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Inducing the rhizosphere microbiome by biofertilizer application to suppress banana Fusarium wilt disease

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ABSTRACT

Worldwide, banana production is severely hindered by Fusarium wilt, a devastating disease caused by the soil-borne fungus *Fusarium oxysporum* f. sp. *cubense* (Foc). With no widely adopted efficient method of control or prevention, the emergence of a new Foc variant, tropical race 4 (Foc TR4), has led to the widespread destruction of plantations in Cavendish-producing areas. Recently, banana Fusarium wilt has been controlled by the consecutive application of biofertilizer (BIO) in newly reclaimed fields. In this study we examine the temporal effects of BIO versus compost application in newly converted banana fields on the composition and abundance of the rhizosphere bacterial and fungal communities and the survival of the biocontrol inoculant, *Bacillus amyloliquefaciens* NJN-6. Our findings show that BIO-amended rhizosphere soils increased the abundance of bacteria while decreasing fungal abundance. This corresponded to higher bacterial richness and diversity in the BIO amendment, while no trends were observed with the fungal community. Rhizosphere soil bacterial and fungal community composition were significantly different between BIO and compost amendment and treatment, not time, exhibited the largest impact. Other potential taxa involved in disease suppression were also identified, such as increased abundances of *Sphingobium*, *Dyadobacter*, and *Cryptococcus* and lower abundances of *Fusarium*, *Ralstonia*, and *Burkholderia*. Overall, decreased abundances of *F. oxysporum* and a lack of variability in the abundance of the biocontrol agent NJN-6 over three years contributed to disease suppression, in combination with alterations in fungal and bacterial composition and abundance, pointing to the sustainability of BIO as an amendment for disease suppression.

1. Introduction

Banana (*Musa* spp.) is one of the most important fruit crops in the world, and is a source of income in many developing countries (Dita et al., 2010). However, its production is severely hindered by Fusarium wilt, a devastating disease caused by the soil-borne fungus *Fusarium oxysporum* f. sp. *cubense* (Foc) (Butler, 2013; Ploetz, 2006). The emergence of a new Foc variant, tropical race 4 (Foc TR4), has led to the widespread destruction of plantations in Cavendish-producing areas globally, especially in China, Australia, Indonesia, Malaysia, and the Philippines (Ploetz, 2015). The pathogen can survive in soil as chlamydospores for decades, with no known efficient method of control or prevention. Therefore, once the pathogen is established in soil, new plantations must be developed from disease-free areas in order to maintain banana production (Ploetz, 2000; 2006). However, due to the re-emergence of banana Fusarium wilt, newly reclaimed areas are usually only productive for 3–6 years or less (Nel et al., 2007; Stover, 1962). This widespread loss of production and arable land for banana cultivation presents an urgent need for the development of novel, sustainable strategies in disease prevention for both current and future banana plantations.

Fusarium wilt disease is principally managed by soil fumigation (Meldrum et al., 2013), fungicides, (Nel et al., 2007), and resistant

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cultivars (Hwang and Ko, 2004). However, in recent years, with the discovery of novel natural pathogen antagonists, efforts to develop biological control mechanisms have become increasingly popular (Fravel et al., 2003; Weller et al., 2002). For example, *Trichoderma* spp., *Pseudomonas* spp. and *Bacillus* spp. were widely reported as antagonistic microbes for the biological control of banana Fusarium wilt (Saravanan et al., 2003; Thangavelu et al., 2004; Yuan et al., 2012). Furthermore, biocontrol agents combined with organic materials have been shown to enhance the activity of biocontrol microbes, offering better disease control than microbes alone (Kavino et al., 2010; Qiu et al., 2012; Shen et al., 2013). In our previous study, the application of a novel biofertilizer (BIO) consisting of *Bacillus amyloliquefaciens* NJN-6 and compost, in severe pathogen-infested soils of a banana orchard (disease incidence > 50%) resulted in high biocontrol efficiency (Shen et al., 2013; Shen et al., 2015a). However, the suitability of this BIO application in the sustainable maintenance of biocontrol efficacy in banana plantations developed from newly reclaimed fields remains largely ignored, as most farmers do not recognize the importance of pre-control of this soil-borne disease from cultivation onset (Fu et al., 2016).

Soil suppression of disease induced by organic amendments and biocontrol agents have been widely described (Bonanomi et al., 2010; Raaijmakers et al., 2009) and are more frequently related solely to the soil microbiota (Bonilla et al., 2012; Kinkel et al., 2011), mainly by general and specific suppression mechanisms (Postma et al., 2008). General soil suppression relates to the biomass, activity and diversity of microbes at the community-level and was reported to negatively correlate with invader survival (Bonilla et al., 2012; Garbeva et al., 2006; van Elsas et al., 2012). Specific suppression is thought to involve a few specific microbial groups (Mendes et al., 2011), and is primarily attributed to a microbial antagonistic mechanism (Weller et al., 2002). Additionally, the resident soil microbial community ultimately influences the success of pathogen establishment (Berendsen et al., 2012). Thus, the composition of the resident microbial community in the rhizosphere, where microbes interact with pathogens and influence the result of infection near the root surface (Raaijmakers et al., 2009), likely plays a role in pathogen invasion success. For disease suppression, the combination of organic and biological amendments relies on the hypothesis that the microbial community can be manipulated to promote soil suppressiveness against soil-borne plant diseases. Therefore, in order to further understand disease suppression, the composition of the rhizosphere microbial community and the presence of specific antagonistic microbial populations is needed in order to develop new strategies to promote plant and soil health in newly reclaimed fields.

In our previous study, banana Fusarium wilt disease was efficiently pre-controlled by consecutive applications of biofertilizer (BIO) from the initiation of banana planting in newly reclaimed fields (Fu et al., 2016). The effects of BIO application on the composition of rhizosphere microbial communities were evaluated by culture-dependent DGGE fingerprinting of the 16S rRNA gene and Biolog. The results suggested that manipulation of the composition of the rhizosphere culturable microbial community could be invoked for disease suppression. However, due to methodological limitations, this study only focused on a small fraction of the soil microbes.

In this study, we expanded upon the previous culture-based study by using high-throughput sequencing of the bacterial 16S rRNA gene and fungal ITS region to investigate how rhizosphere bacterial and fungal communities respond to organic and biological amendments in newly reclaimed banana plantations. We hypothesized that enhanced soil suppressiveness was due to a combination of improved soil general and specific suppression mechanisms and the stable survival of a biocontrol agent. To address this we compared the differences in the composition of rhizosphere microbial communities after consecutive applications of biofertilizer (BIO) and common compost and explored the potential disease suppression mechanisms within the observed variations in the community composition of the overall microflora and among populations of specific rhizospheric microbes.

2. Materials and methods

2.1. Field description

Field experiments, including one treatment applied with BIO and a control amended with common compost (matured pig manure compost; CK), were performed at Wenqiu village in Lingao County (19°812′N, 109°680′E), Hainan Province, China, over four successive years (2009–2012). The sites were previously cultivated with *Eucalyptus* and was reclaimed for a banana plantation in 2009, followed by the reclamation of two new adjacent fields sequentially in 2010 and 2011. This provided the opportunity to collect soil samples in which BIO was applied from 1 to 3 years post-reclamation simultaneously. Detailed information regarding the field experiment are available in our previous report (Fu et al., 2016).

2.2. Soil sampling and DNA extraction

Rhizosphere soil sampling was conducted in August 2012 based on visual observations of banana Fusarium wilt symptoms. The roots of healthy trees from the BIO-treated and compost control areas for one, two, and three years post-reclamation were collected and designated as BIO1H, BIO2H, and BIO3H, respectively, and CK1H, CK2H, and CK3H, respectively. Detailed soil collection protocols were described in our previous paper (Fu et al., 2016). Briefly, for rhizosphere soil, all soil loosely adhered to the plant roots was shaken off and discarded. Soil tightly bound to the roots was rinsed using sterile saline solution and centrifuged at 12,000 rpm for 10 min. The pellet was collected as rhizosphere soil and stored at −70 °C until DNA extraction. Total rhizosphere soil genomic DNA was extracted using the PowerSoil DNA Isolation Kit (Mobio Laboratories, Carlsbad, CA, USA), following the manufacturer's instructions. The concentration and quality of the DNA was determined using NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA).

2.3. Quantitative PCR analysis

Quantitative PCR (qPCR) was used for the quantification of total bacteria, fungi, *F. oxysporum* and biocontrol agent NJN-6 (*Bacillus amyloliquefaciens*) in the rhizosphere soil samples. Abundances of bacteria and fungi were quantified with primers Eub338F/Eub518R and ITS1f/5.8s, respectively (Table S1), according to Fierer et al. (2005). Standard curves were generated using 10-fold serial dilutions of a plasmid containing a full-length copy of the 16S rRNA gene from *Escherichia coli* and the 18S rRNA gene from *Saccharomyces cerevisiae*. The abundance of *F. oxysporum* was determined using the SYBR Green assay with the primers FOF1 and FOR1 (Jiménez-Fernández et al., 2010; Table S1), targeting the rRNA internal transcribed spacer (ITS). A serial dilution from 10^8 to 10^2 gene copies μl^{-1} of the ITS gene from the Foc-TR4 strain was used as a standard. For the biocontrol agent NJN-6, group-specific primers Ba425F and Ba641R (Table S1) were designed for the qPCR assay based on a specific region by comparison with other *Bacillus* genomes in GenBank. The standard curve was generated using a 10-fold dilution series of plasmid DNA containing a fragment of the genome from *B. amyloliquefaciens*. Assays were carried out on a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA,