this study, has been reported for its antifungal activity against *Phytophthora capsici* [32]; however, as that study was conducted on *in vitro* bioassays, caution must be exercised in drawing conclusions on biological interactions that occur under *in vivo* conditions.

Studies that have evaluated the α -diversity of rhizosphere communities in agricultural crops have reported variations in the diversity, presumably because of the different factors to be considered at the time of the study (e.g., temperature, plant age, sampling season, crop rotation). Similar to the results reported in this study on bacterial richness and diversity, in plants of the Solanaceae and Piperaceae families a high bacterial diversity has been reported regardless of whether the plants were healthy or diseased [28, 35], but this was not so for evenness, which significantly declined when the crop became diseased [55]. In fungal communities, similar to what occurred in this study, a higher α -diversity has been reported in healthy plants than in diseased plants [35, 62, 72]. It is worth mentioning that the agronomic practices in the plots sampled in this study were organic, so soil microorganisms had not been exposed to agrochemicals.

In terms of community structure, several studies showed changes in the β -diversity of bacterial and fungal rhizosphere communities, caused by plant pathogen infestation [66]; our results also demonstrated that the β -diversity was segregated according to plant health condition. These differences in microbial communities may have occurred for several reasons, such as modification of soil properties due to attack and colonization of *C. annuum* plants by pathogens (e.g., pH, nutrient solubility, O₂, CO₂, moisture), which triggered a modification of the ecological niche and resulted in the recruitment of microorganisms that exert either deleterious or beneficial effects on the plants [16].

The differential abundance test showed no significant difference in the bacterial rhizosphere regardless of plant health condition, whereas the abundance in the fungal rhizosphere differed significantly between plant conditions. In the rhizosphere of healthy plants, a significant difference was observed in the genus *Mortierella*, members of which have been reported as plant growth promoters, as well as antibiotic and phytohormone producers, thereby improving resistance to phytopathogens in plants of agricultural importance [42, 48, 74]. Moreover, in the rhizosphere of wilted plants, significant differences were shown in several fungal taxa, particularly in some genera that have been reported as phytopathogenic agents in various crops. This has led us to hypothesize that this increase in abundance of *Thanatephorus* and *Fusarium* species is responsible for triggering the symptomatology that affects the plants, and which subsequently allows other opportunistic pathogens (e.g., *Rhizopus*, *Curvularia*, *Cladosporium*, and *Alternaria*) to jointly infect the plant until its decay.

The genus *Rhizopus* was found with a higher abundance in the rhizosphere of the wilted plants, and this pathogen has been previously associated with the deterioration of crops in chili pepper [1, 19] and other plants, such as soybean [2], apple [61], and sugar beet [24]. Similarly, *Thanatephorus*, *Fusarium*, and *Alternaria* have been found to be dominant fungal genera in rhizosphere soil of chili pepper [5, 18, 50, 64, 69], soybean [2], mung beans [13], and maize [43] as causal agents of wilt, root rot, leaf spot, and/or fruit and seed rot. Also, *Curvularia* is a plant pathogen [63]; *C. lunata* has been reported as the causal agent of maize leaf spot [36], leaf spot disease of *Clerodendrum indicum* [45], and root rot of strawberry [70]. Finally, *Cladosporium* species have been reported as pathogenic fungi of members of the Solanaceae family such as tomato [65] and chili pepper [29], in which they cause foliar damage. Surprisingly, *Phytophthora* was not found in the rhizosphere of wilted or healthy chili pepper plants, while other studies carried out even in the same region have reported it as the causal organism of pepper wilt [52].

Diseases caused by soil-borne plant pathogens can be difficult to control for a variety of reasons, as many soil-borne pathogens produce persistent resistance structures that can survive in the soil for many years. Even in the absence of a susceptible host, the agricultural practices focused on reducing pathogens are either unsuitable or insufficient, as well as the selective pressure from the other microorganisms in the rhizosphere, because of competition for nutrients and essential elements [49]. However, as many other groups of microorganisms affect plant health, their pathogenic action could occur in conjunction. For example, it has been demonstrated that pathogens have the ability to secrete effector molecules that can affect the communication between plants and beneficial microorganisms,

and that they can also recruit other microorganisms that compete against native microorganisms and help in the colonization of the host [6, 59].

In summary, our findings provide evidence that wilt-disease in *C. annuum* has an impact on reducing diversity and changes in the structure of bacterial and fungal rhizosphere communities. We found a complex and diverse microbial community, composed of bacterial members with a homogeneous abundance. In contrast, the abundance of some members of the fungal community was a little more heterogeneous. Several of the fungal genera we found have been reported as phytopathogens in chili pepper and other plants where a change in their individual abundance was observed, increasing significantly as the chili pepper plants wilted. In addition, further experiments should be done that isolate the pathogens found as well as the potentially beneficial microorganisms from the rhizosphere soil, to study their importance and focus on the interactions within soil microbial communities in an effort to elucidate possible biocontrol strategies. Finally, it would be interesting to explore, through metatranscriptomic analysis, the metabolic capabilities that are triggered in the rhizosphere when a plant enters a state of disease, in order to review the mechanisms of action exerted by both potentially pathogenic microorganisms and those that confer resistance to the plants.

Availability of data and materials

The datasets generated and analyzed during the current study are available in the NCBI Sequence Read Archive (SRA) repository under the BioProject with accession code PRJNA728362.

Authors' contributions

RG-E, LNM-C, and GDA-Q designed the research; LNM-C conducted the project administration; GDA-Q conducted the rhizosphere soil sampling; RG-E performed the processing and preparation of the samples; ZYM-R performed bioinformatic analysis and prepared the figures; RG-E wrote the original draft; RG-E, LNM-C, ZYM-R, CGL, and GDA-Q participated in the analysis of data, reviewed and edited the manuscript. All authors read and approved the final manuscript.

Declarations

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LNM-C, ZYM-R, CGL, and GDA-Q participated in the analysis of data, reviewed and edited the manuscript. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no conflict of interest.

Ethics Approval

Not applicable.

Consent for Publication

Not applicable.

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Figures

(a)

(b)

Figure 1

Heat map depicting bacterial (a) and fungal (b) diversity based on the relative abundances.

Figure 2

Venn diagrams showing the distribution of (a) bacterial and (b) fungal genera shared by the rhizospheres of both healthy and wilted *C. annuum* L. plants, and those exclusive to either condition.

Figure 3

α -diversity index of bacterial (a) and fungal (b) communities.

Figure 4

PCoAs built with weighted unifrac and pairwise Bray-Curtis dissimilarity matrices of bacterial (a) and fungal (b) communities.

Figure 5

Volcano plot of differential abundance analysis of bacterial (a) and fungal (b) communities. Dots in red represent genera with differentially significant abundance.

Supplementary Files

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