Linking land management, the soil microbiome and suppression of Fusarium wilt disease

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Linking land management, the soil microbiome and suppression of Fusarium wilt disease

De koppeling van landbeheer, het microbioom in de bodem en de onderdrukking van Fusarium ziekte

(met een samenvatting in het Nederlands)

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Table of Contents

Chapter 1

General Introduction

Wu Xiong

General Introduction

Plant pathogens cause great loss of food production across the world. This problem is even more important given the need to produce more food for a growing human population. Given the negative effects of current pesticide-based management strategies, there is an urgent need to develop more sustainable alternatives. This thesis examines the potential of bio-organic fertilizer amendment as a way to enhance soil suppressiveness against phytopathogens. I especially address the question of how fertilizer treatment induces changes in the soil microbiome, and which of these alterations increase the natural potential of soils to keep plants healthy without agrochemical inputs.

Fusarium wilt as model of soil-borne disease

Soil-borne diseases are caused by pathogens residing in the soil and cause serious economic losses to a wide range of agricultural crops (Bailey *et al.*, 2004; Li *et al.*, 2014a; Xiong *et al.*, 2015a). Soil-borne pathogens are difficult to control because 1) they can survive in soil for long periods in the absence of the host crops; 2) they often have wide host ranges; 3) chemical control is often not practical; and 4) selecting resistant crop varieties is a tedious and inefficient process (Dr *et al.*, 2014). Fusarium wilt is one of the most widespread and devastating fungal diseases and is caused by the soil-borne fungus *Fusarium oxysporum* (Gordon and Martyn, 1997). This cosmopolitan species is highly diverse and comprises a wide range of strains varying in their host range, virulence and ecological preferences. *Fusarium oxysporum* strains are ubiquitous soil inhabitants, potentially impacting a wide variety of economically important plant species, and such strains are grouped into forma specialis according to their host range, with each forma specialis corresponding to a particular host specificity. Examples include *F. oxysporum* f. sp. *cubense,* which causes Panama disease in banana (Ploetz, 2006), *Fusarium oxysporum* f. sp. *cucumerinum*, the causal agent of cucumber wilt disease (Ahn *et al.*, 1998), and *Fusarium oxysporum* f. sp. *vanillae* (Pinaria *et al.*, 2010), responsible for stem rot disease of vanilla, the model plant species investigated in this thesis.

Fusarium oxysporum is a genetically heterogeneous species, comprised of both pathogenic and nonpathogenic strains (Gordon and Martyn, 1997). *Fusarium oxysporum* has also many non-virulence strains that have be linked with Fusarium wilt suppression. Non-pathogenic *Fusarium oxysporum* strains have also been reported to be prevalent in soils that are suppressive to Fusarium wilt disease, and some non-pathogenic *Fusarium oxysporum* strains have been demonstrated to have antagonistic effects on pathogenic strains of this species (Fravel *et al.*, 2003). Indeed, it has been suggested that non-pathogenic *Fusarium oxysporum* strains could serve as efficient bio-control agents against Fusarium wilt diseases (Lemanceau and Alabouvette, 1991; Mandeel and Baker, 1991).

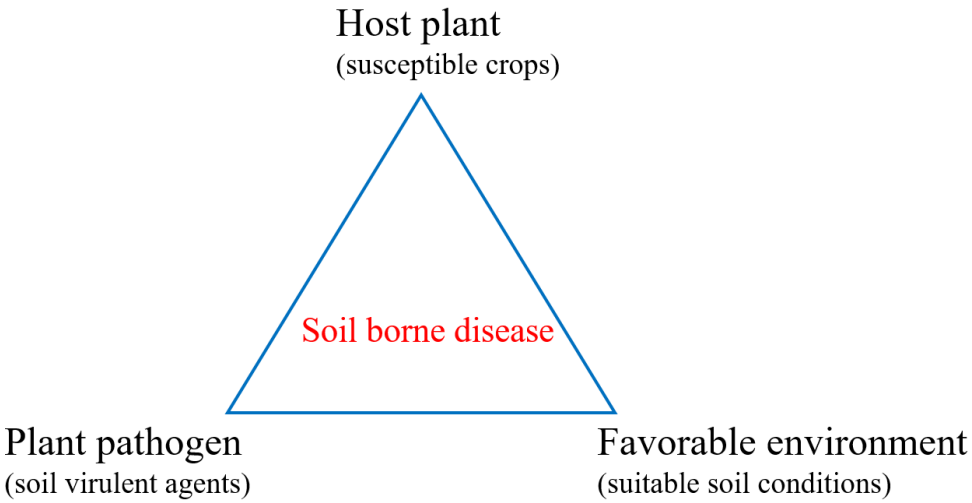

Figure 1. Schematic representation of the three factors required for successful development of soil-borne diseases (from Francl, 2001).

In order to develop an effective soil management strategy of disease suppression, it is not only important to understand interactions between plants and pathogens, but also how soil communities develop and change and how this relates to the ability of a pathogen to survive and thrive in a given environment. The three necessary causal factors of disease have been termed the disease triangle, comprised of host (susceptible plant), pathogen (soil borne pathogen) and environment (suitable soil conditions: physical/chemical conditions of the soil (moisture, pH, organic matter content, etc.) (Figure 1) (Francl, 2001). The disease triangle involves complex interactions that lead to the development of plant disease. This complexity of interactions occurs in the highly heterogeneous soil environment, which further complicates the picture.

Continuous cropping and Vanilla and Tomato as model plants

With the rapid human population increase in the world, intensification of agriculture is often required to provide a more stable and abundant food supply to feed the growing populations (Tscharntke *et al.*, 2012; Struik and Kuyper, 2017). Continuous cropping refers to a system in which a given crop is planted repeatedly in soils that have previously supported the same or similar plant species (Shipton, 1977). Because of limited arable lands and expansive populations in China, continuous cropping systems are commonly used for the production of grain (Zhang and He, 2004; Chen *et al.*, 2009), as well as cash crops (Guo *et al.*, 2014; Zhang *et al.*, 2013). Continuous cropping systems are also able to save labor resources and generally easier for farmers to carry out. However, there are also some disadvantages: for annual crops, such as cucumber and potato (Qiu *et al.*, 2012; Lu *et al.*, 2013), continuous cropping often results in yield decline and increases in soil-borne disease. Perennial plants, such as apple and peach (Mazzola and Manici, 2012; Trout et al., 2003), also suffer from similar suppressed growth after planting in soil that has previously supported the same plant species. In this thesis, I used the perennial crop of vanilla and annual crop of tomato as model plants.

Vanilla (*Vanilla planifolia*), a herbaceous perennial vine with high economic value (an important spice crop), is cropped widely in tropical and subtropical regions (Minoo *et al.*, 2008). However, various types of wilts caused by pathogenic fungi have been reported in all of the vanilla-growing countries (Thomas *et al.*, 2003) that utilize continuous cropping systems. The most prominent problems are associated with vanilla stem and root rot disease caused by *Fusarium oxysporum* f. sp. *vanillae*. In previous investigations (Xiong et al., 2015b), I evaluated the bacterial and fungal communities in fields subjected to four cycles of vanilla growth. I found that continuous cropping of vanilla increases fungal diversity and strongly shifts fungal community structures, the relative abundance of pathogen *Fusarium oxysporum* accumulated with increasing years of continuous cropping and was significantly and positively correlated with vanilla Fusarium wilt disease. By contrast, the relative abundance of some potentially beneficial bacteria, including *Bradyrhizobium* and *Bacillus*, decreased over time.

Tomato, *Solanum lycopersicum* L., is an important vegetable crop cultivated worldwide (Singh and Siddiqui, 2015). However, it is seriously affected by soil-borne pathogens, especially *Fusarium oxysporum* f. sp. *lycopersici* (Shanmugam *et al.*, 2015) and *Ralstonia solanacearum* (Smith) (Wei *et al.*, 2011), leading to Fusarium and bacterial wilt diseases, respectively. These diseases are both important widespread throughout tomato cultivating regions. Here again, continuous cropping systems for tomato have been associated with a buildup of disease pressure, with dramatic effects on yield.

Disease suppressive soil

Understanding the characteristics of naturally disease suppressive soils will help us to protect crops against plant pathogens in a sustainable way, for instance by reducing the necessity for fungicide application. A soil is generally referred to as being disease suppressive if the pathogen 1) does not establish or persist, 2) establishes but causes little or no damage, or 3) establishes and causes disease for some time after which the disease becomes less important (Dignam *et al.*, 2016). A disease-suppressive soil can naturally suppress plant pathogens, because it possesses specific properties that keep pathogens in check (Cook and Rovira, 1976; Cha *et al.*, 2016). In naturally occurring disease suppressive soils, the soil microbiome plays an important role in inhibiting soil-borne pathogens (Schlatter *et al.*, 2017).

Two main types of disease-suppressive soils have been described: generally and specifically disease-suppressive soils (Baker and Cook, 1974). General suppression is related to the total activity of the soil microbial community. The action of the community leads to competition for resources with pathogens or production of anti-microbial substances against pathogens. Such disease suppressiveness is not transferable between soils. On the other hand, specific suppression is due to the effects of specific microorganisms that are antagonistic against a given plant pathogen. A key characteristic of such suppressiveness is that is transferable, *i.e.* that you can inoculate disease-suppressive soil into non-suppressive soil to make it disease suppressive (Weller *et al.*, 2002). As shown in Fig. 2, the development of specific suppression of various plant pathogens has four general phases: 1) pathogen entry, 2) pathogen increase, 3) pathogen decline, and 4) maintenance of low pathogen density, which

typically is induced by a disease outbreak that occurs in field soils during continuous cultivation of a susceptible host plant (Raaijmakers and Mazzola, 2016).

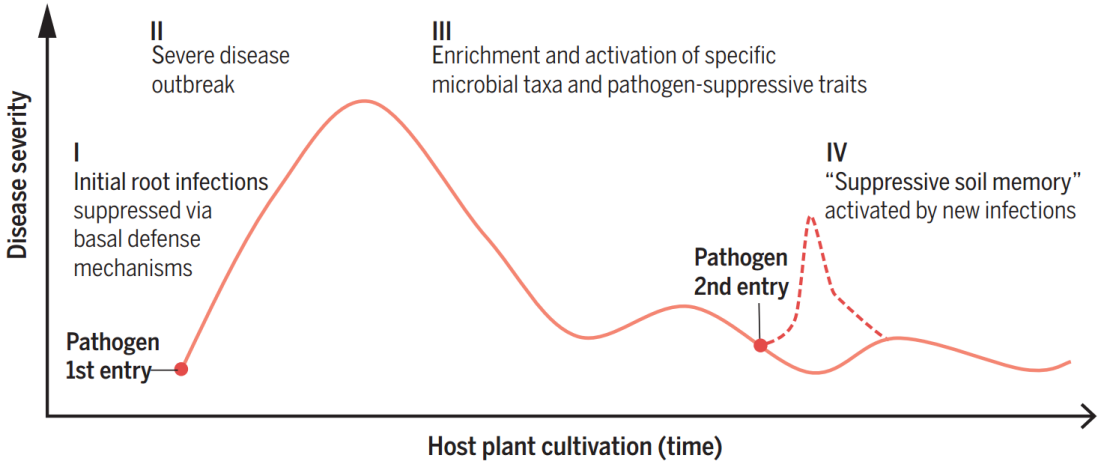

Figure 2. Development of disease-suppressive soil under continuous cropping (Raaijmakers and Mazzola, 2016).

Perhaps the most famous example of natural disease suppressive soil is the development of suppression against the causal agent of "take-all decline" in wheat. In response to wheat monoculture cultivation, soil can become "immune" to subsequent outbreaks of take-all disease several years after the disease was very severe (Shipton, 1975). The mechanisms for this disease suppression are now rather well understood, with the important role of Fluorescent Pseudomonads being well documented (Wong and Baker, 1984; Schlatter *et al.*, 2017). This is a very specific example of the development of disease suppression against wheat take all during long-term monoculture cultivation. Similarly, Fusarium wilt disease suppression soils can be induced by continuous cropping of targeted crops such as watermelon (Larkin *et al.*, 1993). However, long-term continuous cropping of some other economic crops such as vanilla and banana in tropical China agricultural systems does not seem to build up Fusarium wilt disease suppression (Shen *et al.*, 2017; Xiong et al., 2015b). Although some instances of pathogen suppression have been observed in such systems, it is still unclear how management practices or other factors contribute to the development of such suppressive soils. Numerous studies have been dedicated to examining the main drivers behind the development of disease 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).

The rhizosphere and the rhizosphere microbiome

The rhizosphere is a intriguingly complex and dynamic niche, and understanding its ecology and evolution is important to enhancing plant heath and plant productivity (Philippot *et al.*, 2013). The rhizosphere is a dynamic interface between plant roots and the soil. It represents a chemically complex environment that supports the development and growth of diverse microbial communities (Haldar and

Sengupta, 2016). Rhizosphere microbial communities have been coined the second genome of the plant and have a deep impact on plant growth and phenotype (Berendsen *et al.*, 2012). Rhizosphere microbes can have a positive, neutral or even negative impact on the plant (and this impact is environment specific). This community includes a wide range of microorganism such as bacteria, fungi, protists and nematodes, and is responsible for numerous beneficial functions, including helping the plant to respond to abiotic stresses (Yang *et al.*, 2009), promoting plant growth (Ahemad and Kibret, 2014) and affording disease suppression (Mendes *et al.*, 2014).

The microbiota associated with plant roots are generally highly distinct from the complex microbial community present in the surrounding bulk soil (Lundberg *et al.*, 2012). The bulk soil microbial community is relatively less affected than the rhizosphere community by the plant and may be more affected by soil managements. Bulk soil serves as a microbial seed bank (Lennon and Jones, 2011), harboring a genetic and phenotypic diversity of microbial communities that are capable of being resuscitated following environmental change. The changes brought about in the communities of the bulk soil will have an effect on the assembly and the final composition of rhizosphere communities. de Ridder-Duine and his colleagues investigated the rhizosphere bacterial composition of the wild plant *Carex arenaria* (sand sedge) from 10 natural sites in the Netherlands, they found that the bacterial community structure within the rhizosphere of *C. arenaria* is determined by bulk soil community composition (de Ridder-Duine et al., 2005). In addition, a study by Mendes et al. (2014) showed the rhizospheric microbial community of soybean was selected from the bulk soil under agricultural management in Amazon forest soils. Our recent study showed that continuous application of different organic amendments can suppress tomato wilt disease by inducing the development of healthy rhizosphere microbiota through the alterations to the bulk soil microflora (Liu *et al.*, 2017). This study provided insights into soil-borne disease suppression by soil management on bulk soil microbial community, which in turn led to the development of rhizospheric soil microflora that confer disease suppression. Studies on how plants select rhizosphere communities from the bulk soil microbial community will undoubtedly improve our knowledge into soil management strategies toward soil-borne disease suppression.

Soil management practices to combat Fusarium wilt diseases

As stated above, long-term survival of the Fusarium pathogen in soil and the evolution of new pathogenic strains makes management of Fusarium wilt difficult (Egel and Martyn, 2007). However, there are a number of conventional management options, such as screening for resistant cultivars, application of fungicides, crop rotation, grafting, soil fumigation, soil solarisation, *etc*. Although those methods may not be entirely effective alone or when used in combination, they can help lessen disease severity of Fusarium wilt.

Screening for resistant cultivars against Fusarium wilt disease can be highly effective and of economic value. Important examples include the selection of newly developed genotypes of chickpea for resistance against Fusarium wilt (Ahmad *et al.*, 2010), and cavendish banana cultivars resistant to banana panama disease (Hwang and Ko, 2004). However, there is no complete resistance to all

Fusarium races within economic and commercial crops. In addition, the evolution of newly virulent strains of Fusarium is very fast, which makes long-term control of Fusarium wilt very difficult.

Application of fungicides is one of the most efficient methods against Fusarium wilt disease. The systemic fungicide hexaconozole can provide strong inhibition in mycelial growth and spore germination of *Fusarium oxysporum* in *in vitro* experiments (Taskeen-Un-Nisa *et al.*, 2011). DMI fungicides, including the imidazoles (prochloraz) and the triazoles (propiconazole and cyproconazole/propiconazole), have also shown great effectiveness in inhibiting the pathogen *Fusarium oxysporum* f.sp. *cubense* (Nel *et al.*, 2007) both *in vitro* and in greenhouse experiments. However, the overuse of fungicides often comes at a large environment risk, particularly if fungicidal residues persist in the soil or water. Impacts can include public health effects, domestic animal poisoning and contaminated products, destruction of beneficial natural predators and parasites and pesticide resistance in pests (Pimentel and Burgess, 2014).

Crop rotation, an agriculture practice of growing different types of crops in the same field in a sequential order, is one of the most efficient and environmentally friendly soil managements for controlling Fusarium wilt disease. For instance, the pineapple-banana rotation can significantly reduce banana Fusarium wilt disease in tropical China (Wang *et al.*, 2015a). Also, relevant to this thesis, black pepper-vanilla rotations can control vanilla Fusarium wilt disease (Xiong *et al.*, 2016). Crop rotation can improve soil physical and chemical conditions and also improve soil biodiversity though changing crop residues and rooting patterns. Crop rotation can disrupt the accumulation of soil-borne pathogens, possibly through mechanisms such as absence of a host plant for the given pathogen, resistance of previous crop residues or increasing soil microbial biomass and activity (Dill-Macky and Jones, 2000; McDaniel and Grandy, 2016). However, use of rotations can also reduce profits, as farmers would rather keep planting the most profitable crop in a continuous cropping system. In addition, this method is sometimes labor-intensive or time-consuming.

In summary, Fusarium wilt diseases can be controlled to some extent with screening of resistant cultivars (Hwang and Ko, 2004; Pietro *et al.*, 2003; Pinaria *et al.*, 2010), fungicides (Nel *et al.*, 2007) and crop rotation (Wang *et al.*, 2015a; Xiong *et al.*, 2016). However, these measures are often impractical due to labor- and monetary-costs or the low efficiency of available chemical control treatments, not to mention detrimental environmental impacts.

Bio-organic fertilizers

Intensive agriculture plays an important role in meeting the food demands of our growing human population, but has resulted in an increased dependence on environmentally deleterious chemical fertilizers and pesticides (Ju *et al.*, 2009; Tilman *et al.*, 2001). Replacing chemical fertilizers and hazardous pesticides by the application of more organic fertilizers and more efficient biocontrol strategies would help meet the growing world food demand, while reducing negative impacts on the environment. There is thus an urgent need for alternative control strategies. One promising method is the application of so-called bio-organic fertilizers. Several definitions have been proposed over years for the term “bio-organic fertilizer”. This term generally refers to organic fertilizer products or substances containing living microorganisms that stimulate plant growth (Lesueur *et al.*, 2016). In this

thesis, bio-organic fertilizers are considered as a mix of composted organic waste or materials combined with plant beneficial microbes.

An additional issue related to modern farming practices involving increased livestock production is the large amount of animal waste, especially with the increasing demand for meat and livestock products in recent years. In China, animal waste disposal is becoming an increasingly serious problem (Chen *et al.*, 2008), calling for strategies to recycle the nutrients contained in animal waste and manure. Thus, conversion to increased use of animal waste or other organic materials (such as straw) as fertilizer in agricultural systems not only helps to reduce the dependency on chemical fertilizer application, but also decreases the problems associated with animal waste disposal.

Bio-fertilizers can improve soil health by providing various types of organic matter and nutrients, direct suppression of pathogens or via modification of the indigenous microbial community. While direct suppression is well described (Wang *et al.*, 2013; Zhang *et al.*, 2015), very little is known about the effects of bio-fertilizers on soil resident microbial communities and their ability to suppress diseases. This thesis therefore seeks to evaluate the functionality of both the introduced beneficial microbes and alterations of the native soil microbiota in relation to Fusarium wilt disease suppression.

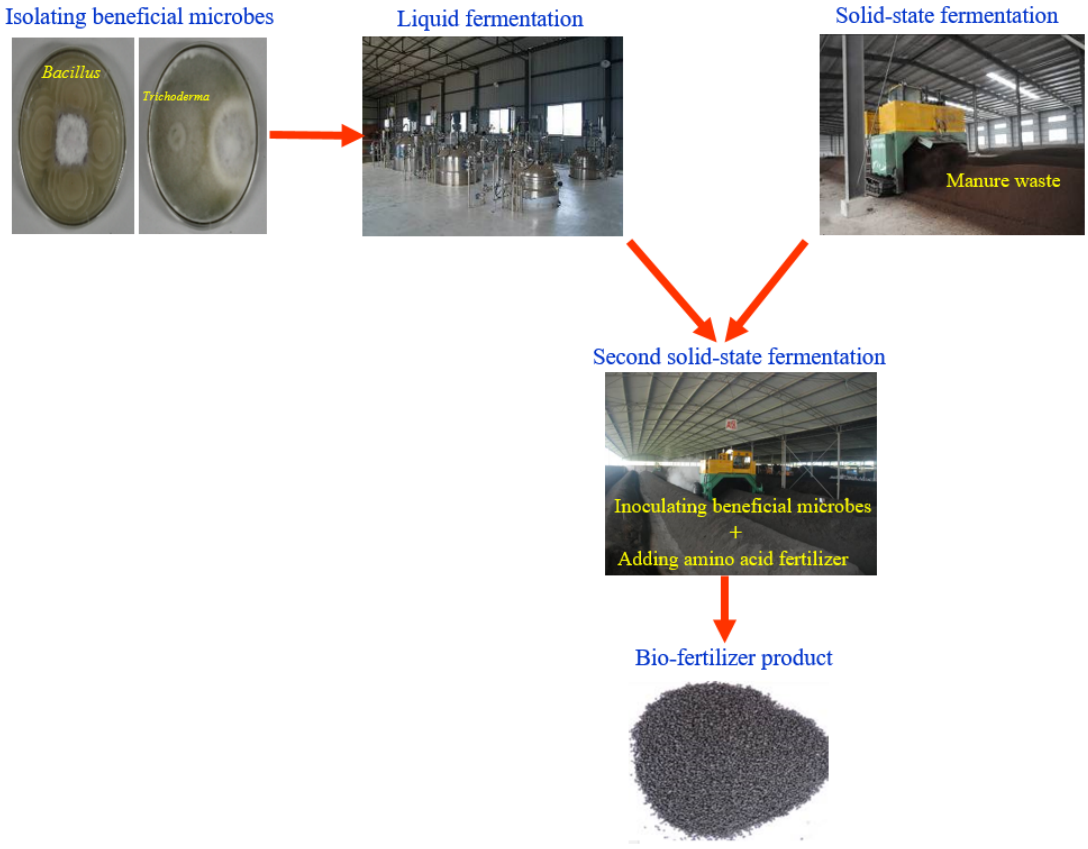

Figure 3. Basic process of producing bio-organic fertilizer. (Shen et al. 2013: Patent US8518428)

Beneficial microorganisms contained in bio-organic fertilizers often promote plant growth and health. Beneficial microorganisms that are commonly used as the biological component of the fertilizer can have a wide range of intended functions, including nitrogen fixation, phosphorus solubilisation,

phosphate mobilization, organic matter mineralization, plant growth promotion via phytohormone stimulation or biological control against soil-borne pathogens (Pii *et al.*, 2015). With respect to improving bio-control strategies, many beneficial microbes, such as *Bacillus*, *Trichoderma* and *Pseudomonas* have been examined for their potential ability to suppress *Fusarium* wilt disease (Liu *et al.*, 2014c; Vinale *et al.*, 2008; Lemanceau and Alabouvette, 1991). Although some success has been observed when inoculating beneficial microbes into the disease-conducive soil, most applications have remained somewhat disappointing (Saravanan *et al.*, 2003), with low inoculum survival as a major reason these poor results.

Bio-fertilizers have the potential to increase the plant health and productivity while reducing chemical fertilizers and pesticides application. I performed part of my PhD at the Jiangsu Provincial Key Lab for Organic Solid Waste Utilization, Nanjing Agricultural University, Nanjing, China, which has developed a new technique for transforming agricultural wastes into commercial bio-fertilizer products (Shen et al. 2013: Patent US8518428) (Figure 3), combining beneficial microbes with organic waste or materials (**Chapter 4**). Application of such bio-organic fertilizers forms the backbone of this thesis.

Protists in the soil food-webs

Soil microbial communities are the main drivers of plant productivity and heath. Many bacterial and fungal species have been reported to provide important services to plants, such as plant protection (disease suppression). Despite the growing body of knowledge concerning the functions involved in keeping plants healthy, the factors of driving community composition and function still remain elusive. This knowledge gap impedes progress in improving specific functions of the microbiome required to promote and restore soil health. One potential reason why this knowledge gap remains is the tendency to focus on specific aspects of the microbial community, often a target species of interest, as related to disease suppression. Also, most soil microbial ecology studies typically consider bacteria and/or fungi as isolated components of the total soil community. However, these microbes are embedded within complex soil food webs (Bardgett and van der Putten, 2014). In particular, soil protists form a multifunctional group that is likely to drive several functions of the soil microbiome, potentially providing the lever needed to understand and manage soil community function. In this thesis (**Chapter 6**), I include such multi-trophic interactions by also focusing on soil protists, which represent a broad range of eukaryotic lineages that play central roles in soil ecology.

Protists encompass most eukaryotic lineages with the exception of plants, animals and fungi (Geisen, 2016a). They are abundant and extremely diverse in soil, where they carry out a range of functions directly relevant for soil functioning. First, protists are among the main predators of soil bacteria and fungi, thereby acting as a driving force of microbial community composition and turnover (Figure 4). Indeed, the activities of protists have been shown to regulate microbial taxa responsible for plant growth and health (Jousset, 2012; Rosenberg *et al.*, 2009). Other protist lineages include autotrophs, parasites or even predators of soil animals (Geisen *et al.*, 2015a). By acting across a range of trophic levels, protists may serve as a functional link between the different components of the soil microbiome. Thanks to their multifarious functional potential, protist communities respond rapidly to

altered ecological conditions and are useful bio-indicators of soil quality (Foissner, 1997). However, despite of their important roles as indicators and regulators of soil microbial processes, protists are often overlooked in microbial ecology studies.

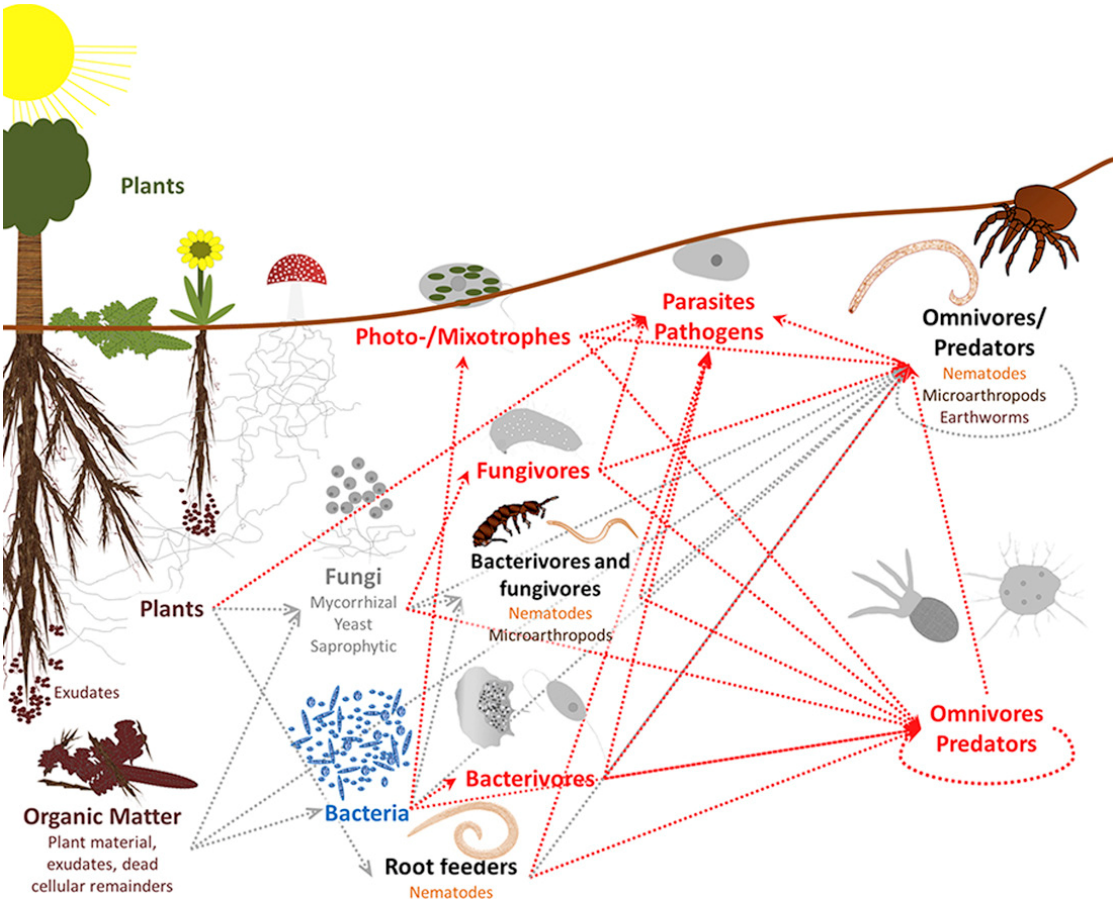

Figure 4. Conceptual soil food web focusing on the neglected functional diversity of soil protists (Geisen, 2016a).

The soil food-web describes the trophic relationships between organisms living that live in the soil. They range in size from viruses, single-celled bacteria, fungi and protists to the more complex nematodes and micro-arthropods, to the visible small vertebrates, insects, and earthworms. The interactions of these different soil-borne groups result in a highly complex functional food web that impacts plant growth and ecosystem functioning. Most studies to date have necessarily simplified the complexity of natural ecosystems, and typically focusing on the diversity of a single trophic group, neglecting the fact that biodiversity at multiple trophic levels is needed when evaluating ecosystem multi-functionality (Soliveres *et al.*, 2016). In my thesis, I placed protists as a central hub in the soil microbiome, linking diverse bacterial and fungal communities. My studies suggest that reshaping protist communities may provide the leverage effect needed to reliably enhance bacterial and fungal microbiota at the service of improved soil health. It should however be noted that my studies still do not include the full trophic complexity of soil communities, leaving out some important components

(such as nematode and earthworm) that might be important as related to bio-fertilizer amendments and disease suppression.

The development of molecular technologies, especially high-throughput sequencing (HTS) technologies (Geisen, 2016b), offers powerful new strategies for the rapid study of soil organisms including soil protists and nematodes with high throughput, low price and high accuracy. Combined with morphological identification for the visible small vertebrates, insects, and earthworms, it is now possible to investigate the full range of trophic levels taxa in soil ecosystems. I propose that investigating soil communities across multiple trophic levels will help us better to gain a more comprehensive mechanistic understanding of disease suppression in the future.

Outline of this thesis

Intensive agriculture plays an important role in meeting the food demands of the growing human population across the world. Because of limited arable lands and expansive populations in China, intensive agricultural strategies, such as long-term monoculture, are commonly practiced in the production of grain (Zhang and He, 2004; Chen *et al.*, 2009) and cash crops (Guo *et al.*, 2014; Zhang *et al.*, 2013). However, long-term monoculture of high economic value crops often results in a deterioration of soil quality, crop yield reduction and serious soil-borne diseases, especially Fusarium wilt diseases.

In most chapter of this thesis, I used Fusarium wilt of vanilla (*Vanilla planifolia* Ames) as a model system (Except **Chapter 5** of tomato), vanilla is an economically valuable spice crop widely cultivated in tropical regions (Minoo *et al.*, 2008), and Fusarium wilt disease, caused by the soil-borne fungus *Fusarium oxysporum* f. sp. *vanillae* (Koyyappurath *et al.*, 2016; Pinaria *et al.*, 2010), is a serious threat resulting in large economic losses in all vanilla-cropping regions. In previous work, it was determined that that the growth of vanilla is seriously hindered under the long-term monoculture systems (Xiong et al., 2015b). This growth decline under long-term continuous cropping may be attributed to alterations of the soil microbial community, *i.e.*, the reduction of the potentially beneficial microbes and the accumulation of the *Fusarium oxysporum* pathogen. In this thesis, I addressed different strategies of disease suppression to control Fusarium wilt disease. The main goal of this research is to provide insights into the mechanisms of Fusarium wilt disease suppression, thereby yielding the necessary information to advise farmers on how to best improve soil health (disease suppression) with suitable agricultural management measures.

Long-term monoculture cropping systems are not only associated with pathogen build-up over time and decreased plant growth. In some systems, disease-suppressive soil can be developed over time, in which the soils community impedes the success of the pathogen. The most famous example of the development of disease-suppressive soils is that case related to “take-all decline” associated with wheat monoculture. After several years of monoculture with heavy disease incidence, this soil becomes “immune” to subsequent outbreaks of take-all (Shipton, 1975). In line with Shipton’s findings, it has been observed that, in some cases, long-term monoculture of vanilla can indeed lead to the suppression of Fusarium wilt disease. In **Chapter 2**, I 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. However, other near-by soils respond very differently, with high disease incidence even after long periods of monoculture cultivation. In this chapter, I 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. 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.

As mentioned above, vanilla is often seriously affected by the soil-borne Fusarium wilt disease. Fungicides have traditionally been used as part of integrated control strategies for Fusarium

wilt disease. However, this method is usually environmentally unfriendly. In **Chapter 3**, I found that the black pepper-vanilla crop rotation system significantly reduced vanilla Fusarium wilt disease; resulted in a lower abundance of *Fusarium oxysporum* in the vanilla rhizosphere soil and increased putatively plant-beneficial fungal groups such as *Trichoderma* and *Penicillium* genus. Such crop rotation therefore appears to be a useful strategy to control vanilla Fusarium wilt disease occurrence.

Fusarium wilt disease can be controlled to some extent with crop rotation (Wang *et al.*, 2015a; Xiong *et al.*, 2016), screening of resistant cultivars (Hwang and Ko, 2004; Pietro *et al.*, 2003; Pinaria *et al.*, 2010) or use of fungicides (Nel *et al.*, 2007). However, these measures are often impractical due to high labor and monetary costs or the low efficiency of available chemical control treatments. There is thus a need for alternative control strategies. One promising method is the application of bio-fertilizers, a mix of beneficial microbes combined with organic materials. However, the mechanisms underlying disease suppression by the use of bio-fertilizers remain elusive. In **Chapter 4**, in order to assess the importance of direct antagonism and modified soil microbiota on suppression of Fusarium wilt disease, I conducted a pot experiment with chemical, organic and biologically enhanced fertilizers. I tracked the impact of the different fertilizer amendments on disease incidence and measured the pathogen density as well as changes in the soil microbiota. Alterations in bacterial abundance and community structure after bio-fertilizer application were determined to be key factors in constraining the pathogen, *Fusarium oxysporum*. In particular, bio-fertilizer application increased the abundance of indigenous microbial groups with reported antifungal activity, such as *Lysobacter* spp., which could play an important role in controlling this pathogen. The microbes introduced in the bio-fertilizer treatments (*e.g.* *Bacillus* and *Trichoderma* spp.) induced suppressiveness via alteration of the soil microbiome rather than by direct pathogen inhibition. These results contrast with the commonly held paradigm of disease suppression using beneficial microbes, opening up new perspectives for the promotion of soil health. In addition to seeking antagonistic microbes based on their direct inhibitory activity, disease suppression may also be achieved by introducing keystone species that reshape soil microbiome structure and function.

The rhizosphere is a critical interface supporting the exchange of resources between plants and their associated soil environment, where microorganisms and processes are important for plant growth and health (Peiffer *et al.*, 2013; Bakker *et al.*, 2015). **Chapter 5** utilized an alternative model system, namely Fusarium wilt of tomato, to examine this issue. The experiment extended over three seasons and included four fertilization treatments (chemical fertilizer, organic fertilizer, amino acid organic fertilizer, and bio-organic fertilizer) and was conducted in a highly diseased tomato field to evaluate how microbial changes from bulk soil into rhizosphere induce disease suppression. Bio-fertilizers induced changes in the bulk soil communities, which in turn translated to more functional rhizosphere communities to finally help in the suppression of the tomato wilt disease.

Soils are extremely complex systems. Thus, studies that focus on single groups or organisms (bacteria or fungi) will typically lead to an underestimation of the importance of soil management on soil functions, such as disease suppression. In **Chapter 6**, I used the same experiments examined in **Chapter 4** to investigate how soil amendments affect protist communities and inferred potential interactions with bacteria and fungi. Results showed that specific fertilization treatments impacted both

the structure and function of the protist communities. Organic fertilizer amendment strongly reduced the relative abundance of plant pathogenic protists and increased bacterivorous and omnivorous protists. The addition of individual biocontrol bacteria and fungi further altered the soil protist community composition, and eventually function. Network analysis integrating protist, bacterial and fungal community data, placed protists as a central hub in the soil microbiome, linking diverse bacterial and fungal populations. Given their dynamic response to soil management practices and key position in linking soil microbial networks, protists may provide the leverage between soil management and the enhancement of bacterial and fungal microbiota at the service of improved soil health.

A summary and general discussion of the findings presented in the thesis, and the implications thereof, is provided in **Chapter 7**. In this chapter, I first discuss how microbial communities contribute to soil-borne disease suppressiveness. Then, I compare the strengths and limitations of different management strategies to control Fusarium wilt disease in agriculture eco-systems. In particular I discuss how bio-organic fertilizer application can serve as a highly effective means of controlling soil-borne disease, while at the same time reducing reliance on chemical fertilizers and hazardous pesticides in agricultural. Furthermore, I discuss soil protists as an integral part of soil ecological networks and examine how protist communities are linked to soil health and soil productivity.

Chapter 2

Title: Disentangling the contribution of soil fungal and bacterial communities to soil disease suppressiveness

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

http://www.sciencedirect.com/science/article/pii/S0038071717300664

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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.

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 (Cook and Rovira, 1976; Cha *et al.*, 2016). 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 *F. oxysporum* pathogen densities in soils (Xiong et al., 2015b). *F. oxysporum* 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. 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.*, 2015c). However, bacterial and fungal communities are rarely investigated together in disease suppressive soils (Cha *et al.*, 2016; Mendes *et al.*, 2011; Penton *et al.*, 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 disease. 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 2,200 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 hour) 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. 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 from two to 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 gram 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'-AYTGGGYDTAAAGNG-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_2O. The following primer pairs used: AFP308R (5'-CGAATTAACGCGAGTCCCAAC-3') and ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-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 expected errors > 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 (version 7.0) (Kõljalg *et al.*, 2013) and the bacterial representative sequences were matched against the RDP database (version 9) (Wang *et al.*, 2007) using the naïve Bayesian classifier implemented in Mothur with a 80% confidence threshold (Schloss *et al.*, 2009).

We estimated fungal and bacterial diversity using the Chao1 richness and phylogenetic diversity (PD) indices (Faith, 1992). PD was calculated based upon neighbor-joining phylogenetic trees generated with the Mothur pipeline (Schloss *et al.*, 2009). To explore variation in fungal and bacterial community structures across the soil samples analyzed, weighted UniFrac distance was also performed in Mothur. PCoA (Principal Coordinate Analysis) was performed on distance matrices, and coordinates were used to draw 2D graphical outputs. Analysis of molecular variance (AMOVA) was performed to evaluate the significant differences in community structures between disease-suppressive and -conducive soils. For Mantel tests, Bray-Curtis and Euclidean distance were used to construct dissimilarity matrices of communities and soil characteristics respectively via the vegan package of R (version 3.2.2). The Mantel tests were used to calculate the correlation between the fungal or bacterial community and soil characteristics.

The linear discriminant analysis (LDA) effect size (LEfSe) method was used to evaluate bacterial and fungal taxa significantly associated with disease-suppressive and -conducive soils (Segata *et al.*, 2011).The alpha value employed for the factorial Kruskal–Wallis test was 0.05, and the threshold employed on the logarithmic LDA score for discriminative feature was 2.0.

2.6. Network analyses

We used the phylogenetic Molecular Ecological Network (pMEN) method to construct interaction networks for disease-suppressive and -conducive soils (Zhou *et al.*, 2010, 2011; Deng *et al.*, 2012). In this study, the 300 most abundant fungal and bacterial OTUs from disease-suppressive and -conducive soil samples were used for the network constructions. The Random Matrix Theory (RMT) was used to identify automatically the appropriate similarity threshold (St) prior to network construction. All analyses were performed using the Molecular Ecological Network Analyses Pipeline (http://ieg2.ou.edu/MENA/main.cgi), and network graphs were visualized using Cytoscape 2.8.2 software.

2.7. Statistical analyses

Soil physicochemical characteristics, *Fusarium oxysporum* abundance, fungal and bacterial total abundances, alpha diversity indices, and the taxa (phyla and genus) in disease-suppressive and -conducive soils were compared using Student's t-test. Analysis of disease incidence from the pot experiment was performed using Tukey's HSD multiple range test. Spearman's rank correlation coefficient was used to evaluate the relationships between alpha diversity indices and soil characteristics. All analyses were performed in SPSS v20.0 (SPSS Inc., USA).

2.8. Sequence Accession Numbers

Sequences are available in the NCBI Sequence Read Archive (SRA) database under the accession number SRX960429.

3. Results

3.1. Soil characteristics and total Fusarium oxysporum abundance in the two vanilla planting sites

Soil characteristics for the two vanilla fields are summarized in Table 1. Compared with conducive soil (Xinglong site), suppressive soil (Gaolong site) had a significantly ($P < 0.05$; Student's t-test) higher OM, available N, P and Fe contents. In contrast, conducive soil showed a significantly ($P < 0.05$; Student's t-test) higher soil pH, EC, and the content of available K. *F. oxysporum* was present in both soils, with the suppressive soil harbouring a lower *F. oxysporum* density (1.5×10^5 copies g^{-1} soil) as compared to the conducive soil (2.8×10^5 copies g^{-1} soil) (Fig. S1).

Table 1 Summary of soil characteristics at sites cultivated with vanilla that were disease-suppressive and -conducive to Fusarium wilt.

Vanilla sites	pH	EC (μs/cm)	OM (g/kg)	Available N (mg/kg)	Available P (mg/kg)	Available K (mg/kg)	Available Fe (mg/kg)
Gaolong (Suppressive soil)	6.32 ± 0.21	116.62 ± 14.79	24.90 ± 2.05*	83.26 ± 4.99*	120.81±15.50*	146.90±50.15	64.88 ± 1.09*
Xinglong (Conducive soil)	6.96 ± 0.10*	175.94 ± 8.14*	20.20 ± 1.89	69.62 ± 7.06	92.34±8.27	221.44±44.69*	54.34 ± 2.64

Values are means ± standard deviation, disease-suppressive and -conducive soils (n = 1).

* represents significance ($P < 0.05$) between disease-suppressive and -conducive soils according to Student's t-test.

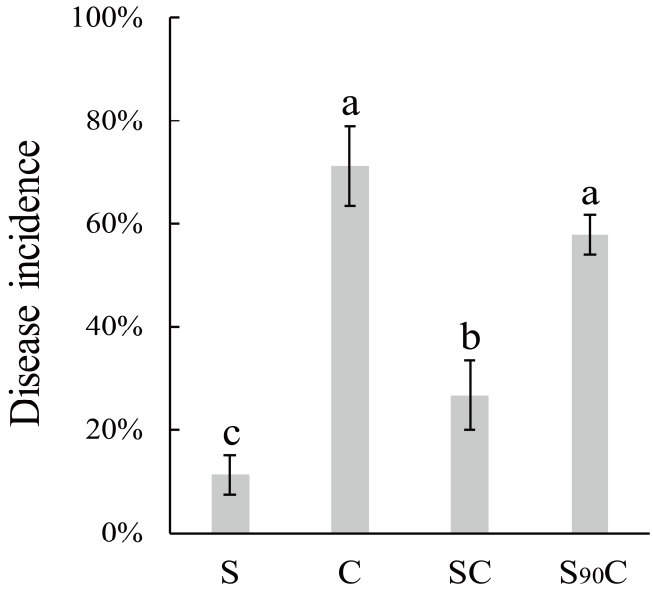

Fig. 1 Disease incidence of seedling vanilla in suppressive soil (S), conducive soil (C), conducive soil amended with 50% (w/w) of suppressive soil (SC) and conducive soil amended with 50% (w/w) of heat-treated suppressive soil ($S_{90}C$).

Different letters above the bars indicate statistically significant differences according to Tukey's HSD test (n = 3, $F_{3,11} = 68.60$, $P < 0.001$, one-way ANOVA).

3.2. Disease-suppressiveness of the two vanilla planting sites

In accordance with what would be expected from field observations, disease incidence was significantly lower for pots with suppressive soil, with only 11% disease incidence compared to 70% disease incidence in the conducive soil ($P < 0.001$; Tukey's HSD test) (Fig. 1). In order to assess whether soil microbial communities or physicochemical properties determined disease suppression, we transferred suppressive soil to the conducive one. Transferring 50% of the suppressive soil into the conducive soil significantly reduced disease incidence from 70% to 27% ($P < 0.05$; Tukey's HSD test). Transferring the same proportion suppressive soil after heat treatment, however, did not have a

significant impact on disease incidence (P = 0.09; Tukey's HSD test). Together, these results indicate that soil microbial communities, rather than chemical properties, are responsible for the differences in Fusarium wilt disease suppression between the two soils.

3.3. Microbial community abundances, diversity and structure

Fungal and bacterial abundances, richness (Chao1) and phylogenetic diversity are summarized in Table 2. We observed significantly higher fungal abundance, richness and phylogenetic diversity in suppressive soil samples (P < 0.05; Student's t-test). In contrast, conducive soil samples contained higher bacterial abundance and diversity.

Table 2 Richness and phylogenetic diversity indices of fungi and bacteria from disease-suppressive and -conducive soils.

	Treatments	Abundance*	Richness (Chao1)	Phylogenetic diversity
Fungi	Suppressive soil	9.04±0.08 a	1483.30±106.98 a	274.54±19.44 a
	Conducive soil	8.69±0.12 b	1085.81±72.09 b	200.56±16.05 b
Bacteria	Suppressive soil	10.51±0.15 B	5917.91±188.29 B	222.81±23.67 B
	Conducive soil	10.92±0.10 A	6816.49±78.56 A	302.19±12.06 A

Values are means ± standard deviation, disease-suppressive and -conducive soils (n = 1). Means followed by a different letter for a given factor are significantly different (P < 0.05; Student's t-test). * Fungal or bacterial copy numbers were $\log_{10}$-transformed in abundance.

As shown in Fig. 2, the weighted-UniFrac PCoA showed that both fungal and bacterial communities from suppressive soil were clearly separated from the conducive soil (AMOVA: Fs = 39.40, P < 0.001 for fungi; Fs = 30.39, P < 0.001 for bacteria). In addition, the weighted-UniFrac distance within suppressive soil was significantly (P < 0.05; Student's t-test) higher than in the conducive soil for the bacterial community system, whereas it was not significant (P = 0.69; Student's t-test) for the fungal community system (Fig. S2).

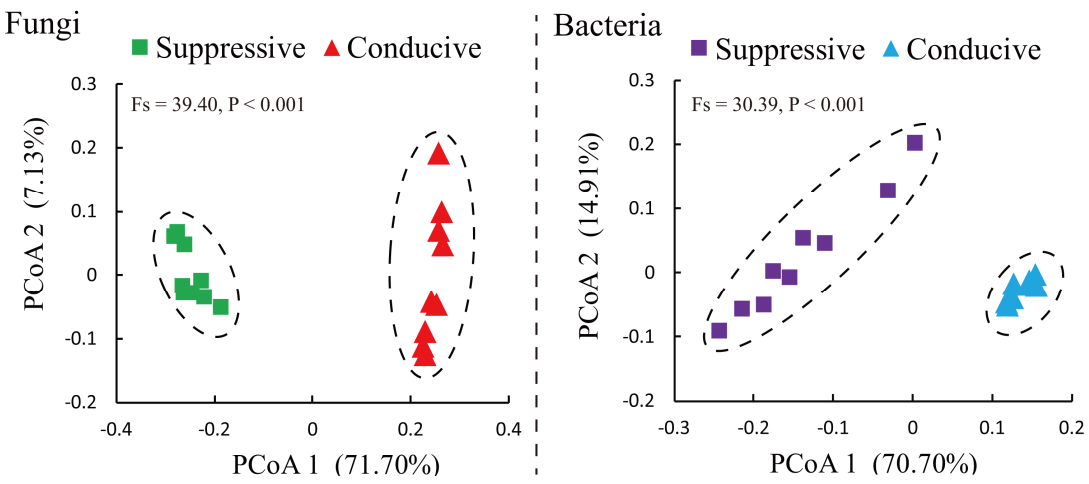

Fig. 2 UniFrac-weighted principle coordinate analysis of fungal and bacterial community structures in disease-suppressive and -conducive soils.

3.4. Taxonomic composition

A total of 474,250 ITS1 sequences and 736,751 V4 16S rRNA sequences were analyzed across the 18 soil samples, and sequences were grouped into 4,071 fungal and 10,191 bacterial OTUs, respectively. Fungal OTUs were predominantly associated with the phyla Ascomycota, Basidiomycota and Zygomycota, and these three phyla accounted for 88.23% of the total fungal sequences (Fig. 3). Zygomycota and Basidiomycota were more abundant in suppressive soil than that in conducive soil, whereas the Ascomycota showed the opposite trend. Bacterial sequences were classified into a total of 21 different phyla, with the most dominant phyla being Acidobacteria (25.56%), Proteobacteria (22.99%), Verrucomicrobia (6.44%), Bacteroidetes (4.17%), Actinobacteria (1.74%) and Firmicutes (1.26%) (Fig. 3). Moreover, Acidobacteria, Verrucomicrobia, Actinobacteria and Firmicutes were relatively more abundant in suppressive soil, whereas Proteobacteria and Bacteroidetes were more prevalent in conducive soil.

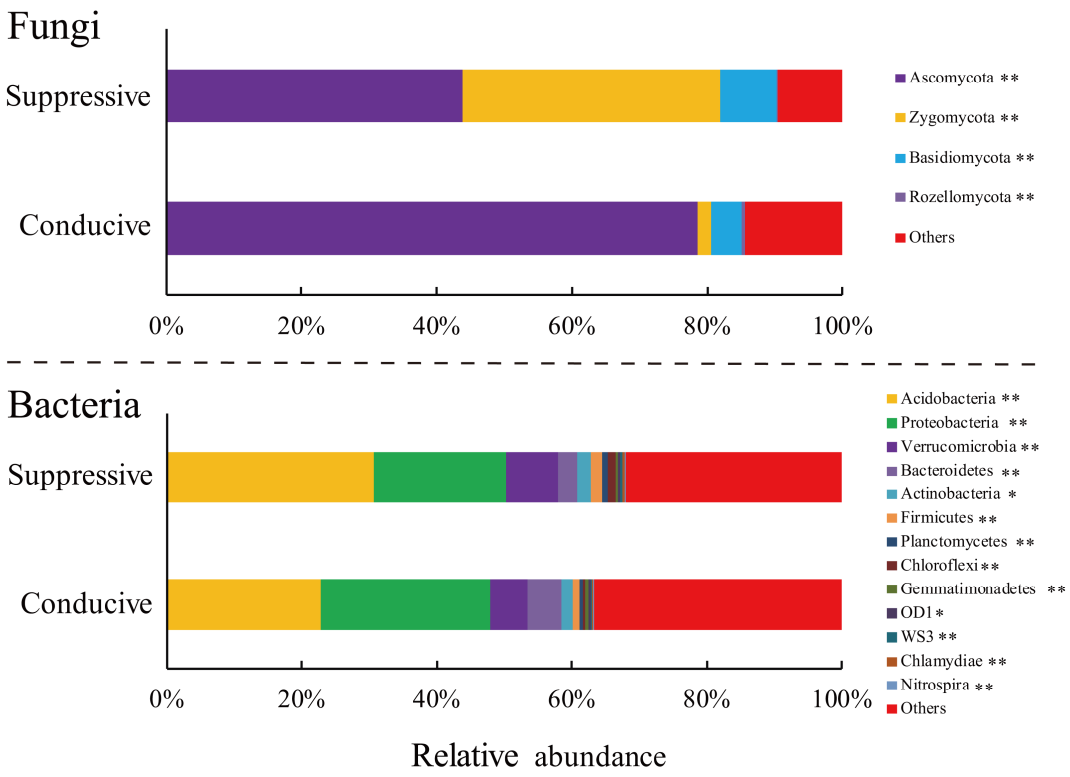

Fig. 3 The average relative abundances of fungal and bacterial phyla from disease-suppressive and -conducive soils.

Others includes phyla below 0.1% of relative abundance and the unclassified phyla. * and ** represent significance ($P < 0.05$ and $P < 0.01$) between disease-suppressive and -conducive soils according to Student's *t*-test (n = 1).

We used LEfSe to further analyze the association of the top 40 abundant fungal and bacterial genera with the disease-suppressive and -conducive soils. The fungal genera, *Mortierella, Ceratobasidium*, *Gliocladiopsis*, *Cylindrocladium*, *Staphylotrichum* and *Gymnopus* were more abundant in the suppressive soil (Fig. 4). *Mortierella* was hyper-dominant in the suppressive soil, accounting for 37.38% of the total fungal sequences in suppressive soil (only 1.82% in conducive soil), whereas *Fusarium* was the most dominant genus comprising 17.20% of total fungal genera in conducive soil (only 0.50% in suppressive soil) (Table S1). With respect to bacteria, 19 genera out of the top 40 bacterial genera were more abundant in conducive soil samples (Fig. 4), such as *Ohtaekwangia*, *Ignavibacterium*, *Solirubrobacter* and some groups of *Acidobacteria* (*Gp4*, *Gp6*, *Gp17* and *Gp22*), whereas the Acidobacteria groups *Gp2*, *Gp1*, *Gp3*, *Gp13* and *Ktedonobacter* were more abundant in the disease-suppressive soil.

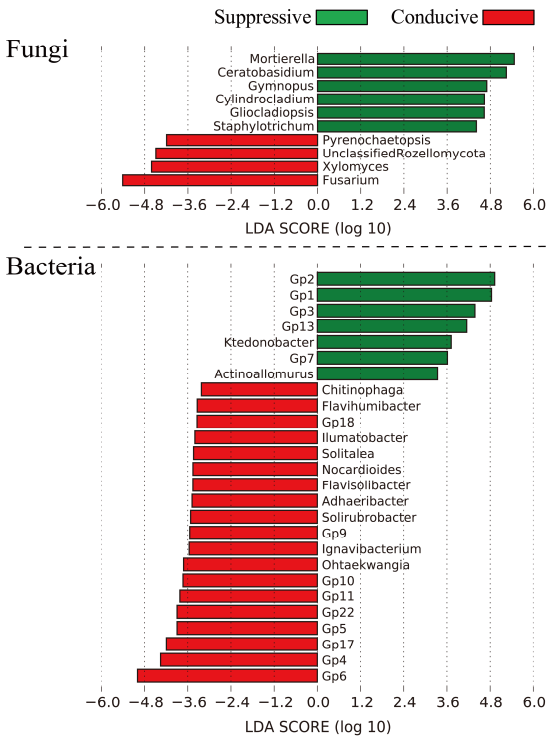

Fig. 4 Histogram of the LDA scores computed for differentially abundant fungal and bacterial genera between the disease-suppressive and -conducive soils.

3.5. Fungal and bacterial community networks

In the fungal phylogenetic Molecular Ecological Networks (pMENs), disease-suppressive and -conducive soils resulted in networks with similar sizes (225 and 230 nodes, respectively; Fig. 5). For suppressive and conducive networks, the average connectivity (connectivity or degree of distribution is the number of links of a node to other nodes) was 6.85 and 5.48, respectively (Table 3); the average path length (path length is the shortest path between two nodes) was 3.28 and 2.42, respectively. The suppressive soil (0.34) had a higher value of average clustering coefficient (clustering coefficient describes how close among the neighbors of a node) than the conducive soil (0.27). Moreover, suppressive and conducive soils harbored 24 and 37 modules with modularity values 0.56 and 0.50, respectively. Modularity measures the degree to which the network is organized into clearly delimited modules, networks with high modularity have denser connections between the nodes within modules but sparser connections between nodes in different modules. Suppressive soil (771 links) had higher link numbers than conducive soil (630 links). Strikingly, the positive link/negative link ratio (P/N) in suppressive soil (P/N = 15.76) was much higher than that in conducive soil (P/N ratio = 1.68), demonstrating that the suppressive soil contained more positive co-occurrence relationships than the conducive soil. In line with the community overview data, the 3 most abundant nodes in suppressive soil (OTU_2, OTU_5 and OTU_81) belonged to Mortierellales, whereas the most abundant species in the conducive soil network (OTU_3: *Fusarium pseudensiforme*) belonged to *Fusarium* genus. It is worth noting that this OTU does not correspond to the pathogen *F. oxysporum*, suggesting that this co-generic species may also play a role in the vanilla Fusarium wilt disease patterns.

Table 3 Major topological properties of the empirical phylogenetic Molecular Ecological Networks (pMENs) of fungal and bacterial communities in disease-suppressive and -conducive soils and their associated random pMENs.

Fungal community

Soil traits	Empirical networks								Random networks[d]		
	No. of original OTUs[a]	Similarity threshold (S_t)	Network size (n)[b]	R^2 of Power law	Avg connect (avgK)	Avg path length (GD)[c]	Avg clustering coefficient (avgCC)	Modularity (No. of modules)	Avg path distance (GD)	Avg cluster coefficient (avgCC)	Modularity (M)
Suppressive	300	0.86	225	0.85	6.85	3.28	0.34	0.56 (24)	2.91±0.11	0.11±0.01	0.31±0.01
Conducive	300	0.89	230	0.73	5.48	2.42	0.27	0.50 (37)	3.00±0.12	0.08±0.01	0.36±0.01

Bacterial community

Soil traits	Empirical networks								Random networks[d]		
	No. of original OTUs[a]	Similarity threshold (S_t)	Network size (n)[b]	R^2 of Power law	Avg connect (avgK)	Avg path length (GD)[c]	Avg clustering coefficient (avgCC)	Modularity (No. of modules)	Avg path length (GD)	Avg cluster coefficient (avgCC)	Modularity (M)
Suppressive	300	0.90	186	0.87	4.25	3.46	0.24	0.62 (21)	3.21±0.16	0.04±0.01	0.45±0.01
Conducive	300	0.86	225	0.85	3.56	4.34	0.24	0.71 (21)	3.89±0.15	0.02±0.01	0.53±0.01

[a] The number of OTUs that were originally used for network construction using the random matrix theory (RMT)-based approach.

[b] The number of OTUs (i.e., nodes) in a network. [c] GD, geodesic distance.

[d] The random networks were generated by rewiring all of the links of a pMEN with the identical numbers of nodes and links to the corresponding empirical pMEN.

With respect to bacteria, networks with 186 and 225 nodes were constructed from suppressive and conducive soil samples, respectively (Fig. S3 and Table 3). The average connectivity was 4.25 and 3.56, and the average path length was 3.46 and 4.34 for suppressive and conducive networks, respectively. The suppressive and conducive soil networks had the same average clustering coefficient value (0.24), and the modularity value in suppressive networks (0.62) was lower than that in conducive networks (0.71). The total link numbers in suppressive and conducive soils were 395 and 401 (P/N = 2.31 for suppressive soil and P/N = 1.49 for conducive soil), respectively.

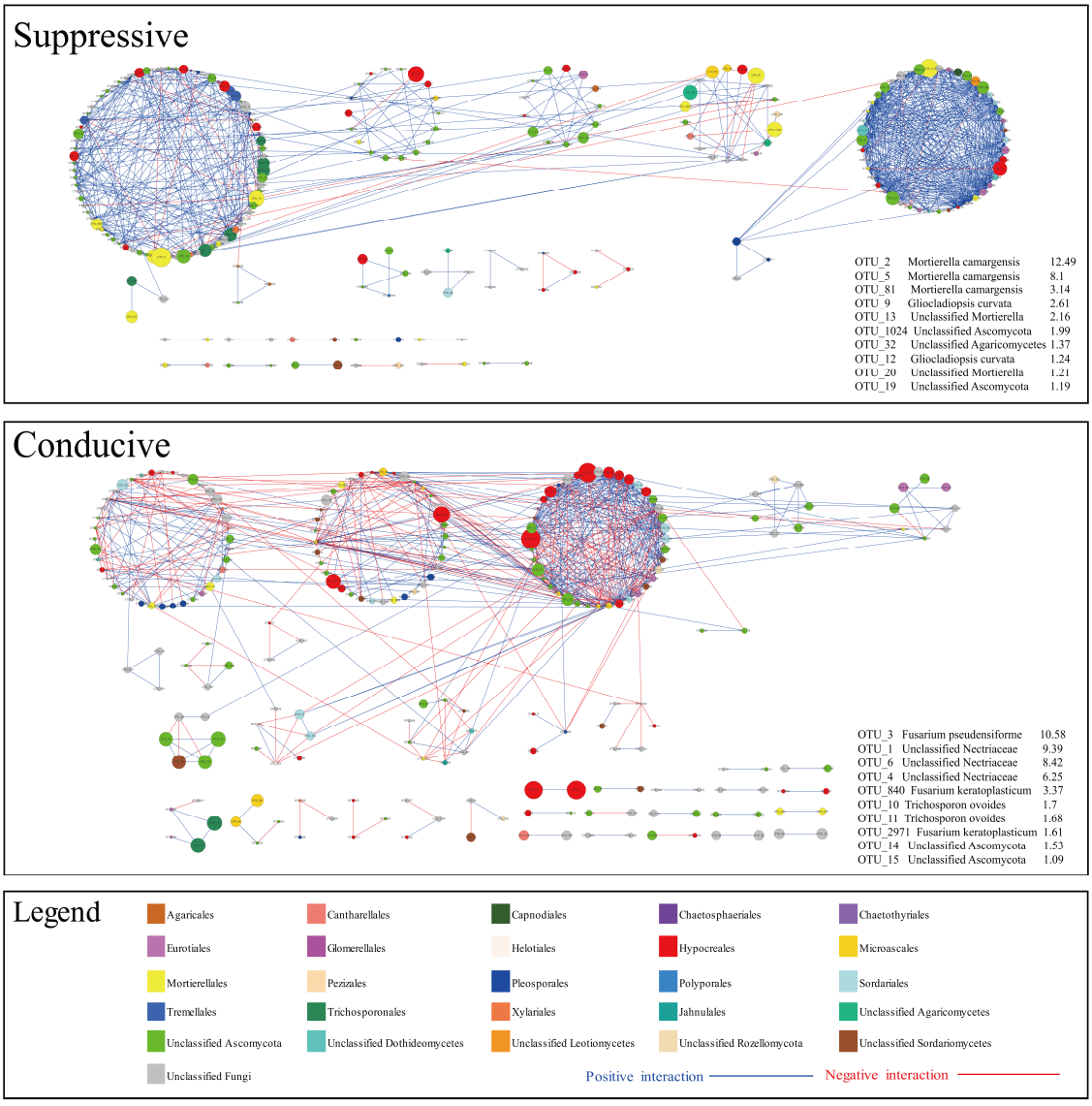

Fig. 5 Network plots of fungal communities in disease-suppressive and -conducive soils.
The node size is proportional to an OTU's relative abundance. Classification of the 10 most abundant OTUs from disease-suppressive and -conducive soils were showed in the figure with the relative abundance (%) behind. Node colors indicate different phylogenetic associations. Lines connecting nodes (edges) represent positive (blue) or negative (red) co-occurrence relationships.

3.6. Effects of soil chemical properties on microbial communities

Mantel tests revealed that soil chemical properties were significantly correlated with fungal and bacterial communities (R = 0.51, $P < 0.01$ for fungi and R = 0.57, $P < 0.001$ for bacteria). In addition, Spearman's rank correlation coefficient was used to evaluate the relationships between the soil properties and alpha diversity indices (Table S2). Bacterial richness and phylogenetic diversity showed positive correlations with soil pH and EC, whereas the fungal richness and phylogenetic diversity were negatively correlated with these two soil properties. In contrast, fungal richness and phylogenetic diversity were positively correlated with available N, P and Fe contents.

4. Discussion

Fusarium wilt disease is an economically important fungal disease of many crops, including cucumber (Klein *et al.*, 2016), watermelon (Ling *et al.*, 2011), banana (Wang *et al.*, 2015a) and vanilla (Pinaria *et al.*, 2010). Characterizing microbial communities in disease-suppressive soil is an important first step toward understanding the potential impacts of these communities on plant fitness (Rosenzweig *et al.*, 2012). In the present study, we compared the soils from two similar orchards that were free from Fusarium wilt disease prior to vanilla cultivation. Since the initiation of vanilla cultivation, these two orchards have diverged in terms of the incidence of Fusarium wilt disease and the density of *Fusarium oxysporum*, thereby yielding one disease-conducive and one disease-suppressive soil. Both soil fungal and bacterial communities varied in abundance, diversity, structure, taxonomic composition and microbial interactions in relation to vanilla Fusarium wilt disease suppression. Results showed the suppressive capacity of the soil was linked to the resident microbial communities, and this could be transferred from one soil to the other.

Based on the qPCR results, *Fusarium oxysporum* absolute abundance differed by a factor of less than two between the conducive and suppressive soils. This variation may explain a part of the difference in disease incidence between the two soils (Pinaria *et al.*, 2010; Xiong et al., 2015b). This is in line with previous findings that total *Fusarium oxysporum* abundance significantly correlates with vanilla Fusarium wilt disease. However, the difference we detected is too small by itself to drive the sharp contrast in disease incidence between conducive (11%) and suppressive (70%) soils. We hypothesize that full manifestation of the disease is impeded by the actions of the resident microbial community in the suppressive soil. Indeed, mixing the two soils in equal quantity resulted in a low Fusarium wilt disease incidence, while mixing conducive soil with heat-treated suppressive soil resulted in disease incidence levels comparable to the conducive soil alone. Together, these findings indicate that not only the pathogen absolute abundance, but also other microbial communities, determined the significant difference in disease incidence.

The suppressive soil harbored a significantly higher fungal abundance and diversity than the conducive soil. This is in line with a previous study that highlighted the importance of fungal diversity for soil health in an intensive potato cropping system (Manici and Caputo, 2009). Conversely, our suppressive soil showed lower bacterial diversity. This result is in contrast with previous results from Rosenzweig et al. (2012), who showed that more diverse bacterial communities were found in potato common scab-suppressive soil. The lower bacterial diversity in our suppressive soil may be due to its

lower pH, as we previously observed that bacterial diversity was positively related to soil pH (Liu *et al.*, 2014a; Shen *et al.*, 2013a).

Microbial taxonomic composition strongly varied between disease-suppressive and -conducive soils. Ascomycota and Basidiomycota were the most abundant fungal phyla identified in the conducive soil, in agreement with our previous work investigating microflora in the vanilla continuous cropping soil (conducive soil) (Xiong et al., 2015b). This result is also in agreement with a previous study in which Ascomycota and Basidiomycota were the top two prevalent fungal phyla in a continuous cropping peanut system (Li *et al.*, 2014b). In contrast, members of the phylum Zygomycota strongly dominated the soil fungal community in suppressive soil. This was somewhat surprising, as this phylum is often rare in agricultural soils (Wang *et al.*, 2015a; Xu *et al.*, 2012). We speculate that the dominant Zygomycota species may play a role in disease suppressiveness. For instance, they may inhibit *Fusarium* pathogen via competition for niche space and resources (Pal and Gardener, 2006). For bacteria, the phyla Actinobacteria and Firmicutes were more abundant in our suppressive soil, and these two phyla are known for species that produce high levels of secondary metabolites (Kim *et al.*, 2011; Palaniyandi *et al.*, 2013). Previous studies have also found higher abundances of Actinobacteria and Firmicutes in *Rhizoctonia*-suppressive soil (Mendes *et al.*, 2011). Our results suggest that members of these phyla may play a similar role in *Fusarium* wilt disease suppression.

The linear discriminant analysis (LDA) effect size (LEfSe) method revealed some of the specific microbial groups (at the genus level) associated with vanilla Fusarium wilt disease-suppression. For fungi, *Fusarium* was the most dominant genus in conducive soil, comprising 17.20% of the total fungal sequences. *Mortierella* was the most abundant genus in the suppressive soil, accounting for 37.38% of the total fungal sequences. Previous studies have shown that some species of *Mortierella* can produce antibiotics, and several isolates have been investigated as potential antagonistic agents against various plant pathogens (Tagawa *et al.*, 2010; Wills and Lambe, 1980). This taxon may serve as an important indicator of *Fusarium* wilt disease suppression in vanilla cropping systems. For bacteria, the structure of the Acidobacteria phylum highly discriminated between suppressive and conducive soils. *Gp4* and *Gp6* were more abundant in the conducive soil with higher pH, while other groups of Acidobacteria such as *Gp1*, *Gp2* and *Gp3* were more prevalent in suppressive soil with low pH. These results were in line with several previous studies demonstrating the importance of pH as a global regulator of Acidobacterial communities, with *Gp4* and *Gp6* being positively correlated with pH, and *Gp1*, *Gp2* and *Gp3* associated with low pH (Bartram *et al.*, 2014; Jones *et al.*, 2009).

In additional to microbial community traits, soil characteristics may also be important indicators of disease suppression. In this study, suppressive soil had moderately higher OM, available N, available P and available Fe contents compared to the conducive soil. (Liu *et al.*, 2014b) found that *Fusarium* abundance was negatively correlated with soil OM in a potato monoculture system. Research by (Shen *et al.*, 2015c) also indicated that higher soil available P was associated with lower banana Fusarium wilt disease incidence in naturally suppressive soil. It may be that higher soil OM, available P, N and Fe stimulates plant growth and general plant health, and thereby enhancing the plant's capacity to resist disease. In our pot experiments, however, these soil chemical properties by themselves were not sufficient to induce disease suppression, as pasteurized suppressive soils could not transfer suppressive

capabilities. We propose that the process of degradation from suppressive to conducive soil may induce feedback loops between soil properties such as pH and microbial communities. Slightly lower pH in the suppressive soil may have selected for specific microbiota such as *Gp1*, which often acts as a plant growth-promoting bacterium (Kielak *et al.*, 2016). The altered microbial community may in turn contribute to a higher disease suppression in this system. Future studies using a time series in long-term experiments would be helpful for disentangling the relationships between soil characteristics, microbiota and Fusarium wilt disease.

Microbial molecular ecological networks revealed distinct patterns within the microbial communities of suppressive- and conducive-soils. Not surprisingly, the putatively beneficial microbe *Mortierella* spp. held a dominant position in the suppressive soil. In contrast, *Fusarium* species had a dominant position in the conducive soil. However, most *Fusarium* sequences were not affiliated with the wilt pathogenic species itself (*Fusarium oxysporum*), suggesting that high disease incidence may also be associated with an increased abundance of other co-generic species. Future studies would be required to determine the specific role of these species in promoting Fusarium wilt disease, either via facilitation of the pathogen or as pathogenic agents themselves. The suppressive soil showed a higher number of positive co-occurrence relationships than the conducive soil for both fungal and bacterial networks (especially in fungal network). More positive interactions may suggest more cooperation in the complex microbial community ecosystems (Zhang *et al.*, 2014). Although high levels of cooperation may be linked to a higher community function, such interactions can also lead to a destabilization (Coyte *et al.*, 2015). In addition, the suppressive soil had higher average connectivity than the conducive soil for both fungal and bacterial networks. A highly connected network may provide resistance to disturbance (Scheffer *et al.*, 2012) up to a critical threshold that is still undefined at our study site. Now that we have determined the differences between suppressive and conducive soils with respect to microbial communities and soil properties, this opens up future research perspectives for understanding transitions developing between conducive and suppressive states.

5. Conclusions

We showed that similar soils that differ in their abilities to suppress vanilla Fusarium wilt disease have contrasting patterns of fungal and bacterial community structure and diversity. Although the *F. oxysporum* pathogen was present in both soils, it only leads to Fusarium wilt disease in the conducive soil. We propose that fungal communities may play a particularly important role in keeping *F. oxysporum* infection under control in the suppressive soil. The genus *Mortierella*, which accounted for 37% of the total fungal sequences, may be a key player in *F. oxysporum* suppression. Further studies identifying *Mortierella* spp. isolates with effective disease suppression ability and revealing the functional mechanisms involved in *Mortierella-F. oxysporum* interactions may open new avenues for the development of informed bio-control strategies.

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Supplementary data

Table S1 Prevalent fungal and bacterial genus in disease-suppressive and -conducive soils.

Fungal genus	Suppressive	Conducive	Bacterial genus	Suppressive	Conducive
*Mortierella***	37.38±12.98	1.82±0.90	*Gp6***	6.97±0.76	10.51±0.84
*Fusarium***	0.50±0.13	17.20±4.75	*Gp2***	7.71±0.90	1.85±0.33
Trichosporon	4.15±1.62	3.41±0.69	*Gp1***	5.73±0.84	1.08±0.12
*Gliocladiopsis***	3.85±1.12	0.03±0.05	*Gp3***	3.48±0.55	1.53±0.27
*Scedosporium**	1.21±0.25	1.80±0.67	*Gp4***	1.62±0.27	2.42±0.29
*Viridispora***	1.92±0.71	0.24±0.21	*Gp17***	0.62±0.07	1.25±0.09
Aspergillus	0.84±0.82	1.45±0.32	*Gp5***	0.83±0.12	1.02±0.11
Acremonium	0.73±0.34	0.83±0.36	*Gp13***	1.50±0.19	0.49±0.08
*Cryptococcus***	0.95±0.43	0.08±0.06	*Flavobacterium***	0.24±0.11	0.68±0.23
*Zopfiella***	0.24±0.28	0.93±0.43	*Gemmatimonas***	0.38±0.05	0.54±0.07
*Unclassified Mortierellales***	0.76±0.23	0.15±0.13	*Gp22***	0.26±0.06	0.60±0.12
*Arthrographis**	0.72±0.67	0.18±0.07	*Gp7***	0.68±0.10	0.35±0.04
Humicola	0.32±0.09	0.57±0.38	*Gp11***	0.18±0.03	0.40±0.04
Unclassified Bionectriaceae	0.53±0.22	0.35±0.17	*Gp10***	0.12±0.03	0.34±0.04
*Gibberella**	0.09±0.08	0.61±0.55	*Terrimonas*	0.23±0.07	0.25±0.07
Unclassified Rozellomycota	0.14±0.07	0.41±0.15	*Gp16*	0.20±0.06	0.26±0.08
Penicillium	0.26±0.29	0.30±0.08	*Ohtaekwangia***	0.05±0.03	0.27±0.08
*Staphylotrichum***	0.42±0.19	0.01±0.01	*Gp15*	0.16±0.03	0.17±0.03
*Unclassified Tulasnellaceae***	0.12±0.06	0.36±0.21	*Nitrospira***	0.18±0.04	0.08±0.04
Ceratocystis	0.22±0.12	0.20±0.14	*Nocardioides***	0.07±0.03	0.15±0.03
*Gibellulopsis***	0.03±0.02	0.33±0.22	*Solitalea***	0.05±0.02	0.14±0.05
Alternaria	0.27±0.27	0.10±0.08	*Solirubrobacter***	0.02±0.01	0.13±0.03
Westerdykella	0.22±0.10	0.17±0.16	*Adhaeribacter***	0.04±0.01	0.13±0.04
*Pyrenochaetopsis***	0.01±0.01	0.29±0.22	*Conexibacter***	0.12±0.04	0.06±0.01
*Geminibasidium***	0.24±0.09	0.01±0.02	*Ktedonobacter***	0.18±0.03	0.02±0.00
*Rhizoctonia**	0.18±0.17	0.04±0.06	*Mycobacterium***	0.10±0.03	0.04±0.02
*Xylaria**	0.18±0.16	0.03±0.04	*Gp18***	0.03±0.01	0.07±0.01
Unclassified Sarcosomataceae	0.12±0.08	0.09±0.13	*Ignavibacterium***	0.01±0.01	0.07±0.01
*Candida**	0.07±0.04	0.12±0.04	*Gp9***	0.03±0.01	0.05±0.01
*Myrothecium***	0.05±0.04	0.13±0.06	*Chitinophaga**	0.00±0.00	0.07±0.08
*Scytalidium***	0.13±0.07	0.02±0.01	*Flavisolibacter***	0.00±0.00	0.06±0.02
*Gymnopus**	0.14±0.14	0.00±0.00	*Ilumatobacter***	0.01±0.01	0.05±0.01
*Ceratobasidium***	0.13±0.06	0.00±0.00	*Actinoallomurus***	0.07±0.04	0.01±0.00
*Conlarium***	0.11±0.05	0.02±0.01	*Gp20**	0.01±0.01	0.03±0.01
Chaetomium	0.04±0.04	0.09±0.09	*Chthonomonas_Armatimonadetes _gp3*	0.03±0.02	0.02±0.01
*Cylindrocladium**	0.11±0.13	0.00±0.00	*Flavihumibacter***	0.00±0.00	0.03±0.02
*Cylindrocarpon***	0.02±0.02	0.11±0.09	*Gp25*	0.02±0.01	0.02±0.01
Neopestalotiopsis	0.07±0.04	0.04±0.05	*Streptomyces*	0.01±0.01	0.02±0.01
*Xylomyces***	0.00±0.00	0.11±0.09	*Kitasatospora***	0.03±0.02	0.01±0.01
*Lophiostoma**	0.01±0.02	0.10±0.11	*Geothrix***	0.03±0.01	0.01±0.01

Values are means ± standard deviation, disease-suppressive and -conducive soils (n = 1). * represents significance ($P < 0.05$) and ** represents significance ($P < 0.01$) between disease-suppressive and -conducive soils according to Student's t-test.

Table S2 Spearman's rank correlation coefficient between soil characteristics and alpha diversity indices.

	Fungal richness	Fungal PD	Bacterial richness	Bacterial PD
pH	-0.834**	-0.853**	0.689**	0.715**
EC	-0.732**	-0.744**	0.835**	0.783**
OM	0.686**	0.675**	-0.773**	-0.812**
Available N	0.606**	0.554*	-0.700**	-0.667**
Available P	0.697**	0.668**	-0.764**	-0.771**
Available K	-0.598**	-0.629**	0.515*	NS
Available Fe	0.770**	0.815**	-0.689**	-0.743**

PD = phylogenetic diversity, NS = not significant, * $P < 0.05$ and ** $P < 0.01$.

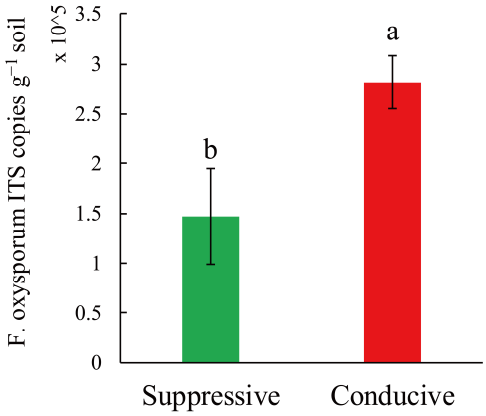

Fig. S1 Total *Fusarium oxysporum* abundance in disease-suppressive and -conducive soils. Different letters above the bars indicate statistically significant differences according to Student's t-test (n = 1).

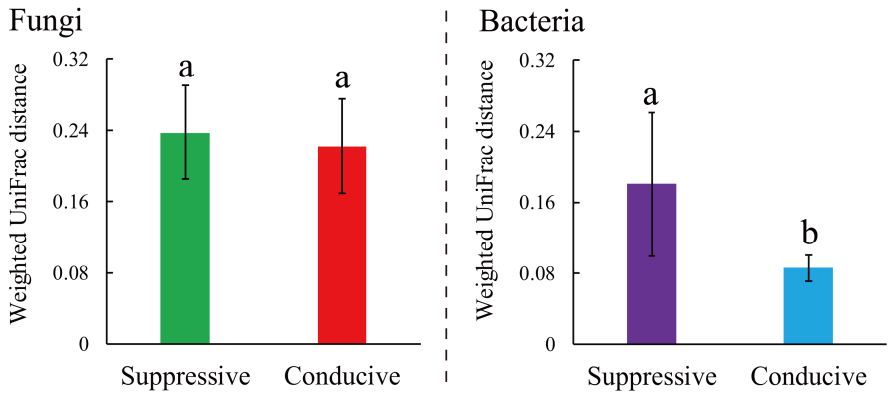

Fig. S2 Within weighted UniFrac distances among the disease-suppressive and -conducive soil samples in fungal and bacterial communities. Means followed by the same letter for a given factor are not significantly different ($P < 0.05$; Student's t-test) (n = 1).

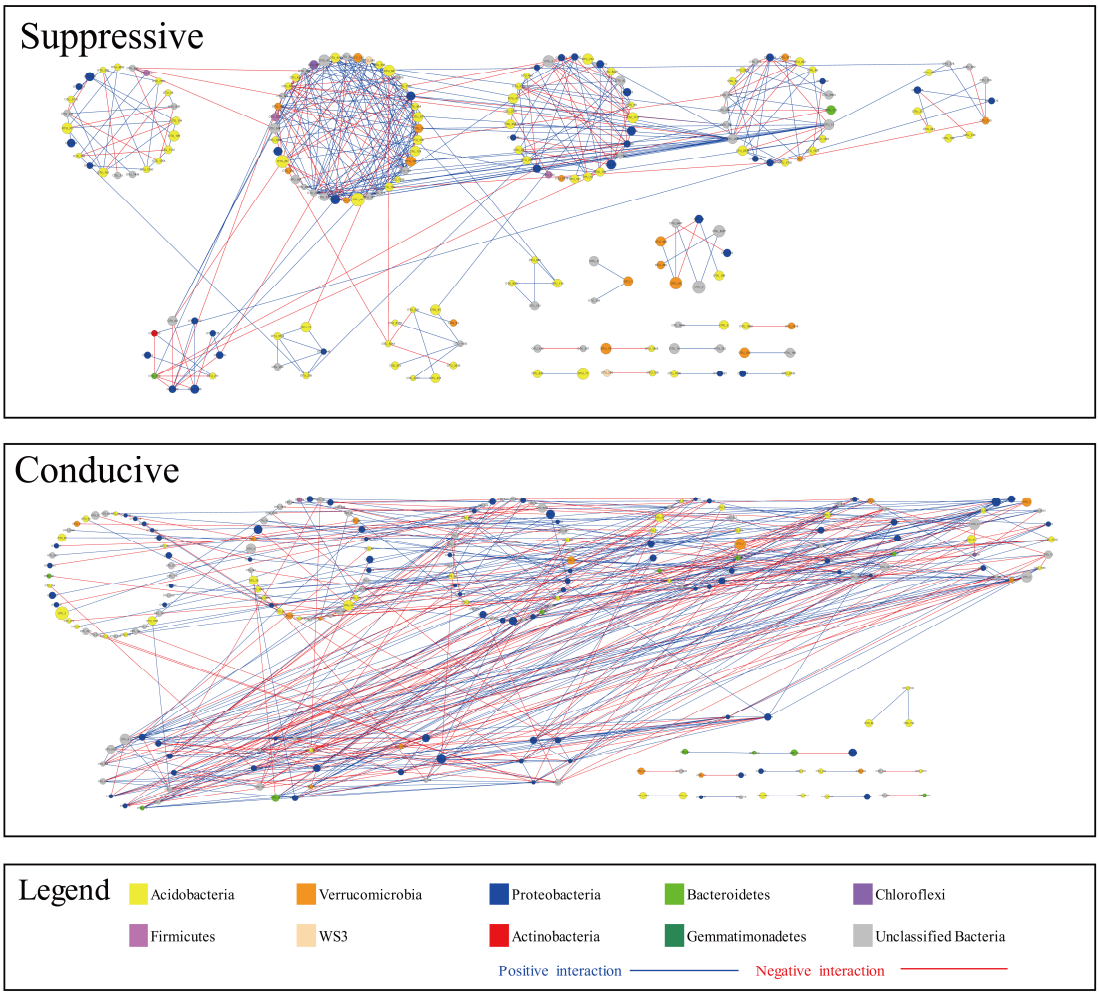

Fig. S3 Network plots of bacterial communities in disease-suppressive and -conducive soils.

The node size is proportional to an OTU's relative abundance. Node colors indicate different phylogenetic associations. Lines connecting nodes (edges) represent positive (blue) or negative (red) co-occurrence relationships.

Chapter 3

Title: Enhancement of soil microbiome functionality by crop rotation

Subtitle: Comparison of fungal community in black pepper-vanilla and vanilla monoculture systems associated with vanilla Fusarium wilt disease

https://www.frontiersin.org/articles/10.3389/fmicb.2016.00117/full

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Abstract

Long-term vanilla monocropping often results in the occurrence of vanilla Fusarium wilt disease, seriously affecting its production all over the world. In the present study, vanilla exhibited significantly less Fusarium wilt disease in the soil of a long-term continuously cropped black pepper orchard. The entire fungal communities of bulk and rhizosphere soils between the black pepper-vanilla system (*i.e.*, vanilla cropped in the soil of a continuously cropped black pepper orchard) and vanilla monoculture system were compared through the high throughput sequencing. The results showed that the black pepper-vanilla system revealed a significantly higher fungal diversity than the vanilla monoculture system in both bulk and rhizosphere soils. The UniFrac-weighted PCoA analysis revealed significant differences in bulk soil fungal community structures between the two cropping systems, and fungal community structures were seriously affected by the vanilla root system. In summary, the black pepper-vanilla system harboured a lower abundance of *F. oxysporum* in the vanilla rhizosphere soil and increased the putatively plant-beneficial fungal groups such as *Trichoderma* and *Penicillium* genus, which could explain the healthy growth of vanilla in the soil of the long-term continuously cropped black pepper field. Thus, cropping vanilla in the soil of continuously cropped black pepper fields for maintaining the vanilla industry is executable and meaningful as an agro-ecological system.

Introduction

Vanilla (*Vanilla planifolia*), a herbaceous perennial vine with high economic value, has been widely cropped in tropical and subtropical regions (Minoo *et al.*, 2008). However, the long-term monoculture of this crop often results in the occurrence of soil-borne Fusarium wilt disease, seriously affecting its production worldwide in vanilla-cropping regions (Jayasekhar *et al.*, 2008; Pinaria *et al.*, 2010; Xiong et al., 2015b) and leading to significant economic losses over the last decade. Fungicides and biological control agents have been traditionally suggested as integrated control strategies for vanilla Fusarium wilt disease (Sandheep *et al.*, 2012; Tombe and Sitepu, 1986); however, these methods are usually environmentally unfriendly or inefficient. Thus, exploring an effective method for controlling the vanilla Fusarium wilt disease is extremely important for maintaining the vanilla production. Meanwhile, in tropical China, multiple continuous cropping fields for other tropical crops, such as black pepper and banana, have suffered serious successive cropping obstacles and were given up for growing the same crops (Wang *et al.*, 2013; Xiong *et al.*, 2015a). Because of these associated problems, farmers naturally grow different crops in these fields. By some chance, after our field investigation, an interesting phenomenon was always observed where vanilla with the lowest Fusarium wilt disease incidence (DI) grew in the continuously cropped black pepper field. The causes of the disease decline might be very complex, such as improved soil physical and chemical properties and land management practices (Hilton *et al.*, 2013; Navarro-Noya *et al.*, 2013). However, the detailed mechanisms involved in the healthy vanilla growth associated with the soil microflora variation under the soil of long-term continuously cropped black pepper fields remain unclear.

Exploring continuously cropped field soil for other crop growth is meaningful and sustainable to agro-ecological systems. Meanwhile, to our limited knowledge, few studies have focused on the long-term continuously cropped soil supporting other crop growth; thus, how the variation in soil microbiota under long-term continuously cropped soil could support other crop growth is even less well understood. Soil microorganisms play critical roles in regulating soil fertility, global nutrient cycling, and plant health (Fierer *et al.*, 2012), which might be directly linked to the maintenance of plant health in agro-systems. Within soil ecosystems, the immediate surroundings of the plant root, *i.e.*, the rhizosphere, is a dynamic interface supporting the exchange of resources between plants and their associated soil environment (Peiffer *et al.*, 2013). Rhizosphere microbiota, considered as the second genome of the plant, are significantly influenced by plant roots (Philippot *et al.*, 2013). The main source of microbial communities in the rhizosphere is the adjacent root-free soil, called the bulk soil; hence, the changes brought about in the communities of the bulk soil will have an effect on the assembly and the final composition of rhizosphere communities (Mendes *et al.*, 2014).

The development of high-throughput sequencing, particularly Illumina MiSeq sequencing (Metzker, 2010; Shokralla *et al.*, 2012), offers a powerful strategy for uncovering the complex and diverse soil microbial communities with high throughput, high accuracy, and considerably lower cost. The internal transcribed spacers (ITS1) region has been widely used in the analysis of soil fungal communities (Xu *et al.*, 2012; Lu *et al.*, 2013). The functional diversity of soil fungi and their capacity to colonize diverse microhabitats can influence pathogen levels and play a significant role in improving plant health (Penton *et al.*, 2014). Given that vanilla Fusarium wilt disease is caused by a fungal

pathogen, exploring the fungal community involved in the healthy growth of vanilla in black pepper-vanilla agro-ecosystems is quite important.

Thus, in this study, we hypothesize that long-term continuous cropping black pepper orchards harbored a unique soil fungal community associated with healthy vanilla growth. To test this hypothesis, we used pot experiments to evaluate the persistent ability of the soil of long-term continuously cropped black pepper fields to support vanilla healthy growth; and fungal community of the bulk and rhizosphere soils in the black pepper-vanilla and vanilla monoculture systems was accessed by the Illumina MiSeq sequencing.

Materials and methods

Experiment descriptions

The experimental site is located at the Spice and Beverage Research Institute, Wanning City, Hainan Province, China (110°19'E-110°22'E, 18°72'N-18°76'N). It is an area with a tropical monsoon climate, a mean annual temperature of 24.5 °C and a mean annual precipitation of 2201 mm. The experimental soil was collected in April 2013 from the 20-years continuously cropped black pepper orchard. The soil was mixed thoroughly and transferred to the greenhouse with an average temperature of 30 °C and an average humidity of 72% at the Spice and Beverage Research Institute. Meanwhile, the soil approximately 200 m away from the black pepper orchard collected from the 21-year continuously cropped vanilla orchard showing serious vanilla Fusarium wilt disease (Xiong et al., 2015b) was considered a control. Both the black pepper and vanilla orchard soils are sandy loam in texture and developed from the same parent material. The experiment was performed using a randomized complete block design in three replicates, where each block had 6 pots for each treatment, and each pot contained 15 kg soil with 3 seedling vanillas. The agronomic management and fertilization regime were uniform during the next 18 months (April, 2013 to October, 2014). Vanilla Fusarium wilt disease was monitored immediately after the seedlings were transplanted into the pots based on the observation of typical wilt symptoms. The DI was calculated as the percentage of infected plants among the total number of plants (Wei *et al.*, 2011). It is worth noting that we also used continuously cropped banana and coffee orchards soil to cultivate vanilla in pots. We found the continuously cropped black pepper soil showed the lowest vanilla Fusarium wilt disease and the highest plant biomass (vanilla shoot dry weight). Hence, we got the two vanilla cropping regimes, *i.e.*, black pepper-vanilla system and vanilla monoculture system for the subsequent research.

Soil sample collection and DNA extraction

After removing the vanilla plants from the pots, the bulk soil samples obtained for each replicate from the black pepper-vanilla and vanilla monoculture systems were referred to as “BB” and “VB”, respectively. All six bulk soil samples were passed through a 2 mm sieve, thoroughly homogenized and divided into 2 subsamples: one was air-dried for a soil characteristic analysis according to our previous methods (Xiong et al., 2015b), and the remainder was stored at -80 °C for DNA extraction. For the vanilla rhizosphere soil samples, six vanilla plants were randomly selected from each replicate in the black pepper-vanilla and vanilla monoculture systems, the roots were vigorously shaken to dislodge the loosely adhering soil, and the soil remaining attached to the root

system was considered to be rhizosphere soil. The rhizosphere soil was collected using the following protocol: the roots were cut into pieces of approximately 1 cm length and carefully mixed, 20 gram of roots were pooled into a 500 mL vol. flask containing 200 mL ddH_2O and washed on a shaking platform for 20 min at 180 rpm, the washing buffer was subjected to centrifugation (10,000 g, 10 min), and then the resulting pellet was obtained and defined as the rhizosphere soil. The rhizosphere soil from the black pepper-vanilla system and vanilla monoculture system are referred to as "BR" and "VR", respectively. All 6 rhizosphere soil samples were stored at -80 °C for DNA extraction.

Total DNA was extracted from the 12 soil samples using a MoBioPowerSoil™ DNA Isolation Kit (Mo Bio Laboratories Inc., Carlsbad, CA, USA) according to the manufacturer's instructions. The genomic DNA concentration and purity were measured using NanoDrop ND-2000 (NanoDrop Technologies, Wilmington, DE) spectrophotometry.

PCR amplification and Illumina MiSeq sequencing

The fungi-specific primers ITS1F (CTTGGTCATTTAGAGGAAGTAA) (Gardes and Bruns, 1993) and ITS2 (GCTGCGTTCTTCATCGATGC) (White *et al.*, 1990) were selected to target the ITS1 region. These primer pairs were modified for pyrosequencing by adding the forward Illumina Nextera adapter, a two-base-pair ''linker'' sequence, and a unique 7-bp barcode sequence to 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 amplification was performed in a 25 µl reaction: 2.5 µl of 10 × reaction buffer, 10 µM of each primer, 2.5 mM dNTPs, 40 ng of template and 0.625 units of Takara Pyrobest (Takara Biotechnology Co., Ltd., Japan). Amplifications were performed with the following temperature regime: 4 min of initial denaturation at 94 °C, followed by 35 cycles of denaturation (94 °C for 30 s), annealing (50 °C for 45 s), extension (72 °C for 1 min), and a final extension at 72 °C for 7 min. The PCR products were purified using a PCR Purification Kit (Axygen Bio, USA). Then, paired-end sequencing was performed on an Illumina MiSeq sequencer at Personal Biotechnology Co., Ltd (Shanghai, China).

Quantification of the *Fusarium oxysporum* and fungal abundances

Real-time quantitative polymerase chain reaction (qPCR) was performed according to Chen et al., (2014) for quantifying the soil *Fusarium oxysporum* and fungi abundances using the SYBR Premix Ex Taq Kit on the ABI PRISM 7500 Real Time PCR System (Applied Biosystems, Germany). The 20 µl reaction mixture contained 10 µl of the *Premix Ex Taq™* (2×) (Takara), 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_2O. The specific primer set of *F. oxysporum* and soil fungi was AFP308R (CGAATTAACGCGAGTCCCAAC)/ITS1F (CTTGGTCATTTAGAGGAAGTAA) (Lievens *et al.*, 2006) and ITS2 (GCTGCGTTCTTCATCGATGC)/ITS1F (CTTGGTCATTTAGAGGAAGTAA), respectively. The thermal conditions were set as follows: 30 s at 95 °C for initial denaturation, 40 cycles of 5 s at 95 °C, and 34 s at 60 °C. The standard curve was obtained using a 10-fold dilution series of plasmid DNA containing a fragment of the ITS region of *F. oxysporum* and ITS1 gene from the *Fusarium oxysporum* f. sp. *vanillae* and soil samples, respectively. All amplifications were performed in triplicate. The specificity of the products was confirmed by a melting curve analysis and agarose gel electrophoresis. The copy numbers were $\log_{10}$-transformed to normalize the values prior to statistical analysis.

Sequencing data analysis

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). The split sequences for each sample were merged using FLASH V1.2.7 (Magoč and Salzberg, 2011), and low-quality sequences were then discarded using QIIME. The sequences retained for each sample were processed following the established UPARSE pipeline (Edgar, 2013). Briefly, the sequences with expected errors > 0.5 or a length shorter than 200 bp were removed. After discarding the singletons, the remaining reads were assigned to OTUs with a threshold of 97% identity level. Then, the chimaera removal processes were performed. Finally, the fungal representative OTUs were classified using the UNITE database (Kõljalg *et al.*, 2013).

The diversity within each individual sample was estimated using non-parametric Shannon diversity indices. Shannoneven was used to measure the evenness of each sample (Schloss *et al.*, 2009). A principal coordinate analysis (PCoA) based on weighted UniFrac metric matrices was performed to explore the differences in fungal community structures among all of the soil samples (Lozupone *et al.*, 2006). A permutational multivariate analysis of variance (Anderson, 2001) was performed to assess the effect of the cropping regime, soil compartment and their interactions on the fungal community structure (abundance of OTUs and genus) using the adonis function of the R vegan package with 999 permutations.

Statistical analyses

The soil physicochemical characteristics and vanilla Fusarium wilt DI between the black pepper-vanilla and vanilla monoculture systems were compared using Student's t-test. For other parameters in our study, one-way analyses of variance (ANOVA) with Turkey's HSD multiple range test were performed for multiple comparisons. All of the statistical analyses were performed using SPSS v20.0 (SPSS Inc., USA).

Sequence Accession Numbers

The sequence data have been deposited in the NCBI Sequence Read Archive (SRA) database with the accession number SRP062990.

Results:

Soil physical and chemical properties in the black pepper-vanilla and vanilla monoculture systems

The results of soil physical and chemical properties are summarized in **Table S1**. When compared with the vanilla monoculture system, black pepper-vanilla system presented a significantly ($P < 0.05$) higher available N content. In contrast, the vanilla monoculture system revealed higher soil pH and the contents of organic matter and available P.

Fusarium wilt DI and fungal abundance in the two vanilla cropping systems

As shown in **Table 1**, the black pepper-vanilla system significantly reduced vanilla Fusarium wilt DI to 15.56%, whereas the value was over 60% in the vanilla monoculture system. The qPCR results showed that the ITS copies of *F. oxysporum* were significantly lower in the black pepper-vanilla system compared with those from the vanilla monoculture system in both bulk and rhizosphere soils

(**Table 1**). Strikingly, the *F. oxysporum* populations significantly increased from the bulk soil to the vanilla rhizosphere soil in both the black pepper-vanilla and vanilla monoculture systems. In addition, the fungal ITS gene copy numbers in bulk soil showed no significant difference between the black pepper-vanilla and vanilla monoculture systems (**Table S2**). Meanwhile, the fungal ITS gene copy numbers in the rhizosphere soil samples from the black pepper-vanilla system (8.15×10^9 copies g^{-1} soil) were significantly higher than those from the vanilla monoculture system (2.89×10^9 copies g^{-1} soil).

Table 1 Vanilla Fusarium wilt disease incidence and pathogen abundance.

Cropping regime	Soil compartment	Disease incidence (%)	*Fusarium* RA	*F. oxysporum* RA	Log_{10} *F. oxysporum* ITS copies g^{-1} soil
Black pepper-vanilla system	Bulk soil (BB)	15.56±3.85 b	6.79±2.23 b	5.51±2.75 b	4.80±0.08 d
	Rhizosphere soil (BR)		10.36±1.31 b	8.66±1.15 b	5.60±0.15 b
Vanilla monoculture system	Bulk soil (VB)	62.22±10.08 a	10.23±0.82 b	5.59±0.37 b	5.13±0.13 c
	Rhizosphere soil (VR)		26.18±7.54 a	22.89±6.80 a	6.18±0.09 a

Values are means ± standard deviation. RA = Relative abundance.

Means followed by the same letter for a given factor are not significantly different ($P < 0.05$; Turkey's HSD test where there are more than two treatment levels and Student's t-test where there are two treatment levels).

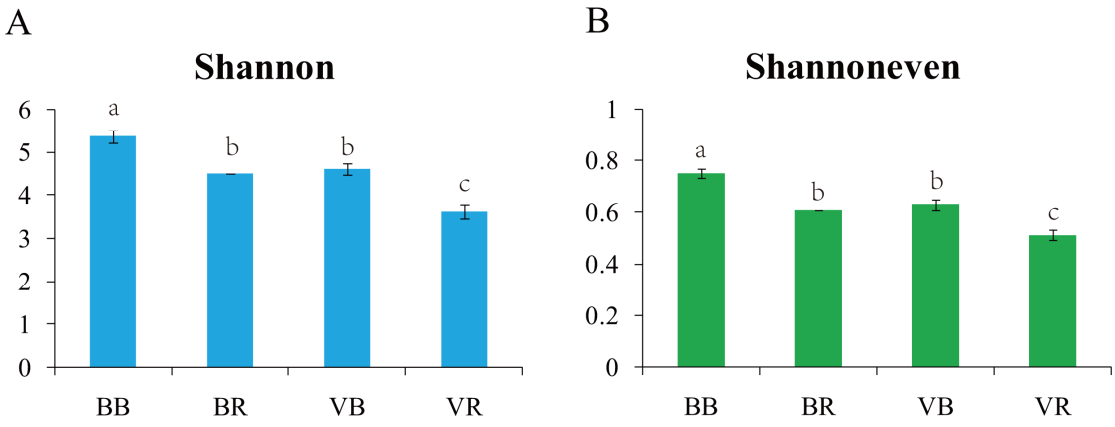

Figure 1 Shannon diversity indices (A) and Shannoneven indices (B) for the bulk soil and vanilla rhizosphere soil of the black pepper-vanilla and vanilla monoculture systems.

Bars represent the standard error of the three replicates, and different letters above the bars indicate a significant difference at the 0.05 probability level according to the Turkey's HSD test. "BB" and "VB" represent the bulk soil from the black pepper-vanilla system and the vanilla monoculture system, respectively. "BR" and "VR" represent the rhizosphere soil from the black pepper-vanilla system and the vanilla monoculture system, respectively.

Overall diversity of fungal community

After quality filtering, the pyrosequencing-based analysis of the fungal ITS1 genes resulted in the recovery of 1,260,032 high-quality sequences across the 12 samples (**Table S3**). The coverage from all samples was above 99%, indicating that the sequencing reads were sufficient for this analysis (**Table S3**). In both the bulk and rhizosphere soils, the black pepper-vanilla system had a significantly higher fungal diversity (Shannon) and evenness (Shannoneven) values than the vanilla monoculture system (**Figure 1**). In addition, in both the black pepper-vanilla and vanilla monoculture systems, the fungal community diversity and evenness significantly decreased from the bulk soil to the vanilla rhizosphere soil.

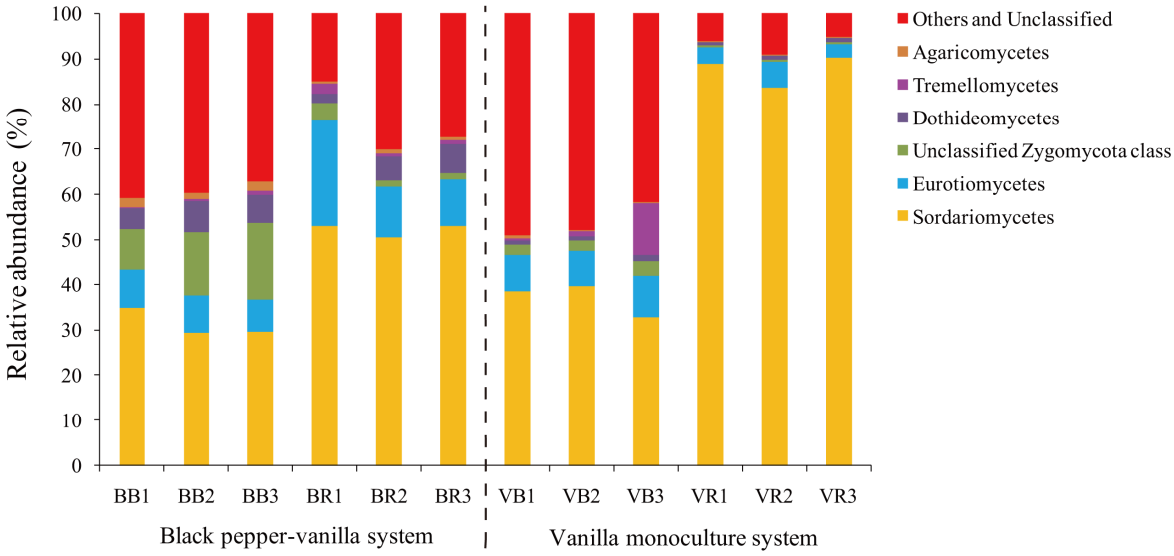

Figure 2 Relative abundances of the main fungal classes in the bulk soil and the vanilla rhizosphere soil of the black pepper-vanilla and vanilla monoculture systems.

The "Others and Unclassified" comprised the unclassified and low-abundance classes (RA < 0.1%). "BB" and "VB" represent the bulk soil from the black pepper-vanilla system and the vanilla monoculture system, respectively. "BR" and "VR" represent the rhizosphere soil from the black pepper-vanilla system and the vanilla monoculture system, respectively.

Fungal community composition

To verify the differences observed in the fungal communities from the black pepper-vanilla and vanilla monoculture systems, the relative abundances (RA) of the different classes and genera from the bulk and rhizosphere soils were compared (**Figure 2 and 3**). In the present study, fungal OTUs across the 12 soil samples were observed predominantly from the 6 classes (Sordariomycetes, Eurotiomycetes, unclassified Zygomycota class, Dothideomycetes, Tremellomycetes, and Agaricomycetes), accounting for 71.10% of the total fungal sequences. Compared with bulk soil, the relative abundance of the class Sordariomycetes in the vanilla rhizosphere soil significantly increased in both the black pepper-vanilla and vanilla monoculture systems. At the genus level, in the bulk soil, compared with the vanilla monoculture system, the black pepper-vanilla system had a higher relative

abundance of *Mortierella*, *Aspergillus*, *Acremonium*, and *Chaetomium*. As for the rhizosphere soil, the *Fusarium* genus was significantly more abundant in the vanilla monoculture system than in the black pepper-vanilla system; moreover, the relative abundance of *Fusarium oxysporum* (OTU level) exhibited a similar trend (**Table 1**). In addition, the relative abundances of the genera *Haematonectria*, *Trichoderma*, and *Penicillium* were significantly higher in the black pepper-vanilla system with a lower *Gibellulopsis* abundance.

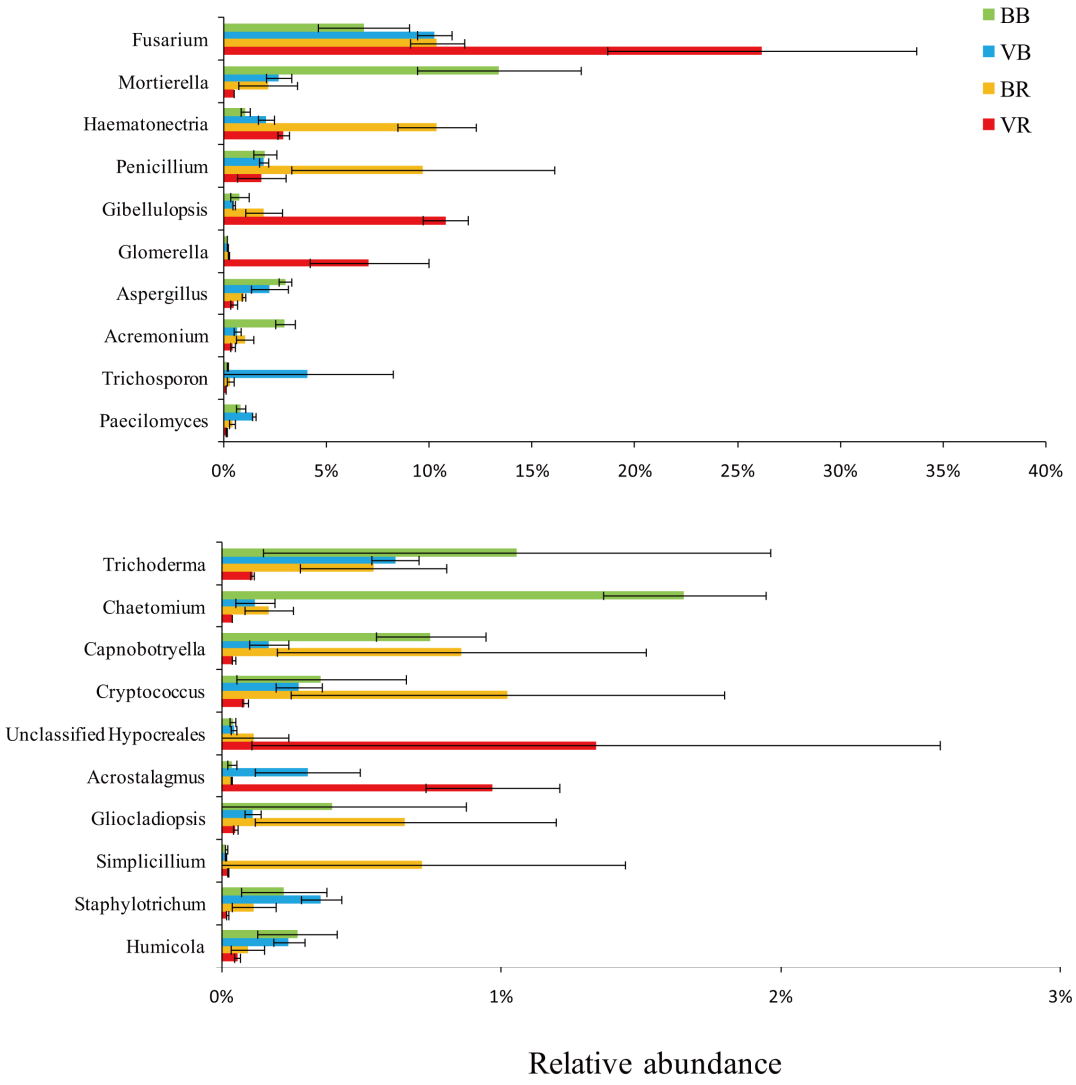

Figure 3 Relative abundances of the top 20 fungal genera in bulk soil and vanilla rhizosphere soil of the black pepper-vanilla and vanilla monoculture systems.

Bars represent the standard error of the three replicates. “BB” and “VB” represent the bulk soil from the black pepper-vanilla system and the vanilla monoculture system, respectively. “BR” and “VR” represent the rhizosphere soil from the black pepper-vanilla system and the vanilla monoculture system, respectively.

Table 2 PERMANOVA analysis.

Source	Df	Abundance of Genera		Abundance of OTUs	
		Sums of sqs	Pseudo-F	Sums of sqs	Pseudo-F
Cropping regime (CR)	1	0.18	11.79***	0.42	7.64***
Soil compartment (SC)	1	0.25	15.80***	0.40	7.30***
CR*SC	1	0.09	5.54***	0.20	3.68***
Residuals	8				

*indicate significant correlations ($P < 0.05$); ** indicate significant correlations ($P < 0.01$); *** indicate significant correlations ($P < 0.001$).

Fungal community structure

A permutational multivariate analysis of variance confirmed that the cropping regime, soil compartment, and their interactions were significant factors of variation for the fungal community structure in terms of both the relative abundance of OTUs and relative abundance of genera (**Table 2**).

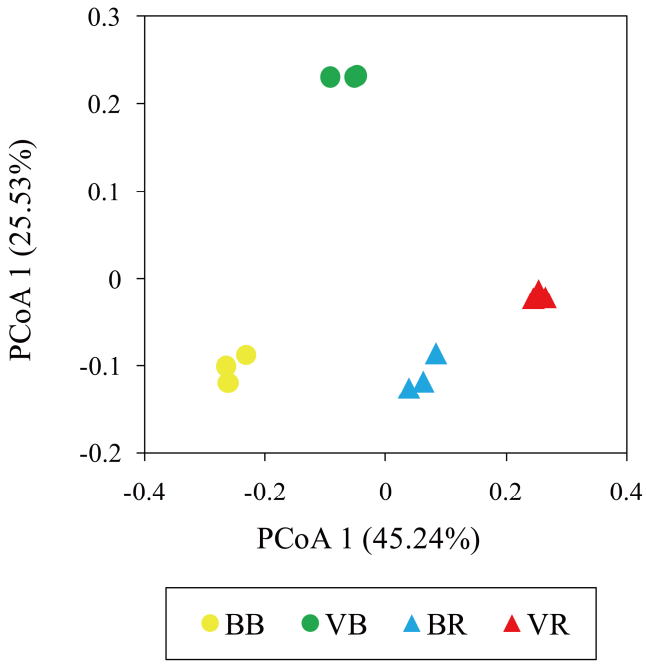

Figure 4 UniFrac-weighted principle coordinate analysis of fungal community structures in the bulk soil and vanilla rhizosphere soil of the black pepper-vanilla and vanilla monoculture systems.

"BB" and "VB" represent the bulk soil from the black pepper-vanilla system and the vanilla monoculture system, respectively. "BR" and "VR" represent the rhizosphere soil from the black pepper-vanilla system and the vanilla monoculture system, respectively.

To further compare the variations in fungal community structure between the black pepper-vanilla and vanilla monoculture systems samples, a UniFrac-weighted PCoA was employed. As shown in **Figure 4**, the bulk soil samples from the black pepper-vanilla system were clearly separated from the vanilla monoculture system, suggesting strong differences in fungal community structures between the different crop regime systems. In addition, the fungal communities in the rhizosphere soils from the two vanilla cropping systems were close together, suggesting that fungal community structures were seriously affected by the vanilla root system.

Discussion

Obstacles to the continuous cropping of vanilla have always been observed on Hainan Island (Xiong et al., 2015b). In the present study, pot experiments confirmed that long-term continuously cropped black pepper orchard soil showed significantly lower vanilla Fusarium wilt disease, implying that crop rotation is an effective management practice to reduce soil-borne plant disease in agro-systems (Wang et al., 2015). In addition, it will also help us to take advantage of the large area of black pepper continuous cropping soil in tropical China (Xiong *et al.*, 2015a; Zu *et al.*, 2014). In this study, the black pepper-vanilla system had no effect on the fungal population abundance in the bulk soil. However, alpha diversity estimates of the fungal communities revealed that the black pepper-vanilla system had a significantly higher fungal diversity and evenness than the vanilla monoculture soil (**Figure 1**). The possible reasons are as follows: residues of black pepper decomposed in the soil and different root exudates could provide more available nutrient to soil microbes, thus improving species richness, heterogeneity, and diversity of the fungal community (Xuan *et al.*, 2011). Furthermore, we could find a negative relationship between the soil fungal diversity and vanilla Fusarium wilt disease, which could support the idea that microbial diversity is a key factor in controlling pathogen invasion (Elsas *et al.*, 2012). In addition, in the present study, the fungal community diversity significantly decreased from the bulk soil to the vanilla rhizosphere soil in both the black pepper-vanilla and vanilla monoculture systems; as explained by Mendes et al., (2014) plants can select a constant rhizosphere community from highly contrasting reservoirs of bulk soil communities.

The UniFrac-weighted PCoA analysis revealed significant variations in the bulk soil of fungal community structures between the black pepper-vanilla and vanilla monoculture systems (**Figure 4**). Our results agreed with the findings of Wang et al., (2015) where the crop regime system was the major determinant factor for microbial community structures. The significant variations in bulk soil community structures among the different cropping systems might be attributed to significant differentiations in soil physicochemical characteristics (**Table S1**), as soil physicochemical properties have significant impacts on microbial community structures (Lauber *et al.*, 2008). Compared with the vanilla monoculture system, black pepper-vanilla system revealed a significantly higher available N content, as nitrogen play a pivotal role in plant growth and might indirectly enhance plant disease suppressiveness (Hayat *et al.*, 2010). In addition, the UniFrac-weighted PCoA analysis suggested fungal community structures were also seriously affected by the vanilla root system, which was

consistent with the many previous studies that plant play a key role in shaping the microbial community structures in the rhizosphere (Edwards *et al.*, 2015; Philippot *et al.*, 2013).

The black pepper-vanilla system was shown to have a significant effect on the fungal community compositions in both bulk and rhizosphere soils. Sordariomycetes was the most abundant fungal class (**Figure 2**), which was generally consistent with the many early studies that found Sordariomycetes to be the most common fungal class in different agricultural systems (Chen *et al.*, 2012; Li *et al.*, 2014b). Compared with bulk soil, the abundance of Sordariomycetes significantly increased in vanilla rhizosphere soil in both the black pepper-vanilla and vanilla monoculture systems, as reported by Zhang et al., (2006) who found that members of the Sordariomycetes are ubiquitous in virtually all ecosystems as pathogens and endophytes of plants.

Deeper taxonomic analyses were performed to explore the fungal community compositions of rhizosphere soil in the black pepper-vanilla system associated with vanilla growth. The black pepper-vanilla system showed significantly lower *Fusarium* and *F. oxysporum* abundance in the vanilla rhizosphere soil, which could be the most important reason for significantly lower vanilla Fusarium wilt disease in the black pepper-vanilla system (Pinaria *et al.*, 2010). The *F. oxysporum* abundance is lower in the black pepper-vanilla system might be because that continuous cropping black pepper soil had not previously been used to cultivate vanilla, however, if vanilla is continuously cropped in that soil which could also increase the pathogen load eventually (Xiong et al., 2015b). In addition, in present study, the *F. oxysporum* populations significantly accumulated from the bulk soil to the vanilla rhizosphere soil in both the black pepper-vanilla and vanilla monoculture systems. Synthesized from the above results, we conclude that iterative crop rotation might be necessary to interrupt the accumulation of *F. oxysporum* abundance to suppress vanilla Fusarium wilt disease.

Some putatively plant-beneficial fungal groups, such as the genera *Trichoderma* and *Penicillium*, increased in the vanilla rhizosphere soil under the black pepper-vanilla system. *Trichoderma* spp. are known to have an effective antagonistic effect against vanilla Fusarium wilt disease (Jayasekhar *et al.*, 2008; Vijayan *et al.*, 2009). *Penicillium* is also a famous biocontrol agent for the biological control of Fusarium wilt disease (Larena *et al.*, 2003); however, this has not yet been reported in vanilla systems. Moreover, *Haematonectria* was the most abundant genus, accounting for 10.33% of the total fungal genera in vanilla rhizosphere soil in the black pepper-vanilla system, which could occupy the rhizosphere niche to avoid pathogen invasion (Qiu *et al.*, 2013). Combining the other variations in fungal genera in the black pepper-vanilla and vanilla monoculture systems and the complex interactions among these microorganisms could explain the status of vanilla Fusarium wilt disease in agro-ecosystems.

In conclusion, compared with the vanilla monoculture system, black pepper-vanilla system harboured a significantly lower abundance of *F. oxysporum* in vanilla rhizosphere soil, increased the putatively plant-beneficial fungal groups and the fungal diversity, which could explain the decrease in vanilla Fusarium wilt disease in the soil of the long-term continuously cropped black pepper orchard. These results suggested that sustainable agricultural management regime, such as crop rotation might be a meaningful strategy to prevent vanilla Fusarium wilt disease occurrence and will be our future research focus.

Acknowledgments

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Supplementary materials

Table S1 Soil physicochemical characteristics.

Field sites	pH	EC (μs/cm)	Organic matter (g/kg)	Available N (mg/kg)	Available P (mg/kg)	Available K (mg/kg)
Black pepper-vanilla system	5.63±0.24 b	313.33±30.99 b	17.65±1.27 b	141.61±4.83 a	201.90±14.81 b	201.34 ± 16.83 a
Vanilla monoculture system	6.23±0.14 a	392.33±27.75 a	24.77±1.13 a	93.30±9.18 b	321.25±14.68 a	188.29±7.23 a

Values are means ± standard deviation (n = 1). Means followed by the same letter for a given factor are not significantly different ($P < 0.05$; Student's t-test).

Table S2 Fungal abundance.

Cropping regime	Soil compartment	Log_{10} ITS gene copies g^{-1} soil
Black pepper-vanilla system	Bulk soil (BB)	8.63±0.18 c
	Rhizosphere soil (BR)	9.91±0.01 a
Vanilla monoculture system	Bulk soil (VB)	8.72±0.20 c
	Rhizosphere soil (VR)	9.46±0.04 b

Values are means ± standard deviation (n = 3). Means followed by the same letter for a given factor are not significantly different ($P < 0.05$; Turkey's HSD test).

Table S3 Average good quality sequences and coverages for each treatment.

Cropping regime	Soil compartment	Good quality sequences	Coverage (%)
Black pepper-vanilla system	Bulk soil (BB)	99431±15698 ab	99.44±0.01 c
	Rhizosphere soil (BR)	89801±4483 b	99.56±0.04 b
Vanilla monoculture system	Bulk soil (VB)	114151±3093 a	99.52±0.02 b
	Rhizosphere soil (VR)	116627±2670 a	99.63±0.03 a

Values are means ± standard deviation (n = 3). Means followed by the same letter for a given factor are not significantly different ($P < 0.05$; Turkey's HSD test).

Chapter 4

Title: Enhancement of soil microbiome functionality by bio-organic fertilizer amendment

Subtitle: Bio-fertilizer application induces soil suppressiveness against Fusarium wilt disease by reshaping the soil microbiome

https://www.sciencedirect.com/science/article/pii/S0038071716306538

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Abstract

Fusarium wilt disease is a growing problem in agriculture systems. Application of bio-fertilizers containing beneficial microbes represents a promising disease control strategy. However, the mechanisms underlying disease suppression remain elusive. Here, in order to assess the importance of direct antagonism and modified soil microbiota on suppression of Fusarium wilt disease, we conducted a pot experiment with chemical, organic and biologically enhanced fertilizers, we tracked the impact of those fertilizer amendments on disease incidence, and measured the pathogen density and changes in soil microbiota. Alterations in bacterial abundance and community structure after bio-fertilizers application were determined to be key factors in constraining the pathogen, *Fusarium oxysporum*. In particular, bio-fertilizer application increased the abundance of indigenous microbial groups with reported antifungal activity, such as *Lysobacter* spp., which could play a keystone role in controlling this pathogen. The microbes introduced in the bio-fertilizer treatments (*e.g. Bacillus* and *Trichoderma* spp.) induced suppressiveness via alteration of the soil microbiome rather than direct pathogen inhibition. These results contrast with the commonly held paradigm of disease suppression using beneficial microbes and open up new perspectives for the promotion of soil health. In addition to seeking antagonistic microbes based on their direct inhibitory activity, disease suppression may also be achieved by introducing keystone species that reshape soil microbiome structure and function.

1. Introduction

Fusarium wilt disease is one of the most serious soil-borne diseases in agriculture systems and is a major limiting factor in many cropping systems (Klein *et al.*, 2016; Wang *et al.*, 2015a). This disease can be controlled to some extent with crop rotation (Wang *et al.*, 2015a; Xiong *et al.*, 2016) and screening of resistant cultivars (Hwang and Ko, 2004; Pietro *et al.*, 2003; Pinaria *et al.*, 2010) or fungicides (Nel *et al.*, 2007). However, these measures are often impractical due to labor- and monetary-costs or the low efficiency of available chemical control treatments. There is thus a need for alternative control strategies. One promising method is the application of bio-fertilizers, a mix of beneficial microbes combined with organic material. Bio-fertilizers combines the advantages of recycling organic waste, introducing beneficial microbes and providing organic material that will create additional niches for beneficial indigenous microbes (Fu *et al.*, 2017; Qiu *et al.*, 2012).

Bio-fertilizers are often enriched with microbial isolates such as *Bacillus* and *Trichoderma* spp., selected based on their potential ability to suppress Fusarium wilt disease under laboratory conditions (Wang *et al.*, 2013; Zhang *et al.*, 2016). However, direct applications of such potentially beneficial species often results in a poor disease suppression due to their low survival in soil (Saravanan *et al.*, 2003). The secondarily fermented bio-fertilizers, a combination of bio-control agents and mature compost, could enhance resident beneficial microbe density in soil and be more efficient in controlling soil-borne disease than solely antagonistic microbe (Zhang *et al.*, 2011). In addition, compost can stimulate the activities of soil microorganisms that are antagonistic to plant pathogens (Akhtar and Malik, 2000; Mehta *et al.*, 2014).

Bio-fertilizers can improve soil health by direct suppression of pathogens or via modification of the indigenous microbial community. While direct suppression is well described, very few is known about the effect of bio-fertilizers on soil microbial communities and their ability to suppress diseases. We hypothesised that changes in disease suppression after bio-fertilizer application is the result of the combined effects of the introduced microbes (direct inhibition of the pathogen) and alterations of soil microbiota.

To investigate this hypothesis, we used Fusarium wilt of vanilla (*Vanilla planifolia* Ames) as a model system. Vanilla is an economically valuable spice crop widely cultivated in tropical regions (Minoo *et al.*, 2008), and Fusarium wilt disease, caused by the soil-borne fungus *Fusarium oxysporum* f. sp. *vanillae* (Jayasekhar *et al.*, 2008; Pinaria *et al.*, 2010; Xiong et al., 2015b), is a serious threat resulting in large economic losses in vanilla-cropping regions. In order to assess the relative importance of introduced microbes versus reshaping of the soil microbiome for disease suppression, we amended natural soil with bio-fertilizers containing *Bacillus* (bacterial-enriched biofertilizer) or *Trichoderma* (fungal-enriched biofertilizer), and compared these treatments to the amendment with organic fertilizer without microbial inoculation and nutrient addition via mineral fertilizer. We tracked the impact of the four fertilizer amendments on disease incidence, measured the pathogen density, and examined bacterial and fungal community by Illumina MiSeq sequencing.

2. Materials and methods

2.1. Experimental design

We collected the experimental soil in October 2014 from a 20-year continuously cropped vanilla field showing serious Fusarium wilt disease (before we collected the soil, the field was abandoned for over six months without vanilla cropping). We collected the field soil with a depth of about 20 cm using shovels, mixed it thoroughly and immediately transferred it to a greenhouse with an average temperature of 30 °C and an average humidity of 72% at the Spice and Beverage Research Institute, Wanning City, Hainan Province, China (110°19'E-110°22'E, 18°72'N-18°76'N). A pot experiment was performed using a randomized complete block design with four replicates for each treatment, where each replicate had four pots, and each pot contained 12 kg soil with three vanilla seedlings from the rapid multiplication nursery (please see more details in (Xiong et al., 2015b; Zhao *et al.*, 2015)). Four fertilization treatments were designed as follows: organic fertilizer (OF), which is a mixture of an amino acid fertilizer (Wang et al., 2013) and chicken manure compost in a 1:1 weight ratio (after first fermentation), two secondarily fermented bio-fertilizers inoculated with either *Bacillus amyloliquefaciens* W19 (bacterial enriched bio-fertilizer, B_BIO) or *Trichoderma guizhouense* NJAU 4742 (fungal enriched bio-fertilizer, F_BIO) respectively, as previously described (Wang et al., 2013; Yuan et al., 2016). The bio-fertilizers contained approximately 1.0×10^9 and 5.0×10^7 CFU of *Bacillus amyloliquefaciens* and *Trichoderma guizhouense* g^{-1} dry weight of bio-fertilizer, respectively. Each pot was supplemented with 360 g OF or bio-fertilizers. A control treatment was set up with chemical fertilizer (CF) containing the same NPK concentrations (*i.e.*, 13.25 g urea, 13.75 g P_2O_5 and 4.10 g K_2O) as OF. After the fertilization treatments, the vanilla seedlings were planted in pots and covered with some sterile coco fiber. Fusarium wilt disease was based on appearance of typical wilt symptoms, and the cumulative disease incidence was calculated as the percentage of infected plants relative to the total number of plants.

2.2. Soil sample collection and DNA extraction

After 12 months we removed vanilla plants, and collected 12 random soil cores from pots with a depth of about 5 cm, which were pooled to yield one composite sample per replicate. The soils were passed through a 2 mm sieve, thoroughly homogenized and divided into 2 subsamples. One subsample was air-dried for the analysis of soil characteristics, according to previously established methods (Xiong *et al.*, 2015a), and the other subsample was stored at -80 °C for DNA extraction. For each soil sample (16 in total: 4 treatments * 4 replicates), total DNA was extracted from 0.5 gram 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) spectrophotometer.

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

The *Fusarium oxysporum*, bacterial and fungal abundances were quantified by quantitative polymerase chain reaction (qPCR), according to previously described protocols (Xiong *et al.*, 2016, 2015a). Briefly, we set up 20 µl reaction mixtures 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_2O. The specific primer sets were as follows: AFP308R

(CGAATTAACGCGAGTCCCAAC) and ITS1F (CTTGGTCATTTAGAGGAAGTAA) (Lievens *et al.*, 2005) for *Fusarium oxysporum*; 515F (GTGCCAGCMGCCGCGGTAA) and 806R (GGACTACVSGGGTATCTAAT) (Caporaso *et al.*, 2012) for bacteria; and ITS1F (CTTGGTCATTTAGAGGAAGTAA) (Gardes and Bruns, 1993) and ITS2 (GCTGCGTTCTTCATCGATGC) (White *et al.*, 1990) for fungi. The PCR thermalcycling conditions were set as follows: 30 s at 95 °C for initial denaturation, 40 cycles of 5 s at 95 °C, and 34 s at 60 °C. The standard curves were also generated according to previously described protocols (Xiong *et al.*, 2016, 2015a). The specificity of the amplification products was confirmed by melting curve analysis and agarose gel electrophoresis. Copy numbers were $\log_{10}$-transformed to normalize the values prior to further statistical analysis. Although the used primer set (AFP308R and ITS1F) may also amplify non-pathogenic strains of *F. oxysporum*, we showed in previous studies that the total soil *Fusarium oxysporum* abundance detected with this primer set was highly correlated with vanilla *Fusarium* wilt disease severity (Xiong *et al.*, 2016, 2015a). Therefore, even if we could not differentiate pathogenic and non-pathogenic *F. oxysporum* by the current primers sets, our qPCR measurements are a a robust indicator for the pathogen (Fu *et al.*, 2017; Qiu *et al.*, 2012).

2.4. PCR amplification and Illumina MiSeq sequencing

We used the general bacterial primers 515F and 806R to amplify the V4 hypervariable region of the bacterial 16S rRNA gene. For fungal communities, the fungal-specific primers ITS1F and ITS2 were used to target the ITS1 region. These 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 amplification was performed following previously established protocols (Xiong *et al.*, 2016, 2015a), 27 and 25 cycles were performed to amplify fungal and bacterial templates, respectively. Each sample was amplified in triplicate, after purification with a PCR Purification Kit (Axygen Bio, USA), the PCR products were pooled in equimolar concentrations of 10 ng μl^{-1} before sequencing. Paired-end sequencing of bacterial and fungal amplicons were performed on the Illumina MiSeq sequencer at Personal Biotechnology Co., Ltd (Shanghai, China).

2.5. Bioinformatics analysis

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). In brief, sequences with expected errors > 0.5 or a length shorter than 200 bp were removed. After discarding the singletons, the remaining reads were assigned to operational taxonomic units (OTUs) with 97% similarity threshold. Then, the chimera sequences were removed. Finally, the bacterial representative sequences were matched against the RDP database (Wang *et al.*, 2007; Cole *et al.*, 2009) and the fungal representative OTUs were classified using UNITE database (Kõljalg *et al.*, 2013) using the naïve Bayesian classifier implemented in Mothur with a 80% confidence threshold (Schloss *et al.*, 2009).

We estimated fungal and bacterial diversity using the Chao1 richness and phylogenetic diversity (PD) indices (Faith, 1992). Shannon evenness was calculated to evaluate species evenness. We used weighted (based on abundances of taxa) and unweighted (sensitive to rare taxa) UniFrac metric matrices to explore the variations in bacterial and fungal community structures among all the soil samples (Lozupone *et al.*, 2006). PCoA (Principal Coordinate Analysis) was performed on distance matrices and coordinates were used to draw 2D graphical outputs. Analysis of similarity (ANOSIM) was performed to evaluate the significant differences in bacterial and fungal community structures between the four fertilizer treatments (Clarke, 1993). Heat maps were generated based on the 60 most abundant bacterial and fungal genera in R (version 3.2.2) with the gplots package. Venn diagrams were constructed to visualize shared and unique OTUs across the treatments in Mothur.

2.6. Statistical analyses

The fold change of each genus in the OF, B_BIO or F_BIO treatments relative to the CF treatment was calculated using the following formula: (X-CK)/CK, X is the relative abundance of the genus in the OF, B_BIO or F_BIO samples, CK represents the average relative abundance of each genus in the CF treatment. One-way analyses of variance (ANOVA) with the Duncan multiple range test were performed for multiple comparisons, and spearman's rank correlation coefficient between the abundant bacterial and fungal genera with the *F. oxysporum* abundance were calculated using SPSS v20.0 (SPSS Inc., USA). Linear models (LM) to examine the relationships of microbial indicators with *F. oxysporum* abundance and the relative importance for each of the predictors in this model were tested in R (version 3.2.2). For the initial microbial indicators, we chose microbial (bacteria and fungi) abundance, richness, phylogenetic diversity, evenness, weighted and unweighted structure (PCoA1), and relative abundance of the *Bacillus* and *Trichoderma*. We used a stepwise model selection using the Akaike information criteria (AIC) to select the model with best explanatory power (step function in R), and the relative importance for each of the predictors in the model was determined using the "relaimpo" package in R. The linear regression analyses between the *F. oxysporum* abundance with the selected microbial indicators were also tested in R.

2.7. Sequence accession numbers

All raw sequences data are available in NCBI Sequence Read Archive (SRA) database under the accession number SRP090114.

3. Results

3.1. Effect of bio-fertilizers on vanilla Fusarium wilt disease incidence

As compared to the chemical fertilizer (CF) treatment, both the bacterial enriched bio-fertilizer (B_BIO) and the fungal enriched bio-fertilizer (F_BIO) treatments significantly (Duncan test, $P < 0.05$) reduced the cumulative incidence of vanilla Fusarium wilt disease (Fig. 1). The F_BIO treatment showed the lowest cumulative disease incidence (DI) among the four fertilizer treatments with a cumulative DI value of 18.75%. Interestingly, there was no significant difference in cumulative DI between the CF and organic fertilizer (OF) treatments (Duncan test, $P > 0.05$), and no significant difference in cumulative DI between the F_BIO and B_BIO treatments (Duncan test, $P > 0.05$).

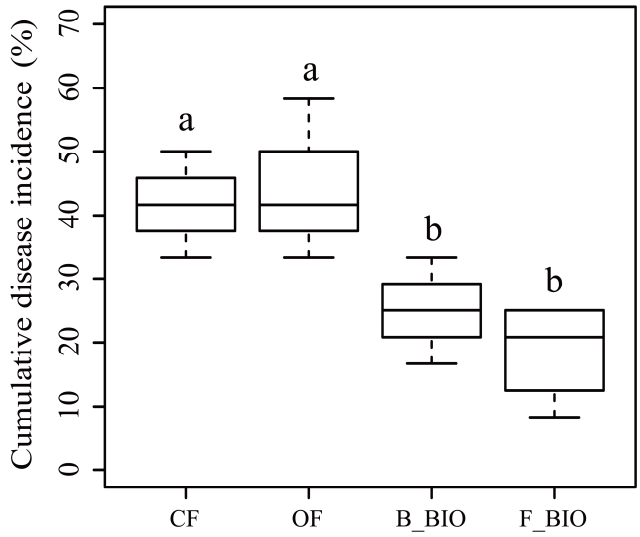

Fig. 1 The cumulative disease incidence of vanilla Fusarium wilt in the four fertilizer treatments. CF = chemical fertilizer, OF = Organic matter fertilizer, B_BIO = bacterial enriched bio-fertilizer and F_BIO = fungal enriched bio-fertilizer. Different letters above the bars indicate a significant difference at the 0.05 probability level according to the Duncan test.

3.2. Effect of bio-fertilizers on soil chemical properties

Soil chemical characteristics among the four fertilizer treatments are shown in Table S1. Soil pH did not vary much between the four treatments (ranging from 7.51 to 7.67). OF and the two bio-fertilizer treatments had significantly (Duncan test, $P < 0.05$) higher organic matter and available N contents as compared to the CF treatment. Interestingly, fungal enriched bio-fertilizer (F_BIO) showed significantly (Duncan test, $P < 0.05$) lower organic matter content compared to the OF and bacterial enriched bio-fertilizer (B_BIO). There were no significant differences for available P across the four treatments.

Table 1 Bacterial and fungal abundance and α-diversity indexes for the four fertilizer treatments.

	Treatment	Abundance*	Richness (Chao1)	Faith's PD	Evenness
Bacteria	CF	11.84±0.29 c	6088.24±132.79 c	251.77±7.27 c	0.867±0.002 c
	OF	11.90±0.16 bc	6663.64±117.80 ab	263.66±15.49 bc	0.877±0.002 ab
	B_BIO	12.14±0.07 ab	6830.04±89.61 a	306.61±20.94 a	0.879±0.002 a
	F_BIO	12.23±0.08 a	6632.25±102.99 b	277.04±11.45 b	0.875±0.001 b
Fungi	CF	8.00±0.06 c	1192.47±115.53 c	197.38±22.21 b	0.685±0.004 a
	OF	8.04±0.14 bc	1277.05±76.01 bc	235.30±19.34 a	0.460±0.022 c
	B_BIO	8.20±0.04 a	1424.72±83.82 a	255.30±29.42 a	0.594±0.024 b
	F_BIO	8.16±0.11 ab	1346.84±62.36 ab	234.05±21.04 a	0.606±0.018 b

CF = chemical fertilizer, OF = Organic matter fertilizer, B_BIO = bacterial enriched bio-fertilizer and F_BIO = fungal enriched bio-fertilizer. Values are means ± standard deviation (n = 3). * Bacterial or

fungal copy numbers were log_{10}-transformed in abundance. Means followed by the same letter for a given factor are not significantly different ($P < 0.05$; Duncan test).

3.3. Effect of bio-fertilizers on microbial abundance, diversity and community structure

Total bacterial and fungal abundances were significantly (Duncan test, $P < 0.05$) higher in the three organic fertilizer treatments (OF, B_BIO and F_BIO) compared to the chemical fertilizer treatment (Table 1). Interestingly, the F_BIO treatment harboured the highest number of 16S rRNA gene copies, while the B_BIO had the highest ITS gene copy numbers. Compared with CF, the three organic fertilizer treatments had significantly (Duncan test, $P < 0.05$) higher Chao1 richness and Faith's phylogenetic diversity (PD) in both bacteria and fungi. The bacterial bio-fertilizer (B_BIO) had the highest Chao1 richness and Faith's PD values of both bacteria and fungi among the four fertilizer treatments. In addition, B_BIO exhibited the highest bacterial evenness, whereas the CF treatment had the highest fungal evenness. Venn diagrams showed 357, 253, 616 and 372 unique bacterial OTUs in the CF, OF, B_BIO and F_BIO treatments respectively, while 210, 185, 242 and 187 unique OTUs were detected for fungi, respectively (Fig. S1). Moreover, the B_BIO and F_BIO treatments harboured the highest number of shared bacterial OTUs (5,727) among the pairwise treatments, whereas B_BIO and OF exhibited the highest number of shared fungal OTUs (1,072) among the pairwise treatments.

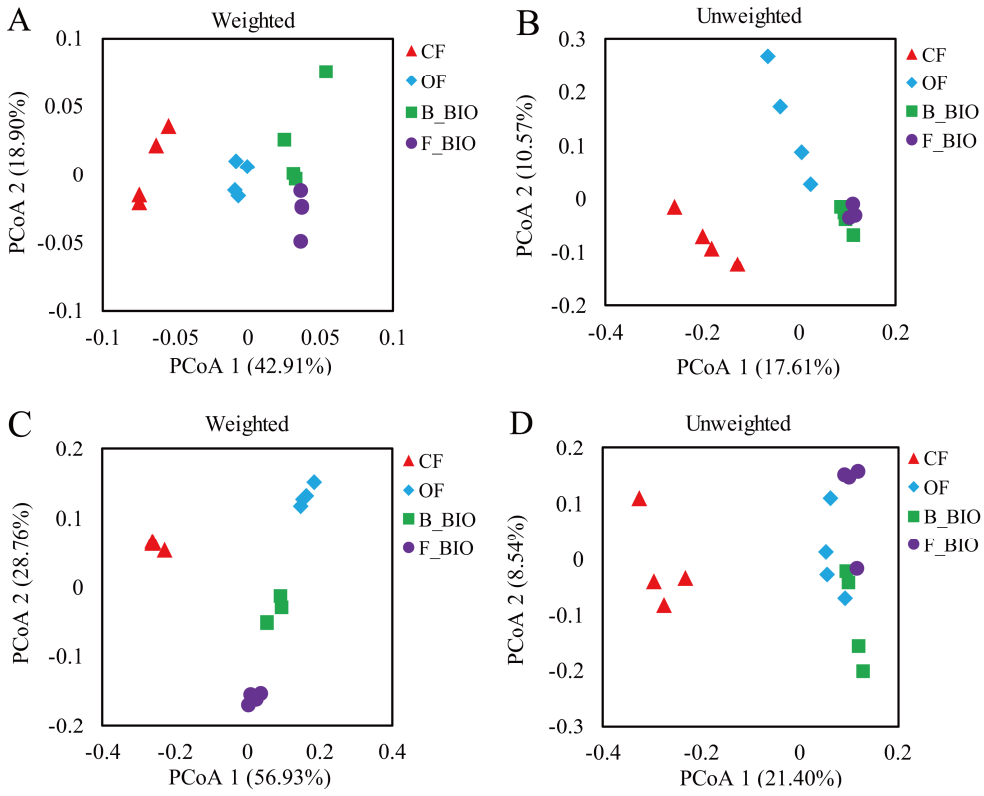

Fig. 2 Microbial community structures in the four fertilizer treatments. UniFrac-weighted principle coordinate analysis of bacterial (A) and fungal (C) community structures, UniFrac-unweighted principle coordinate analysis of bacterial (B) and fungal (D) community structures.
CF = chemical fertilizer, OF = Organic matter fertilizer, B_BIO = bacterial enriched bio-fertilizer and F_BIO = fungal enriched bio-fertilizer.

Unconstrained PCoA of weighted and unweighted UniFrac distances revealed differences in soil microbial communities across the the four fertilizer treatments (Fig. 2). Both weighted and unweighted UniFrac distances revealed that the CF treatment was clearly separated from the other three fertilizer treatments along the first component (PCoA1) for both bacterial and fungal communities (ANOSIM, CF vs OF, B_BIO and F_BIO, $P < 0.05$). In bacteria, weighted UniFrac distances showed that F_BIO treatment was separated from B_BIO treatment (ANOSIM, F_BIO vs B_BIO, $P < 0.05$) along the second component (PCoA2), whereas F_BIO and B_BIO treatments grouped tightly together based upon the unweighted UniFrac distances (Fig. 2A and B). For fungi, CF, OF, F_BIO and B_BIO treatments showed significant differences based upon the weighted UniFrac distances (ANOSIM, CF vs OF vs F_BIO vs B_BIO, $P < 0.001$), while using unweighted UniFrac distances, OF, F_BIO and B_BIO treatments grouped together (Fig. 2C and D).

3.4. Effect of bio-fertilizers on microbial taxonomic composition

The overall taxonomic complexity of the microbial community at the phylum level is presented in Fig. S2. The Proteobacteria, Actinobacteria, Acidobacteria, Bacteroidetes, Gemmatimonadetes, Firmicutes were the 6 most abundant phyla across all samples in rank order, together accounting for 71.34% of the total bacterial sequences. Compared with the chemical fertilizer (CF) and organic matter fertilizer (OF), the two BIO-fertilizer treatments had a significantly (Duncan test, $P < 0.05$) higher relative abundance of Proteobacteria and significantly (Duncan test, $P < 0.05$) lower levels of Actinobacteria abundance. The F_BIO treatment had the highest Acidobacteria abundance, whereas CF had the lowest Acidobacteria abundance. The Ascomycota, Basidiomycota, Zygomycota were the top 3 most abundant fungal phyla across all samples in rank order. Compared to the CF treatment, the OF and the B_BIO treatments had a significantly (Duncan test, $P < 0.05$) higher Ascomycota abundance.

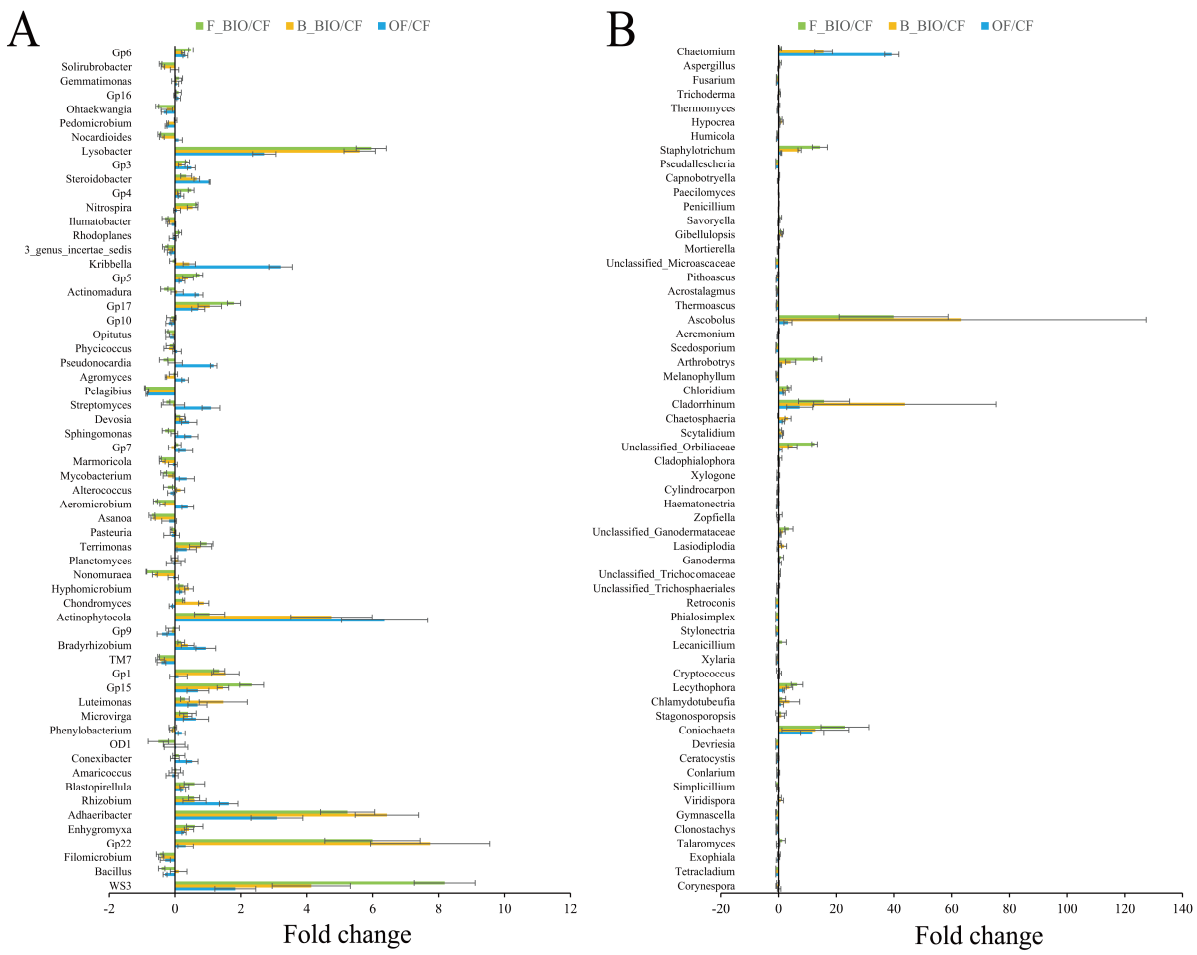

Fig. 3 The fold change of bacterial (A) and fungal (B) genera in F_BIO, B_BIO and OF treatments relative to the control (CF) treatment.

CF = chemical fertilizer, OF = Organic matter fertilizer, B_BIO = bacterial enriched bio-fertilizer and F_BIO = fungal enriched bio-fertilizer. Error bars represent the standard deviation of the four replicates.

An overview of the 60 most abundant bacterial and fungal genera among the four fertilizer treatments are shown in heat map plot (Fig. S3). Based on the 60 most abundant bacterial genera, F_BIO and B_BIO treatments grouped tightly together and showed different patterns of bacterial community structure relative to the CF and OF treatments. For the fungi, OF and the two BIO-fertilizer treatments had similar community structure patterns. For further analysis of bacterial and fungal genera across the four fertilizer treatments, we used fold change to calculate the variations in the relative abundance of the genera in the OF, B_BIO and F_BIO treatments compared to CF. As shown in the Fig. 3A, some taxa from the phylum Acidobacteria, such as *Gp6*, *Gp3*, *Gp4*, *Gp17* and *Gp1*, were of greater relative abundance in the OF and the two BIO-fertilizer treatments in comparison with the CF treatment. Moreover, the fold change of *Lysobacter* relative abundance in the OF, B_BIO and F_BIO treatments was significantly (Duncan test, $P < 0.05$) higher 2.7, 5.7 and 6.0 times relative to the CF treatment, respectively (Fig. 3A and Fig. 4C). The fold change of *Nitrospira* relative abundance in the two BIO-fertilizer treatments was over 50% compared to the CF treatment (Fig. 3A). For fungi, *Chaetomium* were relatively more abundant in the OF treatment, while *Fusarium* levels were lower in

the two BIO-fertilizer treatments (Fig. 3B). Moreover, ANOVA analysis showed that *Bacillus* abundance was significantly higher in the B_BIO treatment compared to the OF and F_BIO treatments (Duncan test, $P < 0.05$), but was not significantly different from the CF treatment (Fig. 4A). The relative abundance of *Trichoderma* did not show significant differences across the 4 fertilizer treatments (Fig. 4B).

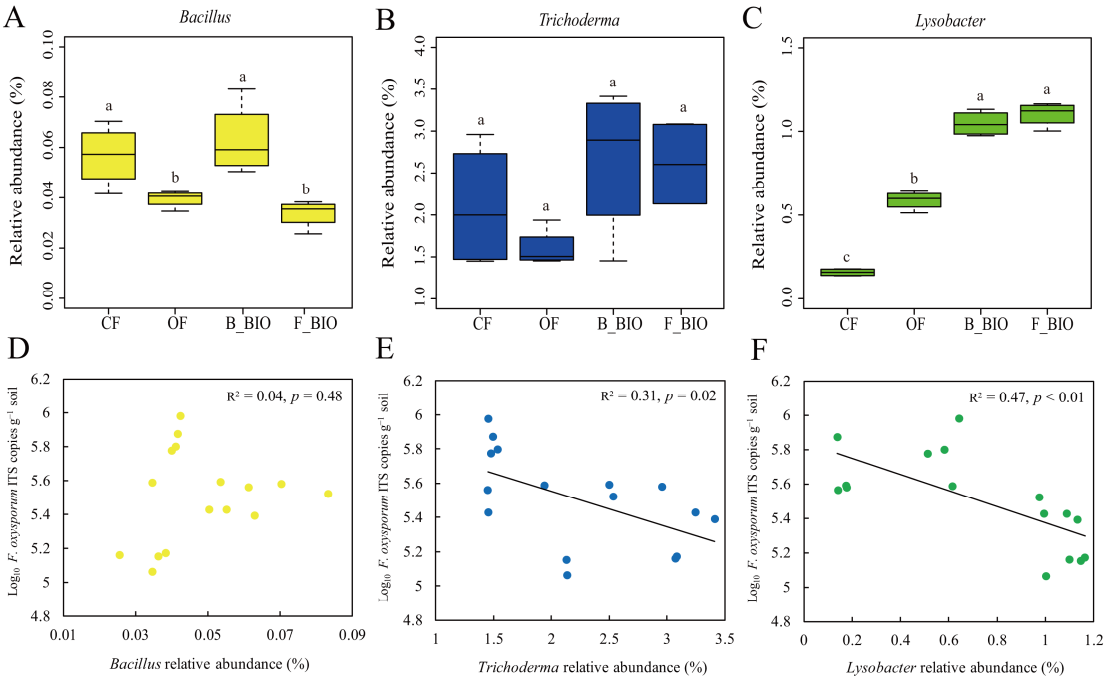

Fig. 4 The relative abundance of *Bacillus* (A), *Trichoderma* (B) and *Lysobacter* (C) genera in the four fertilizer treatments and the linear regression relationship between the *Bacillus* (D), *Trichoderma* (E) and *Lysobacter* (F) genera and the *F. oxysporum* abundance.

CF = chemical fertilizer, OF = Organic matter fertilizer, B_BIO = bacterial enriched bio-fertilizer and F_BIO = fungal enriched bio-fertilizer. Different letters above the bars indicate a significant difference at the 0.05 probability level according to the Duncan test.

Table 2 Linear models (LM) for the relationships of the selected microbial indicators with *F. oxysporum* pathogen abundance and the relative importance for each of the predictors in the model.

	Df	F	*P*-value	Relative Importance
Bacterial abundance	1	71.93	< 0.001 *** ↓	15.36%
Bacterial richness	1	7.52	0.03 * ↓	9.55%
Bacterial PD	1	5.04	0.07	4.25%
Fungal evenness	1	7.21	0.04 * ↓	14.98%
Fungal PD	1	5.11	0.06	3.29%
Bacterial structure (weighted PCoA1)	1	35.53	< 0.001 *** ↓	14.27%
Bacterial structure (unweighted PCoA1)	1	4.68	0.07	15.38%
Fungal structure (unweighted PCoA1)	1	2.83	0.14	10.30%
Trichoderma relative abundance	1	2.58	0.16	8.58%
Residuals	6			
		Model summary: $R^2 = 0.96$, AIC = -26.56		
		Total proportion variance explained by model: 95.96%		

P-values are the results from ANOVAs. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. PD means phylogenetic diversity. "↑" and "↓" behind the *P*-value represent a positive and negative relationship between each predictor and *F. oxysporum* pathogen abundance, respectively.

3.5. Relationship between microbial indicators and F. oxysporum abundance

Bacterial abundance, bacterial richness, bacterial PD, fungal evenness, fungal PD, bacterial structure (weighted PCoA1), bacterial structure (unweighted PCoA1), fungal structure (unweighted PCoA1), and *Trichoderma* relative abundance were selected from our initial microbial indicators in the linear model, showing the best explanatory power for *F. oxysporum* abundance. *Bacillus* was not retained in this linear model. Importantly, the bacterial abundance ($F = 71.93$, df =1, $P < 0.001$) and weighted bacterial structure ($F = 35.53$, df =1, $P < 0.001$) constrained *F. oxysporum* pathogen density most (Table 2), both having a relative importance of over 10%. In addition, based on the linear regression analyses between the *F. oxysporum* abundance with selected microbial indicators, we found bacterial abundance ($R^2 = 0.48$, $P < 0.01$), weighted ($R^2 = 0.38$, $P = 0.01$) and unweighted bacterial structure ($R^2 = 0.41$, $P < 0.01$), and *Trichoderma* relative abundance ($R^2 = 0.31$, $P = 0.02$) have significantly negative relationships with *F. oxysporum* abundance (Fig. 5). Furthermore, we used spearman's rank correlation to evaluate the relationship between the *F. oxysporum* abundance with the 60 most abundant bacterial and fungal genera across the four fertilizer treatments (Table S2). The relative abundance of *Gp6*, *Gp4*, *Lysobacter*, *Nitrospira*, *Rhodoplanes*, *Aspergillus*, *Thermomyces*, *Hypocrea*, *Trichoderma*, *Penicillium* and *Mortierella* revealed a significantly ($P < 0.05$) negative relationship with *F. oxysporum* abundance, while there was no significantly negative relationship between the *Bacillus* abundance with *F. oxysporum* abundance, even though *Bacillus* levels were highest in the B_BIO treatment.

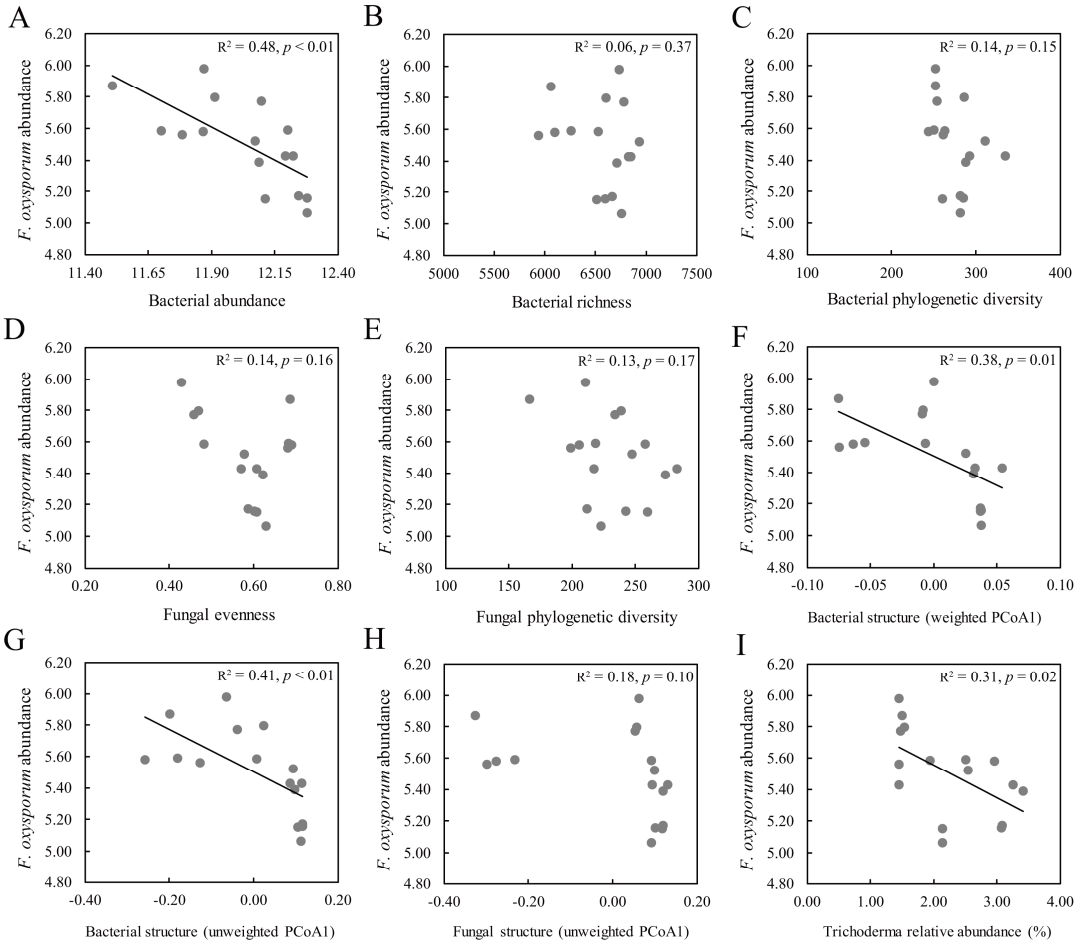

Fig. 5 The linear regression relationship between microbial indicators (selected from the linear models) and the *F. oxysporum* abundance. Bacterial abundance (A), bacterial richness (B), bacterial phylogenetic diversity (C), fungal evneness (D), fungal phylogenetic diversity (E), bacterial structure (weighted PCoA1) (F), bacterial structure (unweighted PCoA1) (G), fungal structure (unweighted PCoA1) (H), *Trichoderma* genera relative abundance.

4. Discussion

In the current study, we sought to examine the mechanisms linking soil amendments (bio-fertilizers) with suppression of *F. oxysporum*. With vanilla used as the wilt-susceptible crop, we observed that the two bio-fertilizer treatments significantly reduced the Fusarium wilt disease incidence as compared to the chemical and organic matter fertilizer treatments. However, no significant difference in disease incidence between the chemical fertilizer and organic fertilizer treatment was observed, suggesting that organic matter amendment alone was not sufficient to induce soil suppressiveness against Fusarium wilt disease. This finding is in line with the findings of Bonanomi et al. (2010) who, using a meta-analysis, showed that organic matter amendment was ineffective or even conducive to other pathogens. Our next question was whether the inoculated *Bacillus* and *Trichoderma* spp. provide a direct suppression against *F. oxysporum* pathogen?

Even though *Bacillus* levels were higher in the B_BIO treatment, there was no significantly negative relationship between *Bacillus* and *F. oxysporum* abundance. In addition, the relative abundance of *Trichoderma* was not different across the four fertilizer treatments, and within the linear models, *Trichoderma* relative abundance did not significantly restrict *F. oxysporum* density. Our results therefore suggest that the introduced microbes (*Bacillus* and *Trichoderma* spp.) had only a limited survival capacity in our study system, and their abundance had only a marginal direct effect on *F. oxysporum* density and thereby the suppression of Fusarium wilt disease.

The soils with the different fertilizer amendments exhibited distinct microbial communities and these differences may be related to the observed patterns of *F. oxysporum* pathogen abundance and Fusarium wilt disease incidence. In our one-year pot experiments, 16S rRNA and ITS gene copy numbers for the OF and two bio-fertilizer treatments revealed higher bacterial and fungal abundance (biomass) compared to the chemical fertilizer treatment, which is consistent with previous observations that organic compost amendment can activate microbial biomass and activity (Jannoura *et al.*, 2013). In addition, the OF and two bio-fertilizer treatments harboured significantly higher bacterial and fungal richness and phylogenetic diversity than CF. Some previous studies support the view that long-term organic compost amendment activates bacterial diversity as compared with conventionally used synthetic chemical fertilizers (Chaudhry *et al.*, 2012). However, some other studies have not revealed any significant effects on microbial richness in organic farms compared to conventional farms (Sugiyama *et al.*, 2010). In addition, other findings revealed that the alpha diversity of bacteria significantly increased, whereas fungal diversity decreased after two years of bio-fertilizer application (Shen *et al.*, 2015a). Thus, the impacts of chemical versus organic fertilizer on patterns of microbial diversity remain unclear, and differences found between different studies may be related to the use of different experimental systems and management definitions (Hartmann *et al.*, 2015). In our attempt to link microbial diversity or abundance with pathogen density, our linear model analysis showed that bacterial abundance significantly constrained *Fusarium* pathogen density. Higher bacterial abundance or diversity of soil microbial communities can play an important role in the capacity of soils to suppress soil-borne plant diseases (Boer *et al.*, 2003; Elsas *et al.*, 2002).

We observed significant differences in microbial community structure after bio-fertilizers applications, and PCoA results revealed that all four fertilizers treatments harboured structurally distinct microbial communities. The microbial structure within the CF treatment was distinctly different from the OF and the two bio-fertilizer treatments for both bacterial and fungal communities, which is in line with previous observations that organic and inorganic fertilizers often result in distinct soil microbial community structures (Marschner *et al.*, 2003; Zhang *et al.*, 2012). For fungi, the OF, F_BIO and B_BIO treatments showed significant differences based upon weighted UniFrac distances (based on abundances of taxa), although this was not the case when using unweighted UniFrac distances (sensitive to rare taxa). For bacteria, weighted UniFrac distances grouped the F_BIO treatment separately from the B_BIO treatment, and the F_BIO and B_BIO treatments grouped tightly together based upon unweighted UniFrac distances. Taken together, these results suggest that the application of the two bio-fertilizers to soil selects for some of the same soil microbes regardless of the biological agent included. The soil microbial populations stimulated by the bio-fertilizer applications in

the two bio-fertilizers may further impact microbial interactions in the soil and may also harbour potential antagonistic capacities to help reduce plant disease (Akhtar and Malik, 2000).

Application of organic fertilizer or bio-fertilizer was a major factor shaping the soil community taxonomic composition, as observed previously in the application of various organic fertilizers (Marschner *et al.*, 2003; Shen *et al.*, 2015b; Zhang *et al.*, 2012). Given the fact that the limited read lengths of the Illumina MiSeq sequencing do not allow for robust taxonomic characterization to the species level, we focused our examination of microbial composition changes at the genus level, as we sought to relate the differences between the four fertilizers treatments to disease suppression. *Lysobacter*, *Gp6* and *Gp4* of the Acidobacteria and *Nitrospira* were significantly overrepresented in the F_BIO and B_BIO treatments, and these genera were negatively correlated with *F. oxysporum* abundance. The genus *Lysobacter*, which is known to produce a wide range of extracellular enzymes and other metabolites with antagonistic activities against many soil-borne diseases (Gómez Expósito *et al.*, 2015; Jochum *et al.*, 2006), was six times higher in the two bio-fertilizer treatments compared as compared to the CF treatment. This genus was clearly stimulated in soils treated with the bio-organic fertilizers, and it may play a keystone role in the observed suppression of vanilla Fusarium wilt disease by these bio-organic fertilizers. Some taxa of the Acidobacteria that we found to be overrepresented in the bio-fertilizer treatments, such as *Gp4* and *Gp6*, have previously been found to be more abundant in potato common scab disease-suppressive soil as compared to conducive soil (Rosenzweig *et al.*, 2012). Thus, these taxa may be indicative of disease suppression, but additional research would be necessary to demonstrate such a role. Genera within the *Nitrospira* (Nitrospirae) are known for their nitrite- and ferrous iron-oxidizing activities, which impact the N and Fe cycles in soil (Attard *et al.*, 2010; Xu *et al.*, 2015). However, it is not yet known if such activities help support vanilla plant growth or indirectly enhance the suppression against to Fusarium wilt disease.

Our results also suggest that the microbes introduced into the bio-fertilizers (*Bacillus* and *Trichoderma* spp.) have little direct antagonistic effects on the pathogen. Rather, these introduced species may function as keystone community members that stimulate other potentially antagonistic species already present in the soil. This more complicated picture of bio-control action is in line with the notion that disease suppression is typically a complex phenomenon, most likely determined by complex microbial consortia (Mendes *et al.*, 2011). In addition, the secondarily fermentation may have also changed organic matter structure (Guo *et al.*, 2012), and more studies are needed to focus on the organic matter structure associated with disease suppression after bio-fertilizer applications.

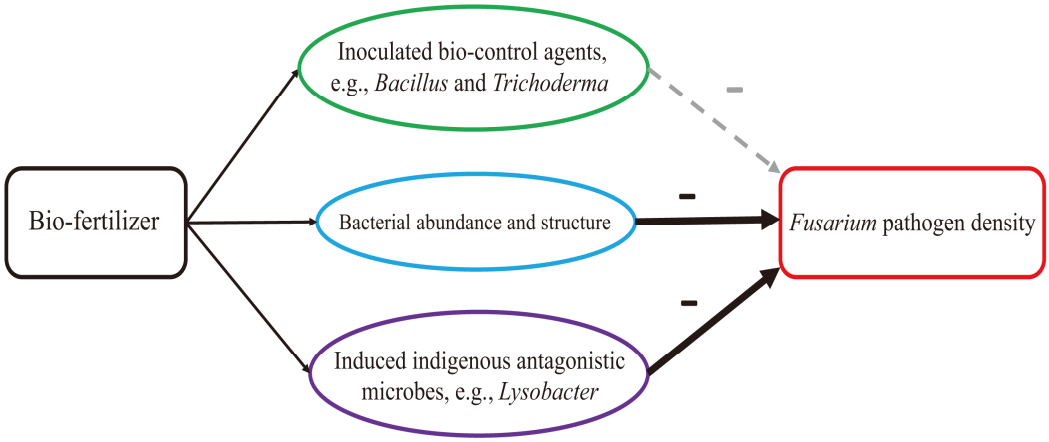

Fig. 6 A conceptual cartoon summarizing the direct influence of bio-fertilizer treatments on soil microbial community and their influence on *F. oxysporum* pathogen inhibition.

5. Conclusions

The agricultural management of soil ecosystems towards a state of increased disease suppressiveness represents one of the methods by which sustainable disease control may be achievable (Dignam *et al.*, 2016). From our results, the application of the secondarily fermented bio-fertilizers significantly reduced the incidence of vanilla Fusarium wilt disease. Fig. 6 represents a conceptual cartoon summarizing our findings of the relative influence of the inoculated microbes and induced microbial community on *F. oxysporum* pathogen inhibition. According to the results presented above, the abundance of the inoculated *Bacillus* and *Trichoderma* spp. in the bio-fertilizer had only a marginal effect on *F. oxysporum* constriction. In contrast, changes in bacterial abundance and community structure after amendment with bio-fertilizers appeared influence *F. oxysporum* population density. Most strikingly, the density of the indigenous *Lysobacter* spp. populations was negatively correlated with pathogen density (Fig. 6 and Fig. 4F). Thus, the effectiveness of bio-fertilizers seems to be a combination of the actual antagonistic activities of the inoculated bio-control agents as well as the promotion of beneficial microbial groups already resident in the soil. We propose that these additional beneficial effects should also be kept in mind in the design of bio-organic fertilizers and their use in sustainable strategies for plant disease protection.

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Supplementary data

Table S1 Soil physicochemical characteristics for the four fertilizer treatments.

Treatment	pH	Organic matter (g/kg)	Available N (mg/kg)	Available P (mg/kg)	Available K (mg/kg)
CF	7.67±0.01 a	57.69±0.91 c	117.00±11.89 b	46.49±1.72 a	434.00±20.12 bc
OF	7.54±0.03 b	73.46±4.36 a	151.41±9.05 a	45.08±1.29 a	463.75±12.61a
B_BIO	7.51±0.08 b	71.62±1.22 a	163.56±5.21 a	46.08±2.04 a	452.25±7.76 ab
F_BIO	7.58±0.03 b	67.63±0.99 b	157.31±7.65 a	46.20±2.96 a	414.75±6.95 c

CF = chemical fertilizer, OF = Organic matter fertilizer, B_BIO = bacterial enriched bio-fertilizer and F_BIO = fungal enriched bio-fertilizer. Values are means ± standard deviation (n = 3). Means followed by the same letter for a given factor are not significantly different ($P < 0.05$; Duncan test).

Table S2 Spearman's rank correlation coefficient between the abundant bacterial and fungal genus with the *F. oxysporum* abundance.

Bacterial genus	Spearman's rhot	RA (%)	Fungal genus	Spearman's rho	RA (%)
Gp6	-0.549*	5.91	*Chaetomium*	NS	21.08
Solirubrobacter	0.829**	1.63	*Aspergillus*	-0.796**	9.35
Gemmatimonas	NS	1.45	*Fusarium*	NS	8.91
Gp16	NS	1.16	*Trichoderma*	-0.536*	2.24
Ohtaekwangia	0.542*	1.11	*Thermomyces*	-0.545*	1.92
Pedomicrobium	NS	0.98	*Hypocrea*	-0.624**	1.4
Nocardioides	0.862**	1.01	*Humicola*	NS	1.04
Lysobacter	-0.777**	0.72	*Staphylotrichum*	-0.786**	0.82
Gp3	NS	0.69	*Pseudallescheria*	NS	1.09
Steroidobacter	NS	0.65	*Capnobotryella*	-0.818**	0.65
Gp4	-0.606*	0.48	*Paecilomyces*	0.500*	0.64
Nitrospira	-0.814**	0.44	*Penicillium*	-0.562*	0.62
Ilumatobacter	0.693**	0.42	*Savoryella*	-0.577*	0.61
Rhodoplanes	-0.734**	0.31	*Gibellulopsis*	-0.824**	0.53
3_genus_incertae_sedis	0.518*	0.31	*Mortierella*	-0.618*	0.49
Kribbella	0.620*	0.29	*unclassified_Microascaceae*	0.701**	0.62
Gp5	-0.795**	0.25	*Pithoascus*	NS	0.46
Actinomadura	0.770**	0.24	*Acrostalagmus*	NS	0.43
Gp17	-0.809**	0.23	*Thermoascus*	NS	0.51
Gp10	NS	0.22	*Ascobolus*	-0.764**	0.26
Opitutus	NS	0.21	*Acremonium*	-0.537*	0.28
Phycicoccus	0.567*	0.21	*Scedosporium*	NS	0.32
Pseudonocardia	0.864**	0.21	*Arthrobotrys*	-0.784**	0.19
Agromyces	0.558*	0.19	*Melanophyllum*	NS	0.21
Pelagibius	NS	0.19	*Chloridium*	-0.680**	0.17
Streptomyces	0.708**	0.19	*Cladorrhinum*	-0.621*	0.12
Devosia	NS	0.15	*Chaetosphaeria*	NS	0.13
Sphingomonas	0.805**	0.15	*Scytalidium*	NS	0.09
Gp7	NS	0.15	*unclassified_Orbiliaceae*	-0.858**	0.09
Marmoricola	0.809**	0.14	*Cladophialophora*	NS	0.09
Mycobacterium	0.826**	0.15	*Xylogone*	NS	0.09
Alterococcus	NS	0.14	*Cylindrocarpon*	NS	0.08
Aeromicrobium	0.893**	0.15	*Haematonectria*	NS	0.08
Asanoa	0.826**	0.15	*Zopfiella*	NS	0.07
Pasteuria	NS	0.13	*unclassified_Ganodermataceae*	-0.792**	0.07
Terrimonas	-0.718**	0.12	*Lasiodiplodia*	NS	0.06
Planctomyces	NS	0.13	*Ganoderma*	NS	0.07
Nonomuraea	0.907**	0.14	*unclassified_Trichocomaceae*	-0.579*	0.06
Hyphomicrobium	NS	0.12	*unclassified_Trichosphaeriales*	NS	0.07
Chondromyces	-0.670**	0.11	*Retroconis*	NS	0.09

Actinophytocola	NS	0.11	*Phialosimplex*	0.587*	0.09
Gp9	NS	0.1	*Stylonectria*	0.732**	0.07
Bradyrhizobium	NS	0.1	*Lecanicillium*	-0.640**	0.05
TM7	NS	0.1	*Xylaria*	NS	0.05
Gp1	-0.804**	0.09	*Cryptococcus*	NS	0.04
Gp15	-0.790**	0.09	*Lecythophora*	-0.840**	0.04
Luteimonas	NS	0.08	*Chlamydotubeufia*	NS	0.03
Microvirga	NS	0.08	*Stagonosporopsis*	NS	0.03
Phenylobacterium	0.525*	0.08	*Coniochaeta*	-0.524*	0.02
OD1	0.567*	0.08	*Devriesia*	NS	0.04
Conexibacter	NS	0.08	*Ceratocystis*	NS	0.02
Amaricoccus	NS	0.07	*Conlarium*	NS	0.02
Blastopirellula	-0.517*	0.06	*Simplicillium*	0.728**	0.02
Rhizobium	NS	0.06	*Viridispora*	NS	0.02
Adhaeribacter	-0.652**	0.05	*Gymnascella*	NS	0.03
Enhygromyxa	-0.612*	0.06	*Clonostachys*	NS	0.02
Gp22	-0.675**	0.05	*Talaromyces*	NS	0.02
Filomicrobium	0.564*	0.05	*Exophiala*	NS	0.02
Bacillus	NS	0.05	*Tetracladium*	NS	0.02
WS3	-0.808**	0.05	*Corynespora*	NS	0.02

RA (%) means the average relative abundance of the genus in the four fertilizer treatments combined.

NS = not significant, * and ** represent significance at $P < 0.05$ and 0.01, respectively.

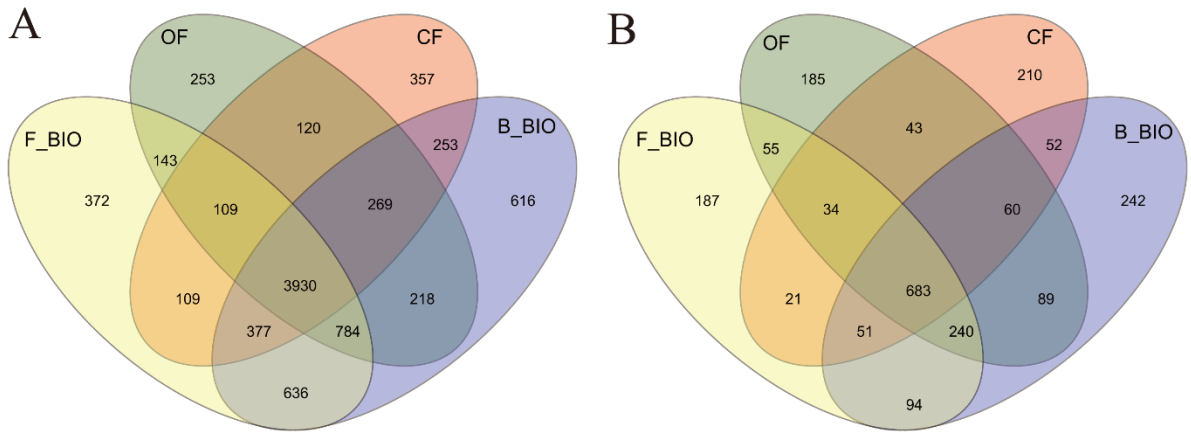

Fig. S1 Venn diagrams showed shared unique OTUs of bacteria (A) and fungi (B) in the soils from the four fertilizer treatments.

CF = chemical fertilizer, OF = Organic matter fertilizer, B_BIO = bacterial enriched bio-fertilizer and F_BIO = fungal enriched bio-fertilizer.

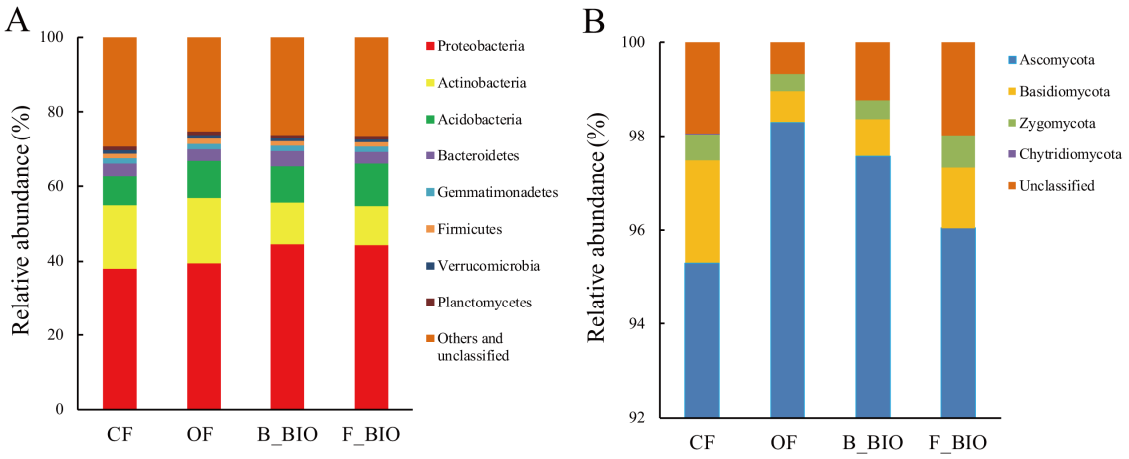

Fig. S2 The relative abundance of bacterial (A) and fungal (B) phyla in the soils from the four fertilizer treatments.

The "others" comprised low abundance bacterial phyla (less than 0.5%). CF = chemical fertilizer, OF = Organic matter fertilizer, B_BIO = bacterial enriched bio-fertilizer and F_BIO = fungal enriched bio-fertilizer.

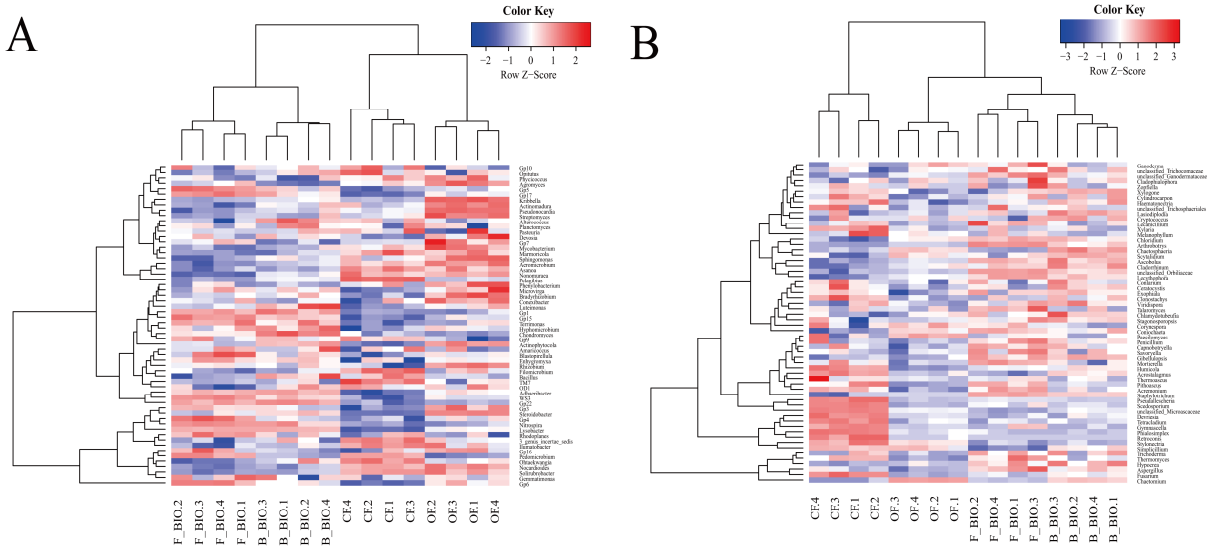

Fig. S3 Heat map of the top 60 classified bacterial (A) and fungal (B) genera of soil samples collected from the four fertilizer treatments.

CF = chemical fertilizer, OF = Organic matter fertilizer, B_BIO = bacterial enriched bio-fertilizer and F_BIO = fungal enriched bio-fertilizer.

Chapter 5:

Title: Enhancement of the rhizosphere soil microbiome for disease suppression by management of the bulk soil microbiome

Subtitle: Continuous application of different organic additives can suppress tomato disease by inducing the healthy rhizospheric microbiota through alterations to the bulk soil microflora

https://link.springer.com/article/10.1007/s11104-017-3504-6

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Abstract

Organic additives are widely applied to suppress soil-borne diseases. However, how different organic additives alter bulk soil microflora and thereby induce the healthy rhizospheric microflora remains unclear. In this paper, a 3-season field experiment containing four fertilization management programs (chemical fertilizer, organic fertilizer, amino acid organic fertilizer, and bio-organic fertilizer) was conducted in a tomato production agroecosystem with high disease incidence to evaluate the induced legacy. The bacterial and fungal microflora of bulk and rhizosphere soil influenced by different management programs were performed on the Illumina MiSeq platform. Soil amended with organic fertilizer, amino acid organic fertilizer, and bio-organic fertilizer progressively and significantly suppressed tomato diseases in comparison with chemical fertilizer, and bio-organic fertilizer presented the best efficacy in all seasons. Interestingly, rhizospheric and bulk soil bacterial and fungal communities of the different fertilization management programs were separated from each other. Six bacterial and ten fungal rhizospheric genera positively correlated with the same genera observed in bulk soil showing significant relationships with tomato disease incidence were observed, and functional strain SQR9 can be detected in the bulk and rhizosphere soils of bio-organic fertilizer treatments. Additionally, the redundancy analysis results showed the genera in treated chemical fertilizer bulk soil were dominated by *Ralstonia* and *Fusarium*. This study provided insights into soil-borne disease suppression by bulk soil management and confirmed that alterations to the bulk soil microbiota by different organic additives played distinct roles in the formation of rhizospheric soil microflora for the suppression of disease.

Introduction

Tomato (*Solanum lycopersicum* L.) is one of the most important vegetable crops cultivated worldwide (Singh and Siddiqui, 2015). However, bacterial and Fusarium wilts caused by *Ralstonia solanacearum* (Smith) (Wei *et al.*, 2011) and *Fusarium oxysporum* f. sp. *lycopersici* (Shanmugam *et al.*, 2015), respectively, are two common diseases throughout tomato growing areas. Seriously, these two pathogens often infect tomato roots concurrently under field conditions, resulting in complex disease occurrence and severe yield loss (Liu *et al.*, 2012).

The main cause for soil-borne disease incidence is an unbalanced soil micro-ecosystem (Avis *et al.*, 2008). Most soil-borne pathogens are adapted to survive in bulk soil, while the rhizosphere is the playground and infection court where pathogens establish a parasitic relationship with the plant (Raaijmakers *et al.*, 2009). The pathogens need to grow saprophytically in the rhizosphere to reach their host or to achieve sufficient numbers on their host before they can infect host tissue (Berendsen *et al.*, 2012). It has been reported that plants actively recruit beneficial soil microorganisms in their rhizosphere to counteract pathogen assault (Mendes *et al.*, 2011) and that rhizospheric microbial communities are influenced by soil type, plant development, fertilizer management, and other environmental factors (Horwath *et al.*, 1998; Chaparro *et al.*, 2014). The outcome of these factors is the development of a rhizospheric microbial community that differs markedly from the source communities in bulk soil (Minz *et al.*, 2013). Bakker *et al.* (2015) has demonstrated that different soil amendments could manipulate different bulk microbial communities, which further shows that legacy effects of prior selections in microbiotas may continue to influence rhizospheric microbial community composition. Thus, there is a need to study the relationship between the legacy effects from bulk soil to the rhizosphere on plant disease.

Chemical soil fumigation, organic amendments, and biocontrol have been suggested as control methods for bacterial and Fusarium wilts (French, 1994; Bonanomi *et al.*, 2010). Chemical soil fumigation can reduce the abundance of pathogens quickly, but pathogens might rebound to a higher abundance again (Gamliel *et al.*, 2000). An application of organic amendments could effectively control most soil-borne pathogens (Bailey and Lazarovits, 2003), however, an increase in disease incidence was also observed in several other cases after the addition of organic amendments (Mazzola *et al.*, 2001). Our previous study revealed that application of bio-organic fertilizer could control tomato disease via the manipulation of the rhizospheric microbial communities (Wang *et al.*, 2015b). Recently, more studies have been performed to examine the relationships between rhizospheric microorganisms and plant diseases, which recently became popular in plant pathology (Cai and Liao, 2003). However, how bio-organic fertilizer alters bulk soil microflora and induces the development of rhizospheric microflora remains unclear.

In this study, a 3-season field experiment was conducted in a tomato production agroecosystem with high disease incidence to evaluate the suppression of tomato disease by the following four fertilization management programs: chemical fertilizer, organic fertilizer, amino acids organic fertilizer, and bio-organic fertilizer. Afterwards, bulk and rhizospheric soil microbiotas were surveyed using Illumina MiSeq sequencing to seek out whether rhizospheric microbiotas affected by the different

fertilizer management programs exhibited varying disease suppression and how these effects were induced by bulk microbiotas alterations.

Materials and methods

Field site and experiment description

The field experimental site was located at the Nanjing Institute of Vegetable Science, Nanjing, China (31°43' N, 118°46' E). This region has the tropical monsoon climate with an average annual temperature and precipitation of 15.4 °C and 1106 mm, respectively. Tomato has been continuously cropped in the field for several years and has suffered from high bacterial and Fusarium wilt disease presence. The oven-dried soil had a pH value of 7.08, and the contents of organic matter, total N, NH_4-N, NO_3-N, available P, and available K were 28.4 g/kg, 2.04 g/kg, 52.7 mg/kg, 544 mg/kg, 110 mg/kg, and 277 mg/kg, respectively.

A 3-season field experiment was performed from March 2014 to June 2015 and included the following four treatments: (1) CF treatment, soil amended with chemical fertilizer; (2) OF treatment, soil amended with organic fertilizer (chicken manure compost); (3) AOF treatment, soil amended with amino acids organic fertilizer; and (4) BF treatment, soil amended with bio-organic fertilizer. Each treatment had three randomized independent replications. The chicken manure compost was produced by Nantong Huinong Co. Ltd, Jiangsu, China by composting chicken manure at 30-70 °C for more than 20 days. The bio-organic fertilizer was produced by solid state fermentation according to Liu *et al.* (2016). In brief, pre-compost matured chicken manure added with 0.2 ml g^{-1} of compound liquid amino acids for 4 days (the pH of the mixture was 5.0-6.0), then antagonistic strain *Bacillus amyloliquefaciens* SQR9 (Cao *et al.*, 2011) was inoculated into the mixture for a secondary fermentation with 4 days. After fermentation, the SQR9 cell concentration in the bio-organic fertilizer was greater than 1×10^8 CFU g^{-1} dry weight. The fertilizer without antagonistic bacteria inoculation and produced with the same procedure was regarded as amino acids organic fertilizer. All treatments were adjusted to the same amount of N (225 kg/ha), P (65 kg/ha) and K (150 kg/ha) for each season using mineral fertilizers as necessary. The N (urea) and K (K_2SO_4) fertilizers were applied as basal and supplementary fertilizer, while the P (calcium superphosphate), organic fertilizer, amino acids organic fertilizer and bio-organic fertilizer were only used as basal fertilizers. A detailed fertilization scheme is shown in Table S1.

Assay of tomato disease incidence and yield

Two months after the tomato plantlets were transplanted into the field, a bioassay for disease incidence including bacterial wilt disease, Fusarium wilt disease and the two-wilt disease complex was performed until the end of the experiment and was based on observations of typical wilt symptoms, including foliage chlorosis, necrosis and drooping of the leaves. Disease incidence was expressed as the percentage of diseased plants per total number of plants. For total tomato fruit yield of each plot, all mature tomato fruits were harvested and weighed. The disease incidence and fruit yield of each crop season (1^{st}: spring season; 2^{nd}: autumn season; 3^{rd}: spring season) were analyzed in this study.

Soil sampling, DNA extraction, Real-Time PCR assay and soil chemical analysis

Soil sampling was performed in June 2015 during tomato harvesting. Briefly, 6 healthy tomato plants were randomly selected in each replicate plot, 3 of which were pooled to minimize the variations. The bulk and rhizospheric soil samples were obtained according to Bakker *et al.* (2015). Thus, 6 bulk and rhizospheric soil samples were collected for each fertilizer treatment (*i.e.* 3 independent biological replicates * 2 times of sampling replicates). Finally, 5 bulk and rhizospheric soil samples for each fertilizer treatment were randomly chosen for subsequent DNA extraction using the UltraClean Soil DNA Isolation Kits (MoBio Laboratories Inc, Carlsbad, USA) according to the manufacturer's protocol. The quality and concentration of the DNA samples were determined using a spectrophotometer (NanoDrop 2000, USA). Total numbers of bacteria and fungi were quantified by Real-Time PCR with primers Eub338 and Eub 518 and primers ITS1f and 5.8s, respectively, according to Liu *et al.* (2016). The copy numbers of SQR9 was also quantified by Real-Time PCR with primers SQR9F (5'-CATGAGATGGCGGGCTTT-3') and SQR9R (5'-CGCATCCTCCCTGTCTTTG-3') according to Qiu *et al.* (2013). Each sample was performed in three replicates, and the results were expressed as log (copies g^{-1}) dry soil. All bulk soil chemical properties were determined according to Bao (2010).

Illumina MiSeq sequencing

The DNA of each soil sample served as the template for the amplification of the 16S rRNA gene and the ITS region. The V4 region of the bacterial 16S rRNA gene was amplified using primers 520F (5'-AYTGGGYDTAAAGNG-3') and 802R (5'-TACNVGGGTATCTAATCC-3') (Claesson *et al.*, 2009), and ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3') (Gardes and Bruns, 1993) and ITS2 (5'-GCTGCGTTCTTCATCGATGC-3') (White *et al.*, 1990) were used for the ITS1 region of the fungal ITS gene. The barcodes of soil samples used to distinguish each sample in the sequencing programs in this study are provided in Table S2. The programs of amplification and sequencing of the 16S and ITS genes were performed at Personal Biotechnology Co, Ltd. (Shanghai, China) on the Illumina MiSeq instrument (USA). All sequences were deposited in the NCBI Sequence Read Archive database with the accession number (SRP067366).

Bioinformatics and Statistical analysis

Quality control and annotation of the raw sequences were performed according to Liu *et al.* (2016). A total of 36,473 16S rRNA and 41,614 ITS gene sequences for each sample were randomly selected for further bacterial and fungal microbial community analysis, respectively. To compare the similarities and differences of the bacterial and fungal community structures among all soil samples, principal coordinate analysis (PCoA) based on the Bray-Curtis distance metric was performed using Mothur (Liu *et al.*, 2016) and analysis of molecular variance (AMOVA) was performed to evaluate the significant differences in bacterial and fungal community structures among the four fertilizer treatments. In addition, Pearson's correlation coefficient was used to evaluate the correlation between abundant bacterial and fungal genera in rhizospheric soils (relative abundance > 0.1%) and tomato disease incidence. Afterwards, the relative abundances of genera, which showing significant differences with disease incidence were further selected and compared between the different fertilizer management programs in bulk and rhizospheric soils. Furthermore, Pearson's correlation coefficient was used to

evaluate the correlation between the relative abundances of those genera in bulk and rhizospheric soil samples (*Ralstonia*, *Fusarium* and the genera which showing significantly relationships between bulk and rhizospheric soil samples were showed in the Fig. 4). In order to examine the relationships among the bulk soil bacterial and fungal genera, samples and environmental variables, redundancy analysis (RDA) was carried out via the vegan package of R (version 3.2.2), bioEnv procedure was performed to select the best subset of environmental variables. In addition, the Mantel test was used to calculate the correlation between the selected soil characteristics and the selected microbial genera. One-way analyses of variance (ANOVA) with the Duncan multiple range test for multiple comparisons were performed in SPSS v18.0 (SPSS Inc, USA).

Results

Effects of different fertilization management programs on disease incidence and tomato yield

Disease incidence in the spring crop season of 2014 and 2015 was significantly higher (20-75%) than those in the autumn season (0-10%) of 2014 (Fig. 1a), and the disease incidence trends of all the seasons was similar [DI (CF)> DI (OF)> DI (AOF)> DI (BF)]. In the third season, the OF, AOF and BF treatments significantly ($P < 0.05$) reduced disease incidence to 30%, 16% and 6%, respectively. These results indicated that the bio-organic fertilizer application more effectively controlled the outbreak of wilt disease in tomato plants compared to the other treatments. In contrast to the disease incidences, tomato yields with the different fertilization treatments were significantly higher than CF in all crop seasons (Fig. 1b). For the third crop season, the application of organic fertilizer, amino acid organic fertilizer and bio-organic fertilizer significantly ($P < 0.05$) increased yield by 13%, 45% and 70%, respectively, compared to the CF treatment. These results indicated that the fertilization treatments (OF, AOF, and BF) progressively suppressed disease incidence and increased tomato crop yields compared to the CF treatment.

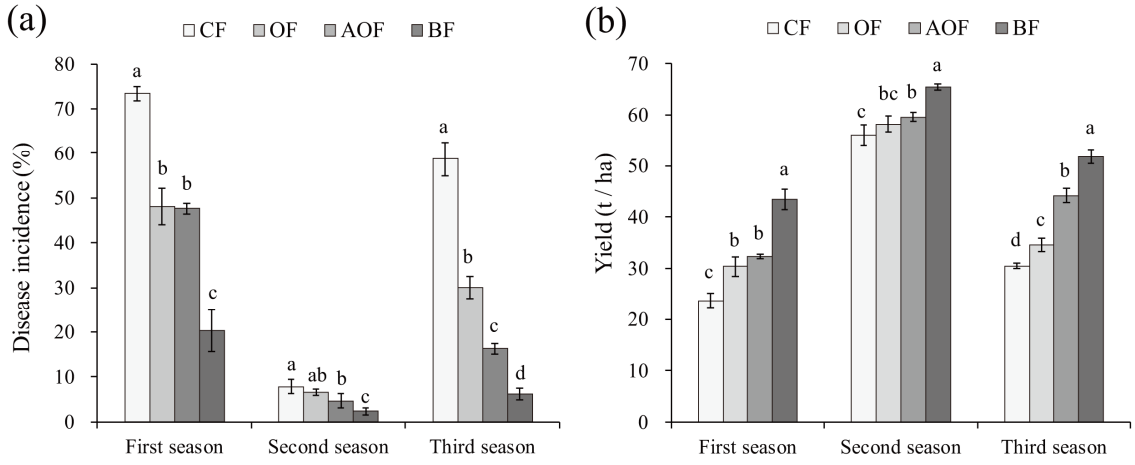

Figure 1 Effects of different fertilization management programs on disease (a) and yield (b) of tomato. Fertilization management programs: chemical fertilizer (CF), organic fertilizer (OF), amino acids organic fertilizer (AOF) and bio-organic fertilizer (BF).

Total bacterial, fungal abundances and SQR9 copy numbers

Compared to the CF treatment, the treatments of BF, AOF, and OF significantly ($P < 0.05$) increased bulk soil bacteria abundances (Fig. 2a). Similarly, rhizospheric bacteria abundances were also significantly ($P < 0.05$) enhanced in the BF, AOF, and OF treatments. Moreover, no significant differences with fungi were observed in bulk or rhizospheric soils, regardless of fertilization management program (Fig. 2b). These results showed that the management programs that contained organic fertilizer (organic fertilizer, amino acid organic fertilizer and bio-organic fertilizer) had a positive effect on the abundance of bulk and rhizospheric bacteria rather than fungi compared to the CF treatment. The copy numbers (Log10 copies) of SQR9 in bulk and rhizosphere soils of BF treatment were 4.48 and 5.17 (Fig. 2c), respectively, while it cannot be detected in the other treatments of CF, OF and AOF.

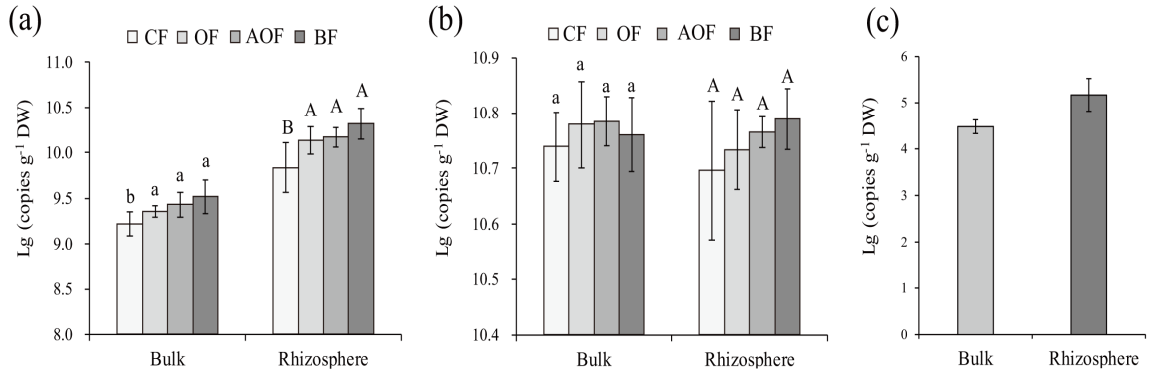

Figure 2 Total bacterial (a) and fungal (b) microbial copies in bulk and rhizospheric soil with the different fertilization management programs. The copy numbers of SQR9 in the bulk and rhizosphere soil of BF treatment (c).

Bars with different letters indicate significant differences as defined by Duncan's test ($P < 0.05$). Fertilization management programs: chemical fertilizer (CF), organic fertilizer (OF), amino acids organic fertilizer (AOF) and bio-organic fertilizer (BF).

Sequencing results

As shown in Table S3, after basal quality control, a total of 2,171,613 16S rRNA and 3,548,914 ITS sequences were obtained for all soil samples. The number of high quality sequences per sample varied from 36,743 to 127,938 for bacteria and from 41,614 to 141,100 for fungi. Moreover, at the 97% similarity cut-off level, 7,579 bacterial and 4,517 fungal OTUs were obtained.

Microbial community structures

PCoA based on the Bray-Curtis distance metric revealed that the bulk soil bacterial (Fig. 3a) and fungal (Fig. 3b) communities significantly differed ($P < 0.001$) from those in the rhizosphere along the first component. Interestingly, the rhizospheric soil bacterial communities from the four fertilizer treatments were distinct ($P < 0.05$) from each other along the second component, showing the same separation tendency ($P < 0.05$) as in the bulk soil. Similarly, the rhizospheric soil fungal communities for the different fertilization managements were well separated ($P < 0.05$) from each other along the second component with the same alteration trends ($P < 0.05$) for bulk soil samples.

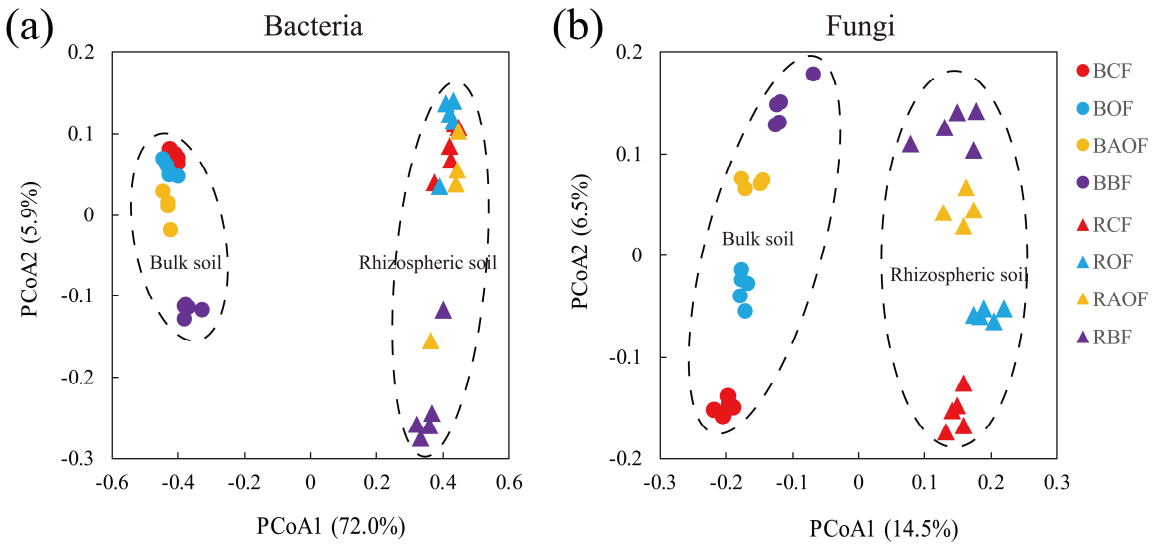

Figure 3 The bacterial (a) and fungal (b) microbial community structures of the different treatments. Fertilization management programs: chemical fertilizer (CF), organic fertilizer (OF), amino acids organic fertilizer (AOF) and bio-organic fertilizer (BF); bulk soil amended with chemical fertilizer (BCF), organic fertilizer (BOF), amino acids organic fertilizer (BAOF) and bio-organic fertilizer (BBF); rhizosphere soil amended with chemical fertilizer (RCF), organic fertilizer (ROF), amino acids organic fertilizer (RAOF) and bio-organic fertilizer (RBF).

Genera abundance analysis

As shown in the Table 1, clear positive correlations between disease incidence and the relative abundances of *Bacillus* ($P < 0.05$), *Ralstonia* ($P < 0.05$), *Fimetariella* ($P < 0.01$), *Fusarium* ($P < 0.01$), *Gliomastix* ($P < 0.01$), *Guehomyces* ($P < 0.01$), *Humicola* ($P < 0.01$), *Penicillium* ($P < 0.01$), and *Trichoderma* ($P < 0.01$), were observed. In contrast, negative correlations were observed for the genera *Chitinophaga* ($P < 0.05$), *Enterobacter* ($P < 0.05$), *Pseudomonas* ($P < 0.05$), *Pseudoxanthomonas* ($P < 0.05$), *Debaryomyces* ($P < 0.01$), *Phialemonium*, and *Purpureocillium* ($P < 0.01$) (Table 1).

Among these genera, the treatment of application of PGPR-containing organic fertilizer (BF) significantly ($P < 0.05$) increased the abundance of *Chitinophaga*, *Pseudomonas*, *Debaryomyces*, *Phialemonium* and *Trichoderma* and decreased the abundance of *Humicola* compared to the other treatments (CF, OF and AOF) in bulk soil (Fig. S1a). Moreover, the lowest value of *Fusarium* was observed in the BF treatment, which was significantly ($P < 0.05$) lower than that in CF. Additionally, the AOF treatment showed the lowest relative abundance of *Ralstonia* and the highest relative abundance of *Penicillium* and *Purpureocillium* among all treatments.

Table 1 Pearson's correlation coefficient between the relative abundance of rhizosphere abundant genus and tomato disease incidence and their relative abundance in corresponding bulk soils.

	Disease incidence	Their relative abundance in corresponding bulk soils
Bacillus	0.51*	0.03
Chitinophaga	-0.64**	0.66**
Enterobacter	-0.46*	-0.14
Pseudomonas	-0.46*	0.78**
Pseudoxanthomonas	-0.54*	0.60**
Ralstonia	0.56*	0.03
Debaryomyces	-0.83**	0.91**
Fimetariella	0.57**	0.1
Fusarium	0.83**	0.36
Gliomastix	0.78**	0.23
Guehomyces	0.88**	0.70**
Humicola	0.63**	0.48**
Penicillium	0.81**	-0.21
Phialemonium	-0.47*	0.55**
Purpureocillium	-0.71**	0.87**
Trichoderma	0.66**	0.1

Statistical significance at *$P < 0.05$ and **$P < 0.01$.

Similar to bulk soil, the same variation trends of the relative abundances of *Chitinophaga*, *Pseudomonas*, *Pseudoxanthomonas*, *Debaryomyces*, *Fusarium* and *Guehomyces* were observed in the BF treated rhizosphere, and the highest and lowest values of the former four and latter two were observed (Fig. S1b). The AOF treatment significantly ($P < 0.05$) enriched the relative abundances of *Debaryomyces* and *Purpureocillium* compared to the CF and OF treatments. In addition, the organic fertilizer containing treatments (OF, AOF and BF) significantly reduced the relative abundance of *Fusarium* compared to CF and amended with bio-organic fertilizer showed significantly lower relative abundances of *Ralstonia* and *Fusarium* compared to other treatments in the rhizosphere (Fig. S1b).

Although the correlation coefficients of the relative abundance of *Ralstonia* and *Fusarium* between bulk and rhizospheric soils were not significant ($P > 0.05$), the two genera of the potential pathogens still showed a positive relationship (Table 1 and Fig. 4). Particularly, 8 genera of *Chitinophaga*, *Pseudomonas*, *Pseudoxanthomonas*, *Debaryomyces*, *Guehomyces*, *Humicola*, *Phialemonium*, and *Purpureocillium* in bulk soil had a significant ($P < 0.01$) and positive relationship with the corresponding genera in rhizosphere soil, and no genus showed a significantly ($P < 0.05$) negative relationship between the bulk and rhizospheric soils **(Table 1)**, indicating that the bulk microbiota is crucial and critical to the development of the rhizospheric microflora and suppressing tomato disease.

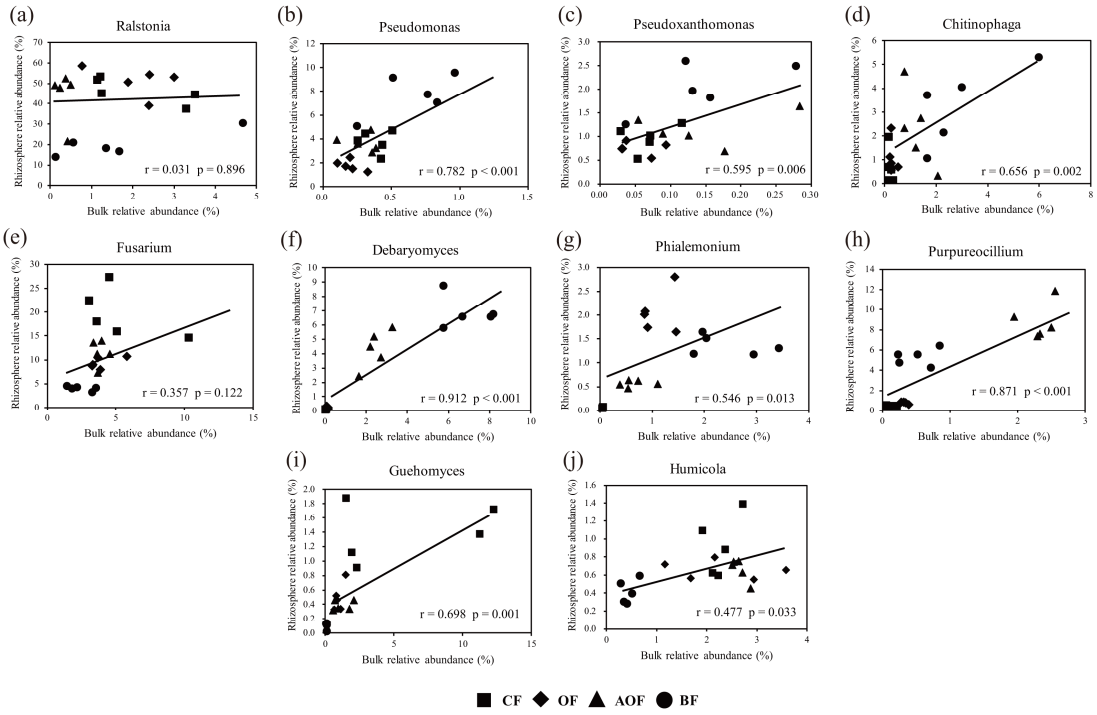

Figure 4 Pearson's correlation coefficient between rhizospheric abundant genera and the same bulk abundant genera.

Fertilization management programs: chemical fertilizer (CF), organic fertilizer (OF), amino acids organic fertilizer (AOF) and bio-organic fertilizer (BF).

Effects of soil chemical properties on bacterial or fungal taxa

The three crop seasons with the different fertilization management programs changed the soil chemical properties (Table 2). The fertilization management programs containing organic fertilizer (OF, AOF and BF) significantly ($P < 0.05$) increased soil OM, TP and AP contents compared to the CF treatment. In addition, fertilizer management programs containing amino acids (AOF and BF) significantly ($P < 0.05$) enhanced soil EC and decreased soil TK and NH_4-N concentrations compared to the fertilizer management programs that did not contain amino acids (CF and OF). Moreover, the BF treatment showed significantly ($P < 0.05$) higher NO_3-N, TN, and AK contents and a lower pH value compared to the other treatments.

Table 2 Physicochemical properties of bulk soil samples under the different fertilization treatments.

	BCF	BOF	BAOF	BBF
pH	7.64±0.16 a	7.81±0.18 a	7.72±0.10 a	7.18±0.08 b
EC (μS/cm)	309±11 c	341±8 c	531±33 b	806±66 a
NH_4-N (mg/kg)	13.8±0.9 b	15.1±0.6 a	11.5±0.7 c	12.9±0.9 b
NO_3-N (mg/kg)	229±12 b	235±9 b	237±9 b	256±6 a
Total N (g/kg)	1.97±0.04 b	1.97±0.01 b	1.94±0.02 b	2.15±0.05 a
Organic Matter (g/kg)	24.6±0.4 c	28.1±0.3 b	28.3±0.5 ab	29.0±0.8 a
Total P (g/kg)	0.54±0.08 c	0.61±0.02 b	0.60±0.03 b	0.64±0.02 a
Available P (mg/kg)	106±7 b	116±3 a	117±6 a	114±7 a
Total K (g/kg)	5.33±0.28 a	5.27±0.18 a	5.00±0.12 b	4.88±0.30 b
Available K (mg/kg)	155±12 b	159±14 b	164±2 b	194±9 a

Values (means ± SD, n = 3) within the same row followed by different letters are significantly different at $P < 0.05$ according to Duncan's test. Fertilization management programs: chemical fertilizer (CF), organic fertilizer (OF), amino acids organic fertilizer (AOF) and bio-organic fertilizer (BF); bulk soil amended with chemical fertilizer (BCF), organic fertilizer (BOF), amino acids organic fertilizer (BAOF) and bio-organic fertilizer (BBF).

The Mantel test based on the selected soil chemical properties and the abundances of the analyzed microbial genera revealed that the selected soil chemical properties were significantly correlated with variations in the selected bacterial and fungal genera ($r = 0.85$, $P < 0.001$). The redundancy analysis performed to examine the relationship between the analyzed genera from bulk soil and soil chemical properties showed that the two components could explained 49.07% of the total variation (Fig. 5). The second component (RDA2), which explained 16.36% of the variation, separated BF from the other treatments. As shown in Fig. 5, the microbial genera in the BF soil samples were dominated by *Trichoderma*, *Pseudomonas*, *Chitinophaga* and were related to TN, EC and OM contents, while the genera in the CF soil samples were dominated by *Ralstonia* and *Fusarium*. Moreover, the microbial community in the AOF were dominated by *Penicillium*, *Purpureocillium* and *Fimetariella*; and in OF soil samples were dominated by *Bacillus*, *Humicola* and *Fimetariella*.

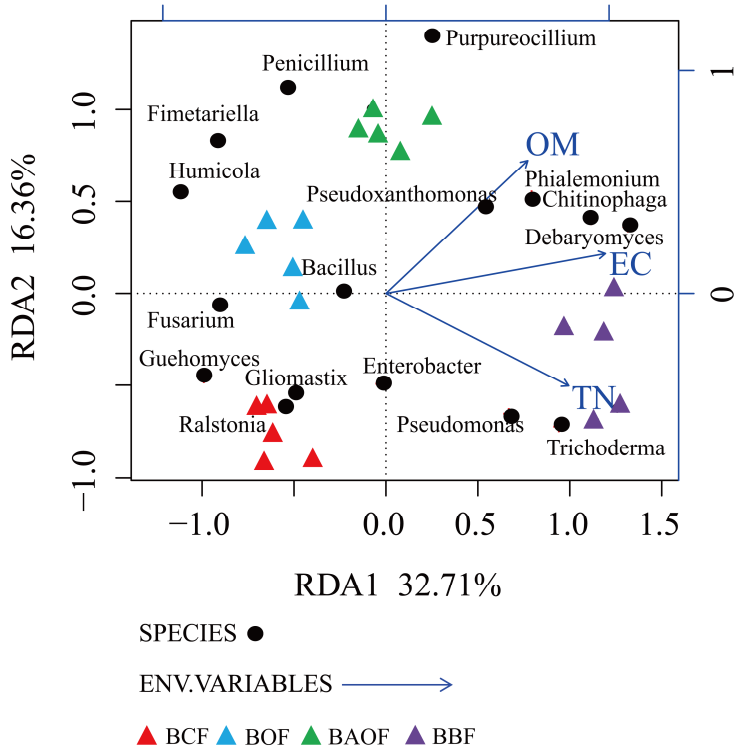

Figure 5 Redundancy analysis of the relationship between the analyzed bulk genera, samples and environmental variables.

Fertilization management programs: chemical fertilizer (CF), organic fertilizer (OF), amino-acids organic fertilizer (AOF) and bio-organic fertilizer (BF). Bulk soil amended with chemical fertilizer (BCF), organic fertilizer (BOF), amino acids organic fertilizer (BAOF) and bio-organic fertilizer (BBF).

Discussion

Applications of organic fertilizer, amino acid organic fertilizer, and bio-organic fertilizer progressively and significantly suppressed plant disease and improved fruit yield compared to the chemical fertilizer. These results were in agreement with previous studies suggested that organic amendments could be used to control diseases caused by soil-borne pathogens (Hoitink and Fahy, 1986; Szczech, 1999). Importantly, the most efficient disease suppression was observed in the BIO treatment, which is in accordance with previous reports where bio-organic fertilizer acted as both an organic fertilizer and a biocontrol agent for different soil-borne diseases in tomato (Wang *et al.*, 2015b), banana (Shen *et al.*, 2013b), and watermelon (Ling *et al.*, 2014) when compared to organic amendments and biocontrol agents applied alone.

The organic fertilizer management programs (OF, AOF and BF) all significantly increased bacterial abundances when compared with CF and was similar to previous studies where higher populations of bacteria in organic treated soils were observed compared with that from a chemical fertilizer treatment (Witter *et al.*, 1993). Interestingly, bacterial abundances in rhizospheric soils of these treatments were also higher compared to CF, indicating that bulk soil management induced legacy in microbial abundance in the rhizosphere. Functional strain SQR9 can be detected in the bulk

and rhizosphere soils of BF treatments, suggesting that SQR9 could survive in the tomato bulk soil and further colonize in the rhizosphere soil. This result was in accordance with the other report as SQR9 could effectively colonize in the rhizosphere soil and suppress the cucumber plant disease (Qiu *et al.*, 2013).

PCoA results revealed that the bacterial and fungal communities in the bulk soils of the different fertilizer treatments were well differentiated from the rhizospheric communities along the first component, which were mainly due to the ability of root exudates to influence the composition of the rhizospheric microbial communities (Chaparro *et al.*, 2014). Interestingly, in both bulk and rhizospheric soils, bacterial and fungal microbial communities of the different fertilization management programs were well separated from each other along the second component. In accordance with our results, Bonanomi *et al.* (2010) also reported that an application of organic amendments significantly shifted the soil microbial community. Moreover, amino acids are excellent C and N sources for microbes (Ge *et al.*, 2009), and may further induce microbial communities shift. Lots of previous researches have confirmed that the PGPR-containing bio-organic fertilizers had an important effect on shaping the microbial community (Shen *et al.*, 2013b; Ling *et al.*, 2014; Wang *et al.*, 2015b). The same alteration trends for the bacterial and fungal communities in both rhizospheric and bulk soil samples were observed. de Ridder-Duine *et al.* (2005) also demonstrated that the rhizosphere microbial composition of the wild plant *C. arenaria* was largely dependent on the microbial composition of the bulk soil. Hence, we can deduce that the different fertilization management programs altered the microbial community in bulk soil first, and then induced their specific rhizospheric microbiotas.

The rhizospheric genera *Ralstonia*, *Fusarium*, *Bacillus*, *Fimetariella*, *Gliomastix*, *Guehomyces*, *Humicola*, *Penicillium*, and *Trichoderma* were positively and significantly ($P < 0.05$) correlated with tomato disease incidence. In contrast, a significantly ($P < 0.05$) negative relationship was observed for genera *Chitinophaga*, *Enterobacter*, *Pseudomonas*, *Pseudoxanthomonas*, *Debaryomyces*, *Phialemonium*, and *Purpureocillium*. In this study, all the tested plants in the different fertilizer treatments were healthy, the healthy plants in high disease incidence treatments contained more relative abundances of the rhizospheric genera *Ralstonia* and *Fusarium*, increasing the risk of plant illness (Wei *et al.*, 2011; Shanmugam *et al.*, 2015). Moreover, studies have confirmed that the genera *Bacillus* (Singh and Siddiqui, 2015), *Penicillium* (Sabuquillo *et al.*, 2005), and *Trichoderma* (Blaya *et al.*, 2013) act as biocontrol agents could induce a resistance capacity of plants that provides protection against of the microbial pathogens (Van der Ent *et al.*, 2009). *Pseudomonas* was reported to suppress plant disease in several studies (Dowling and O'Gara, 1994; Singh and Siddiqui, 2015). *Enterobacter* has been reported for bioactivity against *Fusarium* (Al-Mughrabi, 2010). *Pseudoxanthomonas* is known as a biocontrol agent against *Xanthomonas* (Al-Saleh, 2014). *Debaryomyces* has been reported as a biocontrol agent against *Penicillium expansum* (Dinesh *et al.*, 2009), and *Purpureocillium* has been found exhibiting bio-control of *Meloidogyne incognita* (Singh *et al.*, 2013). However, the mechanism of the above three genera for suppressing *Ralstonia* and *Fusarium* are still unclear, future studies are needed to test such a role. Interestingly, our resulted showed that the relative abundance of *Bacillus* was positively correlated with pathogen abundance and disease incidence as well as *Trichoderma*. While, Xue *et al.* (2015) reported that the relative abundance of *Bacillus* was negatively correlated with

banana disease incidence. Previous study observed when plant infected by pathogens, plant roots would secrete more citric acid and fumaric acid to stimulate the chemotaxis response of plant growth promoting rhizobacteria (Liu *et al.*, 2014c). This may be the reason why more *Bacillus* and *Trichoderma* occurred in the healthy tomato rhizosphere in the higher disease incidence field. Furthermore, among the 16 selected rhizospheric genera, 8 showed a significant ($P < 0.01$) positive relationship with the abundances of the same genera in bulk soil, while none of them showed a significantly ($P < 0.05$) negative relationship. Similar dynamics were observed across the communities, such as the relative abundance of Actinobacteria, Firmicutes, Proteobacteria, Bacteroidetes and Acidobacteria, as the rhizosphere communities developed from bulk soil communities (Bakker *et al.*, 2015). Therefore, our results suggested that the bulk microbial community played the crucial and critical role in manipulating the rhizospheric microflora and suppressing plant disease.

The fertilizer management programs containing organic fertilizer (OF, AOF and BF) significantly ($P < 0.05$) increased soil OM compared to CF, which were consistent with a previous study (Haynes and Naidu 1998). The fertilizer management programs containing amino acids (AOF and BF) significantly ($P < 0.05$) enhanced soil EC and decreased NH_4-N compared to the fertilizer management programs containing no amino acids (CF and OF). Amino acids can form complexes with cations via carboxylate (-COO) and amine (-NH_2) groups (Dalir and Khoshgoftarmanesh, 2014) and might be the reason for the enhancement of soil EC. Yadessa *et al.* (2010) also found that there were significantly ($P < 0.01$) negative correlations between tomato bacterial wilt disease incidence and EC. The higher soil NH_4-N content in the fertilizer treatments with no amino acids may be due to the application of the chemical nitrogen (urea breaks down to NH_4-N) fertilizer (Shen *et al.*, 2013b). Moreover, the BF treatment showed significantly ($P < 0.05$) higher soil NO_3-N content than the CF, OF and AOF treatments. Huber and Watson (1974) reviewed that tomato root disease caused by *Fusarium* pathogen could be reduced by the decreased NO_3-N and the increased NH_4-N contents. In contrast, it was reported that bacterial wilt of tomato increased as NO_3-N increased and was reduced as NH_4-N was reduced (Yadessa *et al.* 2010). Although the processes of disease suppression were different, the possible key factor of disease suppression was to decrease the rhizospheric population of pathogens (Nel *et al.*, 2007). Additionally, the genera in CF bulk soil were dominated by *Ralstonia* and *Fusarium*. While, this phenomena was not observed in OF, AOF and BF treatments. Those results also suggested that soil amended with organic fertilizers have a high efficacy for suppressing the diseases. Strikingly, the application of bio-organic fertilizer showed the highest efficacy.

Conclusion

In the present study, the application of different additives in a three-season field experiment showed organic fertilization management programs (organic fertilizer, amino acid organic fertilizer and bio-organic fertilizer) progressively and significantly decreased soil-borne diseases and enhanced fruit yields of tomato compared to the CF treatment. Bulk soil microbial compositions in agroecosystems were significantly affected by the different fertilizer management programs, and the altered bulk soil microbial communities played a crucial role in manipulating rhizospheric soil microflora, which was related with tomato disease. Compared to the CF treatment, OF, AOF and BF had a high efficacy for

suppressing the tomato disease, while BF possessed the highest efficacy. This study provides insights into the soil-borne disease suppression by bulk soil management which can induce healthy rhizospheric soil microflora.

Acknowledgements

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Supplementary data

Table S1 Fertilization scheme in this study.

	Fertilizers	Organic addition (kg/ha)	Inorganic N (kg/ha)	Inorganic P (kg/ha)	Inorganic K (kg/ha)	Total N (kg/ha)	Total P (kg/ha)	Total K (kg/ha)
Basal fertilizer	CF	0	120	65	100	120	65	100
	OF	6000	75	20	28	120	65	100
	AOF	6000	49	20	28	120	65	100
	BF	6000	53	20	28	120	65	100
Supplementary fertilizer	CF	0	105	0	50	105	0	50
	OF	0	105	0	50	105	0	50
	AOF	0	105	0	50	105	0	50
	BF	0	105	0	50	105	0	50

Fertilization management programs: chemical fertilizer (CF), organic fertilizer (OF), amino acids organic fertilizer (AOF) and bio-organic fertilizer (BF).

Table S2 Barcodes of the soil samples used in this study.

Name	Sequence		Sample name
	Bacterial 16S rRNA	Fungal ITS	
barcode	ACACAGT	ACACAGT	BCF-1
barcode	ACAGTCA	ACAGTCA	BCF-2
barcode	ACTCTGA	ACTCTGA	BCF-3
barcode	ACTGACT	ACTGACT	BCF-4
barcode	AGACTCT	AGACTCT	BCF-5
barcode	AGTCACA	AGTCACA	BOF-1
barcode	TCAGAGA	TGACTGA	BOF-2
barcode	TGACTGA	TGAGACT	BOF-3

barcode	TGAGACT	TGTCAGT	BOF-4
barcode	TGTCAGT	TGTGTCA	BOF-5
barcode	TGTGTCA	CAACTGT	BAOF-1
barcode	CAACTGT	CATCAGA	BAOF-2
barcode	CATCAGA	CATGTCT	BAOF-3
barcode	CATGTCT	CACAAGT	BAOF-4
barcode	CACAAGT	CACACTG	BAOF-5
barcode	CACACTG	CACTTGA	BBF-1
barcode	CACTTGA	CACTGAT	BBF-2
barcode	CACTGAT	CAGATCA	BBF-3
barcode	CAGATCA	CAGACAT	BBF-4
barcode	CAGACAT	CAGAGTC	BBF-5
barcode	CAGAGTC	CAGTACT	RCF-1
barcode	CAGTACT	CAGTCTA	RCF-2
barcode	CAGTCTA	CTAGACA	RCF-3
barcode	CTAGACA	CTTGAGT	RCF-4
barcode	CTTGAGT	CTCACGA	RCF-5
barcode	CTCACGA	CTCAGAC	ROF-1
barcode	CTCAGAC	CTCTCAG	ROF-2
barcode	CTCTCAG	CTCTGTA	ROF-3
barcode	CTCTGTA	CTGATGT	ROF-4
barcode	CTGATGT	CTGTAGA	ROF-5
barcode	CTGTAGA	GAACACT	RAOF-1
barcode	GAACACT	GATCTCA	RAOF-2
barcode	GATCTCA	GACATCT	RAOF-3
barcode	GACATCT	GACAGTA	RAOF-4
barcode	GACAGTA	GACTACA	RAOF-5
barcode	GACTACA	GACTCAC	RBF-1
barcode	GACTCAC	GAGTCGT	RBF-2
barcode	GAGTCGT	GTACAGA	RBF-3
barcode	GTACAGA	GTAGTCT	RBF-4
barcode	GTAGTCT	GTAGTCT	RBF-5

Fertilization management programs: chemical fertilizer (CF), organic fertilizer (OF), amino acids organic fertilizer (AOF) and bio-organic fertilizer (BF). Bulk soil amended with chemical fertilizer (BCF), organic fertilizer (BOF), amino acids organic fertilizer (BAOF) and bio-organic fertilizer (BBF). Rhizosphere soil amended with chemical fertilizer (RCF), organic fertilizer (ROF), amino acids organic fertilizer (RAOF) and bio-organic fertilizer (RBF).

Table S3 Retained sequences and OTUs that were used for further analysis after removing short, ambiguous, and low quality reads of soil samples in this study.

Sample	Retained sequences	
	Bacterial 16S rRNA genes **(OTUs 7579)**	Fungal ITS sequences **(OTUs 4517)**
BCF1	57,583	80,969
BCF2	36,743	87,208
BCF3	45,816	86,108
BCF4	43,683	75,822
BCF5	56,247	76,142
BOF1	49,570	68,940
BOF2	50,994	60,572
BOF3	39,432	73,268
BOF4	67,210	71,329
BOF5	64,993	60,399
BAOF1	61,070	73,074
BAOF2	60,392	68,114
BAOF3	55,631	91,711
BAOF4	52,615	96,314
BAOF5	45,327	100,005
BBF1	62,254	97,331
BBF2	56,733	82,786
BBF3	127,938	81,386
BBF4	43,581	78,750
BBF5	55,562	118,707
RCF1	56,822	141,100
RCF2	58,985	133,688
RCF3	58,475	121,243
RCF4	64,900	121,392
RCF5	51,435	103,617
ROF1	49,647	107,858
ROF2	46,547	125,146
ROF3	44,582	114,087
ROF4	44,357	111,252
ROF5	42,785	103,481
RAOF1	41,378	68,899
RAOF2	54,737	93,859
RAOF3	54,483	92,337
RAOF4	59,376	89,929

RAOF5	51,143	92,993
RBF1	56,824	80,785
RBF2	55,438	41,614
RBF3	53,430	59,310
RBF4	41,585	71,301
RBF5	51,310	46,088
Total	**2,171,613**	**3,548,914**

Fertilization management programs: chemical fertilizer (CF), organic fertilizer (OF), amino acids organic fertilizer (AOF) and bio-organic fertilizer (BF). Bulk soil amended with chemical fertilizer (BCF), organic fertilizer (BOF), amino acids organic fertilizer (BAOF) and bio-organic fertilizer (BBF). Rhizosphere soil amended with chemical fertilizer (RCF), organic fertilizer (ROF), amino acids organic fertilizer (RAOF) and bio-organic fertilizer (RBF).

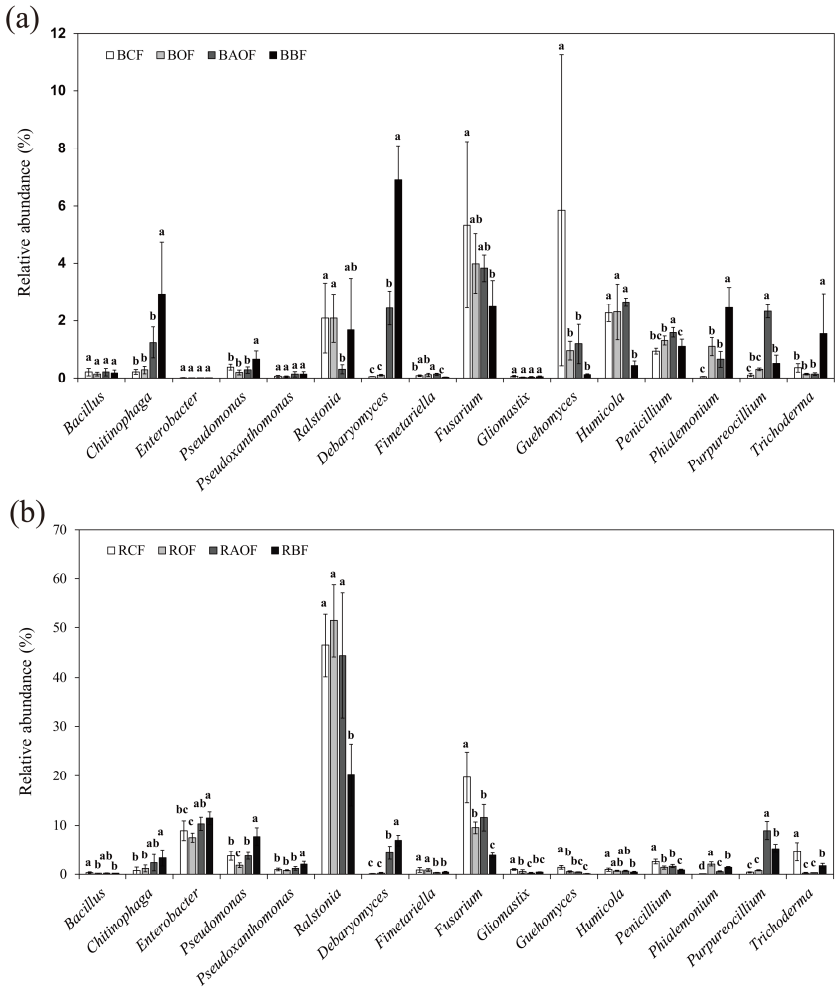

Figure S1 Bulk (A) and rhizospheric (B) relative abundance of the analyzed genera.

Fertilization management programs: chemical fertilizer (CF), organic fertilizer (OF), amino acids organic fertilizer (AOF) and bio-organic fertilizer (BF). Bulk soil amended with chemical fertilizer (BCF), organic fertilizer (BOF), amino acids organic fertilizer (BAOF) and bio-organic fertilizer (BBF). Rhizosphere soil amended with chemical fertilizer (RCF), organic fertilizer (ROF), amino acids organic fertilizer (RAOF) and bio-organic fertilizer (RBF).

Chapter 6

Title: Placing the microbiome in a multitrophic context

Subtitle: Soil protist communities form a dynamic hub in the soil microbiome

https://www.nature.com/articles/ismej2017171

Wu Xiong, Alexandre Jousset, Sai Guo, Ida Karlsson, Qingyun Zhao, Huasong Wu, George A. Kowalchuk, Qirong Shen, Rong Li and Stefan Geisen

Abstract

Soil microbes are essential for soil fertility. However, most studies focus on bacterial and/or fungal communities, while the top-down drivers of this microbiome composition, protists, remain poorly understood. Here, we investigated how soil amendments affect protist communities and inferred potential interactions with bacteria and fungi. Specific fertilization treatments impacted both the structure and function of protist communities. Organic fertilizer amendment strongly reduced the relative abundance of plant pathogenic protists and increased bacterivorous and omnivorous protists. The addition of individual biocontrol bacteria and fungi further altered the soil protist community composition, and eventually function. Network analysis integrating protist, bacterial and fungal community data, placed protists as a central hub in the soil microbiome, linking diverse bacterial and fungal populations. Given their dynamic response to soil management practices and key position in linking soil microbial networks, protists may provide the leverage between soil management and the enhancement of bacterial and fungal microbiota at the service of improved soil health.

Introduction

Protists are an often overlooked component of the soil microbiome. They are abundant and extremely diverse in soil, where they carry out a range of functions (Foissner, 1997; Geisen, 2016a). Protists are among the main consumers of soil bacteria and fungi, but also algae and nematodes (Geisen, 2016a, 2016b; Seppey *et al.*, 2017). Protist activity directly increases plant performance by enhancing the microbial loop (Bonkowski, 2004) and stimulating plant growth-promoting rhizobacteria (Jousset, 2012; Rosenberg *et al.*, 2009). Further, soil protist communities encompass a range of plant- and animal-pathogenic species (Geisen *et al.*, 2015c). Given their functional diversity, protists exert control over various soil organisms and are likely of critical importance for soil fertility. However, we lack an understanding of how protist communities are structured, how targeted soil management can alter protist communities, how such changes might affect protist functioning, and how they are in turn linked to their potential bacterial and fungal prey. Here, we experimentally examined under controlled greenhouse conditions if applications of organic versus conventional fertilizers could modify the taxonomic and functional composition of protists, as well as their putative interactions within the soil microbiome. Furthermore, we added either a bacterial (*Bacillus amyloliquefaciens*) or fungal (*Trichoderma guizhouense*) plant-protective agent (Wang *et al.*, 2013; Zhang *et al.*, 2016) to organic fertiliser (subsequently termed OF+B and OF+T, respectively) to study if these biocontrol agents also impact protist communities (Xiong *et al.*, 2017 and Supplementary Information for additional details).

Results and Discussion

Protist communities differed significantly between all treatments one-year post application. Organic fertilizer-treated soil contained a fundamentally different protist community structure compared with the chemical fertilizer (CF) treatment (UniFrac-weighted and -unweighted PCoA: **Fig. 1A** and **Fig. S2**; RDA: **Fig. S6**), which is in line with former studies (Heger *et al.*, 2012; Murase *et al.*, 2015). Organic fertilizer (OF) amendment enhanced the relative abundance of the most abundant protist taxonomic groups, *i.e.* Stramenopiles, Alveolata, Rhizaria, Excavata and Amoebozoa (**Fig. 1B**), most of which are predators of other microbes. This can be explained by the fact that organic fertilizer provides a wider resource spectrum than chemical fertilizer, which thereby promotes a higher biomass and diversity of bacteria and fungi (Xiong *et al.*, 2017). This impact on primary consumers may foster diverse and active protist consumers, as previously observed in studies of paddy rice (Murase *et al.*, 2015). Organic fertilizer enriched with beneficial microbes (*Bacillus* and *Trichoderma*) caused a further shift in the protist community composition by reducing Stramenopiles, Alveolata and Excavata, and most strongly Rhizaria and Amoebozoa in the OF+B treatment (**Fig. 1B**). As a result, protist richness (observed OTUs), diversity (phylogenetic and Shannon) and evenness (Shannon) were lower in the OF+B and OF+T treatments as compared to the OF treatment (**Fig. S1**). These results suggest that the added microbes differentially affected specific protist taxonomic groups (Jousset *et al.*, 2006; Pedersen *et al.*, 2011), possibly by producing inhibitory compounds. *Bacillus* species are known to produce a broad range of secondary metabolites such as cyclic lipopeptide antibiotics (Alvarez *et al.*, 2012) and various volatile compounds that can deter or even kill potential protist predators (Mazzola *et al.*, 2009; Schulz-Bohm *et al.*, 2017). *Trichoderma* may also have affected protists via the production

of antimicrobial compounds (Cai *et al.*, 2013) or by reducing fungal prey, but information on protist-fungal interactions is too scarce for reliably interpreting these data (Geisen, 2016a). Introduced microbes may also exert indirect effects; the lower diversity of protists and lower relative abundances of Stramenopiles, Alveolata and Excavata in the two microbe-enriched organic fertilizer treatments may be linked to the bacterial genus *Lysobacter*, which increased approximately 6 fold in OF+B and OF+T treatments compared to the CF treatment (Xiong *et al.*, 2017). Members of this genus are known to produce a broad range of bioactive secondary metabolites that can inhibit soil organisms (Gómez Expósito *et al.*, 2015).

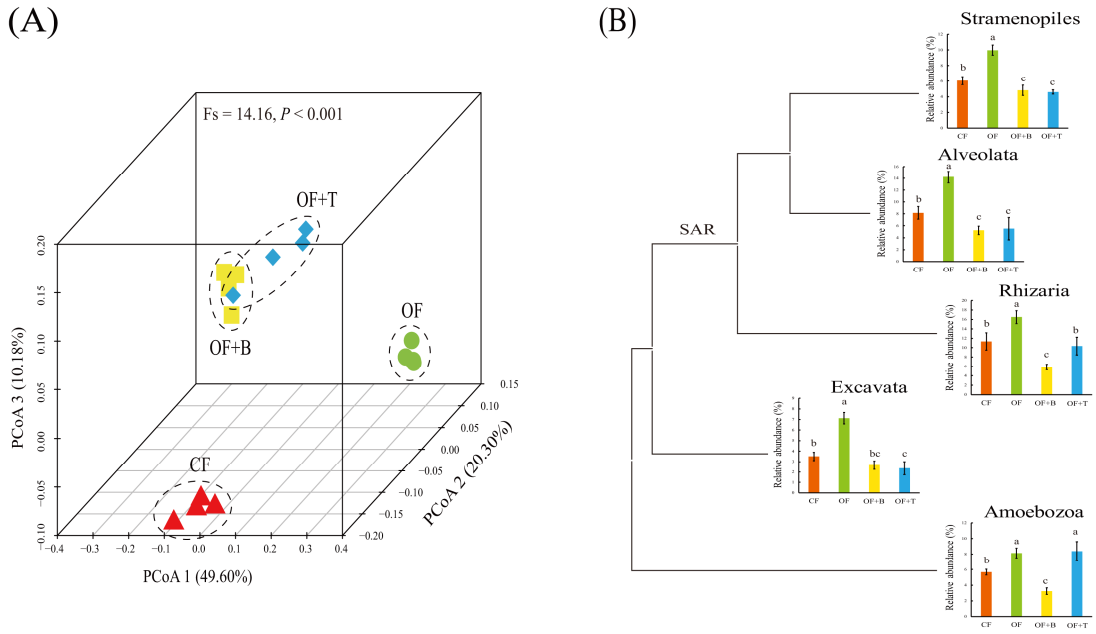

Fig. 1 Protist community changes induced by fertilizations. (A) UniFrac-weighted principle coordinate analysis (PCoA) of soil protist community in the four fertilizer treatments and (B) relative abundance of five main protist taxonomic groups (Stramenopiles, Alveolata, Rhizaria, Excavata and Amoebozoa) with coarse phylogenetic affinities in the four fertilizer treatments.
CF = chemical fertilizer, OF = Organic matter fertilizer, OF+B = *Bacillus* enriched organic fertilizer and OF+T = *Trichoderma* enriched organic fertilizer. Different letters above the bars indicate a significant difference at the 0.05 probability level according to the Tukey's test.

Changes in protist taxonomic community compositions induced by fertilizer regime subsequently resulted in shifts in the relative proportion of protist functional groups **(Fig. 2)**. Several potentially plant-pathogenic *Pythium* spp., a group of widely distributed pathogens of thousands of plant species (Schroeder *et al.*, 2012), were indicative for CF treatment **(Fig. 2B)**. In line, putative protist plant pathogens, including *Pythium* spp., were significantly reduced in all three organic fertilizer treatments (OF, OF+B and OF+T) compared to CF treatment (**Fig. 2A, Table S2**). This may be due either to direct inhibition by the introduced beneficial microbes, the stimulation of antagonistic microbes, or simply the promotion of a range of protists that consume or outcompete *Pythium* in

organic matter. Apicomplexa, known as obligate parasites of vertebrates and invertebrate hosts (Kopečná *et al.*, 2006), were lower in all organic fertilizer treatments as compared to the CF treatment (**Table S2**). These results suggest a relatively "pathogen and parasite" driven food-web in chemical fertilizer-treated soils. In contrast, soils treated with OF were not only higher in saprotrophs, but also in phototrophic algae (**Fig. 2A**), indicative of less disturbed soils and increased soil carbon inputs (Zancan *et al.*, 2006). The indicator taxa for OF were mainly omnivores consuming other eukaryotes (**Fig. 2B**), suggesting a more complex food web. The addition of *Bacillus* into OF, but not of *Trichoderma*, significantly reduced the saprotrophic and phototrophic protists. More targeted studies would be necessary to examine the functional changes in the protist communities after applying different bio-control agents and their long-term impact on soil functioning. It has to be mentioned that we used a conservative approach to assign the classified protists into different functions, focusing merely on feeding mode. As any inference tool, our database approach should be interpreted with caution. It is necessarily reliant on the limited number of characterized reference species (Berney *et al.*, 2017), and may therefore be biased toward specific functions as some elements of functional variation. Current efforts to expand and improve this taxonomic and functional database (Berney *et al.*, 2017) will further improve this tool, and we anticipate that this approach will become more useful in the future to advance studies that seek to more meaningful integrate protists in soil microbiome research.

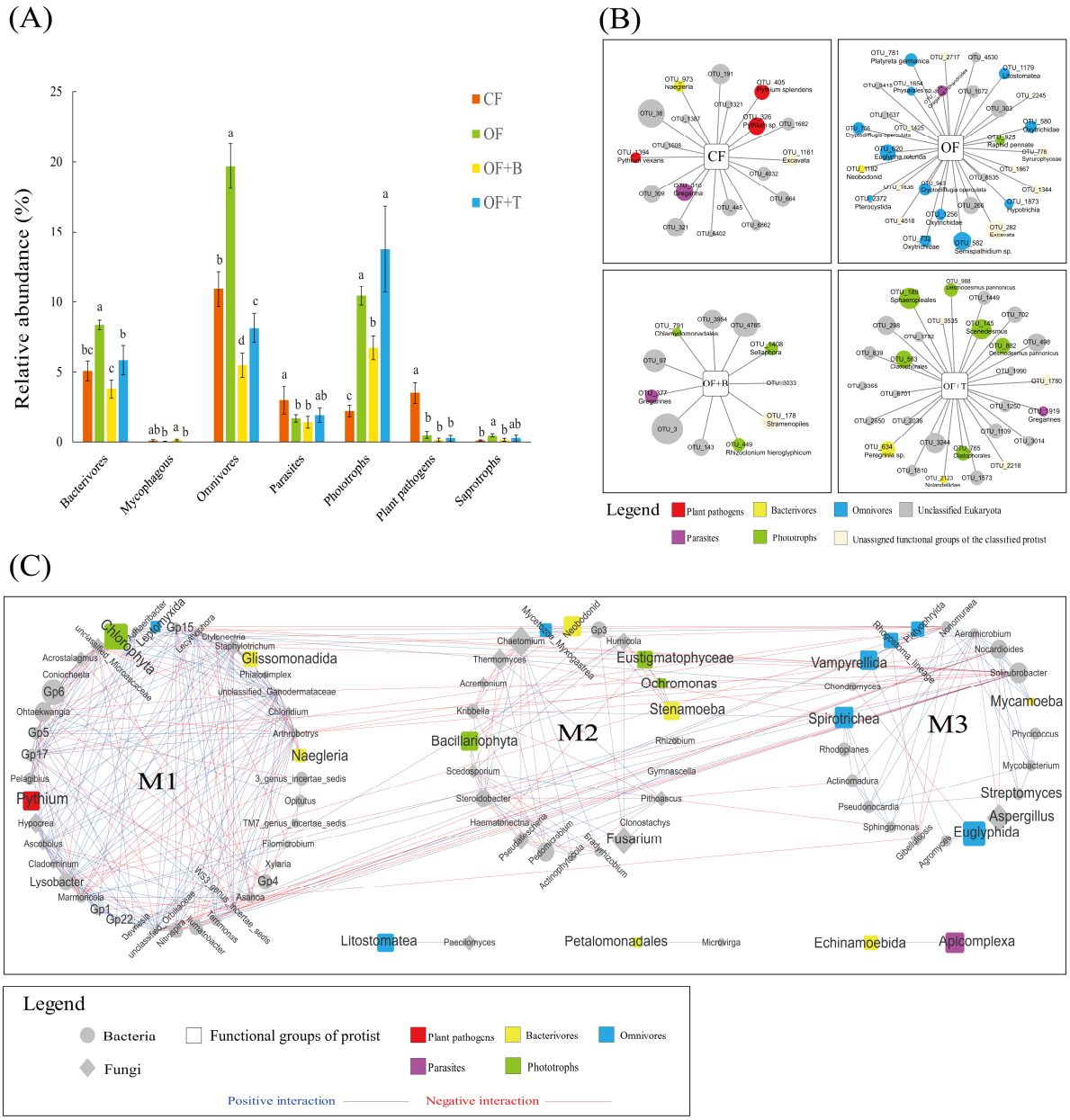

Fig. 2 Protists form a functional and dynamic hub in the soil microbiome induced by fertilizations. (A) Overview of the relative abundance of protist functional groups in the four fertilizer treatments. CF = chemical fertilizer, OF = Organic matter fertilizer, OF+B = *Bacillus* enriched organic fertilizer and OF+T = *Trichoderma* enriched organic fertilizer. Different letters above the bars indicate a significant difference at the 0.05 probability level according to the Tukey's test. **(B) Protist indicator OTUs for the four fertilizer treatments.** Circles represent protist OTUs and circle sizes correspond to their average relative abundance (log transformation) across all the samples. **(C) Correlation-based network analysis showing potential interactions between abundant bacterial and fungal genera as well as protist functional groups.** The node size is proportional to a taxon's average relative abundance (log transformation) across all the samples. Lines connecting nodes (edges) represent positive (blue) or negative (red) co-occurrence relationships.

Finally, we examined the role of protists as an integral part of soil ecological networks. By constructing a combined co-occurrence network encompassing bacteria, fungi and functional groups of protists, we found that protists form distinct hubs in the soil network, linking a range of bacterial and fungal taxa (supplementary information and **Fig. 2C**). Three main modules emerged in our constructed network, with protists present in all modules. Module 1 was dominated by bacteria such as *Lysobacter* and several Acidobacteria groups and contained five protist nodes from distinct taxonomic and functional groups (the mainly plant pathogenic *Pythium*, the bacterivorous Glissomonadida and *Naegleria*). Module 2 was phototroph-dominated (such as Bacillariophyta and Eustigmatophyceae). Module 3 was dominated by omnivorous protists (such as Spirotrichea, Vampyrellida and Euglyphida). Each module appeared to be generally associated with a specific range of functions (Zhou *et al.*, 2011), suggesting interactions between similarly functioning microbes that provide either a stimulatory or inhibitory loop for soil functioning. Parasitic protist taxa, including the potential plant pathogenic *Pythium* and animal parasitic Apicomplexa, were present, but poorly connected to other microorganisms in the network. This disconnected position is likely related to the dependence of these organisms on plants (Xu *et al.*, 2012) and soil animals (Geisen *et al.*, 2015a) rather than other soil microbes.

Our study highlights the multi-trophic nature of the soil microbiome. This study is one of the first to link taxonomically assigned protist taxa to functional groups that are embedded within soil food-webs. Soil amendments strongly impacted protist communities one-year after application, with addition of organic material and beneficial microbes leading to profound changes of protist community composition, and eventually function. This study also serves as a plea to the scientific community to better integrate protists into microbiome studies. Given their large impacts on multiple soil functions, we propose that manipulation of soil protist communities offers new avenues to promote soil health, plant performance and other ecosystem services.

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Supplementary Information

Materials and methods

Experiment description and soil sample collection

To assess the influence of different soil amendments on protist communities, we extracted DNA samples (16 in total: 4 fertilizer amendments * 4 replicates), which were previously used to examine bacterial and fungal communities (see detailed experimental design in Xiong *et al.*, 2017). Briefly, the four fertilizer amendments were: chemical fertilizer (CF), organic fertilizer (OF), two organic fertilizers (regard as OF+B and OF+T) contained approximately 1.0×10^9 and 5.0×10^7 CFU of *Bacillus amyloliquefaciens* and *Trichoderma guizhouense* g^{-1} dry weight of organic fertilizers, respectively (Wang *et al.*, 2013; Zhang *et al.*, 2016). We chose these amendments to induce variation in soil microbial communities, allowing us to examine the importance of various protist groups. We set up a pot experiment using a randomized complete block design with four replicates per treatment. Each replicate had four pots, and each pot contained 12 kg soil with three vanilla cutting seedlings. As for CF treatment, each pot was supplemented with the same NPK contents (*i.e.*, 13.25 g urea, 13.75 g P_2O_5 and 4.10 g K_2O) as OF. Each pot in OF treatment was supplemented with 360 g organic fertilizer, a mixture of an amino acid fertilizer and chicken manure compost in a 1:1 weight ratio (Xiong *et al.*, 2017). After 12 months we removed vanilla plants, and collected 12 random soil cores, which were pooled to yield one composite sample per replicate. For each 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.

Quantification of 18S rRNA gene abundance

We quantified 18S rRNA gene abundance using a eukaryote universal primer combination 1389F (TTGTACACACCGCCC)/1510R (CCTTCYGCAGGTTCACCTAC) (Amaral-Zettler *et al.*, 2009) to target the V9 region of the 18S rRNA gene by quantitative polymerase chain reaction (qPCR). We set up 20 µl reaction mixtures 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_2O. The thermalcyling conditions were set as follows: 30 s at 95 °C for initial denaturation, 40 cycles of 5 s at 95 °C, and 34 s at 60 °C. The standard curve was obtained using a 4-fold dilution series of total DNA of *Saccharomyces cerevisiae*. 18S rRNA gene copy numbers were calculated using the following equation (Whelan *et al.*, 2003): SSU rRNA gene copies = 6.02×10^{23} (copies mol^{-1}) × DNA amount (g) × rDNA copy number / (Genome size (bp) × 660 (g mol^{-1} bp^{-1})). The specificity of the amplification products was confirmed by melting curve analysis and agarose gel electrophoresis. Copy numbers were $\log_{10}$-transformed to normalize the values prior to further statistical analysis.

Illumina MiSeq sequencing

We used the same primer sets (1389F and 1510R) as in the qPCR approach for Illumina MiSeq sequencing. This primer pair was 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 amplification was performed in a 25 µl volume: 5 µl of 5 × reaction buffer, 5 µl of 5 × GC buffer, 2 µl dNTPs (100 mM), 1 µl of each primer (10 uM), high-fidelity DNA polymerase 0.25 µl, 2 µl of DNA template and 8.75 µl of ddH_2O. Amplifications were performed with the following temperature regime: 2 min of initial denaturation at 98 °C, followed by 30 cycles of denaturation (98 °C for 15 s), annealing (55 °C for 30 s), extension (72 °C for 30 s), and a final extension at 72 °C for 5 min. Each sample was amplified in triplicate, after purification with a PCR Purification Kit (Axygen Bio, USA), the PCR products were pooled in equimolar concentrations of 10 ng μl^{-1}. Paired-end sequencing was performed on the Illumina MiSeq sequencer at Personal Biotechnology Co., Ltd (Shanghai, China). All raw sequences data are available in NCBI Sequence Read Archive (SRA) database under the accession number SRP100006.

Bioinformatics and statistical analyses

Raw sequences were processed according to the previously established protocols (Xiong *et al.*, 2016) with some modifications. In brief, sequences with expected errors > 0.5 or a length shorter than 100 bp were removed. After discarding singletons, the remaining reads were assigned to operational taxonomic units (OTUs) with 97% similarity threshold, followed by removal of chimeras using UCHIME (Edgar *et al.*, 2011). Finally, OTUs were matched against the trimmed V9 region (V9_PR2) database (Vargas *et al.*, 2015; Guillou *et al.*, 2012). After removing sequences belonging to bacteria, archaea and unknown organisms, we retained a total of 110,930 sequences for the 16 samples (average 6,933 reads per sample). Thus, an OTU table of eukaryotic sequences was retained. The unclassified eukaryotic sequences in the retained eukaryotic OTU table were further blasted against Silva (v123) (Pruesse *et al.*, 2007) to remove false positive eukaryotic species. Furthermore, we discarded plant (Streptophyta), animal (Metazoa), fungal and unclassified Opisthokonta sequences to generate the retained and conservative eukaryotic OTU table (36,584 sequences with 1,902 OTUs for the 16 samples, average 2,282 reads per sample).

The diversity (α-diversity) of protist community was estimated using the phylogenetic diversity (PD) index (Faith, 1992), number of observed OTUs (richness), and the non-parametric Shannon index. Protist community evenness was defined as the Shannon evenness index. A principal coordinate analysis (PCoA) based on weighted (based on abundances of taxa) and unweighted (sensitive to rare taxa) UniFrac distance metrics (Lozupone *et al.*, 2006) was performed to explore the differences in protist community structures (β-diversity). Analysis of molecular variance (AMOVA) was performed to evaluate significant differences in protist community structure across the four fertilizer treatments. In order to examine protist OTUs that were significantly associated with the four treatments, indicator species analysis (Dufrêne and Legendre, 1997) was conducted using the "labdsv" package of R (version 3.2.2), and visualized with Cytoscape 3.5.1 (Shannon *et al.*, 2003). In order to obtain an equivalent sequencing depth for the above analyses (including α-diversity index, PCoA and indicator species analysis), each sample from the retained eukaryotic OTU table was rarefied to 542 sequences (the lowest number in one of the OF+T replicates). We used the un-rarefied OTU table for all other analyses. To examine the relationships among the four fertilizer samples, functional protist groups (hellinger transformation) and environmental variables (log transformation), redundancy analysis (RDA) was performed using the "vegan" package of R (version 3.2.2), and significance was

tested using the "envfit" function using 999 permutations. One-way analyses of variance (ANOVA) with Tukey's HSD multiple range test were performed in SPSS v20.0 (SPSS Inc., USA) for multiple comparisons.

Network analyses

We used the phylogenetic Molecular Ecological Network (pMEN) to get deeper insights into potential microbial interactions within soil samples obtained from the four fertilizer treatments (Zhou *et al.*, 2010, 2011; Deng *et al.*, 2012). For these analyses, we chose the 60 most abundant bacterial genera, 60 most abundant fungal genera and 65 assigned functional protist taxa (**Table S2**) for network constructions. Similarity matrices were calculated based on Spearman rank correlation. The Random Matrix Theory (RMT) was used to identify the appropriate similarity threshold (St = 0.8) prior to network construction. All analyses were performed using the Molecular Ecological Network Analyses Pipeline (http://ieg2.ou.edu/MENA/main.cgi), and the network graphs were visualized using Cytoscape 3.5.1 (Shannon *et al.*, 2003).

Results

Effect of different soil amendments on 18S rRNA gene abundance, protist community diversity and structure

Soils amended with organic fertilizer (OF), *Bacillus* enriched organic fertilizers (OF+B) and *Trichoderma* enriched organic fertilizers (OF+T) harboured significantly ($P < 0.05$, Tukey's test) higher abundances of eukaryotic 18S rRNA gene copy numbers (about 6.0×10^8 18S rRNA gene copies g^{-1} soil) than soils treated with chemical fertilizer (CF) (4.6×10^8 18S rRNA gene copies g^{-1} soil) (**Fig. S1A**). There were no significant differences in the abundance of 18S rRNA gene copies among the three organic amendment treatments (OF, OF+B and OF+T). High-throughput sequence analysis recovered the highest number of OTUs from the OF treatment (318), while the fewest OTUs were recovered from the OF+B treatment (182) (**Fig. S1B**). OF had the highest phylogenetic diversity, Shannon and evenness indices, while OF+B was lowest for these indices (**Fig. S1C, D** and **E**). When compared with OF, the OF+B and OF+T treatments had significantly ($P < 0.05$, Tukey's test) lower α-diversity indices (phylogenetic diversity, observed OTUs, Shannon and evenness). In addition, PCoA of both weighted- and unweighted-UniFrac distances revealed significant differences in soil protist β-diversity across the four fertilizer treatments (**Fig. 1A** and **Fig. S2**) (Amova analysis: Fs = 14.16, $P <$ 0.001 for weighted; Fs = 1.93, $P < 0.001$ for unweighted, **Table S1**).

Effect of different soil amendments on protist community taxonomic and functional composition

The organic matter input and beneficial microbes significantly modified the taxonomic community composition of soil protists (**Fig. 1B and Fig. S4**). From the 1,902 retained and conservatively assigned eukaryotic OTUs, 57.29% could not be assigned into known protist groups and remained unclassified (**Fig. S3**). Rhizaria (accounting for 25.76% of the assigned protist sequences, i.e. 11.00% of the retained eukaryotic sequences), Alveolata (19.39%; 8.28%), Stramenopiles (14.89%; 6.36%), Amoebozoa (14.87%; 6.35%), Archaeplastida (14.54%; 6.21%) and Excavata (9.15%; 3.91%) (**Fig. S3**), together accounted for 98.60% of the assigned protist community, i.e. 42.12% of the total eukaryotic sequences (the five main and important protist groups: Rhizaria, Stramenopiles, Alveolata,

Amoebozoa and Excavata were presented in **Fig. 1B**). In addition, three rare groups remained (**Fig. S4**): Hacrobia (0.61% of the assigned protist sequences; 0.26% of the retained eukaryotic sequences), Apusozoa (0.40%; 0.17%) and Opisthokonta (0.39%; 0.16%). Compared with chemical fertilizer, OF harboured significantly ($P < 0.05$, Tukey's test) higher relative abundances of Stramenopiles, Alveolata, Rhizaria, Excavata and Amoebozoa. Compared with OF, the addition of beneficial microbes (*Bacillus* and *Trichoderma*) significantly ($P < 0.05$, Tukey's test) reduced relative abundance of Stramenopiles, Alveolata, Excavata and Rhizaria (**Fig. 1B**), with Stramenopiles, Alveolata and Excavata showing no significant ($P > 0.05$, Tukey's test) difference between OF+B and OF+T. When compared to OF, OF+B also exhibited significantly ($P < 0.05$, Tukey's test) lower relative abundance of Amoebozoa, while Amoebozoa stayed constant in OF+T. In addition, OF+T significantly increased ($P < 0.05$, Tukey's test) relative abundance of Archaeplastida as compared to OF, while Archaeplastida stayed constant in OF+B (**Fig. S4**).

In order to better predict potential functions of the different protist communities among the four fertilizers, we conservatively assigned classified protist OTUs into seven functional groups (detailed taxonomic classification of functional protist groups is given in **Table S2**). As shown in **Fig. 2A**, bacterivores (13.48% of the assigned protist OTUs, i.e. 5.76% of the retained eukaryotic community), omnivores (25.88%; 11.06%), phototrophs (19.38%; 8.28%), parasites (4.71%; 2.01%), plant pathogens (2.60%; 1.11%), saprotrophs (0.60%; 0.26%) and mycophagous (0.14%; 0.06%), together accounting for 66.80% of the assigned protist OTUs, i.e. 28.53% of the retained eukaryotic sequences across all samples. Thus, only 33.20% of the taxonomically classified protist sequences could not reliably be assigned into functional groups (**Fig. S5**). Compared with CF, OF significantly ($P < 0.05$, Tukey's test) increased the relative abundance of bacterivores, omnivores, phototrophs and saprotrophs **(Fig. 2A)**, while significantly ($P < 0.05$, Tukey's test) reduced the relative abundance of parasites and plant pathogens. Compared with OF, the addition of beneficial microbes (*Bacillus* and *Trichoderma*) significantly ($P < 0.05$, Tukey's test) reduced relative abundance of bacterivores and omnivores (most strongly in OF+B), while parasites stayed constant in both OF+B and OF+T. In addition, plant pathogens seemed to be even lower in OF+B and OF+F as compared with OF, but these differences were not significant. We also found that with the addition of *Bacillus* in to OF, it significantly ($P < 0.05$, Tukey's test) reduced the relative abundance of saprotrophs and phototrophs, whereas this was not the case with the addition of *Trichoderma*.

Three different potential plant pathogenic protists were identified in our samples: the oomycetes *Pythium* and *Phytophtora*, and the phytomyxean Plasmodiophorida (**Table S2**). Compared to CF treatment, OF and the two beneficial microbe-enriched organic fertilizers (OF+B and OF+T) were significantly ($P < 0.05$, Tukey's test) lower in the relative abundance of the genus *Pythium* (with 8.08% of the classified protist sequences, i.e. 3.45% of the retained eukaryotic sequences in CF; versus less than 0.50% of the retained eukaryotic sequences in both OF, OF+B and OF+T). As compared with OF, *Pythium* seemed to be even lower in OF+B and OF+F, but these differences were not significant. There was no significant difference in relative abundance of *Phytophthora* among the four fertilizer treatments (0.02% of the retained eukaryotic community all samples together), and *Plasmodiophorida*

was only detected in OF (0.21% of the retained eukaryotic community in OF) (**Table S2**). In addition, CF hosted a higher relative abundance of potentially parasitic Apicomplexa than OF, OF+B and OF+F.

Several protist OTUs were selected as being indicative for the different fertilizer regimes (**Fig. 2B, Table S3**). Chemical fertilizer was associated essentially with parasites and pathogens, including three potentially plant pathogenic *Pythium* OTUs (OTU_326, OTU_405 and OTU_1394), one potential parasitic Gregarine (OTU_316) and one bacterivorous Naegleria (OTU_973). The organic fertilizer treatment harboured the highest number of indicator OTUs, most of which belonged to Omnivores (12 taxa), such as *Semispathidium* sp. (OTU_582), *Platyreta germanica* (OTU_781) and *Euglypha rotunda* (OTU_620). The *Bacillus*-enriched organic fertilizer was associated with a low number of OTUs (11), including one parasite, and three belonging to phototrophs. Similarly, the *Trichoderma*-enriched organic fertilizer was associated with a few OTUs encompassing phototrophs, bacterivores, and parasites.

Co-occurrence network between abundant bacterial and fungal genera and protist functional groups

In the final network of the 93 nodes with 21 functionally classified protists, we obtained 3 highly connected modules with over 20 nodes (**Fig. 2C**). In the context of a co-occurrence network, a module refers to a group of microbial nodes that are well connected amongst themselves, but are less linked with the nodes belonging to other modules. Module 1 revealed a module dominated by bacteria (21 out of the 42 nodes belonged to bacteria), such as *Lysobacter* and several Acidobacteria groups, with five protist nodes from distinct taxonomic and functional groups (the mainly plant pathogenic *Pythium*, the bacterivorous Glissomonadida and Naegleria, the omnivorous Leptomyxida and the phototrophic Chlorophyta). Module 2 was phototroph-dominated (3 out of the 6 assigned functional protists belonged to phototrophs, such as Bacillariophyta, Eustigmatophyceae and Ochromonas). In addition to phototrophs, module M2 also contained some free-living protists: two bacterivores (Neobodonid and *Stenamoeba*), and one omnivore (Myxogastrea). Furthermore, module M3 was dominated by omnivorous protists (5 out of the 6 assigned functional protists belonged to omnivores). It contained abundant soil protists from the Ciliophora (such as Spirotrichea) and Rhizaria (Vampyrellida and Euglyphida), as well as several important bacterial and fungal genera such as *Streptomyces* and *Aspergillus*. Throughout the network, *Pythium*, the main potential group of protist phytopathogen in the current context, showed few connections with other nodes, with only one positive and direct link with fungal *Phialosimplex*. Similarly, the parasitic Apicomplexa showed only one positive link with the bacterivorous Echinamoebida. In sum, high-level protist affiliations strongly matched their position in the network, suggesting a phylogenetic conservation of their ecological role.

Effects of soil chemical properties on functional groups of protists

Redundancy analysis (RDA) indicated that soil chemical properties (soil pH, organic matter (OM), available N, P and K contents) were one of the main drivers for the functional composition of protists, explaining 59.91% of the total variation (**Fig. S6**). CF was separated from OF, OF+B and OF+T by the first component (RDA1: explaining 36.32% of the total variation), and OF was separated from OF+T and OF+B by the second component (RDA2: explaining 11.54% of the total variation).

Parasites and plant pathogens were highly present in CF and were more related with soil pH and negatively related with organic matter and available N. Moreover, phototrophs were more linked to soil available N. RDA results inferred that soil chemical properties play very important roles in reshaping functional groups of protists.

Supplementary Tables

Table S1 Amova analysis based on the weighted- and unweighted-UniFrac distances for the four fertilizer soil samples.

Groups	Weighted		Unweighted	
	F_{Score}	*P*-value	F_{Score}	*P*-value
CF vs OF vs OF+B vs OF+T	14.16	< 0.001	1.93	< 0.001
CF vs OF	14.96	0.032	1.96	0.012
CF vs OF+B	13.83	0.033	1.71	0.007
CF vs OF+T	6.60	0.021	1.95	0.008
OF vs OF+B	27.23	0.023	1.94	0.024
OF vs OF+T	8.09	0.012	2.06	0.020
OF+B vs OF+T	16.92	0.028	1.99	0.032

CF = chemical fertilizer, OF = Organic matter fertilizer, OF+B = *Bacillus* enriched organic fertilizer and OF+T = *Trichoderma* enriched organic fertilizer.

Table S2 The relative abundance of each taxon from the functional groups of protist in the four fertilizer soil samples.

Functional groups	Taxon	CF	OF	OF+B	OF+T
Bacterivores	Naegleria	0.79±0.26 a	0.09±0.02 b	0.09±0.04 b	0.03±0.07 b
	Mycamoeba	0.11±0.02 a	0.14±0.04 a	0.01±0.02 b	0.00±0.00 b
	Cyrtolophosidida	0.26±0.16 a	0.41±0.10 a	0.14±0.05 a	0.29±0.22 a
	Nassophorea	0.04±0.05 a	0.41±0.41 a	0.01±0.01 a	0.02±0.03 a
	Oligohymenophorea	0.00±0.00 a	0.04±0.03 a	0.04±0.01 a	0.16±0.18 a
	Phyllopharyngea	0.00±0.00 a	0.03±0.03 a	0.00±0.00 a	0.00±0.00 a
	ATCC50593-Flamella-WIM80-lineage	0.26±0.05 a	0.18±0.07 a	0.14±0.06 a	0.42±0.28 a
	Filamoebidae	0.05±0.04 a	0.12±0.02 a	0.04±0.01 a	0.19±0.19 a
	Vexilliferidae	0.00±0.00 a	0.02±0.02 a	0.00±0.00 a	0.00±0.00 a
	Stenamoeba	0.36±0.12 b	1.37±0.46 a	0.37±0.13 b	0.97±0.20 a
	Echinamoebida	0.57±0.32 a	0.47±0.20 a	0.24±0.08 a	0.57±0.39 a
	Nolandida	0.15±0.03 ab	0.15±0.05 b	0.04±0.01 b	0.35±0.18 a
	Apusozoa	0.20±0.06 a	0.20±0.05 a	0.12±0.07 a	0.17±0.08 a
	Euglenales	0.00±0.00 a	0.00±0.00 a	0.01±0.01 a	0.00±0.00 a

	Petalomonadales	0.02±0.03 a	0.25±0.08 a	0.14±0.05 ab	0.12±0.16 ab
	Eubodonid	0.00±0.00 b	0.09±0.03 a	0.04±0.03 b	0.00±0.00 b
	Neobodonid	0.72±0.22 bc	2.39±0.26 a	1.09±0.25 b	0.54±0.27 c
	Gruberellidae	0.00±0.00 a	0.02±0.03 a	0.00±0.00 a	0.00±0.00 a
	Paravahlkampfia	0.00±0.00 a	0.05±0.05 a	0.01±0.01 a	0.00±0.00 a
	Choanoflagellida	0.02±0.03 a	0.05±0.04 a	0.02±0.01 a	0.05±0.07 a
	Nucleariidea	0.11±0.16 a	0.11±0.06 a	0.12±0.07 a	0.03±0.07 a
	Limnofilida	0.05±0.07 a	0.18±0.14 a	0.11±0.02 a	0.16±0.12 a
	Spongomonadida	0.03±0.03 a	0.03±0.02 a	0.01±0.02 a	0.00±0.00 a
	Thaumatomonadida	0.21±0.18 ab	0.58±0.21 ab	0.17±0.10 b	0.90±0.62 a
	Glissomonadida	0.84±0.30 a	0.52±0.16 ab	0.31±0.07 b	0.22±0.13 b
	Tremulida	0.00±0.00 a	0.02±0.04 a	0.00±0.00 a	0.00±0.00 a
	Bicoecea	0.18±0.09 a	0.30±0.22 a	0.12±0.09 a	0.44±0.37 a
	Spumella	0.04±0.05 a	0.04±0.05 a	0.02±0.02 a	0.02±0.03 a
	MAST	0.06±0.09 a	0.12±0.10 a	0.38±0.14 a	0.18±0.27 a
Mycophagous	Grossglockneriidae	0.09±0.07 ab	0.02±0.03 b	0.13±0.06 a	0.00±0.00 b
Omnivores	Spirotrichea	1.28±0.42 b	3.95±0.98 a	1.26±0.16 b	0.28±0.20 b
	Rhogostoma-lineage	0.65±0.26 ab	1.08±0.56 a	0.23±0.15 b	0.17±0.17 b
	Platyophryida	0.30±0.03 b	0.95±0.16 a	0.12±0.06 b	0.14±0.11 b
	Heterotrichea	0.00±0.00 a	0.00±0.00 a	0.02±0.01 a	0.05±0.10 a
	Litostomatea	0.77±0.25 b	2.20±0.10 a	0.52±0.20 b	0.69±0.43 b
	Mycetozoa-Dictyostelea	0.01±0.03 a	0.02±0.05 a	0.00±0.00 a	0.00±0.00 a
	Mycetozoa-Myxogastrea	0.17±0.02 b	0.67±0.18 a	0.20±0.09 b	0.10±0.08 b
	Acramoebidae	0.08±0.08 a	0.02±0.02 a	0.02±0.03 a	0.00±0.00 a
	Paramoebidae	0.10±0.06 a	0.11±0.12 a	0.10±0.05 a	0.07±0.13 a
	Dermamoebida	0.02±0.05 a	0.04±0.01 a	0.00±0.00 a	0.00±0.00 a
	Arcellinida	0.41±0.22 b	0.86±0.15 a	0.20±0.10 b	0.41±0.11 b
	Glaeseria	0.02±0.03 a	0.03±0.03 a	0.01±0.01 a	0.00±0.00 a
	Leptomyxida	0.29±0.08 a	0.17±0.11 ab	0.03±0.01 b	0.14±0.16 ab
	Centroheliozoa	0.25±0.06 a	0.42±0.27 a	0.18±0.05 a	0.20±0.15 a
	Vampyrellida	0.93±0.32 b	2.05±0.23 a	0.53±0.14 b	0.82±0.27 b
	Euglyphida	5.38±0.85 ab	6.85±0.94 a	1.89±0.58 c	4.83±0.91 b
	Protaspa-lineage	0.08±0.03 a	0.06±0.04 a	0.06±0.03 a	0.07±0.14 a
	Tectofilosida	0.01±0.02 a	0.00±0.00 a	0.01±0.01 a	0.00±0.00 a
	Allogromiidae	0.18±0.14 a	0.21±0.12 a	0.10±0.08 a	0.16±0.18 a
Parasites	Apicomplexa	2.83±1.00 a	1.37±0.35 b	1.21±0.40 b	1.66±0.68 ab
	Prokinetoplastidae	0.06±0.05 a	0.12±0.06 a	0.13±0.07 a	0.15±0.11 a
	Metamonada	0.04±0.07 a	0.14±0.08 a	0.01±0.01 a	0.05±0.09 a
	Ichthyosporea	0.00±0.00 b	0.05±0.04 ab	0.08±0.04 a	0.00±0.00 b
	Haptoglossa	0.06±0.09 a	0.02±0.02 a	0.01±0.01 a	0.07±0.13 a
Phototrophs	Chlorophyta	1.49±0.47 c	6.36±0.36 b	5.40±0.68 b	11.27±3.01 a

	Rhodophyta	0.00±0.00 a	0.04±0.05 a	0.00±0.00 a	0.02±0.03 a
	Bacillariophyta	0.35±0.18 c	2.23±0.33 a	1.00±0.18 bc	1.11±0.59 b
	Ochromonas	0.00±0.00 b	0.34±0.15 a	0.02±0.01 b	0.17±0.12 ab
	Poterioochromonas	0.17±0.07 a	0.19±0.17 a	0.09±0.03 a	0.03±0.07 a
	Eustigmatophyceae	0.21±0.16 b	1.30±0.55 a	0.18±0.06 b	1.15±0.41 a
Plant pathogens	Plasmodiophorida	0.00±0.00 b	0.21±0.16 a	0.00±0.00 b	0.00±0.00 b
	Phytophthora	0.05±0.05 a	0.00±0.00 a	0.00±0.00 a	0.05±0.09 a
	Pythium	3.45±0.80 a	0.31±0.17 b	0.15±0.12 b	0.23±0.19 b
Saprotrophs	Rhizidiomyces	0.00±0.00 a	0.08±0.08 a	0.01±0.01 a	0.00±0.00 a
	Labyrinthulea	0.10±0.04 a	0.42±0.19 a	0.14±0.12 a	0.28±0.25 a

CF = chemical fertilizer, OF = Organic matter fertilizer, OF+B = *Bacillus* enriched organic fertilizer and OF+T = *Trichoderma* enriched organic fertilizer. Values are means ± standard deviation (n = 3). Different letters above the bars indicate a significant difference at the 0.05 probability level according to the Tukey's test.

Table S3 Taxonomic affiliation of the protist indicator species for the four fertilizer treatments.

Fertilizer regime	OTU ID*	Functional groups	Taxonomic affiliation
CF	OTU_191 (0.94)	—	Eukaryota;unclassified;unclassified;unclassified;unclassified;unclassified;unclassified;unclassified
	OTU_973 (0.16)	Bacterivores	Eukaryota;Excavata;Discoba;Heterolobosea;Heterolobosea_X;Vahlkampfiidae;Naegleria;unclassified
	OTU_405 (0.34)	Plant pathogens	Eukaryota;Stramenopiles;Stramenopiles_X;Oomyceta;Oomyceta_X;Oomyceta_XX;Pythium;Pythium+splendens
	OTU_326 (0.51)	Plant pathogens	Eukaryota;Stramenopiles;Stramenopiles_X;Oomyceta;Oomyceta_X;Oomyceta_XX;Pythium;unclassified
	OTU_1321 (0.07)	—	Eukaryota;unclassified;unclassified;unclassified;unclassified;unclassified;unclassified;unclassified
	OTU_316 (0.58)	Parasites	Eukaryota;Alveolata;Apicomplexa;Apicomplexa_X;Gregarines;Gregarines_X;Gregarina;unclassified
	OTU_6402 (0.06)	—	Eukaryota;unclassified;unclassified;unclassified;unclassified;unclassified;unclassified;unclassified
	OTU_445 (0.44)	—	Eukaryota;unclassified;unclassified;unclassified;unclassified;unclassified;unclassified;unclassified
	OTU_1387 (0.08)	—	Eukaryota;unclassified;unclassified;unclassified;unclassified;unclassified;unclassified;unclassified
	OTU_1682 (0.19)	—	Eukaryota;unclassified;unclassified;unclassified;unclassified;unclassified;unclassified;unclassified
	OTU_309 (0.59)	—	Eukaryota;unclassified;unclassified;unclassified;unclassified;unclassified;unclassified;unclassified
	OTU_1161 (0.17)	—	Eukaryota;Excavata;unclassified;unclassified;unclassified;unclassified;unclassified;unclassified
	OTU_321 (0.76)	—	Eukaryota;unclassified;unclassified;unclassified;unclassified;unclassified;unclassified;unclassified
	OTU_1608 (0.06)	—	Eukaryota;unclassified;unclassified;unclassified;unclassified;unclassified;unclassified;unclassified
	OTU_664 (0.32)	—	Eukaryota;unclassified;unclassified;unclassified;unclassified;unclassified;unclassified;unclassified
	OTU_6862 (0.13)	—	Eukaryota;unclassified;unclassified;unclassified;unclassified;unclassified;unclassified;unclassified
	OTU_38 (5.63)	—	Eukaryota;unclassified;unclassified;unclassified;unclassified;unclassified;unclassified;unclassified
	OTU_1394 (0.11)	Plant pathogens	Eukaryota;Stramenopiles;Stramenopiles_X;Oomyceta;Oomyceta_X;Oomyceta_XX;Pythium;Pythium+vexans
	OTU_4032 (0.25)	—	Eukaryota;unclassified;unclassified;unclassified;unclassified;unclassified;unclassified;unclassified
OF	OTU_580 (0.15)	Omnivores	Eukaryota;Alveolata;Ciliophora;Spirotrichea;Hypotrichia;Oxytrichidae;unclassified;unclassified
	OTU_1425 (0.05)	—	Eukaryota;Rhizaria;Foraminifera;Rotaliida;unclassified;unclassified;unclassified;unclassified
	OTU_732 (0.14)	Omnivores	Eukaryota;Alveolata;Ciliophora;Spirotrichea;Hypotrichia;Oxytrichidae;unclassified;unclassified
	OTU_925 (0.09)	Phototrophs	Eukaryota;Stramenopiles;Stramenopiles_X;Bacillariophyta;Bacillariophyta_X;Raphid-pennate;unclassified;unclassified
	OTU_1637 (0.06)	—	Eukaryota;unclassified;unclassified;unclassified;unclassified;unclassified;unclassified;unclassified
	OTU_2245 (0.03)	—	Eukaryota;Rhizaria;Cercozoa;unclassified;unclassified;unclassified;unclassified;unclassified
	OTU_1838 (0.07)	—	Eukaryota;Alveolata;Ciliophora;Colpodea;Colpodida;unclassified;unclassified;unclassified
	OTU_1654 (0.07)	Omnivores	Eukaryota;Amoebozoa;Conosa;Mycetozoa-Myxogastrea;Stemonitales-Physarales;Stemonitales-Physarales_X;Stemonitales-Physarales_XX;Stemonitales-Physarales_XX+sp.
	OTU_2372 (0.04)	Omnivores	Eukaryota;Hacrobia;Centroheliozoa;Centroheliozoa_X;Pterocystida;unclassified;unclassified;unclassified
	OTU_4518 (0.05)	—	Eukaryota;Stramenopiles;Stramenopiles_X;Chrysophyceae-Synurophyceae;unclassified;unclassified;unclassified;unclassified
	OTU_6535 (0.06)	—	Eukaryota;unclassified;unclassified;unclassified;unclassified;unclassified;unclassified;unclassified
	OTU_266 (0.43)	—	Eukaryota;unclassified;unclassified;unclassified;unclassified;unclassified;unclassified;unclassified
	OTU_1873 (0.08)	Omnivores	Eukaryota;Alveolata;Ciliophora;Spirotrichea;Hypotrichia;unclassified;unclassified;unclassified
	OTU_2717 (0.08)	—	Eukaryota;Alveolata;Ciliophora;Colpodea;Colpodida;unclassified;unclassified;unclassified
	OTU_1179 (0.12)	Omnivores	Eukaryota;Alveolata;Ciliophora;Litostomatea;unclassified;unclassified;unclassified;unclassified
	OTU_1675 (0.10)	Parasites	Eukaryota;Alveolata;Apicomplexa;Apicomplexa_X;Gregarines;Gregarines_X;Gregarina;Gregarina+niphandrodes
	OTU_3415 (0.07)	—	Eukaryota;unclassified;unclassified;unclassified;unclassified;unclassified;unclassified;unclassified
	OTU_4530 (0.10)	—	Eukaryota;unclassified;unclassified;unclassified;unclassified;unclassified;unclassified;unclassified
	OTU_1256 (0.12)	Omnivores	Eukaryota;Alveolata;Ciliophora;Spirotrichea;Hypotrichia;Oxytrichidae;unclassified;unclassified

	OTU_1344 (0.11)	—	Eukaryota;Excavata;Discoba;Euglenozoa;Kinetoplastida;unclassified;unclassified;unclassified
	OTU_1182 (0.06)	Bacterivores	Eukaryota;Excavata;Discoba;Euglenozoa;Kinetoplastida;Neobodonid;unclassified;unclassified
	OTU_756 (0.15)	Omnivores	Eukaryota;Amoebozoa;Lobosa;Tubulinea;Arcellinida;Arcellinida_X;Cryptodifflugia;Cryptodifflugia+operculata
	OTU_781 (0.18)	Omnivores	Eukaryota;Rhizaria;Cercozoa;Endomyxa;Vampyrellida;Leptophryidae;Platyreta;Platyreta+germanica
	OTU_1867 (0.07)	—	Eukaryota;Stramenopiles;Stramenopiles_X;Chrysophyceae-Synurophyceae;Chrysophyceae-Synurophyceae_X;Clade-C;unclassified;unclassified
	OTU_778 (0.14)	—	Eukaryota;Stramenopiles;Stramenopiles_X;Chrysophyceae-Synurophyceae;Chrysophyceae-Synurophyceae_X;unclassified;unclassified;unclassified
	OTU_582 (0.59)	Omnivores	Eukaryota;Alveolata;Ciliophora;Litostomatea;Haptoria;Spathidiidae;Semispathidium;Semispathidium+sp.
	OTU_620 (0.36)	Omnivores	Eukaryota;Rhizaria;Cercozoa;Filosa-Imbricatea;Euglyphida;Euglyphidae;Euglypha;Euglypha+rotunda
	OTU_943 (0.16)	Omnivores	Eukaryota;Amoebozoa;Lobosa;Tubulinea;Arcellinida;Arcellinida_X;Cryptodifflugia;Cryptodifflugia+operculata
	OTU_282 (0.85)	—	Eukaryota;Excavata;unclassified;unclassified;unclassified;unclassified;unclassified;unclassified
	OTU_1072 (0.17)	—	Eukaryota;unclassified;unclassified;unclassified;unclassified;unclassified;unclassified;unclassified
	OTU_303 (0.48)	—	Eukaryota;unclassified;unclassified;unclassified;unclassified;unclassified;unclassified;unclassified
OF+B	OTU_377 (0.20)	Parasites	Eukaryota;Alveolata;Apicomplexa;Apicomplexa_X;Gregarines;Gregarines_X;Gregarina;unclassified
	OTU_791 (0.09)	Phototrophs	Eukaryota;Archaeplastida;Chlorophyta;Chlorophyceae;Chlorophyceae_X;CW-Chlamydomonadales;unclassified;unclassified
	OTU_143 (0.48)	—	Eukaryota;unclassified;unclassified;unclassified;unclassified;unclassified;unclassified;unclassified
	OTU_4785 (2.90)	—	Eukaryota;unclassified;unclassified;unclassified;unclassified;unclassified;unclassified;unclassified
	OTU_178 (0.48)	—	Eukaryota;Stramenopiles;Stramenopiles_X;unclassified;unclassified;unclassified;unclassified;unclassified
	OTU_3 (11.83)	—	Eukaryota;unclassified;unclassified;unclassified;unclassified;unclassified;unclassified;unclassified
	OTU_3954 (0.85)	—	Eukaryota;unclassified;unclassified;unclassified;unclassified;unclassified;unclassified;unclassified
	OTU_449 (0.18)	Phototrophs	Eukaryota;Archaeplastida;Chlorophyta;Ulvophyceae;Cladophorales;Cladophorales_X;Rhizoclonium;Rhizoclonium+hieroglyphicum
	OTU_3033 (0.03)	—	Eukaryota;unclassified;unclassified;unclassified;unclassified;unclassified;unclassified;unclassified
	OTU_1408 (0.18)	Phototrophs	Eukaryota;Stramenopiles;Stramenopiles_X;Bacillariophyta;Bacillariophyta_X;Raphid-pennate;Sellaphora;unclassified
	OTU_97 (1.63)	—	Eukaryota;unclassified;unclassified;unclassified;unclassified;unclassified;unclassified;unclassified
OF+T	OTU_882 (0.30)	Phototrophs	Eukaryota;Archaeplastida;Chlorophyta;Chlorophyceae;Chlorophyceae_X;Sphaeropleales;Desmodesmus;Desmodesmus+pannonicus
	OTU_988 (0.22)	Phototrophs	Eukaryota;Archaeplastida;Chlorophyta;Chlorophyceae;Chlorophyceae_X;Sphaeropleales;Desmodesmus;Desmodesmus+pannonicus
	OTU_1792 (0.11)	—	Eukaryota;unclassified;unclassified;unclassified;unclassified;unclassified;unclassified;unclassified
	OTU_1810 (0.12)	—	Eukaryota;unclassified;unclassified;unclassified;unclassified;unclassified;unclassified;unclassified
	OTU_1250 (0.13)	—	Eukaryota;unclassified;unclassified;unclassified;unclassified;unclassified;unclassified;unclassified
	OTU_839 (0.19)	—	Eukaryota;unclassified;unclassified;unclassified;unclassified;unclassified;unclassified;unclassified
	OTU_2036 (0.08)	—	Eukaryota;unclassified;unclassified;unclassified;unclassified;unclassified;unclassified;unclassified
	OTU_1990 (0.07)	—	Eukaryota;unclassified;unclassified;unclassified;unclassified;unclassified;unclassified;unclassified
	OTU_3365 (0.08)	—	Eukaryota;unclassified;unclassified;unclassified;unclassified;unclassified;unclassified;unclassified
	OTU_2123 (0.05)	Bacterivores	Eukaryota;Amoebozoa;Lobosa;Tubulinea;Nolandida;Nolandellidae;unclassified;unclassified
	OTU_498 (0.56)	—	Eukaryota;unclassified;unclassified;unclassified;unclassified;unclassified;unclassified;unclassified
	OTU_1919 (0.08)	Parasites	Eukaryota;Alveolata;Apicomplexa;Apicomplexa_X;Gregarines;Gregarines_X;unclassified;unclassified
	OTU_2218 (0.09)	—	Eukaryota;Amoebozoa;unclassified;unclassified;unclassified;unclassified;unclassified;unclassified
	OTU_3535 (0.05)	—	Eukaryota;Amoebozoa;unclassified;unclassified;unclassified;unclassified;unclassified;unclassified
	OTU_2650 (0.08)	—	Eukaryota;unclassified;unclassified;unclassified;unclassified;unclassified;unclassified;unclassified
	OTU_1109 (0.28)	—	Eukaryota;unclassified;unclassified;unclassified;unclassified;unclassified;unclassified;unclassified
	OTU_583 (0.39)	Phototrophs	Eukaryota;Archaeplastida;Chlorophyta;Ulvophyceae;Cladophorales;Cladophorales_X;unclassified;unclassified
	OTU_3014 (0.10)	—	Eukaryota;unclassified;unclassified;unclassified;unclassified;unclassified;unclassified;unclassified

OTU_634 (0.29)	Bacterivores	Eukaryota;Rhizaria;Cercozoa;Filosa-Imbricatea;Thaumatomonadida;Peregriniidae;Peregrinia;Peregrinia+sp.
OTU_1573 (0.22)	—	Eukaryota;unclassified;unclassified;unclassified;unclassified;unclassified;unclassified;unclassified
OTU_6701 (0.05)	—	Eukaryota;unclassified;unclassified;unclassified;unclassified;unclassified;unclassified;unclassified
OTU_3244 (0.87)	—	Eukaryota;unclassified;unclassified;unclassified;unclassified;unclassified;unclassified;unclassified
OTU_702 (0.34)	—	Eukaryota;unclassified;unclassified;unclassified;unclassified;unclassified;unclassified;unclassified
OTU_765 (0.22)	Phototrophs	Eukaryota;Archaeplastida;Chlorophyta;Ulvophyceae;Cladophorales;Cladophorales_X;unclassified;unclassified
OTU_298 (0.61)	—	Eukaryota;unclassified;unclassified;unclassified;unclassified;unclassified;unclassified;unclassified
OTU_189 (0.95)	Phototrophs	Eukaryota;Archaeplastida;Chlorophyta;Chlorophyceae;Chlorophyceae_X;Sphaeropleales;unclassified;unclassified
OTU_1449 (0.20)	—	Eukaryota;unclassified;unclassified;unclassified;unclassified;unclassified;unclassified;unclassified
OTU_1780 (0.19)	—	Eukaryota;Amoebozoa;Lobosa;Lobosa_X;Centramoebida;Acanthamoebidae;Protacanthamoeba;Protacanthamoeba+bohemica
OTU_145 (0.87)	Phototrophs	Eukaryota;Archaeplastida;Chlorophyta;Chlorophyceae;Chlorophyceae_X;Sphaeropleales;Scenedesmus;unclassified

CF = chemical fertilizer, OF = Organic matter fertilizer, OF+B = *Bacillus* enriched organic fertilizer and OF+T = *Trichoderma* enriched organic fertilizer. * OTU ID behind with the relative abundance (%) across all the samples.

Supplementary Figures

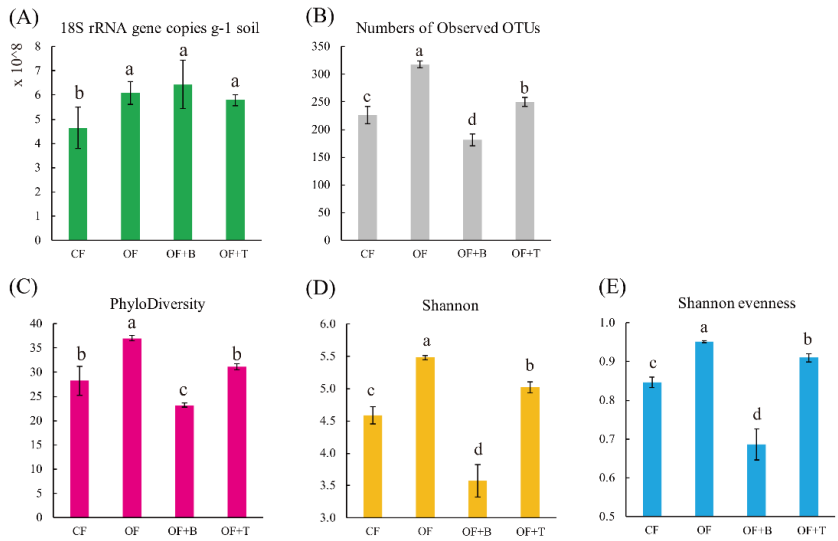

Fig. S1 18S rRNA gene abundance (A) and α-diversity indexes of protist (B, C, D and E) for the four fertilizers.

CF = chemical fertilizer, OF = Organic matter fertilizer, OF+B = *Bacillus* enriched organic fertilizer and OF+T = *Trichoderma* enriched organic fertilizer. "PhyloDiversity" means phylogenetic diversity. Different letters above the bars indicate a significant difference at the 0.05 probability level according to the Tukey's test.

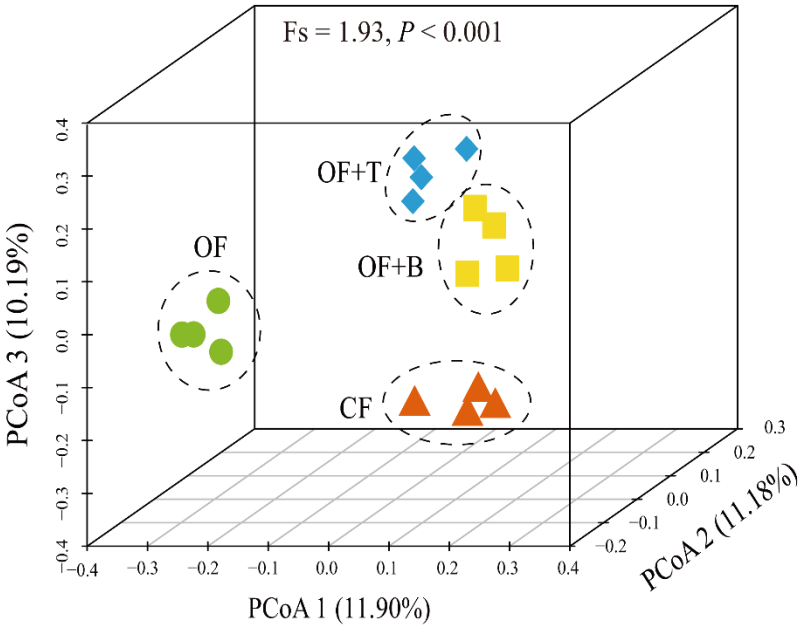

Fig. S2 UniFrac-unweighted principle coordinate analysis of soil protists community structures in the four fertilizer treatments.

CF = chemical fertilizer, OF = Organic matter fertilizer, OF+B = *Bacillus* enriched organic fertilizer and OF+T = *Trichoderma* enriched organic fertilizer.

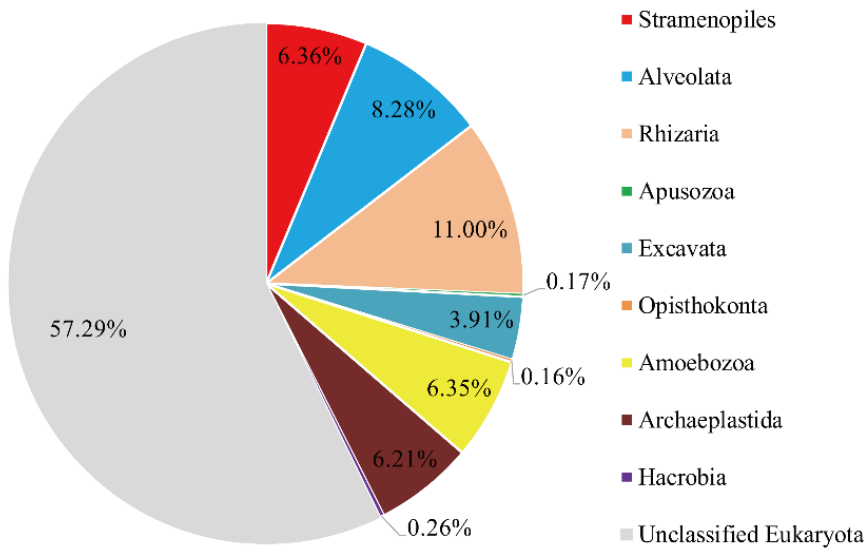

Fig. S3 Overall distribution of phylogenetic composition of protist community.

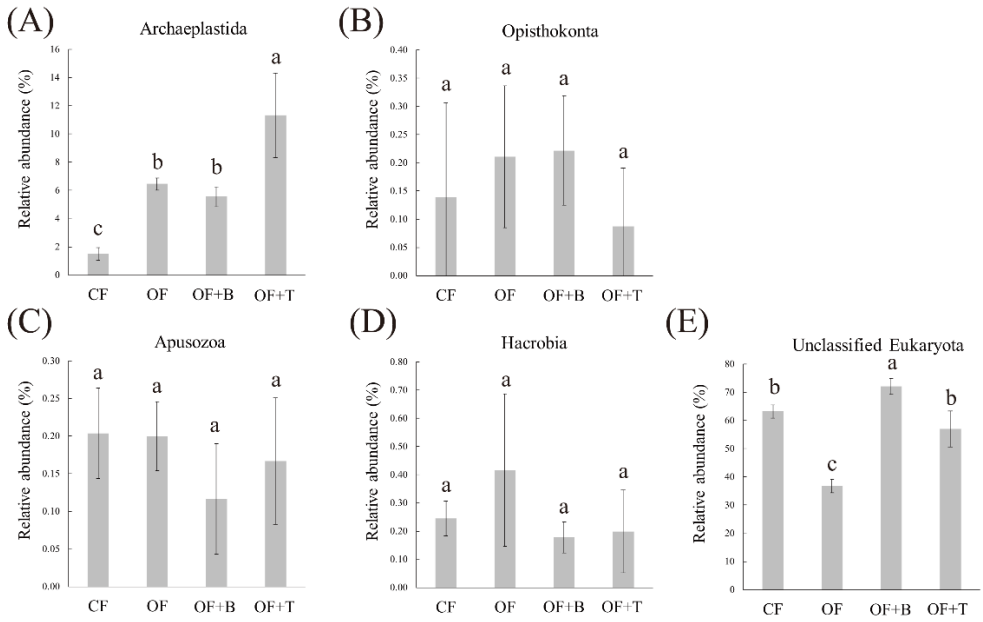

Fig. S4 Relative abundance of Archaeplastida (A), Opisthokonta (B), Apusozoa (C), Hacrobia (D) and unclassified eukaryota (E) in the four fertilizers.

CF = chemical fertilizer, OF = Organic matter fertilizer, OF+B = *Bacillus* enriched organic fertilizer and OF+T = *Trichoderma* enriched organic fertilizer. Different letters above the bars indicate a significant difference at the 0.05 probability level according to the Tukey's test.

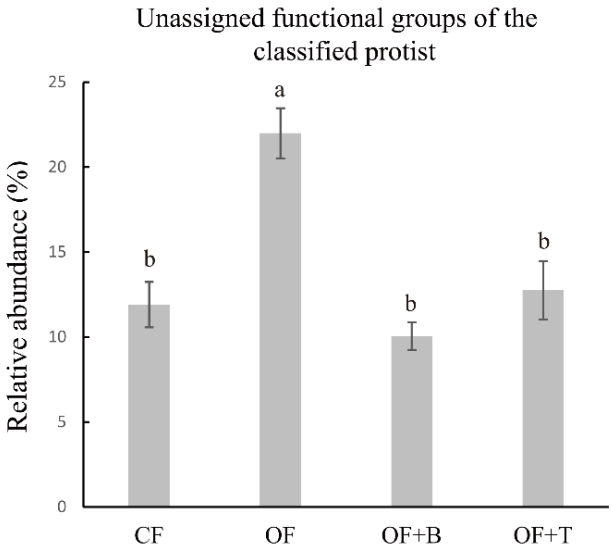

Fig. S5 Unassigned functional groups of the classified protist.

CF = chemical fertilizer, OF = Organic matter fertilizer, OF+B = *Bacillus* enriched organic fertilizer and OF+T = *Trichoderma* enriched organic fertilizer. Different letters above the bars indicate a significant difference at the 0.05 probability level according to the Tukey's test.

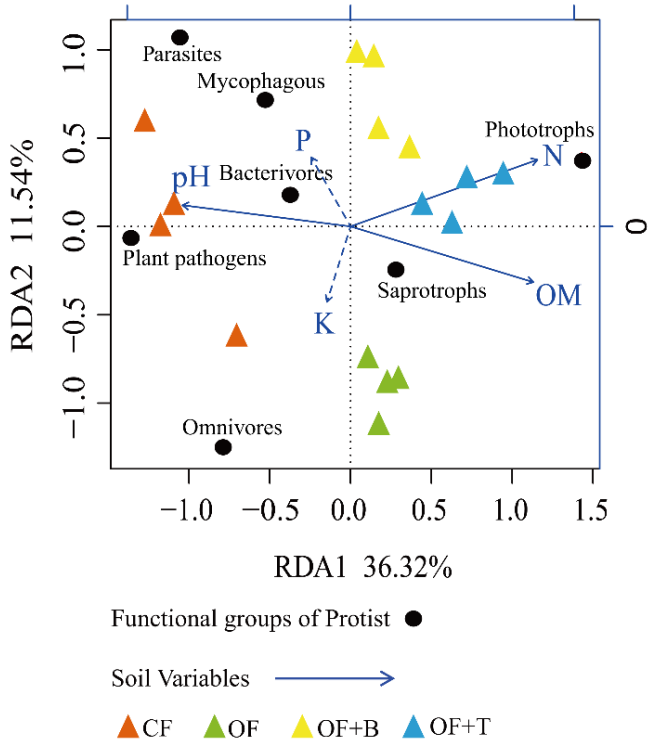

Fig. S6 Redundancy analysis (RDA) of the protist functional groups with soil variables for individual samples from the four fertilizers.

CF = chemical fertilizer, OF = Organic matter fertilizer, OF+B = *Bacillus* enriched organic fertilizer and OF+T = *Trichoderma* enriched organic fertilizer. Soil variables with a significance level of < 0.05 (solid blue line) and no significance ($P > 0.05$, solid blue dash line).

Chapter 7

Summarizing Discussion

Wu Xiong

In this thesis, I first sought to pinpoint which microbiome characteristics that are important for Fusarium wilt disease suppression. I further assessed how different agricultural management strategies (including crop rotation and bio-organic fertilizer) can help enhance microbiome functionality associated with disease suppression. To this end, I highlighted the importance of protists as key players in the soil network and show their importance in determining soil functionality. Together, these results will form the basis of more informed management strategies aiming to specifically improve soil fertility and disease suppressive potential without the use of pesticides.

Microbial communities contribute to the suppression of soil-borne plant disease

Naturally disease suppressive soil is an excellent example of natural microbe-based plant defense, and the study of such soils can help reveal the fundamental mechanisms controlling soil-borne disease and the impact of soil management on this development of disease suppression. Disease suppressive soil is defined as a soil in which disease does not develop despite the presence of the pathogen and a susceptible host (Schlatter *et al.*, 2017). In order to truly speak of a disease suppressive soil, it is first necessary to establish that the disease suppression is attributable to microbial activities, as opposed to simply the physicochemical properties of the soil. In order to show this, most studies rely on soil pasteurization. If the soil microbial community is responsible for suppressiveness, soil pasteurization should result in a loss of suppressive capability. Furthermore, the suppressiveness should be at least partially restorable by adding non-sterilized suppressive soil to the sterilized soil. As an example, when the *Rhizoctonia*-suppressive soil of sugar beet was heat treated at 80°C, disease suppressiveness was almost completely lost (Mendes *et al.*, 2011). Pasteurized Fusarium disease suppressive soil (80 °C, 1 h) lost wilt suppressiveness after one cycle of strawberry, and gamma-irradiation could completely eliminate Fusarium disease suppressiveness (Cha *et al.*, 2016). In this thesis (**Chapter 2**), transferring the same proportion of heat-treated vanilla Fusarium suppressive soil into a conducive soil resulted in a disease incidence similar to that observed for the disease-conducive soil. Together, these various experiments all indicate that soil microbial communities, rather than physicochemical properties, are responsible for disease suppression. In other words, the disease suppressiveness ability can be transferable between soils, which is one of the important characteristics of specific disease suppressive soils (Weller *et al.*, 2002; Schlatter *et al.*, 2017).

Identification of microbial communities in disease-suppressive soil is an important first step toward understanding the potential impacts of these communities on plant health (Rosenzweig *et al.*, 2012) and is a necessary first step to develop an informed management to control of soil-borne diseases (Mazzola 2004). In **Chapter 2**, I first assessed which components of the soil microbiome are most relevant for disease suppression. I compared fungal and bacterial communities from adjacent fields with soils that share similar chemical properties but vary in their ability to suppress Fusarium wilt disease in vanilla. Since the initiation of vanilla cultivation more than 20 years ago, these two orchards diverged in terms of the incidence of Fusarium wilt disease and the density of *Fusarium oxysporum*, thereby yielding one disease-conducive and one disease-suppressive soil. I found that *Fusarium oxysporum* absolute abundance differed by only a factor of less than two between the conducive and suppressive soils, suggesting that the pathogen abundance in bulk soil is not the key driver of disease

incidence. In other words, the pathogen is clearly present in the suppressive soil, but is kept in check and does not cause appreciable damage to the crop (establishes but causes little or no damage). I therefore hypothesized that full manifestation of the disease is impeded by the actions of the resident microbial community in the suppressive soil. I therefore sought to assess which microbial species were associated with the low disease incidence.

One of the main findings of this work was that fungal communities discriminate between conducive and suppressive soils far better than bacterial communities. Specifically, the Zygomecete genus *Mortierella* was hyper-dominant in the vanilla Fusarium wilt disease-suppressive soil, accounting for almost 40% of the total fungal sequences. *Mortierella* species are commonly found in soil environments and have repeatedly been reported to produce antibiotics. Some isolates have even been investigated as potential antagonistic agents against various soil-borne pathogens (Tagawa *et al.*, 2010; Wills and Lambe, 1980). I therefore propose that soil suppressiveness developed together with the increase of *Mortierella* populations, which may have either directly inhibited *Fusarium* pathogen or outcompeted it via resource competition. I also found that the black pepper-vanilla crop rotation system harbored a higher relative abundance of *Mortierella*, which again coincided with lower *Fusarium* wilt disease in comparison with the vanilla monoculture system (**Chapter 3**). Together, these results suggest that this taxon may be a key player of Fusarium wilt disease suppression in vanilla cropping systems. Although not reported in the vanilla suppressive soils, some putatively plant-beneficial fungal groups, such as the genera *Trichoderma* and *Penicillium*, may also play an important role in Fusarium wilt disease suppression in the vanilla rhizosphere soil under the black pepper-vanilla system (**Chapter 3**). *Trichoderma* spp. are known to have an effective antagonistic effect against vanilla Fusarium wilt disease (Jayasekhar *et al.*, 2008; Vijayan *et al.*, 2009), and a strain from this genus was chosen as a bio-control agent in subsequent experiments investigating bio-organic fertilizer impacts on disease and soil-borne microbial communities (**Chapter 4**). *Penicillium* is also well-known for its biological control of Fusarium wilt disease (Larena *et al.*, 2003), although this has not yet been tested in vanilla systems. I therefore propose that further studies on suppressive soils should put a stronger emphasis on both fungi and bacteria, and not restrict the focus purely to bacteria as has commonly been done (Mendes *et al.*, 2011; Rosenzweig *et al.*, 2012).

The bacterial phyla Actinobacteria and Firmicutes were more abundant in vanilla *Fusarium* wilt disease-suppressive soil (**Chapter 2**). Mendes *et al.* (2011) found higher abundances of Actinobacteria and Firmicutes in *Rhizoctonia*-suppressive soil of sugar beet, these two phyla are known to contain species that produce high levels of secondary metabolites (Kim *et al.*, 2011; Palaniyandi *et al.*, 2013) that can inhibit certain plant pathogens. The genus *Lysobacter*, which is known to produce a wide range of extracellular enzymes and other metabolites with antagonistic activities against many soil-borne diseases (Gómez Expósito *et al.*, 2015; Jochum *et al.*, 2006), was six times higher in the two bio-fertilizer treatments as compared to the chemical fertilizer treatment, and showed a significantly negative relationship with *F. oxysporum* pathogen abundance (**Chapter 4**). *Lysobacter* has previously been shown to be more abundant in potato common scab disease-suppressive soil as compared to conducive soil (Rosenzweig *et al.*, 2012). It should be noted that there are many other important disease suppressive agents (or bio-control agents) that have been associated with or isolated from

disease-suppressive soils. Although not specifically highlighted in my study, especially strains of *Pseudomonas* spp. and *Bacillus* spp. (Chung *et al.*, 2008; Wong and Baker, 1984) have been demonstrated to be efficient root colonizers with direct antagonistic effects on plant pathogens via the action of lytic enzymes and antibiotics (Santhanam *et al.*, 2015). It is worth noting that, combined with the high-throughput technologies, future studies are needed to identify and isolate potential bio-control agents such as *Mortierella* spp. and *Lysobacter* spp. with effective disease suppression ability against *Fusarium* pathogens. This would help to unlock the functional mechanisms involved in Fusarium wilt disease suppression and open new avenues for the development of informed bio-control strategies against Fusarium wilt disease.

Microbial interactions play an important role in plant disease suppression. In network analyses, the suppressive soil studied in this thesis exhibited a higher number of positive co-occurrence relationships and higher connectivity than the conducive soil for both fungal and bacterial networks. Such network complexity and connectedness may provide resistance (pathogen endurance ability) to the disturbance up to a critical threshold (Scheffer *et al.*, 2012). Similarly, interactions between arbuscular mycorrhizal fungi and bacteria were highlighted as critical aims for the sustainable management of soil fertility in a previous review analysis (Johansson *et al.*, 2004). Microbial co-occurrence networks of conventionally- and organically-managed agricultural systems have also been shown to contain highly disparate microbe–microbe interaction patterns, and several of the highly connected taxa in such networks represent important plant-associated microorganisms, indicating that these taxa can be indicators of plant health (Hartmann *et al.*, 2015; van der Heijden and Hartmann, 2016). This suggests that agriculture management practices can shift soil microbial networks in relation to plant health.

It is becoming increasingly clear that disease suppression is often a complicated phenomenon that is in most cases involves numerous microbial interactions within complex microbial consortia (Mendes *et al.*, 2011). A field trial with over 900 plants in two field plots revealed that inoculation with a mixture of native bacterial isolates significantly reduced disease incidence of tobacco sudden-wilt (Santhanam *et al.*, 2015). Similarly, a review paper by Compant *et al.* (2010) argued that the combination of multiple biocontrol strains can provide better disease control than application of a single organism. Others have also shown that inoculation of multiple strains can improve disease suppression. Wei *et al.* (2015) examined how the resource competition networks of resident bacterial communities affect invasion resistance to a plant pathogen. Their results showed that microbial communities with a combination of stabilizing configurations (low nestedness and high connectance) and a clear niche overlap with the pathogen most greatly reduced pathogen invasion success and constrained pathogen growth. A priority for future studies will be to examine and understand the mechanisms by which microbial consortia, and the microbial interactions within them, relate to the Fusarium wilt disease suppression.

Developing disease-suppressive soil via soil management

Crop rotation improves disease suppressiveness

Long-term monoculture of some high economic value crops often results in serious outbreaks of soil-borne diseases, resulting in large yield declines (Li *et al.*, 2014b; Lu *et al.*, 2013). Indeed, continuous cropping has been abandoned across large areas in China, for instance for continuous cropping of black pepper or banana, because it has proven impossible to sufficiently control disease outbreaks in such systems (Wang et al., 2013; Xiong et al., 2015). It has been known for centuries that crop rotation can have positive effects in agriculture. In this thesis, I looked into the mechanisms involved in the disease suppression by crop rotation, thereby giving new insight into which rotations might be helpful and why. In **Chapter 3**, my results revealed that the black pepper-vanilla rotation significantly controlled vanilla Fusarium wilt disease (Xiong *et al.*, 2016). Several colleagues have likewise found that a pineapple-banana rotation can also significantly reduce banana Fusarium wilt disease by modulating fungal communities (Wang *et al.*, 2015a). Such examples indicate that proper utilization of strategic crop rotation practices can help control soil-borne Fusarium wilt disease in agriculture systems, offering a very environmentally friendly means of protecting crop production.

Crop rotation can disrupt the accumulation of *Fusarium* pathogens: the black pepper-vanilla system showed significantly lower *Fusarium* and *F. oxysporum* abundance in the vanilla rhizosphere and bulk soil. The most important reasons for significantly lower *F. oxysporum* pathogen abundance in the black pepper-vanilla system are: 1) presence of a non-host plant for the survival of *F. oxysporum* pathogen: continuous cropping black pepper soil had not previously been used to cultivate vanilla; 2) direct suppression against the pathogen by the residue piperine from decomposing black pepper in the soil (Pietro *et al.*, 2003); and 3) indirect suppression by increased antagonistic soil microbial activity, which was induced by the residues or exudates of black pepper. Thus, the possible mechanisms involved in the disease suppressive effects of this crop rotation can be summarized as follows: non-host plant for the specific pathogen, pathogen inhibition due to plant residues and stimulation of soil microbial activity. However, the research presented here also showed that *F. oxysporum* populations significantly accumulated from the bulk soil to the vanilla rhizosphere soil in both the black pepper-vanilla and vanilla monoculture systems. Thus, I propose that iterative crop rotation might be necessary to interrupt the accumulation of *F. oxysporum* abundance to suppress Fusarium wilt disease. However, it should be noted that crop rotation systems can be highly labor-intensive or time-consuming at the field scale, and such practices are especially difficult for perennial plants such as vanilla, apple and black pepper. The use of appropriate crop rotations in integrated land use management, in which both economic and environmental impacts are taken into account (Schönhart *et al.*, 2011), will help dictate the optimally selection of suitable crop rotation strategies to promote soil health.

Bio-organic fertilizer use improves disease suppressiveness

The application of bio-organic fertilizers has the potential to be one of the most beneficial soil management practices for agricultural systems given its ability to reduce reliance on chemical fertilizers and hazardous pesticides. As such, bio-organic fertilizers can play an important role in helping to meet the growing world food demand while reducing the environmental impacts of agriculture. Bio-organic fertilizer treatments have proven to be highly efficient for the control of various soil-borne plant diseases (Fu *et al.*, 2017; Qiu *et al.*, 2012). Bio-organic fertilizers represent a complex mixture of organic materials combined with beneficial microbes, making it rather difficult to disentangle the underlying mechanisms of disease suppression. In examining the impact of bio-organic fertilizers on disease control, it is important to understand that the organic materials of the fertilizer, the inoculated beneficial microbes, or indirect effects on the resident soil microbial communities all represent potential paths to improved disease suppression and plant growth. I will therefore examine the impacts of each of these factors in more details below:

Question 1: How do organic amendments contribute to disease suppression?

In the pot experiment carried out in **Chapter 4**, no significant differences in disease incidence were found between the chemical and organic fertilizer treatments. This suggests that organic fertilizer alone was not sufficient to induce soil suppressiveness against Fusarium wilt disease in our vanilla continuous cropping soil. In this bio-organic fertilizer experiment, soil was collected from a continuously cropped vanilla field exhibiting serious Fusarium wilt disease. The field had been abandoned for over six months without vanilla cropping prior to soil collection. The resting spores of *Fusarium oxysporum* pathogen in vanilla-abandoned soil (*Fusarium oxysporum* spores can survive for long periods in the absence of the plant host) can develop rapidly when the soil amended with sources of organic matter and nutrition. This is one of the possible reasons why organic fertilizer treated soil showed the same level of disease incidence as observed for the chemical fertilizer treatment.

The contents of organic materials and the processes involved in organic matter decomposition in soil are complex. A meta-analysis by Bonanomi *et al*. (2010), who investigated the influence of organic matter decomposition on disease suppressiveness, showed that organic compost can induce a range of responses in the development of disease suppression, from yielding highly suppressive soils, to having little effect or even making the soil more conducive to other pathogens. It is thus difficult to provide a general consensus with respect to the impact of organic compost on disease suppression, especially if the disease (pathogen) is already present in the soil, as is typically the case for continuously cropped vanilla fields. It is, however, possible to improve the effectively of organic fertilizer treatments by combining its application with other management strategies. For instance, Li *et al.* (2016) reported a novel soil fumigation strategy with ammonium bicarbonate, the soil is first fumigated to kill the residual pathogen and then amended with organic compost. This practice efficiently controls cucumber Fusarium wilt disease by reshaping the soil microbial community structure. This may represent a promising soil management strategy for making organic matter applications more effective in reducing disease severity.

Question 2: What is the contribution of the inoculated beneficial microbes to disease suppression?

Many previous studies have demonstrated the direct suppression mechanism by beneficial microbes such as *Pseudomonas* (Weller, 2007), *Bacillus* (Chung *et al.*, 2008) and *Trichoderma* (El_Komy *et al.*, 2015). The widely recognized mechanisms of such biocontrol agents are 1) competition for an ecological niche, nutrients or growth substrates, 2) production of inhibitory compounds or enzymes, and 3) induction of systemic resistance (ISR) in host plants to a broad spectrum of pathogens (Compant *et al.*, 2005). Based on their potential ability to suppress soil-borne disease under laboratory conditions (Wang *et al.*, 2013; Zhang *et al.*, 2016), *Bacillus* and *Trichoderma* are often used as bio-control agents in bio-organic fertilizers used in diverse agricultural systems.

In **Chapter 4**, after one-year of bio-organic fertilizer application, *Bacillus* was significantly higher in the "*Bacillus*-enriched bio-organic fertilizer" treatment as compared to the organic fertilizer alone, and *Trichoderma* was also higher in the "*Trichoderma*-enriched bio-organic fertilizer" treatment as compared to the organic fertilizer treatment, although this result was not significant. Linear regression analyses revealed that *Trichoderma* density was negatively correlated with *F. oxysporum* abundance, but this was not the case for *Bacillus*. These results suggest that the introduced beneficial microbes (*Bacillus* and *Trichoderma* spp.) had only a limited survival capacity in our study system one year after application and that their abundance had only a marginal direct effect on the *F. oxysporum* pathogen. An alternative explanation is that the introduced microbes survive well and constrain the pathogen in the early stages after introduction, but later decrease in numbers. This would lead to suppression of Fusarium wilt disease, but without a correlation between the inoculated strain density after one year and the level of disease suppression. Future experiments that track the densities of the bio-control agents and pathogen would provide useful information related to the importance of direct control of the pathogen by the biocontrol agents in the bio-organic fertilizers. An additional advantage of using bio-organic fertilizers, as opposed to direct application of the biocontrol agent, may be the increased nutrients made available to the inoculated strain, which can enhance survival over time. However, the interaction between the delivery substrate and the survival of biocontrol strains still requires further investigation. Likewise, there is still limited understanding concerning how biocontrol agents survive and interact with the pathogen as nutrition levels in the soil decline. Yang *et al.* (2017) reported that resident species with a high catabolic similarity with the pathogen efficiently reduced the relative density of the pathogen at low resource availability, while fast-growing resident species became more important for the pathogen suppression at high resource availability. Similarly, non-antagonistic soil bacteria showed efficient antagonism against the plant pathogenic fungi *Fusarium culmorum* and *Rhizoctonia solani* on a nutrient-poor agar medium (de Boer *et al.*, 2007). This represents an important research topic related to the effective and predictable use of bio-control agents for controlling plant disease.

Question 3: How do changes in the resident soil community contribute to disease suppression?

In this thesis, I highlighted changes in the resident microbial communities in soil after bio-fertilizer application and showed that such changes had a more important impact on disease suppression than the direct effects of the applied bio-control agents. Increased disease suppression was

associated with increased bacterial abundance, restructuring of the microbial community, and increases in specific microbial taxa with potential antagonistic activities, most notably *Lysobacter*. Our colleagues have also shown similar results when comparing the application of matured pig manure compost versus *Bacillus amyloliquefaciens*-enriched bio-fertilizers for the control of banana Fusarium wilt disease (Fu *et al.*, 2017). Not only did the three years of bio-fertilizer treatment reduce disease incidence, it also increased bacterial biomass and richness, as well as the abundances of *Sphingobium*, *Dyadobacter* and *Cryptococcus* in the banana rhizosphere. Here again, it may be changes in the composition of the native microbial community upon bio-fertilizer application that mediate Fusarium wilt disease suppression.

It is also interesting to note that bio-fertilizer application often had the greatest impact on relatively rare species (please see the PCoA results of the two type bio-fertilizers based upon weighted and un-weighted UniFrac distances in **Chapter 4**). Thus, stimulation of minor microbial populations in soil after bio-fertilizer application can lead to important impacts on disease suppression. Rare species can have an over-proportional role in soil functioning (Jousset *et al.*, 2017). For example, rare species of plant-associated microbes can affect plant productivity and promote the production of antagonistic volatile compounds helping the plant to fend off pathogens (Hol *et al.*, 2015). Revealing the mechanisms by which relatively rare microbes impact disease suppression, especially in the context of amendment with bio-organic fertilizers, remains a future research priority. Nonetheless, recent advances offer new prospects for the development of strategies to manipulate soil communities by the application of bio-fertilizers to improve the suppression of soil-borne diseases.

Management of bulk soil microbial communities can translate to impacts in the rhizosphere

The rhizosphere is defined as the zone around the root, where microorganisms and processes are important for plant growth and health (Bakker *et al.*, 2013; Hinsinger and Marschner, 2006). Manipulation of the plant rhizosphere microbiome could offer an effective means at improving control of soil-borne plant diseases. However, it is often difficult to manipulate rhizosphere microbiome directly as the plant roots develops in the field soil. Fortunately, the rhizosphere microbial community was usually selected from the bulk soil in both natural or agricultural systems (Mendes et al., 2014; de Ridder-Duine et al., 2005). Thus, the work in this thesis proposes that one might best manipulate the rhizosphere microbiome by first manipulating the bulk soil microbial community. In an interesting experiment, Bakker *et al.* (2015) first used defined exogenous chemical amendments to develop disparate bulk soil microbial communities and then allowed for rhizosphere development for two different cultivars of corn. They observed differences in the microbiome responses to different resource amendments, and more importantly, showed that legacy effects of prior selection on microbiomes may continue to influence rhizosphere microbial community structure.

The work in this thesis relies on manipulation of the bulk soil community, which then translates into ultimate effects of plant disease suppression by the rhizosphere microbiome. In **Chapter 3**, I found that the black pepper-vanilla rotation significantly controlled vanilla Fusarium wilt disease by increasing potential plant beneficial microbes such as *Trichoderma* and *Penicillium* in the vanilla rhizosphere from bulk soil (Xiong *et al.*, 2016). In **Chapter 5**, it is shown that continuous application

of different organic amendments can suppress tomato wilt disease by inducing the healthy rhizosphere microbiota through alterations of the bulk soil microflora (Liu *et al.*, 2017). Such findings improve our knowledge regarding how soil management strategies impact general soil communities and how this translates into more effective rhizosphere microbiomes for the control of soil-borne plant diseases.

Soil protists contribute to disease suppressiveness

Protists can directly increase plant performance by enhancing nutrient turnover, serving as the drivers of the microbial loop (Bonkowski, 2004) and stimulating plant growth-promoting rhizobacteria (Jousset, 2012; Rosenberg *et al.*, 2009). Soil protists represent a keystone group, exerting control over various soil organisms. As important predators of bacteria and fungi (Jousset, 2017), protists may be of critical importance for soil disease suppression. Protists therefore may provide new perspectives for managing plant-associated microbial communities to enhance their functionality and their ability to support plant productivity and heath in agricultural systems. In this section, I discuss how soil protists could be involved in promoting plant growth and plant health via multi-trophic interactions.

It is important to note that, soil protists can also act as pathogen themselves. Given their taxonomic and functional diversity, soil protist communities also contain a range of plant- and animal-pathogenic species (Geisen *et al.*, 2015c). For example, *Pythium*, a genus of parasitic oomycotes, is a group of widely distributed pathogens effecting thousands of plant species (Schroeder *et al.*, 2012). In this thesis, it was shown that *Pythium* was significantly decreased in the organic fertilizer and two bio-fertilizer treatments, potentially due to either direct inhibition by introduced beneficial microbes, the stimulation of antagonistic microbes such as *Lysobacter*, or the promotion of bacterivorous and omnivorous protists in the presence of organic matter. In addition, Apicomplexa, obligate parasites of vertebrates and invertebrate hosts (Geisen *et al.*, 2015a), were also lower in the organic fertilizer treatment and the two bio-fertilizer treatments. This effect may also be linked by the differences in soil pH (Dupont *et al.*, 2016) as a result of the different fertilizer applications, and this possibility was confirmed by the RDA analysis. This suggests that soil animals might be more infected by parasites in the chemical fertilizer-treated soils, which might lead to a less functional soil food web (Olsen, 1986). These results suggest a relatively “pathogen and parasite” driven food-web in chemical fertilizer-treated soils, whereas the organic fertilizer systems may promote a more “bacterivorous and omnivorous” protist soil food-web.

Protists can also directly inhibit pathogens, for instance by preying on them. Compared to chemical treatments, organic treatments showed higher relative abundance of bacterivorous and omnivorous protists, with lower relative abundances of plant pathogens, suggesting that the bacterivores and omnivores might consume plant pathogens directly. Protists have traditionally been regarded chiefly as bacterivores and therefore principally involved in the bacterial energy channel of the soil food web. This soil biologist’s perspective has, however, recently been challenged by taxonomic and functional studies showing that a range of protists feed on fungi, other protists and even nematodes (Geisen, 2016a). For instance, Geisen and his colleagues found that a flagellate of the *Cercomonas* genus and some naked amoebae genera can feed or grow on some yeasts and the spores of the plant pathogenic fungus *Fusarium culmorum* (Geisen *et al.*, 2016). Geisen and his colleagues

further revealed that the soil-borne testate amoeba *Cryptodifflugia operculata* had a highly specialized and effective pack-hunting strategy to prey on bacterivorous nematodes (Geisen *et al.*, 2015b). Thus, it is logical to speculate that soil protists may have the potential ability to control soil-borne pathogens such as fungi *Fusarium* sp. and perhaps even plant parasitic nematodes.

Protists also play in integral role within ecological networks in soil. In **Chapter 6**, through building up a combined network encompassing bacteria, fungi and protists, I showed that these microorganism groups, which were typically considered separately, were indeed strongly interconnected with each other. Network analysis placed protists as a central hub in the soil microbiome, linking diverse bacterial and fungal communities. Moreover, these co-occurrence networks exhibited different main modules that were each apparently associated with different functions. For example, module M1 might best be described from a functional point of view and being a "pathogen-suppressive" module. It contained *Lysobacter*, a bacterial genus known for strong antifungal activities that has been proposed to support plant health (Gómez Expósito *et al.*, 2015; Jochum *et al.*, 2006) and which was significantly induced by bio-fertilizers. This module also contained the Acidobacteria groups *Gp6* and *Gp4*, which have also been implicated in disease suppression (Rosenzweig *et al.*, 2012). At the protist level, it contained Glissomonads, a group of small flagellates reported for their bacterivorous activity (Howe *et al.*, 2011). This antagonistic module may be involved in the suppression of plant-pathogenic *Pythium*, with only one connection in the whole network within module one.

At this stage, the underlying mechanisms linking soil amendments and protist community structure remain largely unknown, and we can only speculate whether changes in abiotic factors or bacterial and fungal communities have led to the observed differences. This study is thus a plea to the scientific community to better integrate protists into microbiome studies. Given their large impacts on multiple soil functions, I propose that manipulation of soil protist communities opens up new avenues to promote soil health, plant performance and other ecosystem services. In reality, the real food-web in soil is even more complex than the interaction between bacteria, fungi and protists. It also includes complex assemblages of viruses, nematodes and micro-arthropods, as well the visible small vertebrates, insects, and earthworms (Geisen, 2016a; Geisen *et al.*, 2016). In this light, I hope that this proof-of-concept may stimulate a multi-trophic perspective to research seeking to improve our understanding of soil functionality, especially as related to the suppression of plant diseases (Soliveres *et al.*, 2016).

Conclusions

In this thesis, I first highlight that microbial communities play an important role in the suppression of soil-borne plant diseases. Most notably, especially the dominant fungal *Mortierella* populations and highly connected fungal networks in the naturally Fusarium wilt disease suppressive soil were important biological components of disease suppression. Then, I addressed different strategies of disease suppression to control Fusarium wilt disease including crop rotation and bio-organic fertilizers inoculated with beneficial microbes, with both approaches proving to be efficient and environmentally friendly agriculture managements. I propose that the reconstructed microbial communities affected by these soil managements are directly responsible for the disease suppression. In this thesis, I also highlight how bio-fertilizers can serve as one of the most efficient soil managements used to control Fusarium wilt disease, thereby also reducing the need for chemical fertilizers and hazardous pesticides. In addition to seeking antagonistic microbes based on their direct inhibitory activity, disease suppression may also be achieved by promoting beneficial microbes already resident in the soil.

In addition, I highlight that protists, an often overlooked component of the soil microbiome encompassing most eukaryotic lineages, also play central roles in soil functioning and disease suppression. Results in this thesis demonstrate that protist communities show long-lasting responses to soil management. Treatment with chemical fertilizer promoted plant and animal parasites among the protists, whereas organic fertilizers stimulated bacterivorous and omnivorous groups, suggesting improvements of the detritivorous food web. Network analysis also placed protists as a central hub in the soil microbiome, linking diverse bacterial and fungal communities. Given the complexity of the soil food-web, the work in this thesis suggests that manipulation of higher trophic levels of organisms, such as protists, will offer new avenues to promote soil health and plant performance. In sum, this research will improve the insights in the mechanisms of soil-borne disease suppression, thereby yielding valuable advice for farmers to improve soil health with suitable agricultural management measures.

Future perspectives

The results from this thesis revealed that microbial community differences between Fusarium wilt disease-conducive and disease-suppressive soils, as well as microbial community responses to soil managements, contributed to Fusarium wilt disease suppression. These differences in the soil microbiome were clearly relevant to the level of disease suppression. Besides the evidence from the high-throughput sequencing data, future cultivation-based studies are also needed to identify and characterize the potential bio-control agents such as *Mortierella* spp. and *Lysobacter* spp. and reveal their functional mechanisms in their disease suppression ability against *Fusarium* pathogen. Utilizing the new perspectives afforded by developing multi-omics technologies including metagenomics, meta-transcriptomics, meta-proteomics and meta-metabolomics, should provide insights into the functional activity of soil microbial communities, opening up new possibilities for the development of soil management strategies against Fusarium wilt disease.

In this thesis, protists were identified as a central hub in the soil microbiome, linking diverse bacterial and fungal communities, thereby playing an important role in soil functionality. Protists provide new insights into managing soil microbial communities to enhance their functionality and

ability to support a productive and healthy soil in agriculture. Given the complexity of the soil food web, the work reported here still has not included all the trophic level organisms in the current experiments. High-throughput sequencing for investigating soil bacteria, fungi, protist and nematodes, combined with morphological identification for the visible small vertebrates, insects and earthworms will help us to investigate the full range of soil-borne trophic levels. By tracking food-web responses to different soil managements, we should be able to gain a comprehensive and mechanistic view of soil functionality, thereby providing the necessary knowledge to guide effective and sustainable agricultural management strategies in the future.

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Summary

Due to limited arable lands and world growing populations, intensive agriculture strategy such as long-term monoculture, are commonly practiced in meeting the food demands. However, long-term monoculture often results in the outbreak of serious soil-borne diseases such as Fusarium wilt disease. Soil-borne diseases, such as Fusarium wilt, often lead to seriously economical losses to a wide range of agricultural crops. Some soils can naturally suppress plant pathogens, harboring specific characteristics that keep pathogens in check, referred to as disease-suppressive soil. Soil microbial communities are confirmed as the main drivers of soil borne disease suppression. Understanding which microbial communities are associated with disease-suppression will provide the foundation for soil community manipulation and new opportunities to explore novel strategies to promote plant health in a sustainable way. To date, most studies have focused specifically on bacterial and fungal taxa. Protists, the often overlooked components of soil microbiome, are the main consumers of bacteria and fungi and represent useful bio-indicators for soil quality. In this thesis, I addressed different soil management strategies (including crop rotation and beneficial microbe inoculated bio-organic fertilizers) of disease suppression to control Fusarium wilt disease. This research will improve the insights in the microbial mechanisms of Fusarium wilt disease suppression and give advice for the farmers to improve soil health with suitable agricultural management measures.

The aim of this thesis was to uncover the microbial community characteristics associated with Fusarium wilt disease suppressive and conducive soil and to investigate the microbial community changes induced by different soil managements in relation to disease suppression against Fusarium wilt disease. Furthermore, I sought to examine the role of protists within the soil microbiome and to examine their roles in soil functionality such as plan health or disease suppression. The three main research questions of this thesis were as follows:

1) What are the indicator species and microbial community characteristics in the Fusarium wilt disease suppressive soil?
2) How can we best manipulate the soil microbiome by soil managements, and how do shift in the soil microbiome in response to management contribute to disease suppression, including how alterations in the bulk soil microbiome help promote a healthy rhizosphere microbiome?
3) How do protists respond to the different soil organic amendments, and what are the roles of protists in the soil networks in relation to plant health?

In order to answer these questions, I first sought to uncover the microbial characteristics of the disease suppressive soil? A previous study showed that long-term monoculture seriously hindered the growth of vanilla due to the accumulation of the *Fusarium* pathogen. With a comprehensive investigation of vanilla cropping fields across tropical China, we found that long-term monoculture does not always built up vanilla Fusarium wilt disease. In contrast, it can also induce disease suppressive soil. In **Chapter 2**, I demonstrated that some soils on Hainan Island China, retain a low Fusarium wilt disease incidence under decades of vanilla continuous cropping. This suppressive soil was dominated by the potential antagonistic microbe *Mortierella* and showed a highly connected fungal network in relation with Fusarium wilt disease suppression.

In **Chapter 3**, **4** and **5**, I investigated how different soil managements impact soil-borne disease suppression. In **Chapter 3**, I showed that the black pepper-vanilla crop rotation system can reduce vanilla Fusarium wilt disease due to the decreased *Fusarium oxysporum* pathogen load in both bulk soil and in vanilla rhizosphere. In **Chapter 4**, I found that the alteration in bacterial abundance, community structure and increased *Lysobacter* spp. were the factors most strongly linked to the inhibition of the pathogen *Fusarium oxysporum*. These results open up new perspectives for the promotion of soil health: in addition to seeking antagonistic microbes based on their direct inhibitory activity, disease suppression may also be achieved by introducing keystone species that reshape resident soil microbiome functioning. In **Chapter 5**, it is shown that continuous application of bio-organic fertilizer can suppress tomato wilt disease by inducing the healthy rhizosphere microbiota through alterations to the bulk soil microbiome. The black pepper-vanilla rotation increased potential plant beneficial microbes *Trichoderma* and *Penicillium* in the vanilla rhizosphere from the bulk soil (**Chapter 3**).

Finally, the work in this thesis highlights the importance of protists as key players the soil network and important determinants of soil functionality. First, it demonstrates that protists show different responses to different soil amendment treatments: chemical fertilizer stimulate plant and animal parasitic taxa, while organic fertilizer promoted bacterivorous and omnivorous protists, with the latter suggesting a more complex food web. Subsequently, network analysis revealed protists as a central hub in the soil microbiome, associating with diverse bacterial and fungal taxa linked to plant health and nutrient turnover. In addition, parasitic protist taxa, including the potential plant pathogenic *Pythium* and animal parasitic Apicomplexa, which were significantly reduced in the beneficial microbe-enriched organic fertilizer treatment and were poorly connected to other taxa in the network. This can be explained by the fact that these protists specially attack plants and soil animals, both of which are not included in the current network.

In total, the work presented in this thesis offers several new insights into the development of disease suppression in soils, especially as related to Fusarium wilt disease of vanilla. Disease suppression is affected by management, including crop rotation, type of fertilizer used and introduction of biocontrol agents via bio-organic fertilizers. Increase disease suppression was shown to stem from a number of interrelated mechanisms. Management systems may stimulate important potentially antagonistic species, as was the case with *Mortierella*. Effects can also be broader, including total bacterial biomass or involving more highly connected fungal networks. Furthermore, the work presented here stresses the importance of shifts in the resident microbial communities upon bio-fertilizer application; soil microbiome restructuring represents an exciting new pathway for suppression of Fusarium disease. Finally, critical roles of protists as central hubs in the soil microbiome were shown to play an important part in soil functionality and disease suppression. In total, the insights into the mechanisms of soil-borne disease suppression presented in this thesis can help provide useful advice for farmers in their attempts to promote plant health and improve sustainable soil management systems.

Nederlandse Samenvatting

Vanwege beperkte landbouwgronden en toenemende populatiedruk wordt vaak een intensieve landbouwstrategie, zoals langdurige monocultuur, toegepast om aan de voedselvraag te voldoen. Langdurige monocultuur resulteert echter vaak in het uitbreken van ernstige bodemziekten, zoals Fusarium verwelking. Bodemziekten, waaronder de ziekte van Fusarium, leiden vaak tot ernstige economische verliezen voor een breed scala aan landbouwgewassen. Sommige bodems, zogenaamde ziektewerende bodems, zijn door middel van specifieke kenmerken in staat op natuurlijke wijze plantpathogenen te onderdrukken. Microbiële bodemgemeenschappen spelen hierbij een cruciale rol. Inzicht in welke microbiële gemeenschappen verantwoordelijk zijn voor ziektesuppressie kan bijdragen aan het ontwikkelen van nieuwe strategieën om plant en gewasbescherming op een duurzame manier te bevorderen. Tot op heden hebben de meeste studies zich specifiek gericht op bacteriën en schimmels. Hoewel protisten vaak over het hoofd worden gezien als componenten van het microbioom, zijn het de belangrijkste consumenten van bacteriën en schimmels. Bovendien worden ze beschouwd als nuttige bio-indicatoren voor de bodemkwaliteit. In dit proefschrift worden verschillende bodembeheer strategieën behandeld (waaronder vruchtwisseling en bio-organische meststoffen geïnoculeerd met plant-begunstigende microben) ter bestrijding van Fusarium-verwelking. Dit onderzoek zal onze kennis over de microbiële mechanismen om Fusarium-verwelkingsziekte te inhiberen vergroten. Op basis hiervan worden aanbevelingen gedaan voor boeren om de bodemkwaliteit te verbeteren door middel van geschikte landbouw beheermaatregelen.

Het doel van dit proefschrift was om: te ontrafelen welke microbiële gemeenschapskarakteristieken geassocieerd worden met *Fusariam* werende en bevorderende bodems, en om te onderzoeken wat het effect is van veranderingen in de microbiële gemeenschap als gevolg van verschillende bodembeheer strategieën op de onderdrukking van *Fusarium* verwelkingsziekte. Verder probeerde ik de rol van protisten in het bodem microbioom te onderzoeken, evenals hun rol in bodemfunctionaliteit, zoals plangezondheid of ziektewering. De drie belangrijkste onderzoeksvragen van dit proefschrift luiden als volgt:

1) Wat zijn de indicatorsoorten en kenmerken van de microbiële gemeenschap in Fusarium ziektewerende bodems?
2) Hoe kunnen we het bodem microbioom het best manipuleren door bodembeheer, en hoe dragen verschuivingen in het microbioom in de grond, als gevolg van bodembeheer, bij tot de onderdrukking van ziekten? Specifieker, hoe leidden veranderingen in de 'bulksoil' tot een gezonder microbioom in de rhizosfeer?
3) Hoe reageren protisten op de verschillende organische veranderingen in de bodem, en welke functies vervullen protisten in bodemnetwerken met betrekking tot de gezondheid van planten?

Om deze vragen te beantwoorden, heb ik eerst getracht om de microbiële kenmerken van de ziektewerende bodem te ontrafelen. Een eerdere studie heeft aangetoond dat langdurige monocultuur de groei van vanilleplanten ernstig belemmert, vanwege accumulatie van de *Fusarium* ziekteverwekker. Met een uitgebreid onderzoek naar vanille plantages in tropisch China, ontdekten we dat het lange termijn-monocultuursysteem niet altijd leidt tot ophoping van *Fusarium*, maar in sommige gevallen zelfs een ziektewerende bodem kan induceren. In hoofdstuk 2 heb ik aangetoond dat sommige bodems

op Hainan Island, China, waar al tientallen jaren vanilleplanten in monocultuurplantages wordt verbouwd, een lage prevalentie hebben van Fusarium-verwelkingsziekte. Deze ziektewerende bodem werd gedomineerd door de mogelijk antagonistische microbe *Mortierella* en vertoonde een sterk verbonden schimmelnetwerk.

In hoofdstuk 3, 4 en 5 heb ik onderzocht hoe bodembeheer van invloed kan zijn op de onderdrukking van bodemziektes. In hoofdstuk 3 heb ik laten zien dat een vruchtwisselingschema met zwarte peper-vanilleplanten de prevalentie van Fusariumverwelking kan reduceren als gevolg van een verlaging van het aantal *Fusarium oxysporum* pathogenen, in zowel 'bulksoil' als in de rhizosfeer. In hoofdstuk 4 ontdekte ik dat remming van de ziekteverwekker *Fusarium oxysporum* het sterkst gekoppeld was aan veranderingen in bacteriële abundantie, gemeenschapsstructuur en verhoogde *Lysobacter* spp. Deze resultaten geven nieuwe mogelijkheden voor het verbeteren van bodemgezondheid: naast het zoeken naar antagonistische microben wiens activiteit ziektes onderdrukken, kan ziektesuppressie ook worden bereikt door 'keystone' soorten te introduceren die het functioneren van het microbioom hervormen. In hoofdstuk 5 wordt aangetoond dat langdurig gebruik van organische mest verwelkingsziekte van tomaten kan onderdrukken, doordat de gezonde microbiota in de rhizosfeer worden geïnduceerd door veranderingen in het microbioom in de 'bulksoil'. Op dezelfde manier zorgde vruchtwisseling van zwarte peper-vanilleplant voor verhogingen van de potentiële plant-begunstigende microben *Trichoderma* en *Penicillium* in de vanilleplant-rhizosfeer (Hoofdstuk 3).

Tot slot benadrukt het werk in dit proefschrift het belang van protisten als hoofdrolspelers in het bodemnetwerk en belangrijke determinanten van bodemfunctionaliteit. Ten eerste werd onthuld dat protisten verschillende reacties toonden afhankelijk van het type toegepaste bodembeheer: kunstmest stimuleert parasitaire taxa voor plant en dier, terwijl organische meststof bacterivore en omnivore protisten bevordert, wat een complexer voedselweb suggereert. Vervolgens plaatste netwerkanalyse protisten als een centraal knooppunt in het microbioom van de bodem, met associaties met zowel bacteriën als schimmels. Bovendien waren parasitaire protist taxa, waaronder het mogelijke plant pathogene *Pythium* en parasitaire *Apicomplexa*, significant verminderd en slecht verbonden met andere taxa in het netwerk, bij gebruik van organische meststof.

Als geheel biedt het werk in dit proefschrift een aantal nieuwe inzichten in de ontwikkeling van ziektesuppressie in de bodem, vooral in verband met Fusarium-verwelkingsziekte bij vanilleplanten. De onderdrukking van ziekten wordt beïnvloed door het bodembeheer, waaronder vruchtwisseling, het type meststof dat wordt gebruikt, en de introductie van biologische bestrijders via organische meststoffen. Toename van ziektesuppressie bleek voort te komen uit een aantal onderling gerelateerde mechanismen. Managementsystemen kunnen belangrijke potentieel antagonistische soorten stimuleren, zoals het geval was met *Mortierella*. Effecten kunnen ook breder zijn, inclusief totale bacteriële biomassa of meer sterk verbonden schimmelnetwerken. Bovendien benadrukken de hier gepresenteerde resultaten het belang van verschuivingen in de microbiële gemeenschappen bij de toepassing van organische meststoffen; herstructurering van het bodem microbioom vormt een fascinerende nieuwe aanpak voor de onderdrukking van de ziekte van *Fusarium*. Tot slot werd aangetoond dat protisten als centrale knooppunten fungeren in het bodem microbioom, waarmee ze een

belangrijke rol vervullen in bodemfunctionaliteit en ziektesuppressie. De hier verkregen inzichten over de onderliggende mechanismen van bodemziekte suppressie, kunnen helpen om boeren zinvol advies te geven om de gezondheid van planten te bevorderen en om duurzame bodembeheersystemen te verbeteren.

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Curriculum Vitae

Wu Xiong (熊 武) was born on 17 August 1988 (06 July 1988 of the Chinese lunar calendar), in Yueyang, a city from Hunan province, in the south-central China. In 2008, he started with his 4 years of bachelor major of plant protection in the College of Plant protection, Nanjing Agricultural University, China. During his bachelor study, he known the soil-borne disease was quite a serious problem in China agriculture system. In 2012, he was recommended for the 5 years master-PhD continuous study in College of Resources and Environmental Science, Nanjing Agricultural University, which was supervised by Prof. Qirong Shen and Dr. Rong Li. During the master and PhD study, he realized that animal waste disposal is another serious environmental problem in China agriculture. During the PhD study, with Prof. Shen's help, he combined the two problems together, in which he focused on the topic of organic and bio-organic fertilizers in controlling of soil-borne disease. In 2015, he continued his PhD study in Ecology & Biodiversity at Utrecht University, the Netherlands, supervised by Prof. George A. Kowalchuk and Dr. Alexandre Jousset, in which he focused on the fundamental science of soil microbial ecology. During the PhD study, he linked applied and fundamental science together. He was interested in high-throughput sequencing data and network analysis in microbial ecology. After his PhD study, he wants to continue the research of soil food-web in organic and conventional farming systems in relation with soil functionality and stability.