Molecular Ecological Studies in Agricultural and Natural Ecosystems in Turkey and Japan Focusing on Effect of Environmental Factors on Soil Microbial Diversity

環境因子が根圏微生物群集構造に与える影響に関する分子生態学的研究

－トルコ及び我が国の自然・農業生態系を対象として－

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Index

Chapter I

Introduction

I-1. Importance of soil microbial diversity

Soil microorganisms play important roles in soil functions such as plant productivity, nutrient cycling and biogeochemical cycling. Although the richness of microorganisms has been estimated as to up to 10^6 species per gram of soil (Gans et al. 2005), only a few taxa have been examined in studies of soil microorganisms. In 1992, the United Nations Conference on Environment and Development emphasized the importance of biodiversity in ecosystem functioning, and promoted scientific exploration to improve our understanding of the importance of soil microbial diversity and functions. It has been recognized that soil biodiversity is one of the key mechanisms behind ecosystem functioning (Balvanera et al. 2006; Hector and Bagchi 2007), can contribute to the terrestrial biomass, and is linked with aboveground biodiversity (Wardle et al. 2004). However, the soil microbial community and diversity and the links between biodiversity and functioning remain poorly understood.

In recent years, it has been determined that reduction of soil biodiversity may impair ecosystem functioning, such as nutrient cycling, plant nutrient uptake, and transfer of resources between aboveground and belowground components (de Vries et al. 2013; van der Heijden et al. 2008). Changes in soil communities and the loss of soil biodiversity can threaten ecosystem functioning and sustainability (Wagg et al. 2014). Therefore, it is important to determine how changes of soil biodiversity and community composition

influence ecosystem functioning.

I-2. Molecular ecological methods for understanding soil microbial community

In the past, soil biodiversity was measured using counting techniques such as the most probable number (MPN) technique and the plate count technique. However, these culture-based approaches have become less popular because of the limited number of microorganisms that can be cultured. It is well known that these culture-based approaches estimate only 1–10% of the entire soil microbial flora. In contrast, molecular techniques are now popular because they do not rely on isolation or cultivation of soil microorganisms.

Molecular techniques generally involve DNA extraction from the environment, and are independent of culture methods and taxonomic groups such as species, genera, and families. Molecular techniques allow the detection of microorganisms at the species level. Polymerase chain reaction (PCR) allows the analysis of a limited amount of DNA from environmental sources. In combination with appropriate primers, PCR can amplify the target DNA with high selectivity. Ribosomal RNA gene (rDNA) and internal transcribed spaces (ITS) have often been used to elucidate microbial community and biodiversity. In general, 16S rRNA genes or transcripts have been used as a target for studying the domains Bacteria and Archaea, and 18S rRNA genes and ITS have been used

for fungi. The PCR technique can be used as a starting point in almost every aspect of DNA-based molecular biological research, including sequencing from a clone library, denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (T-RFLP), micro-array and next-generation sequencing.

Sanger sequencing and clone library

A traditional DNA sequencing method called Sanger sequencing was developed in 1977 (Sanger et al. 1977). DNA sequencing of the marker genes allows us to determine the phylogenetic affiliations of studied organisms, including soil microorganisms. However, mixtures of PCR amplicons with different sequences in environmental samples must be separated from each other before DNA sequencing to adapt this technique for environmental microbial ecology. The clone library method can solve this problem. Cloning involves the ligation of the amplified DNA into a plasmid vector, followed by the transformation of competent cells of *Escherichia coli* with the vector. The most common method of ligation of amplified DNA into a vector is T-A cloning (Holton and Graham 1991). After screening of colonies that contain the recombinant vector, colonies derived from individual transformed cells are then picked for extraction (or colony PCR) and subsequent sequence analyses. Obtained sequences can be identified with a taxonomic affiliation based on a comparison with a database such as GenBank (Benson

et al. 2012) and GreenGenes (DeSantis et al. 2006). This vector-based clone library method has been adapted for the molecular microbial ecological survey of bacteria and archaea (Borneman et al. 1996), fungi (O'Brien et al. 2005) and arbuscular mycorrhizal fungal (AMF) communities (Helgason et al. 1999). However, this method is labor-intensive and expensive, and time and research cost therefore tends to limit the number of sequenced-clones obtained from samples.

Denaturing gradient gel electrophoresis (DGGE)

The DGGE technique with PCR amplification of rDNA fragments was introduced into microbial ecology by Muyzer et al. (1993). This technique involves the separation of PCR amplicons on polyacrylamide gel with a linear gradient of a DNA denaturing agent (urea or formamide). Since differences between the sequences influence their denaturation, amplified different DNA fragments are separated during electrophoresis, yielding complex profiles representing the composition of the amplicons. The DGGE technique was adapted to analyze the whole soil bacterial community (Nakatsu et al. 1999), denitrifying bacterial community (Throback et al. 2006), soil fungal community (van Elsas et al. 2000) and the AMF community (Ma et al. 2005). Application of DGGE to PCR amplicons from ribosomal copy DNA by reverse transcriptase PCR (RT-PCR) can indicate the structure or diversity of the active populations in a given

environment (Felske et al. 1996). In addition, after the separation, individual bands can be excised and sequenced for taxonomic identification of specific populations within a community.

Terminal restriction fragment length polymorphism (T-RFLP) analysis

The T-RFLP technique was first described by Liu et al. (1997) and they characterized microbial diversity of genes encoding 16S rDNA. This technique involves digestion of PCR amplicons using restriction enzymes and separation of restricted fragments on gels. In the T-RFLP process, PCR is carried out with fluorescence primers. This makes it possible to detect only terminal fragments, and can demonstrate changes in microbial communities following changes of environmental conditions. The T-RFLP analysis does not require any particular development, which is the main advantage of this technique. The T-RFLP technique has been widely adapted to investigate soil microbial communities such as the whole bacterial community (Lukow et al. 2006), ammonia-oxidizing bacteria (Horz et al. 2000), and the ectomycorrhizal fungal (Rosling et al. 2003) and AMF communities (Dumbrell et al. 2010).

Although PCR-DGGE and T-RFLP were convenient and useful tools for understanding the differences and changes in microbial community structures and

diversities, these approaches cannot easily detect rare taxa with small populations. Relatively recently, metagenomics such as micro-array and next-generation sequencing have been adapted to study soil microbial ecologies.

Micro-array

Micro-array technology is a powerful and high-throughput tool that allows the simultaneous analysis of environmental DNA. Micro-arrays were originally developed for monitoring gene expression (Schena et al. 1995) and was adapted for disease diagnosis (Lindblad-Toh et al. 2000), genome research (MacBeath and Schrieber 2000) and drug discovery (Southern et al. 1999). Micro-arrays have made it possible to measure the expression levels of most genes in a given genome and the abundance levels of microbial taxa in PCR-amplicons using cDNA as the probe target. Several types of micro-array have been developed for soil microbial community detection. For example, phylogenetic oligonucleotide micro-arrays contain sequences from rRNA of specific groups of target organisms, and functional gene arrays contain conserved domains of genes involved in metabolic pathways such as carbon, nitrogen and phosphorus cycling. The micro-array technique has been adapted for studies in soil microbial ecology such as bacterial and archaeal communities (Nuccio et al. 2013), and nitrogen and carbon cycling gene expression (Yergeau et al. 2007). Micro-array technology is high throughput and can

provide substantial information regarding the soil microbial community; however, the detection efficiency is dependent on a preexisting database for preparing probes.

Next-generation sequencing

The next-generation sequencing system can potentially generate 10^5 to 10^7 sequencing reads in parallel, whereas the automated Sanger sequencers can determine the sequences up to 1 kb for only 96 individual specimens at a time. As Chiu and Miller (2016) described, high-throughput next-generation sequencing systems have been commercially developed based on different chemistries and detection techniques, such as Roche 454 sequencer, Illumina sequencers, Applied Biosystems SOLiD sequencer and Life Technologies Ion Torrent. These sequencing technologies can generate sequence reads from fragment libraries of genomes (genome sequencing), pooled cDNA library from reverse transcription of RNA (RNAseq), and pooled PCR amplicons (amplicon sequencing). Next-generation sequencing can recover DNA sequence data directly from environmental samples and identify the microbial taxa in environmental samples with high confidence by comparing obtained sequences with a reference library of known organisms. Next-generation sequencing techniques have been becoming cheaper and have been adapted for the study of soil microbial ecology such as bacterial communities in croplands (Sun et al. 2016), bacterial communities in paddy fields (Daquiado et al.

2016), the whole fungal community (Toju et al. 2015) and the AMF community (Kohout et al. 2015).

I-3. Objectives of this study

In this thesis, four individual studies were carried out, aiming to enhance our knowledge of soil microbial diversity and community from several environments. In Chapter II, we elucidate molecular diversity of indigenous AMF in three different agricultural regions in Turkey. In Chapter III, we discuss the effects of long-term organic fertilization management on the AMF community in semi-arid Mediterranean region. In Chapter IV, we discuss the results of a comprehensive analysis of rhizosphere soil bacterial and AMF communities conducted in grassland under desertification stress. In Chapter V, we consider soil bacterial community compositions in organic paddy fields and the effect of agricultural management. The clone library and sequencing methods were used in Chapters II and III, and next-generation sequencing was used in Chapters IV and V.

Chapter II

Molecular diversity of indigenous arbuscular mycorrhizal fungi in three different agricultural regions of Turkey

II-1. Introduction

Arbuscular mycorrhizal fungi (AMF) are important components of natural and agricultural ecosystems, as they form symbiotic associations with the roots of most terrestrial plants. Symbiotic relationships between AMF and host plant species date from approximately 400–600 million years ago, and are thought to have a critical role in the early establishment of land plants (Remy et al. 1994). AMF are obligate biotrophs that belong to the phylum Glomeromycota (Schussler et al. 2001), and the symbioses formed between AMF and host plants are normally mutualistic (Smith and Read, 2008). Mycorrhizal association involves the transfer of mineral nutrients from the fungus to the host plant in exchange for carbon (C) (Smith and Read, 2008). In particular, it is well known that absorption of phosphorus (P) to the host plant is enhanced by AMF symbiosis (Allen 1991). In addition, AMF provide several other benefits to host plants, including tolerance of water deficiency, mitigation of heavy metal toxicity and protection from pathogens, as well as improvement in soil physical properties due to stabilization of soil aggregates by hyphal elongation (Allen 1991; Newsham et al. 1995; Joner et al. 2000; Augé 2001). AMF can therefore have significant positive effects on agricultural systems, and improve crop growth and productivity.

The Republic of Turkey is one of the world's largest agricultural countries, and the fourth largest producer of vegetables according to FAOSTAT 2010. On the other hand, soil salinization, erosion and missuse, overgrazing, heavy urbanization and industrialization bring about increasing soil degradation problems in Turkey (Camci et al. 2007). According to UNEP (1993), most areas in Turkey can be considered to suffer from desertification or have high potential for desertification. Due to climatic and topographic conditions, soil erosion is a major problem in Turkey and almost 86% of the land suffers

from some degree of erosion (Ozden et al. 2000). Biodiversity investigations of indigenous soil microorganisms such as plant growth-promoting rhizobacteria, symbiotic bacteria and symbiotic fungi are important for the restoration of degraded soil and for sustainable agriculture.

There have, however, been only a few molecular ecological studies of AMF diversity or community composition in Turkish arable soils. By counting the AMF infection rate in the host plant roots in Turkish south Mediterranean dunes, Çakan and Karataş (2006) revealed that rate of AMF association with host plants increased with progressing desertification. Karaarslan and Uyanoz (2011) investigated the relationships between indigenous AMF and soil physical or chemical properties by researching AMF spore density and AMF infection rate in some native plants growing in saline soils around Lake Tuz, Turkey. They found a significant correlation between numbers of AMF spores and soil calcium carbonate (CaCO3) concentration, and indicated that AMF community structures varied to suit the stresses in saline soils. In a field experiment conducted in the Turkish Mediterranean region, Celik et al. (2004) indicated that organic fertilizer or/and inoculation with AMF spores could improve soil physical and chemical properties. It was indicated that the indigenous AMF isolated from the Mediterranean region can significantly contribute to plant growth and P uptake in Turkey (Aka–Kacar et al. 2010; Almaca and Ortas 2010; Ortas 2010). These works have focused on AMF infection rate, spore morphology and density. On the other hand, there has been no effort to investigate AMF diversity through DNA analysis, as far as we know.

In the light of these facts, we aimed to elucidate the indigenous AMF diversity and community composition in 15 plant roots collected from three regions in Turkey, where the climatic conditions, soil characteristics and farming practices differ.

Community composition was determined based on partial nucleotide sequences of the 28S large subunit ribosomal RNA genes (LSU rDNA), which were amplified from DNA extracts of fine root samples.

II-2. Materials and Methods

Sampling Sites

Sampling took place in different geographic regions of Turkey: the East Black Sea, Central Anatolia and the Mediterranean. In the East Black Sea region, samples were collected around two different locations, (i) the Atatürk Tea and Garden Research Institute located in Rize Province (41°01'N, 40°30'E, Site B1–B3), where the regional climate is semi-humid with a 15.8°C mean annual temperature, 2304.8 mm of rainfall and 77.6% humidity and (ii) a hazelnut grove located in Trabzon Province (40°54'N, 40°08'E, Site B4) where the regional climate is again semi-humid with a 15.6°C mean annual temperature, 812.0 mm of rainfall, and 71.3% humidity (TSMS 2009). The Central Anatolian region was represented by soil samples collected at the Kenan Evren Research Farm of Ankara University located in Haymana, Ankara Province (39°37'N, 32°31'E, Site A1–A5), where the climate is typically continental with a 13.0°C mean annual temperature, 462.2 mm of rainfall, and 60.1% humidity (TSMS 2009). The soil samples in the Mediterranean area were collected from various agricultural fields at the East Mediterranean Agricultural research Institute, located in Adana Province, (36°51'N, 35°20'E, Site M1–M6), where the climate is typically Mediterranean with a mean annual temperature of 19.4°C, precipitation of 811.0 mm, and 81.1% humidity (TSMS 2009). According to the classification of the World Reference Base for Soil Resources (WRB), the soils at those sites were classified as Eutric Cambisol in sites B1–B4 in the East Black Sea region, Calcaric Cambisol in sites A1–A5 in Central Anatolia and Molli-Lithic Leptosol in sites M1–M6 in the Mediterranean region (Jones et al. 2005). Selected soil and vegetation characteristics are summarized in Table 1.

Soil and plant root sampling

Bulked samples of 500–750 g soil from the rhizosphere of three plant individuals per species were taken and passed through a 2-mm sieve. The fine roots were collected and frozen at –20°C, whereas the soil samples were stored at 4°C prior to analysis.

Soil physical and chemical properties

Soil particle size and texture were measured using the Bouyoucos hydrometer method (Bouyoucos 1951). Soil pH (H2O) was measured in a 1:2.5 soil:water [weight/volume (w/v)] suspension, using an electrode after shaking for 30 min. Available P (Truog-P) was measured using the vanado-molybdate method after extraction with 0.001 M sulfuric acid (H2SO4) at a ratio of 1:200 (w/v). Total C and nitrogen (N) were analyzed using an MT-700 Mark 2 CN analyzer (Yanaco, Kyoto, Japan).

Molecular analysis

The dried root samples were ground in liquid nitrogen to get a 20 mg sample for total DNA extraction, using the ISOPLANT kit (Nippon Gene, Tokyo, Japan). Part of the LSU rDNA was amplified in a 25-μL reaction, using the KOD-Plus ver. 2 polymerase chain reaction (PCR) mix (Toyobo, Osaka, Japan) with 10 μmol L–1 of each primer and 1 μL of template DNA. Nested PCR amplification rounds were carried out with the LSU rDNA-universal forward primer LR1 (van Tuinen et al. 1998)/the fungal LSU rDNA-specific reverse primer FLR2 (Trouvelot et al. 1999) for the first PCR, and the forward primer FLR3/reverse primer FLR4 (Gollotte et al. 2004) using 1 μL of a 1:100 dilution of the first PCR product as the template for the second PCR. A Takara PCR Thermal Cycler Dice® Gradient (Takara, Ohtsu, Japan) was used, with twice the following

program: initial denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 96°C for 10 s, annealing at 54°C for 30 s, polymerization at 72°C for 30 s, and final elongation at 68°C for 7 min. The PCR products were cloned into the p-GEM® T-Easy Vector (Promega, Tokyo, Japan) and transformed into ECOSTM Competent Escherichia coli DH5α cells (Nippon Gene) following the manufacturer's instructions.

For each sample, 32 recombinant bacterial colonies were randomly chosen from the DNA libraries and purified using a High Pure Plasmid Isolation Kit (Roche, Basel, Switzerland) following the manufacturer's instructions. The nucleotide sequences were determined by dideoxy sequencing at the sequencing facilities of FASMAC Co. Ltd. (Kanagawa, Japan).

Analysis of community composition

To verify AMF identity, the sequences were compared with all known sequences in GenBank using the Basic Local Alignment Search Tool (BLAST; Altschul et al. 1990) on the National Center for Biotechnology Information (NCBI) website. Those sequences showing high similarity to organisms outside the phylum of Glomeromycota were excluded from subsequent analyses. The sequences were aligned in Mega 5.10 (Tamura et al. 2011), using ClustalW (Thompson et al. 1994) and adjustments were made if necessary.

To define operational taxonomic units (OTUs), a distance matrix was computed using DNADIST ver. 3.5c (J. Felsenstein, University of Washington, Seattle, WA, USA). OTUs were defined at 97% sequence identity. Potential phylotype richness (Chao1) and the associated 95% confidence interval (CI) were estimated in the Mothur software (Schloss et al. 2009). The Shannon diversity index was calculated for each sample with a

95% CI based in the 97% sequence identity cutoff.

An additional multiple sequence alignment was assembled in Mega 5.10 that included our new sequences and highly similar ones of formally described AMF species from GenBank. This big alignment was used to create a phylogenetic tree in Mega 5.10, using the neighbour-joining algorithm with 1000 bootstrap replicates for statistical branch support. The tree was rooted with a sequence of *Paraglomus occultum*. These sequence data have been submitted to the DDBJ database (accession numbers AB786346–AB786517).

To compare the AMF communities among sites, the clade type abundances and soil physical and chemical properties were submitted to a canonical correspondence analysis (CCA) using R package vegan (Oksanen et al. 2013).

II-3. Results and Discussion

Soil chemical properties

Soil physical and chemical properties are shown in Table 2. The East Black Sea soils (B1–B4) were acidic and ranged between pH 4.47 and 5.02, while those of the Mediterranean Region (M1–M6) and Central Anatolia (A1–A5) were alkaline and ranged between pH 7.95 and 8.64.

The pH values of the soil from *Camellia sinensis* varied among the fertilizer treatments; the plant compost treatment (B1), the chemical fertilizer treatment (B2), and the control (B3) had a pH of 4.65, 4.90 and 5.02, respectively. The available P, total C, and total N values of the soils in sites B1 and B2 were higher than those of the control (B3).

AMF taxa and diversity

In the present study, a total of 480 clones (32 clones per sample) were amplified, and 424 AMF clone sequences were determined (an average of 28 sequences per sample). The numbers of AMF sequences, species, OTUs (based on 97% similarity), Shannon indices, Simpson indices and estimated phylotype richness (Ace and Chao1) are shown in Table 3. A total of 169 types of AMF sequences were amplified and were ascribed to 57 OTUs.

Estimated phylotype richness of the samples from the Mediterranean (M1–M6) and Central Anatolian region (A1–A5) tended to be higher than those from the East Black Sea (B1–B4). The samples obtained from *C. sinensis* in the plant compost (B1) and the chemical fertilizer treatment (B2) were dominated by only one OTU. The coverage of OTU (OTU obtained/estimated phylotype richness) averaged 85%. Although the samples

obtained from *Zea mays* L. (M2) in the Mediterranean had a low Shannon index, the diversity indices of the samples from this region tended to be higher than those of the other regions. Using BLAST analysis, 38 types of sequence (28 OTUs) had less than 97% sequence similarity to the known AMF sequences in the GenBank database.

No previous study has assessed the AMF assemblages in Turkish arable soils, using a molecular approach. Our 38 sequences with lower than 97% sequence similarity to the known species in the GenBank database suggest that novel and endemic AMF species may exist in Turkish arable land. These sequences were found in all samples except for *C. avellana* (B4) and *P. armeniaca* (A5) sampled in the East Black Sea and Central Anatolia, respectively. All sequences from *C. sinensis* in the East Black Sea had only 83–97% sequence similarity to the known AMF species.

The samples of *T. repens* (A1) and *Z. mays* (A3) obtained from Central Anatolia and *H. annuus* (M1), *Z. mays* (M2), *G. max* (M4), *M. sativa* (M5) and *C. sinensis* (M6) from the Mediterranean involved sequences that showed high similarity (> 97%) to the AMF sequences from potato (*Solanum tuberosum* L.) roots in arable soils in Italy, another Mediterranean country (Cesaro et al. 2008).

Phylogenetic distance analysis

Figure 1 contains the neighbor-joining tree showing the recovered AMF phylotypes. The number of sequences and OTUs with < 97% sequence similarity, and the distribution of clades, is shown in Table 4.

Ten distinct clades are visible in the phylogenetic tree (Fig. 1). Clades Rhi4, Unc2, Aca and Unc3 comprised sequences from the East Black Sea region only. Clade Rhi3 and Cla comprised sequences from the Mediterranean only. Clades Rhi1, Rhi2,

Unc1, and Fun comprised sequences from the Mediterranean and Central Anatolia.

The sequences obtained from *C. sinensis* in the plant compost (B1) and chemical fertilizer treatments (B2) formed different clades from each other (Fig. 1). The sequences found in the plant compost treatment were related to *Acaurospora* (clade Aca) and those in the chemical fertilizer treatment were related to *Rhizophagus* (clade Rhi4). Clade Rhi4 comprised AMF obtained from *C. sinensis* only (B2 and B3), which were related to *Rhizophagus*; however, all sequences in clade Rhi4 had low sequence homologies to known species (< 93% similarity). Clade Aca was only from *C. sinensis* in the plant compost treatment (B1) and was related to *Acaulospora*. These clade Aca sequences also had low sequence homologies to known species (87% similarity). Clade Unc3 was formed from *Corylus avellana* (B4) and *C. sinensis* in the control treatment (B3), and their sequences were related to the AMF species recorded as uncultured glomeromycetes (Verbruggen et al. 2010).

As Table 2 shows, there was a significant difference in soil chemical properties (i.e. available P, total C and N) between the control site (B3) and the two fertilized sites (B1 and B2). The result means that fertilization can drastically decrease AMF diversities associated with *C. sinensis*. This is in accordance with the opinions of Singh et al. (2008), who reported that various cultural practices negatively affect AMF diversity at the genus level, in tea plantations in the colder regions. They also showed that the Shannon-Weaver diversity index at the species level was higher in the natural ecosystem than in the cultivated site.

Clades Rhi2 and Fun had various sequences with < 97% sequence similarity to known species in the GenBank database (Table 4). Clade Rhi2 comprised uncultured Glomeromycota which had been previously identified by sequence analysis (Cesaro et al.

2008), but no spore morphology data have been available for these species.

The clade distribution among the Mediterranean and Central Anatolian sequences was broadly similar. However, as clade Rhi3 consisted of only AMF from the Mediterranean, this region contained more various AMF species related to *Rhizophagus* than did Central Anatolia. Considering that no significant differences were found in the soil chemical properties between these regions, climate and/or parent soil materials may affect AMF diversities.

In this study, the samples collected in the East Black Sea region formed completely different AMF communities from those in the other two regions. As shown in Table 4, there was no common clade between the samples from the East Black Sea region and those in the others. Therefore, the samples from the Black Sea region were excluded from CCA. CCA was performed to investigate differences among samples or regions based on the clade type abundances and soil physical and chemical properties among the samples from the Mediterranean and Central Anatolia. As a result, AMF communities from *M. pumila* (A4) and *P. armeniaca* (A5) whose soils were characterized by a high amount of total C were plotted at points near each other (Fig. 2). It was implied that total C affected clade Rhi2 which was dominant in samples from *M. pumila* (A4) and *P. armeniaca* (A5). It was indicated that clade Rhi3 and Cla have no relation between available P, total C or N (Fig. 2). It is suggested that clade Rhi3, which was comprised only from the samples collected in the Mediterranean region, shows the regional difference. In addition, clade Cla, which was obtained from *G. max* (M4), may reflect host specificity of AMF. The effect of available P on difference of AMF communities among the samples was small compared with total C and N.

Uniqueness of AMF in the East Black Sea region

Why might quite unique AMF communities exist in the East Black Sea region as compared to the other two regions? The soil samples collected from the East Black Sea were characterized by low pH (4.62–5.02; Table 2). While host plant composition is known to be a main driving force for structuring AMF communities (Johnson et al. 1992), alteration in AMF communities due to low soil pH in acid sulfate soil has also been reported (An et al. 2008). The difference in the AMF community composition found in the three Turkish soils is speculated to be due to the acidic soil pH in the East Black Sea region.

In acidic soil, low availability of P, owing to low native P content and high P fixation capacity in acidic soil is known to be one of the main limiting factors for the productivity of tea leaves (Lin et al. 1992). In addition, plants growing on acidic soil are often exposed to relatively severe mineral stress, including toxicity (e.g. aluminium, manganese) and deficiency (e.g. P, potassium, magnesium), as described by Marschner (1991). Excess aluminium, in particular, impairs root elongation, resulting in plant growth depression in acidic soils (Foy 1992). The AMF-root symbiosis may help alleviate some of the problems that plants encounter when grown in acidic soils (Marschner 1991; Sieverding 1991), including Al and Mn toxicity (Maddox and Soileau 1991; Sieverding 1991). The unique AMF species we found in the East Black Sea region in this study are probably adapted to the low pH environment and may contribute to plant growth in this area.

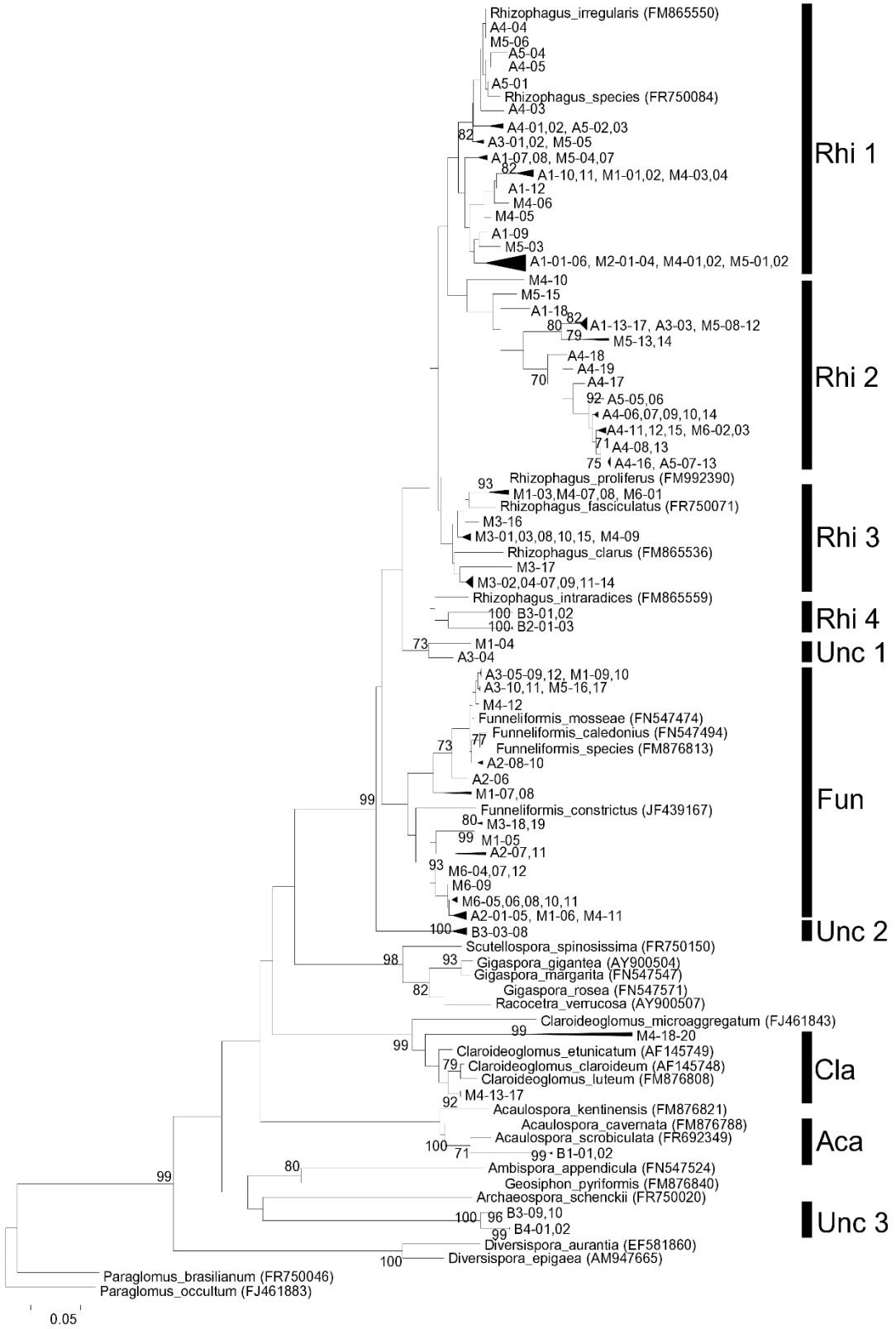

Rhizophagus_irregularis (FM865550)
A4-04
M5-06
A5-04
A4-05
A5-01
Rhizophagus_species (FR750084)
A4-03
A4-01,02, A5-02,03
A3-01,02, M5-05
A1-07,08, M5-04,07
A1-10,11, M1-01,02, M4-03,04
A1-12
M4-06
M4-05
A1-09
M5-03
A1-01-06, M2-01-04, M4-01,02, M5-01,02
M4-10
M5-15
A1-18
A1-13-17, A3-03, M5-08-12
M5-13,14
A4-18
A4-19
A4-17
A5-05,06
A4-06,07,09,10,14
A4-11,12,15, M6-02,03
A4-08,13
A4-16, A5-07-13
Rhizophagus_proliferus (FM992390)
M1-03,M4-07,08, M6-01
Rhizophagus_fasciculatus (FR750071)
M3-16
M3-01,03,08,10,15, M4-09
Rhizophagus_clarus (FM865536)
M3-17
M3-02,04-07,09,11-14
Rhizophagus_intraradices (FM865559)
B3-01,02
B2-01-03
M1-04
A3-04
A3-05-09,12, M1-09,10
A3-10,11, M5-16,17
M4-12
Funneliformis_mosseae (FN547474)
Funneliformis_caledonius (FN547494)
Funneliformis_species (FM876813)
A2-08-10
A2-06
M1-07,08
Funneliformis_constrictus (JF439167)
M3-18,19
M1-05
A2-07,11
M6-04,07,12
M6-09
M6-05,06,08,10,11
A2-01-05, M1-06, M4-11
B3-03-08
Scutellospora_spinosissima (FR750150)
Gigaspora_gigantea (AY900504)
Gigaspora_margarita (FN547547)
Gigaspora_rosea (FN547571)
Racocetra_verrucosa (AY900507)
Claroideoglomus_microaggregatum (FJ461843)
M4-18-20
Claroideoglomus_etunicatum (AF145749)
Claroideoglomus_claroideum (AF145748)
Claroideoglomus_luteum (FM876808)
M4-13-17
Acaulospora_kentinensis (FM876821)
Acaulospora_cavernata (FM876788)
Acaulospora_scrobiculata (FR692349)
B1-01,02
Ambispora_appendicula (FN547524)
Geosiphon_pyriformis (FM876840)
Archaeospora_schenckii (FR750020)
B3-09,10
B4-01,02
Diversispora_aurantia (EF581860)
Diversispora_epigaea (AM947665)
Paraglomus_brasilianum (FR750046)
Paraglomus_occultum (FJ461883)
0.05
Rhi 1
Rhi 2
Rhi 3
Rhi 4
Unc 1
Fun
Unc 2
Cla
Aca
Unc 3

Fig. 1. Neighbor-joining phylogenetic tree of arbuscular mycorrhizal fungi (AMF) sequences (28S rDNA region) obtained during our study, GenBank sequences of known Glomeromycota, and other GenBank AMF sequences highly similar to our sequences. Tree is rooted to *Paraglomus occultum* (FJ461883). Bootstrap values of 70% (1000 replicates) and higher are indicated. Rhi: *Rhizophagus*, Fun: *Funneliformis*, Cla: *Claroideoglomus*, Aca: *Acaulospora*, Unc: Uncultured glomeromycota.

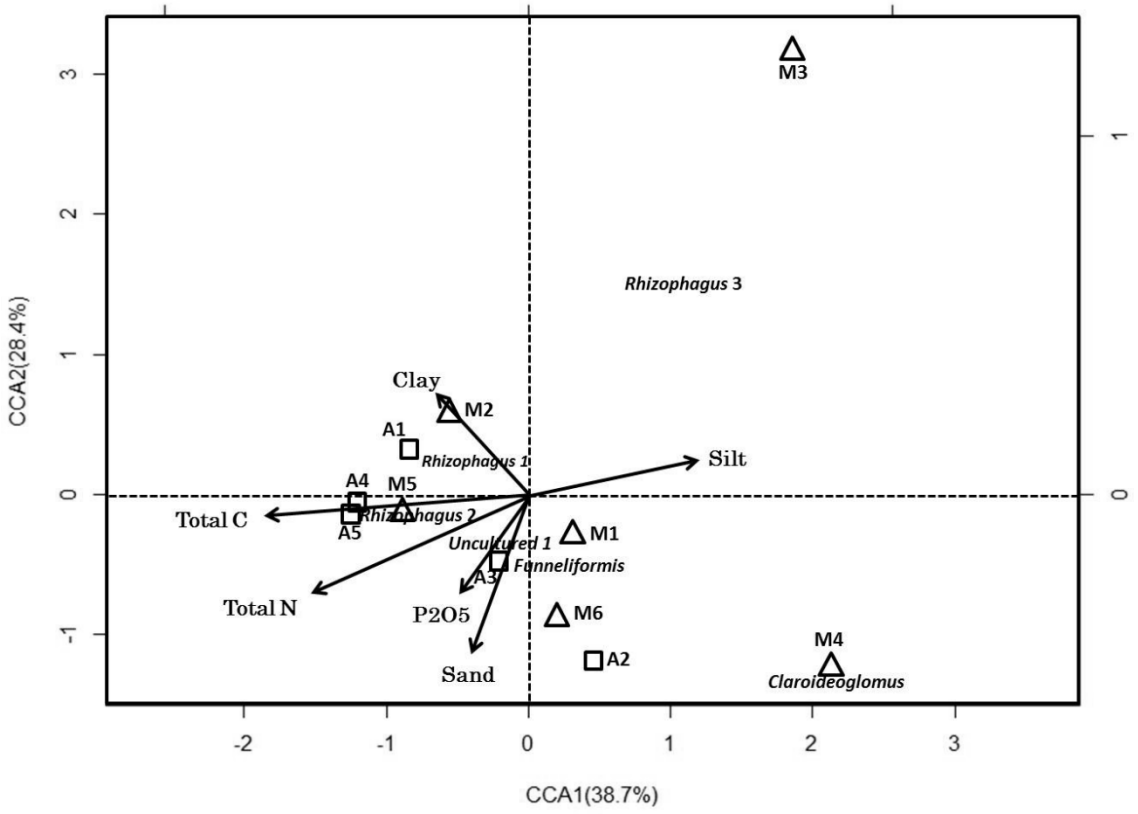


Fig. 2. Canonical correspondence analysis (CCA) of arbuscular mycorrhizal fungal communities (triangles = the Mediterranean region; diamonds = the Central Anatolia region).

Table 1. Selected soil and vegetation characteristics.

Selected soil				Selected vegetation		
Location	Regional climate	Soil group	Soil taxonomy	Plant species	Fertilizer treatment	Site name
East Black Sea region	Semi–humid	Red–yellow podzolic	Inceptisol Entisol	*Camellia sinensis* (L.) Kuntze	Plant compost	B1
		High mountain meadow		*Camellia sinensis* (L.) Kuntze	Chemical fertilizer	B2
		Noncalcareous brown forest soil		*Camellia sinensis* (L.) Kuntze	Control	B3
				Corylus avellana L.		B4
Mediterranean region	Typical Mediterranean	Red Mediterranean soil	Entisol	*Helianthus annuus* L.		M1
				Zea mays L.		M2
				Gossypium spp.		M3
				Glycine max		M4
				Medicago sativa L.		M5
				Citrus sinensis L.		M6
Central Anatolia	Typical continental	Brown soil	Entisol	*Trifolium repens* L.		A1
				Triticum aestivum L.		A2
				Zea mays L.		A3
				Malus pumila Mill.		A4
				Prunus armeniaca L.		A5

Table 2. Soil physical and chemical properties at field sites.

Site	Particle size			Texture class	pH (H_2O)			P_2O_5 (mg/kg–dry soil)			Total C (mg/g–dry soil)			Total N (mg/g–dry soil)		
	Clay (%)	Silt (%)	Sand (%)													
East Black Sea region																
B1	17.67	22.42	59.91	sandy loam	4.65	±	0.01	364.71	±	7.97	67.00	±	1.95	6.73	±	0.17
B2	14.31	24.03	61.66	sandy loam	4.90	±	0.00	516.81	±	6.68	64.97	±	6.62	3.65	±	0.11
B3	12.90	26.56	60.54	sandy loam	5.02	±	0.01	305.75	±	2.42	31.19	±	0.86	2.57	±	0.06
B4	26.54	31.25	42.21	loam	4.47	±	0.01	61.08	±	1.70	55.69	±	0.72	4.53	±	0.06
Mediterranean region																
M1	39.99	38.03	21.98	clay loam	8.23	±	0.02	138.42	±	5.45	34.38	±	0.06	1.24	±	0.02
M2	40.11	38.14	21.75	clay	8.52	±	0.02	107.50	±	2.92	38.10	±	0.12	1.24	±	0.02
M3	37.60	37.77	24.62	clay loam	8.64	±	0.00	87.84	±	2.70	37.61	±	0.21	1.02	±	0.01
M4	39.87	31.53	28.60	clay loam	8.63	±	0.01	111.06	±	2.92	30.79	±	0.18	0.89	±	0.01
M5	40.17	38.20	21.63	clay	8.54	±	0.01	124.05	±	0.45	36.52	±	0.12	1.08	±	0.01
M6	18.18	45.39	36.43	loam	7.95	±	0.01	373.15	±	12.89	45.33	±	0.28	2.47	±	0.03
Central Anatolia region																
A1	36.24	27.75	36.01	clay loam	8.23	±	0.02	68.74	±	9.86	48.75	±	0.10	1.60	±	0.02
A2	20.00	34.00	46.00	N.A.	8.29	±	0.01	50.17	±	1.43	44.51	±	0.22	1.39	±	0.01
A3	45.90	28.37	25.74	clay	8.36	±	0.01	90.70	±	3.00	39.61	±	0.36	1.36	±	0.02
A4	52.29	17.22	30.48	clay	8.41	±	0.01	206.39	±	4.84	55.35	±	0.18	2.10	±	0.02
A5	36.58	21.46	41.96	clay loam	8.25	±	0.00	183.59	±	3.49	55.62	±	0.70	2.42	±	0.09

Values represent mean (n=3) ±SEM

Table 3. Absolute number of AMF clones found at field sites and number of OTUs, diversity indices, potential phylotype richness, and coverage at a taxonomic level equating to 97% sequence similarity.

Site	Richness obtained		Diversity index (±95% confidence interval)		Potential phylotype richness		OTU coverage	
	No. of clones	No. of OTUs (97%)	Shannon	Simpson	Ace	Chao1	Ace (%)	Chao1 (%)
East Black Sea region								
B1	31	1	0.00 ± 0.00	1.00 ± 0.00	0.0	1.0	–	100.0
B2	31	1	0.00 ± 0.00	1.00 ± 0.00	0.0	1.0	–	100.0
B3	29	3	1.09 ± 0.08	0.31 ± 0.02	3.0	3.0	100.0	100.0
B4	27	1	0.00 ± 0.00	1.00 ± 0.00	0.0	1.0	–	100.0
Mediterranean region								
M1	32	9	1.86 ± 0.28	0.17 ± 0.07	15.9	15.0	56.6	60.0
M2	23	2	0.18 ± 0.26	0.91 ± 0.15	0.0	2.0	–	100.0
M3	24	7	1.29 ± 0.46	0.39 ± 0.21	13.2	10.0	52.9	70.0
M4	26	12	2.11 ± 0.40	0.15 ± 0.12	39.6	14.5	30.3	82.8
M5	25	10	1.92 ± 0.39	0.18 ± 0.12	28.4	13.3	35.2	75.0
M6	32	3	0.96 ± 0.29	0.46 ± 0.17	4.5	4.0	66.4	75.0
Central Anatolia region								
A1	31	7	1.42 ± 0.28	0.27 ± 0.09	9.4	6.5	74.5	107.7
A2	31	7	1.26 ± 0.36	0.35 ± 0.12	100.3	17.0	7.0	41.2
A3	25	4	1.14 ± 0.24	0.33 ± 0.10	4.7	4.0	85.1	100.0
A4	31	7	1.18 ± 0.42	0.46 ± 0.20	9.6	8.0	73.1	87.5
A5	26	4	0.59 ± 0.40	0.71 ± 0.22	7.0	4.5	57.1	88.9

Table 4. Clade distribution. The number of clones obtained and OTUs at a taxonomic level equating to 97% sequence similarity. The numbers of clones and OTUs with lower than 97% sequence similarity to known species in the GenBank database are given in parentheses.

Site	Rhi 1		Rhi 2		Rhi 3		Rhi 4		Unc 1		Fun		Unc 2		Cla		Aca		Unc 3		Total	
	Clone	OTU	Clone	OTU	Clone	OTU	Clone	OTU	Clone	OTU	Clone	OTU	Clone	OTU	Clone	OTU	Clone	OTU	Clone	OTU	Clone	OTU
Black Sea region																						
B1																	31(31)	1(1)			31(31)	1(1)
B2							31(31)	1(1)													31(31)	1(1)
B3							9(9)	1(1)					9(9)	1(1)					11(0)	1(0)	29(18)	3(2)
B4																			27(0)	1(0)	27(0)	1(0)
Mediterranean region																						
M1	8(1)	2(1)			3(0)	1(0)			1(1)	1(1)	20(7)	5(3)									32(9)	9(5)
M2	23(1)	2(1)																			23(1)	2(1)
M3					22(1)	6(1)					2(2)	1(1)									24(3)	7(2)
M4	6(0)	3(0)	1(1)	1(1)	3(0)	2(0)					2(0)	2(0)			14(1)	4(1)					26(2)	12(2)
M5	7(1)	5(1)	15(2)	4(2)							3(0)	1(0)									25(3)	10(3)
M6			5(0)	1(0)	1(1)	1(1)					26(0)	1(0)									32(1)	3(1)
Central Anatolia region																						
A1	21(0)	5(0)	10(1)	2(1)																	31(1)	7(1)
A2											31(6)	7(6)									31(6)	7(6)
A3	8(0)	1(0)	4(0)	1(0)					1(1)	1(1)	12(0)	1(0)									25(1)	4(1)
A4	6(0)	3(0)	25(2)	4(2)																	31(2)	7(2)
A5	4(0)	3(0)	22(0)	1(0)																	26(0)	4(0)

II-4. Conclusion

We recorded the phylogenetic composition of the AMF communities in the roots of 13 different plant species collected at three sites in three regions of Turkey. This research showed the first DNA sequence data of AMF from Turkey, half of which appear to have no close similarity (> 97%) to the sequence accessions in the public databases. The AMF communities of the soils from the East Black Sea region with a distinctively lower pH than the other soils separated from all the other AMF assemblages. All sequences from *C. sinensis* in the East Black Sea region had only 83–97% sequence similarity to known AMF species. Although there was no significant difference of the AMF community compositions between the Mediterranean and Central Anatolian samples, some sequences related to *Rhizophagus* (clade Rhi3) indicated regional differences.

Chapter III

Arbuscular mycorrhizal fungal community of wheat under long-term mineral and organic amendments in semi-arid Mediterranean Turkey

III-1. Introduction

Arbuscular mycorrhizal fungi (AMF) are the most common group of mycorrhizal fungi interacting with the roots of most terrestrial plants in nature, including many crops (Allen 1991). The major benefit of AM symbiosis for plants is that AMF collect water and minerals, particularly phosphorus (P), in moisture-deficient or nutrient-poor soils, and transport them through the extraradical hyphae to the root (Smith and Read 2008).

Turkey is one of the major wheat (*Triticum aestivum* L.) producing countries in the world and wheat is the most important cereal produced in Turkey, accounting for 64.5% of total cereal production (Ozkan, Akcaoz, and Fert 2004). Wheat is known to be a facultatively mycorrhizal plant (Molina, Massicotte, and Trappe 1992). Research on the associations between AMF and wheat-AMF associations in Turkey has mainly focused on the effect of AMF inoculation on nutrient uptake, plant yield, and soil fertility (Ortas 2003, 2012). Some previous studies indicated that the indigenous AMF isolated from semi-arid Mediterranean Turkey could significantly contribute to plant growth and P uptake (Almaca and Ortas 2010; Ortas 2010). However, these works used morphological tools such as AMF infection rates, spore morphology, and spore densities, which might not necessarily reflect the total AMF populations associated with the roots.

Molecular ecology methods have been developed that allow direct detection of AMF species in plant roots and, along with the development of molecular classification of AMF, have become promising tools to overcome the bottlenecks in AMF community analysis (Schussler, Schwarzott, and Walker 2001; Kruger et al. 2012; Redecker et al. 2013). Suzuki et al. (2014) investigated for the first time the composition of AMF phylotypes based on the nuclear ribosomal large subunit RNA genes in the roots of various agricultural plants such as tea, clover, wheat, corn, apple, apricot, sunflower, soybean, and alfalfa collected from the East Black Sea, Mediterranean, and Central Anatolian regions of Turkey. Their results showed that AMF communities in the Mediterranean region tended to be more diverse than those in the Central Anatolian region. It was also reported that indigenous AMF in the Mediterranean region improved the growth and nutrient uptake of citrus (Ortas and Ustuner 2014a, 2014b). However, no information is currently available regarding the community compositions of AMF in the wheat rhizosphere under different land managements in semi-arid soils of Mediterranean Turkey.

In this study, we hypothesized that wheat-associated AMF community responds to differences in fertilization practices and source materials of organic fertilizer in a long-term field experiment established in the Cukurova Region, Mediterranean Turkey. The

soil productivity in the field is low owing to xeric climate conditions (i.e., strong seasonal winter/summer rainfall contrast resulting in soil root zone drying during the summer) and exacerbated carbon and nitrogen mineralization in soil (Turgay et al. 2015). The soil type is classified as a Leptosol. A minimal amount is known about AMF community in arable land on Leptosols around the world, although soil type is one of the key factors determining AM community composition (Oehl et al. 2003). The aims of this study were to: (1) elucidate using molecular methods AMF community in Turkish semi-arid arable land on a Leptosol, and (2) compare the impacts of different soil management practices, including organic management, on AMF community.

III-2. Materials and Methods

Site description

The study was conducted in an experimental field on the Research Farm of Cukurova University (37° 0′ 54″ N, 35° 21′ 21″ E, 34 m above mean sea level) in the eastern part of the Mediterranean region of Adana, Turkey. The regional climate is typical Mediterranean with 19.1°C long-term average annual air temperature (ranging from 14.2 to 25.5°C) and 670 mm precipitation. Winter wheat was grown under rainfed conditions where the mean humidity and temperature were 73.2% and 17.8°C, respectively, and total precipitation was 86.3 mm during the wheat growth period. According to the classification of the World Reference Base for Soil Resources, the soil at the experimental site was classified as Molli-Lithic Leptosol (Jones, Montanarella, and Jones 2005). The particle size distribution was 37.5, 31.0, and 31.5% for clay, silt, and sand, respectively. Other soil chemical characteristics under different amendments and wheat yield in 2009 are summarized in Table 1.

Field experiment

The long-term field experiment started in April 1996 using a completely randomized design with four different fertilizer treatments in triplicate (each plot was 10

×20 m), as follows: control without fertilization, traditional chemical fertilization of 160 kg-N ha^{-1} y^{-1} as (NH4)2SO4, 83 kg-K ha^{-1} y^{-1} as K2SO4 and 26 kg-P ha^{-1} y^{-1} as Ca(H2PO4)2 · H2O, farmyard manure at 25 Mg ha^{-1} y^{-1}, and plant compost at 25 Mg ha^{-1} y^{-1}. Preparation of the plant compost material was described previously by Turgay et al. (2015). The organic fertilizers (farmyard manure and plant compost) were uniformly applied annually on the soil surface and were incorporated into the plow layer with a disc harrow. A double cropping system with wheat and maize (Zea mays L.) was introduced to the experimental field. Each plot was moldboard-plowed up to 20 cm depth after each harvest.

DNA extraction and cloning

Three soil samples were collected and mixed well from each plot in May 2009 during the wheat cropping season. Fine wheat roots were obtained using a 2-mm sieve and were washed thoroughly using sterile water and then stored in a refrigerator at 4°C until used. Total DNA was extracted from 50 mg of the root samples using Isoplant (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. Using the extracted DNA as the template, AMF 28S large subunit (LSU) rRNA genes were amplified with a nested polymerase chain reaction (PCR) protocol. The first PCR

amplification was performed using the fungal universal primers LR1 and FLR2 (van Tuinen et al. 1998; Trouvelot et al. 1999), followed by the second PCR amplification using the Glomeromycota-specific primers FLR3 (van Tuinen et al. 1998; Trouvelot et al. 1999) and FLR4 (Gollotte, van Tuinen, and Atkinson 2004). Both reactions were performed using PCR Thermal Cycler Dice Gradient TP600 (Takara, Otsu, Japan) under the same conditions as follows: initial denaturation at 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 15 s, annealing at 50°C for 40 s, polymerization at 72°C for 80 s, and final elongation at 72°C for 10 min. The amplicons generated from each root sample were cloned into the pGEM-T Easy Vector System (Promega, Fitchburg, USA) and were transformed into Takara Escherichia coli DH5α cells. Plasmids were purified using the High Pure Plasmid Isolation Kit (Roche Diagnostics GmbH, Mannheim, Germany). The nucleotide sequences were determined by the Fasmac DNA sequencing service (Atsugi, Japan) and have been deposited in the DNA Data Bank of Japan (DDBJ) under accession numbers AB727771 to AB727848. The sequence results from triplicate plots were combined into one treatment for subsequent analyses. The sequence results were compared with those available in the GenBank database by the Basic Local Alignment Search Tool (BLAST) search engine.

Analysis of sequences

The nucleotide sequences determined above and known mycorrhizal 28S rDNA sequences of related species from GenBank were aligned and edited to the same length in BioEdit 7.0.5.3. All gaps and missing data were deleted. Subsequently, a neighbor-joining phylogenetic dendrogram was constructed in MEGA5 using the Kimura-2 method and bootstrap analysis with 1,000 replications.

Distance matrices were generated with the Jukes-Cantor model using the DNADIST program from Phylip version 3.6.9. Using the data as an input file, we clustered sequences into operational taxonomic units (OTUs) at 99, 97, and 95% similarity levels using the Mothur program version 1.25.1. Rarefaction curves and nonparametric species richness estimators ACE and Chao1 were generated in Mothur software. Based on the results, the frequency of each OTU was calculated and compared among treatments with soil chemical properties using redundancy analysis (RDA) in XLSTAT software.

III-3. Results and Discussion

Arbuscular mycorrhizal fungal taxa and richness

We obtained from the wheat root samples a total of 201 clones that were highly similar to sequences from taxa belonging to the phylum Glomeromycota. Of the total sequences obtained, 52, 48, 49, and 52 clones were from the control, chemical fertilizer, farmyard manure, and plant compost treatments, respectively. Detailed results of sequences obtained are shown in Table 3.

The numbers of AMF sequences, OTUs (based on 97% sequence similarity), and estimated phylotype richness (ACE and Chao1) are shown in Table 2. A total of 69 unique sequences were found. Rarefaction curves for the pooled data were constructed at 99, 97, and 95% levels to assess the sizes on the AMF clone libraries. The curves for 97 and 95% levels showed saturation, suggesting that the number of clone sequences was sufficient to estimate the AMF communities based on 97% OTU level (Figure 1A). Finally, the clones were ascribed to 26 OTUs at the 97% sequence similarity level.

Rarefaction curves were determined at the 97% OTU level for the different agricultural management treatments (Figure 1B). A flattened curve was obtained from the manure amendment treatment, indicating that the number of OTUs approached saturation, and no more additional OTUs could be expected even if the number of samples had been

increased considerably. The curves for the control, chemical fertilization, and plant compost treatments showed a clear leveling off. Therefore, we recognized that the sample sizes were sufficient to recover most potential OTUs that occurred under the different long-term agricultural management treatments.

The plant compost treatment and the control treatment had higher OTU numbers compared with the chemical fertilizer and farmyard manure treatments (Table 2). This suggests that environmental conditions due to different fertilization practices can shape AMF community in different ways. Tanu et al. (2004) reported that the abundance of indigenous AMF propagules was higher in leaf compost-amended soil than in poultry manure-amended soil. Muthukumar and Udaiyan (2002) showed that mycorrhizal response was highly correlated with the type of organic manure applied. They also suggested that cowpea growth and yield in response to organic amendment were influenced by the type of amendment, resulting in proliferation of different AMF within the community.

The farmyard manure treatment showed the least number of AMF OTUs and the highest coverage of estimated richness. This may be because of high nutrient contents in the soil, especially P (Table 1), since higher soil P availability is known to suppress AMF colonization (Sharma and Adholeya 2001) and spore density (Menge et al. 1978).

Phylogenetic analysis of arbuscular mycorrhizal fungi and their distribution

An alignment was conducted between the different Glomeromycota sequences derived from all treatments and twenty-nine reference sequences. Clones that produced the same sequence were represented just once per plot in the alignment. A neighbor-joining tree was generated from the AMF phylotypes (Figure 2). Eight and one AMF sequence types were grouped in the Glomerales and Diversisporales orders, respectively, on the basis of bootstrap values >70%. The AMF sequence types belonging to Glomerales were subdivided into *Rhizophagus*, *Funneliformis*-1, *Funneliformis*-2, Glomeraceae-1, Glomeraceae-2, Glomeraceae-3, *Claroideoglomus*-1, and *Claroideoglomus*-2, all of which were in family Glomeraceae. In the Diversisporales group, one sequence type belonged to *Scutellospora*.

Frequency of the AMF groups in each plot was evaluated (Figure 3 and Figure 5) and the sequence type *Rhizophagus* accounted for 37% of the AMF sequences detected in this study. *Rhizophagus*-related clones were found in all treatments, indicating that *Rhizophagus* spp. are the most common AMF in the area. The farmyard manure treatment showed higher frequency of *Rhizophagus*-related clones (53%) than the control, chemical fertilization, and plant compost treatments (31, 27, and 38%, respectively). After

Rhizophagus, the next-most-frequent sequence type was *Funneliformis*-2, 38 clones of which were found from all treatments except for the control. *Glomus mosseae* (currently *Funneliformis mosseae*) and closely related taxa have been reported as common and typical AMF species in arable fields in Germany and Switzerland (Hijri et al. 2006). The sequence type *Funneliformis*-2 was most dominant under the chemical fertilization (44%), whereas the sequence type *Rhizophagus* was most dominant in the control, manure and plant compost treatments. The incidences of clones belonging to the sequence types *Funneliformis*-1, Glomeraceae-1, Glomeraceae-2, Glomeraceae-3, *Claroideoglomus*-1 and *Claroideoglomus*-2 were less than 10%. The sequence type *Scutellospora* was found only in the chemical fertilization treatment. This corresponds with results from Singh et al. (2008) who reported the absence of large-spored *Gigasporaceae*, including genus *Scutellospora*, in a cultivated ecosite in India. Some taxa in genus *Scutellospora* may be rare or absent in the arable field investigated in this study.

RDA

RDA based on frequency of each OTU revealed that the AMF communities were divided into three groups, namely, the control group, the organic group (farmyard manure and plant compost treatments), and the chemical fertilization group along with the

gradients of soil minerals such as available P, organic C, and available N (Figure 4). The first axis of RDA (60.28% of variance explained, Figure 4) indicated that the long-term chemical fertilization treatment altered the composition of the AMF community more than the organic treatments (farmyard manure and plant compost treatments). The result was similar to that of Verbruggen et al. (2010) who compared AMF community compositions under organic and conventional land managements using terminal restriction fragment length polymorphism community fingerprinting and reported that the difference in the AMF communities between conventionally managed farms and undisturbed, semi-natural grassland was larger than that between organically managed farms and grassland.

Sequence types *Funneliformis*-2 and *Rhizophagus* had the strongest axis1 and axis2 scores, respectively (Figure 4). This indicates that the first axis differentiated the treatments along the dominance gradient of sequence type *Funneliformis*-2 and the second axis differentiated treatments along that of *Rhizophagus*. The sequence types Glomeraceae-1, -2, and -3 also had strong axis2 scores, demonstrating the differences between the control from the farmyard manure and plant compost treatments. Bhadalung et al. (2005) showed different sensitivities among AMF species to fertilization based on an investigation of spore morphology. Our results suggested that the AMF species related

to the sequence type *Rhizophagus* and *Funneliformis*-2 were relatively sensitive to organic fertilizer and chemical fertilizer, respectively.

Among the long-term fertilization amendments, the farmyard manure and plant compost treatments, both of which possessed the dominant sequence type *Rhizophagus*, showed relatively similar AMF communities, whereas the chemical fertilization treatment was very different from all others, dominated by the sequence type *Funneliformis*-2 (Figures 3 and 4). These taxa are commonly found in arable soils (Öpik et al. 2006). The first axis of RDA in which soil available N had a strong axis1 score arranged the AMF communities according to the dominancy of the sequence type *Funneliformis*-2 (60.28% of variance explained, Figure 4). This suggests that AMF related to the sequence type *Funneliformis*-2 (including *F. mosseae*) can dominate under eutrophic conditions. Oehl et al. (2003) reported that spore abundance of *G. mosseae* (currently *F. mosseae*) was higher in mineral and/or mineral + organic fertilizer amendment sites compared with grassland and organic farming sites.

Along the second axis in which soil organic C and available P had a strong axis2 score (29.87% of variance explained), a shift in AMF community composition according to the dominancy of the sequence type *Rhizophagus* was detected. This suggested that the dominancy of *Rhizophagus* species was increased by the annual amendment with organic

fertilizer, regardless of the type of organic matter, and decreased by the addition of chemical fertilizers. This finding corresponds to Toljander et al. (2008), who demonstrated that the occurrence of Glomus intraradices (currently *Rhizophagus irregularis*) was significantly affected by fertilization treatments.

The sequence types Glomeraceae-1, -2, and -3 were not detected (Figure 3) under the chemical fertilization amendment. This indicated that conditions in the long-term chemical fertilization treatment were inhospitable for symbiosis of AMF in family Glomeraceae with wheat, except for genera *Rhizophagus* and *Funneliformis* (i.e., sequence types Glomeraceae-1, -2, and -3).

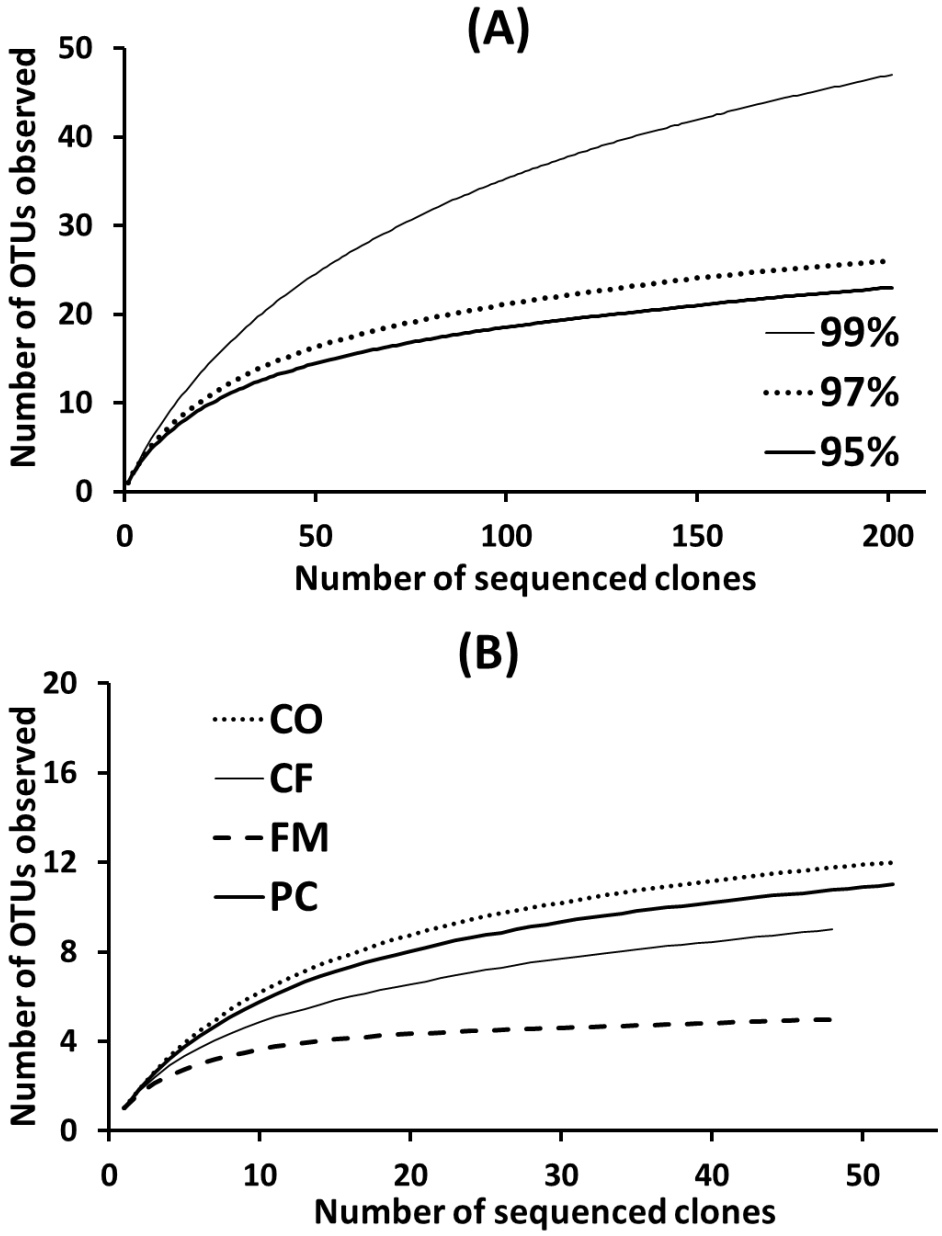


Figure 1. Rarefaction curves of the AMF large ribosomal subunit (LSU) rDNA libraries obtained from wheat roots in this study: for the pooled data at 99, 95 and 97% similarity levels (A) and each treatment at 97% similarity level (B). OTU; operational taxonomic unit, CO; control, CF; chemical fertilizer, FM; farmyard manure, and PC; plant compost.

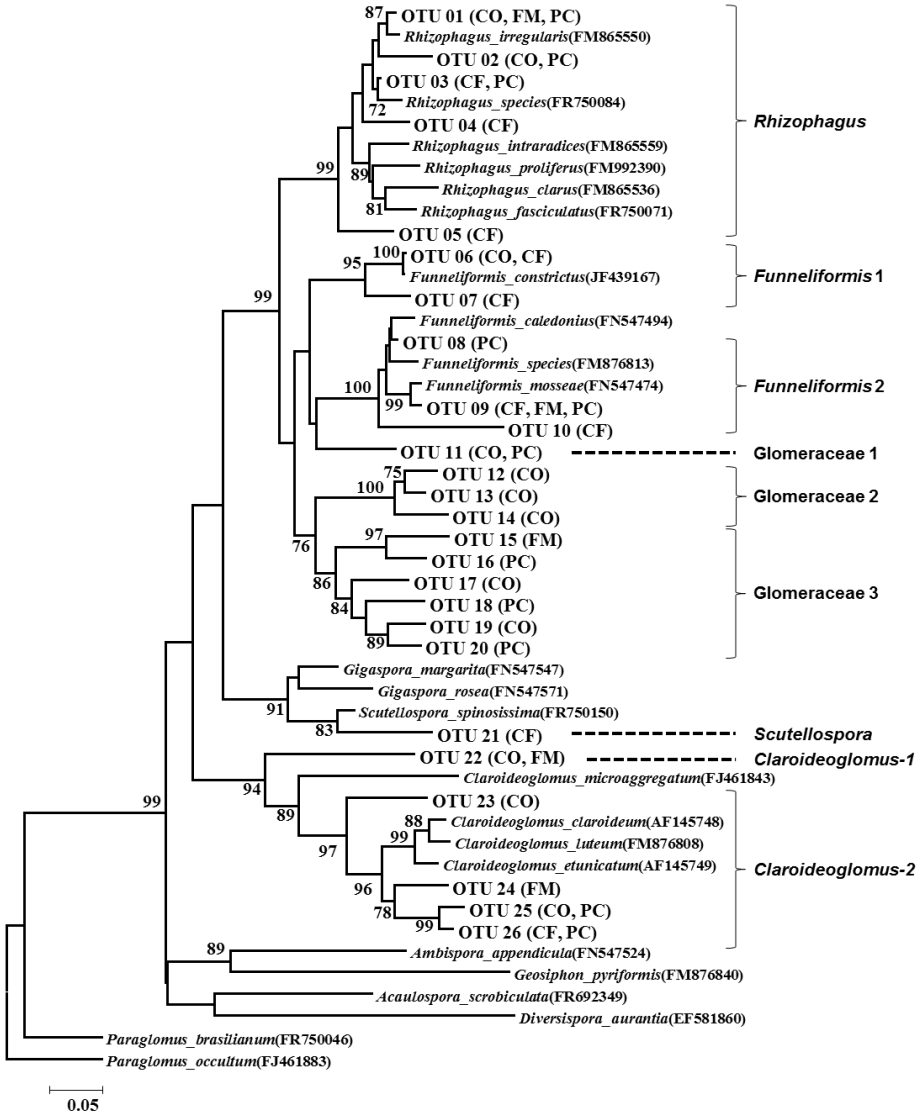


Figure 2. Neighbor-joining tree showing phylogenetic relationships of the AMF sequences obtained in this study and database sequences of known AMF species. The sequences were grouped into operational taxonomic units (OTUs) based on 97% sequence similarity using Mothur software. Parentheses show the treatments from which each sequence was derived. The sequence of *Paraglomus occultum* was used as the outgroup. Numbers above branches denote bootstrap values more than 70% from 1,000 replications. Lines on the right delimit the phylotypes. The scale bar at the bottom left is proportional to branch length. CO; control, CF; chemical fertilizer, FM; farmyard manure, and PC; plant compost.

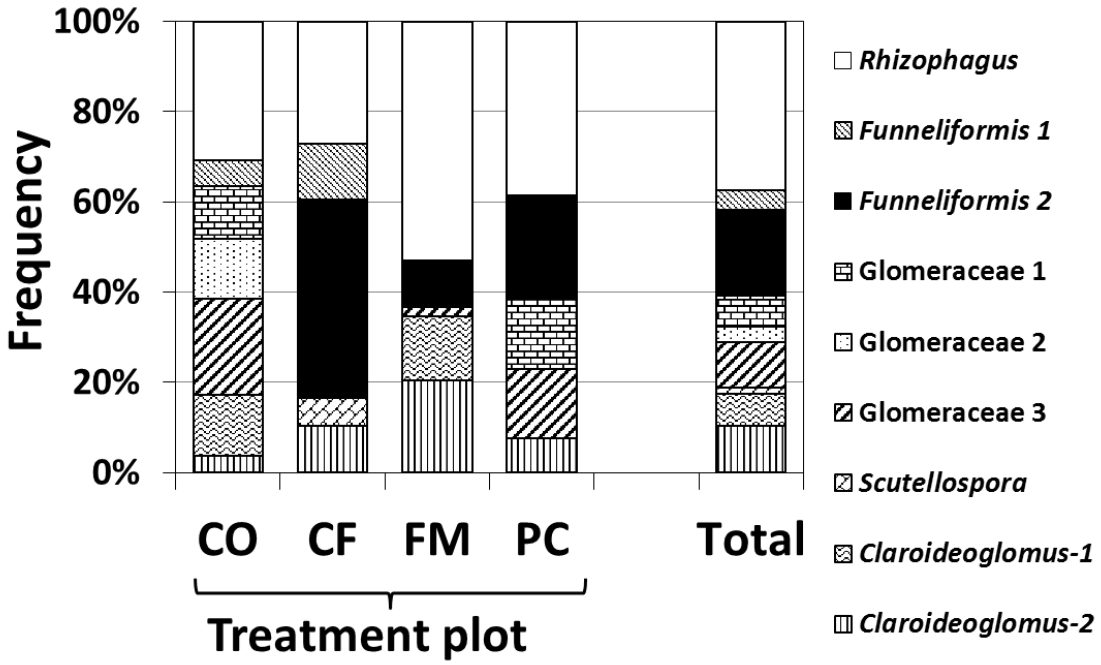


Figure 3. Frequency of the AMF groups detected in wheat root samples collected in this study. CO; control, CF; chemical fertilizer, FM; farmyard manure, and PC; plant compost.

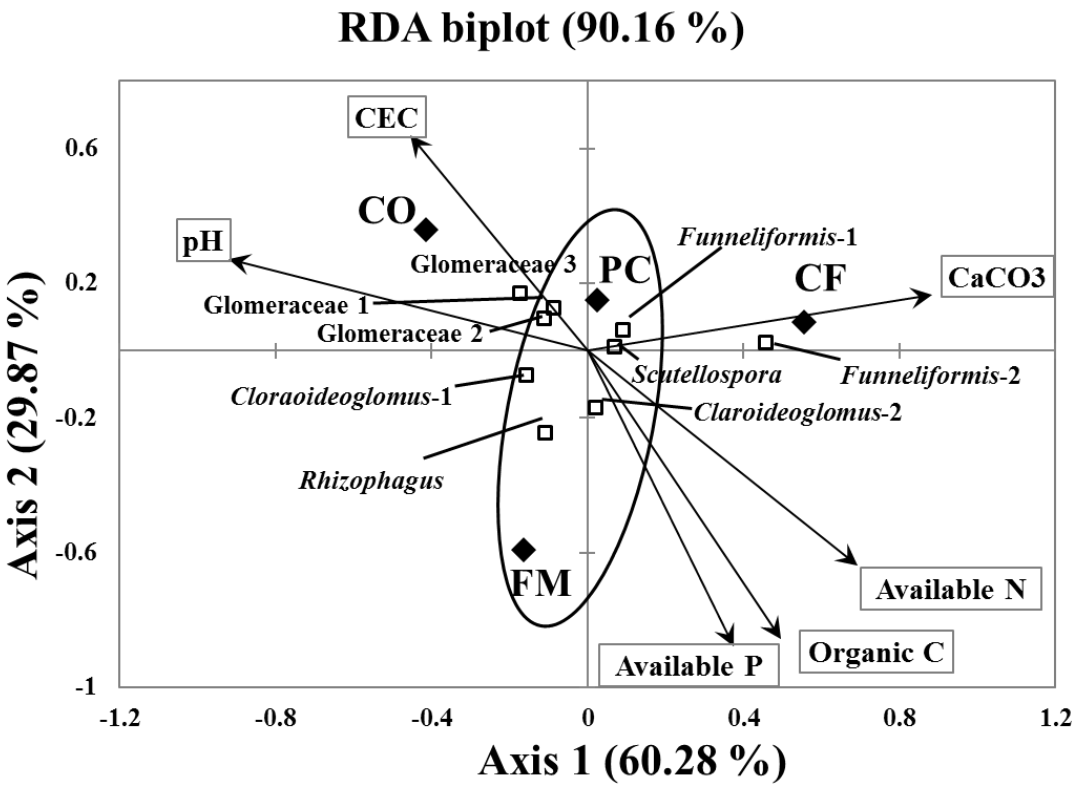


Figure 4. Redundancy analysis (RDA) between AMF communities associated with wheat roots collected in this study and environmental parameters. The percentages in the axes show the percentage of variation explained by the analysis. CO; control, CF; chemical fertilizer, FM; farmyard manure, and PC; plant compost.

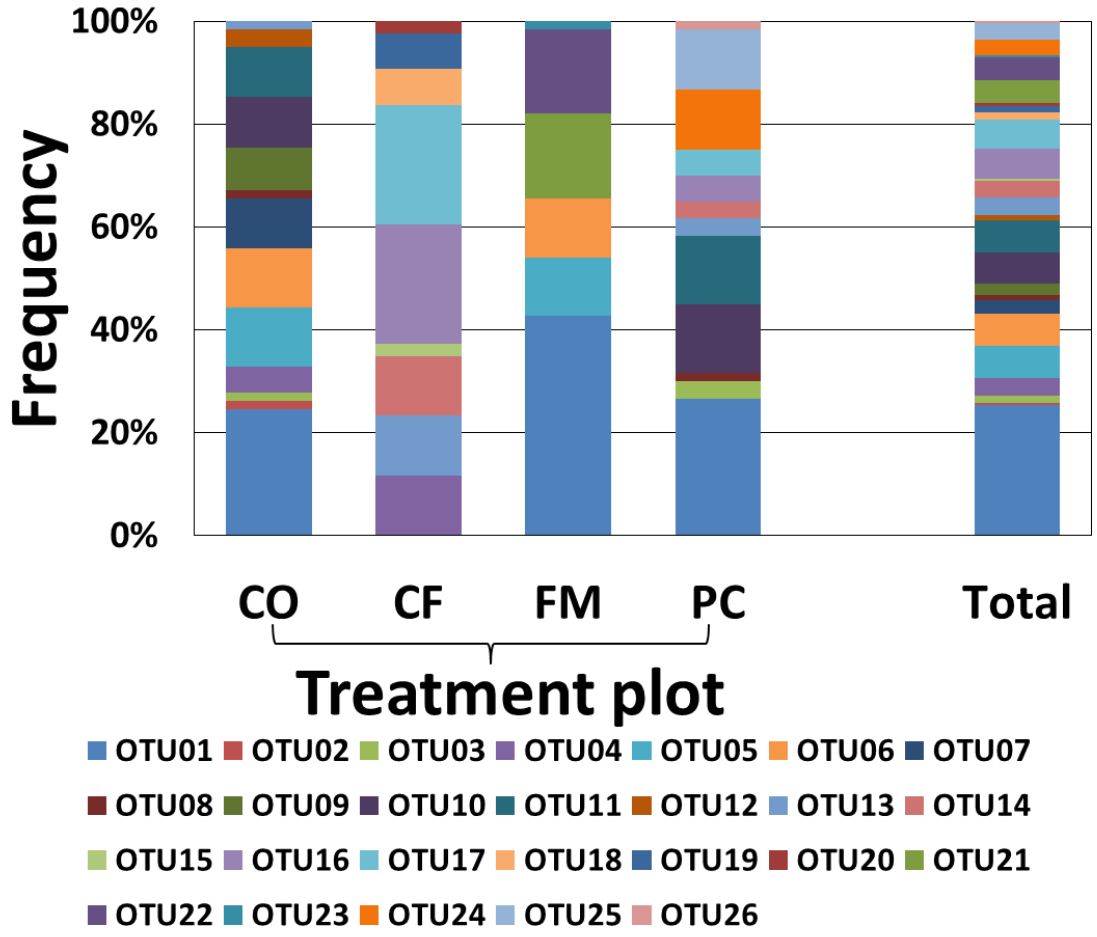


Figure 5. Frequency of the AMF OTUs detected in wheat root samples collected in this study. CO; control, CF; chemical fertilizer, FM; farmyard manure, and PC; plant compost.

Table 1. Soil and yield characteristics in the experimental site[¶]

Treatment[*1]	Soil properties						Wheat yield in 2009
	CEC ($\mu mol_c\ g^{-1}$)	$CaCO_3$ ($mg\ g^{-1}$)	$pH(H_2O)$[*2]	Organic C[*3] ($mg\text{-}C\ g^{-1}$)	Available N[*4] ($mg\text{-}N\ kg^{-1}$)	Available P[*5] ($mg\text{-}P\ kg^{-1}$)	($kg\ ha^{-1}$)
CO	7.54±0.43	3.68±0.05	7.81±0.10	10.8±0.15	37.8±2.4	11.47±0.28	3127±785
CF	6.87±0.50	3.92±0.12	7.72±0.01	15.1±0.37	39.2±0.7	25.17±0.60	7207±1075
FM	6.89±0.60	3.67±0.06	7.77±0.07	16.3±0.43	39.2±4.3	33.76±0.57	6200±1325
PC	7.78±0.10	3.67±0.33	7.79±0.05	12.5±0.37	39.0±1.8	25.05±0.43	4733±1209

[¶] Average ± standard deviation (n=3)

[*1] CO: control, CF: chemical fertilizer, FM: farmyard manure, PC: plant compost

[*2] Measured on a soil:water ratio at 1:2.5 (w/v)

[*3] Organic carbon (C) was calculated by subtracting the inorganic C, which was determined using a modified pressure-calcimeter method (Sherrod et al., 2002), from the total C, which was measured according to the Dumas method using a Variomax CNS elemental analyzer (Elementar GmbH, Hanau, Germany).

[*4] Available nitrogen (N) means sum of NH_4 and NO_3, measured according to a steam distillation method adding Devarda's alloy

[*5] Available phosphorus (P) was measured as indicated by Olsen & Sommers (1982)

Table 2. Description of clone libraries and richnessestimators for AMF communities in wheat (*Triticum aestivum* L.) roots collected in this study. ACE and Chao1 estimators were generated in MOTHUR software at 97% similarity.

Treatment[*1]	No. of clones	No. of OTUs (A)	ACE		Chao1	
			Estimation (B)	Coverage (A/B)	Estimation (C)	Coverage (A/C)
CO	52	12	13.8	0.870	13.0	0.923
CF	48	9	12.1	0.744	10.5	0.857
FM	49	5	5.4	0.926	5.0	1.000
PC	52	11	13.1	0.840	12.0	0.917

[*1] CO: control, CF: chemical fertilizer, FM: farmyard manure, PC: plant compost

Table 3. Classification of the AMF sequences obtained in this study. CO; control, CF; chemical fertilizer, FM; farmyard manure, and PC; plant compost.

Clade	OTU No. (97%)	Number of sequence obtaine CO	CF	FM	PC	Closest relatives	Accession No.	matched/total (similarity%)
Rhizophagus	1	13	0	15	0	Glomus intraradices isolate PACS large subunit ribosomal RNA	EU234492	330/330 (100%)
	1	1	0	0	0	Glomus intraradices isolate PACS large subunit ribosomal RNA	EU234492	329/331 (99%)
	1	0	0	9	5	Glomus intraradices isolate PACS large subunit ribosomal RNA	EU234492	326/330 (99%)
	1	1	0	0	0	Glomus intraradices isolate PACS large subunit ribosomal RNA	EU234492	324/330 (98%)
	1	0	0	2	8	Glomus intraradices isolate PACS large subunit ribosomal RNA	EU234492	328/330 (99%)
	1	0	0	0	2	Glomus intraradices isolate PACS large subunit ribosomal RNA	EU234492	322/330 (98%)
	1	0	0	0	1	Rhizophagus irregularis isolate MUCL43195	FR750079	330/331 (99%)
	2	0	0	0	1	Glomus mosseae isolate BEG 107 large subunit ribosomal RNA gene	AY769968	322/329 (98%)
	2	1	0	0	0	Glomus mosseae isolate BEG 107 large subunit ribosomal RNA gene	AY769968	326/329 (99%)
	3	0	1	0	0	Rhizophagus irregularis DAOM 181602	FR750067	323/330 (98%)
	3	0	1	0	0	Rhizophagus irregularis DAOM 181602	FR750067	324/331 (98%)
	3	0	3	0	0	Rhizophagus irregularis DAOM 181602	FR750067	323/330 (98%)
	3	0	3	0	0	Rhizophagus cf. irregularis FTRS203	FR750085	324/331 (98%)
	3	0	1	0	0	Rhizophagus cf. irregularis FTRS203	FR750085	323/331 (98%)
	3	0	1	0	0	Rhizophagus cf. irregularis FTRS203	FR750085	323/330 (98%)
	3	0	0	0	2	Rhizophagus irregularis DAOM 181602	FR750067	325/331 (98%)
	3	0	0	0	1	Rhizophagus irregularis DAOM 181602	FR750067	322/330 (98%)
	4	0	1	0	0	Rhizophagus irregularis DAOM 181602	FR750067	281/288 (98%)
	5	0	1	0	0	Glomus intraradices isolate Mas Badia large subunit ribosomal	EU234493	305/327 (93%)
	5	0	1	0	0	Glomus intraradices isolate Mas Badia large subunit ribosomal	EU234493	303/327 (93%)
Funneliformis 1	6	3	5	0	0	Glomus constrictum isolate 08_48_12	JF439167	331/333 (99%)
	7	0	1	0	0	Glomus constrictum isolate 08_48_12	JF439167	303/328 (92%)
Funneliformis 2	8	0	0	0	3	Funneliformis caledonium clone Hsp42-13 28S ribosomal RNA	JQ048886	326/333 (98%)
	8	0	0	0	3	Funneliformis caledonium clone Hsp42-13 28S ribosomal RNA (LSU)	JQ048886	326/333 (98%)
	8	0	0	0	1	Funneliformis caledonium clone Hsp42-13 28S ribosomal RNA (LSU)	JQ048886	325/333 (98%)
	9	0	0	3	0	Glomus mosseae 26S rRNA gene	AJ628050	330/332 (99%)
	9	0	0	2	0	Funneliformis mosseae clone Hsp38-11 28S ribosomal RNA	JQ048907	329/332 (99%)
	9	0	1	0	0	Glomus mosseae isolate 24ho102 26S ribosomal RNA gene	GQ330761	331/332 (99%)
	9	0	1	0	0	Funneliformis mosseae clone Hsp38-11 28S ribosomal RNA	JQ048907	328/332 (99%)
	9	0	0	0	1	Glomus mosseae isolate 54DKB01D4 26S ribosomal RNA	GQ330789	330/332 (99%)
	9	0	0	0	3	Glomus mosseae isolate 32dk33R132 26S ribosomal RNA gene	GQ330769	329/332 (99%)
	9	0	4	0	0	Glomus mosseae isolate BEG185-03	AY541909	332/332 (100%)
	9	0	8	0	0	Glomus mosseae isolate FR115 25S ribosomal RNA gene	FJ461844	332/332 (100%)
	9	0	5	0	0	Glomus mosseae isolate BEG185-03	AY541909	331/332 (99%)
	9	0	0	0	1	Glomus mosseae isolate 59sf1171 26S ribosomal RNA gene	GQ330794	331/332 (99%)
	9	0	1	0	0	Glomus mosseae isolate FR115 25S ribosomal RNA gene	FJ461844	331/332 (99%)
	10	0	1	0	0	Glomus mosseae isolate RMM_243 28S ribosomal RNA	AF396788	322/329 (98%)
Glomeraseae 1	11	0	0	0	5	Glomus viscosum isolate BEG50 25S ribosomal RNA gene	FJ461853	324/331 (98%)
	11	0	0	0	1	Glomus aggregatum isolate 08_6_1	JF439190	324/331 (98%)
	11	0	0	0	1	Glomus viscosum isolate BEG50 25S ribosomal RNA gene	FJ461853	323/331 (98%)
	11	0	0	0	1	Glomus viscosum isolate BEG50 25S ribosomal RNA gene	FJ461853	322/330 (98%)
	11	2	0	0	0	Glomus aggregatum isolate Ga-1	JF439131	324/332 (98%)
	11	4	0	0	0	Glomus aggregatum isolate Ga-1	JF439131	325/332 (98%)
Glomeraseae 2	12	2	0	0	0	Uncultured glomeromycete clone FLCC31017 large subunit ribosomal RNA	GQ149222	325/332 (98%)
	13	1	0	0	0	Uncultured glomeromycete clone FLCC31017	GQ149222	320/334 (96%)
	13	2	0	0	0	Uncultured Glomeromycota clone 5-3-5B large subunit ribosomal	JF798537	314/337 (93%)
	14	2	0	0	0	Uncultured Glomus gene for 28S ribosomal RNA	AB561102	328/333 (98%)
Glomeraseae 3	15	0	0	1	0	Uncultured Glomeromycota clone 11-1-3H large subunit ribosomal	JF798531	312/331 (94%)
	16	0	0	0	1	Funneliformis mosseae clone Hsp34-4 28S ribosomal RNA	JQ048912	233/240 (97%)
	17	6	0	0	0	Glomus intraradices isolate B21-18	JF439118	319/334 (96%)
	18	0	0	0	1	Glomus constrictum isolate 08_48_31	JF439180	293/333 (88%)
	19	1	0	0	0	Glomus constrictum isolate 08_48_31	JF439180	324/336 (96%)
	19	1	0	0	0	Glomus constrictum isolate 08_48_31	JF439180	325/336 (97%)
	19	3	0	0	0	Glomus constrictum isolate 08_48_31	JF439180	323/336 (96%)
	20	0	0	0	2	Glomus constrictum isolate 08_48_31	JF439180	310/335 (93%)
	20	0	0	0	1	Glomus constrictum isolate 08_48_31	JF439180	309/335 (92%)
	20	0	0	0	1	Glomus constrictum isolate 08_48_31	JF439180	309/335 (92%)
	20	0	0	0	1	Glomus constrictum isolate 08_48_31	JF439180	312/335 (93%)
	20	0	0	0	1	Glomus constrictum isolate 08_48_31	JF439180	310/335 (93%)
Scutellospora	21	0	2	0	0	Scutellospora sp. S247 gene for large subunit rRNA	AB206251	286/292 (98%)
	21	0	1	0	0	Scutellospora calospora isolate PL114 25S ribosomal RNA gene	FJ461865	283/291 (97%)
Claroideoglomus 1	22	4	0	0	0	Uncultured glomeromycete clone 6j large subunit ribosomal RNA	FJ820862	283/290 (98%)
	22	3	0	0	0	Uncultured glomeromycete clone 6j large subunit ribosomal RNA	FJ820862	284/290 (98%)
	22	0	0	3	0	Uncultured glomeromycete clone 6j large subunit ribosomal RNA	FJ820862	284/290 (98%)
	22	0	0	4	0	Uncultured Glomus partial 28S rRNA gene, clone BROS1-19	FR871374	287/288 (99%)
Claroideoglomus 2	23	1	0	0	0	Glomus claroideum partial 25S rRNA gene	AM040317	250/280 (89%)
	24	0	0	10	0	Glomus claroideum partial 25S rRNA gene	AM040316	295/328 (90%)
	25	1	0	0	2	Glomus claroideum isolate j2-fist 28S ribosomal RNA	AF396801	247/253 (98%)
	26	0	5	0	2	Glomus claroideum isolate j2-fist 28S ribosomal RNA	AF396801	244/253 (96%)
Total sequence		52	48	49	52			

III-4. Conclusion

In this study, AMF community compositions on a Leptosol under a long-term double cropping system with different fertilization practices were investigated in semi-arid Mediterranean Turkey. As with our hypothesis, different fertilization practices affected several ecological indices for the AMF communities. The AMF community richness under no-fertilization treatment and plant compost treatment was higher than those that were amended with farmyard manure or chemical fertilizers. Although different organic amendments supported relatively similar AMF communities, plant compost induced higher AMF richness than farmyard manure fertilization.

Chapter IV

Molecular diversity of arbuscular mycorrhizal fungi and rhizobacterial communities in arid grassland under desertification stress

IV-1. Introduction

Desertification is one of the most important environmental problems worldwide. According to the United Nations Convention to Combat Desertification (UNCCD), desertification is defined as "land degradation in arid, semi-arid, and dry sub-humid areas, resulting from various factors, including climate variations and human activity". The desertification process includes climate change, changes to the hydrological cycle, land misuse, intensive farming, overgrazing, urbanization and food production. Disturbance of natural vegetation cover is the first visible symptom of desertification but the losses of key physicochemical and biological soil properties are known to occur concomitantly (Requena et al. 2001). Desertification is known to be associated with reductions in soil microbial diversity and nutrient cycling activities (Kennedy and Smith 1995). Since land degradation can limit the potential for revegetation of natural plants (Warren et al. 1996), their degradation results in a loss of sustainability. Soil microbial communities are likely to have a strong driving force in these ecosystems. It has been indicated that maintaining a high proportion of biological diversity leads to efficient and stable levels of ecosystem functioning (Naeem et al. 2012), suggesting that losses in microbial diversity may directly impact desert biomass (Hooper et al. 2012).

In addition to rhizospheric bacterial communities, arbuscular mycorrhizal fungi

(AMF) are important soil microorganisms, forming symbiotic associations with roots of most terrestrial plants, and this association is widespread and ancient (Remy et al. 1994). The AMF enhance nutrient acquisition for the host plant by transferring phosphorus from soil in exchange for carbon from photosynthesis (Smith and Read 2008). The AMF also transfer nitrogen, particularly in inorganic forms, to the host plants from decomposing litter (Nuccio et al 2013). In addition, AMF symbiosis includes tolerance of water deficiency, mitigation of heavy metal toxicity and protection from pathogens (Allen 1991; Newsham et al. 1995; Joner et al. 2000).

There are numerous relationships between AMF and bacteria. Some bacterial groups called mycorrhiza helper bacteria (MHB) can assist mycorrhiza formation and interact positively with the functioning of the symbiosis, including AMF spore formation (Frey-Klett et al. 2007). Zhang et al. (2016) suggested that AMF and free-living photosynthetic bacteria positively interacted by providing the carbon or phosphorus that the other microorganism required, depending on soil phosphorus availability. It was suggested that AMF could modify the soil bacterial community and decomposition processes by exporting nitrogen from decomposing litter (Nuccio et al. 2013). In addition, intracellular bacteria hosted in AMF (Desiro et al. 2014) and bacterial communities colonizing the AMF hyphal surface (Scheublin et al. 2010) have been reported. It was

also found that AMF may suppress members of microbial community (Welc et al. 2010), and may be suppressed by other bacteria (Leigh et al. 2011).

Therefore, investigations of indigenous microbial diversities of AMF and rhizobacteria are important for the restoration of degraded soil and for sustainable agriculture in desertified arid ecosystems and understanding the relationship between AMF and rhizobacteria is strongly required. Previous studies have documented patterns of bacterial (Kuske et al. 2002) and fungal composition (Bates et al. 2012) in arid land. However, few studies have been carried out using high-throughput next-generation sequencing to characterize microbial communities within arid ecosystems (Makhalanyane et al. 2015).

In this study, we investigated AMF and rhizobacterial communities in arid perennial grassland under desertification stress in Turkey with ITS and 16S rDNA amplicon sequence analysis using a next-generation sequencer. The objective of the study was 1) to elucidate soil AMF and bacterial communities in degraded arid ecosystems, and 2) to understand the relationships between AMF and bacterial communities under desertification stress.

IV-2. Materials and Methods

Sampling description

To evaluate the effect of desertification on soil microbial biota in Central Turkey, we selected four natural grassland sites with different desertification potentials from the Hasanoglan-Ankara Region and two sites from the Kalecik-Kırıkkale Region (Table 1). Study sites were visited three times, first in early summer of 2015, when plant characteristics such as leafing and flowering allowed floristic observations and thus simple plant identifications, and afterwards, in mid-summer of 2015 and the spring of 2016 to evaluate the effect of seasonal changes on soil microbial diversity.

The degradation status (Table 1) was investigated on June 16th, 2015, when the most common plant genera were present at the study sites. Natural plant diversity and distribution were identified and recorded to elucidate annual and perennial plants present at the sites with different land degradation and desertification potentials (Table 2). Three perennial (*Astragalus microcephalus* Willd, *Verbascum lasianthum* Boiss. ex Bentham. and *Artemisia spicigera* C. Koch), and four annual (*Festuca valesiaca* Schleicher ex Gaudin, *Aegilops biuncialis* Vis., *Stipa ehrenbergiana* Trin. & Rupr. and *Bromus sterilis* L.) plant species were observed in all sites and were therefore selected for subsequent soil microbial diversity analysis.

On June 18th (summer) and April 19th, 2016 (spring), six study sites were visited

for plant and soil sampling. Intact samples of the above mentioned plants were numbered and pressed between sheets of botanical drying paper, transported to the Biology Department of the Science Faculty, Ankara University, dried and stored in the Herbarium of Biology Department for further analysis. The details of the sampling status are shown in Table 3 and Fig. 1.

Approximately 500 g of bulk soil was collected from the rhizosphere of each plant individual in triplicate per species and passed through a 2-mm sieve. The fine roots were collected from each plant and dried after washing. The soil samples were stored at 4°C prior to analysis.

Evaluation of desertification intensity with the MEDALUS Model

The MEDALUS (MEditerranean Desertification And Land Use) model is used to evaluate Environmentally Sensitive Areas (ESA) through qualitative and quantitative estimates of four main qualities (climate, vegetation, soil, and land management), allowing researchers to classify critical, fragile, potential, and unaffected zones of territorial areas. The soil quality index (SQI) considers soil texture, parent material, distribution of rock fragments, soil depth, slope, and drainage. Climate quality index (CQI) is assessed on the basis of the amount of rainfall, aridity, and slope aspect. The VQI covers the factors such as vulnerability to burning, drought resistance; capacity of

protection from erosion and vegetation cover and the management quality index (MQI) is assessed as the product of land use intensity and the enforcement of policy for environmental protection.

In this study, all site characteristics required for the assessments of four indexes mentioned above (SQI, CQI, VQI and MQI) were measured and calculated scientifically according to indicated by Kosmas et al. (1999). The index of Environmentally Sensitive Areas (ESAI) was determined by entering index values obtained to the Desertification Indicator System for Mediterranean Europe (DIS4ME 2004) which is an online knowledge platform developed in DESIRE's Harmonised Information System (DESIRE-HIS, http://www.desire-his.eu/index.html). The table below shows the types of ESAs and corresponding range of the ESAI. The different degrees of vulnerability to land degradation was scored between 1 (low sensitivity) to 3 (high sensitivity).

Amplicon sequencing analysis of AMF communities

The dried root samples were cut into 2-mm pieces and were mixed to obtain a 20-mg sample for total DNA extraction, using the ISOPLANT kit (Nippon Gene, Tokyo, Japan). Isolated DNA was 10 times diluted with ddH2O and used as a template for subsequent PCR. A two-step nested PCR was carried out to obtain the partial ITS region of AMF. In

the first step, the AMF SSU-specific forward primer SSUmAf (Krüger et al., 2009) and complementary sequence of AMF-LSU specific primer FLR3 (Gollotte et al. 2004) were used to amplify partial SSU, whole ITS and partial LSU regions of the rDNA. The first PCR was carried out in a 25-μL volume using KOD-FX NEO mix (TOYOBO, Osaka, Japan). Cycling conditions were 2 min at 94°C, followed by 40 cycles of 10 s at 98°C, 30 s at 56°C and 90 s at 68°C, and final elongation of 7 min at 68°C. The obtained PCR products were purified using QIAquick® Gel Extraction Kit (Qiagen, Venlo, Netherlands) according to the manufacturer's instructions. Purified DNA were eluted into 50 μL of TE buffer and used as a template for the second PCR with eukaryotic primers ITS1-F_KYO1/ITS2-KYO1 (Toju et al., 2012) with the linker sequences for Nextera primers (Illumina Inc., CA, USA). The second PCR was carried out in 25-μL volume using TaKaRa Ex Taq® hot start version (TaKaRa, Shiga, Japan). Cycling conditions were 2 min at 94°C, followed by 20 cycles of 30 s at 94°C, 30 s at 50°C and 30 s at 72°C, and final elongation 5 min at 7 °C. PCR barcode indexing was then performed with Nextera primers. The amplicons were purified with an Agencourt AMPure XP kit (Beckman Coulter, CA, USA) according to the manufacturer's instructions. The purified amplicons were paired-end sequenced on an Illumina MiSeq platform (Illumina) at a read length of 2 × 300 bp using MiSeq reagent kit v3.

Amplicon sequencing analysis of rhizosphere bacterial communities

Soil DNA was obtained from 0.5 g of homogenized soil samples using ISOIL for Beads Beating (Nippon Gene Co., Ltd., Tokyo, Japan), according to the manufacturer's instructions. Isolated DNA was 10 times diluted with ddH2O and used as a template for subsequent PCR. Partial bacterial 16S rDNA (V4 region) was amplified using the bacterial primers 515F/806R with the linker sequences for Nextera primers (Illumina Inc., CA, USA). The PCR amplification was carried out in 25-μL volume using TaKaRa Ex Taq® hot start version (TaKaRa, Shiga, Japan). Cycling conditions were 2 min at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at 50°C and 30 s at 72°C, and final elongation 5 min at 72°C. The PCR products were purified using a High Pure PCR Product Purification Kit (Roche, Basel, Switzerland), and PCR barcode indexing was then performed with Nextera primers. The purified amplicons were paired-end sequenced on an Illumina MiSeq platform (Illumina) at a read length of 2 × 300 bp using MiSeq reagent kit v3.

Data processing

The sequence data were processed and analyzed in QIIME ver. 1.8.0 (Caporaso et al.,

2010). The sequences were paired-end joined and filtered through quality check and chimera check using USEARCH61 (Edgar 2010). The processed sequences were clustered into OTUs using the open reference OTU picking algorithm at 97% similarity with UNITE database (Abarenkov et al. 2010) for fungal identification, and the Greengenes 16S rRNA gene database (released in August 2013) for bacterial identification.

To compare bacterial communities among the samples, principal component analysis (PCA) was performed in R software. Cluster analysis was performed based on the proportions of bacterial community at the genus level in R software with the Ward method.

IV-3. Results

We examined 49 root samples and rhizosphere samples from six natural vegetation areas to reveal the microbial community and relationships between rhizosphere bacterial and AMF communities of seven plant species growing in this degraded area. The samples from seven plants were named “Ast” (*Astragalus*), “Art” (*Artemisia*), “Ver” (*Verbascum*), “Sti” (*Stipa*), “Bro” (*Bromus*), “Fes” (*Festuca*), “Aeg” (*Aegilops*).

According to the result of the site survey (Table 1), our study sites were subdivided into three groups as follows: undesertified (Sites 1 and 2), moderately sensitive (Sites 3 and 4) and highly sensitive (Sites 5 and 6).

The AMF community

A total of 3,201,661 sequences were obtained, and 33 OTUs were obtained at the genus level in this study. On average, 65,340 sequences and 8 OTUs were obtained from each sample. The taxonomic compositions at phylum and genus level are shown in Fig. 2. The proportion of AMF sequences (e.g. phylum Glomeromycota) was approximately 80% (Fig. 2A). Observed AMF families were Archaeosporaceae, Gigasporaceae, Glomeraceae, and genus *Septoglomus incertae sedis*. *Septoglomus* is a new genera of AMF reported by Oehl et al. (2011). At the genus level, nine AMF OTUs were obtained and four AMF

genera were identified molecularly as follows: *Glomus, Rhizophagus, Gigaspora and Septoglomus* (Fig. 2B and C).

Bacterial community composition

A total of 5,858,309 sequences were obtained, and 776 OTUs were obtained at the genus level in this study. On average, 119,557 sequences and 525 OTUs were obtained from each sample.

The proportions of the bacterial community at the phylum level are shown in Fig. 3A. The most dominant three phyla were Firmicutes, Proteobacteria and Actinobacteria, and these three phyla accounted for approximately 80–90% of the total community. Bacteroidetes was the fourth most dominant phylum which accounted for approximately 10% of the total community. The proportions of the bacterial community at the genus level were shown in Fig. 3B and C. The dominant genera were as follows: approximately 9% *Bacillus*, 7% *Rubrobacter* and 5% of *Planococcus*, and 1–2% *Mycobacterium*, *Solirubrobacter*, *Cohnella*, *Arthrobacter*, *Sphingomonas*, *Methylobacterium* and *Clostridium*.

To compare the bacterial community compositions among sites, plant species and sampling seasons, cluster analysis and principal component analysis were conducted

(Fig. 4 and Fig. 5, respectively). At the June sampling, the samples from the highly sensitive area (Site 5 and 6) tended to be clustered and to be differentiated from the undesertified soil samples (Site 1 and 2) (Fig. 4A). Figure 4A also showed that the clusters of samples from moderately sensitive conditions (Site 3 and 4) tended to be merged with undesertified and highly sensitive soil samples. In the April sampling, bacterial communities in the undesertified soil were clearly differentiated from those in the moderately and highly sensitive conditions (Fig. 4B). A similar trend was also found in PCA analysis (Fig. 5). The bacterial communities from Site 2, which have relatively low soil quality compared with Site 1, tended to be similar to those of Sites 4 to 6 (Fig. 5A). Figure 5B clearly shows the difference in the bacterial community between undesertified sites (Sites 1 and 2) and the other sites. However, a consistent difference in bacterial community between the two sampling times was not observed (Fig. 5C), although the bacterial community from the highly sensitive conditions (Sites 5 and 6) showed small differences (Fig. 3C and Fig. 5C).

Genus *Bacillus* and *Planococcus* had strong PC1 scores (Fig. 5). Genus *Rubrobacter* had the highest PC2 score and *Calothrix* had the lowest PC2 score (Fig. 5A and C). This indicates that the first axis differentiated the plots along the dominance gradient of genus *Bacillus* and *Planococcus* and the second axis differentiated plots along

that of *Rubrobacter* and *Calothrix*.

(A)

(B)

(C)

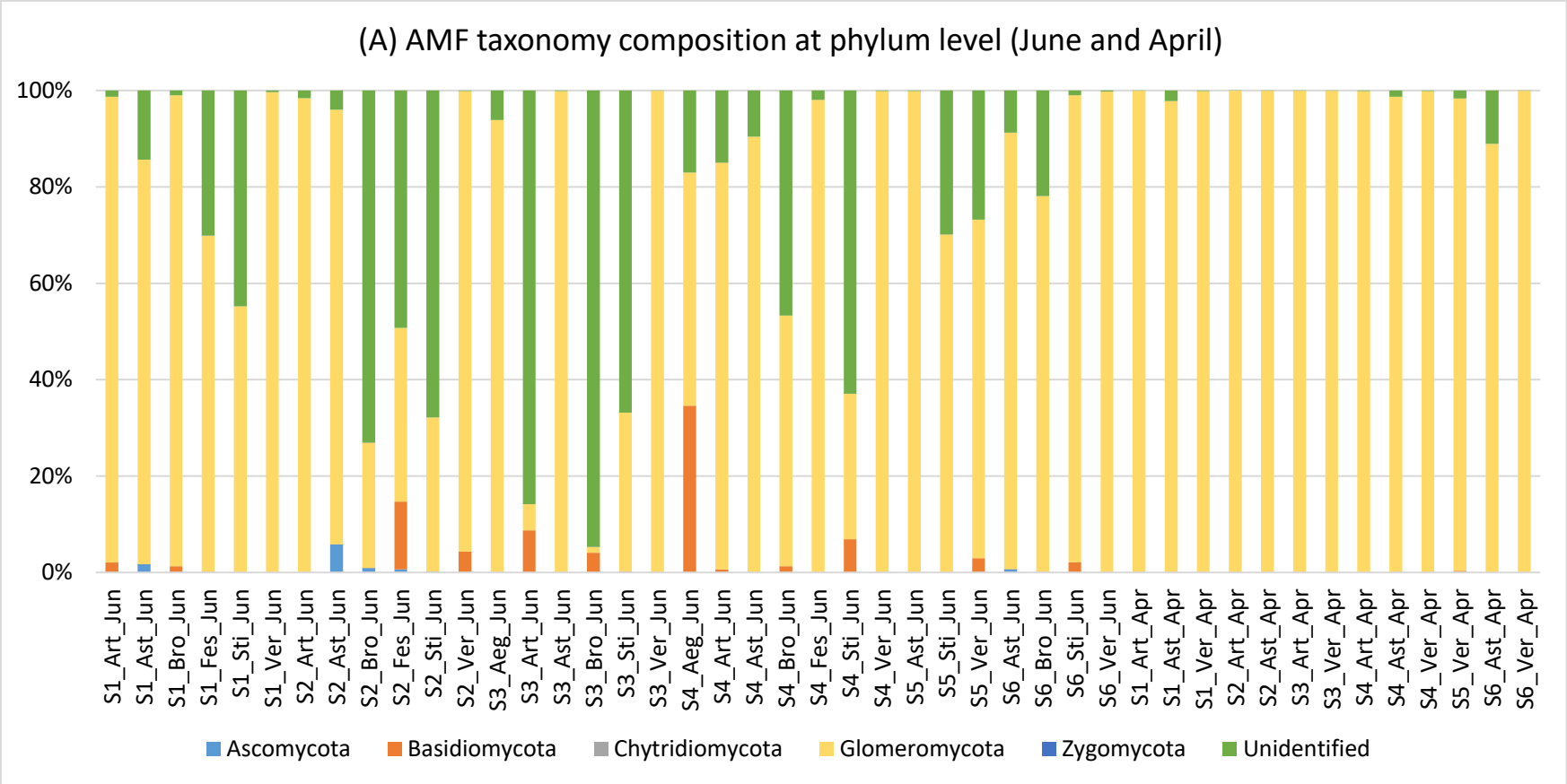
(D)

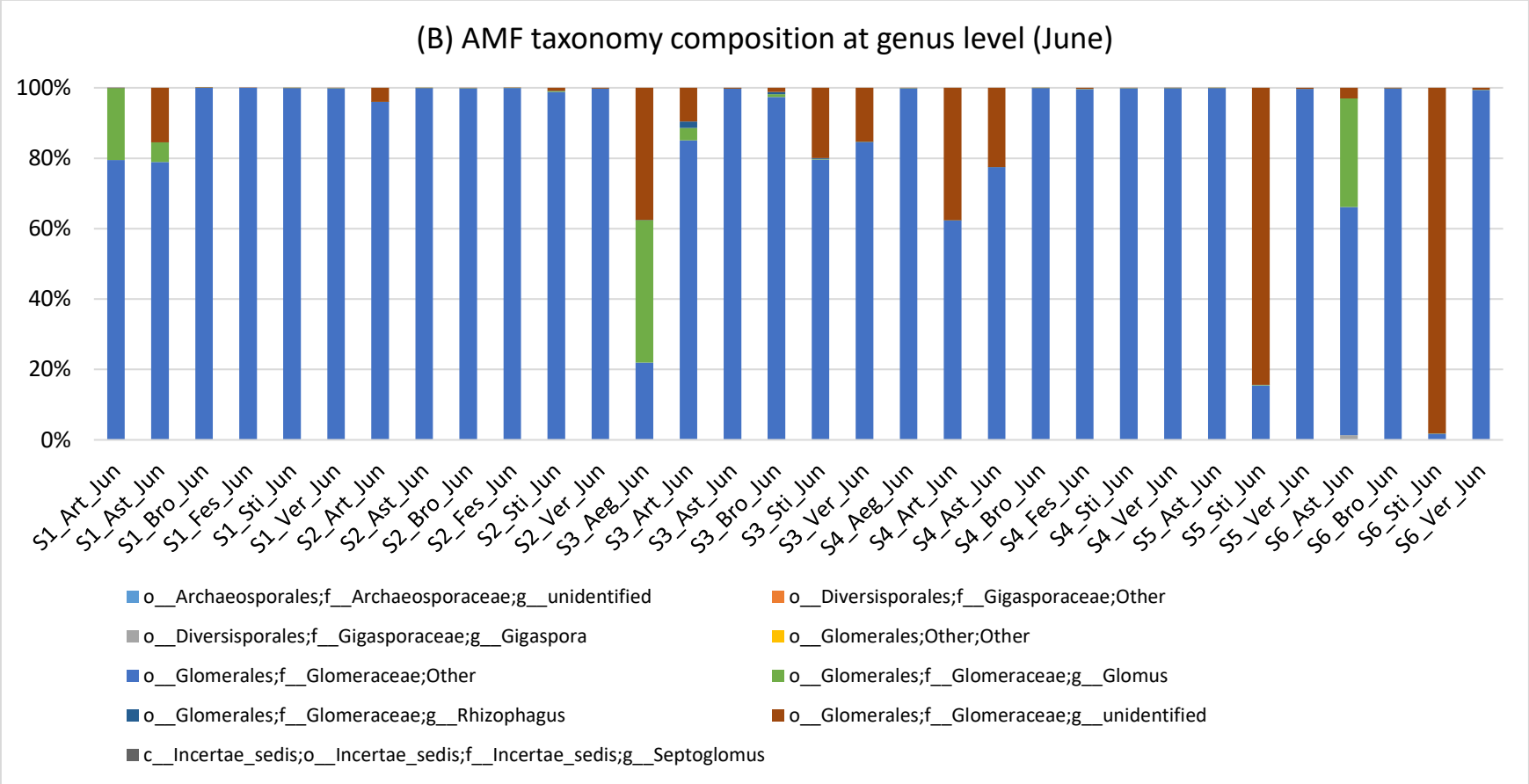
(E)

(F)

(G)

Fig. 1. Photographs of the plants in which symbiotic AMF and rhizobacterial communities were investigated: (A) *Astragalus microcephalus* Willd, (B) *Artemisia spicigera* C. Koch, (C) *Verbascum lasianthum* Boiss. ex Bentham., (D) *Stipa ehrenbergiana* Trin. & Rupr., (E) *Bromus sterilis* L., (F) *Festuca valesiaca* Schleicher ex Gaudin, and (G) *Aegilops biuncialis* Vis.


(A) AMF taxonomy composition at phylum level (June and April)
100%
80%
60%
40%
20%
0%
S1_Art_Jun
S1_Ast_Jun
S1_Bro_Jun
S1_Fes_Jun
S1_Sti_Jun
S1_Ver_Jun
S2_Art_Jun
S2_Ast_Jun
S2_Bro_Jun
S2_Fes_Jun
S2_Sti_Jun
S2_Ver_Jun
S3_Aeg_Jun
S3_Art_Jun
S3_Ast_Jun
S3_Bro_Jun
S3_Sti_Jun
S3_Ver_Jun
S4_Aeg_Jun
S4_Art_Jun
S4_Ast_Jun
S4_Bro_Jun
S4_Fes_Jun
S4_Sti_Jun
S4_Ver_Jun
S5_Ast_Jun
S5_Sti_Jun
S5_Ver_Jun
S6_Ast_Jun
S6_Bro_Jun
S6_Sti_Jun
S6_Ver_Jun
S1_Art_Apr
S1_Ast_Apr
S1_Ver_Apr
S2_Art_Apr
S2_Ast_Apr
S3_Art_Apr
S3_Ver_Apr
S4_Art_Apr
S4_Ast_Apr
S4_Ver_Apr
S5_Ver_Apr
S6_Ast_Apr
S6_Ver_Apr
Ascomycota
Basidiomycota
Chytridiomycota
Glomeromycota
Zygomycota
Unidentified


(B) AMF taxonomy composition at genus level (June)
100%
80%
60%
40%
20%
0%
S1_Art_Jun
S1_Ast_Jun
S1_Bro_Jun
S1_Fes_Jun
S1_Sti_Jun
S1_Ver_Jun
S2_Art_Jun
S2_Ast_Jun
S2_Bro_Jun
S2_Fes_Jun
S2_Sti_Jun
S2_Ver_Jun
S3_Aeg_Jun
S3_Art_Jun
S3_Ast_Jun
S3_Bro_Jun
S3_Sti_Jun
S3_Ver_Jun
S4_Aeg_Jun
S4_Art_Jun
S4_Ast_Jun
S4_Bro_Jun
S4_Fes_Jun
S4_Sti_Jun
S4_Ver_Jun
S5_Ast_Jun
S5_Sti_Jun
S5_Ver_Jun
S6_Ast_Jun
S6_Bro_Jun
S6_Sti_Jun
S6_Ver_Jun
o__Archaeosporales;f__Archaeosporaceae;g__unidentified
o__Diversisporales;f__Gigasporaceae;Other
o__Diversisporales;f__Gigasporaceae;g__Gigaspora
o__Glomerales;Other;Other
o__Glomerales;f__Glomeraceae;Other
o__Glomerales;f__Glomeraceae;g__Glomus
o__Glomerales;f__Glomeraceae;g__Rhizophagus
o__Glomerales;f__Glomeraceae;g__unidentified
c__Incertae_sedis;o__Incertae_sedis;f__Incertae_sedis;g__Septoglomus

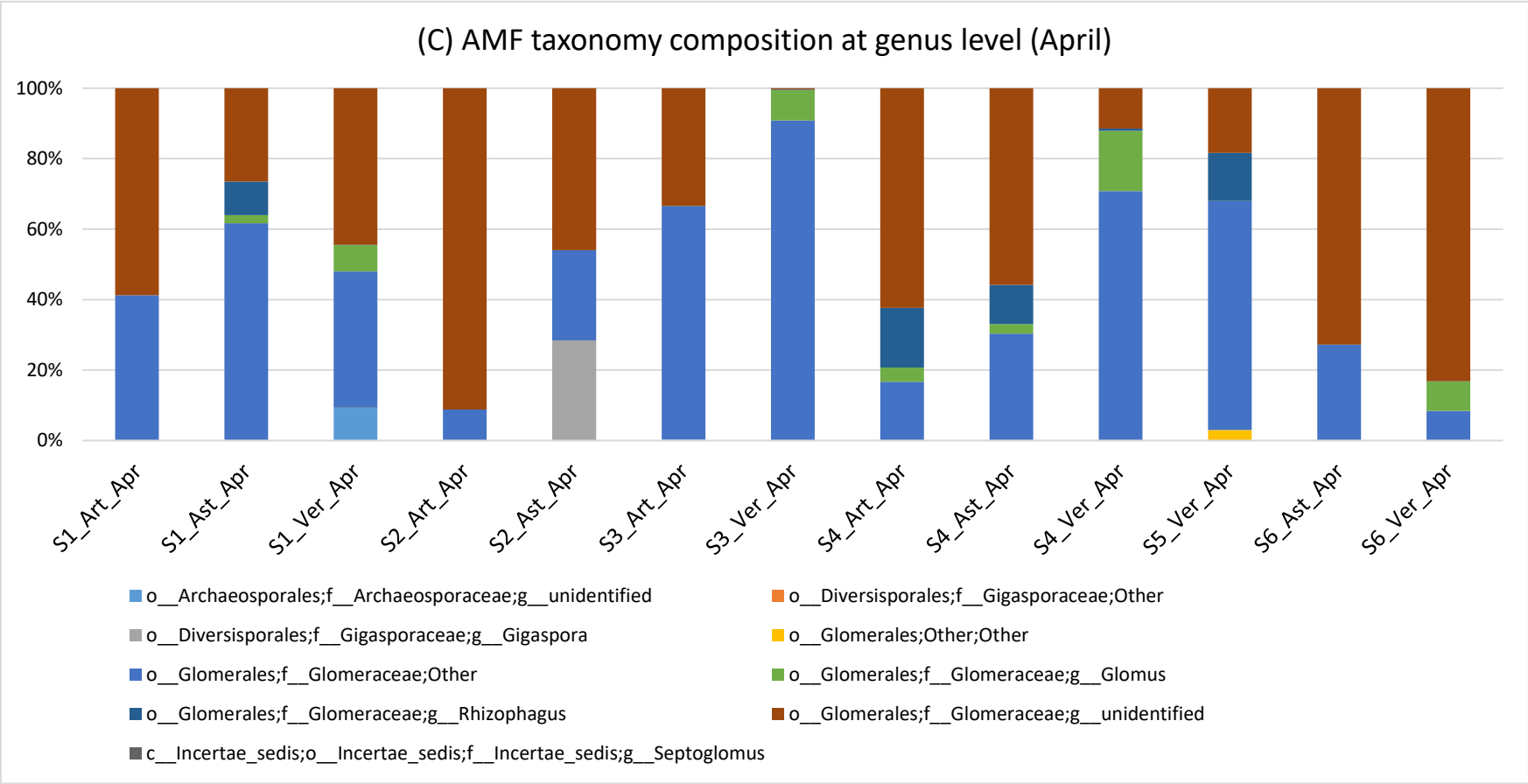
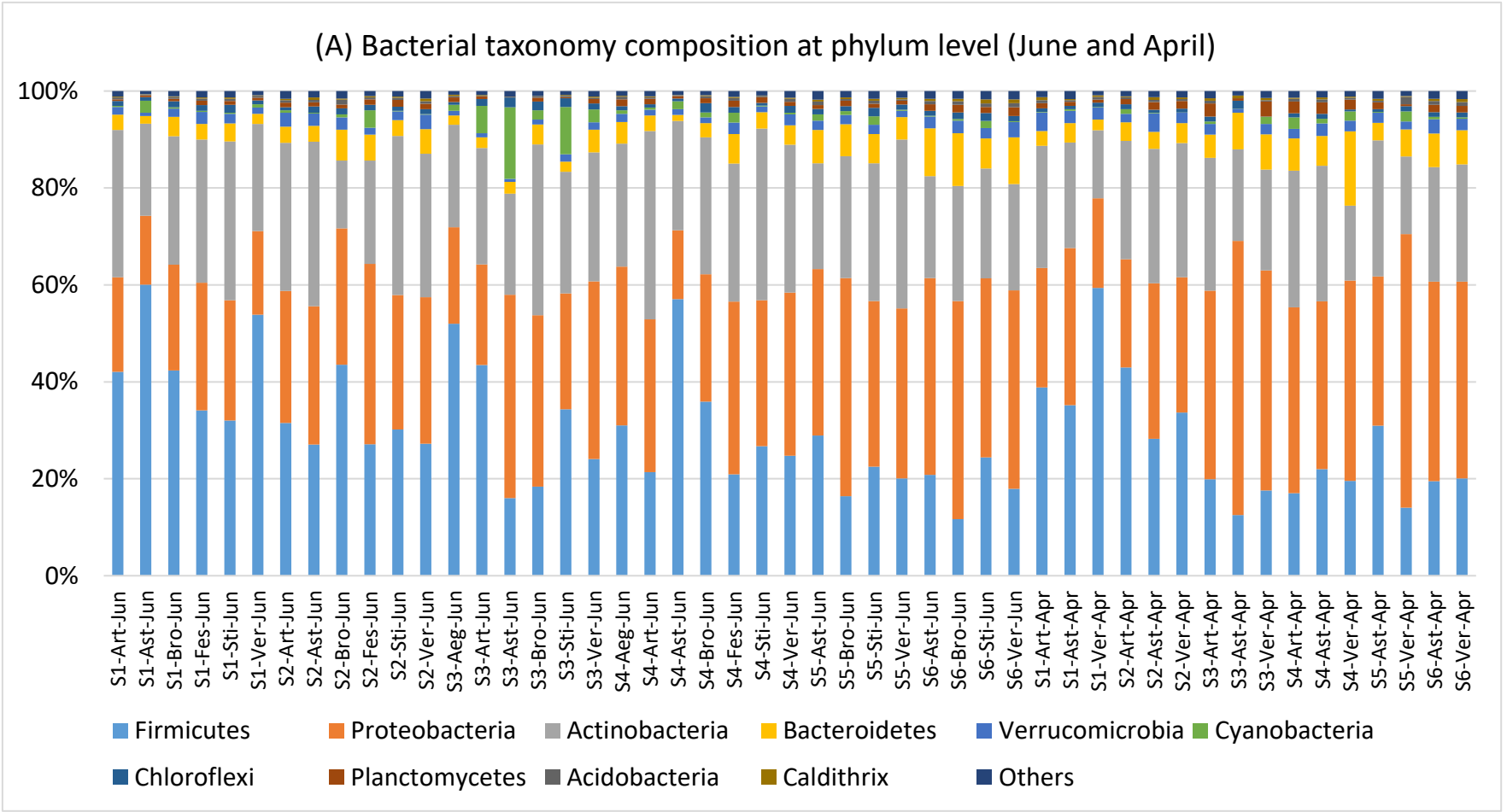
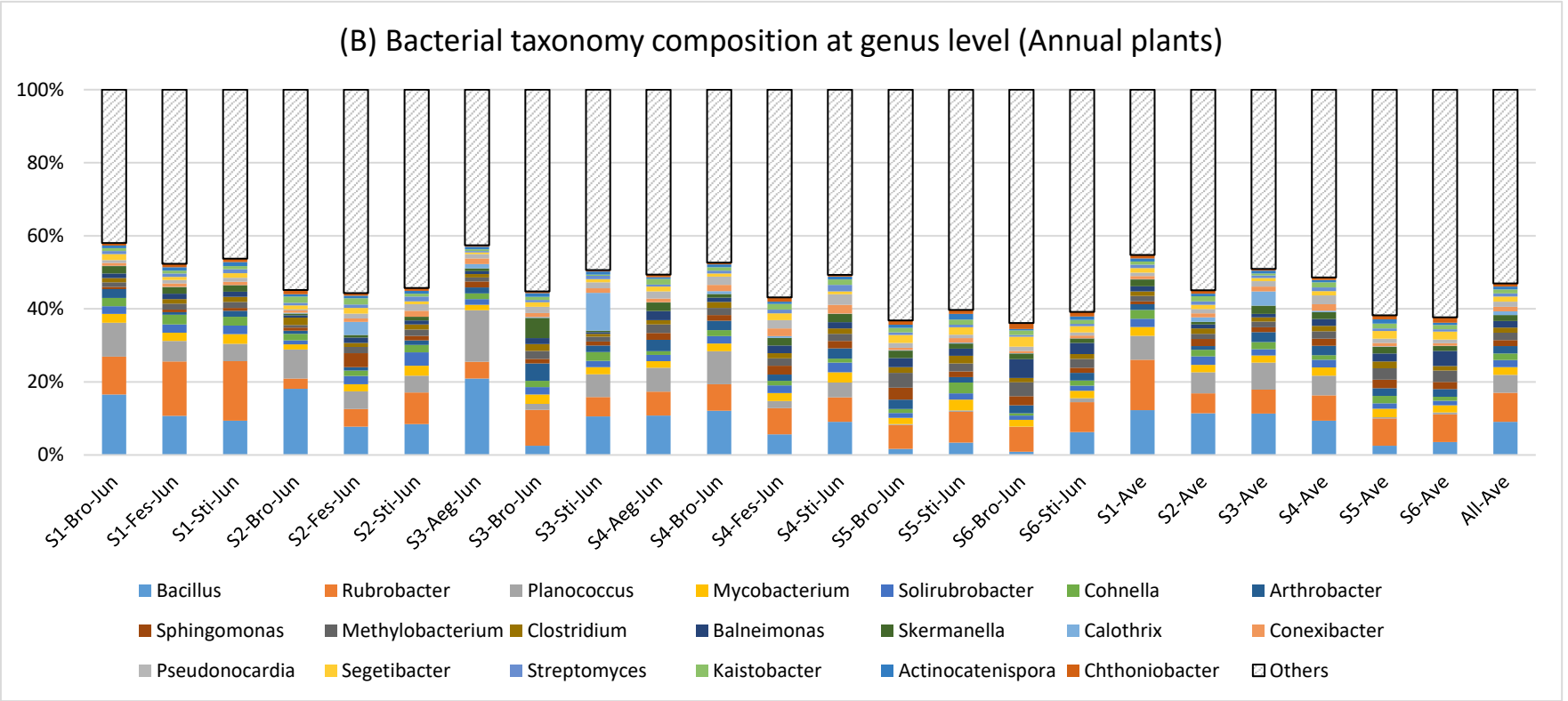


Fig. 2. Taxonomic compositions of AMF communities at the phylum level (A from the June and April sampling periods) and at the genus level (B from the June sampling and C from the April sampling).


(A) Bacterial taxonomy composition at phylum level (June and April)
100%
80%
60%
40%
20%
0%
S1-Art-Jun
S1-Ast-Jun
S1-Bro-Jun
S1-Fes-Jun
S1-Sti-Jun
S1-Ver-Jun
S2-Art-Jun
S2-Ast-Jun
S2-Bro-Jun
S2-Fes-Jun
S2-Sti-Jun
S2-Ver-Jun
S3-Aeg-Jun
S3-Art-Jun
S3-Ast-Jun
S3-Bro-Jun
S3-Sti-Jun
S3-Ver-Jun
S4-Aeg-Jun
S4-Art-Jun
S4-Ast-Jun
S4-Bro-Jun
S4-Fes-Jun
S4-Sti-Jun
S4-Ver-Jun
S5-Ast-Jun
S5-Bro-Jun
S5-Sti-Jun
S5-Ver-Jun
S6-Ast-Jun
S6-Bro-Jun
S6-Sti-Jun
S6-Ver-Jun
S1-Art-Apr
S1-Ast-Apr
S1-Ver-Apr
S2-Art-Apr
S2-Ast-Apr
S2-Ver-Apr
S3-Art-Apr
S3-Ast-Apr
S3-Ver-Apr
S4-Art-Apr
S4-Ast-Apr
S4-Ver-Apr
S5-Ast-Apr
S5-Ver-Apr
S6-Ast-Apr
S6-Ver-Apr
Firmicutes
Proteobacteria
Actinobacteria
Bacteroidetes
Verrucomicrobia
Cyanobacteria
Chloroflexi
Planctomycetes
Acidobacteria
Caldithrix
Others


(B) Bacterial taxonomy composition at genus level (Annual plants)
100%
80%
60%
40%
20%
0%
S1-Bro-Jun
S1-Fes-Jun
S1-Sti-Jun
S2-Bro-Jun
S2-Fes-Jun
S2-Sti-Jun
S3-Aeg-Jun
S3-Bro-Jun
S3-Sti-Jun
S4-Aeg-Jun
S4-Bro-Jun
S4-Fes-Jun
S4-Sti-Jun
S5-Bro-Jun
S5-Sti-Jun
S6-Bro-Jun
S6-Sti-Jun
S1-Ave
S2-Ave
S3-Ave
S4-Ave
S5-Ave
S6-Ave
All-Ave
Bacillus
Rubrobacter
Planococcus
Mycobacterium
Solirubrobacter
Cohnella
Arthrobacter
Sphingomonas
Methylobacterium
Clostridium
Balneimonas
Skermanella
Calothrix
Conexibacter
Pseudonocardia
Segetibacter
Streptomyces
Kaistobacter
Actinocatenispora
Chthoniobacter
Others

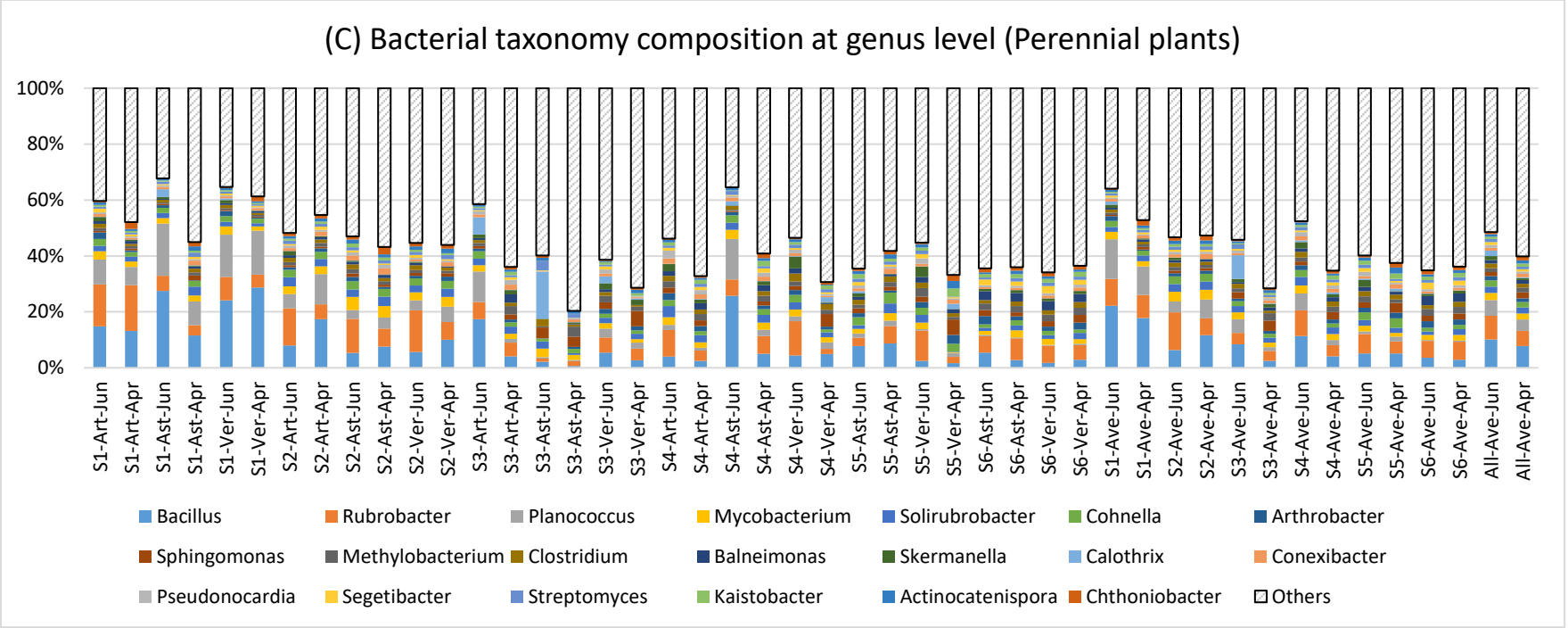
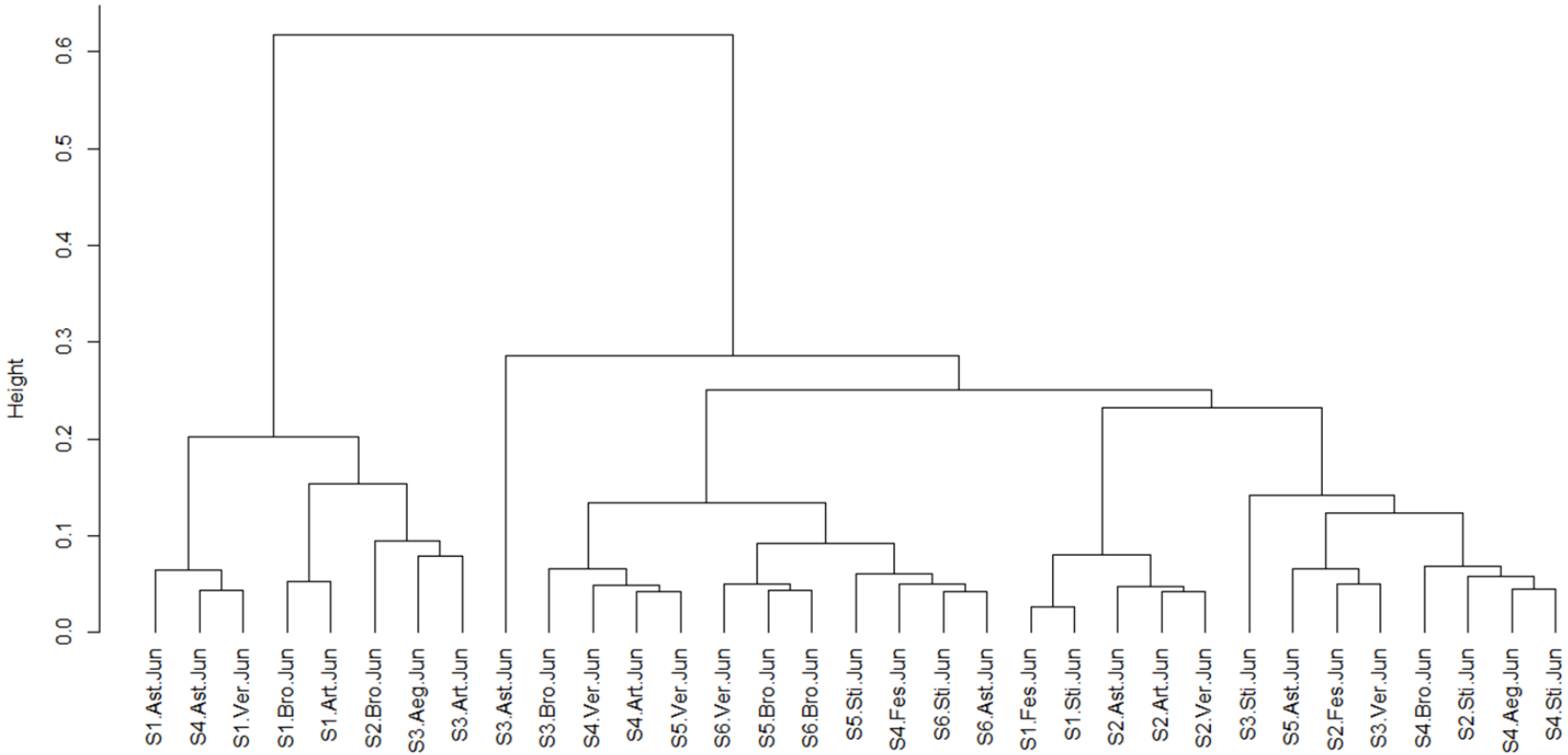


Fig. 3. Taxonomic compositions of the bacterial community at the phylum level (A from the June and April samplings) and at the genus level (B from the annual plants in the June sampling and C from the perennial plants in June and April samplings). The top 20 bacterial taxa are shown individually, and other taxa are compiled into “Others”.

(A)


Height
0.0
0.1
0.2
0.3
0.4
0.5
0.6
S1.Ast.Jun
S4.Ast.Jun
S1.Ver.Jun
S1.Bro.Jun
S1.Art.Jun
S2.Bro.Jun
S3.Aeg.Jun
S3.Art.Jun
S3.Ast.Jun
S3.Bro.Jun
S4.Ver.Jun
S4.Art.Jun
S5.Ver.Jun
S6.Ver.Jun
S5.Bro.Jun
S6.Bro.Jun
S5.Sti.Jun
S4.Fes.Jun
S6.Sti.Jun
S6.Ast.Jun
S1.Fes.Jun
S1.Sti.Jun
S2.Ast.Jun
S2.Art.Jun
S2.Ver.Jun
S3.Sti.Jun
S5.Ast.Jun
S2.Fes.Jun
S3.Ver.Jun
S4.Bro.Jun
S2.Sti.Jun
S4.Aeg.Jun
S4.Sti.Jun

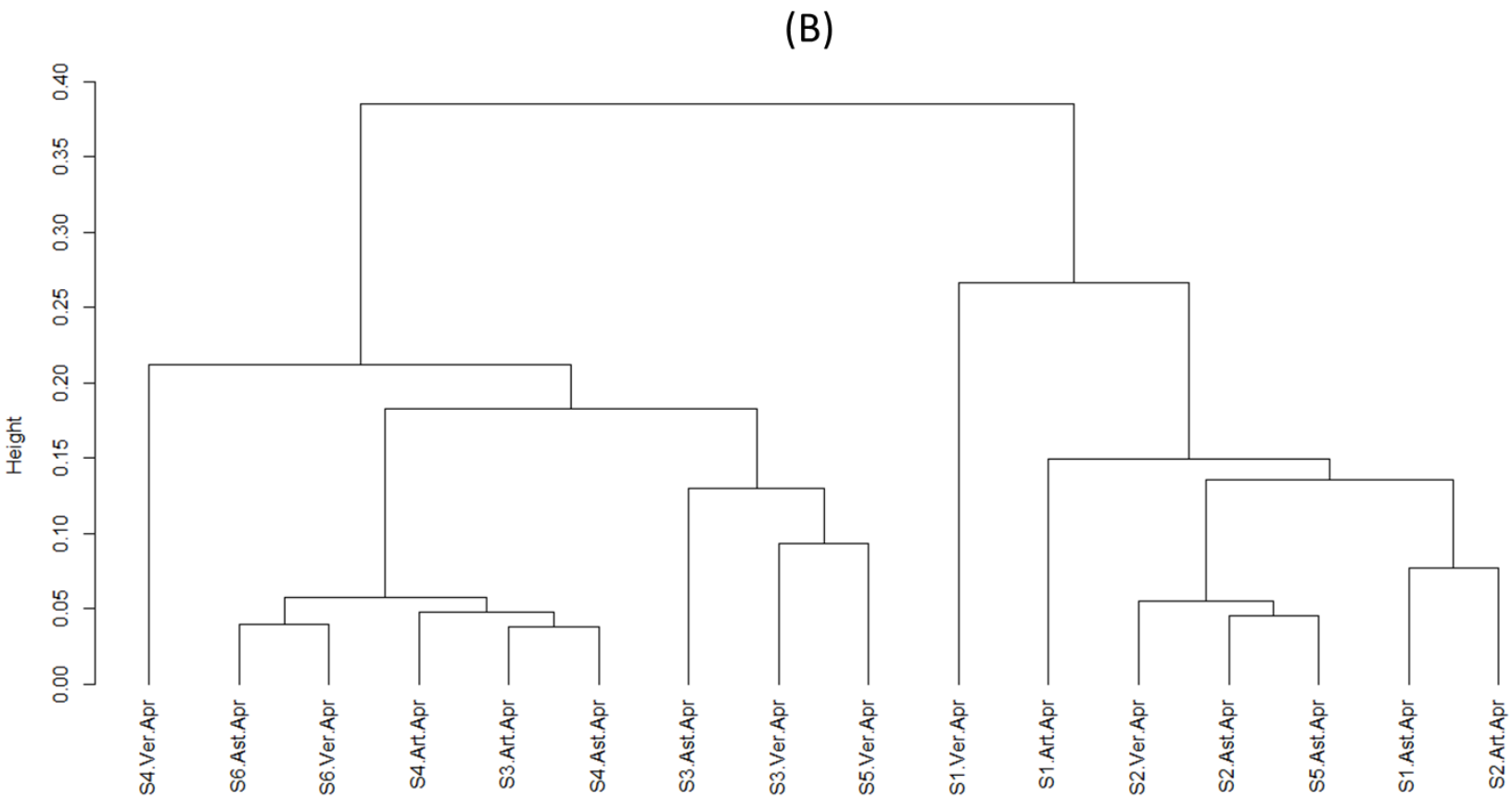
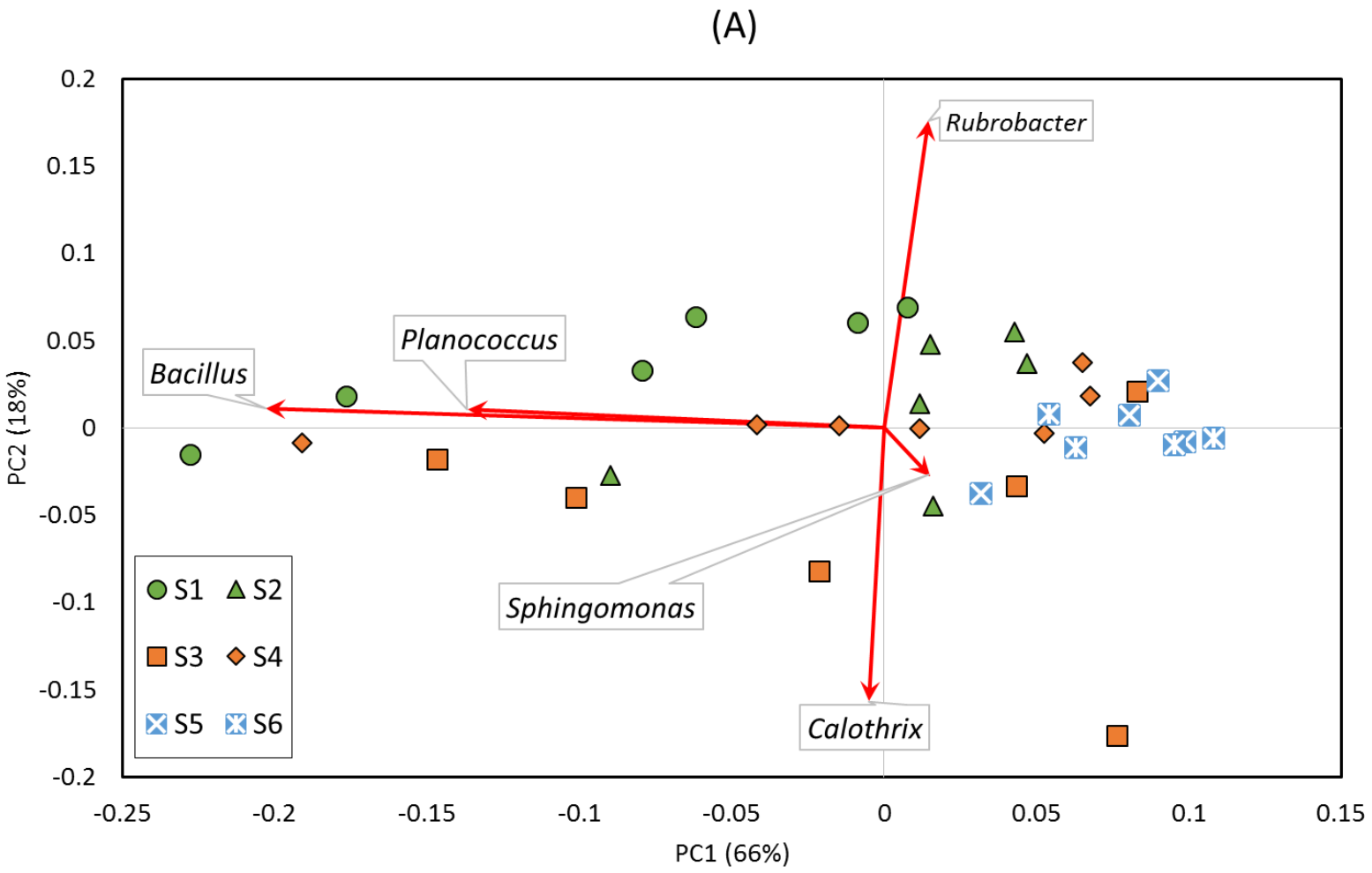
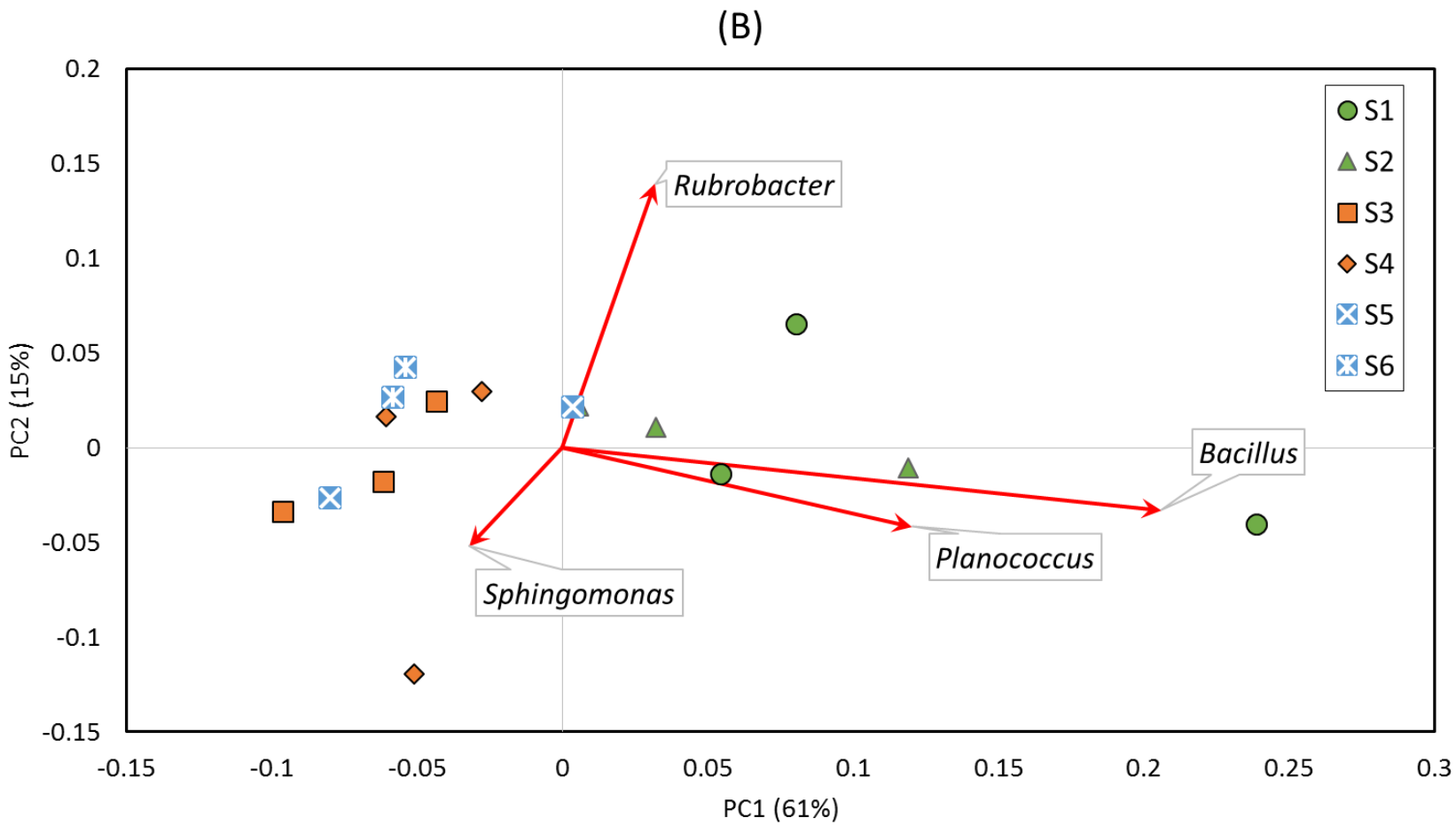


Fig. 4. Cluster analysis of the bacterial communities based on OTUs at 97% similarity level in June (A) and April (B).


(A)
0.2
0.15
0.1
0.05
0
-0.05
-0.1
-0.15
-0.2
PC2 (18%)
-0.25
-0.2
-0.15
-0.1
-0.05
0
0.05
0.1
0.15
PC1 (66%)
Rubrobacter
Planococcus
Bacillus
Sphingomonas
Calothrix
S1
S2
S3
S4
S5
S6


(B)
0.2
0.15
0.1
0.05
0
-0.05
-0.1
-0.15
PC2 (15%)
-0.15
-0.1
-0.05
0
0.05
0.1
0.15
0.2
0.25
0.3
PC1 (61%)
S1
S2
S3
S4
S5
S6
Rubrobacter
Bacillus
Planococcus
Sphingomonas

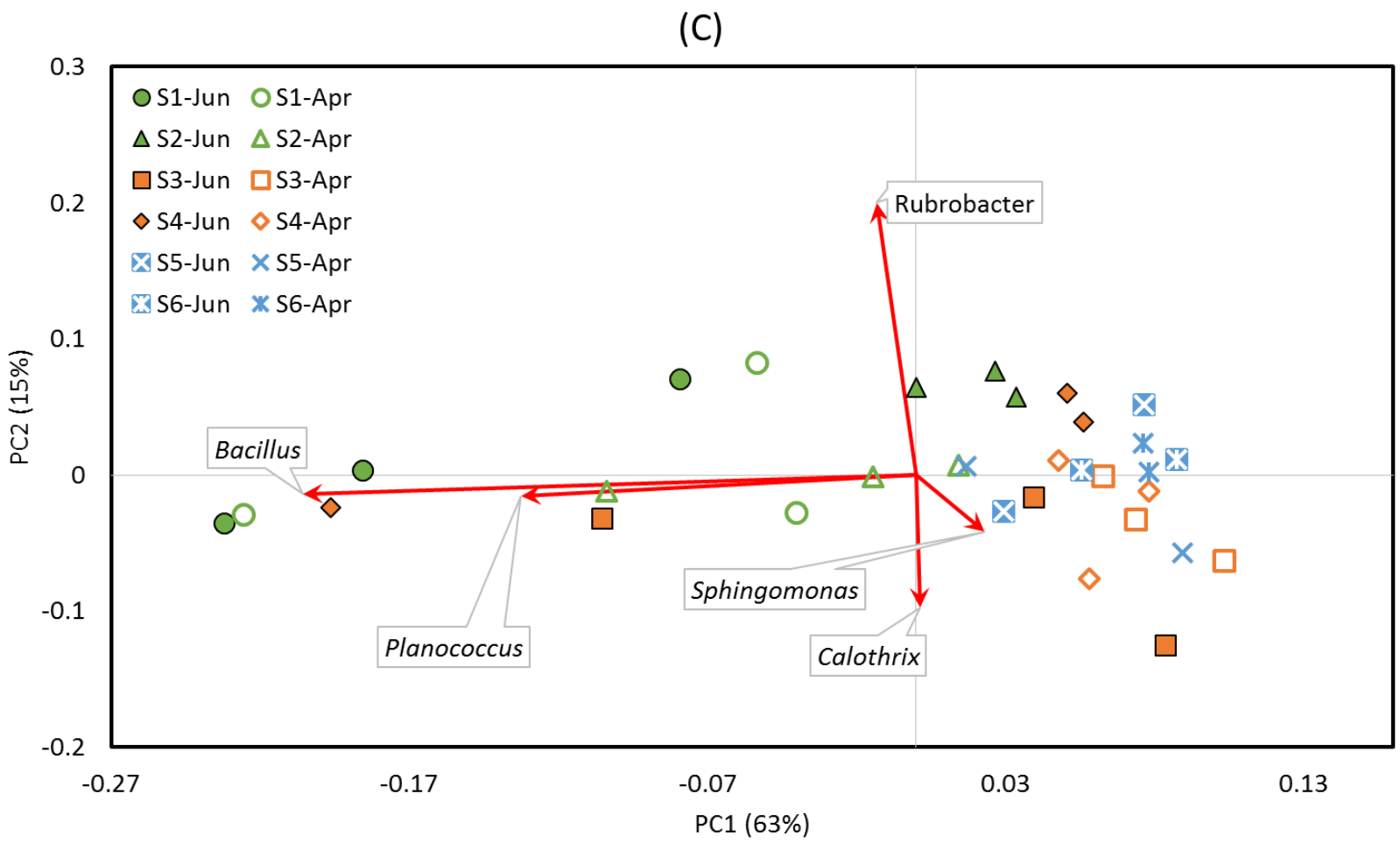

(C)
S1-Jun
S1-Apr
S2-Jun
S2-Apr
S3-Jun
S3-Apr
S4-Jun
S4-Apr
S5-Jun
S5-Apr
S6-Jun
S6-Apr
Rubrobacter
Bacillus
Sphingomonas
Planococcus
Calothrix
0.3
0.2
0.1
0
-0.1
-0.2
PC2 (15%)
-0.27
-0.17
-0.07
0.03
0.13
PC1 (63%)

Fig. 5. Principal component analysis (PCA) based on the proportion of OTUs at 97% similarity level. The percentages in the axis titles show the percentage of variation explained by the analysis. (A) PCA of bacterial community within the June sampling. (B) PCA of bacterial community within the April sampling. (C) PCA of bacterial community from perennial plants comparing between the June and April sampling. Circle, Site 1; triangle, Site 2; square, Site 3; diamond, Site 4; cross, Site 5; and, asterisk, Site 6. Soil qualities are represented by different colors as follows; green, undesertified; red, moderately sensitive; and blue, highly sensitive. The April sampling is shown as open symbols.

Table 1. Evaluation of desertification intensity using the MEDALUS Model.

	Site 1	Site 2	Site 3	Site 4	Site 5	Site 6
Geographic coordinate	N 40.0082, E 33.2169	N 40.0091, E 33.2177	N 39.9983, E 33.1919	N 39.9997, E 33.1956	N 39.9627, E 33.4628	N 39.9628, E 33.4650
Elevation (m)	1430	1420	1220	1230	820	830
Vegetation type	Perennial grassland	Perennial grassland	Perennial grassland	Perennial grassland	Perennial grassland	Perennial grassland
Plant cover	High (>40)	Low (10-40%)	Low (10-40%)	High (>40)	Low (10-40%)	Low (10-40%)
Soil Depth	Deep (>75 cm)	Very shallow (<15 cm)	Very shallow (<15 cm)	Shallow (15-30 cm)	Shallow (15-30 cm)	Very shallow (<15 cm)
Slope Gradient	Very gentle to flat (<6%)	Very step (18-35%)	Gentle (6-18%)	Gentle (6-18%)	Gentle (6-18%)	Gentle (6-18%)
Parent Material	Limestone	Limestone	Limestone	Limestone	Limestone	Limestone
Rock Fragments	Slightly stony to bare (<20%)	Stony (20-60%)	Stony (20-60%)	Slightly stony to bare (<20%)	Stony (20-60%)	Slightly stony to bare (<20%)
Mean annual rainfall	280-650 mm	280-650 mm	280-650 mm	280-650 mm	280-650 mm	280-650 mm
Slope aspect	Plain (<5%)	N	S	N	E	W
Aridity index	50-75 (BGI)	50-75 (BGI)	50-75 (BGI)	50-75 (BGI)	50-75 (BGI)	50-75 (BGI)

Land use intensity	Low	Low	Medium	Medium	High	High
Policy enforcement	Complete (>75%)	Complete (>75%)	Partial (25-50%)	Partial (25-50%)	Partial (25-50%)	Partial (25-50%)
ES Index to desertification	**Area with medium environmental sensitivity (Fragile)**	**Area with medium environmental sensitivity (Fragile)**	**Area with high environmental sensitivity (Critical)**	**Area with high environmental sensitivity (Critical)**	**Area with high environmental sensitivity (Critical)**	**Area with high environmental sensitivity (Critical)**
Vegetation quality	**Medium**	**Low**	**Low**	**Medium**	**Low**	**Low**
Soil quality	**Medium**	**Low**	**Low**	**Medium**	**Medium**	**Low**
Climate quality	**Medium**	**Medium**	**Medium**	**Medium**	**Medium**	**Medium**
Management quality	**Good**	**Good**	**Medium**	**Medium**	**Low**	**Low**

Table 2. Vegetation in the sampling sites in June 2015 where + indicates presence and − indicates absence. The plant genus was identified based on morphological features.

Genus	Site1	Site2	Site3	Site4	Site5	Site6
Astragalus	+	+	+	+	+	+
Verbascum	+	+	+	+	+	+
Artemisia	+	+	+	+	-	-
Festuca	+	+	-	+	-	-
Aegilops	-	-	+	+	-	-
Stipa	+	+	+	+	+	+
Bromus	+	+	+	+	+	+
Acantholimon	-	+	-	-	+	+
Achillea	+	-	-	+	-	-
Ajugo	-	-	+	-	+	-
Allium	-	-	+	+	-	-
Alyssum	+	+	+	-	+	-
Androsace	+	-	-	-	-	-
Anthemis	-	+	-	-	-	+
Arenaria	-	-	+	-	+	-
Asphodelus	-	-	-	-	+	-
Atraphaxis	-	-	-	-	+	-
Briza	-	+	-	+	-	-
Cardoria (Lepidium)	+	-	-	-	-	-
Carlina	-	-	+	-	-	-
Centaurea	+	+	+	-	+	+
Cirsium	+	+	+	+	-	-
Convolvulus	+	-	-	-	+	+
Coronilla	+	-	-	-	-	-
Crupina	+	-	+	+	-	-
Dianthus	-	-	-	-	+	+
Digitalis	-	+	-	-	-	-
Erodium	+	+	+	+	-	-

Eryngium	+	+	+	+	+	+
Erysimum	+	-	+	-	-	+
Euphorbia	-	+	+	+	+	+
Filago	-	-	-	-	+	-
Fumaria	+	-	-	-	-	-
Galium	-	+	-	+	-	-
Globularia	-	-	-	-	+	+
Helianthemum	-	-	-	-	+	-
Herniaria	-	-	+	-	-	-
Hyoscyamus	+	-	-	-	-	-
Juniperus	-	-	-	-	-	+
Lactuca	+	-	-	-	-	-
Marrubium	+	+	-	+	+	-
Minuartia	-	+	-	-	-	-
Morina	-	-	-	+	-	-
Myosotis	-	-	+	-	-	-
Nigella	-	-	-	-	-	+
Onobrychis	+	+	+	+	-	-
Onosma	-	-	-	-	+	+
Ornithogalum	+	+	-	-	-	-
Orobanche	-	+	-	-	-	-
Paracaryum	-	-	-	-	+	-
Paronychia	-	-	+	-	+	+
Phlomis	+	+	-	+	-	-
Poa	+	-	-	-	-	-
Potentilla	+	-	+	+	-	-
Pyrus	-	+	-	-	-	-
Reseda	+	-	-	-	-	-
Rosa	-	+	-	-	-	-
Salvia	-	-	-	-	+	+
Scabiosa	+	-	-	-	-	+
Scorzonera	-	+	-	+	+	+
Sedum	-	+	+	-	-	-
Senecio	+	+	+	+	+	-
Sideritis	-	+	+	+	+	+
Silene	+	-	-	-	-	+

Stachys	+	-	-	+	-	-
Teucrium	+	-	+	+	+	+
Thymus	+	+	+	+	+	-
Tragopogon	+	-	+	-	-	-
Trifolium	+	+	+	+	-	-
Veronica	+	-	-	+	-	-
Xanthium	-	-	-	-	-	+
Ziziphora	-	+	-	-	-	-

Table 3. List of selected annual and perennial plants for rhizosphere soil sampling at different sites where + indicates collected, and – indicates non-collection because of absence of the plant. The results are presented as June 2015 / April 2015.

	Site 1	Site 2	Site 3	Site 4	Site 5	Site 6
Astragalus sp.	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +
Verbascum sp.	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +
Artemisia sp.	+ / +	+ / +	+ / +	+ / +	– / –	– / –
Festuca sp.	+ / –	+ / –	– / –	+ / –	– / –	– / –
Aegilops sp.	– / –	– / –	+ / –	+ / –	– / –	– / –
Stipa sp.	+ / –	+ / –	+ / –	+ / –	+ / –	+ / –
Bromus sp.	+ / –	+ / –	+ / –	+ / –	+ / –	+ / –

IV-4. Discussion

AMF community

The AMF community compositions were completely different between the June and April sampling. In the June sample, AMF communities were mostly simple (Fig. 2B). Although this result indicated a great seasonal change in AMF communities associated with perennial plants in June to April, this result may also be caused by sequencing error and PCR bias. Kohout et al. (2015) investigated AMF communities in temperate grassland using 454-pyrosequencing with the nested PCR method, and reported diverse AMF communities. In addition, the trend of the target region for molecular identification of fungi using next-generation sequencing has shifted from ITS to a phylogenetically-informative marker gene, such as 18S rRNA gene (Yang et al., 2017; Liu et al., 2017). Therefore, further efforts should focus on the confirmation of the results for the AMF community by producing new backup data using different primer pairs, such as targeting 18S rRNA gene.

Bacterial community composition

In this study, most dominant three phyla were Firmicutes, Proteobacteria and Actinobacteria. Using 454-pyrosequencing technology, An et al. (2013) reported that the

phyla Firmicutes, Proteobacteria, Bacteroidetes and Actinobacteria were dominant bacteria in surface sand samples from the Gobi and Taklamakan deserts. Phylum Firmicutes was found to be dominant in different desert conditions, such as the Taklamakan (Puspitasari et al. 2016), Atacama (Piubeli et al., 2015) and Namib deserts (Prestel et al., 2008). Our results support these reports. The genus *Bacillus* (phylum Firmicutes) represented the major genus with 9% of the total sequences. *Bacillus* are well-known as biocontrol agents of plant disease (Emmert and Handelsman, 1999), and are also known to be spore-forming bacteria, and spore production aids in survival under suboptimal conditions (Nicholson 2002). However, it remains unclear whether these spore-forming bacteria were active and alive in the rhizosphere soil in the current study. Further study is required based on bacterial enzymatic and/or ribosomal RNA sequencing analysis to determine the activity of this bacterial community.

In this study, genus *Rubrobacter* (phylum Actinobacteria) was the second dominant group of the total sequences. *Rubrobacter* have been found in arid Australian soil (Holmes et al., 2000), Antarctic soil (Aislabie et al., 2006) and Atacama desert (Connon et al., 2007). Some species of *Rubrobacter* have been isolated from soil (Saul et al., 2006) and hot springs (Chen et al., 2004). Some *Rubrobacter* species have also been shown to be thermophilic and extremely gamma radiation resistant (Ferreira et al., 1999).

In addition, Laiz et al. (2009) isolated five *Rubrobacter* species from biodeteriorated monuments and reported that the isolates reproduced some biodeterioration processes under laboratory conditions. Barnard et al. (2013) showed that an increase of the relative abundance of Actinobacteria (mostly Rubrobacteridae class) with desiccation, and a decrease of the relative abundance of Acidobacteria. The results of the current study support the findings of these studies, as *Rubrobacter* was the major group of rhizobacterial community in our arid grassland, and may have some role in arid environment.

Members of the phylum Cyanobacteria (including genus Calothrix) are known to be photosynthetic taxa, which is important in oligotrophic arid environments as they play an important role in biogeochemical cycling processes such as carbon or nitrogen utilization (Chan et al. 2013). Cyanobacteria have been shown to tolerate desiccation for a prolonged period (Potts 1999). It has also been reported that Cyanobacteria dominate the microalgal community in biological soil crusts in hot deserts (Eldridge and Greene 1994). Although the bacterial community in this study was identified from rhizosphere soil, Cyanobacteria may also have some role in arid ecosystems. Further study is required to investigate the seasonal change of the relative abundance of Cyanobacteria focusing on annual rainfall.

IV-5. Conclusion

We recorded the taxonomic composition of root-symbiotic AMF and rhizobacterial communities in several sites under different degrees of desertification stress in Turkish perennial grassland using high throughput next-generation sequencing. The AMF community was well illustrated in the April sample; however, the June sampling showed substantially lower diversity, suggesting that additional investigation is required to confirm the accuracy of selected primers. Firmicutes (mostly *Bacillus*), Actinobacteria (mostly *Rubrobacter*) and Proteobacteria were the three most dominant bacterial phyla identified in this study. The bacterial communities of rhizosphere soils from relatively undesertified conditions were separated from bacterial assemblages from relatively severe conditions. *Bacillus* and *Rubrobacter* might be key genera that have different driving forces on the rhizobacterial community in arid grassland under desertification stress.

Chapter V

Soil bacterial community variations in organic rice farming revealed by 16S rDNA metagenome analysis

V-1. Introduction

Organic agriculture refers to a farming system that substantially reduces the use of chemical fertilizers, pesticides, and genetically modified organisms, which aims to minimize negative impacts to improve long-term soil sustainability (Gomiero et al. 2011). Nowadays, rice is also cultivated organically. Paddy soil is a group of anthropogenic soils with a long history of rice cultivation and is the most important farming system in Japan because rice is a staple food.

Understanding soil bacterial ecology is very important for organic agriculture, because the diversity and community composition influences the soil ecosystem, nutrient cycling, and agricultural production (van der Heijden et al. 2008). Lopes et al. (2011) compared the microbial diversity of organic and conventional paddy soils and revealed temporal variations in the organic field but not in the conventional field using PCR-DGGE targeting bacterial 16S rDNA. Rui et al. (2009) reported temporal changes in the bacterial community during the decomposition of plant residue in a soil incubation experiment under flood conditions using terminal restriction fragment length polymorphism (T-RFLP) analysis targeting bacterial 16S rDNA. Although these analyses produced useful information, the techniques employed for bacterial community analysis were insufficient for surveying the full extent of microbial diversity.

Next generation sequencing technologies have progressed rapidly in recent years and offer new methods to explore soil microbial communities at high coverage and throughput. However, only a few studies have examined soil bacterial communities in organic rice farming systems using the technology. Daquiado et al. (2016) performed pyrosequencing analysis of soil bacterial 16S rDNA in long-term inorganic and compost fertilization in paddy fields. They found that organic fertilizer amendment activated diverse groups of Gram positive microorganisms and Rhizobiales, which aid nutrient cycling, were influenced by compost application.

In this study, we investigated bacterial communities in organic and conventional paddy fields by 16S rDNA amplicon sequence analysis using a next generation sequencer. The objective of the study was 1) to elucidate changes in soil bacterial communities in paddy field under organic management and 2) to understand the effects of amount of tillage and photosynthetic bacterial inoculation on the bacterial communities.

V-2. Materials and Methods

Experimental field

Experimental plots were established in two adjacent rice fields located in Matsumoto, Nagano, Japan (N 36.2150, E137.8709) in 2014. Soil classification of the rice fields was gravelly gray lowland soil. One of the rice fields had been maintained under conventional management and the other under organic management for 6 years. The soil chemical properties of the experimental fields are shown in Table 1.

For the conventional field, chemical fertilizers were incorporated at 40 kg N ha^{-1}, 96 kg P_2O_5 ha^{-1}, and 56 kg K_2O ha^{-1} on 13th May, 2014, followed by submerging and puddling. Rice seedlings were transplanted on 19th and 20th May. Topdressing of 40 kg-N ha^{-1} and 1.2 kg-K_2O ha^{-1} was carried out on 26th July. Agrochemical treatments were performed as follows: benfuracarb at transplanting, butachlor and pentoxazone on 30th May, cyhalofop butyl, simetryn, benfuresate, and MCPB in late June, cyhalofop butyl and bentazon in early July, and etofenprox and tricyclazole on 22nd July. Mid-summer drainage was carried out from 3rd July to 16th July.

The organic field was tilled on 9th May and 300 kg ha^{-1} rice bran and 400 kg ha^{-1} rapeseed oil cake were incorporated. Half of the field was also tilled in autumn the previous year (23rd October, 2013). After submerging and puddling, the rice seedlings

were transplanted on 23rd May. Bokashi fertilizer made mainly from rice bran and rapeseed oil cake, was topdressed at 100 kg ha^{-1} on 26th May. Mechanical weed control was performed four times in June. Four different treatment plots (each 3.4 m^2) were established in the organic field by the combination of different numbers of tillage (single tillage or double tillage) and photosynthetic bacterial inoculation (*Rhodopseudomonas palustris*) under a 22 factorial design without replication. In the inoculated plots, *R. palustris* cultures (1.6×10^9 MPN mL^{-1}) were inoculated into nursery boxes of rice seedlings at 160 L ha^{-1} just before transplanting. Mid-summer drainage was performed from 21st July to 1st August.

Sampling description and bacterial community analysis

Soil samples were collected at the interhall with a hand shovel just after transplanting (24th May), before the mid-summer drainage (21st June), and at the rice grain-filling stage (21st August). The soil samples were kept at 4°C during transportation. After arriving at the laboratory, the soil samples were homogenized together to produce a single plot sample and a proportion of each sample was stored at -80°C until soil DNA extraction.

Soil DNA was obtained from 0.5 g of homogenized soil samples using ISOIL for

Beads Beating (Nippon Gene Co., Ltd., Tokyo, Japan), according to manufacturer's instruction. Partial bacterial 16S rDNA (V4 region) was amplified using the bacterial primers 515F/806R with the linker sequences for Nextera primers (Illumina Inc., CA, USA). The PCR products were purified using a High Pure PCR Product Purification Kit (Roche, Basel, Switzerland), and PCR barcode indexing was then performed with Nextera primers. The amplicons were purified with a QIAquick® Gel Extraction Kit (Qiagen, Venlo, Netherlands) according to the manufacturer's instructions. The amplicons were paired-end sequenced on an Illumina MiSeq platform (Illumina) at a read length of 2 × 300 bp. Sequencing was performed by Fasmac Co., Ltd. (Kanagawa, Japan).

Data processing

The sequence data were processed and analyzed in QIIME ver. 1.8.0 (Caporaso et al. 2010). The sequence read numbers were normalized to 20, 000 reads per sample by random subsampling. The sequences were paired-end joined and filtered through quality check and chimera check using USEARCH61 (Edgar 2010). The processed sequences were clustered into OTUs using the open reference OTU picking algorithm at 97% similarity with the Greengenes 16S rRNA gene database (released in August 2013).

To compare bacterial communities among the samples, principal coordinate

analysis (PCoA) was performed in QIIME based on UniFrac (Lozupone and Knight, 2005). Cluster analysis was performed based on the proportions of bacterial community at the genus level in Kypolot version 2.0 beta 15.

V-3. Results and Discussion

In total, 743 OTUs were obtained at the genus level in this study and 97% of them had <1% abundance ratio. On average, approximately 500 OTUs were obtained from each plot.

The proportions of the bacterial community at the phylum level are shown in Fig. 1. In all treatments, the proportion of each phylum was usually as follows; approximately 25–30% Proteobacteria, 20% Chloroflexi, 15% Acidobacteria, and 10% Actinobacteria (Fig. 1). At the class level, the dominant classes were as follows: approximately 10–17% Anaerolineae, 10–13% Alphaproteobacteria, 7–11% Betaproteobacteria, and 5–8% Acidobacteria. The proportion of OTUs related to the photosynthetic bacterial inoculum (*Bradyrhizobiaceae* sp.) was approximately 0.3%, and no significant difference was observed between the inoculated and non-inoculated fields both at mid-summer drainage and in the grain-filling period ($P>0.05$, two-way ANOVA).

PCoA based on the frequency of each OTU revealed differences in bacterial communities between conventional and organic farming (Fig. 2). Photosynthetic bacterial inoculation and tillage management had no effect on the bacterial community structure. At transplanting, the bacterial community compositions were mostly similar among the plots despite the differences in field management. After transplanting, the bacterial

communities in the organic plots were clearly differentiated from those in the conventional plot (Fig. 3). There were no significant differences among the treatments (Table 2), meaning that the differences in the bacterial community compositions did not influence the rice production yields in the experimental fields in this study.

Organic amendment impacts the soil bacterial community and changes the community structure in both upland (Chávez-Romeroa et al. 2016) and paddy conditions (Dapuiado et al. 2016). Although our results revealed a similar trend to these studies, organic management had little effect on the bacterial community structure at transplanting in this study. It has been reported that soil bacterial community structure and diversity in paddy soil change temporally during the incubation period (Noll et al. 2005). Our results also revealed a temporal change in bacterial community under organic management.

In addition to the changes in bacterial community structures between organic and conventional management, temporal changes were observed in the bacterial community under organic management (Fig. 3). Rui et al. (2009) reported that the bacterial community structure shifted during plant residue decomposition, and this succession was probably related to resource availability. Our results indicated that organic management affected the bacterial community during the organic carbon decomposition process as a result of the development of soil reduction. Another possibility is the effect of the

additional fertilizer during the cultivation. Doi et al. (2011) reported that rhizosphere bacterial community structures under compost and rice bran treatments clearly differentiated from those of the control. Seasonal changes in the bacterial community structures were also observed after chemical fertilizers had been topdressed during cultivation.

Inoculation of commercial microbial materials containing photosynthetic bacterial strains is sometimes conducted in organic rice farming, since organic farmers generally recognize that photosynthetic bacteria are beneficial in organic rice production. We therefore examined inoculation of *R. palustris* in this study. However, the proportion of related OTUs and the bacterial community structures in soil were relatively similar between inoculated and non-inoculated fields. Harada et al. (2005) reported that *R. palustris* inoculation did not significantly effect a phototrophic purple bacteria population when rice straw was applied, although inoculation could increase rice grain yield. It has also been reported that the inoculation of plant growth-promoting bacteria did not have a significant impact on culturable microbial communities (de Salamone et al. 2012). Our results agree with these studies, but further investigation is required to evaluate the detailed impact of inoculation, considering inoculation methods may influence on the fate of the inoculum.

Although tillage management can affect the bacterial community in cropland (Navarro-Noya et al. 2013), the effect of the amount of tillage has not been reported in paddy field conditions. Our results suggest that the amount of tillage had little effect on the bacterial community.

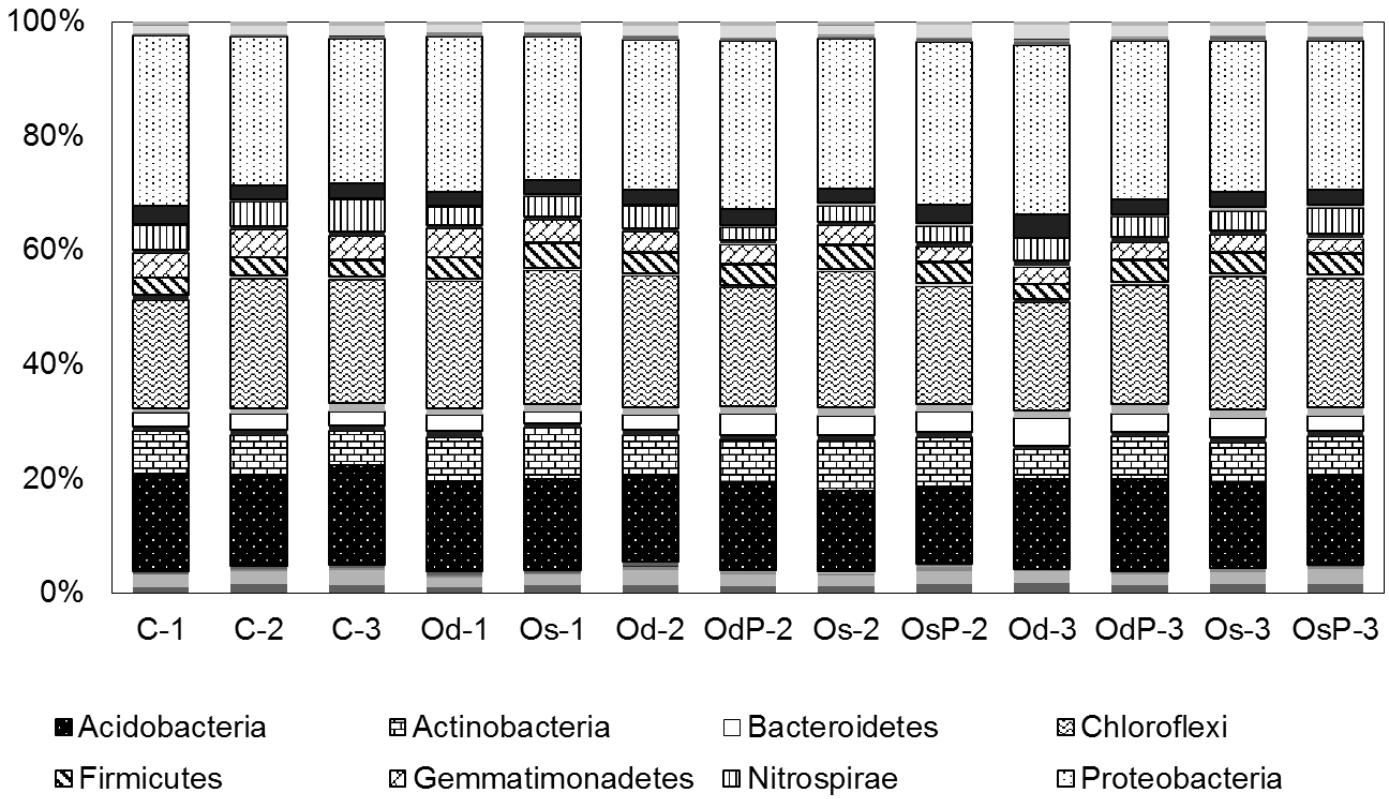


Fig. 1. Frequency of bacterial phylum detected in this study. The phyla with > 0.03% relative abundance are shown in the legends. C, conventional; Os, organic with single tillage; Od, organic with double tillage; P, photosynthetic bacteria inoculation. Sampling period was shown as follows; 1, just after transplanting; 2, before mid-summer drainage; 3, grain-filling period.

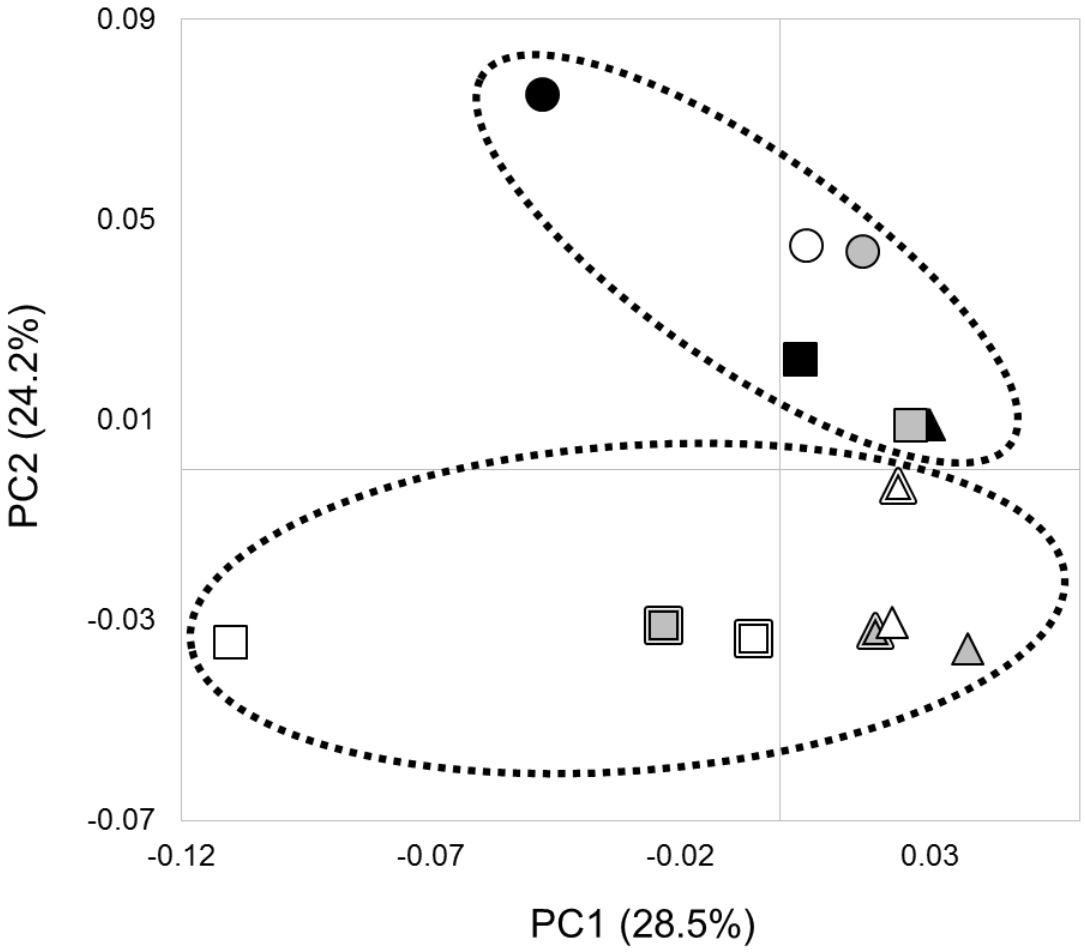


Fig. 2. Principal coordinate analysis (PCoA) based on the proportion of OTUs at 97% similarity level. The percentages in the axes show the percentage of variation explained by the analysis. Circle, conventional; triangle, organic with single tillage; square, organic with double tillage. Photosynthetic bacteria inoculation plots are shown as a double line. Sampling period is represented by different colors as follows; black, just after transplanting; gray, before mid-summer drainage; white, grain-filling period.

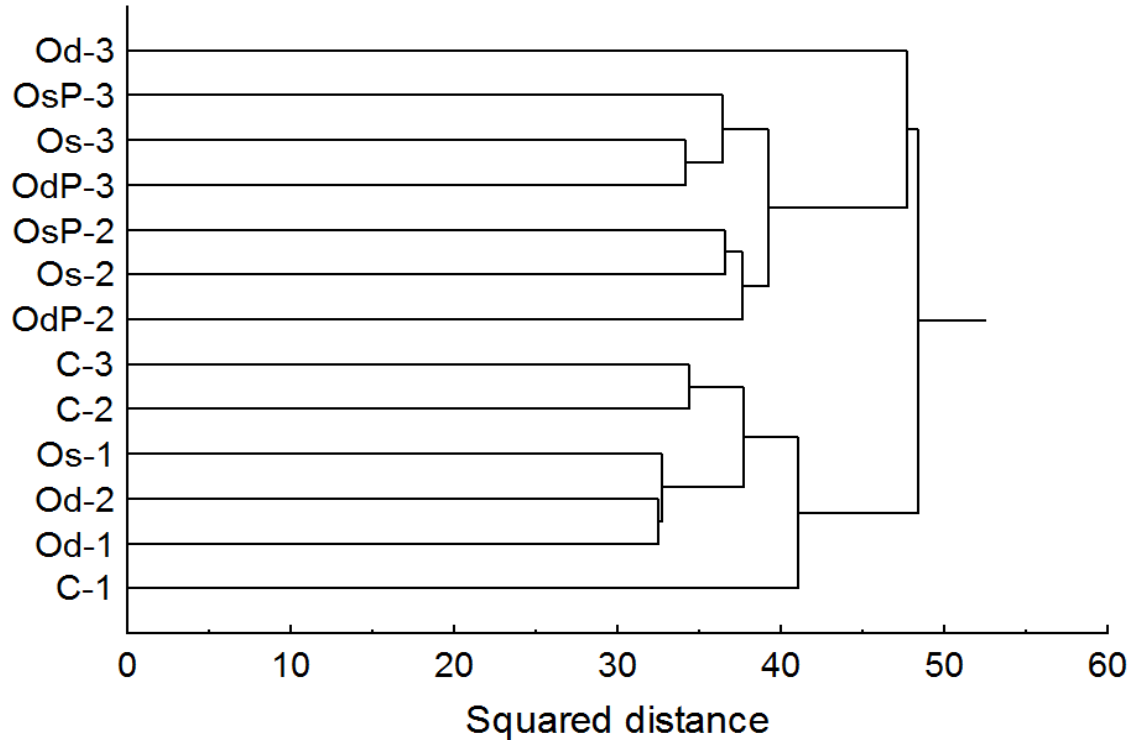


Fig. 3. Cluster analysis of the bacterial communities based on OTUs at 97% similarity level. C, conventional; Os, organic with single tillage; Od, organic with double tillage; P, photosynthetic bacteria inoculation. Sampling periods are shown as follows; 1, just after transplanting; 2, before mid-summer drainage; 3, grain-filling period.

Table 1. Soil chemical properties in the experimental fields

Rice field	pH (H_2O)	EC (mS cm^{-2})	T-C (g-C kg^{-1})	T-N (g-N kg^{-1})	NH_4-N (mg kg^{-1})	NO_3-N (mg kg^{-1})	P_2O_5 (mg kg^{-1})	K_2O (mg kg^{-1})	CaO (mg kg^{-1})	MgO (mg kg^{-1})	CEC (cmol(+) kg^{-1})	Fe_2O_3 (g kg^{-1})
Conventional	5.53	0.03	26.1	2.5	7.3	2.9	449	141	1200	157	7.71	5.43
Organic	5.55	0.03	29.5	2.8	6.7	3.3	396	151	1090	126	8.67	7.80

Table 2. Rice yields in the experimental plots.

Plot	Number of panicles (m^{-2})	Straw yield (kg ha^{-1})	Grain yield (kg ha^{-1})	Brown rice yield (kg ha^{-1})	1000-grain weight (g)
Conventional (C)	360	5230	5945	4663	21.8
Organic					
Double tillage (Od)	417	5448	5653	4297	21.4
Double tillage +PSB (OdP)	422	5618	5832	4564	21.3
Single tillage (Os)	415	5409	5947	4635	21.4
Single tillage +PSB (OsP)	391	4746	4870	3792	21.2

+PSB; photosynthetic bacterial inoculation.

V-4. Conclusion

In conclusion, organic management mainly affected the bacterial community structures after transplanting. During the rice growth period, gradual changes were observed in the bacterial community and the bacterial community under organic management differed from those under conventional management, probably as a result of the decomposition process of organic fertilizers. On the other hand, amount of tillage and photosynthetic bacterial inoculation had little effect on bacterial communities. Our study contributes to understanding the response of bacterial communities to organic management in paddy fields.

Chapter VI

Conclusion

VI-1. Conclusion of this study

To improve our understanding of soil microbial ecology, we carried out molecular microbial studies in four different environments. A summary of these studies is as follows:

Chapter II: This research showed the first DNA sequence data of AMF from Turkey, half of which appeared to have no close similarity (based on a 97% similarity level) to the sequence accessions in the public databases. The AMF communities of the soils from the East Black Sea region, with a distinctively lower pH than the other soils, were separate from all the other AMF assemblages. There was no significant difference in AMF community compositions between the Mediterranean and Central Anatolian samples.

Chapter III: In this study, different fertilization practices affected several ecological indices for the AMF communities. The AMF community richness under the no-fertilization treatment and plant compost treatment was higher than those that were amended with farmyard manure or chemical fertilizers. Although different organic amendments supported relatively similar AMF communities, plant compost induced higher AMF richness than farmyard manure fertilization.

Chapter IV: The AMF community in degraded soil was well illustrated in the April sample; however, the June sampling showed substantially lower diversity, suggesting that additional investigation is required to confirm the accuracy of selected

primers. The bacterial communities of rhizosphere soils from undesertified conditions were separate from bacterial assemblages from relatively severe conditions. *Bacillus* and *Rubrobacter* may be the key genera in arid grassland under desertification stress.

Chapter V: In paddy field soil, organic management mainly affected the bacterial community structures after transplanting. During the rice growth period, gradual changes were observed in the bacterial community. Moreover, the bacterial community under organic management differed from that under conventional management, probably as a result of the decomposition process of organic fertilizers. Conversely, the amount of tillage and photosynthetic bacterial inoculation had little effect on bacterial communities.

VI-2. Future research

Using a next-generation sequencing system, we determined a huge number of soil microbial sequences and elucidated soil microbial community compositions (Chapters IV and V). Compared with other classical methods (such as clone library, DGGE and T-RFLP), next-generation sequencing strongly enhances our ability to illustrate soil microbial community from environmental DNA. However, classical ecological methods such as alpha and beta diversities may not be sufficient for maximizing the potential of such a huge amount of biological information. Therefore, a new approach for processing microbial community information is required. Network analysis could be a good solution

for processing data from next-generation sequencing. Network analysis is a relatively new approach to explore the mathematical, statistical and structural properties of a set of items and the connections between them (Newman 2003). The concept of network analysis has been adapted to studies in several research areas such as humanities (Moody 2001), metabolic pathways (Guimera and Amaral 2005) and food-webs (Krause et al. 2003). However, with a few notable exceptions (for example, Barberan et al. 2012 and Toju et al. 2015), this concept has not been widely applied for soil microbial ecology.

Once we have a technique that can elucidate soil microbial community in depth, the next goal will be to illustrate the interaction of each taxon with other soil microorganisms, the plants providing the rhizosphere, soil physicochemical properties, agricultural management and climate change. Further research should be carried out to develop a new effective ecological approach for understanding the interactions between soil microorganisms and environmental factors.

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Appendix

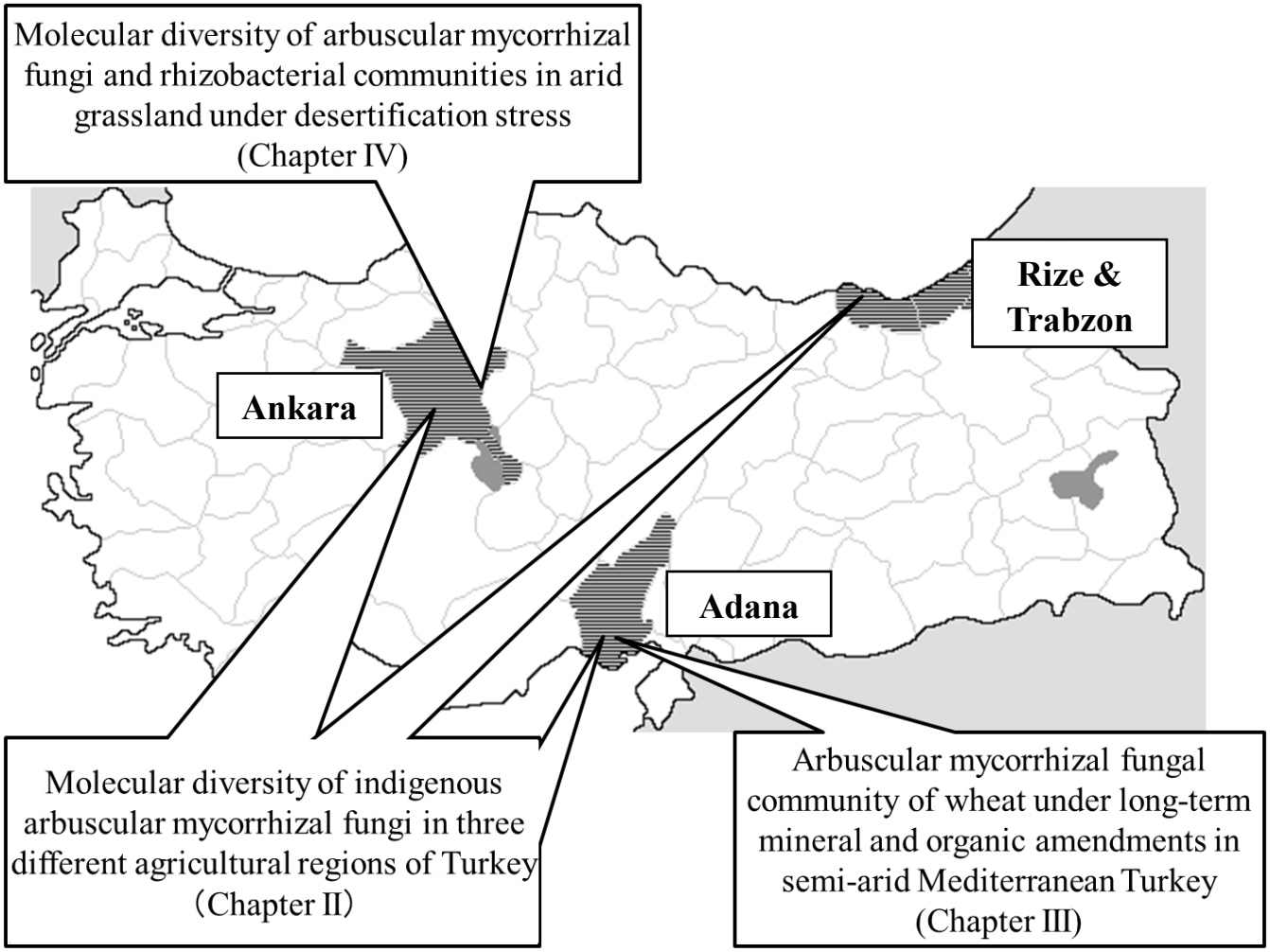

Fig. Study sites in Turkey in this thesis.

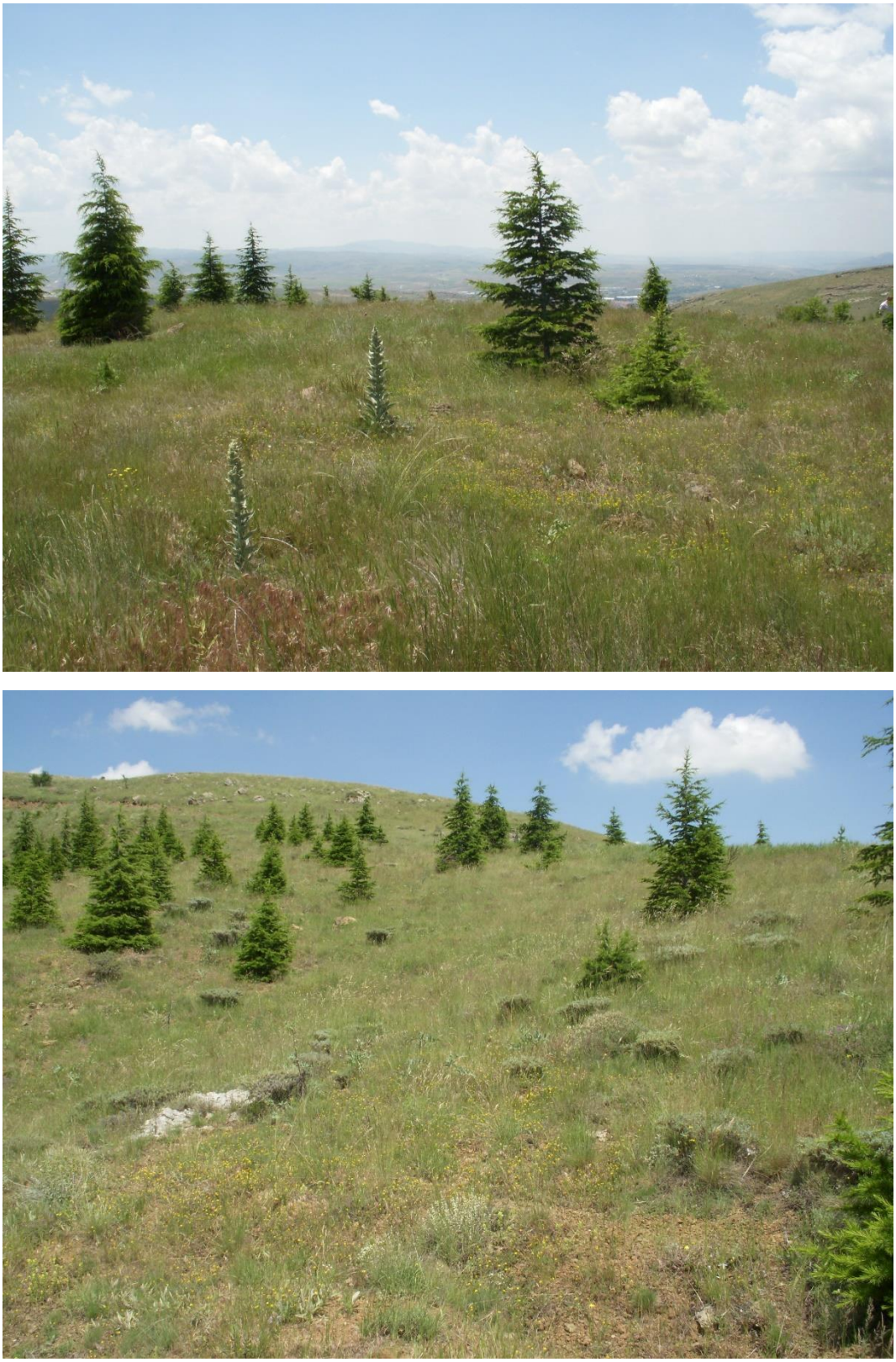

Fig. Photographs of study site in Chapter IV. Site 1 and Site 2 (June 2015).

Fig. Photographs of study site in Chapter IV. Site 3 and Site 4 (June 2015).

Fig. Photographs of study site in Chapter IV. Site 5 and Site 6 (June 2015).

Fig. Photographs of study site in Chapter IV. Site 1 and Site 2 (April 2016).

Fig. Photographs of study site in Chapter IV. Site 3 and Site 4 (April 2016).

Fig. Photographs of study site in Chapter IV. Site 5 and Site 6 (April 2016).

Fig. *Verbascum lasianthum* Boiss. ex Bentham. (Chapter IV)

Fig. *Astragalus microcephalus* Willd (Chapter IV)

Fig. *Artemisia spicigera* C. Koch (Chapter IV)

Fig. *Stipa ehrenbergiana* Trin. & Rupr. (Chapter IV)

Fig. *Aegilops biuncialis* Vis. (Chapter IV)

Fig. *Bromus sterilis* L. (Chapter IV)

Fig. *Festuca valesiaca* Schleicher ex Gaudin (Chapter IV)

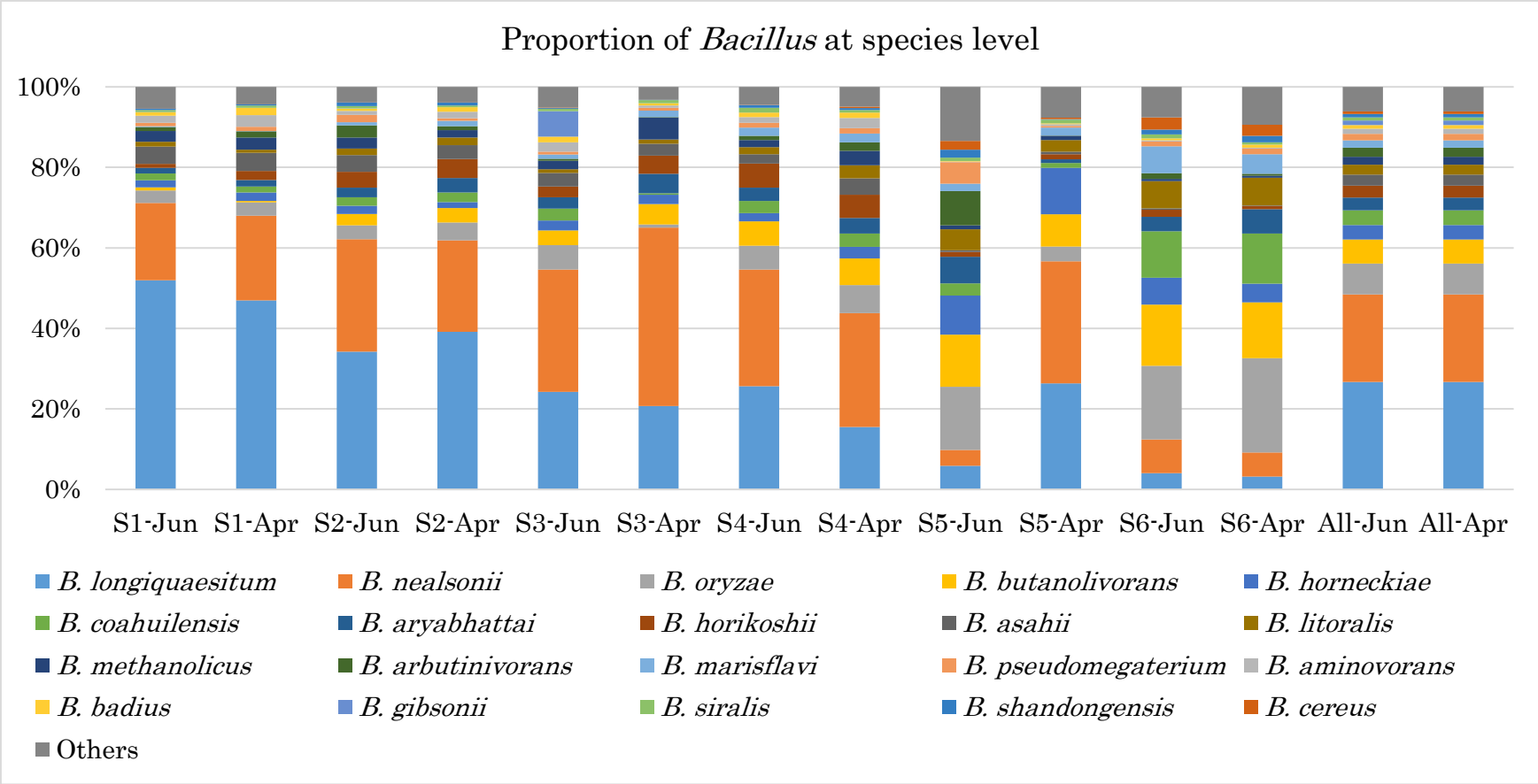

Fig. Taxonomic compositions of *Bacillus* at species level at sites from undesertified (S1 and 2), moderately sensitive (S3 and 4), highly sensitive (S5 and S6). The top 20 *Bacillus* are shown individually, and others are compiled into “Others” (Chapter IV).

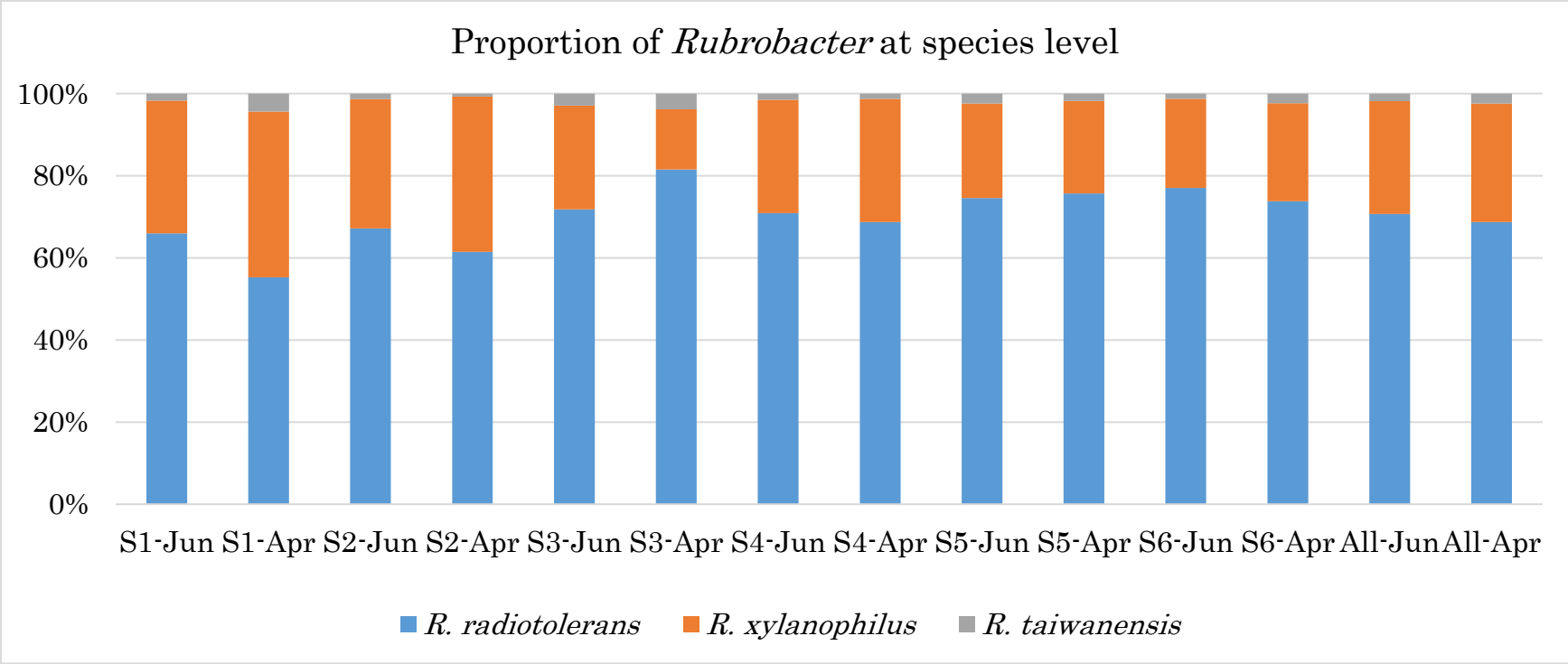

Fig. Taxonomic compositions of *Rubrobacter* at species level at sites from undesertified (S1 and 2), moderately sensitive (S3 and 4), highly sensitive (S5 and S6) (Chapter IV).