DISSERTATION

STRUCTURE AND FUNCTIONS OF ROOT MICROBIOME AND IMPLICATIONS FOR SUSTAINABLE AGRICULTURAL SYSTEM

Submitted by

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ABSTRACT

Plant microbiome is the collective genomes of microorganisms living in association with plants. It has been demonstrated that members of plant microbiome contribute to plant growth, plant nutrition acquisition and productivity, as well disease suppression. Soil as an enormous microbe reservoir closely touches plant roots and provides an opportunity to establish root microbiome. Soil microbial community has been suggested to play roles in promoting plant growth and suppressing soil borne disease. However, our understandings on plant root microbiome, soil microbial community and their effects on plant productivity and health are still unclear.

The purposes of this study were to (1) clarify the assembly rules of root microbiomes by plants, (2) to investigate the effects of soil and root microbiomes on plant productivity, (3) to understand the mechanisms behind suppression of soil born disease by crop rotation from activity of microbiome belowground. Addressing these questions will provide important information on basic science such as plant science and ecology as well as development of sustainable agricultural system.

(1)Soil productivity and structure of bacterial and fungal communities in unfertilized arable soil: Structure of microbial communities in soil and root was analyzed with next-generation sequencing (Illumina Miseq) using 16S rRNA V4 region for bacteria and ITS II region for fungi. We investigated microbial communities in 12 unfertilized arable soils extending over 1000 km in eastern Japan. At the same time, maize were grown in each of these soils and the relationships between soil functions including maize dry weight and microbiomes in soil and maize root were investigated.

Soil bacterial communities shared many operational taxonomic units (OTUs) among farms. An ordination plot based on correspondence analysis revealed convergent distribution of soil bacterial communities across the farms, which seemed to be a result of similar agricultural management practices. Although fungal communities showed lower richness and a lower proportion of shared OTUs than bacterial communities, community structure between the farms tended to be convergent. On the other hand, root communities had lower richness and a higher abundance of specific taxa than the soil communities. Two soil functions, decomposition activity

and soil productivity, were extracted by principal component analysis (PCA) based on eight soil properties. Soil productivity correlated with N mineralization rate, P_2O_5, and maize growth, but not with decomposition activity, which is characterized by C turnover rate, soil organic C, and microbial mass. Soil productivity showed a significant association with community composition, but not with richness and mass of soil microbial communities. Soil productivity also correlated with the abundance of several specific taxa, both in bacteria and fungi. Root communities did not show any clear correlations with soil productivity. These results demonstrate that community composition and abundance of soil microbial communities play important roles in determining soil productivity.

(2) Phylogenetic history and root microbiome structures in twenty plant families: Twenty plants from different families covering the most range of angiosperm phylogeny including monocots and dicots, crops and flower plants, were planted into identical unfertilized arable soil and structure of root microbiomes were examined. Bacterial root microbiomes have distinctive composition from soil community, while fungal root microbiomes have similar structure to soil community. Plant root microbiomes largely diffused among plant species even when grown under identical soil. Twenty plant species preferred different OTUs and this preference characterized the interspecific difference in bacterial root microbiomes. The assembly of bacterial root community is more strongly regulated by phylogenetic hierarchy than fungal community. Beta-proteobacteria and Gamma-proteobacteria showed a close association with host plant phylogeny. Although plant phylogenetic evolution didn't play important roles in the assembly of fungal microbiomes, the present study suggests that symbiotic association with mycorrhizae seems to play important role in structuring fungal microbiomes across plant phylogenetic evolution.

(3) The effect of crop rotation on clubroot disease resistance through changes of soil microbial community and root microbiome: Chinese cabbage seedlings were transplanted in soils after the growth of 20 plant families in the previous chapter to imitate crop rotation and spores of clubroot pathogen (*Plasmodiophora brassicae*) were infected to each soil. Preceding crops had great effects on constitution of soil microbiome, which also altered structure of root microbiomes in Chinese cabbage. The differences in disease damage (DSI) of clubroot among 20 preceding crops were the most evident at the 4^{th} week. The pathogen density at the earlier stage (the 3^{rd} week) was the main cause of differences in disease damage among preceding crop

treatment. The pathogen density (the 3rd week) was mainly determined by the two key microbes, Bacillales and Rhizobiales, which were mutually exclusive in the root community. The former bacteria had a negative effect and the latter bacteria had a positive effect on pathogen density. In addition to these two key microbes, many bacterial groups were involved into suppression of disease damage through two mechanisms: competitive suppression of pathogen proliferation and activation of plant immune response. Our results demonstrate that crop rotation can work as defense mechanisms by inducing changes in microbiome structure in soil and root.

The results in this study prove that microbiomes in soil and plant root are very effective means for the development of low input sustainable agricultural system.

和文要旨

Microbiome (微生物叢)とはある場所に存在する微生物全体を意味し，ヒトや植物などの宿主生物との関わりやその中で発現する遺伝子なども含む広い概念である。植物の葉や茎，根の表面と内部には細菌と真菌を含む多様な微生物群集が棲息していることが知られており，これらの植物マイクロバイオームが土壌からの栄養塩獲得や植物の生長と発育，病害防除などの多くの機能と関連していることが知られている。しかしながら，植物マイクロバイオームがこれらの機能にどのように関係しているかの詳細なメカニズムはよく分かっていない。本研究は，植物栽培と次世代シークエンスを利用した微生物群集解析により，持続可能な農業におけるマイクロバイオームの可能性を探ったものである。植物の根におけるマイクロバイオームは根周辺に棲息する微生物の侵入と定着を通じて形成されるが，本研究では，特に，(1)この形成過程に宿主植物がどのように係わっているか，(2)異なるマイクロバイオームより成る土壌で栽培された植物が栄養塩獲得や成長にどのように影響を受けるか，(3)輪作は土壌病害防除に有効な方法であるが，輪作が根のマイクロバイオームの変化を通じてどのように病害を抑制しているかを究明した。

本論文で得られた概要は以下の通りである。

(1) 関東以北の長期無肥料栽培を行っている 12 カ所の畑土壌について，細菌と真菌の群集構造を比較した。Illumina 社製 Miseq を用い，リボソーマル RNA の特定領域（細菌は rRNA V4 領域，真菌は ITS II 領域）の塩基配列から解析した。リード解析には QIIME を用い，RNA データベースから OTU の系統解析を行い，OTU の群集解析には統計ソフト R を用いた。同時に，土壌を用いたトウモロコシのポット栽培と窒素の無機化力や分解力などの土壌の機能特性も調査した。1000km 以上離れているにも係わらず，土壌の細菌群集はサイト間で収斂する傾向にあり，高い類似性が見られた。根のマイクバイオームは土壌に比べ，多様性が減少し，少数のグループが優占する傾向が見られた。無施肥の土壌で生育させたトウモロコシの乾燥重を土壌生産力の指標とし，関連する要因を調べたところ，土壌の窒素無機化力が最も高い相関を示した。土壌の生産性は土壌の全微生物量と相関を示さなかったが，特定の細菌目(Rhizobiales, iii1-15)の割合と高い相関を示し，これらの細菌は土壌の無機窒素供給力に関係すると思われた。

(2) 同一土壌に異なる 20 科に属する植物を栽培し，生育後の各植物の根のマイクロバイオームの構造を比較した。細菌群集は宿主植物の進化系統と密接な関係が見られたが，真菌にはそのような関係が見られなかった。宿主植物の系統に影響を受けた細菌としてβプロテオバクテリア,γプロテオバクテリアと放線菌(Actinobacteria)があげられる。このことから，植物進化の過程で根は土壌中の特定の微生物を選好してきたことが示された。

(3) 畑作栽培における輪作の土壌病害抑止効果を土壌と根のマイクロバイオームから解析した。20 の異なる科に属する植物栽培後の土にハクサイの実生を移植し，同時にアブラナ科野菜の重要土壌病害であるネコブ病菌の胞子を接種し，その後のハクサイの病害度と根のマイクロバイオーム構成を調べた。その結果，キク目やセリ目の植物の栽植後の土壌ではネコブ病が抑えられ，イネ目の後作では病害が大きい傾向が見られた。こ

れら植物の栽植後の土壌で育ったハクサイ根のマイクロバイオームは大きく異なり，根に Bacillales 目の細菌が多いほどネコブ病菌のハクサイの年での増殖は抑えられ，病害も抑制されることが分かった。ネコブ病菌抑制以外に，植物の病害発生を抑制する微生物群も多く見つかり，輪作による土壌病害抑制には多くの微生物が複雑に関与していることが明らかとなった。

以上の結果は，低投入で持続可能な作物栽培システムに土壌と根のマイクロバイオームは非常に有効であることを示している。

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CHAPTER 1: GENERAL INTRODUCTION

Microbiome in Soil and Root

Microbiome refers to microbial ecosystem, including the microorganisms (bacteria, archaea, lower and higher eukaryotes, and viruses), their genomes (i.e., genes), and the surrounding environmental conditions [1]. The human microbiome is widely known as having extensive functions on their hosts such as maturation of the immune system [2], host obesity [3], and processing of xenobiotics [4]. Fecal microbiota is also reported to play important roles in digestion and nutrition as well as lots of evidences for the link between a host's microbiota, digestion, and metabolism [5]. The microbiome, especially fecal microbiome, is increasingly recognized as a crucial key to understand human health and nutrition.

Plants also harbor a wide diversity of microorganisms both inside and outside their tissues [6]. The collective genomes of these microorganisms living in association with plants were defined as the plant microbiome [7], which is an extremely complex microbial community with various effects on host plants. It has been demonstrated that members of plant microbiome contribute to plant growth modulation [8-10], plant nutrition acquisition and productivity [9, 11-13], as well disease suppression [14, 15]. Microbiomes may also help plants overcome abiotic stresses such as drought and salinity, because it enables more rapid adaptation to a changing environment by reorganizing microbiome constitution than does plant adaptation that is a slow process of genetic change in plants over generations [16].

Plants recruit microbes primarily from potential sources of rhizosphere soil which is being closely touched by plants root. It has been reported that leaf bacterial microbiome shared large proportion of members with root-associated assemblies in Arabidopsis, grapevine and perennial wild mustard. The high share of constituting microbes between leaf and root suggests that soil as an enormous microorganism library might be a primary source of the whole microbiomes [17-19]. These studies imply that root-associated microbiomes play crucial roles in plant microbiome constitution.

Surveys of root-associated microbiomes suggested both biotic and abiotic factors impact assembly of plant root-associated microbiome, several important factors have been reported,

including soil factors [20, 21], geographic locations [22], plant type [23], plant development [19, 24], plant domestication[25, 26], as well as host genotype [19, 20, 27]. On the other hand, the large difference was also observed between compartments of root-soil interface. For instance, the rhizosphere possesses more diverse microbiomes than the endosphere [20, 27, 28], while the majority of entophytic bacterial microbes originate from the rhizosphere where is exposed to involvement of root exudates [9]. Furthermore, a study reported the dynamic changes in microbiome composition during acquisition and colonization period and suggested that rhizoplane acts as a selective gate to endophytic microbes [22]. From these, soil microbial community exists within an inseparable relation with plant root microbiome. However, how plants structure root microbiome from a potential source of soil microbial community is still unknown and thus needs to be investigated systemically.

Importance of Microbiome in Agriculture

Soil productivity and microbial community

Conventional agriculture is the most commonly implemented farming management in the world, aiming to produce high crop yield and quality through input of resources into farm lands. In conventional agriculture systems, synthetic fertilizers are applied for providing mineral nutrition and promoting plant productivity. At the same time those benefits take the high cost of the environment such as degrading natural environments, deteriorating soil quality and increasing biodiversity loss [29-32]. The abuse of the synthetic fertilizers is the main reason of soil degradation, greenhouse gas emissions and waterway eutrophication, posing a great threat to Earth's biogeochemical cycles [33]. Due to these problems, people exerted efforts on developing alternative methods for sustaining agriculture. The alternative strategies are decreased use of synthetic fertilizer, manure application, and cultivation of leguminous crops, while those alternative approaches still disrupt global nitrogen and phosphorus cycles that leads impactions on aquatic ecosystem [34, 35].

Biological fertilization is based on the use of natural inputs such as fertilizers, decaying remains of organic matter, domestic sewage, farmyard manure, and microorganisms [36], through means of soil amending, nitrogen-fixing plants and plant nutrient uptaking microorganisms introducing [37]. Biofertilizer is a substance containing living microorganisms

which can promote plant growth through plant hormone and may help to sustain environmental health and soil productivity [38]. The wide availability and release of biofertilizers were announced in several studies to promote N, P and K cycling [39, 40]. However, since type of microbial inoculants included in biofertilizers are not abroad enough for extensively using on various plant cultivars, and long-term application could cause adverse effects on plant growth and development of native soil organisms [41]. While effects of biofertilizer on plant growth through soil microbes has been known, how soil microbial community causes soil productivity and plant growth are still unclear. To fill a gap between basic knowledge and real agricultural fields, it is essential to understand the relationship between structure and functions of soil microbiomes and their implications for soil fertility and crop productivity in agriculture.

Crop-rotation, soil borne disease and microbial community

Plant diseases caused by soil borne pathogens have reduced yield and quality of many crops worldwide. Many crops are susceptible to several soil borne pathogens that survive in soil for long periods of time and infect plant roots under favorable conditions [42]. Soil borne diseases cause symptoms such as swellings (galls, knots, and clubs), lesions, rotting of root tips, and loss of root cortical tissue for the belowground and wilting, chlorosis, and stunting for the aboveground [43]. Several managements have been used for suppression of soil borne diseases such as using resistant cultivars, pesticides, biofungicides, proper irrigation, crop residue management and organic amendments [44, 45]. Among them, crop rotation is the most commonly used management in agriculture due to its efficiency and facility.

Compared with continuous cropping which results in the accumulation of soil borne pathogens, crop rotation, which is a historical agricultural management, inhibits the spread of soil borne pathogens and mitigates the decline of crop yield and quality leading to great damage of farmers [46, 47]. It has been suggested that crop rotation and residue amendments can enhance activity of soil microbial communities and suppress damages from soil borne diseases [48, 49]. Soil microbial activities have greater effects on incidence of soil borne disease than soil chemical properties (pH, nutrient status, C:N ratio) or other microbiological parameters (microbial biomass, microbial diversity) [50]. Additionally plant cropping system has been suggested to manipulate rhizosphere bacterial community [51]. However, how structure of soil and root

microbial communities is altered by crop rotation and how their changes in microbial community contributes suppression of soil borne disease are still unclear.

Soil microbes play roles in altering plant hormones secretion [10, 52], inducing plant systemic resistance and tolerance [53], resisting pathogens therefore suppressing disease [54], inducting the plant immune system [55, 56], influencing nutrient cycling and availability [57, 58]. It is suggested that soil disease is suppressed by modifying root microbiome associating with reduction in colonization of pathogen into root tissues [54]. Crop rotation can improve disease suppression by enhancing the antibiosis abilities of bacteria living around root zone [59].

Plant root microbiome contribute to plant growth [8-10], nutrition acquisition and productivity [9, 11-13], as well as disease suppression [14, 15]. Bacteria taxa with disease-suppressive activity in rhizosphere microbiome involve in pathogen control [60]. It has been demonstrated that assembly of plant root microbiome is influenced by several associated factors such as plant development, plant domestication, host genotype, geographic locations, as well soil factors [19-22, 24-27, 61-63]. Rhizosphere, the soil zone closely touched with plant root, is an enormous library of microorganism and thus is a primary source for microbes to establish their whole community [17-19]. In spite of many studied aiming to seek the relationships of structure of microbial communities in plant root and in soil, it remains poorly understood how crop rotation suppresses soil borne diseases through interaction between soil and root microbes, although control of soil borne disease through crop rotation could contribute decline in yield loss in agriculture fields.

Disease Suppression and Belowground Microbiomes

There are complex relationships in community dynamics among free-living microbes in soil, root endophytic microbes and pathogenic microbes as the Fig 1-1 shown. The soil in which plants thrive is the most important microbial source for root microbiome. Soil microbes contribute plant individual functions such as plant nutrient uptake, plant diversity and productivity [64, 65], mitigation of abiotic stress [66], plant growth [67] and pest and disease suppression [68, 69]. Furthermore, soil microbes are capable to influence structure and functions of community such as productivity, diversity, and species composition both directly and indirectly [70, 71]. In return, plants also have important effects on structure of soil microbial

community in that change plant species composition induces changes in community structure of soil microbes [72]. Plant root exudates, which are a complex combination of compounds, can mediate and maintain structural changes in soil microbial community [73, 74]. Recent studies have demonstrated that specific root exudates attract specific soil microbes involving in mineral acquisition, plant growth and defense pathogens [72, 75, 76], and thus cultivating their specific self-serving soil microbes has great advantages to plants.

Soil also contains many microbes interacting with pathogens. Arbuscular mycorrhizal (AM) associations have been shown to reduce damage caused by soil-borne plant pathogens [77], while *Bacillus* and *Streptomyces spp.* have been used as biocontrol agents to suppress pathogen proliferation [78]. Beneficial soil microbes can enhance immune responses and defense capacity in plant [79] through the release of antimicrobial or antifungal compounds [80, 81] and protect plants against microbial pathogens and pest insects. It is increasingly recognized that soil biota have extensive antibiotic resistance genes [82] and have a great potential to suppress damage of soil diseases.

Soil is like a buffer pull that mediates plant-pathogen interactions. Soil microbial community as a whole drives the disease suppressive ability. Although scientists understand that soil microbes have great impacts on plant health and disease suppression, the interactions between plant, soil and pathogen remain an open question. To clarify this mechanism the integration of multiple disciplines such as plant science, ecology and soil science is required.

Tools for Microbial Community Analysis

Chloroform fumigation and the use of phospholipid fatty acids are effective ways to estimate microbial mass, while molecular fingerprinting methods such as denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism (T-RFLP) have been used to analyze community composition [83]. However, these methods have a limited ability to dissect the structure of microbial communities. Furthermore, despite the fact that fungi are important members of the soil microbial community, much more attention has been paid to bacteria [27, 61]. The recent development of next generation sequencing (NGS) technology has enabled a deeper resolution of community structure and identification of a large number of low-abundance taxa in bacterial and fungal communities [84].

The NGS technologies provide a massive amount of sequence data and thus require computational tools to handle the huge amount of sequence data [85]. However, development of pipeline tools for microbial community analysis such as mothur [86], RDP [87] and QIIME [88] enables the analysis of sequence raw data easily for everyone.

Especially, QIIME, an open-source bioinformatic pipeline for performing microbiome analysis, enables users to import raw sequencing data generated on the Illumina or other platforms and to provide high-level statistics and publication quality graphics. This process includes demultiplexing and quality filtering, operational taxonomic units (OTUs) picking, taxonomic assignment, and phylogenetic reconstruction, and diversity analyses and visualizations. In this study QIIME was also used for the identification of OTUs and measures of diversity within and between samples across studies. Although the concept of OTU, which is a key concept for microbiome analysis, is increasingly problematic as sequence data accumulate, we employed phylogenetic approaches, which gain in popularity [5].

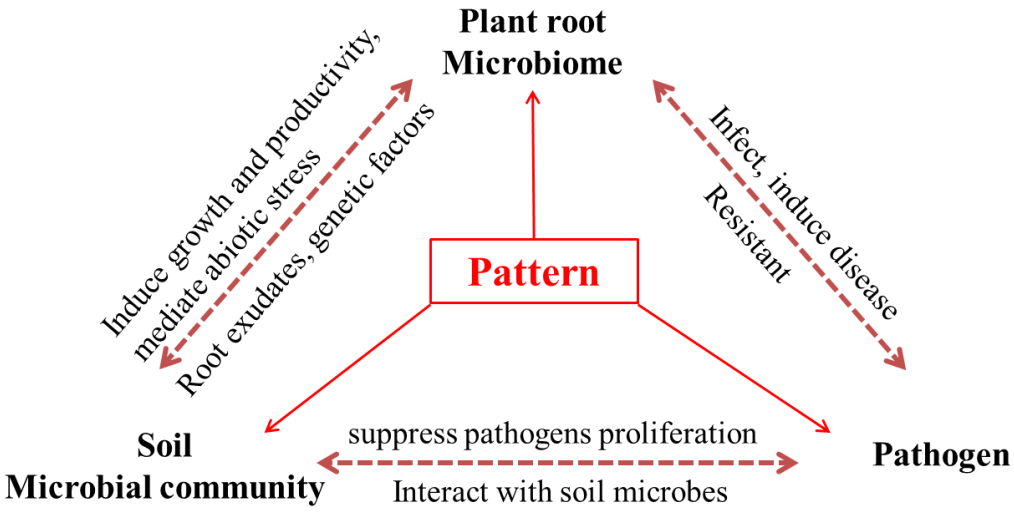


Fig 1-1. Relationships of plant root microbiome, soil microbial community and pathogen.

Objectives of This Study

The importance of soil microbial community to plant health and productivity has been described above. Previous studies reveal that our understandings on plant and soil microbiome, as well as their relations with pathogen are still unclear. The goal of this study was to clarify (1) the assembly rule of plant root microbiome that initially start from recruitment of microbes from a potential source of surrounding soil, (2) plant phylogenetic effects on microbial selection in the process of root microbiome establishment, (3) roles of soil and root microbiomes on plant productivity and (4) suppression of soil borne disease through microbial processes under cultivation of crop rotation.

The above issues are also to give answers to the following specific questions: What is the difference between soil and root microbiomes? Is there any pattern between them? What factors are responsible for their differences? What are differences between bacterial and fungal microbiomes? Which characteristics of microbial communities are responsible for soil productivity? How does crop rotation affect soil and root microbial communities? How does pathogen interact with root microbes? Answers to these issues will have great contribution to development of sustainable agricultural system in addition to improvement of basic science such as plant science and microbial ecology.

To address these questions, we conducted several experiments by using next generation sequencing. The chapter 2 describes a study on how soil productivity is affected by microbial communities by using different unfertilized arable soil and how same plant structure root endophytic microbiomes from those different soils. The chapter 3 focuses on clarifying the pattern of how different host plants structure root endophytic microbiomes from the identical soil in both bacterial and fungal communities. The chapter 4 tried to clarify the mechanisms of crop rotation to suppress soil borne disease, clubroot in Chinese cabbage, through changes in the soil and root microbiomes.

CHAPTER 2: SOIL PRODUCTIVITY AND STRUCTURE OF BACTERIAL AND FUNGAL COMMUNITIES IN UNFERTILIZED ARABLE SOIL

Introduction

Soil harbors diverse microbial communities. The diversity and composition of these communities vary largely between different environments. For example, the structure of microbial communities can be influenced by soil pH [89-94], soil type [95, 96], electrical conductivity (EC) [90, 97], nutrient availability [98], climate [99, 100], and vegetation type [101]. On the other hand, microbial communities play an important role in determining soil functions such as carbon (C) turnover rate, mineralization of nitrogen (N), and pest control [102-104]. As soil properties influence the microbial community, and the microbial community, in turn, shapes various soil functions, there exists a close association between the soil and the microbes.

Soil productivity is strongly influenced by soil microbial communities. Since crop yield in conventional agriculture depends strongly on the utilization of synthetic fertilizers and various kinds of pesticides, the effects of soil productivity on crop yield have been neglected. In contrast, organic farming does not depend on synthetic fertilizers, and thus crop yield essentially relies on soil productivity. While soil microbes contribute to soil productivity through amelioration of soil physical structure and activation of mineral cycling in soil [104], it is not completely clear how the microbial community influences soil productivity. As the structure of soil microbial communities is heavily altered by the quality and quantity of synthetic fertilizers and manures [29, 105], arable lands that have not been exposed to any fertilizers are suitable for investigating the relationships between microbial communities and soil productivity.

Soil microbes play important roles on altering plant hormones secretion [10, 52], inducing plant systemic resistance and tolerance [53], suppressing disease [54], influencing nutrient cycling and availability [57, 58]. Host species, plant development, soil type and geographic factors were also capable of impacting root zone microbial structure [20, 22, 24, 27, 61-63, 106]. It was suggested that soil was a driver of plant root endophyte community structure, which could facilitate plant growth [107]. Even if many studied have been performed to seek the insight of the correlation between plant root and soil microbial communities, it’s remain poorly understood

that how plant structures root endophytic microbiome from different soil microbial environment and also what microbes are closely correlated with plant growth.

The structure of a microbial community is characterized by its mass, abundance, and richness. Chloroform fumigation and the use of phospholipid fatty acids are effective ways to estimate microbial mass, while molecular fingerprinting methods such as denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism (T-RFLP) have been used to analyze community composition [83]. However, these methods have a limited ability to dissect the structure of microbial communities. Furthermore, despite the fact that fungi are important members of the soil microbial community, much more attention has been paid to bacteria [27, 61]. The recent development of next generation sequencing (NGS) technology has enabled a deeper resolution of community structure and identification of a large number of low-abundance taxa in bacterial and fungal communities [84]. In this study, I examined the richness, composition, and abundance of soil bacterial and fungal communities from 12 unfertilized farms extending over 1000 km, where microbial communities have not been exposed to any fertilizers, including manures, for at least the last five years. Maize was used as the plant material to detect differences of root and soil microbial structures.

I first compared the diversity patterns of soil bacterial and fungal communities across the 12 farms. I investigated how spatial variations in bacterial and fungal communities, including root communities, are organized in organic farms. Second, I examined how soil productivity is affected by microbial communities. Third, I investigated how maize structure root microbiomes from different soil microbial communities. Soil productivity is determined by many factors including soil physical and chemical properties, microbial activities, and their interactions. To clarify which soil properties are responsible for soil productivity, we measured C turnover rate when incubated with grass litter, N mineralization rate, and maize growth in each soil, as well as chemical properties such as soil pH, N, phosphorous (P_2O_5), and organic C (Co) contents.

My aim was to clarify which characteristics of microbial communities are responsible for soil productivity and its components. Several specific questions were addressed. Among mass, richness, and composition of communities, which components are involved in soil productivity? Are bacterial or fungal communities more important in determining soil productivity? What is the contribution of root communities to soil productivity? How does plant structure root

microbiomes from different soil microbial communities? What is the difference between soil and root microbiomes? Do microbes show different enriched pattern between soil and root? What microbes are correlated with plant growth?

Materials and Methods

Soil collection

Soils were collected from 12 unfertilized organic farms in eastern Japan with the permission of landowners (Fig 2-1 and Table 2-1). The soils were sampled from the 0–10 cm layer at three different locations (replicates) in each site during April and May 2015. After sieving through a 2 mm mesh to remove roots, macrofauna, and rocks, the soils were subjected to chemical and microbial analysis for assessing the growth of maize, and to DNA extraction for microbial community analysis. The soils were stored at 4 ℃ for the chemical analysis and at -25 ℃ for DNA analysis until processing.

Chemical and microbial analysis

Available N (NO_3^- and NH_4^+) and P were measured via colorimetric assay with a spectrophotometer (Jasco V-630 BIO, Tokyo, Japan). After extraction of 5 g dry soil in 50 mL of 2 M KCl solution, NO_3^- and NH_4^+ were determined using the sodium salicylic acid and indophenol blue methods, respectively [108]. The available P_2O_5 content was determined using the ammonium molybdate-ascorbic acid method, following the extraction of 5 g dry soil in a 50 mL solution of 0.05 M ammonium sulfate [109]. Soil total N content (%) and total C content (%) were determined using an automatic N-C analyzer (Vario EL cube. Elementar, Langenselbold, Germany). The soil pH was determined with a pH meter (Horiba pH Meter D-52, Tokyo, Japan) after dissolving 10 g of dry soil in 25 ml of distilled water.

In addition, three microbial traits, C turnover rate, N mineralization rate, and microbial mass, were measured using fresh soil. C turnover rate was evaluated for the soil incubated with leaf litter from C_3 grass *Dactylis glomerata*, which has high nitrogen content and a high decomposition rate [110]. A polyvinyl plastic pot (19.6 cm^2 area and 3.5 cm height) was filled with 25.0 g fresh soil along with dried leaf litter (0.3 g). Sterilized distilled water was added to all the soils to standardize the moisture content to 60%. The samples were incubated at 25 ℃ for

40 days. The CO_2 efflux rate was monitored using an automatic CO_2 analyzer (Li-6400; Li-Cor Biosciences, Lincoln, NE, USA) equipped with a soil CO_2 emission chamber (LI-6400–09; Li-Cor). The CO_2 concentration inside the chamber was set at 400 μmol during the measurements. The changes in the CO_2 concentration were monitored for 60 s, and the CO_2 efflux from the soil was calculated in μmol CO_2 m^{-2} s^{-1}. As the CO_2 efflux rate reached a peak value at 3 days after the incubation and then rapidly declined, the maximum rate at 3 days was used as the C turnover rate. The N mineralization rate was measured by incubating the soil for 40 days without the addition of grass litter. The available N was measured before and after the incubation, and N mineralization rate was calculated as the difference in available N content during the incubation period (40 days). Microbial mass was measured using the chloroform fumigation method [111]. Fresh soil (8 g) was placed into a 100 mL glass beaker and fumigated with 50 mL chloroform under reduced pressure in a vacuum desiccator for 24 h. After extracting 50 mL of 0.5 M potassium sulfate solution, soil C was measured with an automatic carbon analyzer (Shimadzu TOC-L, Kyoto Japan). Soil microbial C was calculated as the difference between the control and the fumigation treatment multiplied by a correction factor (2.64).

Soil productivity was evaluated using the dry mass of maize grown on each soil. Maize seeds were sown on a wet filter paper for germination, and on July 7, three seedlings were transplanted into pots (113 mm diameter and 184 mm depth) containing each of the soils and grown in a greenhouse. Three pots (replicates) for each soil were prepared. After 20 days, the shoot and root of the maize were harvested. The root parts were washed to remove attached soil, and 2 g of fresh root tip was taken and stored at -25 ℃ for DNA extraction. After drying at 70 ℃ for 48 h, the root and shoot dry weights were measured. As there was a large difference in the bulk density among soils, maize weight was expressed as per g of dry soil in a pot, not as per pot.

DNA extraction

As DNA extraction using commercial extraction kits was difficult for some soil samples from volcanic ash, the DNA was extracted by bead-beating and CTAB-based method of Hoshino and Matsumoto [112] with slight modifications. This method can be applied for DNA extraction from roots. Soil samples (400 mg) were smashed with 0.1 mm glass beads, 800 μL extraction buffer (0.1 M NaCl, 0.1 M Tris–HCl, 0.1 M EDTA, and 0.3 M sodium phosphate), 2% SDS, and 3.2 mg skim milk at 5500 rpm for 40 s. The samples were then subjected to centrifugation at

18,000 ×g for 1 min, and the supernatants were transferred to a new tube and incubated for 5 min with 400 μL of 5% CTAB, 30 μL of 5 M potassium acetate, and 550 μL of chloroform-isoamyl alcohol (24:1). After centrifugation at 10,000 ×g for 5 min, the upper phase was transferred to a new tube and incubated at 55 ℃ for 1 h after mixing it with 800 μL of CTAB precipitation solution (5 g L^{-1} CTAB, 0.04 M NaCl). The samples were centrifuged for 5 min at 18,000 ×g, following which, the supernatant was discarded and the pellet was washed with 500 μL of ethanol (70% v/v). The pellet was dried for 1 h, and the DNA was dissolved in 50 μL of sterile deionized water.

The 100 mg root sample was smashed with a 0.5 mm zirconia bead, 0.2 mm glass beads, and 100 μL of extraction buffer (0.5 M NaCl, 0.1 M Tris, 0.1 M EDTA), following which, it was incubated with 700 μL of 1.3% CTAB, 20 μL of 2-mercaptoethanol, and 700 μL of chloroform-isoamyl alcohol (24:1). After precipitation and purification by the same method mentioned above, DNA was dissolved in 50 μL of sterile deionized water.

PCR amplification and sequencing

For bacteria, a V4 region of 16S ribosomal RNA (rRNA) was used [113]. The primer pair in the 1st PCR amplification was 515F (5′- GTGCCAGCMGCCGCGGTAA -3′) and 816R (5′- GGACTACHVGGGTWTCTAAT -3′) with adaptors. To reduce the chloroplast and mitochondrial amplification, 2.5 pmol μL^{-1} peptide nucleic acid (PNA) clamps were included in the reaction according to Lundberg [113]. For fungi, the ITS2 region of rRNA was used [114]. The primer pair for the 1st PCR was fITS (5′- GTGARTCATCGAATCTTTG -3′) and ITS4 (5′- TCCTSCGCTTATTGATATGC -3′) with adaptors. The PCR conditions were as follows: an initial denaturation at 94 ℃ for 30 s, and 25 cycles at 94 ℃ for 15 s, 50 ℃ for 30 s, and 72 ℃ for 30 s, with a final extension at 72 ℃ for 5 min. After purification by AMPure XP magnetic beads (Beckman-Coulter, Indianapolis, IN, USA), the 1st PCR products were followed by the second PCR with the primer pair, 2nd-F (AATGATACGGCGACCACCGAGATCTACAC- Index2 -ACACTCTTTCCCTACACGACGC) and 2nd-R (CAAGCAGAAGACGGCATACGAG AT- Index1 -GTGACTGGAGTTCAGACGTGTG), for both 16S rRNA and fungal ITS2. The index pair was specific to each sample, for an accurate recognition of the samples. The second PCR conditions were: 94 ℃ for 2 min, 8 cycles at 94 ℃ for 10 s, 60 ℃ for 30 s, 72 ℃ for 30 s, with a final extension at 72 ℃ for 5 min. The second PCR products were purified using the

AMPure XP magnetic beads and pooled in equimolar ratios. After confirming the library quantity, paired-end 2 ×250 bp sequencing of the barcoded amplicons was performed on a MiSeq machine (Illumina Inc, San Diego, CA, USA).

Sequence processing

The sequences obtained from the MiSeq were processed through a custom pipeline developed at Fasmac Inc. (Atsugi, Japan). The raw reads were demultiplexed based on the barcode sequences and filtered by exact matching using Fastx toolkit (fastq_barcode_splitter). If the quality score was less than 20 and the sequence length was less than 40 bases, the reads were discarded. Paired-end reads with a minimum 10 base overlap were merged into full-length sequences by FLASH v1.2.10 [115]. Merged sequences between 246 and 260 bases were used for later processing of 16S rRNA. Chimeric sequences were detected using the UCHIME algorithm from the USEARCH package [116]. Operational taxonomic unit (OTU) generation and phylogenetic assignment were conducted using QIIME (v1.9.0) script with default conditions. OTUs were clustered using UCLUST [117] at a 97% similarity level by a de novo picking method, using Greengenes 16S reference database [118] for 16S V4 data and UNITE reference database [119] for ITS2 data. To obtain the filtered OTU dataset, all OTUs assigned to Archaea, chloroplast, and mitochondria were discarded from the 16S dataset, and only the OTUs assigned to kingdom Fungi were reserved for the ITS2 dataset. The filtered datasets were then normalized by transforming the number of OTU counts to relative abundance values. The raw reads of sequences were deposited into the DDBJ Sequence Read Archive (DRA) database (DRA accession: DRA006580).

Statistical analysis and visualization

The differences in soil properties between the farms were tested with ANOVA and Tukey-Kramer HSD for multiple comparisons. Since the residual variations for all traits were distributed randomly and were independent from the mean values, no transformations of the data for the improvement of normality and homoscedasticity were applied. Since soil properties were highly correlated with each other, a principal component analysis (PCA) based on the correlation matrix was applied to extract independent ordination axis. These statistical analyses were conducted using JMP (v4.0, SAS, Cary NC, USA).

As spurious sequences and unrepresentative OTUs decrease the reproducibility for community assemblage [120], the generated OTUs were filtered at the threshold of 0.01% of the total abundance, and the OTUs above this level were used for the diversity analysis. Community richness was evaluated using the number of OTUs. Although various measures evaluating α- and β- diversity have been proposed, the Shannon-Weaver index (H′) was used in this study, as this method enables the total diversity (γ- diversity) to be partitioned into independent α- (within-site) and β-diversity [121].

$$H' = - \Sigma (N_i/N_t) * \ln (N_i/N_t),$$

where N_i is the number of reads in each OTU and N_t is the sum of OTUs. To avoid $N_i = 0$ during the calculation of logarithm, 1 was added for all N_i.

Soil community structure was evaluated by canonical correspondence analysis (CCA) based on the relative abundance of each taxa. The relative abundance of OTUs belonging to each taxon were summed and calculated at the phylum, class and order level respectively. A permutation ANOVA was used to examine the relationships between community ordination structure by CCA and soil properties. CCA analysis was conducted using the Vegan package in R (v3.1.1) [122].

Small number OTUs possessed large proportion in root microbiome, thus 0.5% threshold OTUs were used for analysis between root and soil microbial communities. α-diversity were calculated by the function “diversity()” using “Shannon” and “Simpson” method of the R package Vegan. The richness of community was evaluated using the number of observed OTUs. Principal coordinate analysis (PCoA) was generated for the community structure dissimilarity analysis based on the Bray-Curtis distances of relative abundance of OTUs by “pcoa()” function of the R package APE [123]. To detect any differences in relative abundance between soil and root communities, a paired t-test was applied for each taxon. The lower taxonomic resolution may clarify the close association between beneficial functions of host plants and specific microbial groups in root communities. Accumulated relative abundance of OTUs at genus level was used to detect correlation with maize growth by Pearson correlation.

Results

Soil microbial community structure

The total diversity across all sites (γ-diversity) was partitioned into α- and β-diversity using the Shannon-Weaver index (Table 2-2). The α-diversity was approximately twice as high as the β-diversity in bacterial and fungal communities (Table 2-2 and Fig 2-2), and thus the total diversity was mostly ascribed to within-farm variation. The bacteria showed a significantly higher α-diversity than fungi ($p < 0.01$, t-test). The total number of OTUs in soil was 5337 for bacteria and 2569 for fungi. The mean number of OTUs in the soil community for single sites was 2785 ± 90 for bacteria and 498 ± 32 for fungi.

In spite of the high richness, the total abundance was largely comprised of the highest-ranked OTUs; the top 100 OTUs accounted for 47.1% of the total abundance for bacteria and 61.9% for fungi, as shown in Fig 2-3. Although the fungal communities had a lower proportion of the OTUs shared among all sites (1.8%) than the bacterial communities (31%), the high-ranked OTUs tended to be shared by a greater number of sites than the low-ranked OTUs, in both bacterial and fungal communities.

Three phyla, Proteobacteria, Acidobacteria, and Actinobacteria, comprised more than 60% of the total abundance in the soil bacterial communities (Fig 2-1). At the order level, the mean abundance of the three dominant groups, Actinomycetales, Rhizobiales and Sphingomonadales, were 6.7% ±0.47, 6.5% ± 0.62 and 4.6% ± 0.61, respectively, as shown in Fig 2-10. More than 80% of the abundance in soil fungal communities was due to three phyla (Ascomycota, Zygomycota, and Basidiomycota), while the order Mortierellales accounted for 18.3% ±1.51 (se) of the abundance.

Soil productivity and microbial communities

The eight soil measurements [soil pH, mineralized N (Nm), P_2O_5, soil organic C (Co), C/ N ratio, microbe C (Cm), C turnover rate (R), and maize dry weight (DW)] showed significant differences at the 0.1% level across the 12 sites (Table 2-1). Pearson correlation analysis (Fig 2-4 and Table 2-3) showed that R was significantly positively correlated with Co (r = 0.68*) and Cm (r = 0.67*). In contrast, the maize DW linearly increased with mineralized N (r = 0.89***) but not with R (r = 0.03). These correlation patterns indicated that the two microbial processes, C

turnover and N mineralization, were not correlated with each other (Fig 2-4). The principal component analysis (PCA) discriminated between these two functions (Table 2-4). The first component (PC1), which explained 39.6% of the total variation, was characterized by Co, Cm, and R, and thus represents microbial decomposition activity, while the second component (PC2), which explained 24.3% variation, was characterized by maize DW, P_2O_5, and Nm, and thus, represents soil productivity.

Richness did not show any significant correlations with decomposition activity (PC1) or soil productivity (PC2) in bacterial or fungal communities. On the other hand, the mass of soil microbes was closely associated with decomposition rate (PC1) but not with soil productivity (PC2). Canonical correspondence analysis (CCA) revealed that soil productivity (PC2) was strongly influenced by the community structure at the order level in both bacteria and fungi (Table 2-5). However, significant effects of community structure were not found at the higher taxonomic levels of class and phylum. The relationships between the abundances of the top 10 taxa at the order level and soil productivity were examined (Fig 2-5). Soil productivity (PC2) was positively correlated with the relative abundances of Rhizobiales (r = 0.651*) and iii1-15 (r = 0.827***), and negatively with Acidobacteriales (r = -0.606*) and Solibacterales (p = -0.839***). For fungi, only Pezizales (r = -0.724**) showed a significant correlation with soil productivity (Table 2-6).

On the other hand, bacterial orders Sphingomonadales (r = 0.631*) and Solibacterales (r = 0.579**) were significantly correlated with the latitude of the sampling site (Table 2-6). Soil pH (PC3 in PCA) was also significantly correlated with the bacterial orders, Saprospirales (r = -0.595*), Acidobacteriales (r = 0.627*), and RB41 (r = -0.753**). These results demonstrate that environmental factors can also have strong effects on the abundance of each taxon, especially in bacterial communities.

Maize root microbiome constructions from different soil and growth correlated microbes

Shannon index, Simpson index and observed OTUs numbers were calculated for α-diversity. The soil community had significantly higher α-diversity than the root community both for bacteria and fungi (Fig 2-6; Table 2-7). The bacteria showed a significantly higher α-diversity than fungi (Table 2-7; Table 2-8). OTUs rank-abundance curve showed that root communities

were less diverse than soil communities in both bacterial and fungal communities; a smaller number of high-ranked OTUs showed higher abundance in the root communities than in the soil; bacterial communities were more diverse than fungal communities (Fig 2-7).

The principal coordinates analysis (PCoA) based on the relative abundance revealed a convergent distribution of maize root bacterial microbiomes, and separated with soil samples (Fig 2-8). Although maize root samples were dispersal at fungal communities, soil samples were clustered tightly and separated with them (Fig 2-8).

To investigate the habitat preference of microbes, I tested the differences of accumulated relative abundance between root and correspondent soil samples. Bacterial phyla Proteobacteria and Actinobacteria significantly enriched in maize root, while Acidobacteria, Gemmatimonadetes, Verrucomicrobia, Nitrospirae and AD3 significantly depleted (Fig 2-9). As for the fungi, phyla Ascomycota and Zygomycota significantly enriched and depleted in maize root respectively (Fig 2-9). At the order level, three of the top 10 bacterial orders, Actinomycetales, Rhizobiales, and Burkholderiales, were significantly enriched at the root (Fig 2-10). Notably, Actinomycetales enriched its abundance by 31.5% (Fig 2-10), whereas six other orders showed a significantly lower abundance. As for the fungi, orders Pleosporales and Hypocreales were significantly enriched at the root, with the order Pleosporales showing an especially high enrichment of 40.0% (Fig 2-10). These results suggest that some specific microbes showed different enriched and depleted pattern at root and soil compartment, indicating that these microbes may have their specific habitat preference and/ or deliberately selected by maize plants.

To detect correlations of maize growth and root microbes, we analyzed the correlations between maize DW and relative abundance of microbes at genus level in root, at which level microbe functions were well understood. Bacterial genus *Methylibium*, fungal genera *Leptodontidium* and *Pseudobotrytis* were significantly positive correlated to maize DW, while bacterial genus *Streptomyces*, fungal genera *Geopora* and *Pyrenochaeta* showed significant negative correlations to maize DW (Fig 2-11).

Discussion

Structure of bacterial and fungal communities

Although detailed analyses of the community structure of soil microbes have been lagging because of a lack of appropriate methods, recent advances in NGS analysis have helped reveal a picture of extremely diverse soil microbial communities [124, 125]. In this study, a 0.4 g soil sample included an average of 2785 bacterial OTUs, which belonged to 150 different taxa at the order level. In contrast to this high within-site diversity, the soil bacterial communities shared many OTUs between farms, even those that were 1000 km apart from each other. Another study had also reported that a small proportion of phylotypes comprises almost half of the total abundance of bacterial communities worldwide [125]. Therefore, sharing of common dominant taxa across spatially distant farms seems to be a universal pattern in soil bacterial communities. On the other hand, soil bacterial communities in this study showed a lower β-diversity than α-diversity (Table 2-2), which indicates the absence of clear community divergence between farms. This result does not agree with other studies which have reported a high β-diversity and community divergence in soil bacteria [126].

Dispersal limitation and environmental selection are predominant factors that cause community divergence across sites [127]. Dispersal limitation increases the community divergence between geographically distant sites, while selection causes community divergence depending on differences in environmental conditions across sites. Therefore, the spatial patterns of α- and β-diversity indicate the relative importance of external factors in shaping the structure of soil microbial communities. Furthermore, it is known that microbes establish distinctive communities in plant roots [28], which can potentially influence the structure of soil microbial communities. Although the latitude of the sampling sites showed a significant effect on the structure of bacterial communities (Table 2-6), the sharing of most OTUs among distant farms suggests that dispersal limitation does not play an important role in the shaping of bacterial communities. On the other hand, based on the abundance, latitude and soil pH had significant effects on the community structure (Tables 2-5, 2-6), and therefore the effects of environmental selection seemed to be prevalent in the bacterial communities in spite of their low β-diversity. In agricultural lands, soil bacterial communities show a large divergence between conventional and organic farms [128, 129], between different tillage regimes [130, 131], and between nitrogen

treatments [132, 133]. Therefore, the low β-diversity of bacterial communities seems to be related to similar management conditions at the farms examined in this study, which could cause directional selection and resulting convergence of the communities. Although fungal communities consisted of a smaller number of OTUs (an average of 577) per soil sample and a lower proportion of shared OTUs among the farms than the bacterial communities, α-diversity was still higher than β-diversity and soil communities were still less divergent in the ordination plot (Fig 2-8), suggesting the importance of cultivation management on the structure of fungal communities.

Soil productivity and microbial communities

Soil productivity is one of the most important soil functions and is closely associated with microbial structure and activity [104, 134, 135]. Although soil productivity in agricultural lands is influenced by many factors, the cycling of mineral nutrients such as C, N, and P should play an important role in determining the soil productivity in unfertilized soils [98, 104]. Maize DW was not correlated with C turnover rate but was correlated with N mineralization rate, demonstrating the importance of N cycling in determining soil productivity (Fig 2-4).

Inorganic N in the soil under unfertilized conditions is supplied mainly via organic N mineralization and microbial N fixation. Most inorganic N is released from organic matter in soil during microbial decomposition and a part of the inorganic N is reabsorbed by microbes for their growth [136]. Therefore, active microbial activities may lead to low N mineralization through reabsorption of inorganic N, as shown in Fig 2-4. Carbon turnover rate and N mineralization rate, both of which are derived from microbial processes, are not necessarily correlated with each other. In fact, a significant correlation between soil respiration rate and soil productivity has been reported in some studies [134, 137], but not in others [138].

N fixation is the other source for N supply to soil. N fixation in the soil is performed by free-living bacteria as well as symbiotic bacteria [139]. Rhizobiales, which showed a significant positive correlation with soil productivity, contains various members with N-fixation capabilities [125, 140, 141]. On the other hand, order Solibacterales, which showed a negative correlation with soil productivity, is reported to be a potential indicator for soil degradation [142]. Therefore, soil productivity and supply of inorganic N in soil should be strongly affected by the

composition of microbial communities, especially by the abundance of microbial groups with low demands for N and N-fixing ability.

Differences between maize root and soil microbiomes

Maize root had distinctive communities from the soil, for both bacteria and fungi (Fig 2-8). We observed an increased abundance of the bacterial orders Actinomycetales, Rhizobiales, and Burkholderiales in maize root, which is in accordance with previous studies at rhizosphere [27, 143, 144]. Actinomycetales is reported to confer beneficial functions, including plant disease suppression and plant growth promotion, to host plants [145]. Rhizobiales contributes to nutrient cycling, production of phytohormones, and plant growth promotion [146, 147]. On the other hand, maize root enriched the fungal orders Pleosporales and Hypocreales. These fungal groups contain a large number of plant-associated pathogens [127, 148], which might have negative effects on the host plants [144]. I also found the fungal genus *Pyrenochaeta* belong to order Pleosporales, negative related with maize dry weight (Fig 2-11), which provided more evidence.

It was suggested that the enrichment of genus *Methylibium* from the order Burkholderiales, was through root exudates [149]. While in our study, the abundance of *Methylibium* in endosphere was positively related to maize growth which hasn't been reported. *Streptomyces* species have shown potentials in disease suppression [150, 151], which show negative correlation with maize growth. The fungal genus *Leptodontidium* significantly positively related to maize growth (Fig 2-11), which was reported includes plant growth promoting strains [152]. The fungal genus *Leptodontidium* belongs to order Helotiales which had high relative abundance in soil microbial community (Fig 2-10). *Geopora* includes important ectomycorrhizal associates that can dominate the communities of some plant taxa [153], while it was negative correlated with maize growth in our results. The presence of distinctive microbial communities in plant roots, likely caused by differential ability of various taxa to colonize root tissues, reflects the ecological interactions between host plants and soil microbes.

Conclusion

The present study revealed extremely high diversity and a disproportionate contribution of a small number of phylotypes to the total abundance in soil bacterial and fungal communities. Soil

productivity and its components showed close associations with the abundances and composition of communities rather than with diversity *per se*. I identified four orders in bacteria Rhizobiales, iii1-15, Acidobacteriales and Solibacterales, and one order in fungi Pezizales that showed significant correlations with soil productivity. Since these taxa were shared among all farms, changes in their abundance through agricultural management could contribute to improvements in soil productivity in farms. Although there is still little information about the most effective farm management practices and soil conditions for altering community composition in a way that leads to increased soil productivity, this study could inform further studies on the relationships between microbial communities and soil functions.

FIGURES AND TABLES

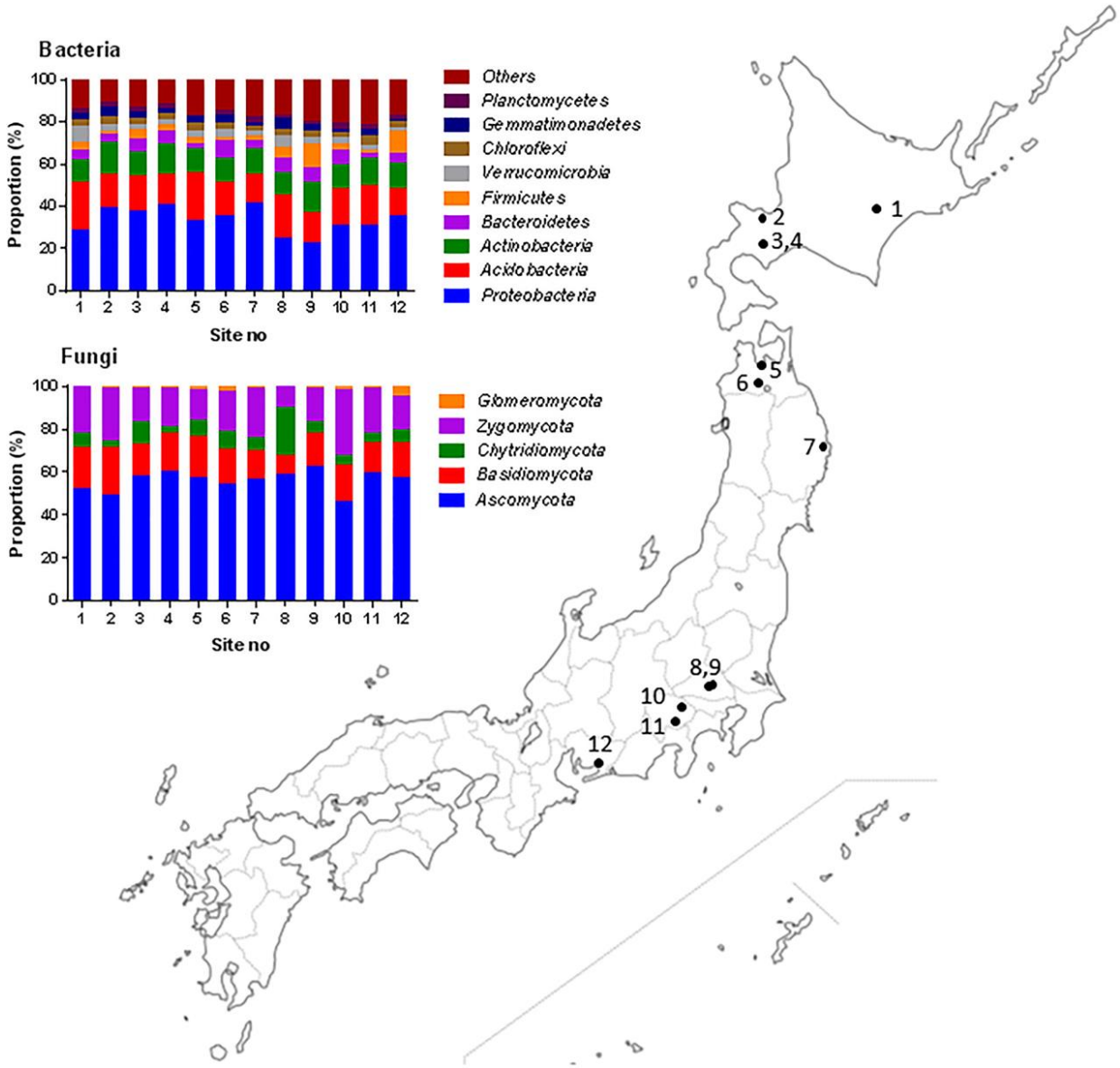


Fig 2-1. Map of the 12 organic farms used in this study and the composition of bacterial and fungal communities at the phylum level in each farm.

Table 2-1. Geographic positions and soil properties in each farm.

Site no	Site code	Latitude	Longitude	Soil [#1] type	Soil pH	Mineralized N (mg 100g soil^{-1})	P_2O_5 (mg 100g soil^{-1})	Soil Organic C (%)	Soil C/N	Microbe C (mg 100g soil^{-1})	C turnover rate (μmol CO_2 m^{-2} h^{-1})	Maize DW (g kg soil^{-1})
1	OR	43.16	140.78	V	5.6	0.8	8.5	2.78	14.12	27.93	1.08	1.61
2	ST	42.87	143.36	F	5.3	4.1	30.1	3.76	12.59	56.86	1.21	4.68
3	M4	42.62	140.79	V	5.9	1.2	12.5	1.31	9.23	28.50	1.16	2.27
4	M9	42.62	140.79	V	6.2	1.5	18.7	2.16	11.64	38.32	1.18	2.91
5	H	40.85	140.67	A	4.7	2	5.1	2.19	10.51	45.66	1.08	2.29
6	N	40.55	140.55	A	6	1.6	23.5	1.80	8.00	36.52	1.23	2.46
7	IZ	39.32	141.45	F	5.9	3.9	29.9	0.91	8.35	28.25	1.47	3.93
8	SE	35.84	139.54	V	5.8	0.1	5	4.23	12.26	61.46	2.53	1.73
9	SI	35.85	139.56	A	6.2	1.5	14.4	2.28	10.28	33.99	1.31	2.53
10	OG	35.9	138.53	V	6.7	4.6	34.5	4.63	11.48	68.87	1.91	3.73
11	Y	35.86	138.39	V	5.8	4.7	1.3	5.78	10.61	97.08	1.95	3.47
12	IM	34.8	137.01	A	6.4	1.4	111.4	1.57	9.55	39.14	1.19	2.53
Significance					***	***	***	***	***	***	***	***
LSD [#2]					0.47	1.3	8.1	0.57	2.42	32.67	1.06	1.19

***: significance at 0.1%

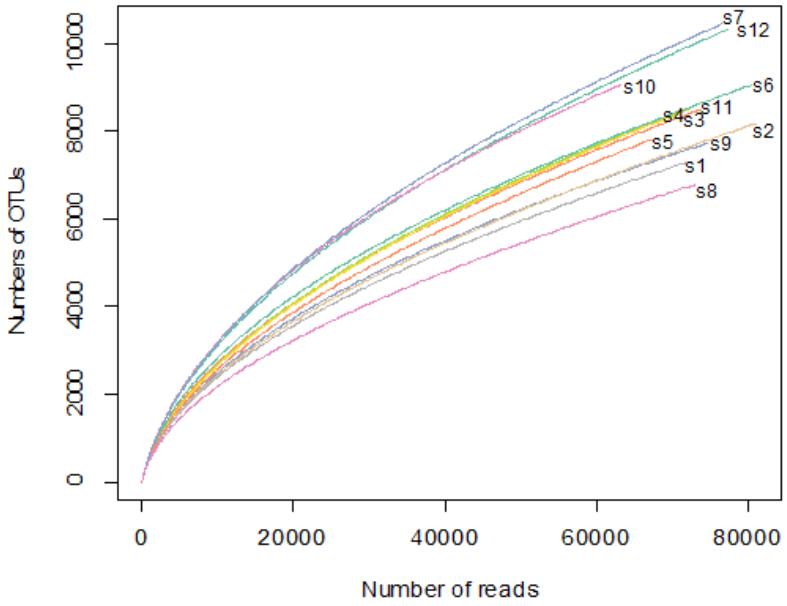
[#1]: Soil type; V: volcanic ash soil, F: brown forest soil, A: alluvial soil

[#2]: Least significant difference (LSD) based on Tukey-Kramer HSD test.

Table 2-2. α-, β-, and γ-diversity, and the number of observed OTUs for soil and root microbial communities. The α-, β- and γ-diversity were calculated at the OTU level.

	Bacteria		Fungi	
	Soil	**Root**	**Soil**	**Root**
α-diversity	5.99	4.54	4.25	2.86
β-diversity	2.48	2.48	2.48	2.10
γ-diversity	8.64	7.02	6.80	4.96
No. of observed OTUs	5337	3791	2569	1082

(A)



(B)

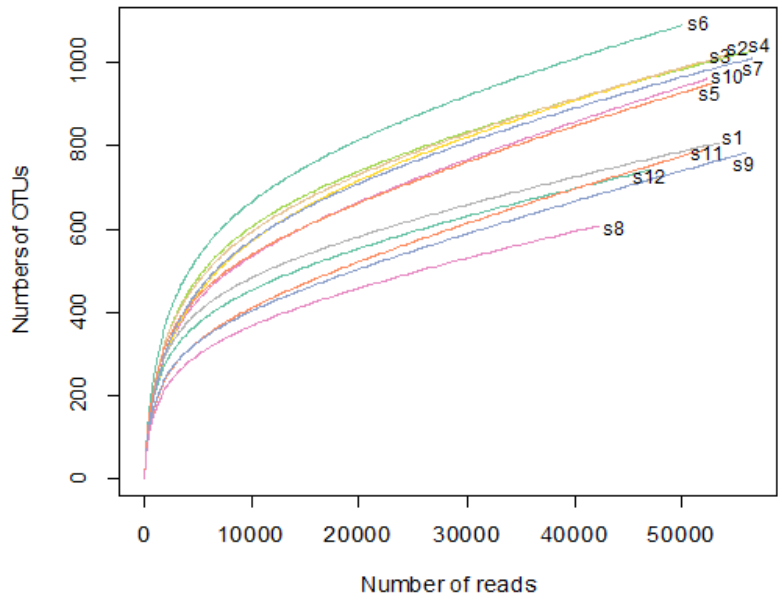


Fig 2-2. Rarefaction curve of soil bacterial 16s rRNA sequences (A) and soil fungal ITS sequences (B).

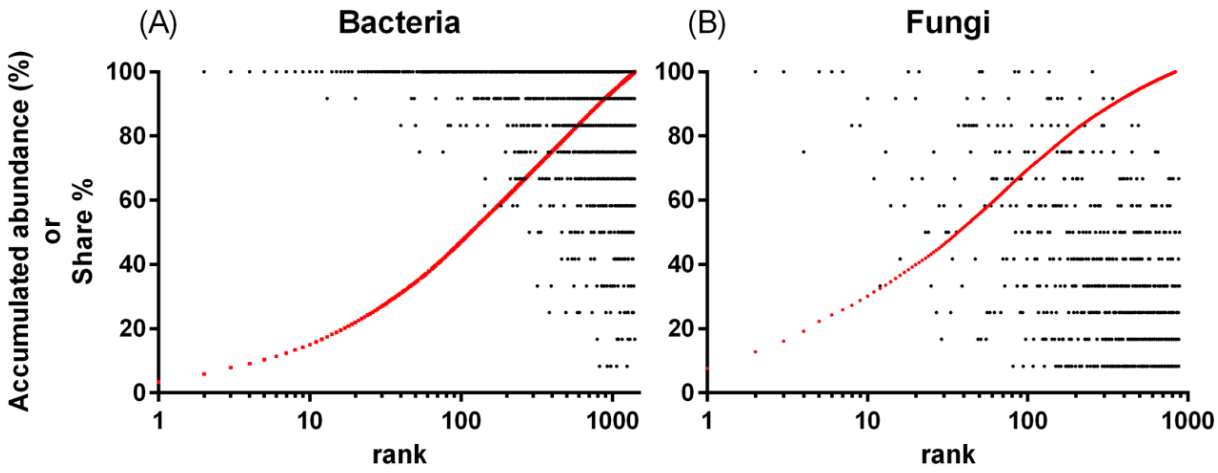


Fig 2-3. Abundance structure and overlap of each OTU in soil bacterial and fungal communities from 12 farms. The proportion of OTUs shared among the 12 farms (black) and the accumulated proportion (red) are shown according to the rank order of each OTU.

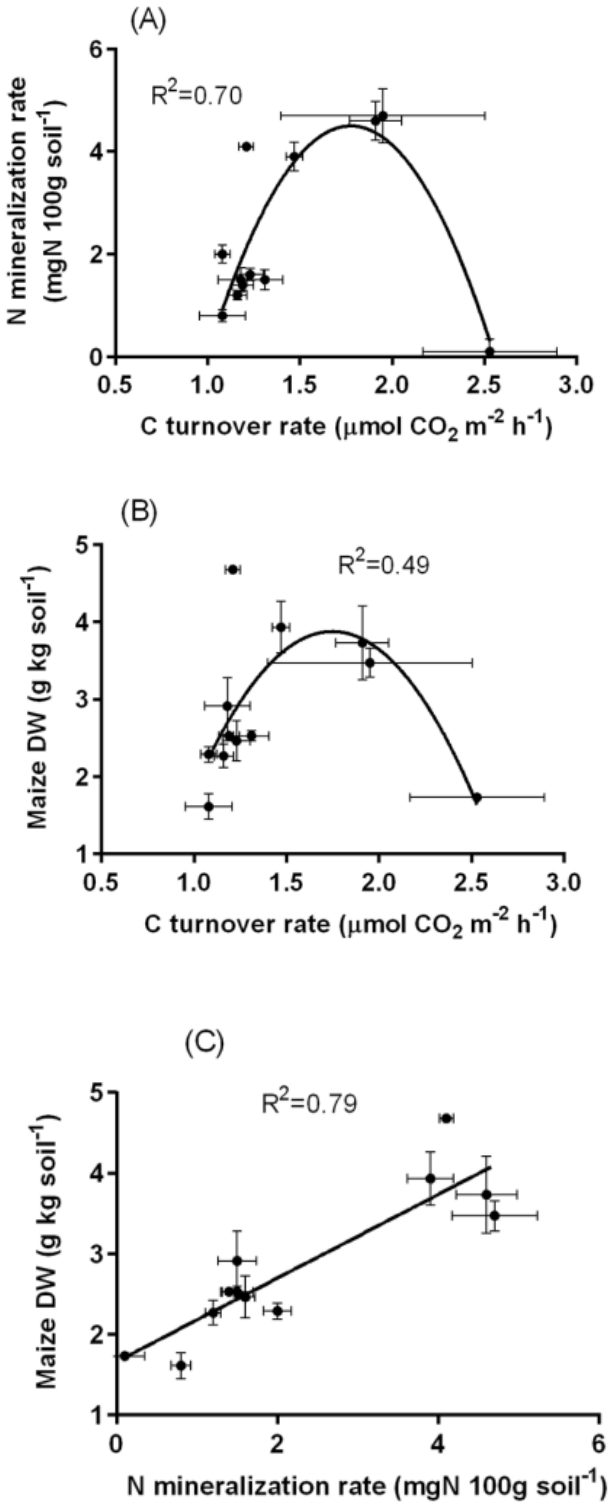


Fig 2-4. The relationships between C turnover rate, N mineralization rate, and maize dry matter weight (DW) in soils from 12 farms.

Table 2-3. Correlation coefficients among eight soil properties and OTU richness in soil bacteril communities. Eight soil properties are mineralized N (Nm), P_2O_5 content, soil organic carbon content (Co), soil C/N ratio, microbial biomass (Cm), C turnover rate (R) and maize dry weight (DW).

	pH	Nm	P_2O_5	Co	C/N	Cm	R	DW
Nm	0.06							
P_2O_5	0.45	0.01						
Co	0.01	0.44	-0.31					
C/N	-0.21	-0.1	-0.26	0.53				
Cm	0.01	0.57	-0.18	0.92***	0.23			
R	0.24	0.15	-0.22	0.68*	0.16	0.67*		
DW	0.08	0.89***	0.15	0.24	-0.1	0.36	0.03	
OTU Richness	-0.5	-0.02	-0.57	-0.07	-0.02	0.02	-0.07	0.06

Table 2-4. Normalized eigen vectors for eight soil properties and the proportion of variation explained (%) by the three PCA components (PC1, PC2, and PC3). The eight soil properties were: soil pH, mineralized N (Nm), P_2O_5 content, soil organic carbon content (Co), soil C/N ratio, microbial biomass (Cm), C turnover rate (R), and maize dry weight (DW).

	PC1	**PC2**	**PC3**
Eigen vectors			
pH	0.04	0.52	-0.83
Nm	0.73	0.65	0.4
P_2O_5	-0.3	0.7	-0.41
Co	1.02	-0.27	-0.13
C/N	0.38	-0.65	0.05
Cm	1.02	-0.02	-0.09
R	0.75	-0.22	-0.56
DW	0.56	0.75	0.44
%	39.6	24.3	16.6

Table 2-5. Results of a permutation ANOVA examining the effect of three PCA components (Table 2-4) and the latitude of farms on the structure of soil bacterial and fungal communities. The evaluation was done by correspondence analysis based on the abundances at three taxonomic levels: phylum, class, and order.

	Bacteria			Fungi		
	Phylum	**Class**	**Order**	**Phylum**	**Class**	**Order**
PC1	0.80	0.68	0.91	0.62	0.92	0.96
PC2	1.26	1.08	2.40*	1.29	0.90	1.85*
PC3	1.17	1.04	2.59**	2.35*	1.69	1.58
Latitude	1.37	1.56	2.00*	1.81	1.17	1.22

The F ratios are shown, along with the significance

* $p < 0.05$;

** $p < 0.01$;

*** $p < 0.001$

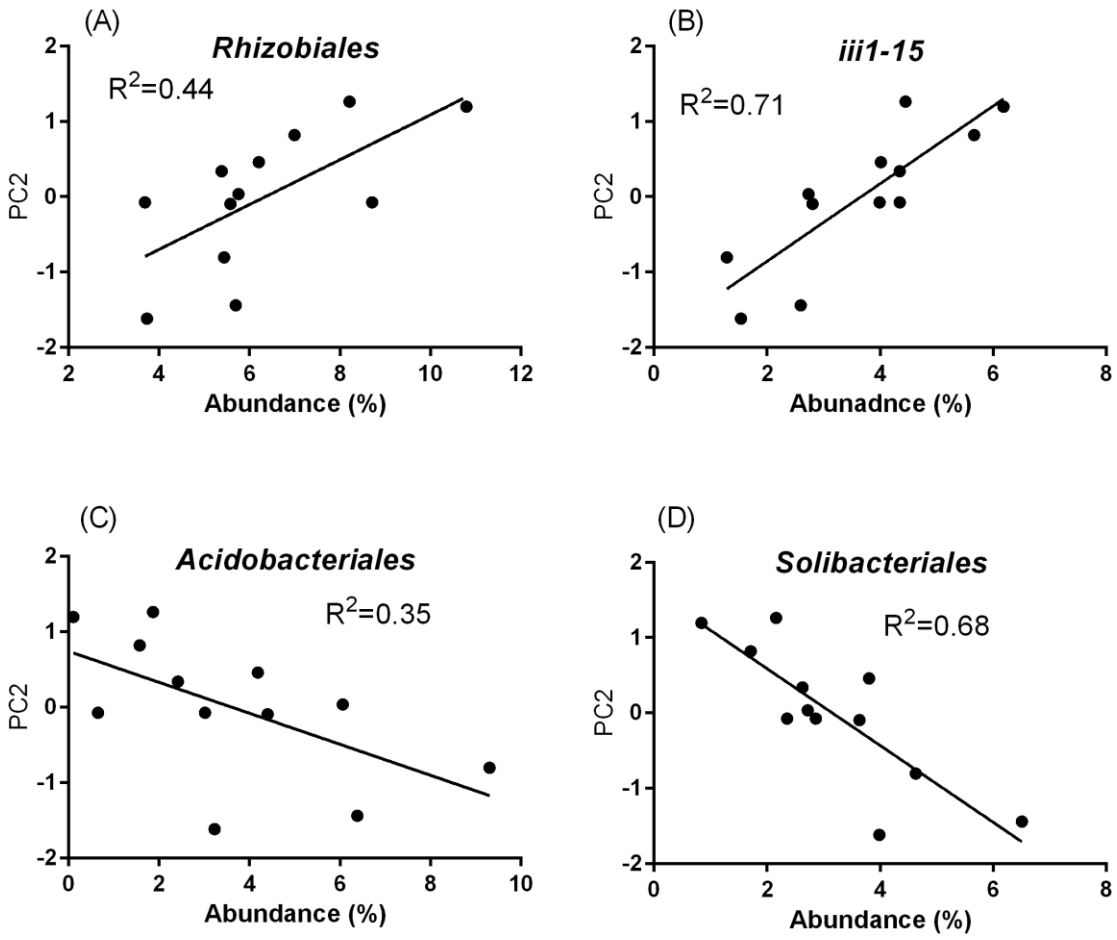


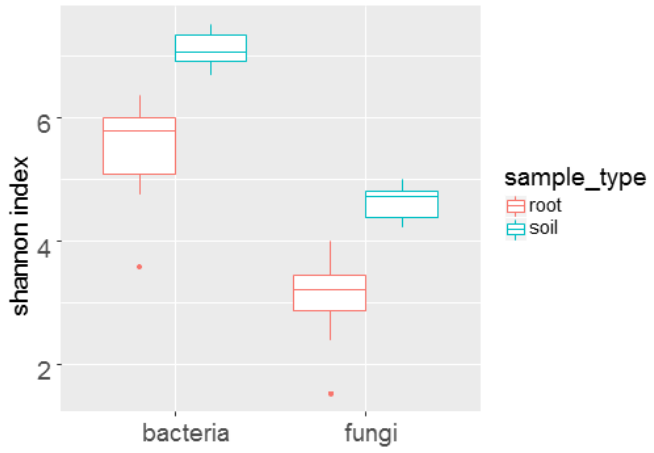
Fig 2-5. The relationships between soil productivity (PC2) and the relative abundance of the four orders (Rhizobiales, iii1-15, Acidobacteriales, and Solibacterales) in soil bacterial communities.

Table 2-6. The relationships of soil properties and the latitude of farms with the whole community structure and the abundance of the top 10 orders in soil bacterial and fungal communities. The three principal components, PC1, PC2 and PC3, which represent soil microbial activity, soil fertility and soil pH, respectively, were used as measures of soil properties. The relationships with the whole community structure were tested by permutation ANOVA (F values) and the relationships with each order were tested by Pearson correlation.

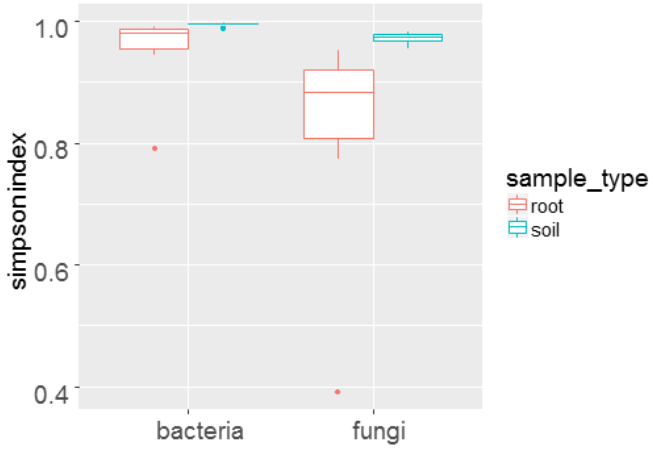
	PC1	**PC2**	**PC3**	**Latitude**
Bacteria				
Whole Community	0.93	3.01*	4.23**	4.31***
Actinomycetales	-0.33	0.18	0.16	0.10
Rhizobiales	-0.20	0.66*	-0.12	-0.03
Sphingomonadales	-0.10	0.03	0.27	0.64*
Burkholderiales	-0.26	0.17	0.53	0.50
Acidobacteriales	0.14	-0.59*	0.64*	0.47
[Saprospirales]	-0.21	-0.15	-0.58*	-0.13
Bacillales	-0.35	0.03	-0.57*	-0.54
iii1-15	-0.09	0.84**	-0.34	-0.31
Solibacterales	-0.08	-0.80**	0.44	-0.62*
RB41	0.16	-0.37	-0.77**	0.50
Fungi				
Whole Community	1.10	2.76**	2.14*	1.32
Mortierellales	0.38	0.50	0.11	0.00
Pleosporales	-0.24	0.30	-0.46	-0.36
Sordariales	-0.10	-0.36	0.56	0.28
Incertaesedis	-0.04	0.11	0.38	0.33
Pezizales	0.28	-0.72	-0.39	-0.23
Saccharomycetales	0.11	-0.55	-0.18	-0.20
Helotiales	-0.14	0.40	0.32	0.37
Cantharellales	0.17	0.42	-0.10	-0.04
Hypocreales	-0.49	0.29	0.06	0.08
Spizellomycetales	-0.52	0.10	-0.11	0.09

*, ** and *** represent significance at 5%, 1% and 0.1% level, respectively.

(A)



(B)



(C)

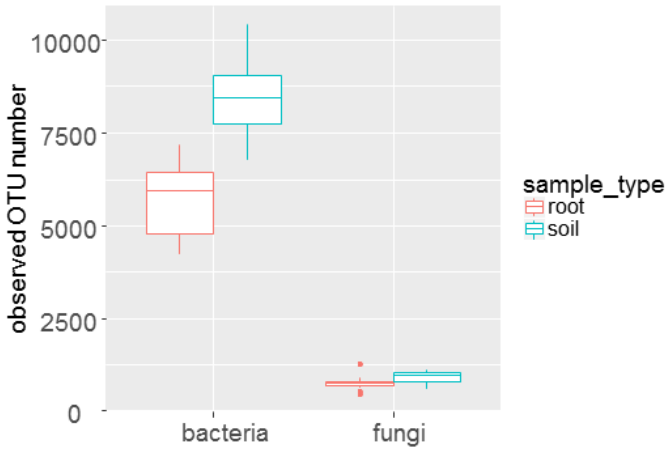


Fig 2-6. α-diversity of microbes in root and soil samples.

Table 2-7. Average Shannon index, Simpson index and observed OTUs numbers of root and soil microbes. Statistical analysis used Paired t-test.

	Bacteria			Fungi		
	Shannon	Simpson	Observed OTUs	Shannon	Simpson	Observed OTUs
Root	5.51	0.96	5696	3.07	0.84	698
Soil	7.09	1.00	8513	4.64	0.97	900
P-value	<0.001***	0.04*	<0.001***	<0.001***	0.01**	0.001**

Table 2-8. Average Shannon index, Simpson index and observed OTUs numbers of bacterial and fungal OTUs. Statistical analysis used ANOVA.

	Shannon	Simpson	Observed OTUs
Bacteria	6.30	0.98	7104
Fungi	3.85	0.90	799
P-value	<0.001***	<0.01**	<0.001***

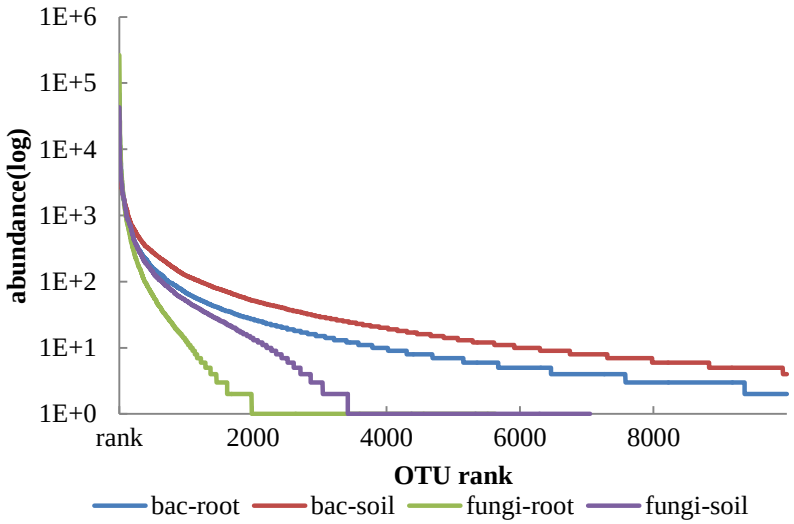


Fig 2-7. Bacterial and fungal OTUs rank-abundance curve of root and soil samples. The main Fig was constructed by the top 10000 abundant OTUs; the top-right Fig was constructed by the top 100.

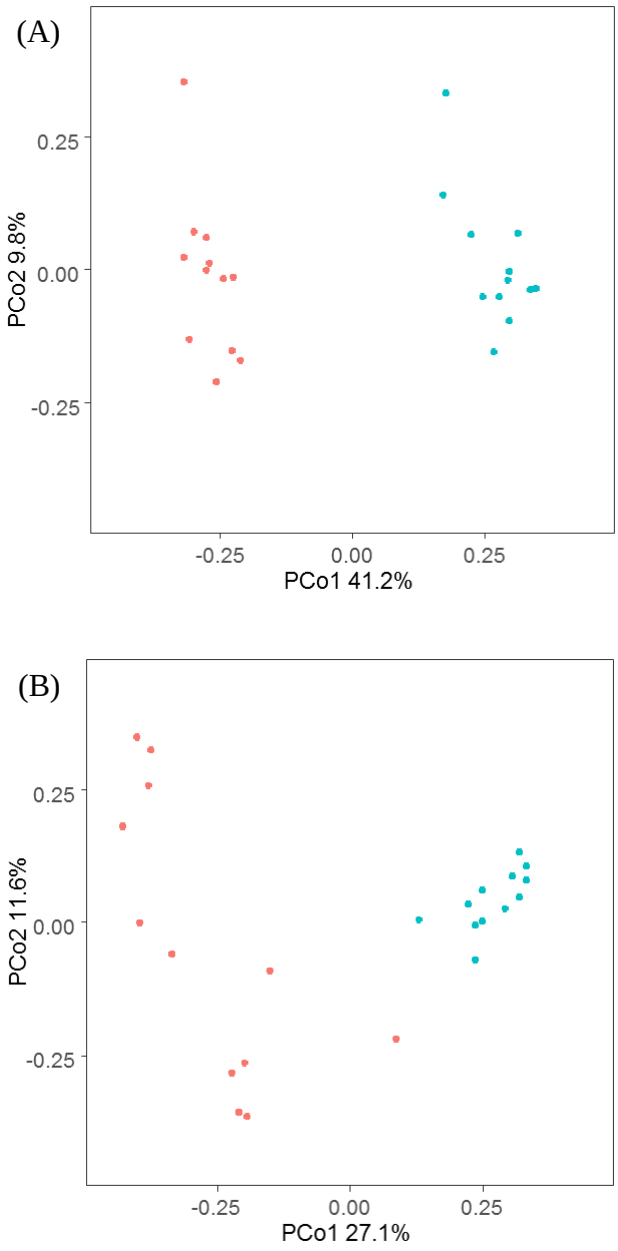


Fig 2-8. Principal coordinates analysis (PCoA) utilizing the Bray-Curtis distances dissimilarity based on relative abundance of OTUs in soil (blue) and root (red) bacterial (A) and fungal (B) communities.

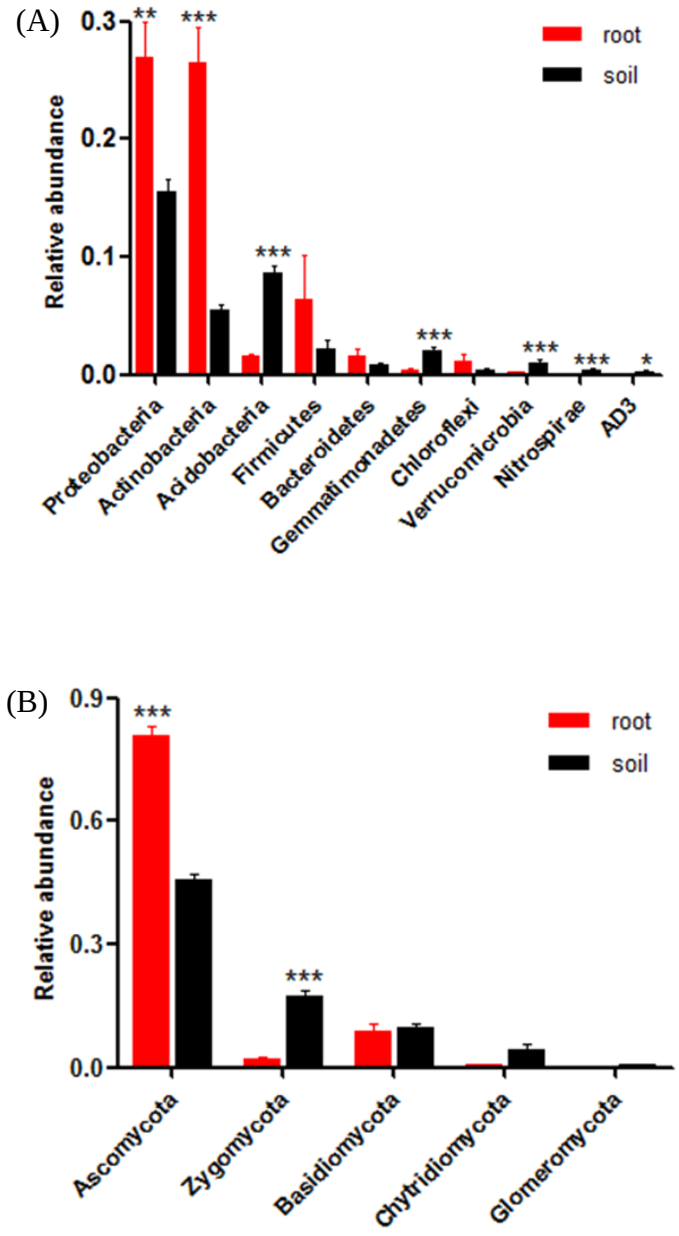


Fig 2-9. The accumulated relative abundance (%) of OTUs at the phylum level in soil (black) and root (red) bacterial (A) and fungal (B) communities. Significant differences between the root and soil communities were detected by a paired t-test (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

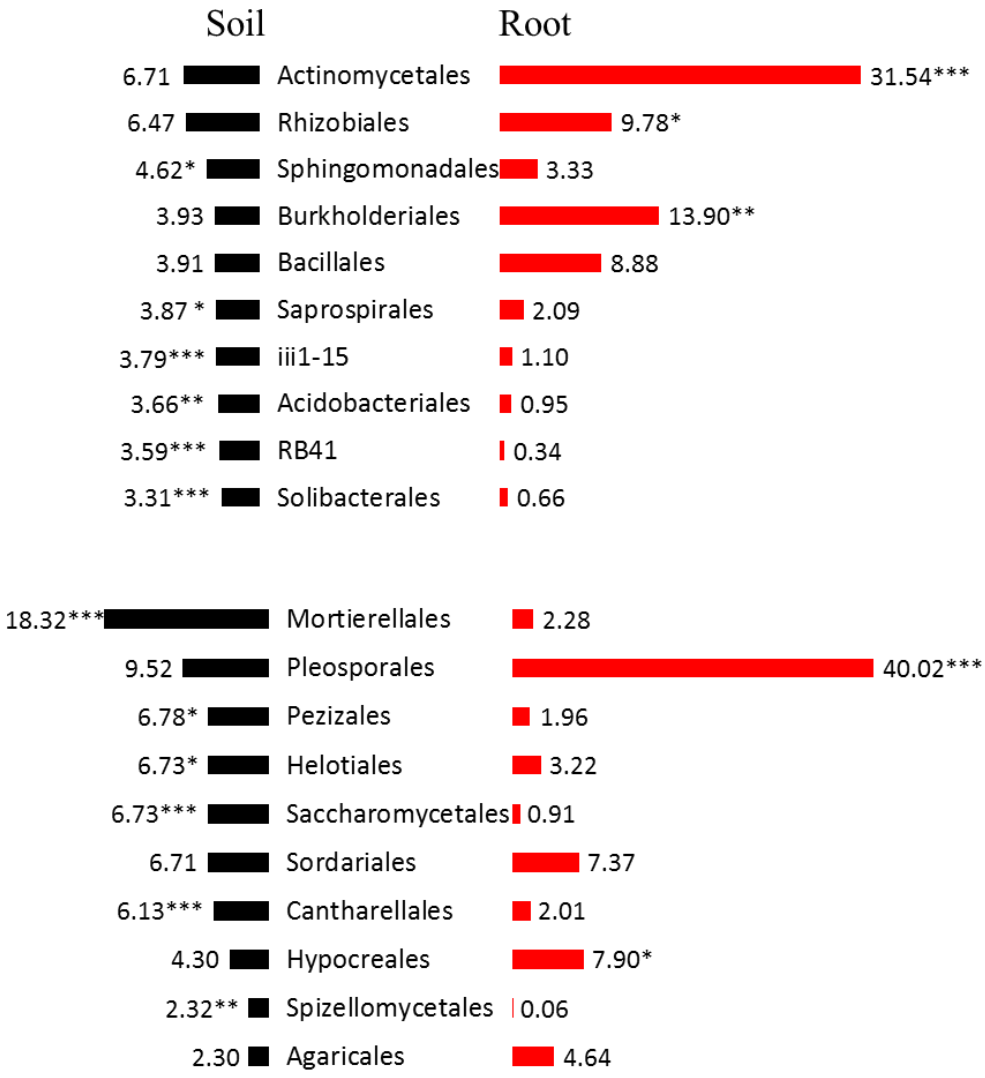


Fig 2-10. The accumulated relative abundance (%) of the top 10 taxa at the order level in soil (black) and root (red) bacterial (A) and fungal (B) communities. Significant differences between the root and soil communities were detected by a paired t-test (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

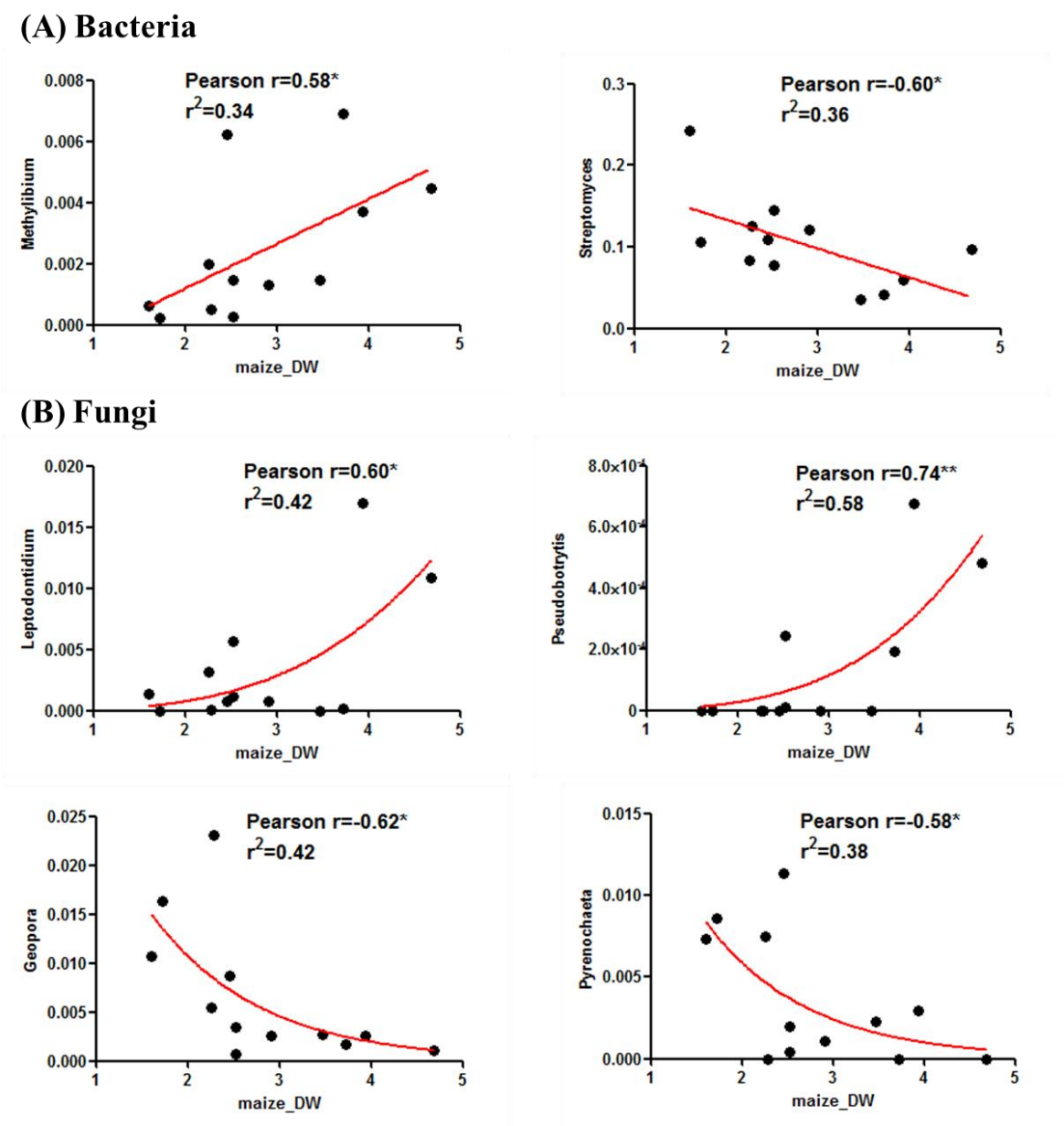


Fig 2-11. Maize growth (maize DW) correlated bacterial and fungal microbes at genus level. Correlation analysis used Pearson's correlation and regression analysis was represented by R-square.

CHAPTER 3: PHYLOGENETIC HISTORY AND ROOT MICROBIOME STRUCTURES IN TWENTY PLANT FAMILIES

Introduction

Plant microbiome is the collective genomes of microorganisms living in association with plants [7], which is an extremely complex microbial community with various effects on host plants. It has been demonstrated that members of plant microbiome contribute to plant growth [8-10], plant nutrition acquisition and productivity [9, 11-13], as well disease suppression [14, 15]. The interactions of plant and microorganisms have stimulated large interests in many academic fields such as plant science and ecology including basic and applied sides.

Plants recruit microbes primarily from potential sources of rhizosphere soil which is being closely touched by plants root. It has been reported that leaf bacterial microbiome shared large proportion with root-associated assemblies in Arabidopsis, grapevine and perennial wild mustard and the high share of constituting microbes between leaf and root suggests that soil as an enormous microorganism library might be a primary source of the whole microbiomes [17-19]. These studies imply that root-associated microbiomes play crucial roles in plant microbiome constitution.

Surveys of root-associated microbiomes suggested several important factors for plant root-associated microbiome constitutions, including soil type [20, 21], geographic locations [22], plant type [23], plant development [19, 24], plant domestication[25, 26], as well as host genotype [19, 20, 27]. On the other hand, the large difference was also observed between compartments of root-soil interface. For instance, the rhizosphere possesses more diverse microbiomes than the endosphere [20, 27, 28], while the majority of entophytic bacterial microbes originate from the rhizosphere where involve root exudates [9]. Furthermore, a study reported the dynamic changes in microbiome composition during microbiome acquisition and suggested that rhizoplane acts as a selective gate to endosphere [22].

It was suggested that plant phylogenetic relation play important roles in structure of root bacterial microbiome in maize and other Poaceae [154] and of phyllosphere bacterial microbiomes of trees [155]. Another study also showed that phylogenetic factor works as a signal on microbiomes structures in marine sponge [156]. However, there is lack of information

on this topic and further investigation is required to fill this gap in root microbiome study. These studies will explore the new understanding of root-associated microbiomes and hint several important functions on host plants by root microbiome.

However, so far studies focused on a narrow range of plant variation such as genotypes within a single species and/ or related species across a broader range of taxonomic range. In addition, most studies paid much attention to bacterial community and neglected fungal community. Furthermore, the recent advancement of next-generation sequencing enables the deeper analysis of complex microbiome studies such as how different host plants structure root endophytic microbiomes from soil.

Based on the past studies and current technical conditions, several queries are posed as follows:

How do plants belonging to various phylogenetic positions structure root entophytic microbiomes from soil microbial community? (2) Do different plants possess divergent root entophytic microbiome assemblies? (3) What factors are responsible for the disparities between different plants? (4) Do plants dominate similar assembling rules in bacterial and fungal microbiomes?

To address these questions, we investigated root microbiomes of 20 plant species, each from different 20 families belonging to 13 orders, grown under identical soil. We assessed the structure of communities both of bacteria and fungi by using next generation sequencing (NGS), by which the sequencing analysis could be generated at greater depth and has a high resolution to detect many low-abundant taxa [84].

Materials and Methods

Plant growth conditions

In this study herbaceous cultivated species including cereals, vegetables and ornamental crops were used because these plants seem to have similar functional types adapted to cultivated fields. Twenty species were selected from 20 different families, *Brassica rapa subsp. Pekinensis*, *Glycine max*, *Abelmoschus esculentus*, *Ipomoea aquatica*, *Basella alba*, *Impatiens balsamina*, *Platycodon grandiflorus*, *Dianthus superbus*, *Celosia argentea*, *Antirrhinum majus*, *Papaver*

rhoeas, *Zea mays subsp*. *Mays*, *Fagopyrum esculentum*, *Solanum lycopersicum*, *Allium fistulosum*, *Cucumis sativus*, *Cryptotaenia canadensis*, *Chrysanthemum coronarium*, *Spinacia oleracea*, *Perilla frutescens* (Fig 3-1; Table 3-1). These species belong to 13 orders (Poales, Asparagales, Ranunclulales, Cucurbitales, Fabales, Malvales, Brassicales, Geraniales, Carophyllales, Lamiales, Solanales. Asterales and Apiales) that cover almost full range of angiosperms phylogeny excluding magnoliids (Fig 3-2).

The seeds of all species were collected from the market. Seeds were surface sterilized by 1% SHS (sodium hypochlorite solution) for 20 min followed by 3 times washing in SDW (sterile distilled water). Sterilized seeds were sown on sterile substrate media in petri dish and incubated in chamber at 22 ℃ with 16 h/8 h day/night period. After one week, five germinated seeds were sown in a single pot (113 mm diameter and 184 mm height) filled with soil on May.

Soils were collected from unfertilized plot of experimental farm in Hirosaki University (40°35'22.9"N 140°28'20.8"E, Hirosaki city, Aomori prefecture, Japan). This plot has been cultivated without any fertilizer and pesticides for five years. The soil is classified as volcanic ash and soil pH (water extraction) was 5.7, organic carbon was 7.89%, organic nitrogen was 0.59%, C/N ratio was 13.44, microbial C was 64.53 mg per 100 g soil and available phosphorous content was 3.0 mg per 100 g soil. The soil was characterized by high organic matter content and low phosphorous availability. The soils were sampled from 0-10 cm layer of three different sites. After sieving through 2 mm mesh to remove roots, macrofauna and rocks, the soils were subjected to plants growing and stored at -80 ℃ for DNA analysis until processing.

Five pots (replicates) were prepared for each of 20 plant species and the control without growing plants and thus total pot number was 105. All pots were raised in a glasshouse after the sowing. Plants were harvest after 7-8 weeks depending on growth rate. At 7-8 weeks after the sowing depending on growth rate, the whole plants including the belowground part were harvested from pot. Soil attaching the root was removed and returned to the pot. After the harvest all pots were used for infection study of Chinese cabbage by clubroot pathogen.

Sample preparation and DNA extraction

The whole plants with attached soil were collected into plastic sampling bag separately. After transferred to the laboratory, root samples were shaken to remove attached soil as much as

possible and washed with tap water, followed by sonication in SDW at 50-60 Hz for 5 min (Ultrasonic Cleaner US-1, AS ONE corporation) to disrupt tiny soil aggregates and attached microbes. Root samples were stored at -80 ℃ until processing.

Root or soil samples of five replicates were mixed together in equal weight and two technical replicates were prepared for each species. Two technical replicates were subjected to followed DNA extraction and sequencing.

Root DNA was extracted by beads-beating and the CTAB (Cetyl trimethyl ammonium bromide) method. Soil DNA was prepared with ISOIL for Beads Beating Kit (Nippon Gene CO., Ltd). Beads beating were processed by Micro Smash MS-100 (Tomy Seiko CO., Ltd)). DNA quantification was assessed with the NanoDrop 2000 (Thermo Fisher Scientific Inc.)

PCR amplification and sequencing

For bacteria, a V4 region of 16S ribosomal RNA (rRNA) was amplified. The primer pair in the 1st PCR amplification was 515F (5′- GTGCCAGCMGCCGCGGTAA -3′) and 816R (5′- GG ACTACHVGGGTWTCTAAT -3′) with adaptors. To reduce the chloroplast and mitochondrial amplification, peptide nucleic acid (PNA) clamps were included in the reaction according to Lundberg [113]. The appropriation PNA concentration for each plant species was detected by Realtime PCR. The PNA concentrations of each plant sample in 1st PCR amplification are shown in Table 1. The 1st PCR conditions were as follows: an initial denaturation at 95 ℃ for 45 s, and 35 cycles at 95 ℃ for 15 s, 78 ℃ for 10 s, and 50 ℃ for 30 s, with a final extension at 72 ℃ for 30 s.

For fungi, the ITS2 region of rRNA was amplified. The primer pair for the 1st PCR was fITS (5′- GTGARTCATCGAATCTTTG -3′) and ITS4 (5′- TCCTSCGCTTATTGATATGC -3′) with adaptors. The 1st ITS2 PCR condition used was: an initial denaturation at 94 ℃ for 2 min, and 25 cycles at 94 ℃ for 30 s, 50 ℃ for 30 s, and 72 ℃ for 1 min, with a final extension at 72 ℃ for 5 min.

After purification by Fast Gene Gel/ PCR Extraction Kit (NIPPON Genetic CO., Ltd) , the 1st PCR products were followed by the 2nd PCR with the primer pair, 2nd-F (AATGATACGGCGA CCACCGAGATCTACAC- Index2 -ACACTCTTTCCCTACACGACGC) and 2nd-R (CAAGC AGAAGACGGCATACGAGAT- Index1 -GTGACTGGAGTTCAGACGTGTG), for both 16S

rRNA and fungal ITS2. The index pairs were specific for each sample, for an accurate recognition of the samples. The 2nd 16S rRNA PCR condition were: an initial denaturation at 94 ℃ for 2 min , 12 cycles at 94 ℃ for 30 s, 60 ℃ for 30 s, 72 ℃ for 30 s, with a final extension at 72 ℃ for 5 min. The 2nd ITS2 PCR condition were: 94 ℃ for 2 min, 12 cycles at 94 ℃ for 30 s, 60 ℃ for 30 s, 72 ℃ for 1 min, with a final extension at 72 ℃ for 5 min. The 2nd PCR products were purified using the AMPureXP magnetic beads (Beckman-Coulter, Indianapolis, IN, USA) and pooled in equimolar ratios. After confirming the library quantity, paired-end 2 x 250 bp sequencing of the barcoded amplicons was performed on a MiSeq machine (Illumina Inc, San Diego, CA, USA).

Sequence processing

The sequences obtained from the MiSeq were processed through a custom pipeline developed at the Bioengineering Lab. Co., Ltd. (Atsugi, Japan). The raw reads were demultiplexed based on the barcode sequences and filtered by exact matching using Fastx toolkit (fastq_barcode_splilter). If the quality score was less than 20 and the sequence length was less than 40 bases, the reads were discarded. Paired-end reads with minimum 10 base overlap were merged into full-length sequences by FLASH. Merged sequences between 246 and 260 bases were used for later processing of 16s rRNA. The UCHIME algorithm was used for detecting chimeric sequences. OTU generation and phylogenetic assignment were conducted by QIIME script with default conditions. OTUs were clustered using UCLUST at a 97% similarity level by a de novo picking method, using Greengenes 16S reference database for 16S V4 data and UNITE reference database for ITS2 data. To get filtered OTU dataset, all OTUs assigned to Archaea, chloroplast, and mitochondria were discarded from the 16S dataset, and only the OTUs assigned to kingdom Fungi were reserved for the ITS2 dataset. The filtered datasets were then normalized by transforming the number of OTU counts to relative abundance. To remove low abundance OTUs, we used 0.5% as threshold, which means only OTUs possess at least 0.5% in at least one sample were reserved for further analysis.

Statistical analysis

As spurious sequences and unrepresentative OTUs decrease the reproducibility for community assemblage [120], the generated OTUs were filtered at the threshold of 0.5% of the total abundance, and the OTUs above this level were used for the statistical analysis.

Shannon-weaver diversity index was used as a measure of α-diversity (diversity within sample) using the R package Vegan. The richness of community (the number of observed OTUs) was also used as a measure of α-diversity. The β-diversity (diversity between samples) was obtained by Bray-Curtis dissimilarity based on relative abundance by the function "vegdist" in the R package Vegan. Microbial community structure was analyzed by principal coordinate analyses (PCoA). The Bray-Curtis distances matrix was calculated from the relative abundances of OTUs and then PCoA scores were calculated using the "pcoa" function in the R package Ape. Permutational multivariate analysis of variance (PERMANOVA) was performed with the function "adonis" in the R package vegan. The difference between plant families, as well between sample types (root and soil) were tested by ANOVA. The change in relative abundance between soil and root was tested for each OTU using paired t-test. Heatmap were constructed using custom scripts and the function heatmap.2 from the R package gplots. For better visualization, all data was Log_2 -transformed.

To construct phylogenetic relationship among 20 plant species, ribulose-bisphosphate carboxylase (*rbcL*) gene present in chloroplast was used, because *rbcL* sequence can establish reliable phylogeny at the family level [157, 158]. Chloroplast *rbcL* DNA sequences of 20 plant species were downloaded from GenBank, their sequences were aligned and pairwise evolutionary distances was calculated by the proportion of nucleotide differences between each pair of 20 species in MEGA v. 6.0 (2). Phylogenetic tree was generated by maximum likelihood method with 1000 times bootstrap replications. Dissimilarity of the whole community was calculated from Bray-Curtis distance based on relative abundances of OTUs. Manhattan distance based on the relative abundance of single OTUs was also calculated to detect phylogenetic signal of each OTU. Mantel correlation was calculated between evolutionary distance based on *rbcL* sequence and dissimilarity matrix of community structure. Dendrogram was constructed by hierarchical cluster analysis by the function "hclust" in the R package. Clustering was conducted by unweighted pair group method with arithmetic mean (UPGMA).

To test the relationship between plant phylogeny and root microbiomes, mantel correlation between evolutionary distance and community distance was performed by the function "mantel()" in the R package vegan. We also compared the topology of the two trees by the functions "dendlist()" and "tanglegram()" in the R package dendextend. To visualize connections between the two dendrograms cluster rotation on the hinges were performed without changing their

topology and a tanglegram plot was created by crossing lines. The Fowlkes-Mallows (FM) index was calculated to test the similarity of species arrangement between the plant phylogenetic tree and tree of microbiome structure[159]. FM index ranges from 0 to 1, and a higher value indicates a greater similarity between the plant phylogenetic evolution and root microbiomes. Network files and statistics of root enriched and depleted OTUs were generated by make_otu_network.py script in QIIME and passed into Cytoscape for networks generation. The correlation-based network was analyzed by the CoNet app in Cytoscape v.3.6.1.

Results

Structure of root microbiomes in 20 plant families

In this study, 145,181 OTUs for bacteria and 17,653 OTUs for fungi were detected. Mean observed OTU number of root community per sample were 6,241 for bacteria and 1,009 for fungi, while that of soil communities were 8,989 for bacteria and 1,242 for fungi. Bacteria community had higher OTU richness than fungal communities. OTU richness showed a significant difference among 20 plant species for fungi but not for bacteria. However, most OTUs contain only a few reads. OTUs that contain less than five reads occupied 94% of the total OTUs for bacteria and 90% for fungi. Root samples possessed significantly lower Shannon index than soil samples in both bacterial ($p=0.002^{**}$) and fungal ($p=0.048^{*}$) communities (Fig 3-3a, b; Table 3-2). In addition, root microbiomes in dicots were significantly higher than those of monocots.

Heatmap representing abundance pattern of OTUs showed that abundant OTUs were inconsistent between 20 plant species in bacterial communities but tended to be consistent over 20 plant species in fungal communities (Fig 3-4). OTUs that were both enriched or depleted in root compared with soil OTUs, tended to be less common among 20 plant species in bacterial communities than in fungal communities. Monocots had similar bacterial communities. Consequently, soil sample showed separated from root samples in bacteria, but was clustered together in fungal communities. These results show that bacterial root microbiomes have distinctive composition from soil community while fungal root microbiomes have similar structure to soil community.

PCoA analysis (Fig 3-5a) showed the second axis (PCo2) separated the soil sample from the root samples in bacterial communities. Acidobacteria and Gemmatimonadetes characterized the differences across PCo2. On the other hand, the first axis (PCo1) separated 20 plant species, especially monocots. Bacterial phyla, Betaproteobacteria, Chloroflexi, Bacteroidetes and Actinobacteria characterized the differences in PCo1 (Fig 3-5a; Fig 3-6a).

0.5% of the relative abundance was set as a threshold level and the OTUs below this level were omitted from the analysis. The OTUs obtained were 161 for bacteria and 130 for fungi and these OTUs occupied 70.0% of the total abundance for bacteria and 92.1% for fungi. Root bacterial communities had 119 enriched and 45 depleted OTUs compared to bulk soil (t-test, $p<0.05$) (Fig 3-7). Among them, 28 of enriched OTUs and 17 of depleted OTUs were species specific (yellow) among 20 plant species. Most enriched OTUs were shared with only a few plant species, but depleted OTUs tended to be shared by many species (Fig 3-7, Fig 3-8). These results suggest that 20 plant species had different OTU preference when recruiting microbes from soil and this difference characterized difference in bacterial root microbiomes among plant species.

Fungal communities were separated in three groups across the first and the second PCoA axis and soil sample was clustered within root samples (Fig 3-5b). Zygomycota, Ascomycota and Glomeromycota characterized the differences. The compositional difference of root microbiomes reflected the score distribution in PCoA (Fig 3-5b; Fig 3-6b).

These results suggest that 20 plant species structured their root bacterial and fungal microbiomes from soil microbial community with different pattern, and bacteria had more different structure of microbiomes than fungi.

Plant host effects on microbiome assembly

Since closely related organisms share more genes than distantly related organisms, comparison between species has been examined with consideration for their phylogenetic relations. However, since host plants and root microbiomes do not share common genes, it is assumed that root microbiomes are assembled independently from host plant phylogeny. I examined how plant phylogenetic evolution influences assembly of root microbiomes by three methods: (1) mantel correlation between evolutionary distance of host plant and dissimilarity of microbiome, (2)

comparison of topology between plant phylogenetic tree and microbiome dissimilarity tree and (3) variation partitioning of community structure between and within taxonomic groups. The evolutionary distances among host plants estimated from chloroplast *rbcL* genes showed a significant correlation with community dissimilarity of root microbiomes for bacteria (r=0.44**; Fig 3-9a) but not for fungi (r=-0.03, Fig 3-9b). The significant mantel correlation was found among 18 plant species within dicot (r=0.27*; Fig 3-9c), although the correlation became smaller. These results indicate that closely related plants have more similar roots communities than distantly related plants in bacteria, but not in fungi.

The two trees, phylogenetic tree of host plants and microbiome tree based on compositional dissimilarity, were arranged through cluster rotation on the hinges without changing their topology (Fig 3-10). Twenty plant species tended to take similar positions in the two trees as shown by the lines connecting the identical species between the two trees. The entanglement index of connecting lines, which ranges from 0 (no entanglement) to 1.0 (full entanglement), was 0.17, showing the plant species have small entanglement between the two trees. The Fowlkes-Mallows Index, a measure of similarity of species position between two trees, was 0.409, which rejected the null expectation of random assortment of 20 plant species between two trees ($p<0.05$). This result also demonstrates high association between phylogenetic tree of host plants and assembly of bacterial root communities.

Community ordination analysis can recapitulate structural differences of microbial communities. The PCoA analysis revealed that twenty plant species separated across the first axis both for bacterial and fungal communities (Fig 3-5). The first PCoA scores showed a significant mantel correlation with evolutionary distance of host plants (r=0.43**) for bacteria but not for fungi (Fig 3-11). Twenty plant families explained 97% of the total variation in the first PCoA scores for bacteria and 83% for fungi. This variation was further partitioned into between-class, between-order, and within-order base on phylogenetic information of twenty plants (Table 3-1). The proportions explained by the between-class, between-order and within-order variation were 52.5%, 33.9% and 10.5% for bacteria and 0.01%, 53.1% and 29.5% for fungi (Fig 3-12). The bacterial community had higher contribution between the class level and smaller contribution within order than fungal community. These results show that the assembly of bacterial root community is more strongly regulated by phylogenetic hierarchy than fungal community.

Key microbes responsible for phylogenetic effects

Next, I identified bacterial members responsible for host plant phylogeny effects. The mantel correlations of community dissimilarity at class level revealed that the two class, Betaproteobacteria and Gammaproteobacteria, showed significant correlations with evolutionary distance (r=0.433, p=0.013*; r=0.404, p=0.045*) as shown in Fig 3-13. At the correlation at OTU level, eight OTUs showed significant correlations with evolutionary distance (r>0.4, p<0.05*). These OTUs belonged to the class, Betaproteobacteria, Gammaproteobacteria, Alphaproteobacteria, Chloroflexi and Sphingobacteriia. As to 18 dicot families, significant correlations were found for 18 OTUs, which belong to the class, Chloroflexi, Gammaproteobacteria, Armatimonadia and Anaerolineae. Phylum Proteobacteria and classes Betaproteobacteria and Gammaproteobacteria were significantly abundant in monocots than in dicots. The classes Alphaproteobacteria and Chloroflexi were significantly abundant in dicots than in monocots.

The mantel correlation can identify important taxa responsible for host plant effects on changes in root microbiomes but cannot uncover the whole pictures of their change along phylogenetic divergence. I investigated the abundance changes of major bacterial taxa along the PCo1 score since this axis significant correlated with evolutionary distance and thus represents arrangement of plant families across phylogenetic divergence. Four major classes, Betaproteobacteria, Actinobacteria, Gammaproteobacteria and Alphaproteobacterial, occupied 84.5% of the total abundance and 68.1% of total number of OTUs. Betaproteobacteria, the most abundant class, showed a significantly negative correlation with PCo1 (Table 3-3; Fig 3-14). At OTU level, three OTUs belonging to the genus, Burkholderia and Ralstonia, showed significantly negative correlations, while two OTUs showed significantly positive correlations with PCo1. The OTUs in Betaproteobacteria responded to host plant phylogeny in both positive and negative directions. Actinobacteria showed a positive correlation with PCo1 (r=0.42**). One OTU belonging to Streptomyces showed a highly positive correlation with PCo1 and most other OTUs also showed positive correlations although they are not significant. Gammaproteobacteria showed a negative correlation with PCo1 (r=-0.41**) and two OTUs had significantly negative correlations. Although Alphaproteobacteria did not show a significant correlation with PCo1 at bacterial class level, one negatively and two positively significant correlations were found at OTU level. Many OTUs showed correlations with contrasting directions.

There were other classes also showed significant correlations with PCo1 (Table 3-3), including [Saprospirae], Mollicutes, Chloroflexi, Anaerolineae, Opitutae, Deltaproteobacteria, Cytophagia, Armatimonadia, Acidobacteria-6, [Chloracidobacteria], Gemmatimonadetes and Chloroflexi, although these classes didn't occupy large proportion of the total abundance. Six of them had absolute correlation coefficients higher than 0.4. As the Fig 3-15 shows, Betaproteobacteria not only negatively correlated with PCo1 scores, but also the other five classes which had positive correlations with PCo1 scores. These microbes correlated with each other and some other classes. However, the Gammaproteobacteria didn't closely correlate with other classes. These results demonstrate that specific bacterial groups have gained directional preference for specific plant taxa along plant phylogeny and interact together.

Despite significant differences among 20 plant species, fungi OTUs did not show significant correlations with evolutionary distance except three (Fig 3-16). However, mycorrhizal symbiotic association influenced the abundance of some taxa (Fig 3-17). The fungal class Sordariomycetes ($p=0.024$) and Glomeromycetes ($p=0.020$) showed significantly higher abundance in mycorrhizal plants than nonmycorrhizal plants. While the taxa assigned to Pezizomycotina ($p=0.006$), was significantly abundant in nonmycorrhizal plants than mycorrhizal plants. These results suggest mycorrhizal symbiotic association impacted the plant root fungal microbiome constitutions.

Discussion

The results of this study revealed the variation in structure of root microbiomes both in bacteria and fungi among 20 plant families covering the most range of angiosperm phylogeny (Fig 3-1, 3-2). Particularly, I focused how different plants recruit microbial members from potential microbial pool in soil and how plant phylogeny influences assembly rule of root microbiomes.

Root endophytic microbiomes and plant phylogenetic history

There was a large disparity in Shannon diversity, especially in bacterial microbiomes, among 20 plant families (Fig 3-3), showing that different plant species were characterized by distinctive root microbiomes in both bacteria and fungi (Fig 3-5a, 3-6a). Furthermore, abundant OTUs consisting of community were inconsistent among plant species (Fig 3-4a). OTUs enriched in the

root relative to the soil were shared by few plant species (Fig 3-7a, 3-8) and thus 20 plant species had a large difference in preference for OTUs in bacteria. Consequently, the proportion of the variation in microbiome composition explained by plant species reached to the high proportion (83.0%) for bacteria. These results agreed with other studies reporting important effects of host plants on structure of root microbiomes [25, 160], although a larger effect of season rather than of host plants on root microbiomes was reported in Agave [161]. On the other hand, fungi showed more consistent enrichment and depletion pattern of OTUs in root microbiomes than bacteria (Fig 3-4b). However, plant species was still an important determinant of root microbiome variation, which explained 72.1% of the total variation (Fig 3-5b). The result also agreed with other study reporting important effects of host plants on root fungal communities [162].

One of the important results in this study is that the large interspecific variation in bacterial root microbiomes was strongly controlled by plant phylogenetic evolution. I constructed phylogenetic tree of 20 plant families based on chloroplast *rbcL* gene, which has been widely used for inferring plant phylogenetic relations above the family level [163]. The comparison between plant phylogenetic tree and tree of root bacterial microbiome dissimilarity revealed a strong connection of two trees (Fig 3-10). Mantel test showed the significant correlation between the evolutionary distance and dissimilarity distance of root bacterial communities ($r=0.44$; $p=0.004$**; Fig 3-9a, 3-9c). The significant correlation was also found among only dicot plants ($r=0.27$; $p=0.041$*; Fig 3-9c). These results agreed with the study reporting important effects of host plant phylogeny on root microbiome structure [154, 164]. However, these studies examined small range of angiosperm phylogeny such as plants including only 6 families or less, and thus it was not well clarified how root microbiomes are influenced by plant phylogenetic relations.

In contrast, by covering a wide range of phylogeny, 20 plant families belonging to 13 orders included in the two classes (monocots and dicots), I found firm evidence showing close association between plant phylogenetic evolution and constitution of bacterial root microbiomes. The significant correlation was also detected among 18 species only within dicots. Among OTUs, those belonging to the class Betaproteobacteria tended to have significant correlation with evolutionary distance ($r=0.433$, $p=0.013$*). The Betaproteobacteria has been reported to include members with functions such as nitrogen fixing, nutrient cycling and plant growth promotion [165, 166]. Members of Gammaproteobacteria also show significant correlation ($r=0.404$,

p=0.045*), which was also reported to have function of nodule formation of legumes [167]. The phylum, Chloroflexi, which contains a diverse assemblage of organisms with various metabolic lifestyles [168], was significantly correlated with phylogenetic dissimilarity of dicots (r=0.372, p=0.037*). These bacterial groups seem to gain nitrogen-fixing symbioses several times along land plants evolution [169]. I speculate that nitrogen fixing ability might play a crucial role in evolution of root bacterial microbiome constitutions.

Selection of root endophytic microbiomes from soil microbial community

I found soil microbial community is more diverse than root endophytic microbiomes in both bacteria and fungi (Fig 3-3, Table 3-2), which support the idea of soil as primary microbial source for plant establishing entophytic bacterial microbiomes [17-19, 62]. Although soil and root endophytic microbiomes shared many bacterial taxa, great variation was detected between soil and root bacterial microbiome constitutions (Fig 3-4a, 3-6a), which support previous studies [20, 22, 25]. Bacterial phyla Acidobacteria and Gemmatimonadetes were significantly depleted in root communities, Firmicutes also showed low relative abundance (Fig 3-5a, 3-6a). Furthermore, most OTUs depleted in the root were shared by different plants, while OTUs enriched in root were less shared between many species (Fig 3-7, 3-8), which suggest plants might share disfavored bacteria in the root but not for preferred OTUs. These results support the idea that rhizosphere and/ or rhizoplane play selective role in acquisition of endophytic bacteria by the root [22], and suggest that plants might have the capability of screening disfavored and selecting preferred bacterial microbes.

As to the fungal community, soil community showed similar constitution with root communities (Fig 3-4b, 3-5b, 3-6b). Our results partially agreed with the studies reporting that plant genotypes have significant effects on fungal endophytic communities of A. thaliana, but not that the endosphere plays obvious roles on selecting fungal microbes [170]. In addition, fungal endophytes are suggested to have functions influencing plant health through bioactive compounds [171].

Microbiome structure of monocots and dicots

From our study, monocots and dicots showed a large divergence in root microbiomes of bacteria. Root microbiomes in dicots were significantly diverse than those of monocots (Fig 3-3a,

Table 3-2). Other study also found higher richness of microbial community in herbaceous than in monocot grasses [172]. Monocot and dicot largely differed in composition of root microbiomes (Fig 3-4a, 3-5a, 3-6a). Bacterial phylum, Proteobacteria (Betaproteobacteria) were significantly abundant in monocots than in dicots (Fig 3-5a, 3-6a), which agreed with the study showing a slight difference in the abundance of Proteobacteria between dicots and monocots [172]. Chloroflexi was significantly abundant in dicots than in monocots (Fig 3-5a, 3-6a), which has been suggested evolving in anoxygenic photosynthesis system [173-175]. Monocots and dicots exude different compounds from root under the condition of iron deficiency. Monocots release phytosiderophores to chelate with Fe while dicots release protons to increase rhizodermal cells and resulting reducing capacity [176]. These functional differences between monocot and dicot may cause different assembly of root microbiomes.

Mycorrhizal symbiosis and fungal microbiome

In contrast to bacterial root microbiomes, fungal root microbiomes did not show a significant mantel correlation with evolutionary distance at the whole community level (Fig 3-9b, 3-9c), as well as at lower taxonomic level each OTU level (Fig 3-13). However, the presence or absence of mycorrhizal symbiotic association had great effects on structure of fungal community. The abundances of Glomeromyco and Sordariomycetes were significantly greater in mycorrhizal plant families than in non-mycorrhizal families (Fig 3-17). The fungal class, Glomeromycota, includes members involving into arbuscular mycorrhizal (AM), which is the prime candidate interacting with the first terrestrial plants [177]. Sordariomycetes were reported as pathogens and endophytes of plants involved in decomposition and nutrient cycling [178]. Moreover, members of Pezizomycetes are widely exist in many plants [179]. Many taxa belonging to Pezizomycetes have been recognized as ectomycorrhizal symbionts [180, 181] and as root symbionts of orchid [182]. Nevertheless, I detected significant higher abundance of Pezizomycetes in non-mycorrhizal plants than in mycorrhizal plants (Fig 3-17 b).

The fitness conflict between host plants and symbionts has been proposed and alters with host genotypes and ecological factors [171]. Mycorrhizal symbiotic association is host specific and has been elaborated during the evolutionary co-adaptation with partners [183]. Although plant phylogenetic evolution didn't play important roles in the assembly of fungal microbiomes, the

present study suggests that plant symbiotic association with mycorrhizae seems to play important role in structuring fungal microbiomes across plant phylogenetic evolution.

Conclusion

Twenty plant families had distinctive root microbiomes compositions from soil microbial community in bacteria, but had similar microbiome compositions in fungi. Both plants root and soil microbiomes were more diverse for bacteria compared to fungi. Plant root microbiomes largely diffused among plant species even when grown under identical soil and had different assembly pattern in bacteria and fungi. Plant phylogenetic divergence has great effects on bacterial microbiome assemblies. Mycorrhizal symbiotic association plays a role in structuring fungal microbiomes across plant phylogenetic evolution.

FIGURES AND TABLES

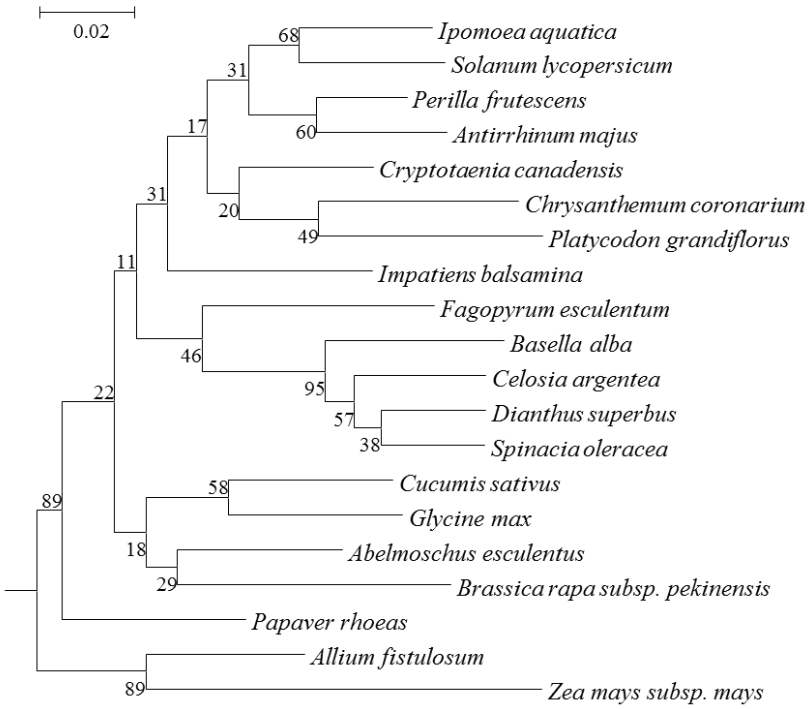


Fig 3-1. Phylogenetic correlation of twenty plant species used in the experiment. The phylogenetic tree was generated based on plant chloroplast *rbcL* gene by maximum likelihood analysis, 1000 times bootstrap replications. Binomial Nomenclature was used to represent each plant.

Table 3-1. Information of twenty plant species used in the experiment including basic information, taxonomic information and PNA concentration used in 16s rRNA 1st PCR program.

No.	Abbr.	Common Name	Class	Order	Family	Genus	Species	PNA
1	I.A.	Water spinach	E	Solanales	Convolvulaceae	Ipomoea	Aquatica	2.5
2	S.L.	Tomato	E	Solanales	Solanacea	Solanum	Lycopersicum	2.5
3	P.F.	Perilla	E	Lamiales	Lamiacea	Perilla	Frutescens	2.5
4	A.M.	Snapdragon	E	Lamiales	Plantaginaceae	Antirrhinum	Majus	2.5
5	Cr.C.	Canadian honewort	E	Apiales	Apiacea	Cryptotaenia	Canadensis	2.5
6	Ch.C.	Crown daisy	E	Asterales	Asteraceae	Glebionis	Coronaria	5
7	P.G.	Platycodon	E	Asterales	Campanulaceae	Platycodon	Grandiflorus	2.5
8	I.B.	Garden balsam	E	Geraniales	Balsaminaceae	Impatiens	Balsamina	2.5
9	F.E.	Buckwheat	E	Caryophyllales	Polygonaceae	Fagopyrum	Esculentum	5
10	B.A.	Creeping spinach	E	Caryophyllales	Basellaceae	Basella	Alba	2.5
11	C.A.	Plumed cockscomb	E	Caryophyllales	Amaranthaceae	Celosia	Argentea	5
12	D.S.	Fringed pink	E	Caryophyllales	Caryophyllaceae	Dianthus	Superbus	5
13	S.O.	Spinach	E	Caryophyllales	Chenopodiideae	Spinacia	Oleracea	2.5
14	C.S.	Cucumber	E	Cucurbitales	Cucurbitaceae	Cucumis	Sativus	5
15	G.M.	Soybean	E	Fabales	Fabaceae	Glycine	Max	2.5
16	A.E.	Okra	E	Malvales	Malvaceae	Abelmoschus	Esculentus	2.5
17	B.R.	Chinese cabbage	E	Brassicales	Brassicaceae	Brassica	Rapa	2.5
18	P.R.	Corn poppy	E	Ranunclulales	Papaveraceae	Papaver	Rhoeas	5
19	A.F.	Welsh onion	M	Asparagales	Amaryllidaceae	Allium	Fistulosum	2.5
20	Z.M.	Maize	M	Poales	Poaceae	Zea	Mays	2.5

No. was sorted by phylogenetic position.
Abbr.: abbreviation, represented by the first letters of genus and species name
E: Eudicot; M: Monocot
PNA: μM, same mPNA and pPNA concentration were used in 1st PCR

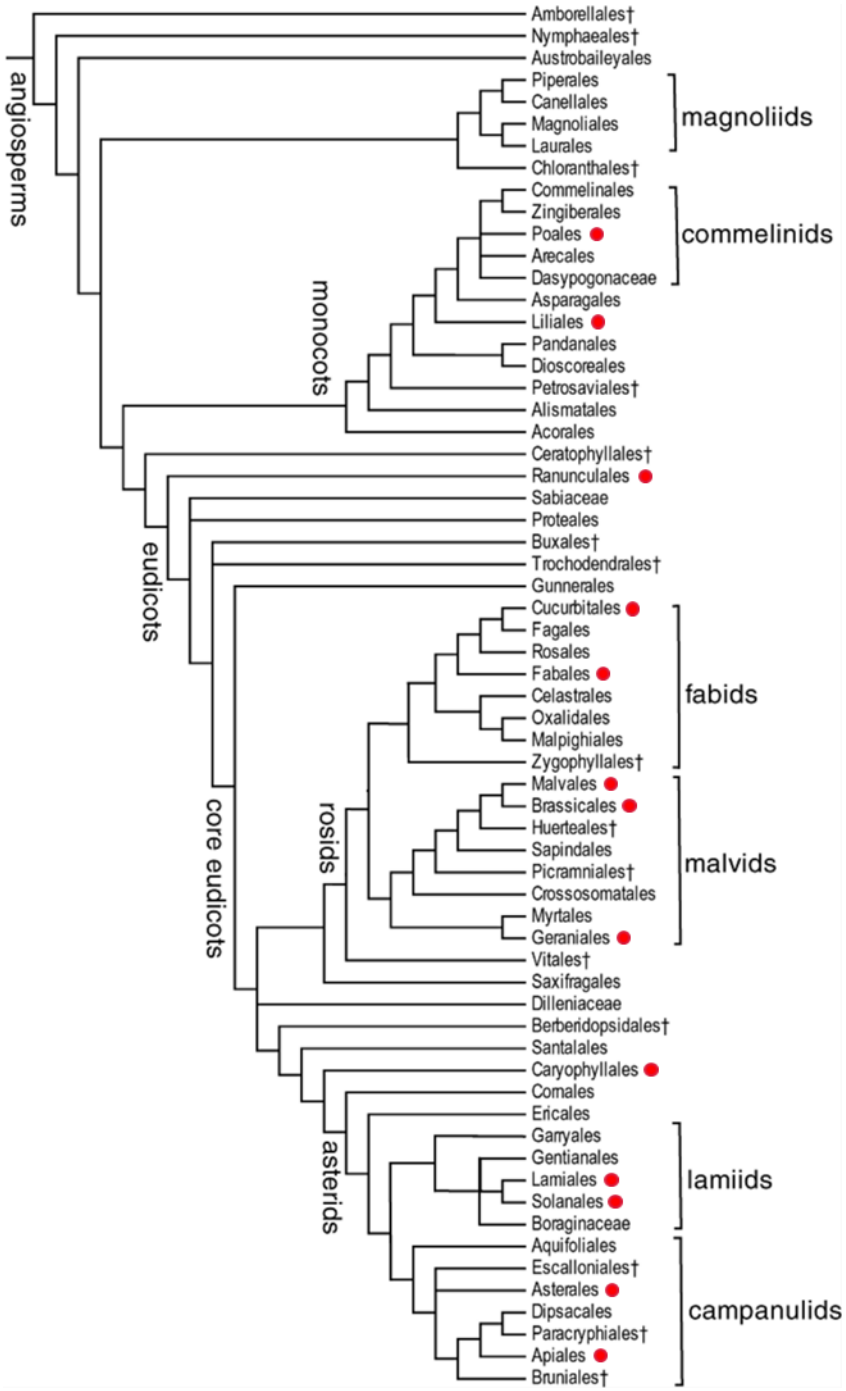


Fig 3-2. Distributions of the 13 plant orders in a summarized phylogenetic tree representing interrelationships of the APG III orders and some families of flowering plants [184]. Plant orders used in our experiment were marked by red circles. Newly-recognized-for-APG orders are denoted (†). Some eudicot families not yet classified to order are not shown.

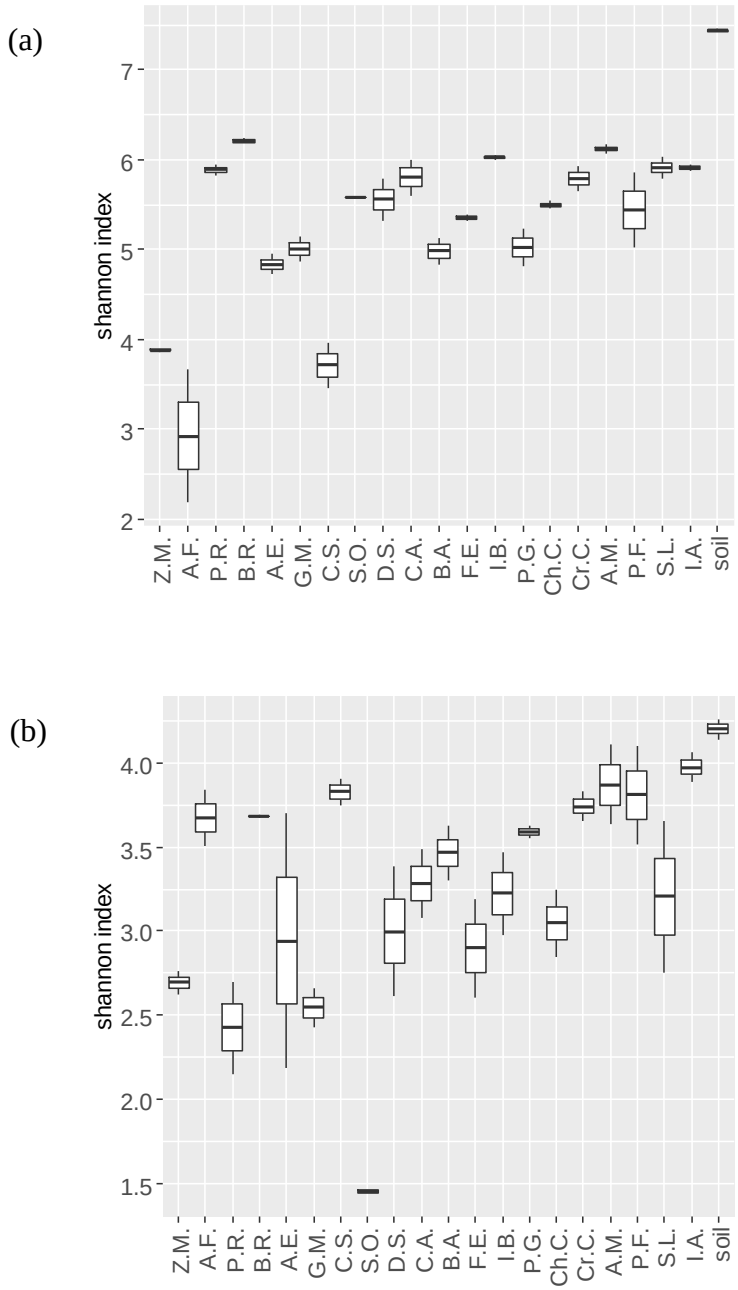


Fig 3-3. Shannon index of twenty plant species root and bulk soil bacterial (a) and fungal (b) communities. The x axes were sorted by phylogenetic positions of plants. Mean ± SE were shown in the figures.

Table 3-2. Shannon index and observed OTU numbers of twenty plant species root and bulk soil bacterial and fungal communities. Significant effect was tested by the ANOVA (* p<0.05; ** p<0.01; *** p<0.001).

	Bacteria		**Fungi**	
	Shannon index	Observed OTUs	Shannon index	Observed OTUs
Root	5.27	6241	3.22	1009
Soil	7.44	8989	4.20	1242
Pr(>F)	0.002 **	0.045 *	0.048 *	0.308
Monocots	3.41	5861	3.18	1092
Dicots	5.48	6284	3.22	1000
Pr(>F)	<0.001 ***	0.67	0.911	0.588

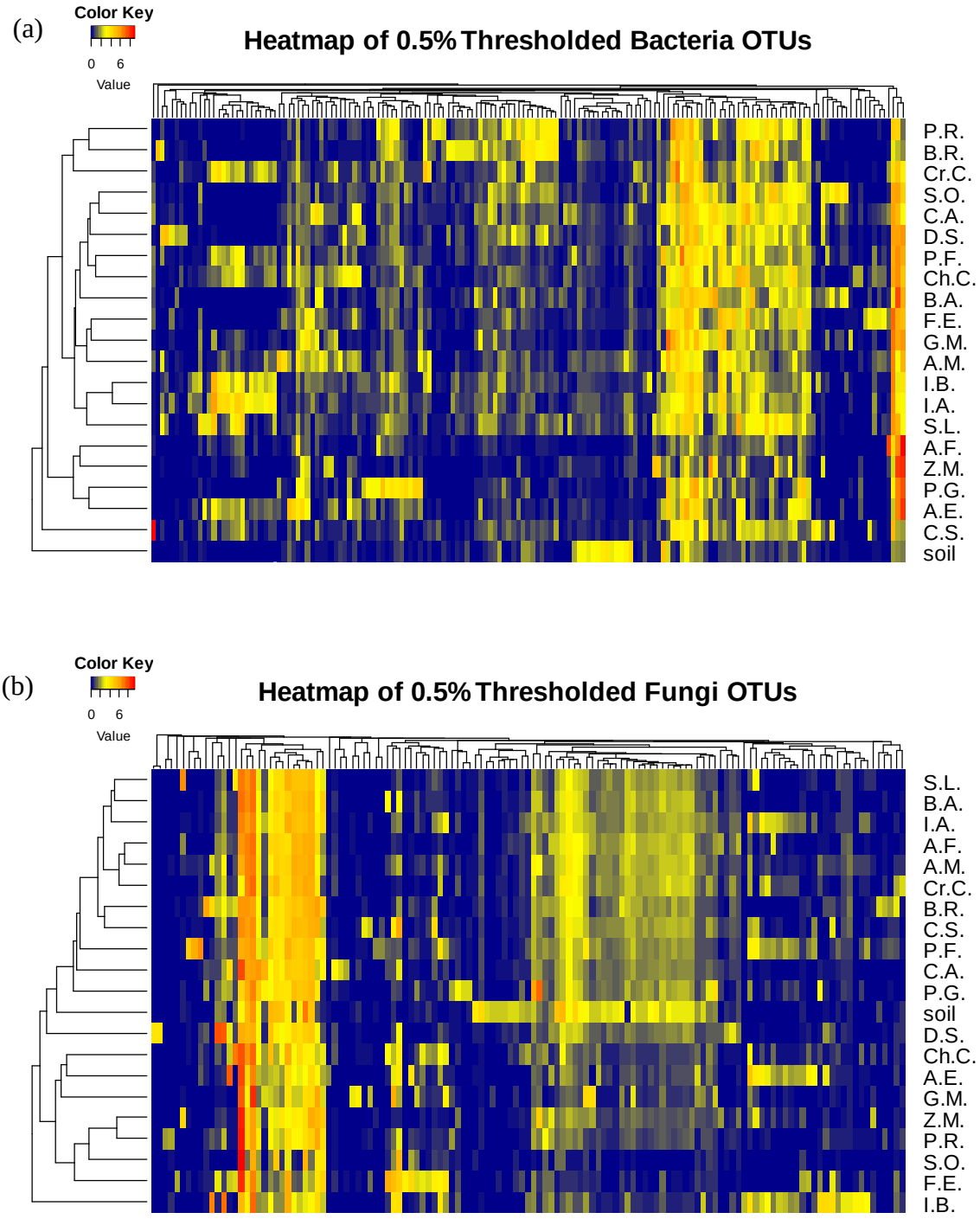


Fig 3-4. Heatmaps of bacterial (a) and fungal (b) 0.5% thresholded OTUs of twenty plant species root and bulk soil samples. All data were log normalized. Column represents relative abundance of each OTU. Row represents each sample. Samples and OTUs were clustered by Bray–Curtis similarities.

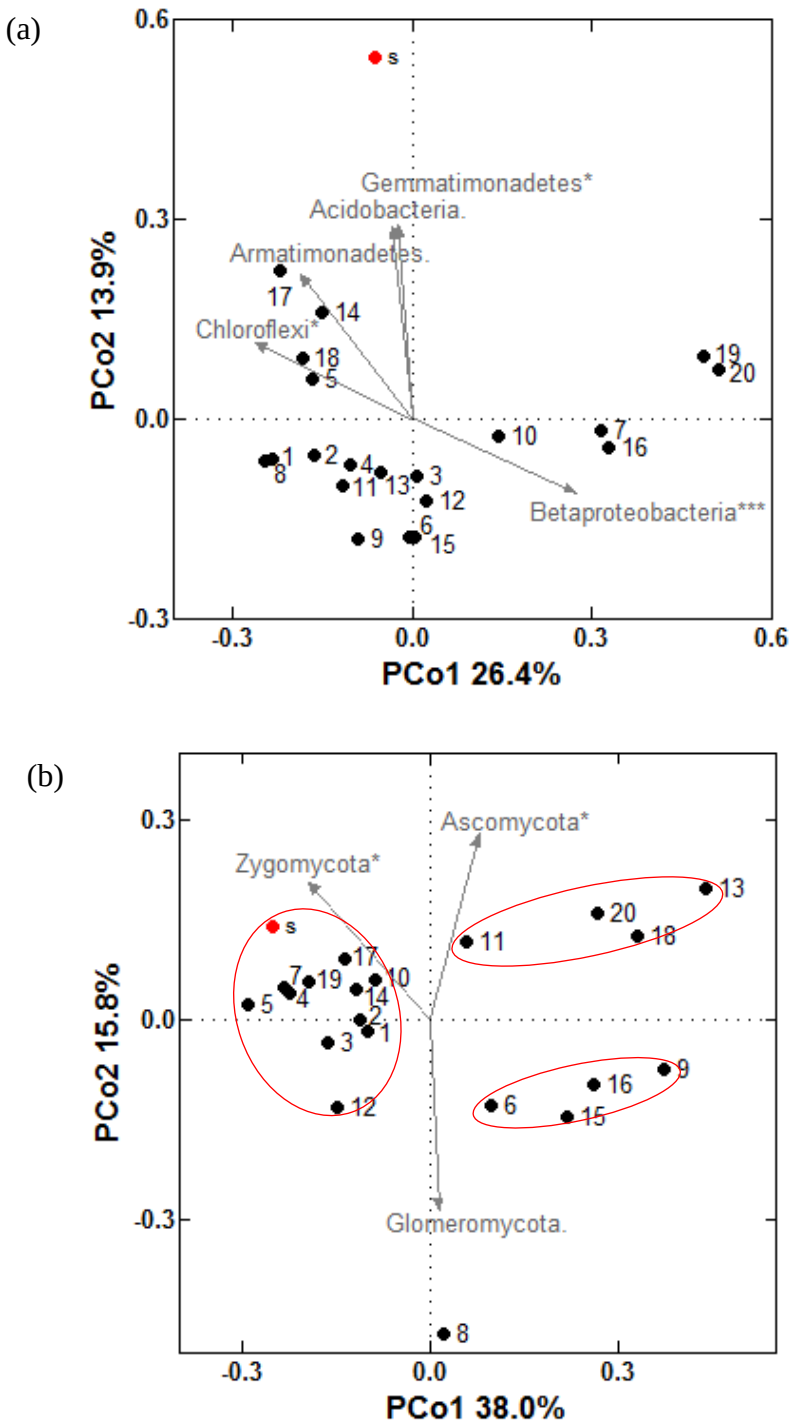


Fig 3-5. Principal coordinate analysis (PCoA) of bacterial (a) and fungal (b) communities of twenty plant species root and bulk soil samples based on Bray-Curtis distances. The arrows represent direction and strength of the gradient. The significances are based on random permutations of the data (‘.’0.1, ‘*’0.05, ‘**’0.01, ‘***’0.001), only P-values less than 0.1 were shown in the figures.

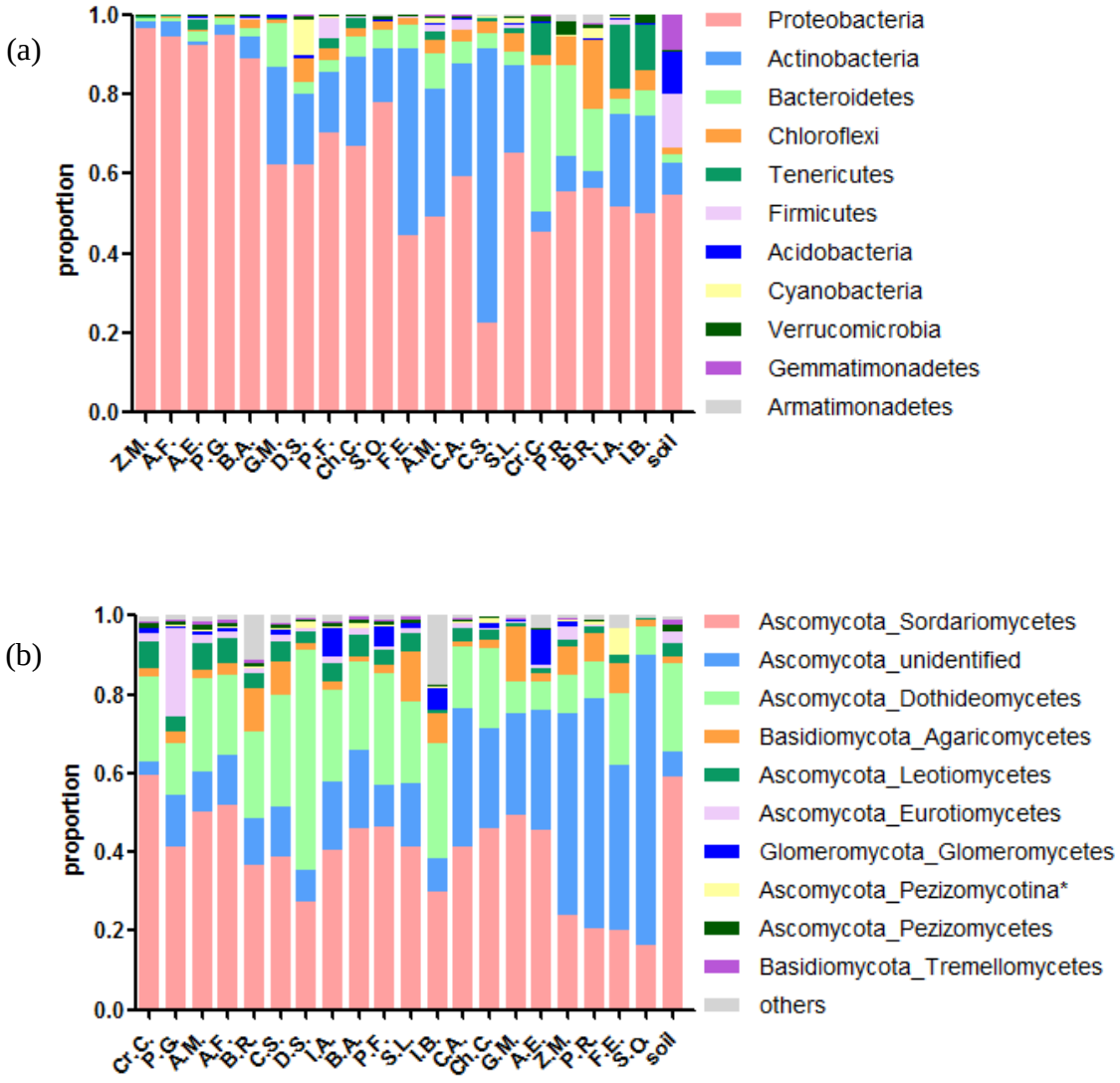


Fig 3-6. Bacterial (a) and fungal (b) community distributions of twenty plant species root and bulk soil samples. Root samples were sorted by PCo1 scores. Phylum and class taxonomic levels were used on bacteria and fungi respectively.

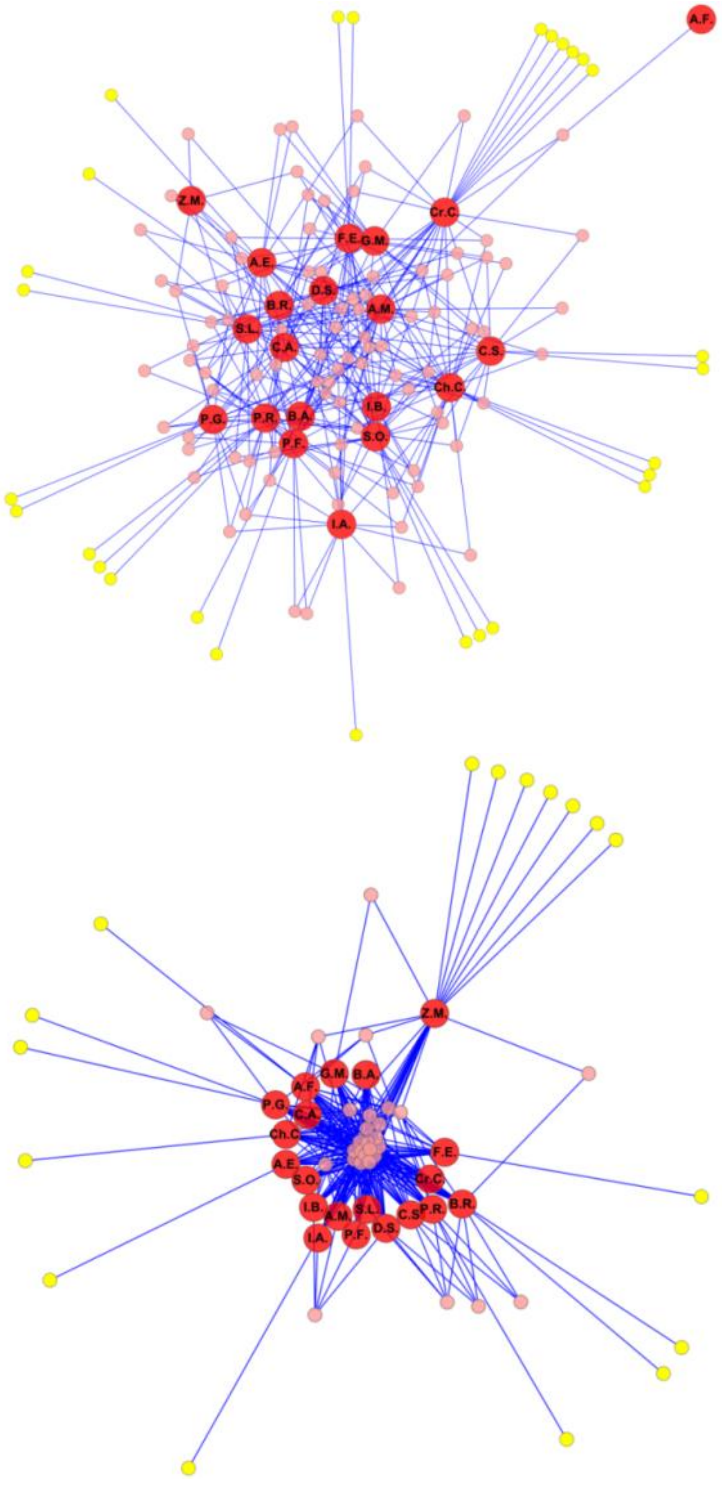


Fig 3-7. Networks based on habitat preference analysis by T-test of bacterial OTUs relative abundance between twenty plant species root and bulk soil samples. (a) Pattern of root preferred OTUs. (b) Pattern of root depleted OTUs. Red points: twenty plant species; pink points: OTUs shared by different plant species; yellow points: plant specific OTUs.

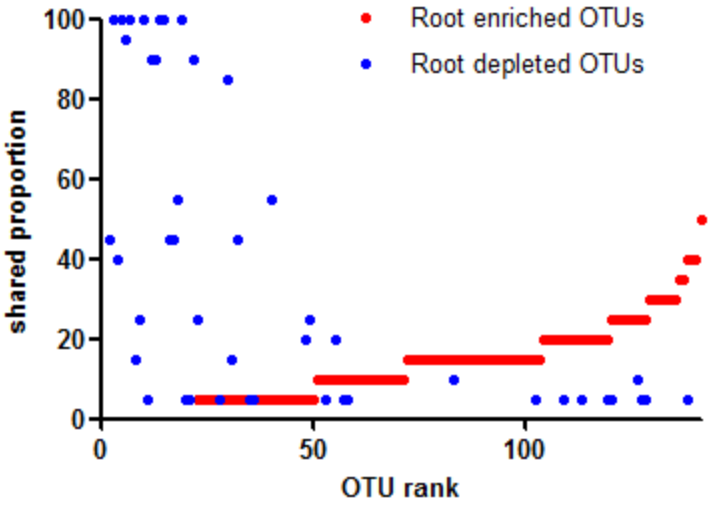
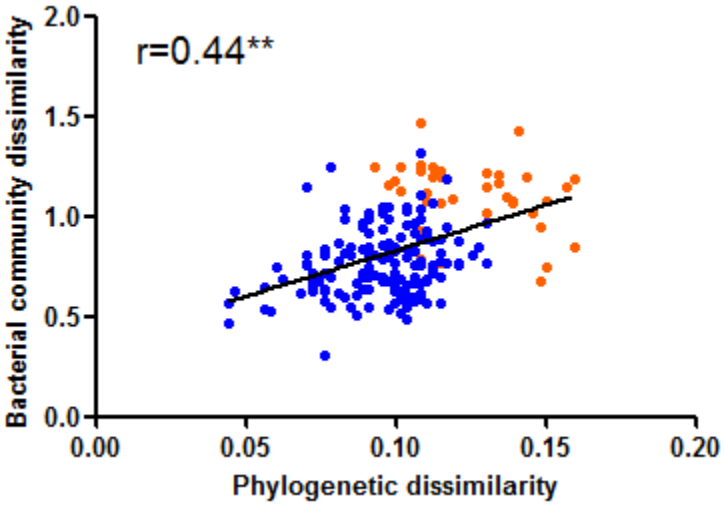
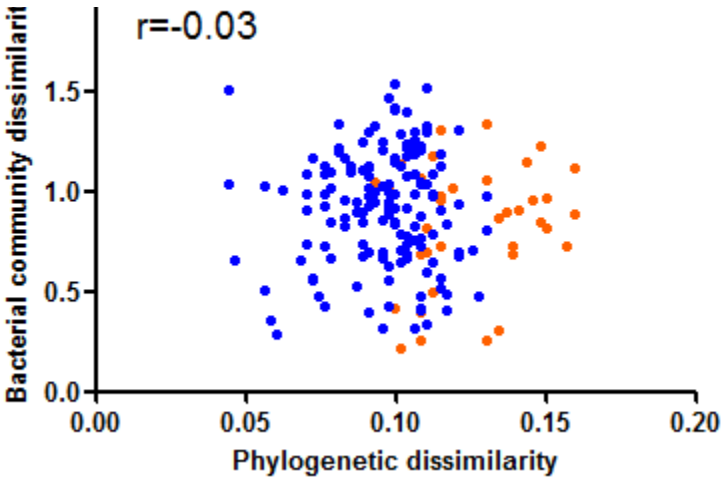


Fig 3-8. The shared proportion of OTUs enriched and/or depleted in root compartment.

(a)



(b)



(c)

Compared factors	All 20 plants	Dicots
Phylogenetic & Bacteria	0.44 **	0.27 *
Phylogenetic & Fungi	-0.027	0.01

Fig 3-10. Correlations between evolutionary distance of host plants (blue: correlations between dicots only; red: correlations correlated to monocots) and bacterial (a) and fungal (b) microbiome dissimilarity (Manhattan distance). Mantel statistic based on pearson's product-moment correlation (c) on twenty plant species and only dicots. The PERMANOVA was applied to test the significant effect (* $p<0.05$; ** $p<0.01$; *** $p<0.001$).

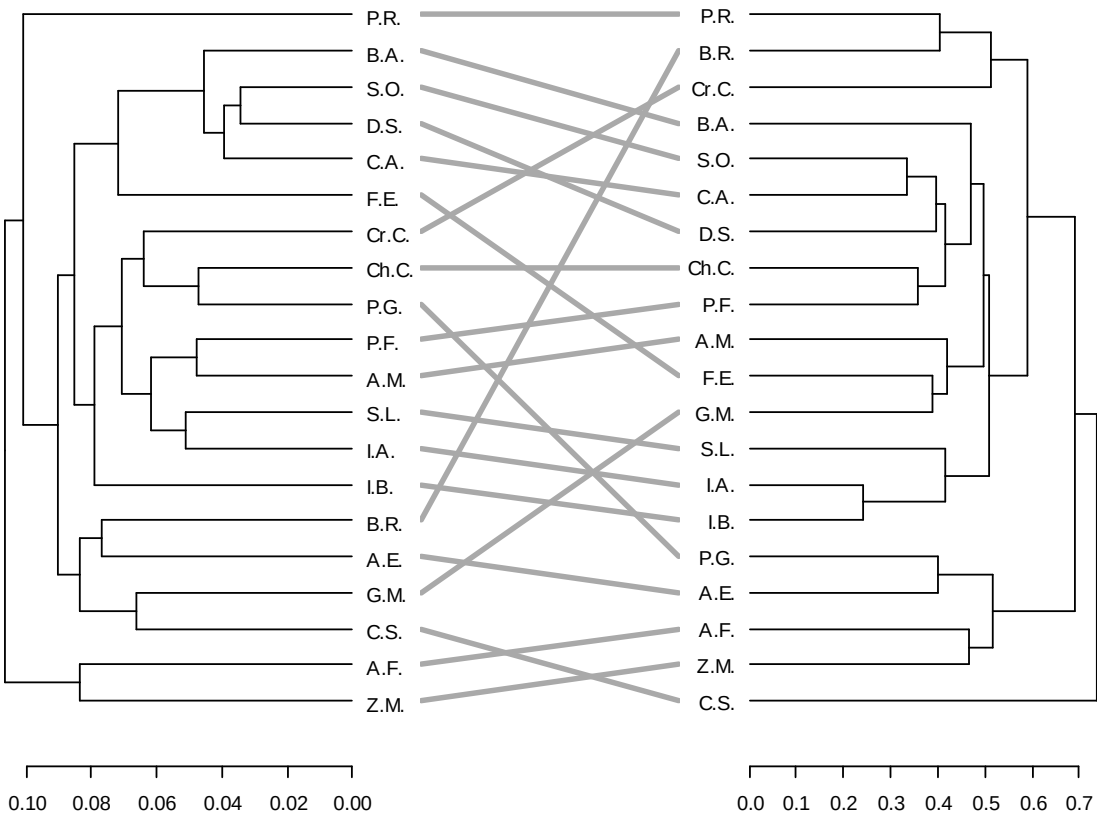


Fig 3-10. Comparison of topology between plant phylogenetic tree and bacterial microbiome dissimilarity tree based on Bray-curtis distances.

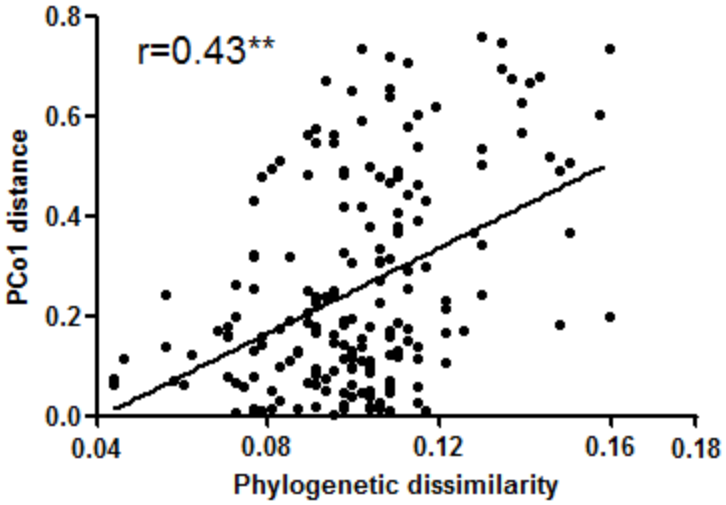


Fig 3-11. Mantel Correlations between PCo1 scores and bacterial microbiome on twenty plant species. Mantel statistic based on pearson's product-moment correlation.

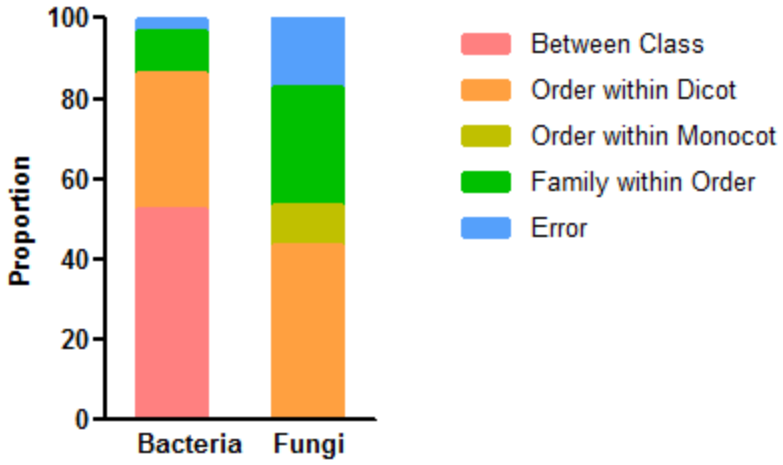


Fig 3-12. Variation partitioning of community structure between and within taxonomic groups.

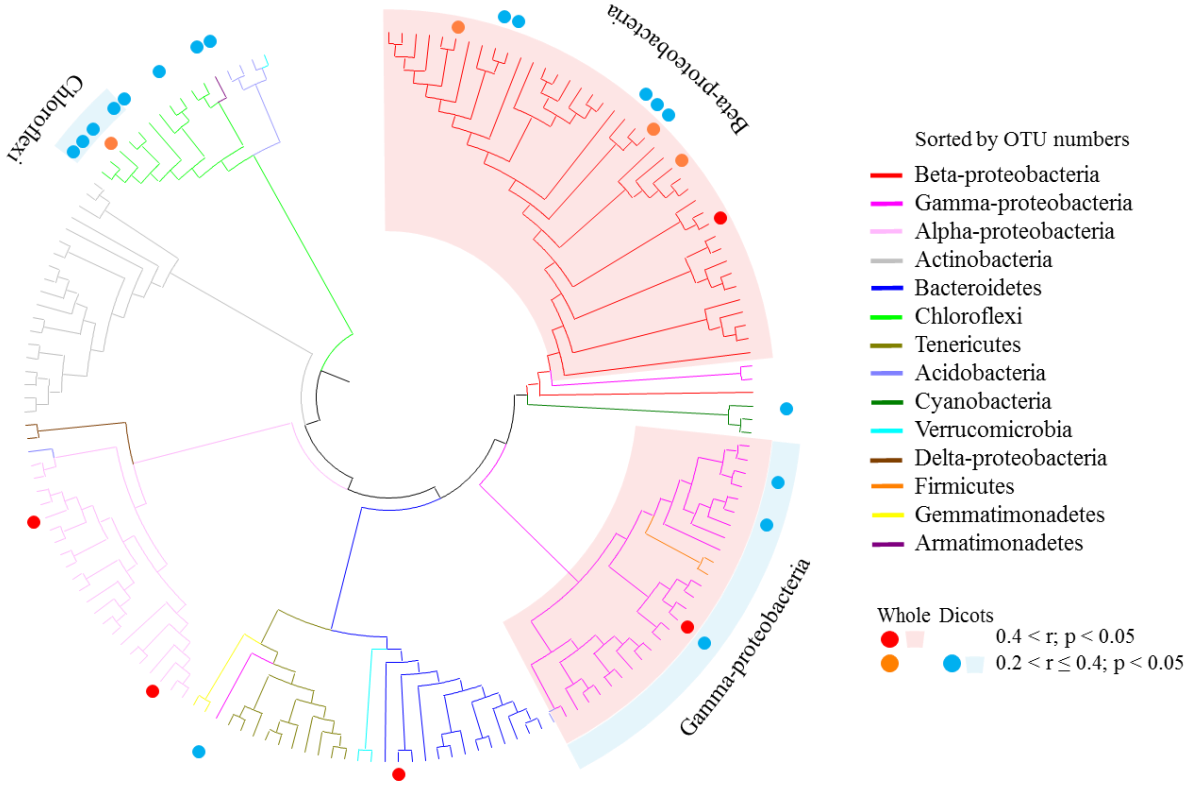


Fig 3-13. Plant evolutionary history correlated root bacterial microbes of whole plants (inner ring) and dicot families (outer ring). The correlations were detected by Mantel test based on manhattan dissimilarity of relative abundance of each single OTU, and OTUs belong to the same class. The PERMANOVA was applied to test the significant effect.

Table 3-3 PCo1 scores correlated bacterial classes based on Pearson product-moment correlations. PCo1 scores were calculated by each replicate, PCo1 scores and relative abundances of both two replicates were used for statistical analysis. Only classes show significant correlations were shown in the table (* $p<0.05$; ** $p<0.01$; *** $p<0.001$).

Class	Pearson r
Betaproteobacteria	-0.91***
Actinobacteria	0.42**
Gammaproteobacteria	-0.41**
[Saprospirae]	0.45**
Mollicutes	0.36*
Chloroflexi	0.53***
Anaerolineae	0.41**
Opitutae	0.52***
Deltaproteobacteria	0.38*
Cytophagia	0.37*
Armatimonadia	0.38*
Acidobacteria-6	0.39*
[Chloracidobacteria]	0.34*
Gemmatimonadetes	0.33*

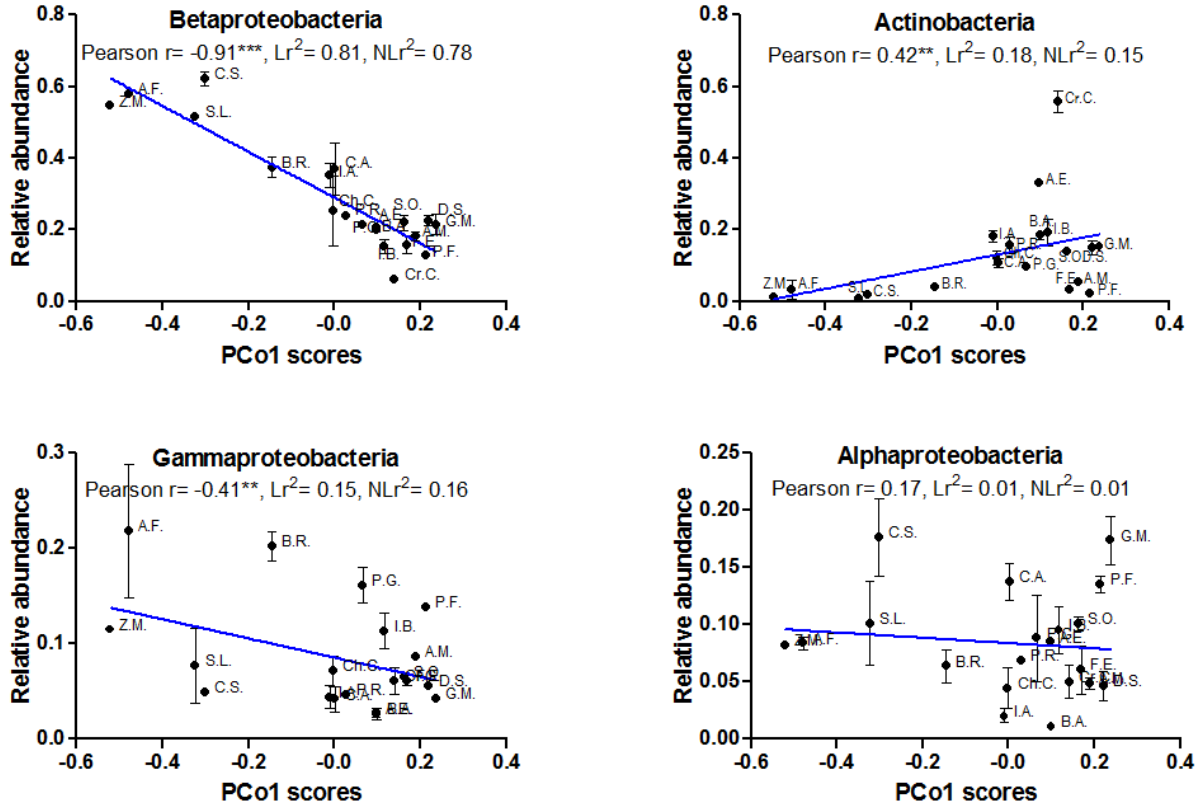


Fig 3-14. Correlations of PCo1 scores (mean of two replicates) and top four bacterial classes.

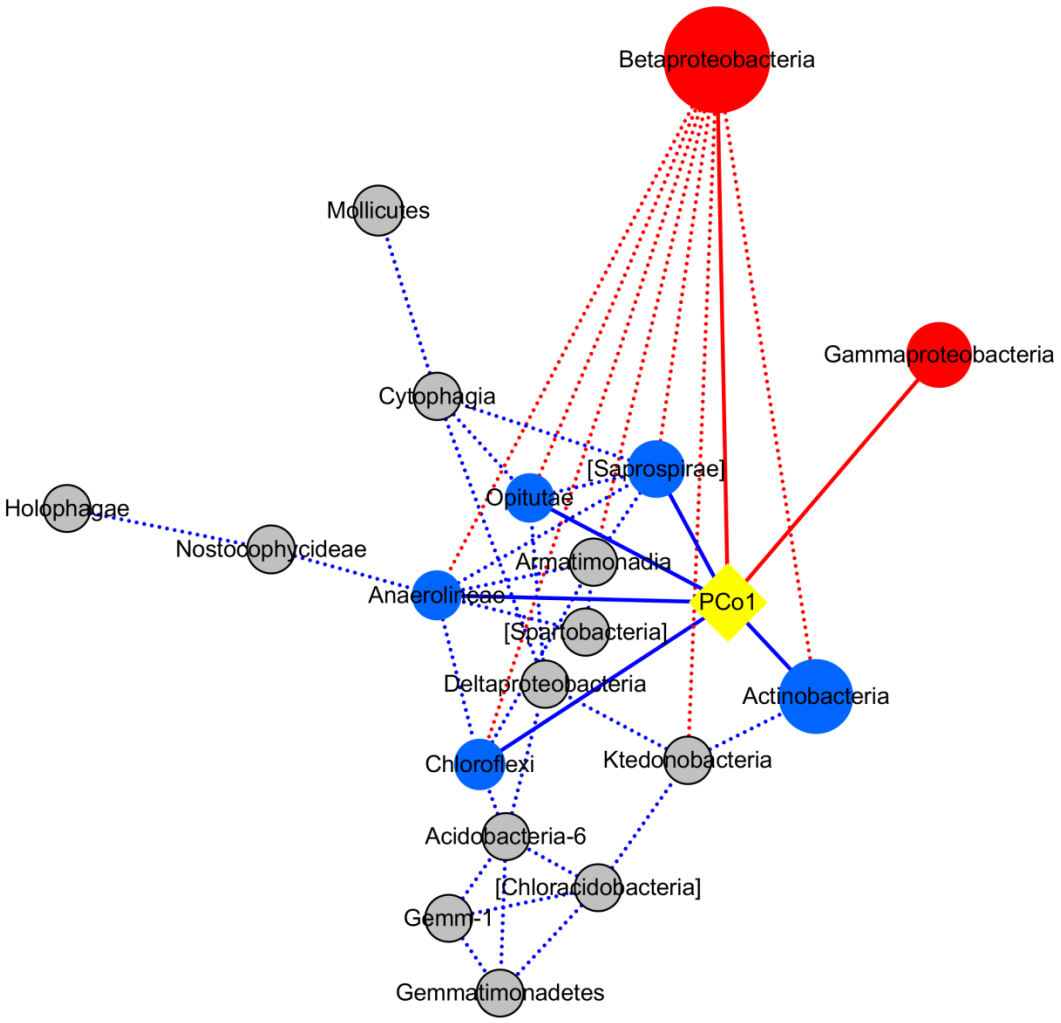


Fig 3-15. Correlation-based network analysis of the relative abundances of bacterial class and PCo1 score. The analysis used Pearson correlation with threshold at |r|>0.4 and Fisher's Z with P-value threshold at 0.05. The size of each circle represents average relative abundance. The blue and red color circles correspond to co-presence and mutual exclusive orders respectively, which directly significantly correlated with PCo1 scores. Red lines indicate negative correlations, and the blue lines indicate positive correlations. Solid lines indicate correlations between bacterial classes and PCo1 scores, and dotted lines indicate correlations between bacterial classes.

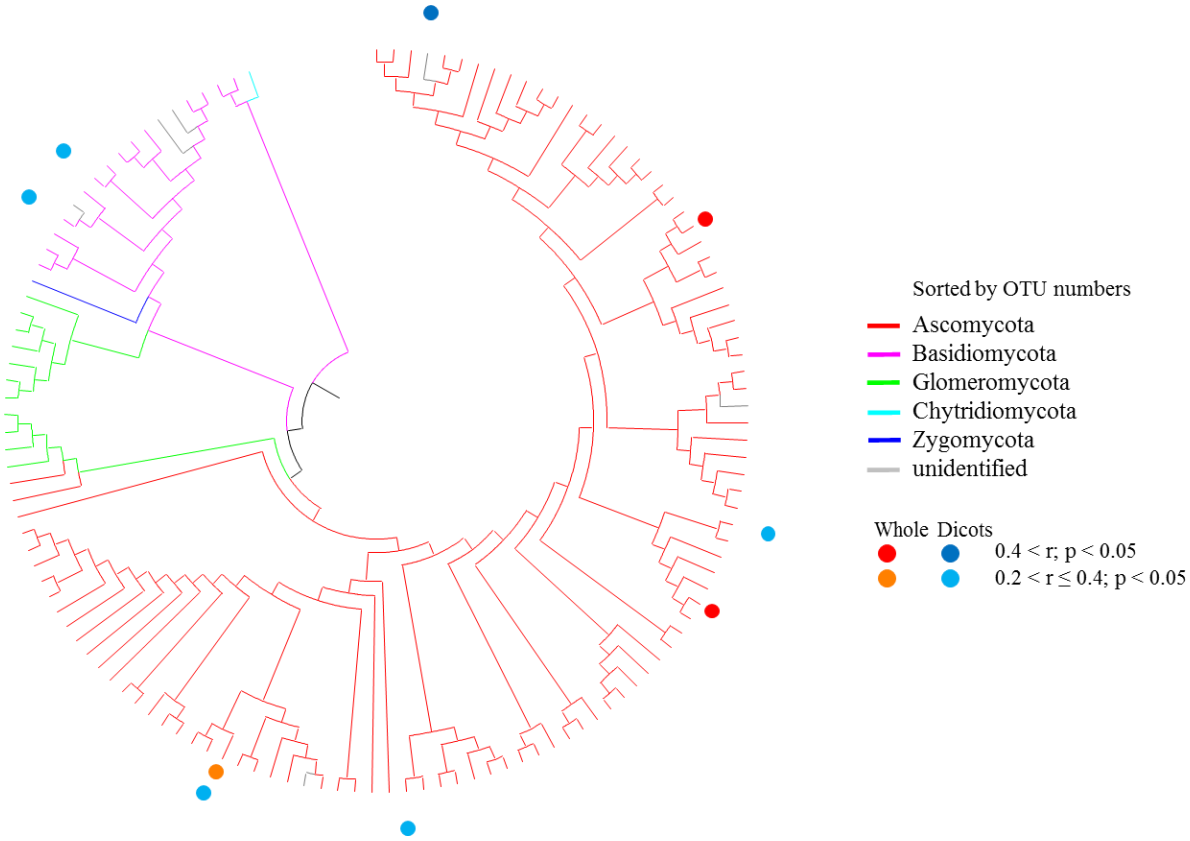
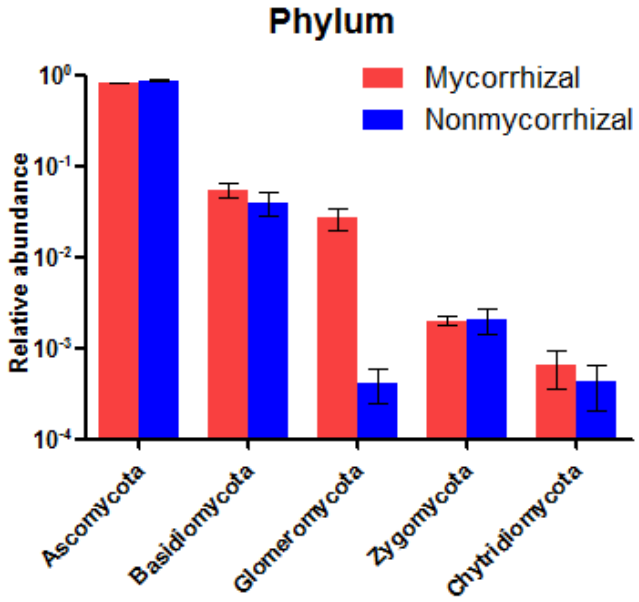


Fig 3-16. Plant evolutionary history correlated root fungal microbes of whole plants (inner ring) and dicot families (outer ring). The correlations were detected by Mantel test based on manhattan dissimilarity of relative abundance of each single OTU, and OTUs belong to the same class. The PERMANOVA was applied to test the significant effect.

(a)



(b)

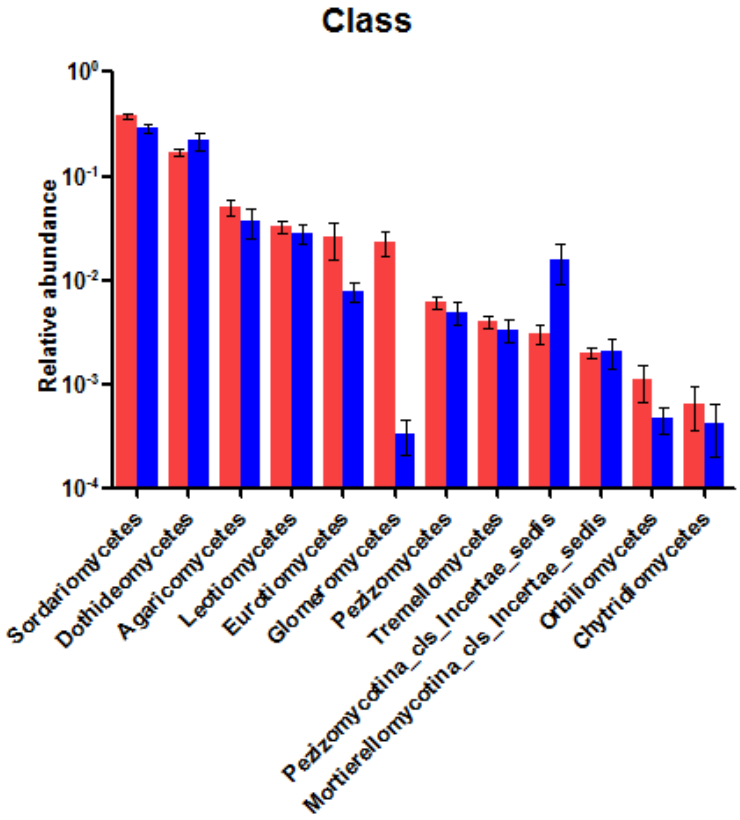


Fig 3-17. Relative abundance of fungal phyla (a) and classes (b) from plants with mycorrhizal/ nonmycorrhizal symbiotic associations. Y-axes were logarithmic scaled.

CHAPTER 4: THE EFFECT OF CROP ROTATION ON CLUBROOT DISEASE RESISTANCE THROUGH CHANGES OF SOIL MICROBIAL COMMUNITY AND ROOT MICROBIOME

Introduction

Clubroot, which is caused by soil borne protist *Plasmodiophora brassicae*, is one of the most serious diseases that afflict crops of the family Brassicaceae worldwide and is the major source of disease-induced crop loss in this family [185]. The clubroot-infected plants become stunted, leaves chlorotic and necrotic, with typical gall symptom on root system, therefore impact yield and cause economic costs. Chinese cabbage has been one of the most important vegetable crops in Asia that is highly suffered from clubroot disease [186].

Several managements such as soil liming [187], biofungicide [188] and resistant cultivars [189] have been conducted to control clubroot disease. Among them crop rotation is one of the most efficient methods widely used. Crop rotation has been considered to restrict damage by soil borne diseases through altering constitution of soil microbiome including pathogens. However, it has not been well understood how crop rotation alter structure of soil microbial community and how the changes in microbial community restrict crop damage by soil borne diseases.

To deepen our understanding of mechanisms underlying disease control by crop rotations, we designed a model system where 20 taxonomically different plants were grown in single pots as previous crops and then Chinese cabbage seedlings were raised with infection of clubroot pathogens under the soil with influences of different previous crops. We followed the changes in abundance of clubroot pathogen and in structure of microbiomes of soil and root during the experiment. In this study we addressed the following three questions:

1. What are the effects of crop rotation on structure of microbiomes in soil and root of Chinese cabbage?

2. How is the abundance of clubroot pathogen influenced by soil and root microbes during colonization and infection stages?

3. How are the differences in disease damage of Chinese cabbage among previous crop treatments caused through the interactions among soil microbial community, root microbiome and pathogen?

Materials and Methods

Soil preparation by pre-cropping of 20 plant species

Original soils were collected from the Hirosaki University farm (40°35'22.9"N 140°28'20.8"E, Hirosaki city, Aomori prefecture, Japan), which has been managed by unfertilized organic farming approach (no fertilizer, no pesticides and no herbicides) for more than 5 years. The soils were sampled from 0-10 cm layer of three different places. After sieving through 2 mm mesh to remove roots, macrofauna and rocks, the soils were used for the experiment and the rest was stored at -80 ℃ for DNA analysis of soil microbial communities.

In this study cultivated plant species including cereals, vegetables and ornamental crops were used because these plants seem to have similar functional types that are adapted to growth in cultivated fields. Twenty plant species were selected from different families belonging to 13 orders included in two classes (monocot and dicot). The species used were *Brassica rapa subsp. Pekinensis*, *Glycine max*, *Abelmoschus esculentus*, *Ipomoea aquatica*, *Basella alba*, *Impatiens balsamina*, *Platycodon grandiflorus*, *Dianthus superbus*, *Celosia argentea*, *Antirrhinum majus*, *Papaver rhoeas*, *Zea mays subsp*. *Mays*, *Fagopyrum esculentum*, *Solanum lycopersicum*, *Allium fistulosum*, *Cucumis sativus*, *Cryptotaenia canadensis*, *Chrysanthemum coronarium*, *Spinacia oleracea*, *Perilla frutescens* as shown in Table 4-1 and Fig 4-1. The seeds of all species were collected from the market. Five germinated seeds of each plant species were planted at five positions as shown in Fig 4-2 in a pot (113 mm diameter and 184 mm height) filled with prepared soil on May. Five pots (replicates) were prepared for each plant species and were raised in a glasshouse. In addition, the five pots without plants were prepared as the control. Depending on the difference in growth rate among the species, the whole plants including the belowground were harvested at 7-8 weeks after sowing depending on growth rate. Soil attaching the root were removed as much as possible and returned to the pot. The 105 pots in each replicate of each plant species was used for infection study of Chinese cabbage by clubroot pathogen.

Growth of Chinese cabbage

Chinese cabbage (*Brassica rapa subsp. Pekinensis*) seeds were surface sterilized by 1% SHS (sodium hypochlorite solution) for 20 min, followed by 3 times washing in SDW (sterile distilled water). Sterile seeds were sown on sterile substrate media in petri dish and cultured in chamber at 22 ℃ with 16 h/8 h day/night period for one week, 4 seedlings were transplanted into each of the 105 pots exposed to pre-cropping treatment (five replicates by 20 plant species including the control) and raised in a glasshouse during 2-5 weeks. The nutrient solution (KNO_3 0.51 g/L+ KH_2PO_4 0.14 g/L, 30 ml/pot) were applied at one week after transplanting.

Resting spore suspension preparation and inoculation

Clubroot pathogen, *Plasmodiophora brassicae*, was obtained from clubroot-infected Chinese cabbages (Brassica oleracea) grown in a farmer's field in Hirosaki city. For preparation of resting spores of *P. brassicae*, clubroot galls were cut from infected root and approximately 3 g of tissues were immersed in 50 ml of sterilized distilled water (SDW) for 2 h to soften the tissue and then homogenized followed by filtering through 8 layers of gauze. The filtrate was centrifuged at 3000 rpm for 15 min and the pellet was recovered and diluted by SDW. The concentration of suspension was calibrated to 10^7 mL^{-1} by microscopy haemocytometer observation. The spore suspension of 30 mL was injected on the surface of soil in each pot soon after the transplant of Chinese cabbage seedlings.

Sampling and measurement of Disease Severity Index (DSI)

Harvests and infection measurement were conducted at four developmental stages starting from the third week after the sowing at weekly interval. At each harvest one of the four plants raised in a single pot was selected and the whole plants with attached soil were carefully taken from a pot without harming other plants. The sampled plants were transferred to the laboratory, gently shaken to remove attached soil as much as possible and washed with tap water.

A disease severity index (DSI) was used for evaluation of disease damage according to Wang et al. (2012) [190] as follows: no symptoms (0), gall only formed on fibrous root (1), small galls formed on lateral roots (3), galls on lateral roots or small galls on the main root (5), many large galls on lateral roots or galls on the main root (7) and severe galls formed on the main root leading to partial degradation and the plant nearly or already dead (9).

Sample preparation and DNA extraction

Root samples were shaken to remove attached soil as much as possible and washed with tap water, followed by sonication in SDW at 50-60 Hz for 5 min (Ultrasonic Cleaner US-1, AS ONE corporation) to disrupt tiny soil aggregates and attached microbes. Root samples were stored at -80 ℃ until processing.

5 replicate samples were mixed in equal weight respectively to get mixed samples. 2 replications of each mixed sample were subjected to followed DNA extraction.

Root DNA was extracted by DNeasy Plant Mini Kit (Qiagen, Valencia, CA). Soil DNA was prepared with ISOIL for Beads Beating Kit (Nippon Gene CO., Ltd). Beads beating were processed by Micro Smash MS-100 (Tomy Seiko CO., Ltd)). DNA quantification was assessed with the NanoDrop 2000 (Thermo Fisher Scientific Inc.)

qPCR of *P. brassicae* pathogen density

To determine the density of *P. brassicae* in root tissues, qPCR reaction was performed in a 10 μL reaction volume and quantified using DNA Engine Peltier Thermal Cycler (BioRad, Hercules, CA, USA) with six replicates for each sample. The thermocycling profile consisted of an initial denaturation step of 10 min at 95 ℃, then 44 cycles of 15 s at 95 ℃, 30 s at 58 ℃ and 30 s at 72 ℃. Standard curve developed by serial dilutions of purified DNA after PCR amplification from DNA extracted from clubroot galls by Fast Gene Gel/ PCR Extraction Kit (NIPPON Genetic CO., Ltd). PCR and qPCR reactions were conducted using the specific primers Pb4-1 (5′- TACCATACCCAGGGCGATT -3′) and PbITS6 (5′- CAACGAGTCAGCTTGAATGC -3′) described by *Sundelin et al*. [191].

PCR amplification and next-generation-sequencing

To detect bacterial microbiome consititution, a V4 region of 16S ribosomal RNA (rRNA) was amplified by primer pair 515F (5′- GTGCCAGCMGCCGCGGTAA -3′) and 816R (5′- GGACT ACHVGGGTWTCTAAT -3′) with adaptors in the 1st PCR amplification. To block the chloroplast and mitochondrial amplification, peptide nucleic acid (PNA) clamps were included in the reaction according to Lundberg [113]. The appropriation PNA concentration was detected by Realtime PCR, 2.5 μM mPNA and pPNA were used in 1st PCR amplification. The 1st PCR

conditions were as follows: an initial denaturation step of 95 ℃ for 45 s, then 35 cycles of 15 s at 95 ℃, 10 s at 78 ℃, 30 s at 50 ℃, and 30 s at 72 ℃.

After purification by Fast Gene Gel/ PCR Extraction Kit (NIPPON Genetic CO., Ltd), the 1st PCR products were followed by the 2nd PCR with the primer pair, 2nd-F (5′- AATGATACGGC GACCACCGAGATCTACAC- Index2 -ACACTCTTTCCCTACACGACGC -3′) and 2nd-R (5′- CAAGCAGAAGACGGCATACGAGAT- Index1 -GTGACTGGAGTTCAGACGTGTG -3′). The index pairs were specific for each sample, for an accurate recognition of the samples. The 2nd 16S rRNA PCR condition were: an initial denaturation at 94 ℃ for 2 min, 12 cycles at 94 ℃ for 30 s, 60 ℃ for 30 s, 72 ℃ for 30 s, with a final extension at 72 ℃ for 5 min. The 2nd ITS2 PCR condition were: 94 ℃ for 2 min, 12 cycles at 94 ℃ for 30 s, 60 ℃ for 30 s, 72 ℃ for 1 min, with a final extension at 72 ℃ for 5 min. The 2nd PCR products were purified using the AMPureXP magnetic beads (Beckman-Coulter, Indianapolis, IN, USA) and pooled in equimolar ratios. After confirming the library quantity, paired-end 2 x 250 bp sequencing of the barcoded amplicons was performed on a MiSeq machine (Illumina Inc, San Diego, CA, USA).

Sequence processing

The sequences obtained from the MiSeq were processed through a custom pipeline developed at the Bioengineering Lab. Co., Ltd. (Atsugi, Japan). The raw reads were demultiplexed based on the barcode sequences and filtered by exact matching using Fastx toolkit (fastq_barcode_splilter). If the quality score was less than 20 and the sequence length was less than 40 bases, the reads were discarded. Paired-end reads with minimum 10 base overlap were merged into full-length sequences by FLASH. Merged sequences between 246 and 260 bases were used for later processing of 16s rRNA. The UCHIME algorithm was used for detecting chimeric sequences. OTU generation and phylogenetic assignment were conducted by QIIME script with default conditions. OTUs were clustered using UCLUST at a 97% similarity level by a de novo picking method, using Greengenes 16S reference database. All OTUs assigned to Archaea, chloroplast, and mitochondria were discarded from the 16S dataset to get filtered OTU dataset and normalized by transforming the OTU counts number to relative abundance. As spurious sequences and unrepresentative OTUs decrease the reproducibility for community assemblage [120], the generated OTUs was thresholded by 0.06% (in at least one sample), which was

derived from the correlation between abundance in the same OTU in technical replicates (Fig 4-3) [20].

Statistical analysis

α-diversity were calculated by the function diversity() using the "Shannon" method in the R package Vegan. The richness of community was evaluated using the number of observed OTUs. β-diversity were detected by using Log_2 transformed relative abundance to calculate a Bray-Curtis distance dissimilarity matrix using the function "vegdist()" of the R package Vegan. The dissimilarity matrix was used to generate corresponding cluster dendrograms using the function "hclust" of the R package Vegan and clustered by using weighted pair group average (UPGMA) method. Heatmap were constructed using custom scripts and the function "heatmap.2" of the R package gplots. 0.5% threshold dataset was used for hierarchical cluster and heatmap. Principal coordinate analyses (PCoA) utilizing the Bray-Curtis distances dissimilarity based on relative abundance of OTUs using the "pcoa()" function from the R package Ape. Network analysis of interactions between DSI, pathogen density and bacterial taxa was performed using the CoNet app in Cytoscape v.3.6.1. ANOVA was applied to determine differences in the relative abundances of taxa in soil at before and after pre-crops growing, as well differences between different pre-crops. Pearson's correlation was used for correlation analysis by JMP.

Stepwise multiple regression analysis was conducted to detect important microbes that influence pathogen density and DSI by JMP. Mixed direction method was employed, which alternates the forward and backward steps. Threshold Stopping Rule at 0.25 probabilities to enter and leave with includes the most significant term that satisfies Prob to Enter and removes the least significant term satisfying Prob to Leave continues removing terms until the remaining terms are significant and then it changes to the forward direction was used in the analysis.

Results

The structure of microbiomes of the soil and root after pre-cropping

α-diversity

We used Shannon index and the number of observed OTUs as a measure of α-diversity. The soil had a significantly larger mean number of OTUs (8289) than the root community (6227) as shown in Table 4-2. The root community at 3rd week had a significantly greater number of OTUs (6823) than the root community at 4th week (5631). Shannon index also showed significant difference between the soil and root communities, while no significant difference was found between the communities at 3rd week and at 4th week (Table 4-2). Preceding crop had significant effects on Shannon index both of the soil community and the root community ($p=0.045$*, ANOVA; $p<0.001$***, ANOVA), although the number of observed OTUs did not show significant difference between the species of preceding crops ($p =0.83$, ANOVA; $p=0.27$, ANOVA) (Fig 4-4, Fig 4-5). Soil bacterial microbiomes were more diverse than the root microbiomes of Chinese cabbage. Fig 4-6 showed the rank-abundance curve in the soil and the root communities. The root community had greater abundances in the high-ranked OTUs but smaller abundances in the rest of ranks than the soil community, and thus a larger proportion of the abundance was shared by a smaller number of OTUs for the root community (Fig 4-6).

β-diversity

β-diversity, which represents the degree of differentiation between samples, was evaluated by dendrogram of cluster analysis, heatmap diagram and principal coordinate analysis (PCoA). The cluster analysis showed that soil samples were included in a single cluster that was different from the clusters including the root communities of Chinese cabbage, while the root communities produced two clusters consisting of the community at the 3rd week and the community at 4th week (Fig 4-7, Fig 4-8, Fig 4-9). Soil and root communities separated across the first axis of PCoA analysis, while the root communities at 3rd week and 4th week were discriminated across the second axis of PCoA (Fig 4-9). These results demonstrated that the soil communities possessed different structure from the root communities, which also showed differentiation between different developmental stages.

The heatmap diagram revealed different abundance pattern of OTUs between the soil and root communities, which was represented by different pattern of enrichment and depletion of OTUs (Fig 4-8). Compared with the differentiation between the soil and the root communities, the root communities at 3rd week and 4th week, which belonged to the different cluster (Fig 4-7), did not show clear differentiation between them (Fig 4-7, Fig 4-8, Fig 4-9).

There were large differences in abundance at the phyla level between the soil and the root communities. The root community at the 3rd week had higher proportions of Proteobacteria (63.5%), Actinobacteria (22.1%) and Bacteroidetes (8.1%) than the soil community (33.0%, 7.9% and 5.2%, respectively, $p<0.001$***, paired t-test). On the other hand, the soil community had significantly greater abundances for Acidobacteria (22.7%), Firmicutes (8.4%), Gemmatimonadetes (6.1%), Chloroflexi (5.3%) and Verrucomicrobia (4.6%) than the root community (1.3%, 1.3%, 0.2%, 2.3% and 0.4%, respectively, $p<0.001$***, paired t-test) as shown in Fig 4-10 and Fig 4-11. The root communities showed significant changes in the abundances of some phyla from 3rd week to 4th week. Chloroflexi and Verrucomicrobia significantly increased their abundances from the 3rd week to the 4th week ($p<0.001$***, paired t-test; $p<0.001$***, paired t-test), while Bacteroidetes and Firmicutes significantly decreased ($p<0.05$*, paired t-test; $p<0.001$***, paired t-test) (Fig 4-10, Fig 4-11).

Different preceding crops brought about different structure of the soil communities (Fig 4-12, Fig 4-13). The soil community after the growth of soybean (G.M.) showed distinctive structure from other soil communities as shown by the scores of the first axis of PCoA, while the soil community after the growth of buckwheat (F.E.) took the similar position to the control soil. The soil communities with different preceding crops were arranged across the second axis of PCoA.

The root communities with different preceding crops were arranged across the second axis of PCoA (Fig 4-14). As to phyla level Bacteroidetes ($p<0.5$*, ANOVA), Chloroflexi ($p<0.001$***, ANOVA), Acidobacteria ($p<0.01$**, ANOVA) and Verrucomicrobia ($p<0.5$*, ANOVA) showed significant difference among preceding crops (Fig 4-10, Fig 4-11).

The difference in damage by clubroot disease

The two-way ANOVA showed that clubroot disease damage (DSI) significantly differed between development stage, preceding crop species and their interaction (Table 4-3). The mean

DSI was 3.44 at the 3rd week but increased to 46.7 at the 4th week ($p<0.001$***, ANOVA), and sequentially increased to 78.7 at the 6th week (Fig 4-15). Since DSI only showed significant difference between proceeding crops at the 4th week (Table 4-4), we used DSI at the 4th week for the later analysis. The plants after the growth of Z.M. (Maize), S.O. (Spinach), A.F. (Welsh onion) caused high DSI, while the ones after the growth of Cr.C. (Canadian honewort), P.G. (Platycodon), G.M. (Soybean) brought about low DSI (Fig 4-16).

The pathogen density significantly differed between development stages and between preceding crop species and their interaction (Table 4-5). One-way ANOVA showed that the difference in preceding crops produced significant difference in the pathogen density both at the 3rd and 4th weeks (Table 4-6). However, the pathogen densities between the two stages positively correlated with each other (r=0.46*, Fig 4-18). As expected, DSIs during the 3rd to the 4th weeks showed a positive correlation with the pathogen density during this time period (r=0.53***, Fig 4-17), although the pathogen density at the 3rd week had a higher positive correlation with DSI (r=0.67**) than that at the 4th week (r=0.57**). Since the transformation into natural logarithm made the relationships between the pathogen density and DSI more linear and clearer (r=0.73**, Table 4-7; Fig 4-18), the ln-transformed pathogen density was used in the later analysis.

Microbes responsible for Clubroot damage

I examined the relationships of the abundance of each microbial group with pathogen density at the 3rd week and for DSI at 4th week to identify key microbes responsible for clubroot resistance. Each OTU was assigned to taxonomic identity according to its sequence. The proportion of OTUs with taxonomic identity progressively decreased, 99.9% at class level, 92.7% at order level, 70.2% at family level, 28.7% at genus level and 3% at species level (Table 4-8). Because a high proportion of taxonomic identity (92.7%) was attained at the order level and the top 20 orders shared high shares of the total abundance in the root communities, we used the abundance at the order level for the analysis (Table 4-9).

Network diagrams among microbes revealed that the abundances of 20 orders in the soil and the root communities had close and complicated associations with each other and with pathogen density and DSI (Fig 4-19, Fig 4-20, Fig 4-21). To make their relationships simpler and clearer, we conducted stepwise multiple regression analysis where the pathogen density (after transformation into natural logarithm) and DSI were regressed against the abundance of the top

20 orders of the root community at the 3rd and 4th week and of the soil community (Table 4-10, Table 4-11, Table 4-12). In addition, Pearson correlations among them were calculated.

The network diagram showed that pathogen density at the 3rd week were significantly negatively correlated with Ktedonobacterales, Bacillales, RB41, iii1-15 and Gaiellales and positively correlated with Rhizobiales, Enterobacteriales, Cytophagales and Pseudomonadales (Fig 4-19). This correlation pattern indicated that bacterial orders are classified into the pathogen-suppressing and the pathogen-promoting groups. The representative of the former group was Bacillales and the representative of the latter was Rhizobiales. The two orders were mutually exclusive as shown by a highly negative correlation (r=-0.65**) and Bacillales showed the highest negative correlation with pathogen density at 3rd week (r=-0.75**). P.G. (Platycodon), P.R. (Corn poppy) and F.E. (Buckwheat) as preceding crop brought about higher proportion of Bacillales in root communities, while Z.M. (maize) A.F. (Welsh onion) and G.M. (soybean) did its lower proportion. The stepwise regression analysis with pathogen density selected four orders in the root community at 3rd week, which explained 80% of the total variation in the pathogen density (Table 4-10). The selected microbes included Bacillales, Xanthomonadales, Sphingobacteriales and Saprospirales. The stepwise regression with DSI selected five bacterial orders but the proportion of the variation explained by selected microbes was merely 55% (Table 4-10).

As to the 4th week, root bacterial groups decreased their impacts on the pathogen density but increased on DSI (Table 4-11), the network correlations also changed (Fig 4-20). A stepwise regression model with the pathogen density selected three orders of the root community and explained only 36% of the variation, while the regression model against DSI selected nine orders of the root community and explained 88% of the total variation (Table 4-11). The progression of developmental stage also altered the correlation network among microbes. The bacterial orders selected by stepwise regression had little correspondence between the 3rd week and the 4th week. While Rhizobiales had consistently highly positive correlations with DSI at the 4th week and with pathogen density at the 3rd week. Bacillales, which had a negative correlation with pathogen density at the 3rd week (Fig 4-20), showed a positive correlation with DSI at the 4th week.

Since microbe members of the root community are recruited from a potential source of soil microbes through host plant selection, the composition of root community is expected to

resemble the community composition of soil in which they grow. However, the abundance of top 20 orders in the root community at the 3rd week did not show any significant correlations with the abundance of respective orders in the soil community except for Bacillales (r=0.476*). This result suggests that soil community does not have great influences on infection processes that occur in root tissues. Actually, the stepwise regression based on bacterial orders in the soil community did have a small effect on the pathogen density at the 3rd week (R^2=0.42) as shown in Table 4-12. However, the regression with DSI explained 81% of the total variation in DSI (Table 4-12).

There are at least two mechanisms underlying disease resistance caused by micorbiomes: competitive suppression of pathogen abundance and activation of plant immune response. In this study pathogen abundance at the 3rd week had a high positive correlation with DSI (r=0.731***) and thus suppression of pathogen abundance by the root microbes actually played important roles in controlling clubroot disease. The high correlation with pathogen abundance at the 3rd week demonstrated that Bacillales was the most effective bacteria that restrict clubroot pathogen abundance in the root of Chinese cabbage (Fig 4-22). On the other hand, Rhizobialles, which had a strong positive correlation with pathogen density (r=0.623**), was mutually exclusive with Bacillales (r=-0.647**). Therefore, pathogen density in the root is regulated by the balance of these two bacterial orders, which was induced by the cultivation of different preceding crops (Fig 4-22). The high correlation of pathogen density with DSI (r=0.731***) indicated that pathogen density is the most important factor in determining plant damage by clubroot. On the other hand, the pathogen density explained merely 53% of the variation in DSI (Fig 4-24) and the rest of the variation was explained by other bacterial orders such as Actynomycetales and Ktedonobacterales etc.. (Fig 4-23). These results indicate that in addition to Bacillales and Rhizobiales, many bacterial orders involved into suppression of clubroot damage.

Discussion

Crop rotation and microbial community changes

Our results provide insight into how crop rotation affects the assembly of microbiomes in soil and root. Preceding crops had great effects on microbiome structure not only on the diversity (Fig 4-4) but also on the composition of communities (Fig 4-13, Fig 4-14), which support

previous studies [48, 192]. With next generation sequencing that enables the analysis of microbial community at finer resolution, deep understanding of how preceding crops alter composition of soil and root communities was obtained. The relative abundance of Proteobacteria, Acidobacteria, Actinobacteria and Gemmatimonadetes in the root community significantly changed with preceding crops, while all bacterial phyla in soil significantly influenced by different species of preceding crops. The studies reporting that root exudates containing root-specific metabolites have critical ecological impacts on soil macro and microbiota [74, 193] suggest one mechanism behind the changes in structure of soil and root communities after preceding crops.

Root microbiomes in Chinese cabbage had lower OTU richness and a larger proportion of high-ranked OTUs than soil microbiomes (Table 4-2, Fig 4-6, Fig 4-8). Acidobacteria, Firmicutes and Gemmatimonadetes, were top three enriched phyla in soil, while Proteobacteria, Actinobacteria and Bacteroidetes were enriched in root (Fig 4-10, Fig 4-11, Fig 4-12). Our results including previous chapters indicate that bacterial phyla have a large difference in habitat preference between soil and root. Moreover, preceding crop treatment modified abundance pattern among phyla (Fig 4-10, Fig 4-11, Fig 4-12). The significant changes in relative abundances of microbes in root communities were found among Chinese cabbage seedlings grown under soil with different history of preceding crops. (Fig 4-8, Fig 4-14). Plants rescue microbes from soil according to genetic factor [20]. However, our results demonstrate that although plant effects as preceding crops were taken over the microbiome structure of succeeding crops, which proves effects of crop rotation on disease suppression. In a previous chapter plant family had different root microbiomes depending on their phylogenetic history. However, there were not any clear correlations between preceding crops and succeeding crops in terms of the abundances of top 20 orders of the root communities. These results suggest that effects of preceding crops on root microbiomes of Chinese cabbage do not result from difference in root microbiome structure among preceding crops but from other factors such as difference in chemical substance exuded from root of different plants.

Developmental stage also had significant effects on structure of root microbiomes (Fig 4-8, Fig 4-9, Fig 4-14). The observed number of OTUs in Chinese cabbage root microbiome significantly decreased from the 3rd to the 4th week (Fig 4-5, Table 4-2), and developmental difference was also found on the relative abundances of main bacterial phyla such as

Chloroflexi, Verrucomicrobia, Bacteroidetes and Firmicutes. These results were consistent with the study reporting substantial effects of plant development on structure of rhizosphere and root microbiomes [24].

Pathogenesis of Clubroot disease

The DSI of clubroot disease substantially increased from the 3rd week to 4th week and to the 6th week (Fig 4-15). DSI significantly correlated with the pathogen density (Fig 4-17), which agreed with the previous study reporting increase in clubroot DSI with time and with environmental factors such as temperature [194]. In this study, the significant difference in DSI due to preceding crop treatment was only found at the 4th week (Table 4-4, Fig 4-16), because pathogen (*Plasmodiophora brassicae*) density, the main cause of disease damage, was more variable at the earlier stage (the 3rd week) among preceding crops than the 4th week (Table 4-7, Fig 4-18). Several studies have shown linear relationships among resting spore density, root hair infection and disease severity [195, 196]. In this study, where resting spores were infected to Chinese cabbage when planting seedlings, the pathogen proliferation in root and disease severity were inseparable, because rapid infection of *Plasmodiophora brassicae* pathogen density to root tissues may cause hysteretic development of clubroot disease symptom.

Inhibition of clubroot damage and microbiomes

Microbiomes can have two roles on inhibition of disease damage: competitive suppression of pathogen proliferation and activation of plant immune response. These two mechanisms of disease inhibition can be visualized by a regression line of disease damage against pathogen density and the deviation from the regression line, respectively (Fig 4-25). The pathogen density at the 3rd week was mainly determined by the two key microbes, Bacillales and Rhizobiales, which showed sensitive response of their abundance to preceding crops (Fig 4-22). However, pathogen density explained almost half of the variation in DSI at the 4th week and the rest of the variation was due to activation of plant immune response that was induced by many bacterial orders in the root and soil communities (Fig 4-23). These results suggested that in addition to the two key microbes, many bacterial groups are intricately involved into inhibition of clubroot disease damage in Chinese cabbage.

The correlation analysis reveals that microbes in soil and root were composed of complicated network among them and were involved into the two mechanisms of disease inhibition: suppression of pathogen density and activation of plant immune response. The network can be classified into the two groups according to the relation with the density of clubroot pathogen, *Plasmodiophora brassicae*: the group that has a positive relation with pathogen density and the other that has a negative relation (Fig 4-19). Bacillales and Xanthomonadales belonged to the latter group (Table 4-10). Especially, the relative abundance of Bacillales was highly correlated with pathogen density in negative direction and played central roles in connecting the network (Table 4-10, Fig 4-19). *Bacillus subtilis* belonging to Bacillales is well known to produce insecticidal protein and Bacillales is reported to suppress clubroot damage of vegetables in Brassicaecea and is used for a commercial biofungicide "Serenade" with good effectiveness [188]. It was suggested that Bacillales suppressies Clubroot disease via antibiosis and inducing host resistance [197]. These antibiotic effects of Bacillales could explain the effects of Bacillales on pathogen density and DSI (Fig 4-19, Fig 4-20).

On the other hand, Rhizobiales that had the strongest positive correlations with pathogen density at the 3rd week and DSI at the 4th week is a representative member of the other group (Table 4-10, Fig 4-19, Fig 4-20). Rhizobiales is known to provide plants with beneficial functions such as providing various nutrients, phytohormons as well as precursors for essential plant metabolites [198, 199]. It also includes many genera with functions like nitrogen-fixing and methanotrophic [200].

Since Bacillales and Rhizobiales in soil didn't show significant correlations with pathogen density and DSI, that these microbes seem to play roles after entering root tissues.

Actinomycetales was also an important member that has a negative relation with pathogen density and formed many connections with several microbes in both 3rd and 4th week (Fig 4-19, Fig 4-20). Actinomycetales is reported to play a pivotal role in maintaining the biological balance in soil, largely because of their ability to produce antibiotics and other secondary metabolites [201]. Gaiellales involves in a potato soil-borne disease, although the function still unclear [202]. Xanthomonadales, Pseudomonadaceae and Burkholderiaceae were identified as the most dynamic taxa associated with disease suppression [60]. Burkholderiaceae and Xanthomonadales are known to harbor genera and species with activity against plant pathogenic

fungi [203]. Although this study did not clarify roles of these bacterial orders, the complicated network suggests many bacteria involves into suppression of clubroot disease in Chinese cabbage and crop rotation can work as defense mechanisms by inducing changes in microbiome structure.

Conclusion

Clubroot disease damage among 20 preceding crops mostly differed at the 4th week, while the pathogen density at earlier stage was the main cause. Preceding crops had great effects on soil microbiome constitutions, which also altered root microbiomes structure in Chinese cabbage. Two key microbes Bacillales and Rhizobiales were detected in determining pathogen density, which were mutually exclusive each other in root community. Clubroot pathogen density was the main determinant of clubroot disease damage. Several microbes from root and/or soil involved into clubroot disease suppression.

FIGURES AND TABLES

Table 4-1. Taxonomic information of twenty plant species used in the experiment.

No.	Abbr.	Common Name	Class	Order	Family	Genus	Species
1	I.A.	Water spinach	E	Solanales	Convolvulaceae	Ipomoea	aquatica
2	S.L.	Tomato	E	Solanales	Solanacea	Solanum	lycopersicum
3	P.F.	Perilla	E	Lamiales	Lamiacea	Perilla	frutescens
4	A.M.	Snapdragon	E	Lamiales	Plantaginaceae	Antirrhinum	majus
5	Cr.C.	Canadian honewort	E	Apiales	Apiacea	Cryptotaenia	canadensis
6	Ch.C.	Crown daisy	E	Asterales	Asteraceae	Glebionis	coronaria
7	P.G.	Platycodon	E	Asterales	Campanulaceae	Platycodon	grandiflorus
8	I.B.	Garden balsam	E	Geraniales	Balsaminaceae	Impatiens	balsamina
9	F.E.	Buckwheat	E	Caryophyllales	Polygonaceae	Fagopyrum	esculentum
10	B.A.	Creeping spinach	E	Caryophyllales	Basellaceae	Basella	alba
11	C.A.	Plumed cockscomb	E	Caryophyllales	Amaranthaceae	Celosia	argentea
12	D.S.	Fringed pink	E	Caryophyllales	Caryophyllaceae	Dianthus	superbus
13	S.O.	Spinach	E	Caryophyllales	Chenopodiideae	Spinacia	oleracea
14	C.S.	Cucumber	E	Cucurbitales	Cucurbitaceae	Cucumis	sativus
15	G.M.	Soybean	E	Fabales	Fabaceae	Glycine	max
16	A.E.	Okra	E	Malvales	Malvaceae	Abelmoschus	esculentus
17	B.R.	Chinese cabbage	E	Brassicales	Brassicaceae	Brassica	rapa
18	P.R.	Corn poppy	E	Ranunclulales	Papaveraceae	Papaver	rhoeas
19	A.F.	Welsh onion	M	Asparagales	Amaryllidaceae	Allium	fistulosum
20	Z.M.	Maize	M	Poales	Poaceae	Zea	mays

No. was sorted by phylogenetic position.
E: Eudicot; M: Monocot

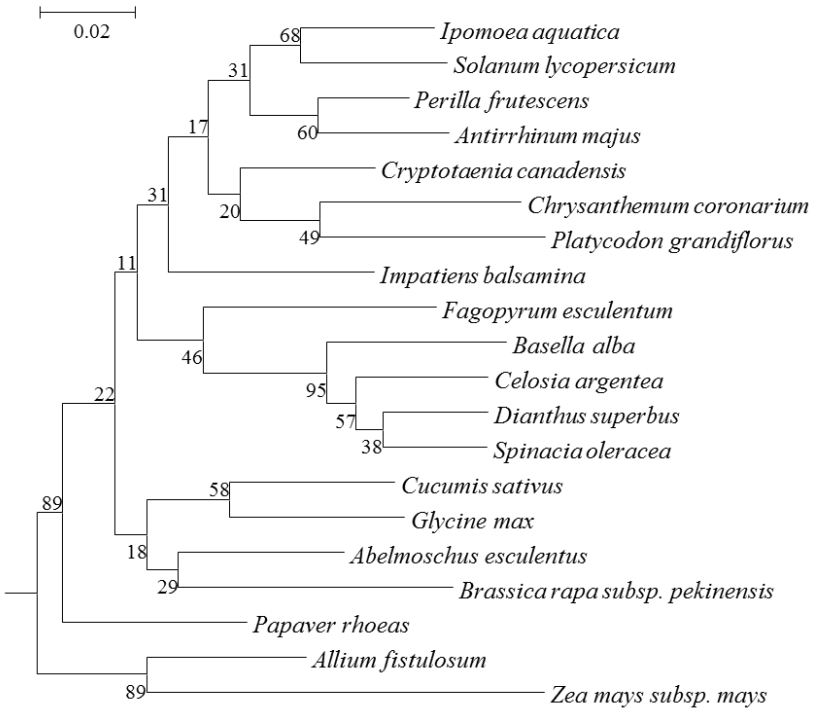


Fig 4-1. Phylogenetic correlation of twenty plant species used in the experiment. The phylogenetic tree was generated based on plant chloroplast *rbcL* gene by maximum likelihood analysis, 1000 times bootstrap replications. Binomial Nomenclature was used to represent each plant.

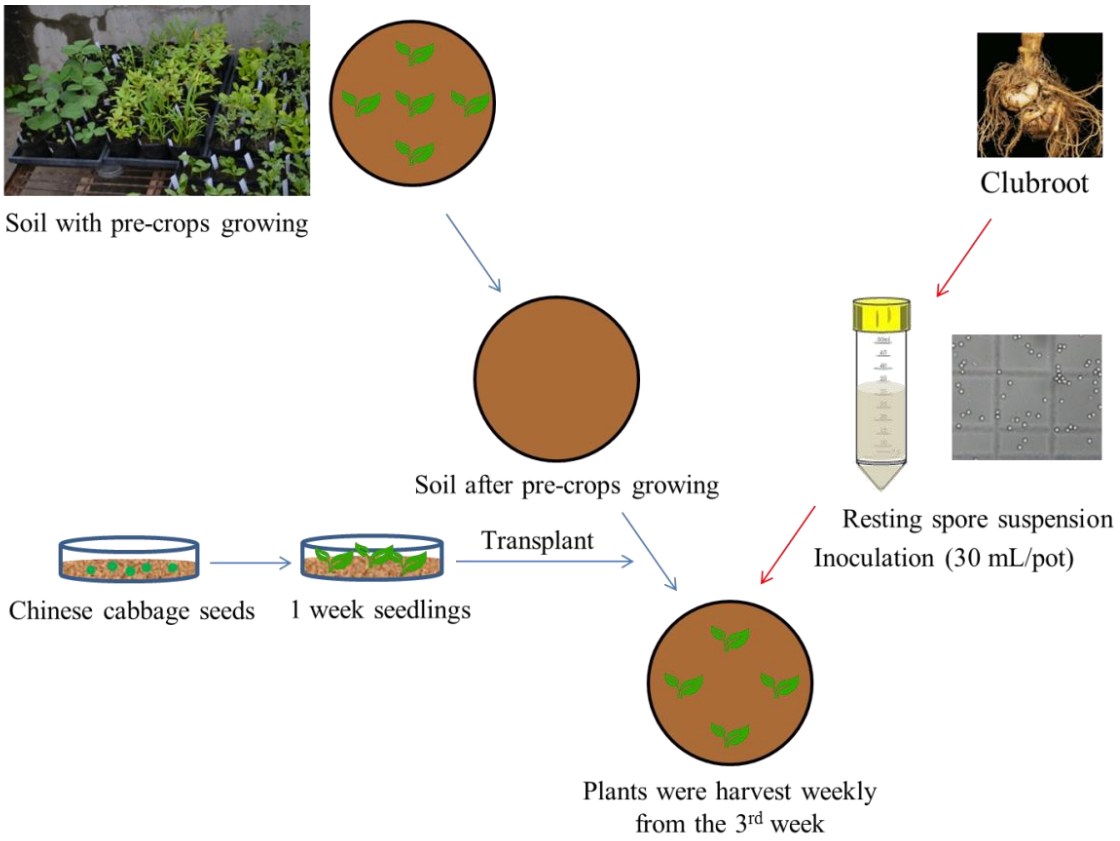


Fig 4-2. The flowchart of experiment design.

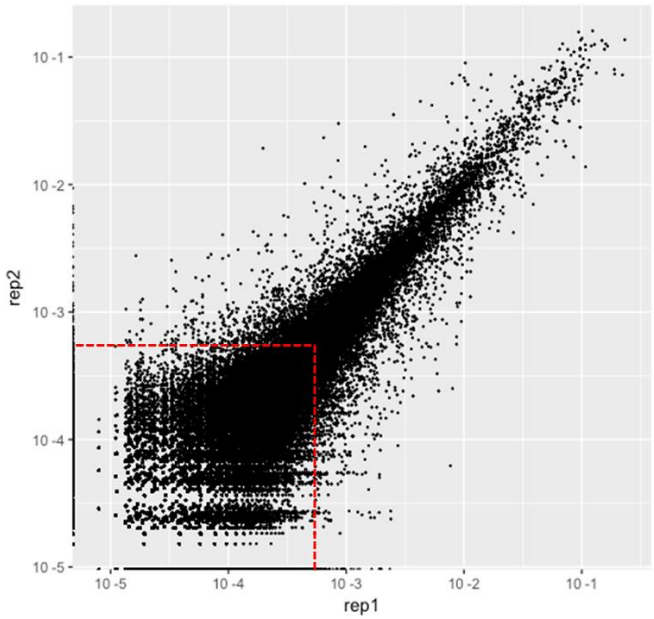


Fig 4-3. Threshold value decisions—to decrease bias of technical replicates. OTUs relative abundance of each replicate were Log_{10} transformed and plotted as x axis (replicate 1) vs y axis (replicate 2), both replicates were PCR-amplified and sequenced from the same sample (mix sample of 5 replicates).

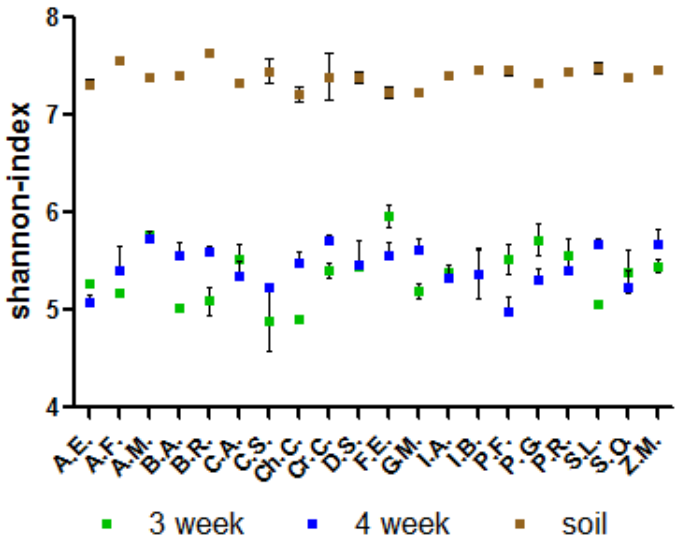


Fig 4-4. α-diversity–Shannon index of soil bacterial communities after twenty different pre-crops growing and root bacterial microbiomes of Chinese cabbage at the 3rd week and the 4th week.

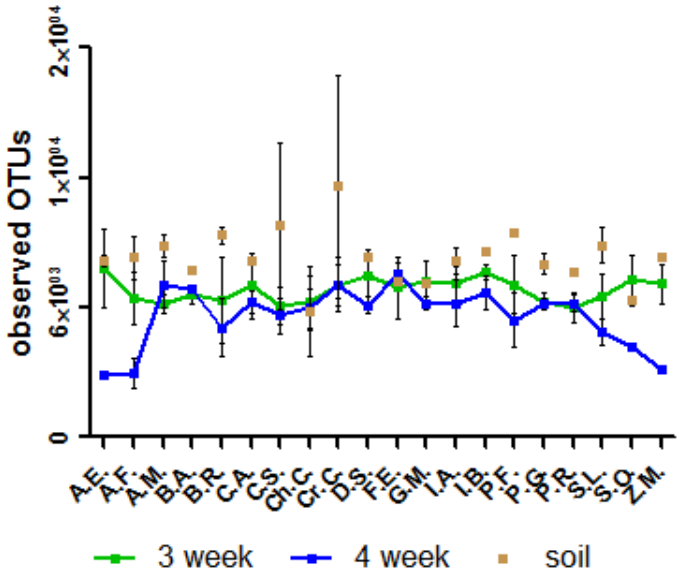


Fig 4-5. α-diversity–observed OTU numbers of soil bacterial communities after twenty different pre-crops growing and root bacterial microbiomes of Chinese cabbage at the 3rd week and the 4th week.

Table 4-2. α-diversity (mean values) of soil and Chinese cabbage root samples. The one-way ANOVA was applied for testing the variances between sample type; the two-way ANOVA for testing the variances of the interaction between development stage and pre-crop species (* $p<0.05$; ** $p<0.01$; *** $p<0.001$).

	Shannon index	Observed OTUs
Soil	7.39	8289
Root	5.39	6227
Pr(>F)	<0.001 ***	<0.001 ***
3rd week	5.35	6823
4th week	5.44	5631
Pr(>F)	0.07	<0.001 ***

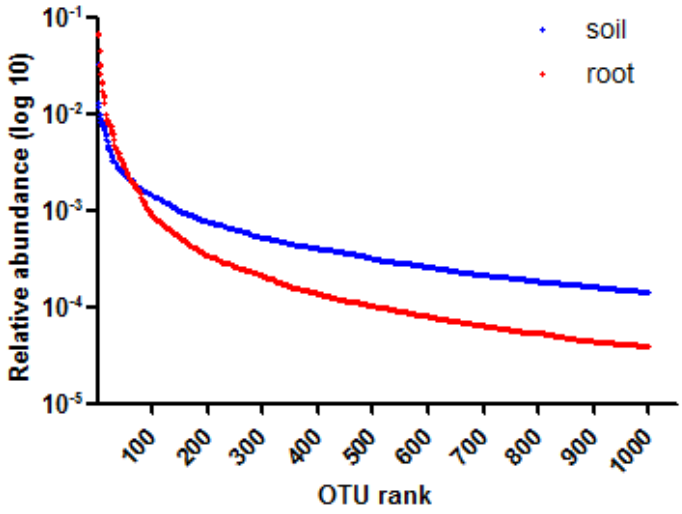


Fig 4-6. Rank abundance curves--OTUs were arranged by rank of total counts number, relative abundance were Log_{10} transformed.

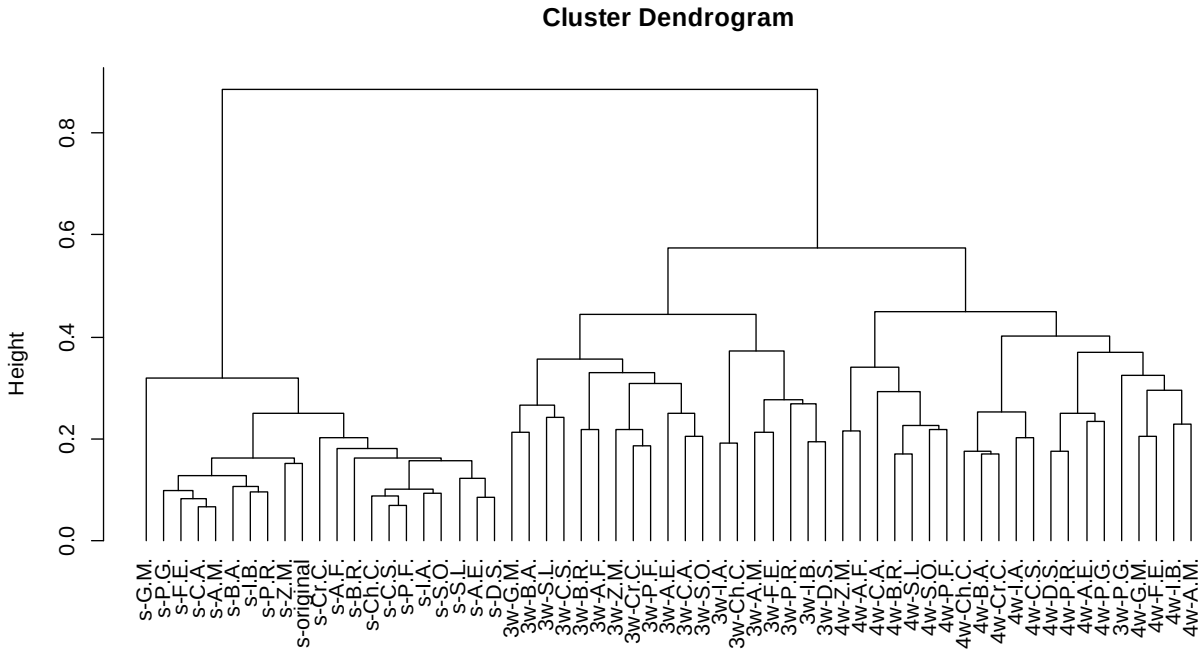


Fig 4-7. Hierarchical clustering of soil and root samples (0.5% threshold). Dendrogram clustered using weighted pair group average (UPGMA) method.

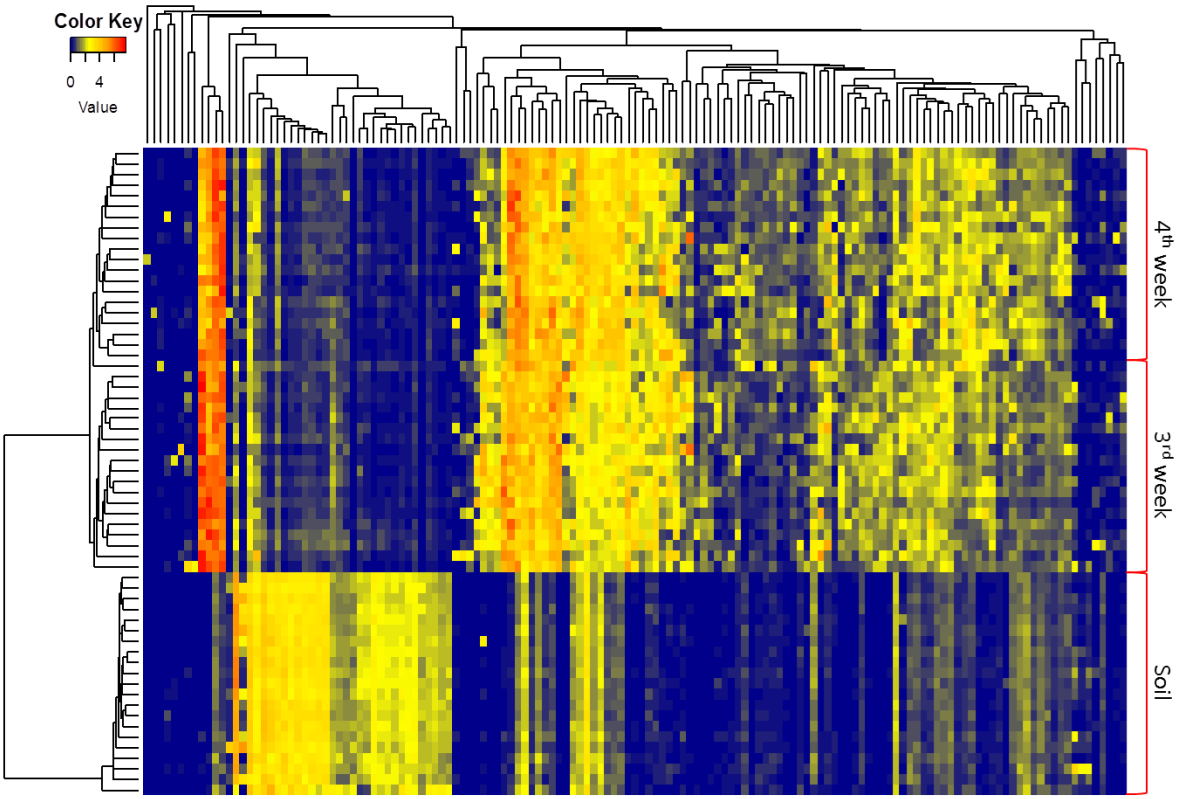


Fig 4-8. Heatmap of the soil and root samples (0.5% threshold). All data were log normalized. Columns represent relative abundance of each OTU. Rows represent each sample of different soil and root. Both samples and OTUs were clustered based on Bray–Curtis similarities.

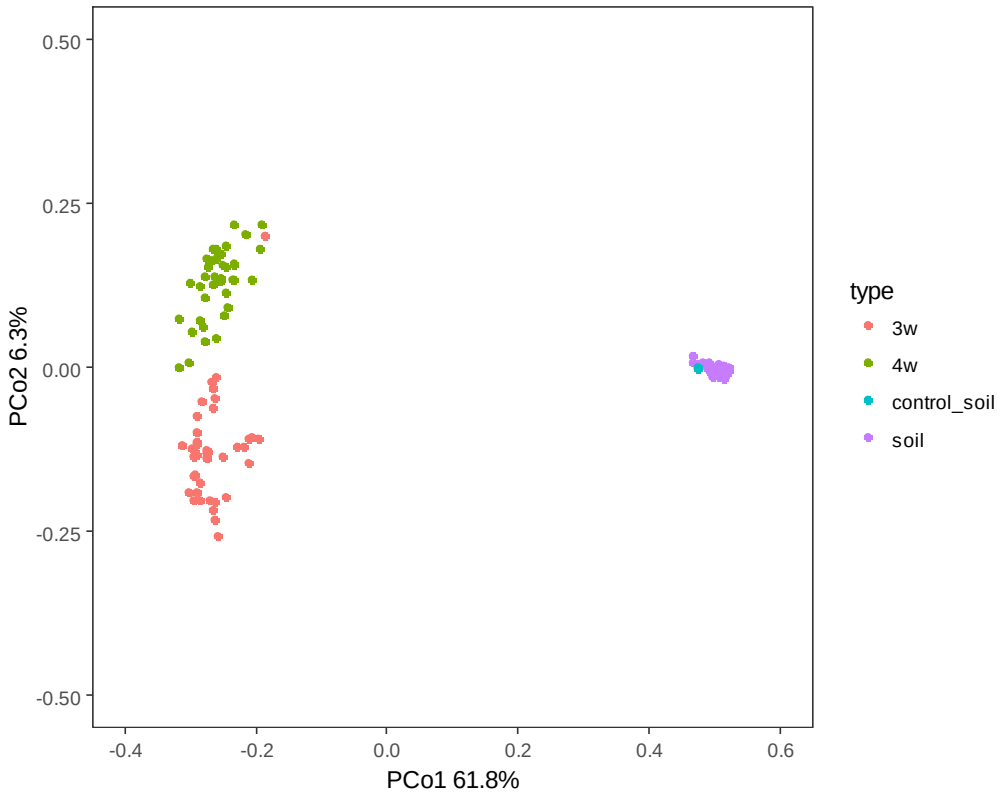


Fig 4-9. Principal coordinates analysis (PCoA) of bacterial community compositions of soil and root samples based on Bray-Curtis distances.

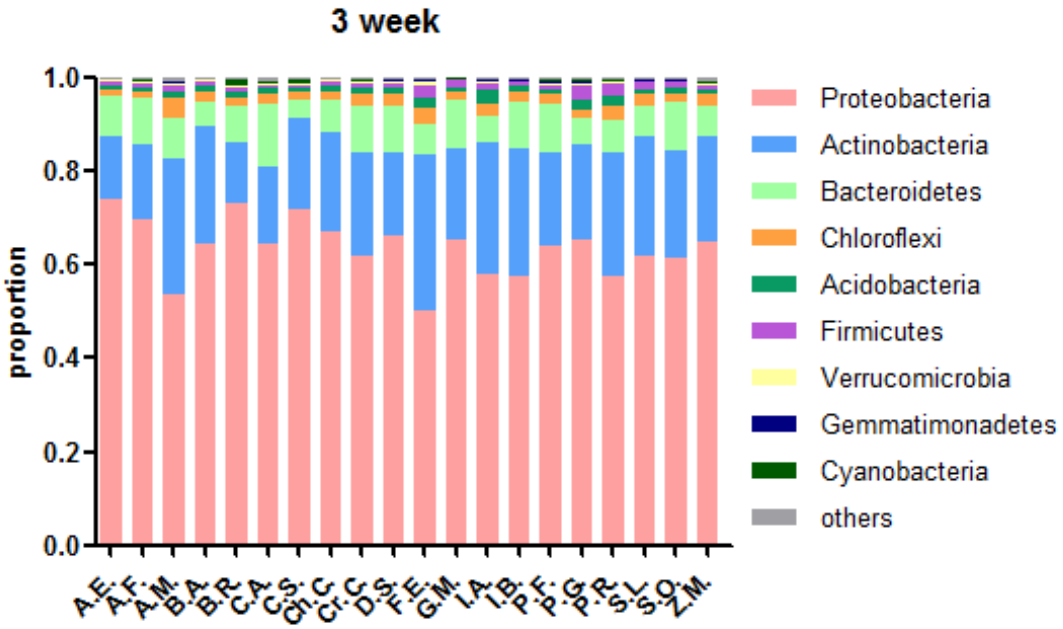


Fig 4-10. Bacterial community distributions of the 3rd week Chinese cabbage root samples at phylum taxonomic level.

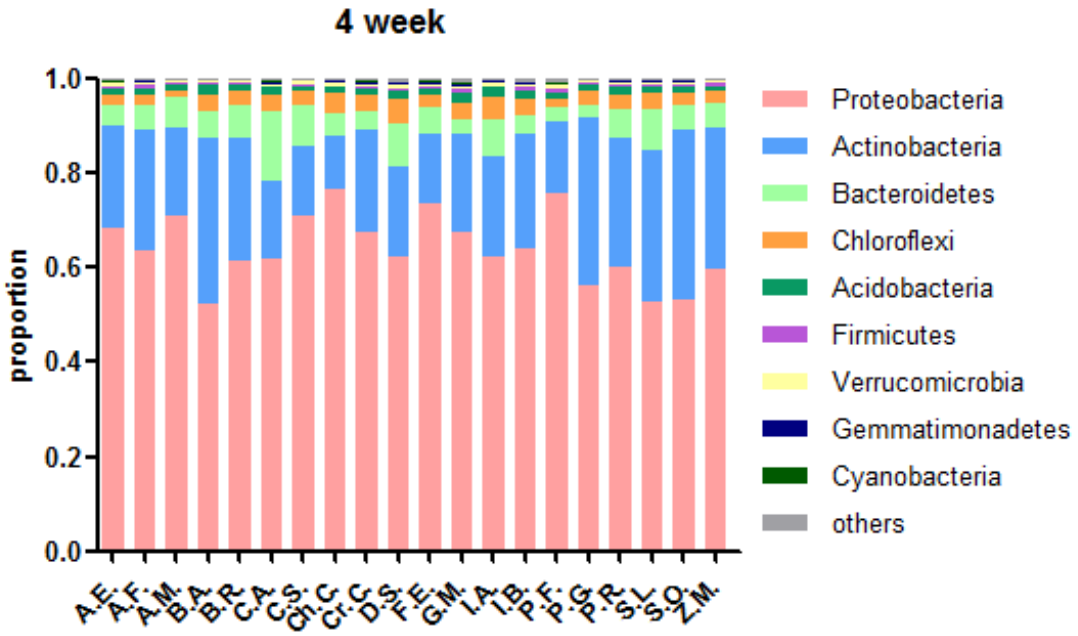


Fig 4-11 Bacterial community distributions of the 4th week Chinese cabbage root samples at phylum taxonomic level.

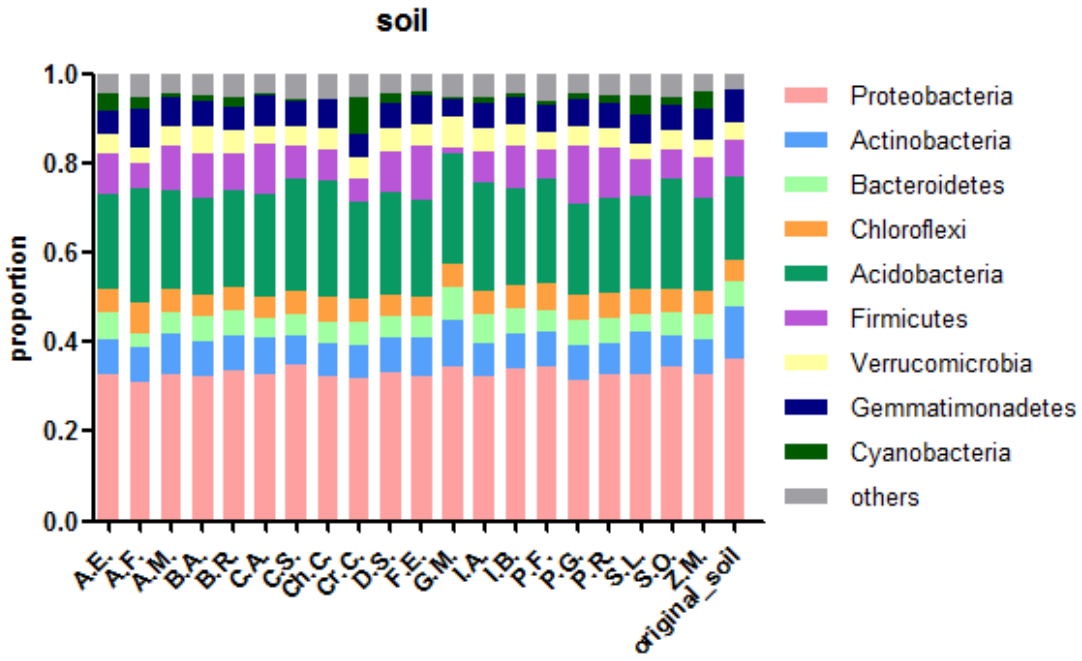


Fig 4-12 Bacterial community distributions of the original soil and after pre-crops growing soil samples at phylum taxonomic level.

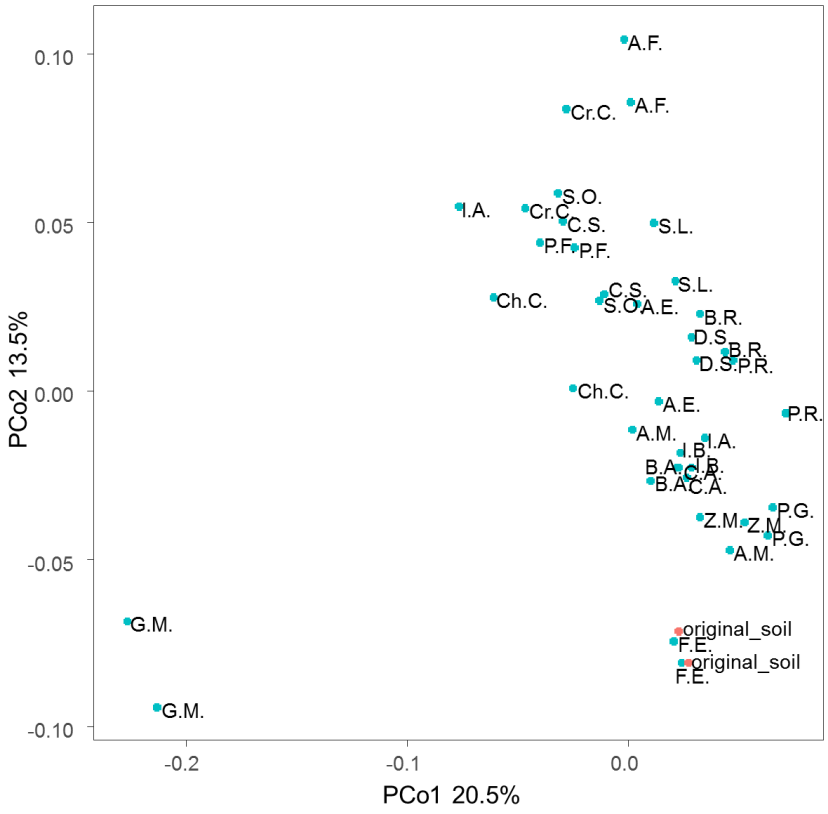


Fig 4-13. Principal coordinates analysis (PCoA) of bacterial community compositions of the original soil (red) and after pre-crops growing soil (blue) samples based on Bray-Curtis distances.

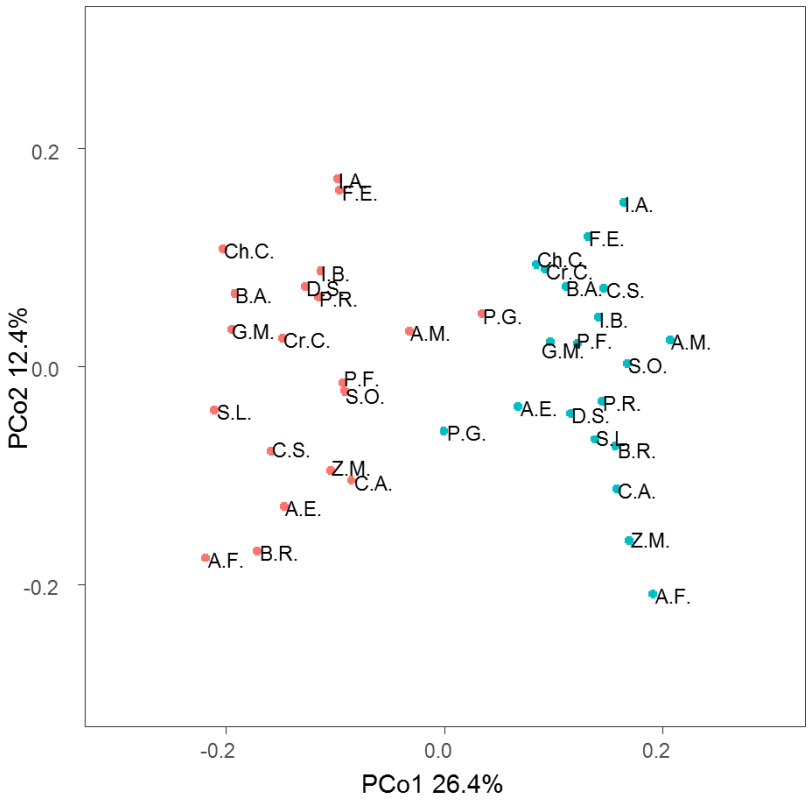


Fig 4-14. Principal coordinates analysis (PCoA) of bacterial community compositions of the 3rd week (red) and the 4th week (blue) root samples based on Bray-Curtis distances.

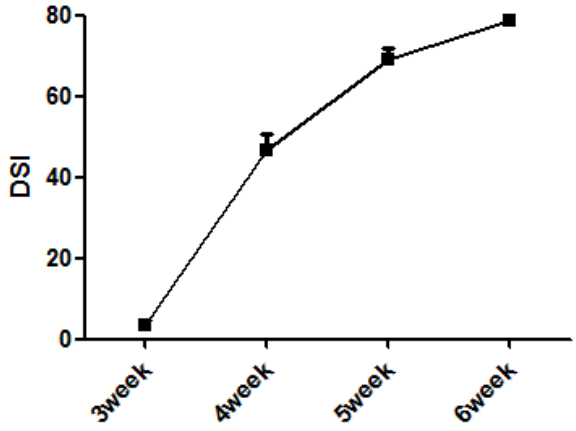


Fig 4-15. DSI of clubroot disease on Chinese cabbage root in different sampling period.

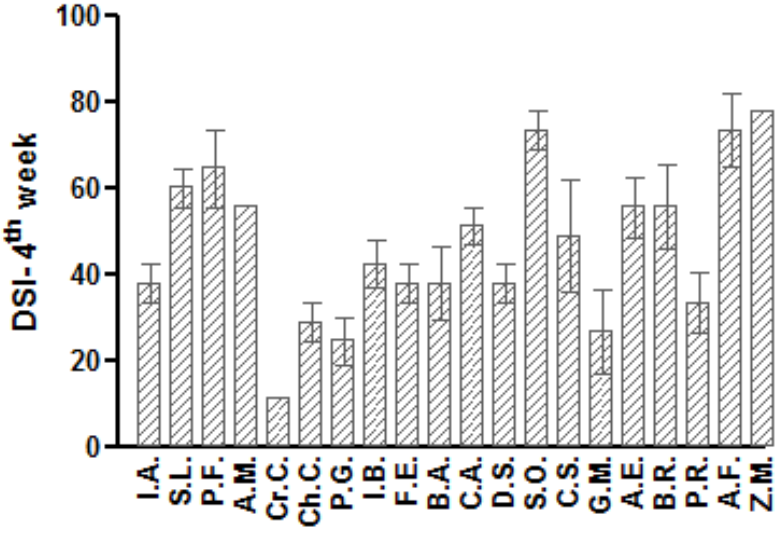


Fig 4-16 DSI of Chinese cabbage with different pre-crop species at the 4th week. All samples were ordered by phylogenetic relations of pre-crop species.

Table 4-3. Variances of development stage, pre-crop species and interaction on DSI by two-way ANOVA.

Factors	F	Pr(>F)
time	381.093	<0.001
precrop	4.389	<0.001
time:precrop	1.872	<0.001

Table 4-4. Variances of pre-crop species on DSI at each development stage by one-way ANOVA.

Sampling time	F	Pr(>F)
3 week	1.182	0.293
4 week	7.386	<0.001
5 week	1.483	0.115
6 week	1.246	0.244

Table 4-5. Variances of development stage, pre-crop species and their interaction on pathogen density by two-way ANOVA.

Factors	F	Pr(>F)
time	20.254	<0.001
precrop	7.335	<0.001
time:precrop	4.413	<0.001

Table 4-6. Variances of pre-crop species on pathogen density at the 3rd and the 4th week by one-way ANOVA.

Sampling time	F	Pr(>F)
3 week	12.54	<0.001
4 week	5.611	<0.001

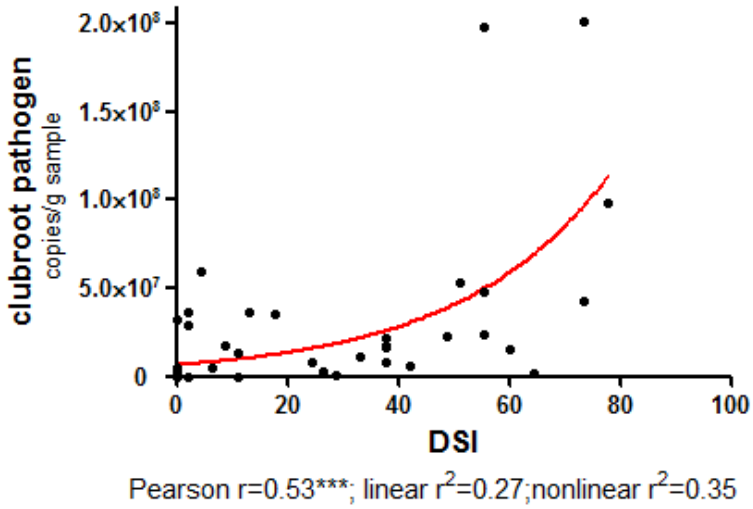


Fig 4-17. Correlation of DSI and clubroot pathogen density at the 3rd and 4th week.

Table 4-7. Correlations of the 4th week DSI and the 3rd and 4th week clubroot pathogen density. Pathogen copy number (copy) and natural logarithm transformed copy number (lncopy) were used in the analysis.

	copy-3w	copy-4w	DSI-4w	lncopy-3w
copy-4w	0.46*			
DSI-4w	0.67**	0.57**		
lncopy-3w	0.85***	0.48*	0.73***	
lncopy-4w	0.37	0.60**	0.69***	0.34

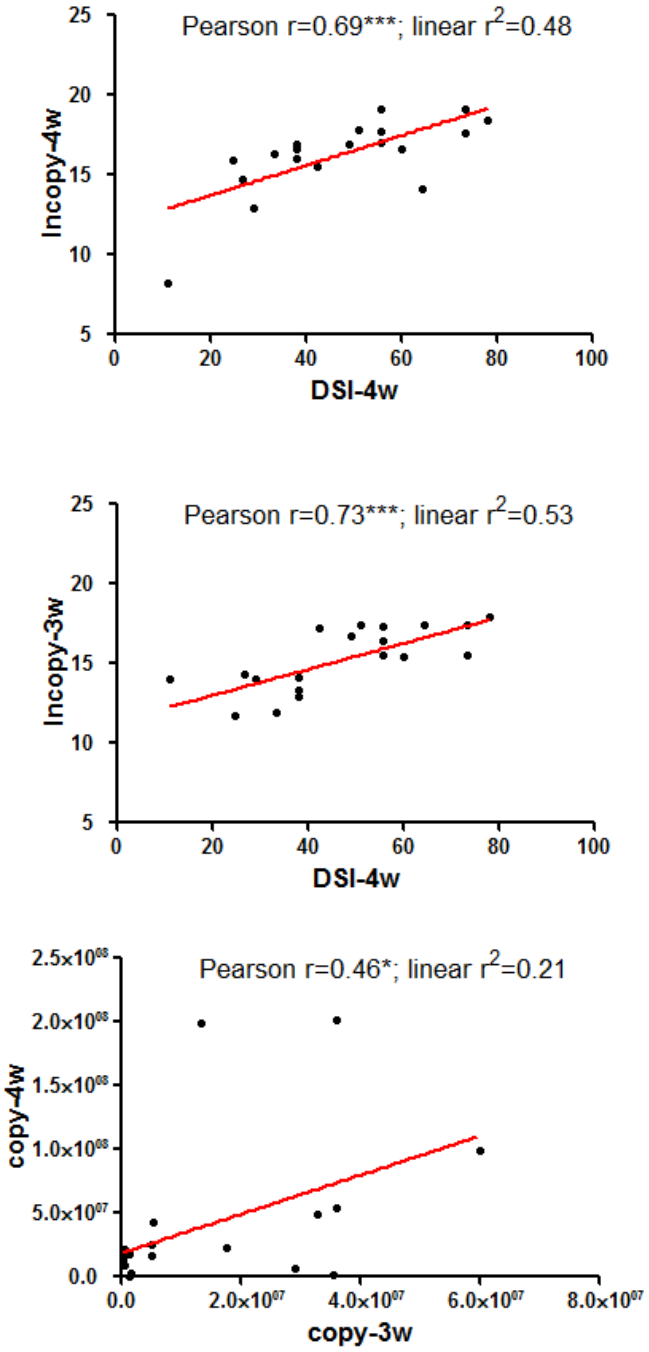


Fig 4-18. Correlations of the 4th week DSI and the 3rd and 4th week clubroot pathogen density. Pathogen copy number (copy) and natural logarithm transformed copy number (lncopy) were used in the analysis.

Table 4-8. Proportions of assigned taxa (with taxonomic information) at different taxonomic level of threshold data.

	phylum	class	order	family	genus	species
Assigned taxa proportion (%)	100.0	99.9	92.7	70.2	28.7	3.0

Table 4-9. Proportions of top twenty orders in soil and root samples.

	No. of total orders	Proportion of top 20 orders
Soil	89	45.4%
Root (3^{rd} week)	87	81.2%
Root (4^{th} week)	90	80.3%

Table 4-10. Stepwise regression analysis and correlation analysis of pathogen density (3rd week) and DSI (4th week) against the abundance of the top 20 orders of the root community at the 3rd week. Top 20 bacterial orders were arranged by their abundances.

Term	lncopy number (3rd week)		DSI (4th week)	
	Estimate	Pearson r	Estimate	Pearson r
Intercept	23.77***		36.8	
Actinomycetales		-0.43		-0.18
Xanthomonadales	-34.42***	-0.36		-0.45*
Rhizobiales		0.62**		0.3
Burkholderiales		-0.07		-0.09
Enterobacteriales		0.45*		0.41
[Saprospirales]	-19.28	0.31		0.03
Sphingomonadales		0.35	1743.03**	0.56*
Ktedonobacterales		-0.45*	-939.23	-0.42
Bacillales	-335.54***	-0.75***		-0.36
Cytophagales		0.59**		0.54*
Pseudomonadales		0.53*	850.35	0.38
Caulobacterales		0.22	-3228.71	0.13
Myxococcales		0.16		0.31
[Roseiflexales]		0.05		0.12
Solirubrobacterales		-0.21		0.02
Sphingobacteriales	170.29	-0.33	-2338.16*	-0.35
RB41		-0.52*		-0.35
iii1-15		-0.6**		-0.34
Gaiellales		-0.53*		-0.25
Methylophilales		-0.28		-0.14
RSquare Adj	0.80***		0.55**	

Table 4-11. Stepwise regression analysis and correlation analysis of pathogen density (4th week) and DSI (4th week) against root microbes at the 4th week. Top 20 bacterial orders were used for analysis and sorted by abundance from high to low.

Term	lncopy number (4th week)		DSI (4th week)	
	Estimate	Pearson r	Estimate	Pearson r
Intercept	23.77***		241.46***	
Burkholderiales		-0.11	-367.85***	-0.53*
Actinomycetales		-0.26	-171.35**	0.02
Rhizobiales		0.41		0.68***
Xanthomonadales		0.21		-0.1
[Saprospirales]		-0.28		-0.35
Sphingomonadales		0.13	-1032.29**	-0.17
Enterobacteriales	-34.42	-0.15	-749.86***	-0.46*
Ktedonobacterales		-0.29		-0.43
[Roseiflexales]		0.21		0.15
Cytophagales		0.05		-0.11
Caulobacterales		0.31		0.04
Pseudomonadales		0.18	-2216.77*	-0.02
Myxococcales		-0.10	2810*	-0.02
Bacillales		0.11		0.43
Solirubrobacterales		-0.16	-6604.47***	0.21
Methylophilales	-19.28*	0.54*	1563.28	0.48*
iii1-15		-0.09		-0.04
RB41		-0.31	-8129.93**	-0.49*
Rhodospirillales		0.42		0.58**
Ellin6067	-335.54	-0.46*		-0.31
RSquare Adj	0.36*		0.88***	

Table 4-12. Stepwise regression analysis and correlation analysis of pathogen density (3rd week) and DSI (4th week) against soil microbes. Top 20 bacterial orders were used for analysis and sorted by abundance from high to low.

Term	lncopy number (3rd week)		DSI (4th week)	
	Estimate	Pearson r	Estimate	Pearson r
Intercept	1.22E+08*		112.69*	
Bacillales		-0.28		-0.04
Rhizobiales		-0.08		-0.29
RB41		<0.01		-0.14
Acidobacteriales	-1.90E+09	0.08	-3332.62***	0.22
iii1-15		0.16		0.17
[Saprospirales]		-0.40	-1443.35*	-0.46*
Burkholderiales	1.49E+09	0.10	2615.13***	0.21
Actinomycetales		-0.15		-0.09
Solibacterales		-0.35		-0.52*
Xanthomonadales		0.06	2326.47*	0.19
[Chthoniobacterales]	-4.1E+09**	-0.44*	-3105.86**	-0.5*
Sphingomonadales		-0.21		-0.26
N1423WL		-0.04		0.05
Gaiellales		0.06		0.07
MND1		0.28	2149.93	0.33
Myxococcales		-0.07		0.1
Nitrospirales		-0.11		-0.14
Ellin6067		0.24		0.13
Nostocales	-8.5E+08*	-0.05	-1245.55***	-0.25
A21b		0.29		0.08
RSquare Adj	0.42*		0.81***	

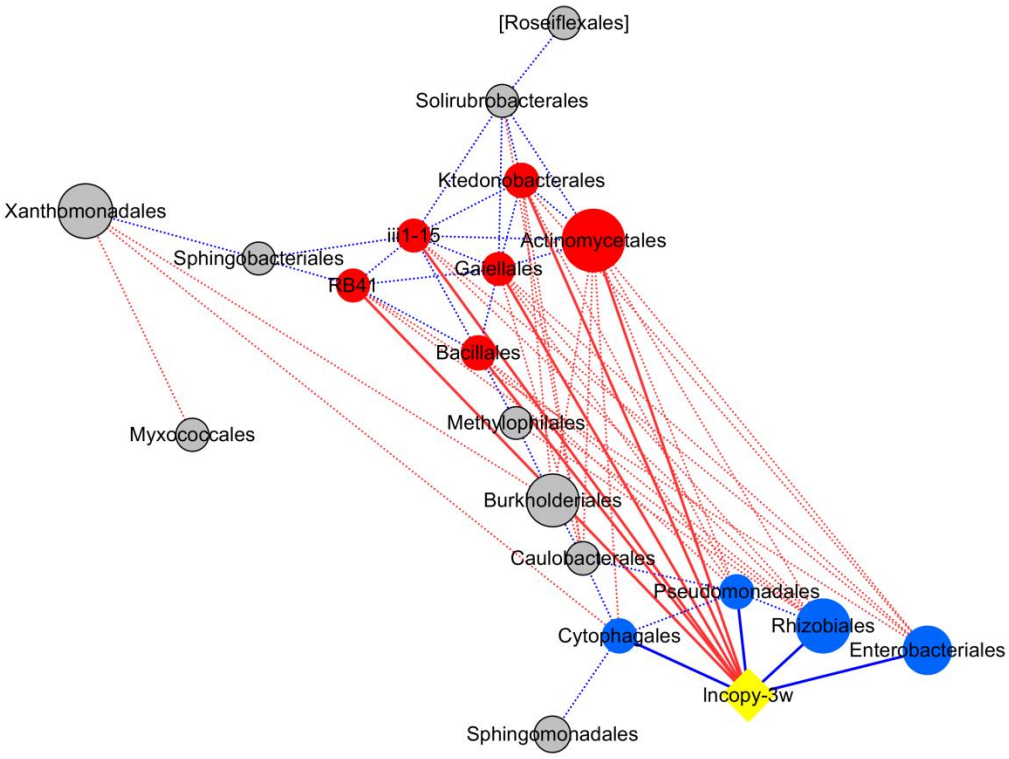


Fig 4-19 Correlation-based network analysis of the relative abundances of root top 20 bacterial orders and pathogen copy number at the 3rd week. The analysis used Pearson correlation with threshold at |r|>0.4 and Fisher's Z with P-value threshold at 0.05. The size of each circle represents average relative abundance (maximum=4). The blue and red color circles correspond to co-presence and mutual exclusive orders respectively, which directly significantly correlated with pathogen density. Red lines indicate negative correlations, and the blue lines indicate positive correlations. Solid lines indicate correlations between bacterial orders and pathogen density, and dotted lines indicate correlations between bacterial orders.

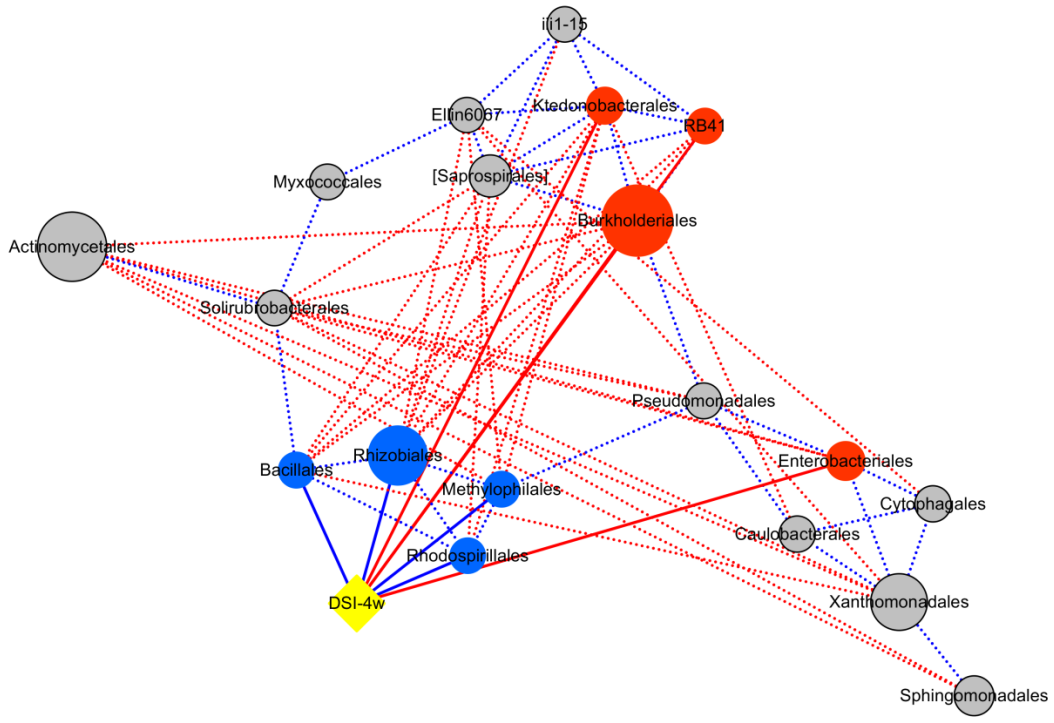


Fig 4-20 Correlation-based network analysis of the relative abundances of root top 20 bacterial orders and DSI at the 4th week. The analysis used Pearson correlation with threshold at |r|>0.4 and Fisher's Z with P-value threshold at 0.05. The size of each circle represents average relative abundance (maximum=4). The blue and red color circles correspond to co-presence and mutual exclusive orders respectively, which directly significantly correlated with pathogen density. Red lines indicate negative correlations, and the blue lines indicate positive correlations. Solid lines indicate correlations between bacterial orders and pathogen density, and dotted lines indicate correlations between bacterial orders.

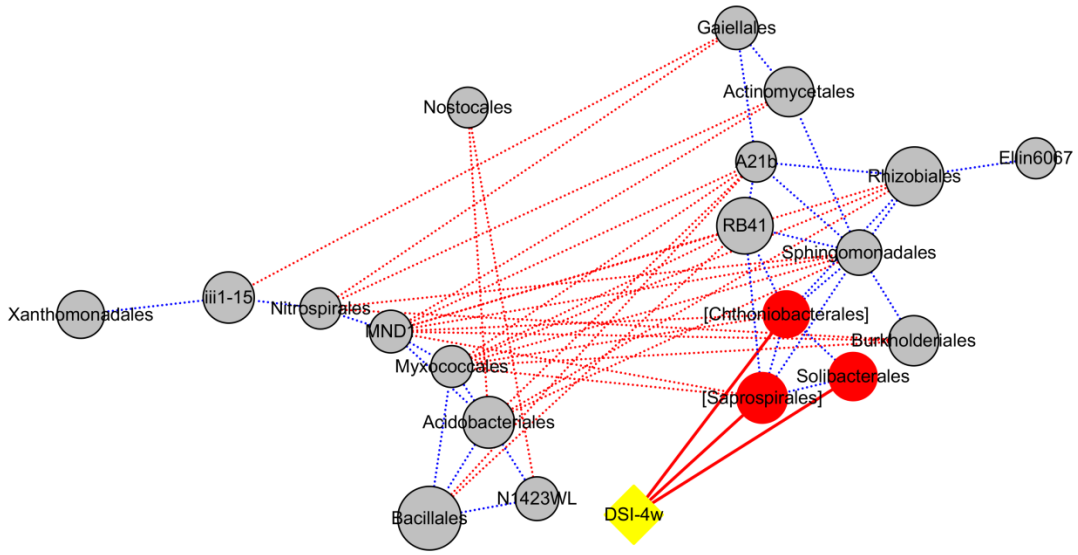


Fig 4-21 Correlation-based network analysis of the relative abundances of soil top 20 bacterial orders and DSI at the 4th week. The analysis used Pearson correlation with threshold at |r|>0.4 and Fisher's Z with P-value threshold at 0.05. The size of each circle represents average relative abundance (maximum=2). The red color circles correspond to mutual exclusive orders, which directly significantly correlated with pathogen density. Red lines indicate negative correlations, and the blue lines indicate positive correlations. Solid lines indicate correlations between bacterial orders and pathogen density, and dotted lines indicate correlations between bacterial orders.

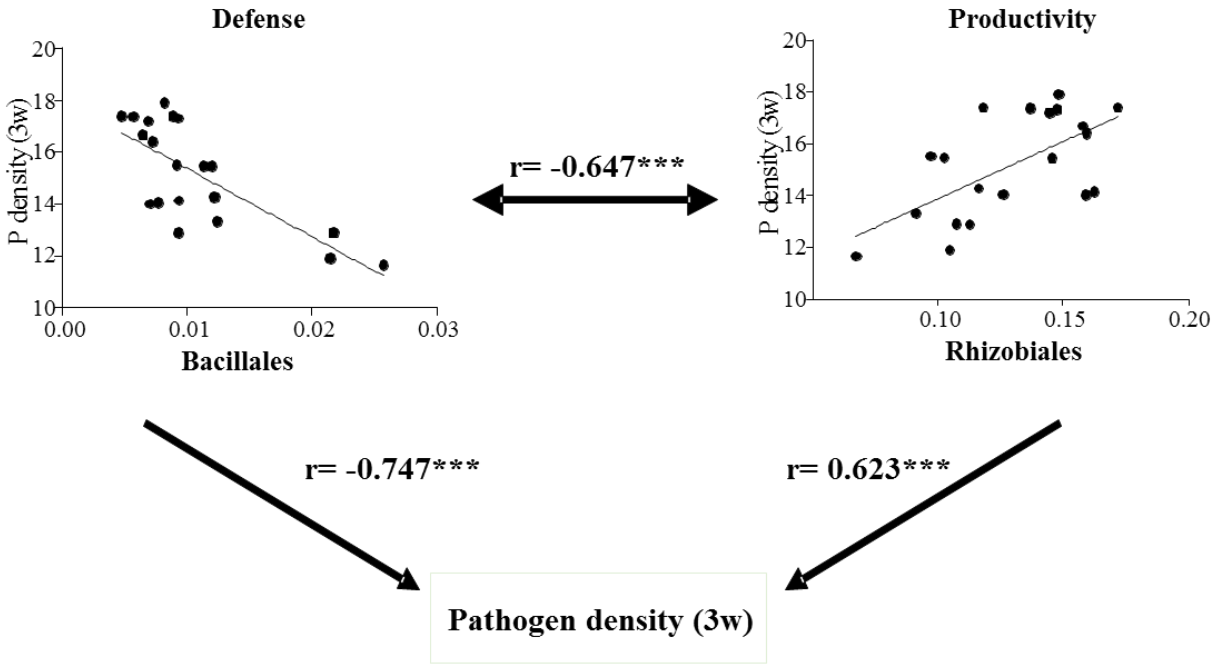


Fig 4-22. The relationships of pathogen density (3rd week) against key microbes, Bacillales and Rhizobilaes of root communities at the 3rd week.

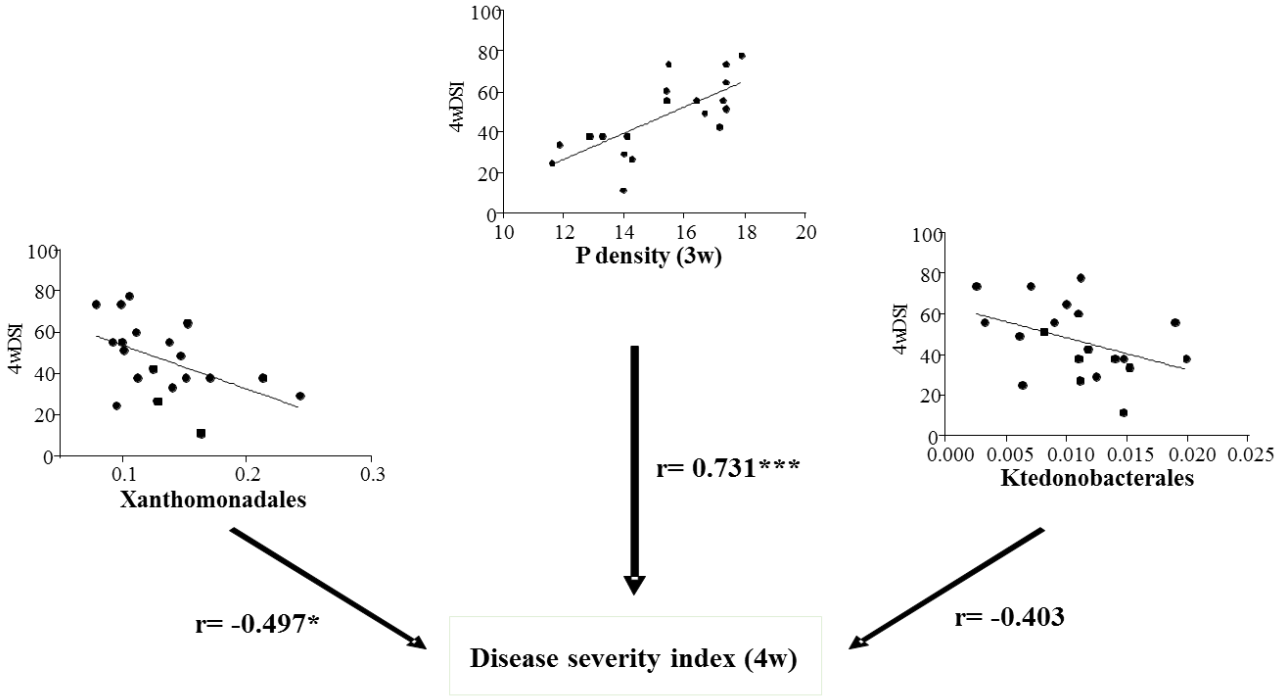


Fig 4-23. Relationships of DSI at the 4th week against pathogen density and key microbes in the root communities.

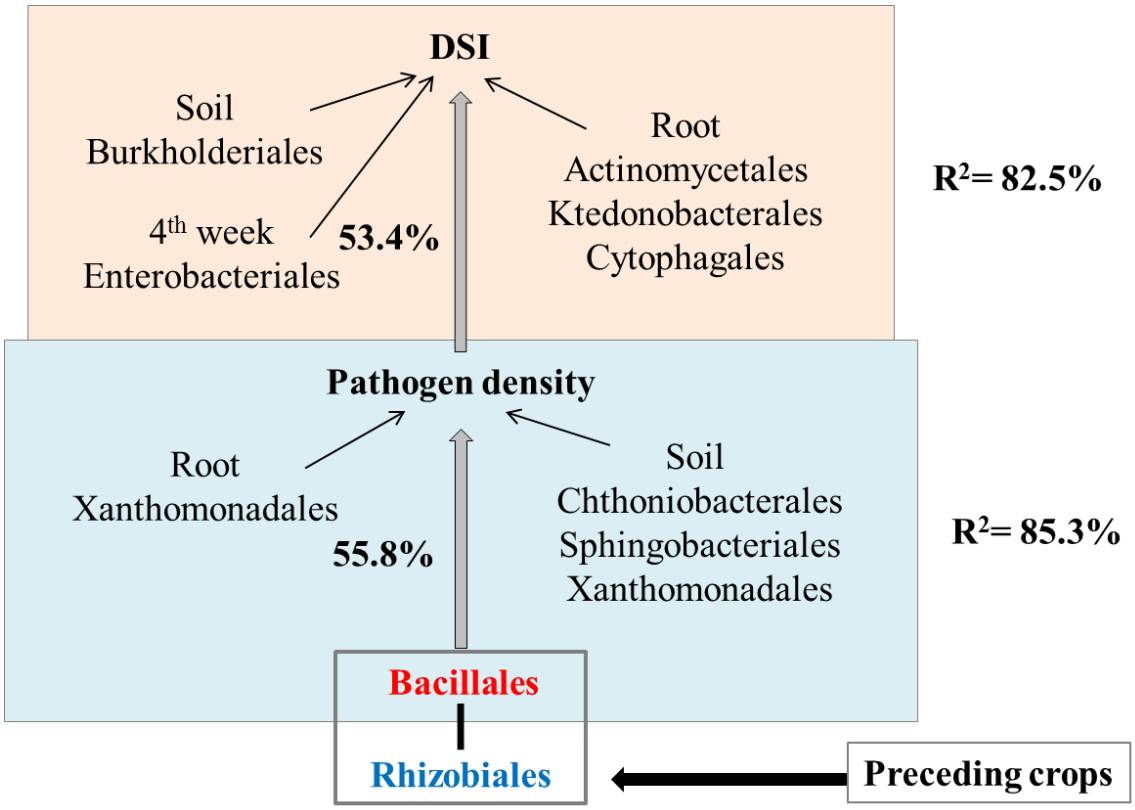


Fig 4-24. The model representing mechanism for inhibition of clubroot damage by soil and root microbiomes.

CHAPTER 5: GENERAL DISCUSSION

Soil Microbial Community and Root Microbiome

Differences between soil and root microbiome

The results of all three experiments demonstrated that plant root microbiome possesses significantly less rich OTUs but a larger hierarchy in relative abundance among OTUs than the soil community despite that root microbiomes share almost the same OTUs with soil microbial community. These results imply that host plants select specific microbial members from soil, which seems to confer benefits to plants, and these selected microbes serve important functions of host plants such as nutrient acquisition and defense to pests. The results obtained from this study agreed with previous studies reporting that root microbial communities are less diverse than those of the soil [204, 205], and support the idea that soil is an important reservoir of microorganisms that potentially confer large benefits to plants [17].

Assembly rules of plant root microbiome

It was suggested that the constitution of plant microbiome have had great effects on plant evolution and environmental change such as plant domestication, plant secondary metabolite, soil type, soil properties, nutrient status, and climatic conditions [25, 206, 207]. In this study effects of plant microbiomes were analyzed by the two opposing experiments: investigation of microbiomes of the single identical plant species (maize) grown under different soils derived from different geographical locations and different soil types (Chapter 2) and the investigation of microbiomes of the identical plant species (Chinese cabbage) when grown under originally identical soil exposed to the cultivation of different preceding crops (Chapter 4).These experiments demonstrated that plant species have their own selecting rules of microbes to construct appropriate root microbiomes, which agreed with previous study [20]. Although soil communities are composed of a great diversity of microbes, preference and selection by host plants become more powerful filter to construct root microbiomes than the constitution of soil origin in which plants grow [160].

Moreover, the experiment in Chapter 3 revealed assembly rules of root microbiomes by host plants. The previous studies have shown that host genotype is one of the main factors in

determining root associated microbiome structure [208, 209]. The study of Chapter 3, which compared root microbiomes among 20 plant families covering almost full range of angiosperm phylogeny, clarified that plant phylogenetic history poses great constraint on constitution of bacterial root microbiomes, but not on fungal microbiomes, which support the idea that plant phylogenetic evolution has crucial effects on root microbiome structure [154, 164].

Plant root exudates are chemical cues to monitor and interact with their surroundings and assumed to be active behaviors by plants to ameliorate surrounding environments [210, 211]. The secretion compounds can specifically stimulate or repress distinct microbial members [212, 213]. Increasing evidences demonstrate that plants predominantly drive and shape the structure of rhizosphere and endosphere microbes [214, 215], and a variety of root exudates released from plants involve assembly of plant microbiomes [211]. The differences in exudation patterns released from different plant accessions determined bacterial assemblages [216]. Therefore, evolutionary constraint by plant phylogeny on constituting root bacterial community may be caused by secondary metabolites released from host plants although their detail is still unclear.

Contributions and Functions of Important Microbes

Bacterial phyla Proteobacteria, Actinobacteria and Bacteroidetes had a large proportion of root bacterial communities for plant species covering a wide range of phylogeny (chapter 3), as well the identical plant species grown in different soils (chapter 2 and 4). Fungal phyla Ascomycota and Basidiomycota comprised a large proportion of root fungal microbiome in both conditions (chapter 2 and 3). Agreed with the study that a small number of bacterial taxa in Proteobacteria and Actinobacteria were highly enriched in the endophytic compartment of Arabidopsis [20]. Actinobacteria members are well known for production of antimicrobial secondary metabolites [217], and many Proteobacteria members for plant-growth-promoting [218]. Actinobacteria, Proteobacteria, Chloroflexi and Bacteroidetes characterized the differences of root microbiome structures between plant species in this study.

Bacterial phyla, Acidobacteria and Gemmatimonadetes, characterized the interspecific differences in bacterial root communities between twenty plants as shown in the chapter 3, while these taxa were significantly depleted in roots of both maize and Chinese cabbage compared with soil microbial communities in the chapter 2 and 4. A previous study also showed that

Acidobacteria and Gemmatimonadetes depleted in Arabidopsis endosphere [20]. Acidobacteria contribute to the carbon cycle to degrade complex plant derived polysaccharides [219] and thus seems to play more important roles in soil than root.

The abundance pattern represented by enrichment and depletion relative to original soil reflects differences in colonizing processes to root tissues [20]. Relative abundances of Proteobacteria, Actinobacteria, Acidobacteria and Gemmatimonadetes in soil bacterial communities changed after growth of different preceding crops, which provide evidence for effects of crop rotation on structural changes in soil microbial community, which could be a cue for suppression of soil borne diseases.

The abundance of the two classes of Proteobacteria, Betaproteobacteria and Gammaproteobacteria showed significant mantel correlations with plant phylogenetic evolutionary distance (chapter 3). These two classes have been reported to include members with functions such as nitrogen fixing, nutrient cycling, nodule formation of legumes and plant growth promotion [165-167]. The abundance of Betaproteobacteria was negatively related with other bacterial classes including Chloroflexi, which contains taxa with various metabolic lifestyles [168] and Actinobacteria, which has been known to promote plant growth, reduce disease symptoms and associated with disease suppressive soils [60, 220, 221]. Therefore, the abundance of Betaproteobacteria seems to be determined by the balances with other members such as Chloroflexi, Actinobacteria and other classes having different functions. Consequently, the balance of these two bacterial groups may be based on the balances between two functions, increasing productivity including nitrogen fixing and increasing defense such as disease suppression.

With respect to the relationship between productivity and bacterial community, Chapter 2 showed that the relative abundances of bacterial orders, Rhizobiales and iii1-15 were positively contributed to soil productivity. Rhizobiales has been reported to include various functional members from nitrogen fixer represented by nodule bacteria in leguminous plants to pathogenic bacteria causing infection to animals and plants [222-224], and the horizontal gene transfer involved into symbiotic and pathogenic events may cause complex evolution of functions in these taxa [225].

Crop rotation had great effects on clubroot suppression. It has been shown that suppression of soil born disease is caused by plant pathogenic bacteria and plant-growth promotion taxa [226-228]. Bacterial orders, Actinomycetales and Bacillales, which were mutual excluded with the clubroot pathogen density, has been shown to be involved in disease suppression [60]. It is known that Bacillales includes the biocontrol strains, *Bacillus*, which produces antibiotics and promotes plant growth [229], and that *Bacillus sp.* also exhibit antagonistic activity against some phytopathogenic fungi [230].

The bacteria orders, Actinomycetales, Bacillales and other orders, were mutual exclusive with the clubroot pathogen density, while the orders, Rhizobiales, Pseudomonadales and other orders had positive association with the density of clubroot pathogen. These antagonistic relations between bacterial groups further support the idea that the balance between different functions such as plant growth promoting (nitrogen fixing) and disease suppressing exists among bacterial members consisting of the community. The correlations of clubroot density with clubroot DSI at the 4^{th} week differed between the 3^{rd} week and the 4^{th} week. These results suggest that plant development have important effects on contribution of constituting microbe on the symptom of clubroot pathogen and their suppression by host plants. This inference is supported by previous studies reporting that shifts of pathogen on microbiome could be compensated by beneficial microbes [231]. Some fungal and oomycete microbes could suppress other microbes even across kingdom [232]. Moreover, plant development and the existence of pathogens or symbiotic bacteria could change root secretions [233, 234], which has been well known as regulator of microbes.

Impacts of Soil Microbial Community on Plant Growth and Health and Applications on Agriculture

This study conducted three experiments: different host plants grown under identical soil (chapter 3), identical plant species grown under various soils from different farms with different soil properties (chapter 2) and identical plants grown under soils after the growth of different preceding crops (chapter 4). All experiments provided firm evidence for the existence of assembly rules of root microbiomes by host plants: the answers to the question how different plants structure root microbiomes from soil, a diverse source of microbial reservoir. The

assembly rule is closely associated with host plants genetic factors, the phylogenetic evolution of host plants and community composition of original soil.

Chapter 2 showed that soil microbial community composition was a decisive driver of soil productivity which greatly influences plant growth. The chapter 4 focused on the effect of crop rotation on soil borne disease suppression. In this study, preceding crops changed soil microbial community and its changes induced root microbiome compositions and interaction of root microbes with pathogen density and resulting disease suppressing, which confirmed the classic idea that crop rotation suppresses soil borne disease through changing soil microbial community compositions.

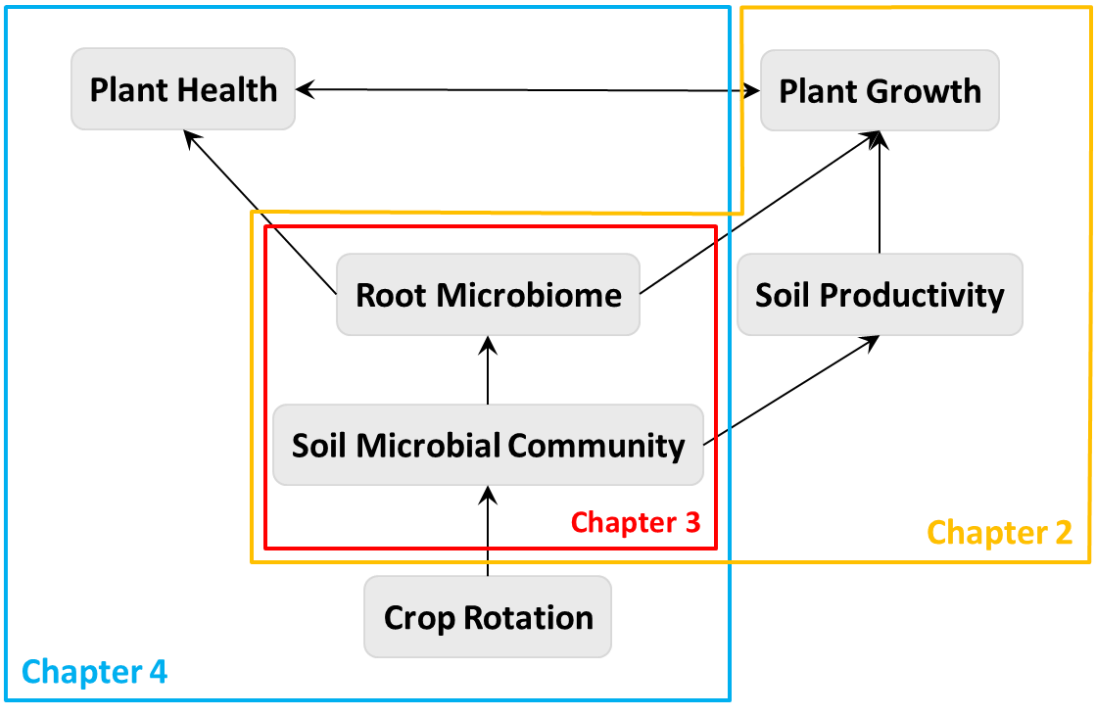
The whole results of this study can provide an integrated story of possibility of root microbiomes on future low input sustainable agricultural system. Soil and root microbiomes can have positive influence plant growth and health, which leads to low or few input of chemical fertilizers and chemical pesticides.

It was previously reported that pathogens and beneficial microbes induce a shift in the structure of the root microbiome [231]. Root exudates can shape soil microbial community [235]. Plants have abilities to defense pathogen attacks through regulations of root secretions which recruit beneficial microbes from soil [72], as well as release of exudates to attract and initiate symbiosis on rhizobia to overcome nitrogen deficiency [236]. The compensation to these stresses were driven by plants on microbial changes, giving a hint that manipulation of soil microbial community might be a feasible way for enhancement of plant health and disease suppressing.

It has been known that adding beneficial microorganisms can maximize plant nutrient uptake and increase plant growth [237, 238], plant tolerance to abiotic stress [239] and suppression of disease [240]. Applying plant growth promoting rhizobacteria (PGPRs) is a possible management technique to minimize niche for proliferation of pathogens and effectively fill vacant niches [241], which could reduce disease damage, produce antibiosis, induce plant systemic resistance, and promote plant growth indirectly through increasing nutrient acquisition [237]. It is more likely to be successful to inoculate PGPRs with low microbial biomass soil which is more particularly and effectively [242] and more efficient with low fertilizer level (N, P and K) [243]. Moreover, the supplementation of N fertilization halted the activity of nitrogen fixation, leading to the advantage of microbes supplying on plant health [244]

Inoculations of combined beneficial microbes enhance nodulation and plant biomass [245]. Other studies suggest the benefits by combining beneficial strains including decreasing fertilizer, increasing nutrients absorption and yield [246]. Application of compost with beneficial strain have also shown the ability to suppress plant pathogens [247]. Formulations of multiple microbes with mutually beneficial roles hint the potential roles on plant productivity and health [241]. The performance of beneficial inoculations is still not consistent with the results of field application study [248]. The questions of what microbes match with what type of soil and how it works need to be understood, and answer to these questions will result sustainable and cost-effective crop production system.

Understanding soil microbial community and root microbiome can lead to regulation of plant growth and health through controlling associated microbial community. Modifying soil microbial community might be a feasible approach to the development of sustainable agriculture. Appending multiple microbes including Biofertilizer and Biocontrol can promote plant growth and suppress disease when they are in a good balance. In addition, modulation of plant metabolites, which requires specific recipes to meet individual needs, might be a new way for the innovation of sustainable agriculture.


Plant Health
Plant Growth
Root Microbiome
Soil Productivity
Soil Microbial Community
Chapter 3
Chapter 2
Crop Rotation
Chapter 4

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