Distinct roles for soil fungal and bacterial communities associated with the suppression of vanilla *Fusarium* wilt disease

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ABSTRACT

Characterizing microbial communities associated with disease-suppressive soil is an important first step toward understanding the potential of microbiota to protect crops against plant pathogens. In the present study, we compared microbial communities in suppressive- and conducive-soils associated with *Fusarium* wilt disease in a vanilla long-term continuous cropping system. Suppressive soil was associated with higher fungal diversity and lower bacterial diversity. The fungal phyla *Zygomycota* and *Basidiomycota*, and the bacterial phyla *Acidobacteria*, *Verrucomicrobia*, *Actinobacteria* and *Firmicutes* were strongly enriched in the suppressive soil. Notably, suppressive soil was dominated by the fungal genus *Mortierella*, accounting for 37% of the total fungal sequences. The hyper-dominance of *Mortierella* spp. in suppressive soil suggests that this taxon may serve as an indicator and enhancer of *Fusarium* wilt disease suppression in vanilla. In addition, Molecular Ecological Network analysis revealed that fungal communities were more connected and showed more co-occurrence relationships in the suppressive versus conducive soils. Our results indicate that fungal communities may be important in the development of soil suppressiveness against vanilla *Fusarium* wilt disease.

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1. Introduction

Some soils, referred to as disease-suppressive, can naturally suppress plant pathogens, suggesting that they harbor specific characteristics that keep pathogens in check (Cha et al., 2016; Cook and Rovira, 1976). Microorganisms, including bacteria and fungi, are the main drivers of soil suppressiveness (Chaparro et al., 2012; Garbeva et al., 2004). Understanding which microbial communities are associated with disease-suppression can provide the foundation for soil community manipulation and new opportunities to explore novel strategies to promote plant health in a sustainable way (Stone et al., 2004).

Vanilla (*Vanilla planifolia*), a high-value cash crop, is widely cropped in tropical and subtropical regions (Minoo et al., 2008). However, this crop is seriously threatened by *Fusarium* wilt disease, caused by *Fusarium oxysporum* f. sp. *vanillae* (Pinaria et al., 2010).

Vanilla is typically grown as a monoculture, resulting in the rapid accumulation of *Fusarium* pathogen densities in soils (Xiong et al., 2015b). *Fusarium* pathogen accumulation can be controlled by crop rotation (Xiong et al., 2016), but such measures are generally impractical due to monetary and labor costs.

In previous field surveys, we discovered that some soils on Hainan Island China, retain a low *Fusarium* wilt disease incidence even after decades of vanilla monoculture. In these soils, the pathogen is present but remains at a low level and does not cause damage to the crop. We hypothesized that distinct soil microbial communities in these soils may explain differences in *Fusarium* wilt disease incidence between the geographically proximate fields, which otherwise share the same climatic conditions, agronomic management and fertilization regimes (Xiong et al., 2015b). Soil microbial communities play an essential role in the suppression of *Fusarium* wilt disease in several other plants, such as strawberry and banana (Cha et al., 2016; Shen et al., 2015). However, bacterial and fungal communities are rarely investigated together in disease suppressive soils (Cha et al., 2016; Mendes et al., 2011; Penton et al.,

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2014) and fungal communities are often overlooked.

In the present work, we aimed for an integrative study of soil microbial communities and analyzed the relative importance of bacterial and fungal communities for the suppression of *Fusarium* wilt. We tested whether suppressive and conducive soils differed in bacterial and fungal abundance, diversity and taxonomic composition. Further, we used association networks to examine the frequency of interactions within microbial communities associated with the suppressive-versus conducive-soils.

2. Materials and methods

2.1. Site description and sampling

We selected two model orchards continuously planted with vanilla for at least 20 years in Hainan province, China. Both two orchards have similar edaphic properties (loam soil), agronomic management history and fertilization regimes. The mean annual temperature and precipitation in this area are 24.5 °C and 2200 mm. However, the two orchards differ strongly in *Fusarium* wilt disease incidence. The first study site (later: suppressive soil), a vanilla orchard located in the town of Gaolong (18°736′N-18°738′N, 110°191′E-110°193′E), has been continuously cropped with vanilla since 1989, yet harbors a low *Fusarium* wilt disease incidence of less than 10% during the last ten years. The second orchard (later: conducive soil) is situated 4.6 km from the first field, in the town of Xinglong (18°698′N-18°700′N, 110°170′E-110°171′E). This orchard has been cropped continuously with vanilla for over 20 years and has a high disease incidence (over 65% over the last three years). Based on the accounts of the local farmers, *Fusarium* wilt was not detected at this site prior to vanilla cropping.

For each site, 9 random subplots (about 60 m^2) were chosen and 10 random cores (0—20 cm in depth) from each subplot were collected using a 2.5 cm diameter (at least 2 m between the cores) in April 2014. The 10 random cores from each subplot were mixed to form one composite sample, resulting in 9 samples per site. The 18 soil samples were placed into separate sterile plastic bags and transported to the laboratory on ice. Each soil sample was sieved through a 2-mm sieve and thoroughly homogenized. One portion of each sample was air-dried for chemical analysis according to our previous methods (Xiong et al., 2015b), and the other portion was stored at −80 °C for subsequent DNA extraction.

2.2. Assessing the disease suppressive ability of soils in pots

For the pot experiments, soils were collected with a shovel in the direct vicinity of the cores used for DNA extraction. Soils were thoroughly mixed for each site. In order to assess whether disease suppression can be attributed to microbial communities rather than differences in physicochemical properties, we performed soil suppressiveness assay based on Mendes et al. (2011) with some modifications. Briefly, we set up four treatments as follows: 1) suppressive soil (S), 2) conducive soil (C), 3) conducive soil amended with 50% (w/w) of suppressive soil (SC), and 4) conducive soil amended with 50% (w/w) of heat-treated (90 °C for 2 h) suppressive soil (S$_{90}$C). For each treatment, the soil was thoroughly mixed and poured into the sterilized pots (15 kg soil per pot). Each treatment contained three replicates, and each replicate consisted of five pots. Three vanilla seedling were planted in each pot (Xiong et al., 2015b). Pots were incubated in a greenhouse (located at the Spice and Beverage Research Institute, Wanning City, Hainan Province, China) at 30 °C and with 72% relative humidity with a randomization of all pots. Vanilla seedlings were monitored daily for the appearance and severity of vanilla *Fusarium* wilt disease. Disease symptoms typically manifested themselves approximately three weeks after planting, and disease incidence was calculated as the percentage of infected plants among the total number of plants.

2.3. DNA extraction, PCR amplification and illumina sequencing

For each composite soil sample, total DNA was extracted from 0.5 g soil using the MoBioPowerSoil™ DNA Isolation Kit (Mo Bio Laboratories Inc., Carlsbad, CA, USA) according to the manufacturer's instructions. Genomic DNA concentration and purity were measured using a NanoDrop ND-2000 (NanoDrop Technologies, Wilmington, DE) spectrophotometry. The primer set: ITS1F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) (Gardes and Bruns, 1993) and ITS2 (5′-GCTGCGTTCTTCATCGATGC-3′) (White et al., 1990) was selected to target the fungal ITS1 region. 520F (5′-AYTGGGYD-TAAAGNG-3′) and 802R (5′-TACNVGGGTATCTAATCC-3′) (Claesson et al., 2009) was used to amplify the V4 hypervariable regions of the bacterial 16S rRNA gene. Primer pairs were modified for sequencing by adding the forward Illumina Nextera adapter, a two basepair "linker" sequence, and a unique 7-bp barcode sequence at the 5′ end of the forward primer, and the appropriate reverse Illumina Nextera adapter and linker sequence at the 5′ end of the reverse primer. PCR was performed following previously published amplication conditions (Xiong et al., 2015b). Briefly, 27 and 25 cycles were performed to amplify fungal and bacterial templates, respectively. Then, the PCR products were then purified using a PCR Purification Kit (Axygen Bio, USA) and pooled in equimolar concentrations of 10 ng μl^{-1} before sequencing. Finally, paired-end sequencing of fungal and bacterial amplicons were carried out on the Illumina MiSeq sequencer at Personal Biotechnology Co., Ltd (Shanghai, China).

2.4. Quantification of the Fusarium oxysporum, bacterial and fungal abundances

We quantified *Fusarium oxysporum*, bacterial and fungal abundances using quantitative polymerase chain reaction (qPCR) according to the established protocols (Xiong et al., 2016, 2015a). Briefly, we set up a 20-μl reaction mixture containing 10 μl of the *Premix Ex Taq^{TM}* (2 ×) (Takara-Bio, Japan), 0.4 μl of each primer (10 μM), 0.4 μl of ROX Reference Dye II (50 ×), 2 μl of template DNA and 6.8 μl of ddH$_2$O. The following primer pairs used: AFP308R (5′-CGAATTAACGCGAGTCCCAAC-3′) and ITS1F (5′-CTTGGTCATTTA-GAGGAAGTAA-3′) (Lievens et al., 2005) for *Fusarium oxysporum*, ITS1F and ITS2 for fungi, and 520F and 802R for bacteria. The PCR thermal conditions were as follows: 30 s at 95 °C for initial denaturation, followed by 40 cycles of 5 s at 95 °C, and 34 s at 60 °C. Standard curves were obtained according to our previous protocols (Xiong et al., 2016, 2015a). The specificity of the amplification products was confirmed by melting curve analysis and visual inspection after agarose gel electrophoresis.

2.5. Bioinformatics analyses

After removing the adaptors and primer sequences, the raw sequences were assembled for each sample according to the unique barcode using QIIME (Caporaso et al., 2010). Split sequences for each sample were merged using FLASH V1.2.7 (Magoč and Salzberg, 2011). The sequences retained for each sample were processed following the established UPARSE pipeline (Edgar, 2013). Briefly, low-quality sequences with a quality score lower than 0.5 or a length shorter than 200 bp were discarded. After discarding singletons, the remaining reads were assigned to OTUs with a threshold of 97% identity level, followed by removal of chimeras using the UCHIME method (Edgar et al., 2011). Finally, the fungal representative OTUs were classified using the UNITE database