Spatial and Temporal Variations in Arbuscular Mycorrhizal Fungal Community Throughout the Plant Root System of *Avena barbata*

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ABSTRACT

The symbiosis relationship between arbuscular mycorrhizal fungi and plant roots are complex and important for plant growth in nutrient exchange between the fungi and the plant host. The rhizosphere is the area where soil is directly in contact with the roots and where the symbiotic interface begins and continues to persist throughout AMF colonization in the roots. I analyzed the AMF species richness and diversity in the rhizosphere through different root sections in the root system across different plant life stages and ages of roots from three timepoints. Root systems were divided in three root sections and compared in their AMF species diversity and relative abundance to explore the how time and space may impact AMF colonization in different parts of the roots. DNA sequencing method was utilized to identify AMF diversity in the rhizosphere at various root sections. After attempting to use microscopy to acquire AMF percent colonization in roots, I found that roots in field soil are colonized by many fungi and it is difficult to count and identify all the types of fungi in the roots. Since there is an entire fungal community in the roots in addition to the presence of AMF, I will use molecular methods to identify fungal species in these roots to complete the larger picture of the entire fungal community that are in the rhizosphere. This study hopes to contribute to the greater understanding of temporal variations in AMF-plant interactions.

KEYWORDS

Glomeromycota, exudates, AMF species diversity, rhizosphere, plant-fungal interactions, temporal variations

INTRODUCTION

Arbuscular mycorrhizal fungi (AMF) are a type of endomycorrhizal fungi that form symbiotic relationships with over 80% of all terrestrial plants by penetrating their hyphae into the living root cell lumen to form arbuscules (Bonfante & Desiro 2015). AMF transport nutrients from the soil to the plant while obtaining carbon source from the plant (Bonfante & Desiro 2015). AMF is highly active in the rhizosphere, which is an important region for carbon cycling and plays a prominent role in the global terrestrial carbon cycle (Treseder & Holden 2014). The species of AMF colonization and the impacts they have on plants depend largely on management intensity and plant species (Dickie et al. 2015; Helgason et al. 1998). Some farmland has a dominant AMF species that made up about 92% of AMF found in the roots of plants compared to a woodland that had a greater diversity of AMF species (Helgason et al. 1998). Since AMF have differential functions, the low diversity of AMF community in crop fields will likely to have limits in functionality, in contrast to woodlands, that negatively affect plants and the ecosystem due to fertilizers used and plowing (Helgason et al. 1998). AMF positively affect crop and soil health, by increasing pant drought tolerance and nutrient uptake, stabilizing soil, and decreasing N leaching (Augé 2001; Augé et al. 2015; Ruiz-Lozano & Azcon 1995; Kaschuk et al. 2009; Ruiz-Lozano 2003; Wu &Xia 2006; Pocel & Ruiz-Lozano 2003; Singh et al. 2013, Paterson & Sim 1999, Cavagnaro et al. 2015). Understanding below round interactions is crucial to maximize the benefit of AMF to agriculture.

Given the importance of AMF, understanding the interactions at the symbiotic interface are critical. Rhizosphere is the region where soil is in direct contact with roots and it is an area of highly active plant-fungal interactions. The symbiotic interface between the plant and AMF is made from AMF hyphae penetrating the living root cells to form arbuscules for nutrient exchange (Bonfante & Genre 2010). The AMF-plant relationship begins when the AMF recognizes the plant exudates, leading hyphae to grow and reach the roots. Once the fungus reaches the root surface, hyphopodia differentiates, this is the structure that attaches to the cell wall and generates the first structure that enters the root (Bonfante & Desiro 2015). When the hyphae first penetrate the plant cell, the cell will “consume” the hyphae to create a barrier between the fungus and the cytoplasm of the root cells (Bonfante & Desiro 2015). The plant cortical cells are heavily colonized by the hyphae and then the inner cortical cells are colonized

where the branching hyphae will become arbuscules in two to three days (Bonfante & Desiro 2015). Arbuscules are complex structures inside the cell lumen of the root and they are responsible for the nutrient transfer to the plant and obtaining carbon from the plant (Bonfante & Genre 2010). The initial growth of hyphae from AMF spores is thought to be caused by root exudation to attract AMF colonization.

Plant roots release different types and abundance of exudates depending on the species and the root section (Neumann et al. 2000). These exudates attract AMF spores to grow hyphae towards plant roots for colonization and the exudates can be made of various secondary metabolites and sugars (Cameron et al. 2013). There are various reason why different roots segments or the entire root system deposit different exudates. Younger roots usually release more exudates than older roots probably because the younger roots are highly active (Hirsch et al. 2013). A phosphate limited plant may release more active exudation than one with adequate amount of nutrient likely to attract AMF in soil for colonization, since AMF can uptake nutrients for the plant (Nagahashi & Douds 1999). The same signal can attract both beneficial and harmful organisms because the same evolutionary forces acted on both types of organisms to adapt to the host species (Cameron et al. 2013). Some root exudates attract favorable bacteria as well as unfavorable worms so root exudations have a wide range of effects on the plant (Cameron et al. 2013). Within the AMF community, we do not yet know if the change in exudation implies that different species of AMF spores are more attracted to the certain range of exudation released. We may use the differences in root exudation to make inference on the variations of AMF species found in the root system.

The age and spatial distribution of roots in soil can determine the functionality and microbial communities in the plants and the rhizosphere around them. The spatial distribution of the roots refers to the kind of soil that the roots are in which can be defined by soil moisture and pH, and AMF spores in that region. Fine root nitrogen uptake and uptake efficiency decrease significantly with an increase in age; sometimes nitrogen uptake can even decrease by half after only one day (Volder et al. 2004). This may indicate the importance in AMF colonization to continue stable nitrogen and other nutrient uptake from soil to maintain productivity. The initial rapid uptake of nitrogen can quickly deplete the nitrogen supply in the soil near the roots, and the area of depletion can be the major factor that causes the decline in nitrogen uptake (Volder et al. 2004). The soil condition and nearby AMF spores can influence the AMF species that will

colonize the roots and the efficiency to do so. Time and space are both crucial factors to consider when investigating microbial communities in roots and soil.

I looked at the spatially and temporal changes in AMF species and abundance throughout the root system of *Avena barbata,* slender wild oat, and implication of such variations with experimentation. I will focus on three sections of the root system: the older part of the root where it's the closest to the shoot, the midsection of the roots, and the root tips. I hypothesize that there will AMF species in the different regions of the root system will be different. Further, I hypothesize that: (i) younger sections will have a higher percentage of colonization due to highly active young roots, (ii) younger sections will have greater diversity, where I will identify AMF down to the genus and species level, whichever that is possible, and (iii) the relative abundance of certain groups of AMF will be higher than other and will persist as a core group of AMF that colonize all parts of the root where there is a group of generalist AMF species (Vályi et al. 2015).

METHODS

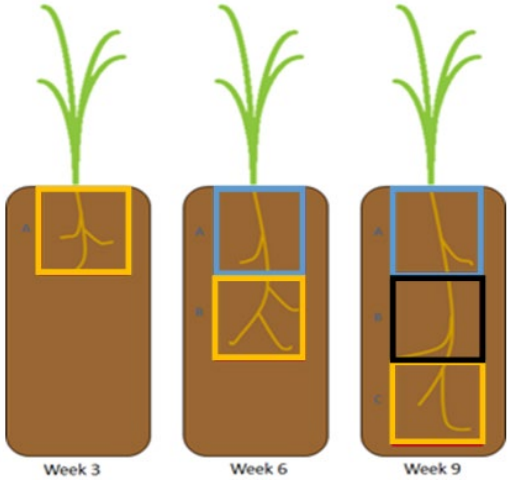
Experimental setup

The experiment was conducted at the Oxford Tract Greenhouse Facility of the College of Natural Resources at University of California, Berkeley, where microcosms were set up in the growth chambers at a constant temperature of 28℃ and 16-hour light periods per day. I used soil from the Hopland Research and Extension Center (HREC) in Hopland, CA that was collected during mid-May and I sieved with 2mm sieve before packing into microcosms. I packed the sieved soil into 10 microcosms to reach bulk density of 1.2g/cm^3 to replicate the soil bulk density at the field site. I germinated *Avena* seeds collected from the previous year by the Firestone Lab by wrapping wet paper towels around the seeds for two to three days. Once germinated, I planted one seedling per microcosm for nine out of the ten microcosms where the tenth one is a control with no seedling. The microcosms were watered every two to three days with the fans turned on occasionally.

Harvests for DNA sequencing and root staining

To find AMF composition and percentage through time and space, I deconstructively sampled the microcosms over a 9-week period where each harvest timepoint was three weeks apart. I harvested three microcosms every three weeks at week 3, 6, and 9 of plant growth to obtain rhizosphere soil, bulk soil, and root samples from each root sections. I decided to analysis these specific time points because it follow the stages throughout the life cycle of the plant (Shi et al. 2015). At the week 3 harvest on June 2, 2017, I treated the entire root system as a "young" root section. At the week 6 harvest, on June 23, 2017 the root system was harvested as two sections, the upper section being "older" and the lower section being the "younger" roots. Finally, at the week 9 harvest on July 14, 2017, the root system will be harvest as three sections: upper section as "old" roots, middle section as "intermediate", and the lowest section as the "youngest" roots (See Figure 1). All soil and root samples were put in phosphate-buffered saline solution immediately after taken out of microcosms. Bulk soil is soil that is farther than 2mm away from roots and rhizosphere soil is less than 2mm away from roots. I collected rhizosphere soil by using the vortex and then centrifuging the tubes with roots.

Figure 1: Root section illustration. The yellow boxes outline the young root sections, the black box outlines the intermediate section, and the blue boxes outline the old root sections.



AMF colonization

Root staining and imaging

To determine the AMF colonization percentage with root staining and visualization, I used the dye WGA 488 to stain the roots and use the confocal and Axioimager microscopes at the CNR Biological Imaging Facility at UC Berkeley to visualize the AMF colonization in the root sections. WGA 488 is a dye that stains the chitin in fungi (Ramonell *et al.* 2005) so I can be more certain to identify AMF structures in roots. Roots were subsampled from the three microcosms from each timepoint and mount on slides using PVLG mountant (INVAM).

Statistical analysis

To determine significant differences of % colonization between root sections, I used ANOVA analysis from the R environment.

AMF Species/Genera Identification

DNA extractions and sequencing

To identify AMF species and genera from rhizosphere and bulk soil samples, I extracted DNA from soil samples using an extraction method utilizing a cetyltrimethylammonium bromide (CTAB) buffer and phenol-chloroform purification (Shi et al. 2015) to obtain DNA from soil samples. Molecular methods are more consistent when identifying AMF species below the genus level (Helgason et al. 1998). I used a Qiagen DNA clean up kit to clean the phenol that remained in the sample from the extraction protocol because phenol can act as a PCR inhibitor. Then I quantified the DNA concentration with PicoGreen using BioRad CFX (Shi et al. 2015). DNA will be amplified by PCR with WANDA (Dumbrell et al. 2011) and reverse primer AML2 that specifically targets AMF DNA (Lee et al. 2008). Then they were sent to sequencing at the Zhou Lab at the University of Oklahoma and receive back through a pipeline with OU.

DNA and statistical analysis

To analyze my sequencing data, I will use the mothur software package to organize my sequencing data and to report the alpha and beta diversity of the various samples (Schloss et al. 2009). Then I will construct an OTU table after using BLAST to assign taxonomy for the AMF species and genera in my samples. I will use ANOVA analysis from the R environment to find if the difference in AMF species composition is statistically significant. For the ANOVA tests that I find to be significantly different, I will use a post hos test to find the detail of the differences.

RESULTS

Microscopy Findings

AMF structures were rarely found in the root microscopy images but other kinds of fungi were observed in the roots. AMF make a unique structure called arbuscle and the presence of arbuscles can confirm AMF presence in roots (Bonfante & Genre 2010). After viewing the roots under a microscope with an AMF expert, Angela Hodge, we found one ambiguous arbuscle structure found in a root from the week 3 timepoint that may be AMF (Figure 4). This was the only potential arbuscle appearance in the roots but there were many different types of fungi that colonized the roots in which I currently could not identify. Although I was not able to identify the types of fungi observed, there were a few types that I saw repeatedly. Type A (Figure 1 & 2) was found mostly in the roots from week 3 timepoint and they seem to have complex hyphal structures that are connected through hyphae from one structure to another. Type B (Figure 3) was much less common than type A fungi and they possess a round and solid structure, and they appeared to have a less complex structure than type A fungi.

Figure 1. **A potential arbuscule.** It was found in a root from week 3 samples (shown by white arrow).

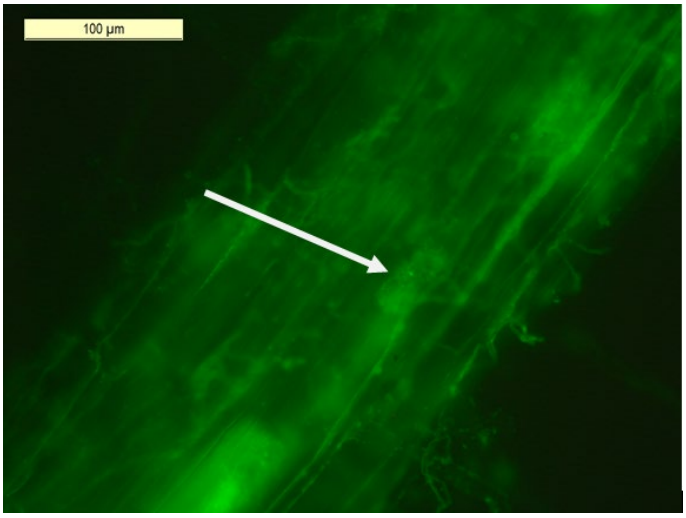


Figure 2 (left). **Type A fungi**. Fungi that is commonly found in the roots and here I will categorize them to be in type A. This image is taken from week 3 roots. **Figure 3 (right):** A magnified image of the type A fungi found in roots from week 3 root section.

Image 2 **Image 3**

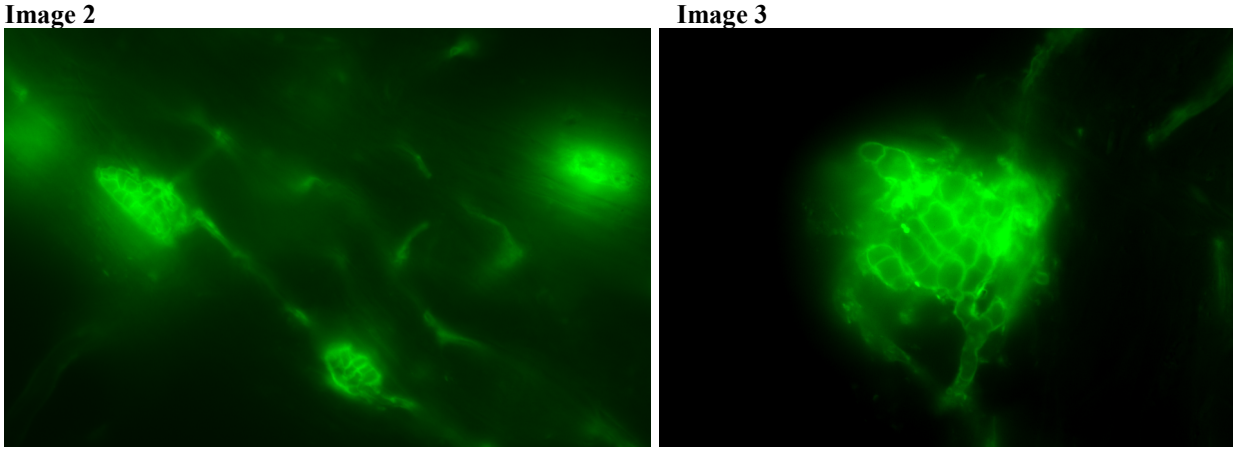
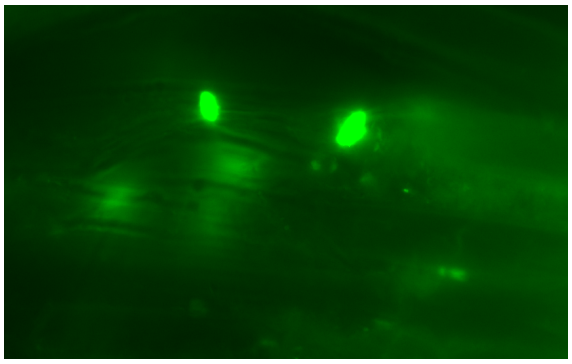


Figure 4. Type B fungi. They were found in the old root section of the week 6 roots. Fungal structure seems to be less complex than type A and they are found less frequently than type A.



Molecular Work Findings

Although I found only one potential arbuscle in the roots, initial PCR and gel electrophoresis analysis showed that AMF are present in the rhizosphere soil. Using gel electrophoresis, the desired length of target AMF DNA region (around 530bp) was visible on the gel when compared to the reference ladders on both ends of the gel (Figure 5 & 6).

Figure 5. **Gel image for samples A5-9, A15-18, B1-14, positive and negative control (start from the right) with ladders on both ends.** Arrows indicate the length of AMF DNA region and each sample takes one column; if there is a band at the desired length of AMF DNA region, there is AMF DNA in the sample. Refer to **Table 1** in **Appendix A: Sample Names and Unique IDs** to identify each sample ID.

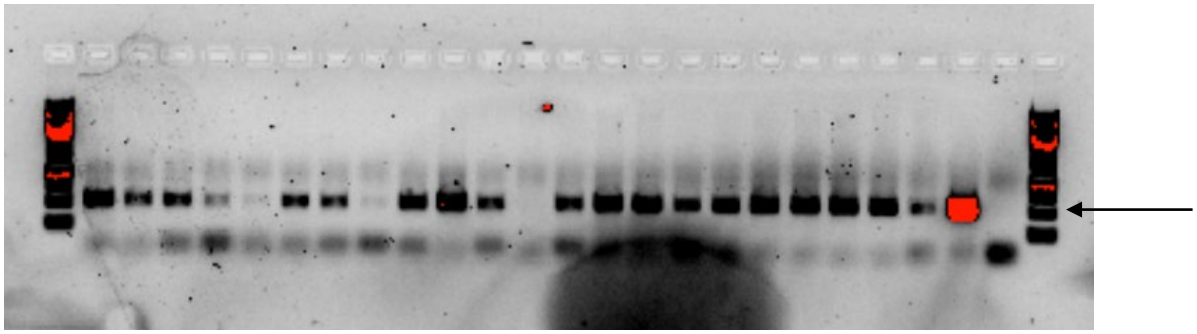
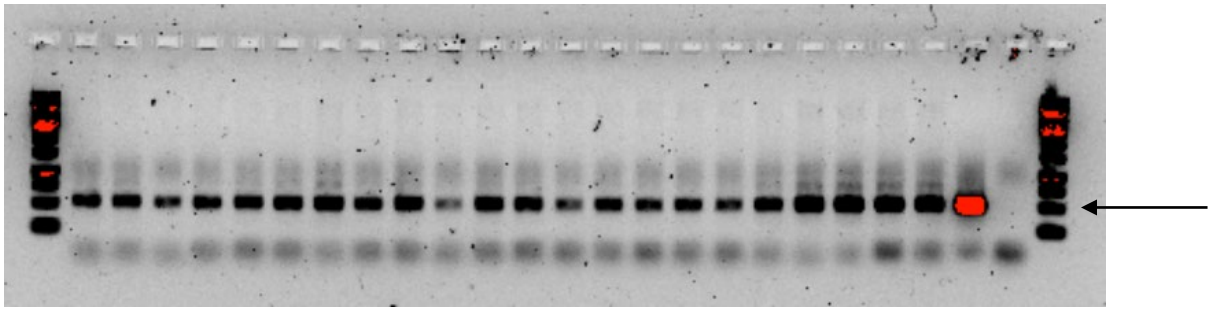


Figure 6. **Gel image for samples B15-36, positive and negative control (start from the right) with ladders on both ends.** Arrows indicate the length of AMF DNA region.



DISCUSSION

My study demonstrated that time plays a prominent role in AMF microbial community throughout the root system of *A. barbata*, a slender oat grass. We are observing the AMF diversity in the rhizosphere and the bulk soil samples from DNA sequencing. The community at the rhizosphere of the roots can demonstrate a good representation of the AMF species and

genera that colonized the root since the rhizosphere soil samples were in contact with the roots. From deconstructive sampling of *A. barbata* roots, we will be able to look at the diversity variations in the different root sections.

AMF % Colonization in Roots

The overall AMF presence observed was not as abundant as other fungi in the root system. The soil used in this study is from a research site collected during the end of spring in May 2017 and the microbe diversity is high in the soil. AMF identification is much more difficult with a large range of fungi in the roots because of the similarity in fungal structures. While viewing the roots with an expert from the field, Angela Hodge, she acknowledged and realized the difficulty of finding and identifying arbuscule structures from the roots collected from the experiment, where the structure is essential to distinguishing AMF from other types of fungi in roots. AMF studies are often done in sterile environments, such as using autoclaved sand and soil as a growth medium, and then inoculating the medium with AMF to ensure the fungi that colonize the roots will be AMF (Hodge et al. 2001). I used field soil instead of sterile growth medium to simulate an environment like the natural setting of fungal community, even though identification problems arose with the large amount of fungi that colonized the roots. This study shows a realistic outcome when studying fungi in natural environments where a wide range of fungi exist in soil in addition to AMF. Observation of the roots showed a large amount of fungi but there are few unidentifiable arbuscule structures, so I can conclude that the fungal community additional to AMF is overwhelmingly larger than that of solely AMF's. Although we did not find much AMF colonization in the roots, the fungi are still very important to the root's microbial community.

Ongoing Research

AMF Species/Genera Diversity

DNA sequences analysis and statistical analysis are following the visualization of the fungi colonization in roots. Microscopy results cannot find abundant AMF presence but using molecular work, I was able to confirm that AMF are present in the samples. After sending the

samples out for DNA sequencing, the sequencing data I recently received are currently being analyzed with the software package mothur (Schloss et al. 2009), then the results of that will be statistically analyzed in R with ANOVA test.

I expect the young root sections will have the highest diversity regardless of the timepoint the young root sections from. Rhizosphere is an area of highly active interactions between fungi and plant roots where the soil is directly in contact with roots. Root exudates are compounds that can shape the microbial community and they are usually made of "sugars, amino acid, carboxylic acids, phenolics, and other secondary metabolites" (Cameron et al. 2013). Root exudates play a big role in triggering AMF colonization in the rhizosphere because AMF-plant relationship begins when the AMF recognizes plant exudates, leading hyphae to grow and reach the roots (Bonfante & Desiro 2015). Plants release exudates such as primary and secondary metabolites to the soil, and these attract AMF hyphae to grow towards plant roots for colonization (Cameron et al. 2013). The difference in exudation can lead to differential colonization throughout the root system. In addition to exudates from roots, the root growth and elongation can have a large impact on AMF colonization. The presence of high root growth activities in young roots can lead to the high AMF diversity in such root sections.

The difference in root age can show the impact of temporal variation on AMF diversity in the different root sections. One study observed that depending on the age and class of the *Lupinus albus* root, it will release a certain exudate predominates: young roots release mainly malate exudation, and older roots release mainly citrate (Neumann et al. 2000; Hirsch et al. 2013). The release of different types of exudates is likely to attract different AMF species, thus, changing the AMF community structure in various root sections. The diversity of AMF in root section can be coupled with the % colonization; increase in % colonization also increases the diversity in the roots. The hypothesized causes for the difference in % colonization perhaps can also explain the diversity difference.

AMF Species/Genera Composition

I expect that there will be several AMF species that will persist throughout the root sections to be a generalist species. These generalists may also be more abundant than other

species found in the roots because they are able to survive in a wider range of conditions than specialist species.

Limitations and Future Directions

Although this study addressed the effects of life stages and age of *A. barbata* roots on AMF colonization, there are a few limitations to the study. My results have data for only one species but AMF community composition has been found to be dependent heavily on host species identity (Helgason et al. 2014). Other grassland species like *Agrostis capillaris* and *Trifolium repens* showed host preference for AMF root colonization and it is likely that *A. barbata* used for this study to have the same property (Vandenkoornhuyse *et al.* 2002). Then the AMF community composition found in this experiment will only address the composition patterns in other *A. barbata* plants but no other plant species, although this will contribute to greater understand the fungal species distribution in the root system.

While this study makes a great starting point to begin studying how time and age of roots can impact AMF colonization, this is merely the beginning to deeper understand of the AMF community. Future research can continue researching the AMF community structure in roots of different ages and throughout life stages but with plants that exist in various ecosystems from around the world. Although AMF species seem to show host plant preference, different host species in the same ecosystem may demonstrate a pattern of AMF colonization due the shared environment. This will require more explorations in site-specific and soil-specific studies. Since there are so many types of fungi that colonized the roots, a follow up study should also examine the general fungal community in the roots and soil by using the 5.8S-FUN/ITS4-FUN primer set to identify the fungal species in the soil and the roots of the host plant (Taylor et al. 2016). This primer set was made from focusing on the 5.8S region of the nuclear ribosomal large subunit (LSU) and target a region in the internal transcribed spacer called ITS4; the primers for each of these regions are called 5.8S-Fun and ITS4-FUN, respectively (Taylor et al. 2016). Using this primer set, I will be able to identify a wider range of fungi community in the soil samples. It will be useful to understand a more general fungal pattern in roots because of the wide range of fungi in soil and their sweeping benefits to the plants.

Broader implications

The time component in the microbial community is crucial to the community composition and the amount of colonization in the root system. The results of this study can contribute to the greater knowledge of AMF and their behaviors throughout the root system depending on the root age and the life stage of the plant, in addition to the specific host species (Vandenkoornhuyse et al. 2002). The strong evidence of the time as a large impact of the AMF and fungal composition in root rhizosphere and root system can reveal the behavior of AMF in relations to their plant host. The AMF diversity and colonization pattern observations can provide the foundation in creating and improving models for understanding the AMF community composition in terms of temporal variations.

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APPENDIX A: Sample Names and Unique ID

Name	Unique ID	Name	Unique ID	Name	Unique ID
W3 Bulk M1R1	A1	W6 Rh M2UR2	B26	W9 Rh M1UR3	C33
W3 Bulk M1R2	A2	W6 Rh M2UR3	B27	W9 Rh M1CR1	C34
W3 Bulk M1R3	A3	W6 Rh M2LR1	B28	W9 Rh M1CR2	C35
W3 Bulk M2R1	A4	W6 Rh M2LR2	B29	W9 Rh M1CR3	C36
W3 Bulk M2R2	A5	W6 Rh M2LR3	B30	W9 Rh M1LR1	C37
W3 Bulk M2R3	A6	W6 Rh M3UR1	B31	W9 Rh M1LR2	C38
W3 Bulk M3R1	A7	W6 Rh M3UR2	B32	W9 Rh M1LR3	C39
W3 Bulk M3R2	A8	W6 Rh M3UR3	B33	W9 Rh M2UR1	C40
W3 Bulk M3R3	A9	W6 Rh M3LR1	B34	W9 Rh M2UR2	C41
W3 Rh M1R1	A10	W6 Rh M3LR2	B35	W9 Rh M2UR3	C42
W3 Rh M1R2	A11	W6 Rh M3LR3	B36	W9 Rh M2CR1	C43
W3 Rh M1R3	A12	W9 Bulk M1UR1	C1	W9 Rh M2CR2	C44
W3 Rh M2R1	A13	W9 Bulk M1UR2	C2	W9 Rh M2CR3	C45
W3 Rh M2R2	A14	W9 Bulk M1CR3	C3	W9 Rh M2LR1	C46
W3 Rh M2R3	A15	W9 Bulk M1CR1	C4	W9 Rh M2LR2	C47
W3 Rh M3R1	A16	W9 Bulk M1CR2	C5	W9 Rh M2LR3	C48
W3 Rh M3R2	A17	W9 Bulk M1UR3	C6	W9 Rh M3UR1	C49
W3 Rh M3R3	A18	W9 Bulk M1LR1	C7	W9 Rh M3UR2	C50
W6 Bulk M1UR1	B1	W9 Bulk M1LR2	C8	W9 Rh M3UR3	C51
W6 Bulk M1UR2	B2	W9 Bulk M1LR3	C9	W9 Rh M3CR1	C52
W6 Bulk M1UR3	B3	W9 Bulk M2UR1	C10	W9 Rh M3CR2	C53
W6 Bulk M1LR1	B4	W9 Bulk M2UR2	C11	W9 Rh M3CR3	C54
W6 Bulk M1LR2	B5	W9 Bulk M2UR3	C12	W9 Rh M3LR1	C55
W6 Bulk M1LR3	B6	W9 Bulk M2CR1	C13	W9 Rh M3LR2	C56
W6 Bulk M2UR1	B7	W9 Bulk M2CR2	C14	W9 Rh M3LR3	C57
W6 Bulk M2UR2	B8	W9 Bulk M2CR3	C15	Blank 1	M7
W6 Bulk M2UR3	B9	W9 Bulk M2LR1	C16	Blank 2	M8
W6 Bulk M2LR1	B10	W9 Bulk M2LR2	C17	Blank 3	M9
W6 Bulk M2LR2	B11	W9 Bulk M2LR3	C18		
W6 Bulk M2LR3	B12	W9 Bulk M3UR1	C19		
W6 Bulk M3UR1	B13	W9 Bulk M3UR2	C20		
W6 Bulk M3UR2	B14	W9 Bulk M3UR3	C21		
W6 Bulk M3UR3	B15	W9 Bulk M3CR1	C22		
W6 Bulk M3LR1	B16	W9 Bulk M3CR2	C23		
W6 Bulk M3LR2	B17	W9 Bulk M3CR3	C24		
W6 Bulk M3LR3	B18	W9 Bulk M3LR1	C25		
W6 Rh M1UR1	B19	W9 Bulk M3LR2	C26		
W6 Rh M1UR2	B20	W9 Bulk M3LR3	C27		
W6 Rh M1UR3	B21	W9 Control R1	C28		
W6 Rh M1LR1	B22	W9 Control R2	C29		
W6 Rh M1LR2	B23	W9 Control R3	C30		
W6 Rh M1LR3	B24	W9 Rh M1UR1	C31		
W6 Rh M2UR1	B25	W9 Rh M1UR2	C32		

Table 1: Sample names and their unique ID. Each of the sample name indicates the timepoint (week 3, 6, or 9), sample type (bulk or rhizosphere soil), the microcosm (M1, 2, or 3), which root section (U for old, C for intermediate, or L for young), and the replicate (R1,2, or 3) each sample is from.