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MYCORRHIZAE IN MAIZE (*Zea mays* L.) CROPPING SYSTEMS RESPOND DIFFERENTLY TO NITROGEN FERTILIZATION UNDER INCREASING CROP ROTATIONAL DIVERSITY

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by

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Arbuscular mycorrhizal fungi (AMF) remain a vital obligate symbiont of nearly all plants. It is well established that the symbiosis between AMF and host plant improves plant nutrient acquisition, alleviates abiotic and biotic environmental stressors, defends against plant pathogens, and contributes to overall plant fitness and productivity through modification of the soil habitat. Modifications include increased soil aggregation and stability, carbon sequestration through provision of fungal wall precursors to soil organic matter (SOM) formation, and enhanced nutrient cycling in the mycorrhizosphere. The goal of this dissertation was to assess how AMF respond to nitrogen (N) fertilization regimes in maize cropping systems of increasing crop rotational diversity. Two, long-term field sites were used to evaluate AMF responses to N application during maize growth. The first site was a conventionally tilled and rainfed site in Elora, Ontario, Canada at the University of Guelph, hereafter referred to as Canadian Nitrogen Study (CNS). CNS evaluates contrasting mineral fertilization rates applied either continuously for 10 years or shocked with a higher/lower N rate once every five years. We demonstrate that soil AMF biomass is more responsive to current season N application rates than

historical N regimes and supports our prior research showing that extramatrical AMF biomass declines with increasing N applied. The second site is a rainfed, no-till maize system managed by the United States Department of Agriculture (USDA) and referred to as Crop Rotation Study (CRS). CRS was sampled seasonally over two years for soil biological and chemical properties and was designed to evaluate soil C and N stocks in diverse rotations with continuous corn under three levels of N fertilization. We found a similar inverse relationship of extramatrical AMF to N application rate as in CNS and demonstrate how N fertilization drove AMF biomass dynamics in the soil. Due to the agronomic importance of maize, it is necessary to cultivate sustainable management practices that contribute to resilient mycorrhizal communities and SOC stabilization.

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For the plants, that always guide.

For the fungi, that consistently fascinate.

For Boggsy, my constant and loving friend. It has always been you and me.

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Chapter 1: Review of the literature and outline of dissertation research

1.1. Introduction

Mycorrhizae (-zas) are mutualistic associations between plant root systems and soil fungi. The word "mycorrhiza" (Greek origin) translates literally to 'fungus-root'. Mycorrhizae can be grouped into many types based on lifestyle, host preference, and root colonization phenotype. These types include endomycorrhizae, ectomycorrhizae, orchid mycorrhizae, arbutoid ectendomycorrhizae, and eroicoid mycorrhizae. Throughout this chapter, only endomycorrhizae and ectomycorrhizae will be emphasized as these are the two dominant mycorrhizal forms in temperate ecosystem soils. Endomycorrhizae, commonly called 'arbuscular mycorrhizal fungi' (AMF), survive as obligate symbionts across a broad host range. Arbuscular mycorrhizal fungi develop hyphae that penetrate cortical root cells (intracellular), form arbuscules, and extend extramatrical hyphae into the soil environment. In contrast, ectomycorrhizae (ECM) have a limited host range confined to mostly woody perennial plants, are not obligate (can grow as weak saprophytes), form hyphae that penetrate between cortical root cells (intercellular) and surround the root with a hyphal sheath or mantle, known as the Hartig net (Massicotte et al., 1989). Ecologically, each of these types of mycorrhizae have a similar role, specifically in nutrient acquisition. Additional roles such as soil aggregation, carbon (C) sequestration, and protection against plant pathogens are common to both types of mycorrhizae but differ in mode and extent. For example, AMF play a large role in phosphorus (P) and nitrogen (N) acquisition for the host plant, contribute to soil aggregation and C sequestration via extramatrical hyphae, and aid in plant stress

resistance. In contrast, ECM mineralize soil organic matter releasing organically bound N and P for uptake by the host, protect plants via their hyphal mantle, and provide a large source of soil C from extramatrical and mantle hyphae (Drijber and McPherson, 2021; Genre et al., 2020). Mycorrhizae are vital to most terrestrial ecosystems and are especially important contributors to the resiliency and sustainability of agroecosystems worldwide (Duarte et al, 2022).

Maize agroecosystems play an important role in grain production, biofuels, and food security throughout the world (Cassman et al., 1999; Tilman et al., 2002). Maize production dominates the Midwest of North America (Sacks and Kucharik, 2011). In 2021, Nebraska alone planted approximately 4.01 million hectares (ha) of maize and 2.26 million ha of soybeans, which is the number one crop in rotation with maize. In 2017, there were 2.14 million ha of irrigated maize systems compared to 1.63 million ha rainfed, and 0.83 million ha of intensive tillage compared to 4.15 million ha of no-till (USDA NASS). Midwest crop production has a global reach, with exports from the US rising 20% in 2021 compared to 2020 and adding $1.6 billion to 2020's $9.2 billion (USDA Foreign Agricultural Service). The importance of maize to global food security necessitates increased future productivity (Cassman et al., 2003; Cassman & Grassini 2020) without compromising sustainability of our current agroecosystems or the environment.

Currently, many efforts are being implemented to improve N management and resiliency of maize agroecosystems in the face of climate insecurity. Climate smart agricultural (CSA) practices include precision N application (i.e., improvement in

nitrogen use efficiency (NUE) through modified timing, placement, and formulation of fertilizers), reduction of inputs into the system to reduce nitrate and soluble organic N leaching into soils and water, crop breeding, increasing crop rotational complexity, and diversifying rotations by adding cover crops (Chandra et al., 2018). One approach is to synthetically engineer microbes already present in these environments to optimize their biological N fixation abilities or nutrient acquisition strategies. A second approach is to apply biologicals or biostimulants either as seed coatings or foliar sprays to enhance NUE and disease resistance (Dellagi et al., 2020; Gargouri et al., 2021; Schroeder et al., 2019; Wen et al., 2021). The goal of all these approaches is to not only increase yields, but to create and sustain agroecosystem resiliency (Garcia et al., 2016; Thijs et al., 2016).

Climate changes associated with increasing atmospheric carbon dioxide (CO_2) concentrations along with other greenhouse gases are causing uncertainty in food production worldwide. Contributors to rising CO_2 include anthropogenic emissions as well as climate feedback cycles, such as the loss of C from terrestrial environments to the atmosphere, from increasing rates of greenhouse gas production (Kweku et al., 2018; Terrer et al., 2018). These increases in air temperature caused by higher atmospheric greenhouse gas concentrations drive losses of soil organic matter (SOM), which ultimately impacts N cycling and SOM storage and quality in the environment (Del Galdo et al., 2006; Hofmockel et al., 2011; Mullen et al., 1999). More extreme and erratic weather events, as well as increased night-time temperatures during the growing season, are symptoms of rising atmospheric gas concentrations (Barrett 2010; Nelson et al., 2016; Singh et al., 2021; Tilman et al., 2002; Van Ittersum et al. 2013). Food production and

security continues to be a major issue worldwide as the climate changes. These food security challenges require intensive investigation to ensure enough resources for humanity while balancing environmental concerns (Butler 2010).

In maize agroecosystems, high inputs of N fertilizer are needed to maintain productivity, but poor NUE can lead to losses of N as greenhouse gases (e.g., nitrous oxide and NH_3) to the atmosphere or through leaching (as nitrate or soluble organic-N) to ground and surface waters thereby impacting water quality (Chen et al., 2021; McLellan et al., 2015; Tenorio et al., 2021). Further understanding and optimizing these maize agroecosystems to improve NUE and SOM storage provides an important step towards maintaining or improving the sustainability of cropping systems.

One approach to evaluate current management practices within maize agroecosystems of the Midwest is through the lens of soil microbial ecology and linkages to maize productivity and soil sustainability. AMF are capable of physiologically altering the plant host, protecting the plant host from drought, disease, pest attack, all while scavenging for nutrients in the soil solution and increasing yields (Higo et al., 2018; Tiemann et al., 2015). The impressive breadth of abilities within this symbiotic relationship set AMF up as a natural and ideal participant in building resilient agroecosystems. To add perspective to the longevity of this soil-fungal-plant relationship, the symbiosis between AMF and host plants predates the symbiosis between N-fixing bacteria and legumes which arose approximately 450 million years ago. Ultimately, understanding and managing the AMF-host plant continuum is a major step towards

harnessing the soil microbiome for a productive and resilient agroecosystem (French, 2017).

1.2. Biology and function of arbuscular mycorrhizal fungi (AMF)

A major contributor to soil quality and more specifically the maize microbiome are AMF. These obligate biotrophs survive and reproduce asexually in the soil environment, and act as an extension of the plant's root system. Through an intricate cascade of molecular signaling, AMF spores in the soil germinate to form hyphae which grow into the cortical cells of plant roots to create arbuscules, which are highly branched structures that allow for nutrient exchange (inorganic minerals, carbon, phosphorus) between the plant and fungus, and vesicles, which are thick-walled lipid storing organs (Begum et al., 2019; Gerdemann, 1968). Hyphae are produced not only within (intraradical) the plant roots, but also extend beyond the root(s) into the soil as resources (e.g., C) are gained from the plant. This soilborne type of hyphae is called the extraradical mycelium (ERM) or extraradical hyphae. Once the symbiotic relationship is established, the plant sends carbohydrates and lipids to the fungus, while AMF scavenges essential nutrients, such as nitrogen (N) and phosphorus (P) from the soil and shuttles them to the plant (Antoine et al., 2021; Drijber and McPherson, 2021; Garcia et al., 2016; Jeske 2012; Olsson et al., 1997). More specifically, inorganic nutrients are taken up from the soil solution by AMF hyphal tips, converted into transportable forms (e.g., polyphosphates, peptides) then transferred via the ERM to arbuscules within root cells of the host plant.

Nutrient and energy transport processes are facilitated by specific membrane transporters at key locations along the symbiosis. To gain entry into the plant cytoplasm, the nutrient is processed to pass through both arbuscular and plant membranes. At no time does the fungal cytoplasm encounter the plant cytoplasm. Each nutrient, e.g., nitrate, ammonium, or phosphorus, has a specific set of membrane transporters. For example, the transportation of nitrate requires an energy intensive group of nitrate/peptide transporters, from the nitrate transporter (NRT) and peptide transporter (PTR) gene families (Drechsler et al., 2018; Drijber & McPherson, 2021; Garcia et al., 2016; Johnson, N. C. 2010; Wipf et al., 2019). The rate of transfer and availability of nutrients depend on a multitude of variables in the soil environment. Continuing to understand this vital symbiosis and how it interacts with and impacts nutrient cycling is important to furthering the resiliency of maize agroecosystems (Antoine et al., 2021).

Arbuscular mycorrhizal fungi are a fundamental component to not only soil biology, but also an important indicator of soil quality (Karlen et al., 1997). As the conversation surrounding soil quality continues to evolve (Lehmann et al., 2020) and is ultimately highly dependent on each unique environment, one theme that persists is the ubiquitous nature of mycorrhizae. Mycorrhizae, specifically AMF, play a major role in soil aggregation through the production of glomalin and provide a large carbon sink via excretions and ERM (Rillig 2004; Rillig & Steinberg 2002). Glomalin acts to stabilize soil aggregates lending resiliency to the soil microbiome contained therein. Maintaining the health and productivity of agroecosystems relies, in part, on a resilient soil microbiome where AMF function as strong contributors to this resiliency.

1.3. Measuring AMF biomass in soils and roots

Traditionally, AMF were detected and visualized in roots and soils through classical staining approaches. Classical staining approaches allowed for visual confirmation and quantification of vesicles, arbuscules, and hyphae in roots and spores in soil. Arbuscular mycorrhizal fungi structures were stained with a variety of dyes including acid fuschin, trypan blue, Sudan IV, chlorazol black E, and black ink. After staining the fungal structures, there are multiple protocols to estimate mycorrhizal colonization of roots using microscopic techniques (Antoine et al., 2021; Trouvelot, 1986). In contrast there are more recent methods that focus on cell biochemistry, such as cell wall membrane phospholipid fatty acid (PLFA) extractions, as well as molecular biology which includes techniques such as qPCR or nested PCR (Bodenhausen et al., 2021; Heller et al., 2022; Thonar et al., 2012).

A primary method used throughout this dissertation is fatty acid methyl ester (FAME) analysis, which is a simplification of the more commonly used PLFA method. The PLFA method specifically measures the abundance and composition of fatty acids associated with phospholipids in cell membranes and it is very useful for bacteria given their single cell status. FAMEs quantify fatty acids from all three major lipid classes: neutral, glyco- and phospholipids in a single extraction, and thus includes storage or transport vesicles from eukaryotic organisms, including fungi. This technique uses a mild alkaline extraction procedure to extract ester-linked fatty acids from samples, which creates a unique “fingerprint” that allows for identification and subsequent quantification

of active microbial biomass and community structure. It is important to note that the biomarkers are measured only in living cells, which represent the living microbial community at the time of measurement. FAMEs give insight into spatio-temporal shifts in the microbial community by hydrolyzing these fatty acids from microbial cells *in situ* (Drijber et al., 2000; Grigera et al., 2007a, b; Jeske 2012). AMF contain a relatively unique fatty acid biomarker, 11-hexadecenoic acid or C16:1cis11, in three major lipid classes: neutral, glyco- and phospho-lipids. AMF are generally quantified by C16:1c11 in the neutral lipid fraction because a few bacteria contain a specific biomarker in their membrane phospholipids and glycolipids make up a small proportion of the total lipid pool (Grigera et al., 2007a, b; Olsson and Johansen, 2000). However, because of issues with the polarity of chloroform used to separate neutral lipids on silica gel (Drijber et al., 2019), it is recommended to either combine the neutral and glycolipid fractions or use FAMEs to quantify AMF given the overall small contribution from bacterial phospholipids. Because of this biomarker's specificity, it allows for greater confidence in assigning fatty acids as biomarkers of specific taxa (e.g., mycorrhizae) compared to bacteria and saprophytic fungi (Frostegård et al., 2011). Specifically, AMF biomass can be quantified from a FAMEs extraction and linked to fluctuations in AMF community structure based on environmental and management practices. It is important to remember that soil is an immensely complex environment, thus each method expanded upon below serves to answer specific questions and hypotheses (Fierer et al., 2021).

1.4. Phylogeny of AMF

Glomeromycota were previously described by morphological characteristics, such as spore size, shape, and wall structure (Oehl et al., 2011). Since the early 2000's, researchers have been able to incorporate molecular approaches into identifying and classifying mycorrhizae. Molecular approaches can include sequencing of specific conserved gene regions that are present throughout *Glomeromycota*. As the systematics of *Glomeromycota* are refined by phylogenetics, the taxonomic classification of this group is still informed by classical morpho-anatomical data. The concomitant nature of organizing and assigning taxonomic rank within *Glomeromycota* continues to develop and evolve through time, especially as molecular and phylogenetic techniques are refined (Hart et al., 2015; Oehl et al., 2011; Öpik & Davison, 2016; Redecker et al., 2013). Another caveat within fungal taxonomic science is an ongoing disagreement on the naming regimes used to classify fungi, leading to a disconnect and mislabeling of new fungal species. Recently, there have been many new additions to *Glomeromycota* including *Polonospora, Scutellospora deformata,* and *Dominika glomerocarpica sp. nov* (Błaszkowski et al., 2021a, b; Guillen et al., 2021). One way to mitigate this is to create a call to action for the scientific community to follow the same taxonomic approach (Tedersoo et al., 2018), which includes using the same primers and sequencing methods as they progress through time. Using the same classification system for scientific research allows for comparison across datasets, continuity in the scientific literature, and a stable approach to compare phylogenies and evolutionary hypotheses.

1.5. Quantification of AMF diversity and community composition

Quantifying AMF in soils has evolved and developed throughout time. Earlier methods included nested polymerase chain reaction (PCR) and cloning (Jacquot et al. 2000; Liang et al., 2008; Renker et al., 2003; van Tuinen et al., 1998). These methods allowed for distinction from other fungi in the roots and soils using eukaryote-specific primers. Greater primer specificity allows easier determination of what species were colonizing root samples. Some examples of primer sets include AM1-NS31 and Glo1-NS31GC for amplification of 18S rDNA fragments and subsequent DGGE analysis (Liang et al., 2008). Other primers include the first internal transcribed spacer (ITS1) of the ribosomal DNA using the primers ITS1f (5′-CTTGGTCATTTAGAGGAAGTAA-3′) and ITS2 (5′-GCTGCGTTCTTC ATCGATGC-3′) (White et al., 1990), the LR1 (5′GCATATCAATAAGCGGAGGA-3′) and FLR2 (5′-GTCGTTTAAAGCCATTACGTC-3′) for the large subunit of the 18S rRNA gene (Trouvelot et al., 1999; van Tuinen et al., 1998), and the subsequent nested primers FLR3 (5′-TTGAAAGGGAAACGATTGAAGT-3′) and FLR4 (5′-TACGTCAACATCCTTAACGAA-3′) (Golotte et al., 2004). These techniques are an important step in investigating AMF community structure, or diversity of root colonization, throughout a field experiment (Renker et al., 2003). The ability to identify AMF in root samples allows for greater phylogenetic confirmation, but limitations to primer specificity include the presence of polymorphisms in the subunits being amplified during PCR and other fungal contamination (Renker et al., 2003). While these limitations are present across most realms of molecular biology, the limited phylogenetic

background of AMF further constrain researchers to using the same methodology to compare results.

Another more recent development in molecular techniques is amplicon or next-generation sequencing. Next-generation sequencing technology has allowed for deeper insights into the composition of soil microbial communities; however, it provides only an identification of 'who' is in the sample, not potential functionality. However, discovering 'who' is present in soil microbial communities is the first step in elucidating potential function. For all experiments discussed in this dissertation, amplicon sequencing was conducted, focused on the variable 9 (V9) region of the 18S single subunit (SSU) rRNA gene (Simon et al., 1992; Taylor et al., 2017). This region, and its associated primers (Euk1391f and EukBrR), provide taxa level distinction, such as between families, genera, and species. In addition to amplification of the 18S rRNA gene region which highlights taxa from a community ecology perspective, it is important to consider the breadth of the potential amplification area (See figure 1.1 below). Within the potential areas to amplify are the internal transcribed spacer (ITS) regions, which are increasingly being used in AMF sequencing (Berruti et al., 2017; Schoch et al. 2012). This dissertation will compare 18S rRNA sequencing results to ITS sequencing results, where applicable, to compare community composition from two different operons.

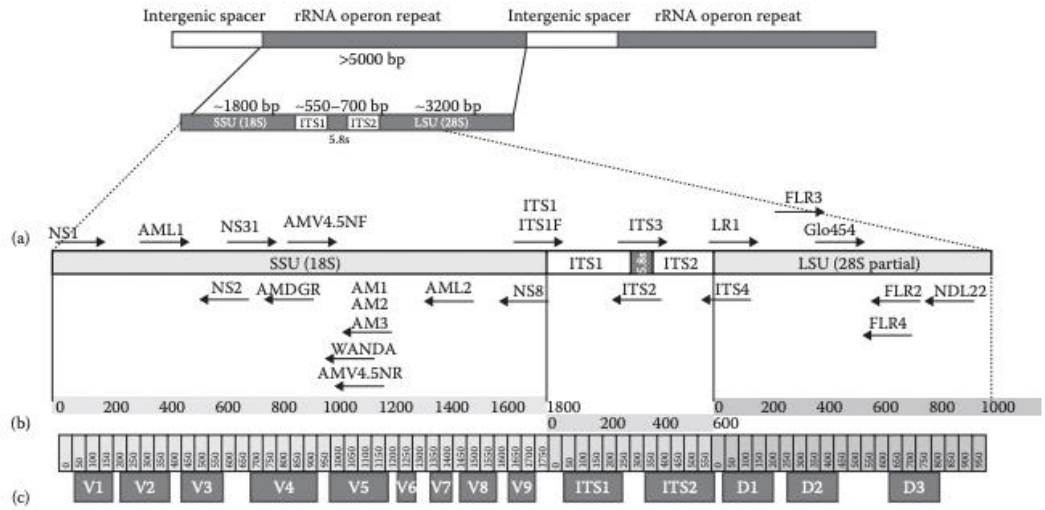

Figure 1.1. The conserved and variable regions of the SSU 18S and LSU 28S partial gene as well as ITS1 and ITS2 regions used for amplicon sequencing. Certain primers are designed around these regions (Taylor et al., 2017).

1.6. AMF biomass and community composition in maize agroecosystems

AMF biomass, community composition, and diversity in maize agroecosystems is important for disentangling historical and current year nutrient cycling and management impacts. Hontoria et al., (2019) found that in a 10-year maize experiment aiming to stimulate indigenous AMF populations with cover crops (barley and vetch) compared to no cover crop, the driving factor of AMF diversity was soil properties, including total organic carbon, soil pH, soil EC, and soil microbial biomass carbon. They also found AMF communities under barley differed from those under fallow, but not under vetch, indicating that AMF communities respond to multiple factors in the soil environment. In this experiment, fallow refers to an unplanted period or season and the cover crops were terminated before maize was planted. These results indicate AMF respond positively to

long-term cover crop management by having increased diversity and biological activity. In another maize study that explored correlations between weed pressure and using existing AMF as a biocontrol, Li et al. (2019) found that the impact of AMF community composition (taxa) on maize growth depended on the 4 weed species (*Abutilon theophrasti* Medik., *Sida spinosa* L., *Setaria pumila* (Poir.) Roemer & J.A. Schultes, and *Chenopodium album* L). These results suggest that certain species of AMF can control and reduce the growth of certain weed species, while not negatively impacting corn growth. Additionally, they found that the specific weed species influenced AMF community structure in soil and in maize roots. This effect of weed species was stronger than other management strategies, such as tillage and cover cropping (Li et al., 2019). A third example concluded that AMF community composition and specific components of SOM in the maize rhizosphere were increased using organic fertilizer but not synthetic N and P fertilizers (Zhu et al., 2016).

Previously, the benefits of AMF were thought to be limited to nutrient poor or low yielding soils, but more recent work has postulated that AMF may span the phosphorus depletion zone around maize roots to meet increased crop nutrient demand even in high-yielding cropping systems (Drijber & McPherson, 2021; Grigera et al., 2007b; Tian et al., 2013). The latter example illustrates the trade-deficit model, where the AMF-plant relationship can shift on a continuum depending on the availability of P and N in the environment (Johnson 2010). Mutualistic relationships are more likely to occur when the host plant is productive despite limited P in the soil, meaning that the plant can produce enough photosynthates to supply energy to AMF and N is non-limiting. Such

mutualisms can shift with P and N availability in the soil solution towards a more C-limited relationship (i.e., commensalism) and even parasitism (Drijber & McPherson, 2021; Johnson, 2010; Johnson et al., 1997). To optimize this AMF-plant relationship for more resilient agroecosystems, a better understanding is needed of the interplay among AMF and local or regional soil/crop management factors, such as fertilization practices, water availability, soil nutrient pools, crop rotations and cover crops, under a changing climate.

1.7. Inorganic nitrogen fertilization

Nitrogen is an essential element to life on earth and can take on many forms in soil. These N forms are determined by decreasing oxidation states including nitrate (NO_3^-), N dioxide (NO_2), nitrite (NO_2^-), nitric oxide (NO), nitrous oxide (N_2O), dinitrogen gas (N_2), ammonia (NH3), and ammonium (NH_4^+) (Robertson & Groffman, 2007; Jackson et al., 2008; Ramirez II, 2020). Soil microorganisms play vital roles in the transformation and cycling of N in agroecosystems (Fierer et al., 2012; Hayatsu et al., 2008; Parihar et al., 2019). Throughout maize systems in the Midwest, common types of fertilizer include more organic forms such as compost, manure, and urea (which is considered organic due to the carbon), and synthetic N sources such as injected ammonia and urea plus ammonium nitrate (UAN). Microorganisms transform existing soil organic matter and externally added N sources through mineralization, nitrification, and immobilization, which can affect the N availability for plants. AMF are important in facilitating effective absorption of nutrients outside of the nutrient depletion zone of plant

roots. Depending on the mobility of nutrients, the nutrient depletion zone can range from ~1-2 mm to up to 5-10 cm from the root (Drijber & McPherson, 2021; Kuzyakov & Razavi, 2019). Once the hyphae have extended beyond the nutrient depletion zone, they are able to uptake nutrients via hyphae and membrane transporters located at the tips of hyphae, which then shuttle the nutrients back to the host plant (Drijber & McPherson, 2021). Improving N uptake by crops would decrease the amount of reactive N remaining in the soils, which are at risk of being lost to the environment. Thus, when making N fertilization management decisions, it is important to consider N losses via microbial processes.

In a field study in eastern Nebraska, Tian et al. (2013) found that although variable N application rates did not reduce AMF colonization of maize roots, AMF community composition varied temporally with N fertilization rate. The diversity of AMF in maize roots was high (up to 26 specific phylotypes) regardless of the N applied, and crop rotation (monoculture maize or maize-soybean rotation) did not influence AMF diversity. Although long-term maize monoculture and high N application rates (300 kg N ha^{-1}) did not impede AMF colonization of maize roots, there was lower AMF community richness and diversity (H') within roots compared to lower N fertilization rates.

In contrast to maize roots, AMF biomass in soil from this same field study was inversely related to N fertilization rate that varied depending on crop and crop rotation (Jeske et al., 2018). This observed variation was most pronounced at early reproductive growth when N demand is high (Grigera et al., 2007a, b; Jeske et al., 2018). This relationship was attenuated when soybean was the prior crop suggesting that prior N

fertilization history, crop rotation, or crop species may have a legacy effect with respect to N mineralization during the current maize growing season. This inverse relationship between AMF in soils and external N inputs has been documented not only in maize agroecosystems, but also grasslands, rice, wheat, and sunflower (Abobaker et al., 2018; Bradley et al., 2006; Johnson et al., 2003; Liu et al., 2013; Zhu et al., 2018).

1.8. Crop rotations

When compared to continuous cropping, more diverse crop rotations provide benefits ranging from increased pest and disease resistance to more resiliency of the entire ecosystem (Karlen et al., 2006; Katsvairo et al., 2002). This may lead one to think that a more diverse crop rotation leads to a more resilient and sustainable approach to agriculture, however, in terms of AMF abundance and diversity, it has been found that long-term monoculture and high N application rates did not reduce AMF colonization of maize roots but did reduce AMF biomass in the soil (Tian et al., 2013). Another example that emphasizes AMF function within crop rotations is the large amounts of nutrient transfer from the soil matrix to the crop, the movement of carbon in the form of plant photosynthates into SOM in the soil, and ultimately C sequestration (Van Der Heijden et al., 2008). These results emphasize the importance of approaching an agroecosystem from a holistic perspective: e.g., AMF's role in productivity and in provision of other ecosystem services, such as C sequestration and improved soil structure; recognizing that soil microbial communities under a particular management system may have system-specific adaptations. Focus on a singular component or outcome may undermine efforts

to achieve sustainable agroecosystems more broadly across different landscapes and climate scenarios.

There are multiple factors that influence agronomic and environmental outcomes from crop rotations, such as quality and quantity of C and N inputs into the soil, crop residue management and sequence, use of cover crops and other soil amendments, climate and seasonal weather patterns, and soil type, namely the chemical, physical and biological makeup of the soil. The C provided by plant root exudates, above and below ground plant residues, and organic amendments are all incorporated, either mechanically or by soil fauna, into the soil environment and processed by the microbial community. Mycorrhizae, more specifically, are important conduits between the plant-root interface and the soil environment, and factor strongly in SOM dynamics (Frey, 2019). AMF hyphal networks are beneficial in distributing plant photosynthates throughout the soil pore network and onto mineral surfaces. The residual AMF necromass and exudates also function in SOM formation and stabilization (Frey, 2019). Further, Tiemann et al. (2015) found that increased quality, quantity, and chemical diversity of residues from high diversity rotations (corn, soybean, wheat, red clover, and rye) increased productivity, resource use efficiency, and nutrient availability. In another study, AMF showed positive impacts on maize yields and aboveground biomass when grown in rotation with cover crops (Higo et al., 2018; Higo et al., 2019). Additionally, King et al. (2018) found that by increasing the functional diversity of crop rotations, there was a subsequent increase in SOC which adds to the resiliency of the soil environment. Functional diversity can be defined as a mix of legumes and non-legumes, annual or perennial plants, and if the

plants were harvested or not. Overall, increasing diversity and 'perenniality' of crop rotations leads to improved carbon inputs into the soil environment.

1.9. Tillage

Tillage is a mechanical disturbance of the soil which ultimately modifies the soil physical environment from its previous state (El Titi, 2002; Koller, 2003). Tillage can be used as a management practice to enhance and incorporate decomposing crop residues into the soil as well as serve as a method to incorporate fertilizers, manures, and pesticides (Kabir, 2005). Some benefits of using tillage include leveling soil, post emergence weed control, and a way to mechanically disrupt or reduce incidences of disease or pests. In contrast, detriments to using tillage can include degradation of the soil and potential environmental pollution (Garcia et al., 2007; Kabir, 2005). Specifically for AMF, tillage of the soil can disrupt hyphae networks and reduce the diversity and/or richness of the AMF species present (Bowles et al., 2017; Chagnon et al., 2013; Jansa et al., 2002; Säle et al., 2015; Wortmann et al., 2008). Soil microbial biomass is typically higher in surface soil due to greater root density and accumulation of above-ground residues, especially under no tillage (Fierer et al., 2003; Turner et al., 2017). Tillage causes a redistribution of soil microorganisms with depth depending on type, i.e., disk, chisel or soil inversion (moldboard plow). This is also true for AMF where AMF biomass in surface soil was found to be higher in no-till systems compared to conventional-till in a wheat-fallow system (Drijber et al., 2000). Overall, when implementing management practices, specifically tillage, it is important to consider sustainability and conservation of

resources and soil biota, such as AMF, and their contribution to SOM and soil structure in maize agroecosystems (Xu et al., 2019).

1.10. Seasonal dynamics of AMF in maize cropping systems

The symbiotic relationship between the AMF community and maize fluctuates throughout time in response to nutrient exchange and environmental factors. The biomass of AMF in soil and roots is closely tied to maize growth stage being highest at early reproduction (Grigera et al., 2007a, b; Tian et al., 2011) and reflects significant transfer of C from the host to the fungus in the soil. The net accumulation of AMF biomass in both soil and maize roots highlights the temporal nature and synergy of plant nutrient demand, nutrient availability, and other edaphic factors during maize vegetative growth.

Gravito & Varela (1993) found that mycorrhizal dynamics in four maize fields closely followed the growth of the host plant. Specifically, there was high mycorrhizal colonization and spore counts observed in young maize plants which decreased slowly until maize maturity. Mycorrhizal sporulation was highest at maize maturity and decreased as the maize plants senesced (Gravito & Varela, 1993). Another maize field experiment found similar results that AMF colonization of the maize plants was highest at flowering/reproductive growth stage (Alvarado-Herrejón et al., 2019). Finally, AMF in the soil can ameliorate drought related stressors to maize plants in a field setting by maintaining maize growth. Overall, AMF provide a positive influence on maize growth and development throughout the growing season by strengthening tolerance mechanisms (Begum et al., 2019).

1.11. Soil microbial ecology: more than a sum of its parts

Soil is one of the most complex systems on Earth, without which planetary functions would falter. As weather events become more extreme and anthropogenic pressures continue to rise due to climate change, we need to look to the soil and its microbial community for solutions. However, a deep understanding of these microbial communities, including AMF, and how they respond to agroecosystem management is needed to achieve sustainable and resilient cropping systems under a changing climate. While it is important to note the discoveries that have been made in how AMF live and operate in soil systems (Antoine et al., 2021; Chagnon et al., 2012; Faust et al., 2012; Kivlin et al., 2011), there are still unknowns surrounding AMF and their interrelationships among the soil biological community and how these communities respond to major disturbances. However, potential mitigation effects that AMF colonization would confer to agroecosystem resiliency via volatile weather include improved crop conditions during drought, improved soil structure to increase water infiltration, and soil water retention during extreme rainfall. It is vital to characterize and analyze these processes as soil microbes are crucial in sustaining and regulating nutrient cycling, biogeochemical processes and soil organic matter turnover, terrestrial greenhouse gas flux, certain ecosystem services, and ultimately plant populations and community biology (Baveye et al., 2016; Frey, 2019; Luo et al., 2020; Sokol et al., 2022; Tedersoo et al., 2020; Wardle et al., 2004). Figure 2 attempts to demonstrate the

complexity of the soil environment and factors that go into shaping soil microbial communities.

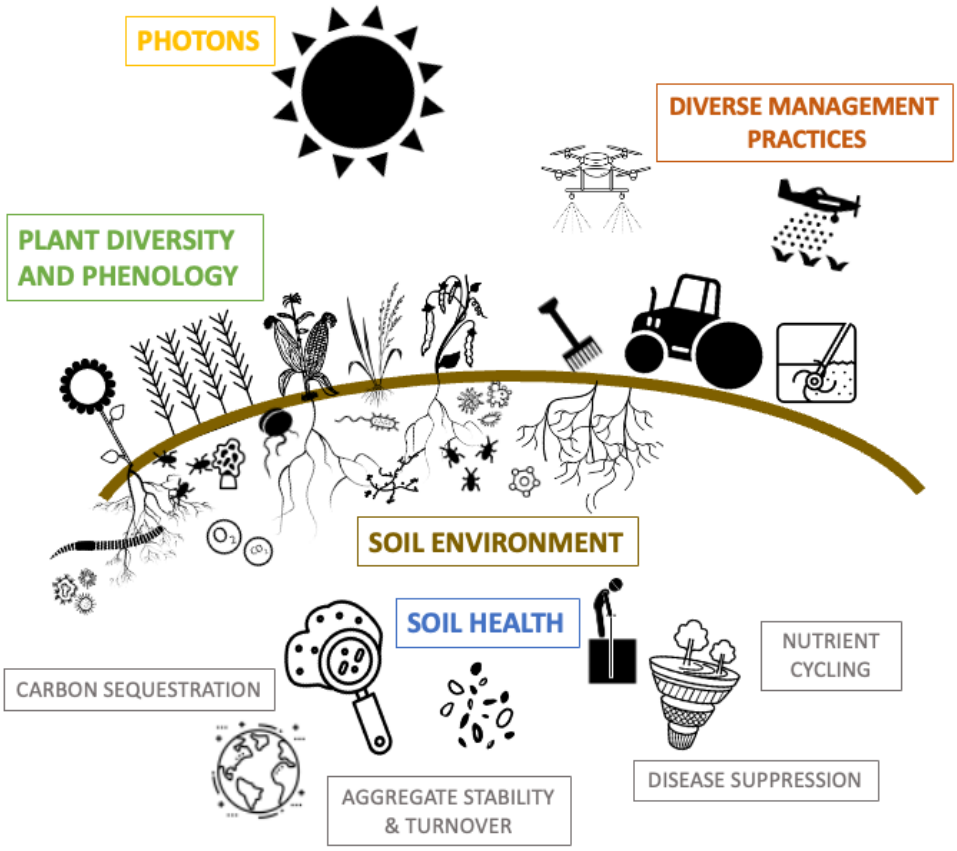

Figure 1.2. Shaping ecology belowground for provision of ecosystem services. Recall, ecology is the interaction of organisms with their environment. This figure is modified from a previous figure I made for a virtual journal club in Stengel et al., 2021.

1.12. Outline of dissertation research

The general aim of this dissertation is to understand how AMF, nitrogen availability, and maize roots interact and impact soil nutrient cycling, soil carbon storage and allocation, and crop yields in agroecosystems. Understanding these tripartite

interactions, particularly within a spatial context (soil depths, proximity to root) and temporal context (over the growing season, from year-to-year), leads to more efficient approaches of maize agroecosystem management while sustaining soils in the changing climate. These symbiotic organisms provide a glimpse into the complex communication occurring in the belowground soil environment and how it translates to aboveground sustainability.

Given previous findings from our lab that the biomass of AMF in soil cropped to maize is inversely related to N fertilization rate, the following chapters of this dissertation attempt to: (1) refine our understanding of this relationship under altered N fertilization regimes and increasing crop rotational diversity; and (2) elucidate tripartite interactions among AMF community composition, soil properties and crop productivity. Greater understanding of these tripartite interactions will better equip researchers and extension educators to guide stakeholders in best management practices for productive and resilient agroecosystems in changing climate.

Chapter 1 of this dissertation serves as a literature review and summarizes how maize agroecosystems and soil nutrient cycling influence subsequent AMF microbial community structure and composition with the addition of variable N fertilization treatments, tillage, and crop rotation management strategies. The hypotheses explored throughout this dissertation are described below.

Chapter 2 explores the relationship between historical N versus current year N fertilization rate on soil and root biomass of AMF at early reproductive growth of maize. Because of the reliance of AMF on plant photosynthate C and the positive relationship

between N fertilization rate and maize yield, we **hypothesize** that current year N fertilization rate would be more influential on soil AMF biomass and root colonization than historical N fertilization rate. In addition, we **hypothesize** that soil AMF community composition better reflects long-term historical N rate over current season N rate due to buildup of AMF inocula. This study; hereafter referred to as the Canadian Nitrogen Study (CNS), took place at a rainfed, conventionally tilled field site planted to continuous maize located in Elora, Ontario, Canada and managed by the University of Guelph. CNS evaluates contrasting mineral N fertilization rates applied either continuously (CON) for 10 years or shocked with a higher (shocked up: SKU) or lower (shocked down: SKD) N rate once every five years. The CON treatments were 0, 28, 57, 115, 188, 230 kg N ha^{-1}. The SKU treatments were 0 to 188 and 57 to 188 kg N ha^{-1} and the SKD treatments were 57 to 0 and 188 to 0 kg N ha^{-1}. Three of the CON application rates, 0, 57, and 188 kg N ha^{-1}, were used to assess current year impact of the 'shock' treatments on AMF biomass and community structure.

Chapter 3 assesses how AMF community composition changes throughout two years (2014 and 2015) of variable N application rates in conjunction with diverse crop rotations. With more diverse inputs into the soil environment via more rotational diversity, we enhance not only AMF development, but the entirety of the soil microbiome. The diverse inputs support more biodiversity in the soil. Thus, we **hypothesize** long-term N fertilization and increasing rotational diversity support a more stable and biodiverse soil environment. We also **hypothesize** that this stabilization of the soil environment molded AMF extraradical mycelium (ERM) biomass and diversity

responses within the growing season, as the AMF were able to draw upon larger pools of nutrients and soil organic matter. This long-term crop rotation study (CRS) had three N application levels applied to seven crop rotations including continuous corn (CCCC) in a no-till, dryland system. Three of the seven crop rotations were studied in addition to CCCC, these were 2-year corn-soybean (CSCS), 4-year corn-oats/clover-sorghum-soybean (COGS), and 4-year corn-soybean-sorghum-oats/clover (CSGO). N fertilizer was applied in the form of urea (46-0-0) and was manually broadcast at 3 rates: 0 (zero), 90 (low), 180 (high) kg N ha^{-1}.

Chapter 4 synthesizes the findings and results from the previous 2 chapters and introduces the next step of evaluating how AMF communities assemble early in the maize growing season through the greenhouse experiment. Additionally, I discuss and compare methodology, potential downfalls, and future directions for this research.

Chapter 5 evaluates how the community structure of AMF changes early in the growing season with N shock fertilization treatments in a controlled, greenhouse environment. It also evaluates the influence of management history on AMF communities in soils versus root associated soils. For this experiment, we **hypothesize** that the AMF ERM development pattern is triggered by the current season soil environment, which considers the diverse residues from crop rotational diversity and N fertilization. Briefly, soil was collected from the CRS field site and used in conetainers to examine AMF community recruitment and composition in early development. This experiment directly examined how AMF and maize seedlings determine and maintain a symbiotic

relationship using soil from 4 crop rotations of CRS (CCCC, CSCS, CSGO, and COGS) and variable N treatments.

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Chapter 2: Current season N fertilization rate, not prior N history, dictates extramatrical hyphal biomass of arbuscular mycorrhizal fungi in soil at maize reproduction

2.1. Abstract

Arbuscular mycorrhizal fungi (AMF) are important symbionts of terrestrial plants, with an evolutionary history dating back more than 450 million years. AMF provide a benefit to the host plant by enhancing nutrient and water acquisition in exchange for sugars and lipids derived from photosynthetically fixed carbon. These interactions, which manifest through AMF hyphal colonization of soil, are not only vital for plant productivity but also contribute to soil health through carbon sequestration and maintenance of soil structure. Our prior research shows an inverse relationship between nitrogen fertilization rate and AMF extramatrical biomass in soil that is expressed most strongly at maize reproduction. The question arises as to whether this inverse relationship holds under N disturbance or stress, where historical N fertilization rates are significantly increased or decreased in the current growing season leading to altered soil N cycling, and hence, AMF response. In collaboration with an ongoing, long-term field study conducted in Ontario, Canada, we investigated the impact of N fertilization rate on soil AMF biomass and community structure in a rainfed, tilled, monoculture maize cropping system where historical N fertilization rates (0, 28, 57, 115, 188 and 230 kg N ha^{-1}) were subjected to a 'shock' N treatment (shocked up (SKU) to 188 kg N ha^{-1} or shocked down (SKD) to 0 kg N ha^{-1}) once every five years. As previously found, AMF biomass in soil was inversely related to current year N fertilization rate regardless of whether that rate was historical or 'shocked'. Percent (%) hyphal colonization of roots was high (~50-

70%) across all N fertilization regimes, with no relationship to prior N history. Amplicon sequencing of the 18S rRNA gene (V9 region) and subsequent alpha diversity measurements (e.g., Chao1 richness and Shannon diversity) showed a slightly more diverse AMF community under historically zero N, as well as a slight increase in diversity (Shannon diversity) under SKD fertilization in the current growing season. These findings were also present in the beta diversity, where SKD fertilization led to identifiable shifts in AMF community structure. Our findings demonstrate that in a monoculture maize agroecosystem, zero to low N fertilization regimes, regardless of N fertilization history, supports higher AMF biomass in soil and a more diverse AMF community. Unfortunately, this comes at the cost of maize productivity under this rainfed system and the potential gains in soil C from extraradical AMF and its byproducts are offset by reduced plant inputs (roots and stover) and their contribution to soil C. Reducing N inputs for economic and environmental benefits and increasing cropping system complexity through rotations and/or cover crops may better balance the interplay between crop productivity and AMF's contribution to plant nutrient acquisition and soil C sequestration.

Abbreviations

AMF, arbuscular mycorrhizal fungi; FAMEs, fatty acid methyl esters; CON, continuous (or historical) N fertilization: CON 0_0, CON 28_28, CON 57_57, CON 115_115, CON 188_188, and CON 230_230 kg N ha^{-1}; SK, shocked (current year) N fertilization; SKD

(shocked down): SKD 188_0 and SKD 57_0; SKU (shocked up): SKU 57_188 and SKU 0_188; ASVs, amplicon sequence variants

Keywords

Arbuscular mycorrhizal fungi (AMF), nitrogen fertilization, AMF extramatrical biomass, long-term continuous maize, FAMEs, 18S rRNA sequencing

2.2. Introduction

Arbuscular mycorrhizal fungi (AMF) remain an ancient and vital symbiont of plants. It is well established that the symbiosis between AMF and plant hosts attenuates environmental stressors, stabilizes soil structure, increases plant productivity, helps in plant defense against pathogens, and contributes to overall fitness under a changing climate (Gamper et al, 2010; Martin et al., 2017; Rillig et al, 2002; Sosa-Hernández et al, 2019). Arbuscular mycorrhizal fungi are best known for increasing plant available P, a poorly mobile soil nutrient, through the production of extensive extramatrical hyphae capable of bridging the P depletion zone around plant roots. The agronomic outcome of this relationship depends on the trade balance between plant photosynthetic carbon delivered to AMF versus supply of P via AMF to the plant host (Johnson, 2010). More recently, AMF have emerged as key players in plant N uptake and thus participate in overall plant productivity beyond their role in P acquisition. In fact, available soil N may have a larger impact on the development of the hyphal network in soil than available soil P (Jeske et al., 2018; Veresoglou et al., 2012; Verzeaux et al., 2017; Zeng et al., 2021).

Because AMF are instrumental to aggregate formation and stability in agroecosystems (Rillig, 2004) as well as the formation of soil organic matter (Frey, 2019), a greater understanding is needed on how N fertilization practices impact the development of the AMF hyphal network in soils of highly productive maize agroecosystems.

Maize is an intensively produced and researched crop that is important for grain production, C sequestration, local as well as worldwide economies, and food security (Cassman et al., 1999; Duvick & Cassman, 1999; Li et al., 2011; Ren et al., 2018; Sacks and Kucharik, 2011; Weinhold et al., 2018). A main management factor in maize production throughout the Western Corn Belt of the United States, is application of large quantities of nitrogen fertilizer (Liebig et al., 2002; Tenorio et al., 2021). These N fertilizers are used to maximize yields of maize; however, there is a need to optimize nitrogen use efficiency (NUE) and the associated N application rates without sacrificing yields to reduce negative environmental impacts due to runoff, leaching (as nitrate or soluble organic-N) and greenhouse gas production (Nasielski et al., 2020; Zhang et al., 2015). Further understanding the impacts of maize cropping systems and associated N management on the soil environment, including soil biotic and abiotic factors that interplay with the soil mycorrhizal community, is necessary for fostering long-term agroecosystem resiliency and sustainability.

The AMF symbiosis is highly context dependent and has variable responses to N fertilization depending on the soil chemical and physical environment, N source and rate, cropping system or plant community, and climatic factors (Han et al., 2020; Zhang et al., 2019). Nitrogen fertilization influences AMF extraradical mycelium (ERM) development

in both unmanaged (grasslands; forests), managed (rangelands) and agroecosystems (Bradley et al., 2006; Egerton-Warburton et al., 2007; Gryndler et al., 2006; Hovland et al., 2019; Soka & Ritchie, 2018; Tian et al., 2013; Van Diepen et al., 2010). In a meta-analysis of field studies ranging from temperate grasslands and forests, boreal forests, agroecosystems, deserts, woodlands, and tropical forests it was found that mycorrhizal abundance (hyphal length, percent colonization, and/or spore count) decreased ~15% under N fertilization and ~32% under P fertilization across studies (Treseder, 2004). Although there was variability in study design and length, there were consistent responses in mycorrhizal abundance to N and P fertilization across studies. Additionally, a separate long-term (27 years) field experiment found that N fertilization decreased the total number of spores and identified species (Bhadalung et al., 2005).

In maize cropping systems, AMF are highly responsive to maize growth stage where both intraradical (Tian et al., 2013) and extraradical biomass (Grigera et al., 2007; Jeske et al., 2018; Tian et al., 2011) peak during late vegetative to early reproductive growth stages when maize has achieved maximum root biomass (Amos & Walters, 2006). This is also when soil AMF biomass is most responsive to N fertilization rate (Jeske et al., 2018) regardless of maize productivity. In contrast, in the same field study, Tian et al. (2013) found that AMF biomass in maize roots, either by fatty acid biomarkers or root colonization structures, was unresponsive to varying agronomic N fertilization rates. This duality suggests that AMF colonize maize roots to the same extent regardless of current season N fertilization regimes and may be more sensitive to limiting nutrients such as phosphorus. Zhu et al., (2016), found through 18S rRNA sequencing that AMF

diversity (Shannon diversity and ACE index) in maize rhizosphere field soil was lower with mineral N fertilization regimes, but increased with manure N application suggesting indirect impacts on the AMF community beyond just provision of N. Additionally, Zhang et al. (2020) found in a maize/soybean intercropping system, N fertilization significantly decreased alpha diversity (Shannon diversity, Simpson, ACE index, and Chao1) of AMF communities in the maize rhizosphere. This study also found no significant differences between monoculture and intercropped maize, indicating strong seasonal influence on AMF community diversity (Zhang et al., 2020). Toljander et al., (2008) examined the mycorrhizosphere of maize in a long-term field experiment and found through cloning and sequencing a significant decrease in AMF richness due to changes in soil pH with variable nutrient applications and amendments (nitrate, ammonium sulphate, calcium, green manure, farmyard manure, and sewage sludge). Lastly, Borriello et al., (2013) also found a decrease in AMF community diversity via 18S (and 28S and ITS) sequencing with N fertilization application. There was a dominance of Glomeraceae present throughout the sequencing data, which suggests that this AMF group were the main colonizers of the maize fields in the experiment. Taken together, these studies confirm the sensitivity of AMF biomass and community composition *in soil* to N fertilization in maize agroecosystems.

Although AMF clearly respond to current season N fertilization (Borriello et al., 2013; Jeske et al., 2018; Tian et al., 2013; Toljander et al., 2008; Zhang et al., 2020l Zhu et al., 2016) it is unknown how prior N fertilization history impacts current year AMF response through alternations in internal soil N cycling. In the study by Jeske et al.,

(2018) it was noted that maize following soybean resulted in an equivalent reduction in soil AMF biomass to adding 100 kg N ha^{-1} urea-N fertilizer suggesting changes to internal soil N cycling with crop rotation. Thus, can historical N fertilization regimes produce a similar outcome? In collaboration with an ongoing, long-term field study conducted in Ontario, Canada, we investigated the impact of N fertilization rate on soil AMF biomass and community structure in a rainfed, tilled, monoculture maize cropping system where historical N fertilization rates (0, 28, 57, 115, 188 and 230 kg N ha^{-1}) were subjected to a 'shock' N treatment (shocked up (SKU) to 188 kg N ha^{-1} or shocked down (SKD) to 0 kg N ha^{-1}) once every five years. These shifting N fertilization regimes may impact both crop productivity (Banger et al., 2020; Nasielski et al., 2020) and soil microbial communities (Tosi et al., 2021), that play important roles in C and N cycling (Moreau et al., 2019). To this end, we examined the interplay among AMF, host plant and N fertilization regime through the lens of AMF biomass and community composition at maize reproduction. We posed two main hypotheses: 1) current year N fertilization rate would be more influential on soil AMF biomass and root colonization than historical N fertilization rate, and 2) soil AMF community composition better reflects long-term historical N rate due to buildup of AMF inocula in the soil.

2.3. Materials and methods

2.3.1. Experimental location

A long-term field experiment (IPNI-2008-CAN-ON29) initiated in 2009 at the Elora Research Station (Elora, Ontario, Canada, 43º38'38" N, 80º24'20" W, 373 m

a.s.l.), University of Guelph, was sampled in 2018 to assess the response of AM fungal biomass to N fertilizer management in rainfed monoculture maize under conventional tillage. Soils are classified as Albic Luvisols with a silt-loam texture (silt 48%, sand 32%, and clay 20%), soil pH of 7.7 and 4.5% soil organic matter (SOM) (Nasielski et al., 2020). The experimental site has mean monthly temperatures ranging from -7.1ºC to 19.8ºC, and a mean annual precipitation of 900 mm, classifying the climate as a humid continental. Leading up to sample collection, the average temperature in 2018 was 6.5 ºC, ranging from -24.8ºC to 31.9ºC, with approximately 527 mm of rainfall (AERDR 2018). After a baseline year where the site received a uniform amount of N fertilizer (57 kg N ha^{-1}), treatment plots (~15 m x 6 m) were set up over tile-drains in a randomized block design with 4 replicates. Treatments consisted of six 'continuous' (CON) N fertilization rates of 0, 28, 57, 115, 188 and 230 kg N ha^{-1} and four 'shock' (SK) treatments where once every five years the N rate was shocked 'down' (SKD; 188 or 57 kg N ha^{-1} to zero N) or shocked 'up' (SKU; 0 or 57 kg N ha^{-1} to 188 kg N ha^{-1} N (Figure 2.1). Nitrogen fertilizer was applied pre-plant as urea ammonium nitrate (UAN) injected mid-row to a depth of 7 cm. In addition to this pre-plant fertilization, all plots received approximately 30 kg N ha^{-1} from a formulated, dry 'starter' fertilizer (NPK 15-15-15 plus 2% zinc). This starter fertilizer was applied at the time of planting in a band 5 cm below and 5 cm beside the seed. For the purposes of this experiment, we have excluded this 30 kg N ha^{-1} from the treatment labels, however, it is included in the Σ N applied over 10 years at the bottom of Figure 2.1. This creates six continuous N levels, CON 0_0, CON 28_28, CON 57_57, CON 115_115, CON 188_188, and CON 230_230. The corresponding shock

treatments are SKD 188_0 and SKD 57_0, and SKU 57_188 and SKU 0_188. In figures throughout the manuscript, the SKD/SKU treatments are shown with the paired CON treatment for comparison. Every plot also received pre-plant phosphorus (0-46-0) and potassium (0-0-60) in addition to the following herbicides: mesotrione, S-metolachlor, and atrazine. All plots were planted with corn (*Zea mays* L., hybrid DKC 39-97) at 79,000 seeds ha^{-1} with 0.76 m rows. In the sampling year, corn was planted on May 9th and harvested on October 18th, 2018. The experimental site was managed with conventional fall tillage, moldboard plow, with a spring secondary tillage. Additional information can be found in Tosi et al., (2021) and Nasielski et al., (2020).

2.3.2. Soil sampling

Soil samples were collected 10 years after the start of the experiment on August 7th, 2018, when the corn crops were in early reproductive growth (stage=R2 to R3). Plot borders (~1 m) were excluded avoid border effects. In each plot, 10 soil cores (0-15 cm depth, ⌀=2 cm) were collected along two perpendicular transects within 10 cm of the crop row, combined into one composite sample, and transported to the lab in coolers. After lab arrival, an aliquot of each homogenized sample was shipped cold, overnight to the University of Nebraska-Lincoln where it was sieved to 4 mm and stored at -20ºC for quantification of soil AMF biomass, AMF community structure, and selected soil chemical properties.

2.3.3. Soil chemical properties and crop growth

Soil properties were measured on the same soil samples analysed for AM fungal biomass and community structure. These analyses included soil pH and electrical conductivity (EC). Soil pH and EC were measured using a soil pH probe and an EC probe, respectively (VWR symPHony SB80PC). Soil pH was calibrated on two-points using standard solutions pH 7 and pH 10. EC was calibrated using a standard solution of 1413 μS/cm, as per the manufacturer's instruction.

Soils were also collected the following spring on May 2019 after the experiment was finalized for baseline soil properties and are reported only for the continuous N input rates (See Tosi et al., 2021 Table S1). The following analyses were conducted by SGS Agri-Food Laboratories Inc. (Guelph, Ontario): soil organic carbon (SOC) and total N from the 0-20 cm depth, and soil pH, extractable P, and exchangeable cations from the 0-15 cm depth.

Additionally, agronomic data was available for the continuous and shocked N fertilization treatments. This data includes yield, total plant biomass, and total N uptake, Additional agronomic performance data is given in Supplemental Figure 2.1. More detailed information regarding sampling and measurements can be found at Nasielski et al., (2020).

2.3.4. Quantification of AMF biomass in soil

The AMF-specific fatty acid biomarker, C16:1*cis*11, was used to quantify AMF biomass in the soil (Olsson, 1999). Five grams of soil was extracted with 0.2 M KOH in

methanol according to the method of (Jeske et al., 2018). The resulting fatty acid methyl esters (FAMEs) were quantified on an Agilent 7890 gas chromatograph fitted with an Ultra 2 HP (Agilent) capillary column (50 m 0.2 mm I.D., 0.33 µm film thickness) using helium as the carrier gas. The injector was maintained at 280ºC and the flame ionization detector at 300 ºC. The oven temperatures were held at 50ºC for 2 minutes, then ramped up by 40ºC min^{-1} to 160ºC for 2 minutes, then ramped up again by 3ºC min^{-1} to 300ºC for 30 minutes. Sample masses of individual FAMEs were calculated from peak areas relative to the internal standard methyl nonadecanoic acid and reported as nmol FAME g^{-1} dry soil or relative abundance (nmol%). The identity of C16:1*cis*11 was confirmed by gas chromatography mass spectrometry on an Agilent 7890 GC with a 5975 mass-selective detector using the same column as described above.

2.3.5. AMF colonization of maize roots

To quantify colonization of AMF in maize roots, roots were stained to count AMF structures, specifically hyphae in the maize roots. Briefly, maize roots were heated in 2-5% KOH for 10-30 minutes in a 90ºC water bath and then rinsed. To acidify the roots, they were then soaked in a 1% HCl solution overnight (1-24 hr). Next, roots were stained in an acidic glycerol/trypan blue solution for 10-30 minutes in a 90ºC water bath. After the staining process, roots were destained using acidic glycerol at room temperature and mounted on slides for counting and quantification. For counting, 10 root sections were mounted on one microscope slide per sample. Ten stops were made per one root section where mycorrhizal hyphae were counted and recorded. Lastly, hyphal colonization was

calculated by % hyphal colonization: (100 – average hyphal counts per sample)/100 (Koske & Gemma, 1989; Trouvelot, 1986).

2.3.6. DNA extraction and sequencing for soil samples

DNA extractions were conducted using the DNeasy PowerSoil™ kit (Qiagen, Valencia, CA, USA). Following the manufacturer's instructions, DNA was extracted in duplicate from approximately 0.25 g soil and quantified using a DS-11 Series Spectrophotometer/Fluorometer NanoDrop (DeNovix, Wilmington, DE, USA). Duplicate extractions of all samples (n=80; 40 soil samples x 2 extractions) were sequenced at the University of Minnesota Genomics Center (UMGC) using high throughput 2x250 base pair sequencing on the Illumina MiSeq sequencing platform (Gohl et al., 2016). The 18S (V9) region of the ribosomal DNA was amplified using the '18S_V9_1391_F_Nextera' (GTACACACCGCCCGTC) and '18S_V9_EukBr_R_Nextera' (TGATCCTTCTGCAGGTTCACCTAC) primers (Banos et al., 2018; Berruti et al., 2017; Hadziavdic et al., 2014; Hart et al., 2015; Öpik, M., et al. 2010; and Stockinger et al., 2010). The UMGC staff performed amplification, library preparation, and sequencing.

2.3.7. Bioinformatics and data analysis

Raw sequencing data was subjected to a quality control pipeline for downstream analyses. UNL's local Holland Computing Center (HCC) and DADA2 (Callahan et al., 2016a, b) were used to demultiplex, denoise, filter, trim, and merge the demultiplexed

paired end reads and ultimately generate amplicon sequence variants (ASVs). For 18S rRNA of soil we obtained a total of 2,613,979 quality filtered and trimmed reads. These reads were used to generate the ASV table. Next, ASVs were aligned to construct a phylogenetic tree, which would be used in taxonomy assignment. For the 18S rRNA gene, the SILVA reference library was used at 99% similarity for taxonomic assignment (Quast et al., 2012; Yilmaz et al., 2014). Sequence tables and taxonomy files were then used to create phyloseq objects uning the phyloseq R package (McMurdie and Holmes, 2013). The ASV table was then subject to community composition, alpha diversity, and beta diversity analyses using R (R Core Team, 2021).

2.3.8. Statistics

Agronomic and soil variables were analyzed using one-way ANOVA in JMP (JMP). The relationship between variables and the continuous N fertilization treatments was also assessed using regression analysis in JMP. Bar graphs were used to compare shocked N treatments with their associated continuous N treatment. The data was adjusted using Tukey's adjustment where levels not connected by the same letter are significantly different ($P<0.05$).

For alpha diversity, we used MANOVA to look at historical N application rate, current year N application rate, as well as the interaction for each of the alpha diversity indices measured. We also ran pairwise comparison within each alpha diversity index. For beta diversity, PERMANOVA was used to evaluate CLR transformed data and associated Manhattan distances.

2.4. Results

2.4.1. Agronomic performance

The agronomic performance of maize from 2018 was evaluated by grain yield, dry stover biomass, and total N uptake (Figure 2.2). This study found that maize grain yields were significantly different ($P<0.0001$) among current year zero N (CON 0_0, SKD 188_0 and SKD 57_0), CON 57_57, and higher N (CON 188_188, CON 230_230, SKU 57_188 and SKU 0_188) fertilization treatments. A similar trend was present with dry stover biomass ($P<0.0001$) and total N uptake ($P<0.0001$, Figure 2.2). Overall, all three variables were significant ($P<0.0001$) across continuous (CON) and shocked (SKD & SKU) N fertilization applications. Two additional variables, grain N content and aboveground maize biomass (Supplemental Figure S2.1) were also significant ($P<0.0001$) across continuous (CON) and shocked (SKD & SKU) N fertilization applications.

2.4.2. Soil chemical properties

Soil pH and electrical conductivity (EC) measured at maize reproduction in 2018 did not differ among N fertilization regimes (Supplemental Figure S2.2), yet some significance was found between blocks for soil pH ($P=0.0198$, mean=7.61, standard deviation=0.12) and EC ($P<0.0001$, mean=300.23, standard deviation=66.8). Soil nitrate and ammonium were measured two months after sampling and none of these variables differed in the N fertilization treatments (Supplemental Figure S2.3). Soil extractable P

(P=0.013) and exchangeable K (P=0.019) were significant across N fertilization application and decreased as more N was applied (Supplemental Figure S2.3 and S2.4, respectively). Additional agronomic and soil variables are reported in Supplemental Figures S2.1 to S2.4.

2.4.3. AMF biomass in soil and hyphal colonization of roots

Four outliers were removed from the AMF biomass dataset based on the 'greater than 3 standard deviations' rule (Personal correspondence). Arbuscular mycorrhizal fungal biomass in soil differed among N fertilization treatments (P=0.0057) with no block influence. Regression analysis (Figure 2.3A) showed a negative relationship between AMF biomass and N fertilization rate from the CON N treatments (P=0.0004; R^2=0.47). When comparing SK to CON N treatments (Figure 2.3B), the SKD treatments better matched CON 0_0 while SKU treatments better matched CON 188_188. In contrast to soil AMF biomass, there were no significant differences in % hyphal colonization of maize roots across CON or SKU/SKD treatments (Figure 2.3C, D).

2.4.4. Community composition

We detected 16 unique ASVs belonging to six genera within four families, *Claroideoglomeraceae, Glomeraceae, Paraglomeraceae* and *Archaeosporales* in soil at maize reproduction (Supplemental Table S2.1; Supplemental Figure S2.5). No sequences were found from the order *Diversisporales*. Of the 16 unique ASVs, we identified four as *Septoglomus*, two as *Rhizophagus*, four as *Claroideoglomus*, one as *Paraglomus*, two as

Ambispora, and three as *Glomus*. Total counts of ASVs (Table 2.1) within each of the six genera in declining order are: *Septoglomus* (1236), *Claroideoglomus* (659), *Rhizophagus* (473), *Paraglomus* (163), *Ambispora* (44) *and Glomus* (25). To explore general trends in ASV counts across N fertilization regimes, treatments were consolidated into the following groups: Total ASV counts (all genera), low CON (0_0, 28_28, 57_57), high CON (115_115, 188_188, 230_230), SKU (0 _57, 57_188), SKD (188_0, 57_0), all low (CON 0_0, 28_28, 57_57, SKD 57_0, SKD 188_0), all high (CON 115_115, 188_188, 230_230, SKU 0_188, SKU 57_188) N fertilization treatments. Overall, ASV counts under low CON N rates were ~1.4 times higher than under high CON N rates (Table 2.1). This trend was also present in the amount of AMF biomass from the soil, quantified by the AMF-specific lipid biomarker C16:1c11 (Figure 2.3A & and B). The genera *Septoglomus*, *Rhizophagus* and *Paraglomus* were favored under low CON N. *Claroideoglomus* and *Ambispora* were more abundant under high CON N. When shocked up, ASV counts were half those of shocked down. The greatest reduction in counts were in *Septoglomus* and *Claroideoglomus*, the two most dominant genera overall. *Ambispora* was the only genus to see a slight increase in counts on shocking up.

In Figure 2.4A, the number of AMF genera found within each CON rate ranged from three (CON 230_230) to five (CON 0_0, 115_115 and 188_188) with the remaining two N rates (CON 28_28 and 57_57) having four genera. No CON rate had all six genera. The relative abundance of dominant AMF genera (*Septoglomus*, *Rhizophagus*, and *Claroideoglomus*) showed no consistent relationship with long-term N (CON) fertilization rate (Figure 2.4A; Supplemental Table S2.1). Although not as abundant as

the prior three genera, *Paraglomus* was favored under low CON fertilization (CON 0_0 to 57_57), while *Ambispora* appeared only under higher CON fertilization (CON 115_115 and 188_188) but was absent from the highest fertilization rate (CON 230_230). *Glomus*, a minor contributor to overall AMF abundance, only appeared in CON 0_0 and CON 115_115 fertilization treatments.

In Figure 2.4B, which shows the shocked (SKD and SKU) and associated CON N fertilization rates, *Septoglomus* dominated across all N treatments, ranging from ~42% to 62% of the AMF community. When shocked down (SKD 188_0 or 57_0) *Septoglomus* relative abundance increased above that of the historical N rate (CON 188_188 or 57_57) rather than reflect the current year zero N fertilization rate (i.e., CON 0_0). Changes in relative abundance of *Septoglomus* on SKU depended on the magnitude of the shock: a modest SKU of 57_188 led to a decline in *Septoglomus* at the expense of increased *Rhizophagus*, the appearance of *Ambispora* and loss of *Paraglomus* while a major SKU of 0_188 led to increased abundance *Septoglomus* over CON 0_0, but less than that of CON 188_188 likely due to the proliferation of *Rhizophagus* and loss of *Claroideoglomus*.

For the other dominant AMF genera, results were mixed depending on the severity and direction of the shock: SKD 188_0 led to an increase in *Claroideoglomus* at the expense of *Rhizophagus* and the complete loss of less abundant genera *Paraglomus* and *Ambispora* (Figure 2.4B; Supplemental Table S2.1). In contrast, SKD 57_0 led to an increase in *Rhizophagus* at the expense *Claroideoglomus* with a small change in relative abundance of *Paraglomus*. Also of note was the modest detection of *Ambispora* (2.84%)

and *Glomus* (1.09%) in SKD 57_0, the later also found in similar abundance in CON 0 (2.07%). Shocking up from zero or low to high N (SKU 0_188 or 57_188) led to a significant loss of *Claroideoglomus* and a gain in *Rhizophagus*. *Glomus*, present in small relative amounts under zero N (2.07%) increased a small amount (2.85%) when shocked up to 188 kg N ha^{-1} largely at the expense of *Claroideoglomus*. Also of note is the absence of *Ambispora* at low CON rates and its appearance when shocked up to higher N rates in line with (SKU 0_188) or even greater (SKU 57_188) than its abundance in CON 188_188.

2.4.5. Alpha diversity

We measured three microbial alpha diversity indices, specifically Chao1 for species richness, Pielou's index for species evenness, and Shannon for overall diversity. We ran a global MANOVA and saw significant differences for all three alpha diversity metrics by N fertilization history (Chao1, P=0.0295; Pielou's, P=0.0251; Shannon, P=0.0353), and current year N fertilization rate (Chao1, P<0.0013; Pielou's, P=0.0012; Shannon, P=0.001) (Supplemental Table S2.2). We also observed an interaction effect of N fertilization history by current year N fertilization rate for Chao1 (P<0.0048), Pielou's evenness (P=0.0220), and Shannon diversity (P<0.0086). Pairwise comparisons were conducted separately for CON N and SKD/SKU N treatments (Supplemental Table S2.3). Overall, historical continuous 0 N kg ha^{-1} fertilization supports a slightly more diverse and enriched AMF community compared to those receiving N fertilization (Supplemental Table S2.3); however, low sample size led to lack of significance between

most treatment pairs. Regression analysis showed a trend for decreasing species richness (Chao1) and overall diversity (Shannon) as N fertilization increased among CON N treatment groups, although the relationship was only significant at $P<0.1$ (Figure 2.5) and N fertilization rate only accounted for ~10% of AMF diversity or richness.

2.4.6. Beta diversity

To visualize relationships among N fertilization treatments, we used principal coordinates analysis (PCoA). Differences among AMF communities were evaluated using PERMANOVA of CLR transformed data with the adonis2 function in the vegan package in R. Community dissimilarity was calculated using the Manhattan distance metric (Supplemental Table S2.4) for AMF communities under two scenarios shown in Figure 2.6: (A) all continuous N fertilization treatments (CON 0_0, 28_28, 57_57, 115_115, 188_188, and 230_230); and (B) shocked (SKD and SKU) and associated continuous (CON) N fertilization treatments (CON 0_0, SKD 188_0, SKD 57_0, CON 57_57, SKU 57_188, SKU 0_188, CON 188_188). In Figure 2.6A, we show that AMF community structure differed among historical N application rates ($P=0.0020$). In Figure 2.6B (and Supplemental Table S2.4) comparing shocked N treatment groups to the continuous (CON) N fertilization rates, AMF community structure was only affected by current year (shock) N fertilization rate ($P=0.0003$) and not prior (CON) N history ($P=0.07$), with no significant interaction between the two.

2.5. Discussion

In this 10-year field experiment, we examined the response of AMF communities to a current year perturbation (shock) of historical rates of N fertilization in monoculture maize at reproduction (stage=R2-3) under tilled, rainfed management. In this simplified system, we hypothesized that current year N fertilization rate, including shock N treatments, would be more influential on soil AMF biomass and root colonization than historical N fertilization rate, and that soil AMF community composition would better reflect long-term historical N rates due to buildup of AMF inocula. The following discusses the outcomes of our hypotheses while factoring in soil and agronomic data as explanatory variables.

2.5.1. Maize productivity dictated by current year N fertilization rate

Throughout this 10-year monoculture maize field experiment, maize yields were affected by CON, SKD, and SKU N fertilization treatments. Agronomic variables (maize grain yield, dry stover biomass, total N uptake, grain N content, and aboveground maize biomass) all showed a 'typical' N response curve (Figure 2.2; Supplemental Figure S2.1) with increasing productivity as N rate increased, plateauing near 200 kg N ha^{-1}. However, when N fertilization rate was shocked down (SKD), all maize productivity measures were the same as CON 0_0 N treatment. This could indicate that internal N cycling (N mineralization from SOM) could not offset yield losses, despite adequate root colonization and the observed increase in soil AMF biomass at zero N fertilization (see Section 2.5.2).

2.5.2. AMF biomass in soil responds to current year N fertilization rate

In agreement with prior literature (Jeske et al., 2018; Tian et al., 2013), AMF biomass in soil was strongly and inversely related to N fertilization rate under historical (CON) application rates (Figure 2.3A; R^2=0.47; P<0.0004), while no differences were observed in hyphal colonization of roots across all N fertilization rates in agreement with Tian et al. (2011; 2013). Although, this inverse relationship was strong for historical N rates, the relationship was less clear for shock N treatments (Figure 2.3B) despite high statistical significance (Adj. R^2=0.71; P<0.0001). High standard deviations likely contributed to this outcome given variations in soil across the field experiment (Nasielski et al., 2020) and spatial clustering of AMF near roots that may or may not be captured during soil coring. There could also be a buffering effect due to altered N cycling and capacity for N mineralization based on years of prior CON N treatments. This is supported by the higher average pH of 7.7 in this agricultural field despite urea-ammonia-nitrate (UAN) based fertilizer being applied for 10 years. Typically, application of ammonia-based fertilizers decreases the soil pH via nitrification, or the conversion of ammonium to nitrate and subsequent H+ released in the soil (Geisseler & Scow 2014; Zhalnina et al., 2015).

This inverse relationship between soil AMF biomass and N fertilization rate has also been shown in other agricultural systems such as grasslands and other commodity crops (Abobaker et al., 2018; Bradley et al., 2006; Johnson et al., 2003; Liu et al., 2013; Zhu et al., 2018).

2.5.3. AMF community in soil dominated by few genera in monoculture maize

In this study we detected genera from three of the four main orders of *Glomeromycota*: *Glomerales*, *Archaeosporales* and *Paraglomerales*, but none from *Diversisporales* (Supplemental Figure S2.5). The most dominant genus of the AMF community was *Septoglomus* (family *Glomeraceae*), which ranged from ~34-62% of the relative abundance across all N fertilization treatments (Figure 2.4). *Claroideoglomus*, the sole genus in the family *Claroideoglomeraceae*, was the second most abundant genus, ranging from ~8-54% of relative abundance across all N fertilization treatments. Both genera each contained four unique ASVs' that we were unable to identify to species based on available databases. *Rhizophagus*, the third dominant genus, ranged from ~5-33% of total abundance and contained two unique ASV's. Although *Claroideoglomus* and *Rhizophagus* are regularly reported as dominant genera in maize cropping systems (Moebius-Clune et al., 2013), *Septoglomus* tends to be a minor component in agricultural systems (Säle et al., 2015) and more prevalent under less disturbed or grassland systems (Säle et al., 2015; Xiao et al., 2020). This could also reflect recent reassignment of several *Glomus sp.* (and few *Funneliformis sp.*) to *Septoglomus* including the type species, *Glomus/Funneliformis constrictum* (Redecker et al., 2013). Also unusual was the very low relative abundance of *Glomus* in our study and the absence of *Funneliformis*, both dominant genera detected in several published studies (Alguacil et al., 2014; Hontoria et al., 2019; Luo et al, 2021; Oehl et al., 2003; Tian et al., 2011; Tian et al., 2013: Zeng et al., 2021). Overall, relative abundances of different genera may differ

between maize systems due to climate and land use, soil type, host plant(s), variable management practices, seasonality of sampling, and taxonomic reassignment.

The high relative abundances of the AMF genus *Septoglomus* throughout all N fertilization treatments may be because *Septoglomus* is more resistant to short-term change in N fertilization regimes (shock). This could be due to the nature of *Septoglomus,* in that they are widely distributed across many environments (Table 2.3) including agroecosystems and form their spores mainly in the rhizosphere and sometimes within the roots (Redecker et al., 2013). The proximity to the plant roots and rhizosphere in which AMF produce spores and subsequently colonize maize roots could be important for efficient colonization in the next growing season. This could also aid in overall AMF function in the soil environment, by setting up AMF for successful growth patterns in the future. In addition, certain genera may present specific morphological features that allow for adaptability to variable N environmental conditions, such as highly melanized spores, sporocarp formation, the ability to float in water or high soil moisture environments, etc. (Redecker et al., 2000; Redecker et al., 2013). Another perspective is the AMF continuum of function, ranging from forming a symbiotic, commensalistic, or parasitic relationship with the maize plant based on the trade balance of soil nutrients available (Johnson, 2010). Arbuscular mycorrhizal fungi typically function as an obligate symbiont; thus, they are reliant on the host plant for photosynthetic carbon, and it is a give-and-take regarding the amount of C shuttled from the plant to the AMF community.

2.5.4. Community response of AMF inconsistent across long-term N fertilization

We expected AMF community composition to reflect long-term rates (CON) of N fertilization in a somewhat consistent manner, i.e., linear, or bell-shaped response of dominant genera. Given this was not observed (Figure 2.4A) we divided the CON N rates into two groups: low CON (0, 28, 57 kg N ha^{-1}) and high CON (115, 188, 230 kg N ha^{-1}) N treatments (Table 2.1). Except for *Claroideoglomus and Ambispora*, all remaining genera had greater ASV counts under low CON compared to high CON N treatments, and this mirrors the negative trend in AMF biomass with increasing N rate (Figure 2.3A). These inconsistencies in relative abundances across CON N fertilization treatments may signal differing ecological optima among soil, plant and symbiont created over narrowly defined N fertilization regimes and resulting plant productivity. This may be confounded with variations among genera in sporulation events (Oehl et al., 2009). Alternatively, we cannot rule out inclusion of AMF 'hot spots' during field sampling despite composite sampling of several soil cores or selectivity during DNA extraction of such small soil mass (0.25 g) given heterogeneity in soil particle sizes.

In addition to the high amounts of *Septoglomus*, the alpha diversity of the AMF communities in the soil was more diverse under CON and lower N fertilization treatments compared to the higher CON and SKU N fertilization treatments. This may indicate that higher amounts of N fertilizer application and shocking the maize system, especially with the SKU treatment, decrease AMF diversity, selecting for specific genera. These genera could be selected for based on their elasticity to environmental stressors (e.g., CON 0_0 being SKU 0_188). In addition to specific genera demonstrating more

elastic behaviors compared to others (e.g., *Septoglomus* being present regardless of N fertilization treatment), these results may be a reflection of each genera's ability to shift through the lifestyle continuum, as previously mentioned (Johnson, 2010; Van Der Heijden & Horton, 2009). A study conducted in a 5-year grassland experiment found through sequencing and N fertilization that there was a similar reduction in AMF species richness and diversity, which ultimately led to a loss of rare AMF species and an increase in *Glomus* species (Egerton-Warburton et al., 2007). Arbuscular mycorrhizal communities are responsive to current seasonal dynamics at maize reproduction as demonstrated in Figure 2.6 A & B, where the AMF community structure displayed in the PCoA analyses show less variance with zero and low N fertilization applications. These shifts could indicate that N management approaches shape the soil AMF community structure long-term. Also, AMF respond more to current year N inputs compared to historical, which have ties to organic matter and other inputs into the soil environment. Arbuscular mycorrhizal communities are shaped by edaphic factors (soil pH, soil moisture, soil chemical and physical properties) as well as agronomic management practices (N fertilization, tillage), however, the underlying question remains as to if AMF could be driving plant community composition, or if plant communities drive AMF community diversity and subsequent function (Guzman et al., 2021; Tedersoo et al., 2020).

Although variation in relative abundance was largely the rule for dominant genera across CON fertilization treatments, some trends were noted for minor genera. Specifically, *Paraglomus* (one ASV, or species) was favored under low CON

fertilization, ranging from ~6% at 57 kg N ha^{-1} to ~13% at 0 and 28 kg N ha^{-1} with only trace amounts (~2.4%) at 188 kg N ha^{-1} (Supplemental Table S2.1). There are multiple studies that suggest more intensive management practices negatively impact *Paraglomaceae*, the family that *Paraglomus* is in (Gosling et al., 2014; Oehl et al., 2016) In contrast, *Ambispora* appeared only under high CON fertilization (115 and 188 kg N ha^{-1}) but was absent from the highest fertilization rate (230 kg N ha^{-1}).

The inconsistency in relative abundance of the different AMF genera across CON rates is mirrored in the alpha diversity metrics (Supplemental Table S2.3) despite reported significant differences for all measures (Supplemental Table S2.2). Low sample size prevented clear trends in species diversity, richness and evenness although Shannon Diversity and Chao1 richness had greater overall means at CON 0 N than for other CON N rates. This was further shown by the slight downward trend in AMF community diversity and richness (Shannon diversity and Chao1), with increasing CON N rate ($P<0.1$).

2.5.5. AMF genera differ in their response to shock N fertilization

Not only was *Septoglomus* the most abundant genus, but its behavior to N shock also depended on whether the shock was up (SKU) or down (SKD). Both SKD 188_0 and SKD 57_0, led to small but significant increases relative abundance of *Septoglomus* suggesting rapid adaptation to reduced N inputs (Xiao et al., 2020) largely at the expense of *Rhizophagus* and *Paraglomus* (Figure 2.4B). In contrast, SKU 0_57 or SKU 0_188 led to a decline in *Septoglomus* with shifts in relative abundance of other genera dependant

on whether the shock was moderate (SKU 57_188) or major (SKU 0_188). With a moderate SKU, an increase in *Ambispora* accounted for most of the change, along with the loss of *Paraglomus*, a minor contributor at CON 188_188. This increase in *Ambispora* was not noted for SKU 0_188, where *Glomus* entered the picture along with an increase in *Rhizophagus* and *Paraglomus*, and decreased abundance of *Claroideoglomus*. Thus, members of each of these genera responded in unique ways depending on the direction and severity of the N fertilization shock. Whether these shifts in relative AMF abundance at maize reproduction under altered N fertilizer regimes were related to inoculum potential left from the prior cropping season, or ability to adapt to resulting changes in soil properties and/or maize productivity requires further study.

2.5.6. The role of environment and seasonal dynamics on AMF communities

Given the inconsistencies we see across AMF community composition in the continuous and shocked N fertilization rates and the complexity of AMF community dynamics, there are many alternate scenarios that can impact AMF biomass and community structure in this study. For example, soil type, climate, exact location of soil sampling, and various management practices, such as the type of N fertilizer and tillage, impact the development and subsequent AMF community structure (Abobaker et al., 2018; Gosling et al., 2014). Another variable is soil moisture, as the soil at this field site is high in SOM and rainfed. These conditions, when compared to an irrigated maize system, can lead to non-optimal soil moisture conditions for more sustained N mineralization synchronized to plant growth. It is unlikely that the AMF community was

influenced by prior cropping systems and residues as this was a long-term, monoculture maize experiment. Additionally, there was only one sampling time at maize reproduction which provides a snapshot in time, whereas previous work from our lab and others has shown changes in AMF biomass, sporulation, and community structure throughout the maize growing season (Alvarado- Herrejón et al., 2019; Gavito & Varela, 1993; Jeske et al., 2018; Tian et al., 2013).

In addition to the N fertilization treatments, it is important to consider other environmental variables such as climate and precipitation not only for agriculture productivity, but also for seasonal shifts of AMF community structure. Environmental variables such as precipitation, specifically at a rainfed field site, greatly impact other processes such as C cycling, N cycling, and SOM stabilization and destabilization (Frey, 2019; Rillig et al., 2001; Van Der Heijden et al, 2008). The soil at this field site, an Albic Luvisol, also interacts with AMF ERM development in the soil. From the soil properties measured, Supplemental Figure S2.3, we saw a significant increase in soil pH as N fertilization increased ($P<0.004$), as well as a decrease in extractable phosphorus (P) and exchangeable potassium (K) ($P<0.0129$ and 0.185, respectively). One reason we may see such an increase in soil pH was briefly described above. As N fertilizer is applied, the nitrification process increases and acidifies the soil, however, in this soil environment the H^+ that are acidifying the soil may be altering the exchange site in the soil (Clark & Zeto, 2000; Geisseler & Scow 2014; Zhalnina et al., 2015). This soil also retains a high amount of SOM (4.5%), which could be due to the moldboard plow technique used in this maize agroecosystem. This tillage type is efficient at incorporating the aboveground

decomposing residues into the soil underneath, thus redistributing the OM into the belowground soil layers.

2.6. Conclusions

This research allowed us to focus on the AMF community, maize yields, and N fertilization regimes in a 10-year, uniform agronomic environment. By reducing the number of variables in this study, such as crop rotation and tillage practices, we were able to solidify previous findings and expand upon how AMF interact with the maize mycorrhizosphere. We found that patterns of AMF biomass in soil from this monoculture maize agroecosystem mirrors previous work in our lab (Jeske et al., 2018). This reinforces that AMF extramatrical biomass development during maize growth is inversely related to long-term N fertilization rate spatially. We also found that there were no significant differences in AMF hyphal colonization of maize roots between the N fertilization treatments, which aligns with another study conducted by Tian et al., 2013. This is evidence that indigenous AMF communities respond similarly to maize in agroecosystems from Nebraska, USA to Ontario, Canada. In terms of community composition, only one amplicon (18S rRNA) was used to assess AMF diversity and relative abundance of genera in soils, which could be a limiting factor to exploring the entirety of the AMF community. Another approach would be to use a variety of primers to amplify different regions, thus areas that may or may not detect difference AMF species. Arbuscular mycorrhizal fungi are notoriously difficult to culture, thus the development of their databases used for sequencing is not as developed as other

culturable organisms, such as bacteria. Future work that would complement this research and explore more of AMF function includes transcriptomics of the root and fungal tips in the soil throughout the growth season. Lastly, in addition to furthering our understanding of AMF community shifts through variable N fertilization treatments in maize agroecosystems, it is important to apply this to the bigger picture of creating sustainable management practices that maintain the biology of the soil as well as food production

2.7. Tables and Figures

Table 2.1. Amplicon sequence variant (ASV) counts of genera present in the treatment groups of the 18S rRNA V9 region amplicon for *Glomeromycota.*

18S Genera	**Septoglomus**	**Rhizophagus**	**Claroideoglomus**	**Paraglomus**	**Ambispora**	**Glomus**	**Total ASVs**
Total ASV counts	1236	473	659	163	44	25	2600
Low CON	399	175	195	105	0	10	884
High CON	258	92	264	5	12	3	634
SKU	144	95	24	24	19	7	313
SKD	435	111	176	29	13	5	769
All low	834	286	371	134	13	15	1653
All high	402	187	288	29	31	10	947

There were 6 AMF genera identified in the soils at maize reproduction represented across the top of the table. The treatment groups are as follows: Total ASV counts (all genera), low CON (0, 28, 57 kg N ha^{-1}), high CON (115, 188, 230 kg N ha^{-1}), SKU (0 or 57 kg N ha^{-1} to 188 kg N ha^{-1} N), SKD (188 or 57 kg N ha^{-1} to zero N), all low (0, 28, 57, 57_0 SKD, 188_0 SKD kg N ha^{-1}), all high (115, 188, 230, 0_188 SKU, 57_188 SKU kg N ha^{-1}) N fertilization treatments. For sequencing, n=80; 40 soil samples x 2 extractions.

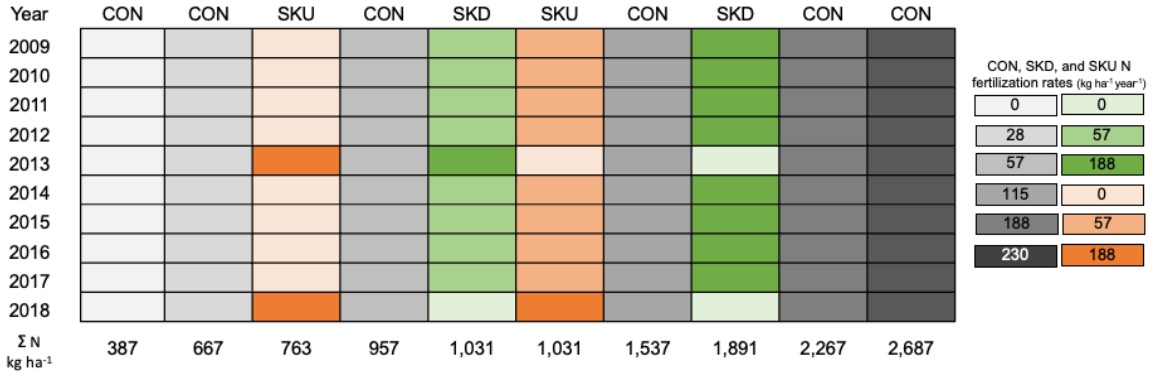
Table 2.3. Functional and environmental attributes of the genera/species assignments found in this experiment.

Previous *Glomus* subgroups	**Current *Glomus* genera**	**Functional and physical attributes**	**Environmental attributes**	**Literature**
Group A, B	*Glomus*	Multi-layer spores. Spores form in a continuum of increasingly complex sporocarps.	Found in many agroecosystems. Contains some species previously thought to be in *Sclerocystis* until 18S sequences revealed position in *Glomus* clade.	Redecker et al., 2000; INVAM
Glomus Group B	*Claroideoglomus*	Multi-layer spores ranging from 1 to 4 layers (L1, L2, etc.). Have subtending hypha.	Found in high abundance in managed ecosystems and is one of the most common genera found throughout the world (from tundra of Alaska to deserts of Namibia)	Bindell, M. et al., 2021; INVAM
Glomus Group Ab	*Rhizophagus*	Spores in roots are highly infective (more so than in soil). Multi-layer spores or varying color with mucilaginous surface layer. Variable distribution throughout host roots possibly due to early colonization in the season. Sometimes form sporocarps.	Arbuscule production seems to peak earlier than in other *Glomus.* Colonization of roots later in the season consisting of almost exclusively intraradical hyphae and aggregates of spores.	Morton & Walker, 1984; INVAM
	Septoglomus	Pigmented spores form singly in soil or as loose clusters. Unclear phylogenetic positioning due to ongoing disagreements among experts.	Widely distributed environmentally and closely related to *Glomus.* Spores mainly formed in rhizosphere and sometimes within roots, abundant glomalin producer.	Redecker et al., 2013; INVAM
	Ambispora	Forms dimorphic spores: acaulosporoid and glomoid morphs. Dimorphic based on SSU data. Somewhat of a taxonomic conundrum.	Found mainly in natural/non-managed ecosystems	Walker, C. 2008; Bindell, M. et al., 2021; INVAM

Table 2.3 continues

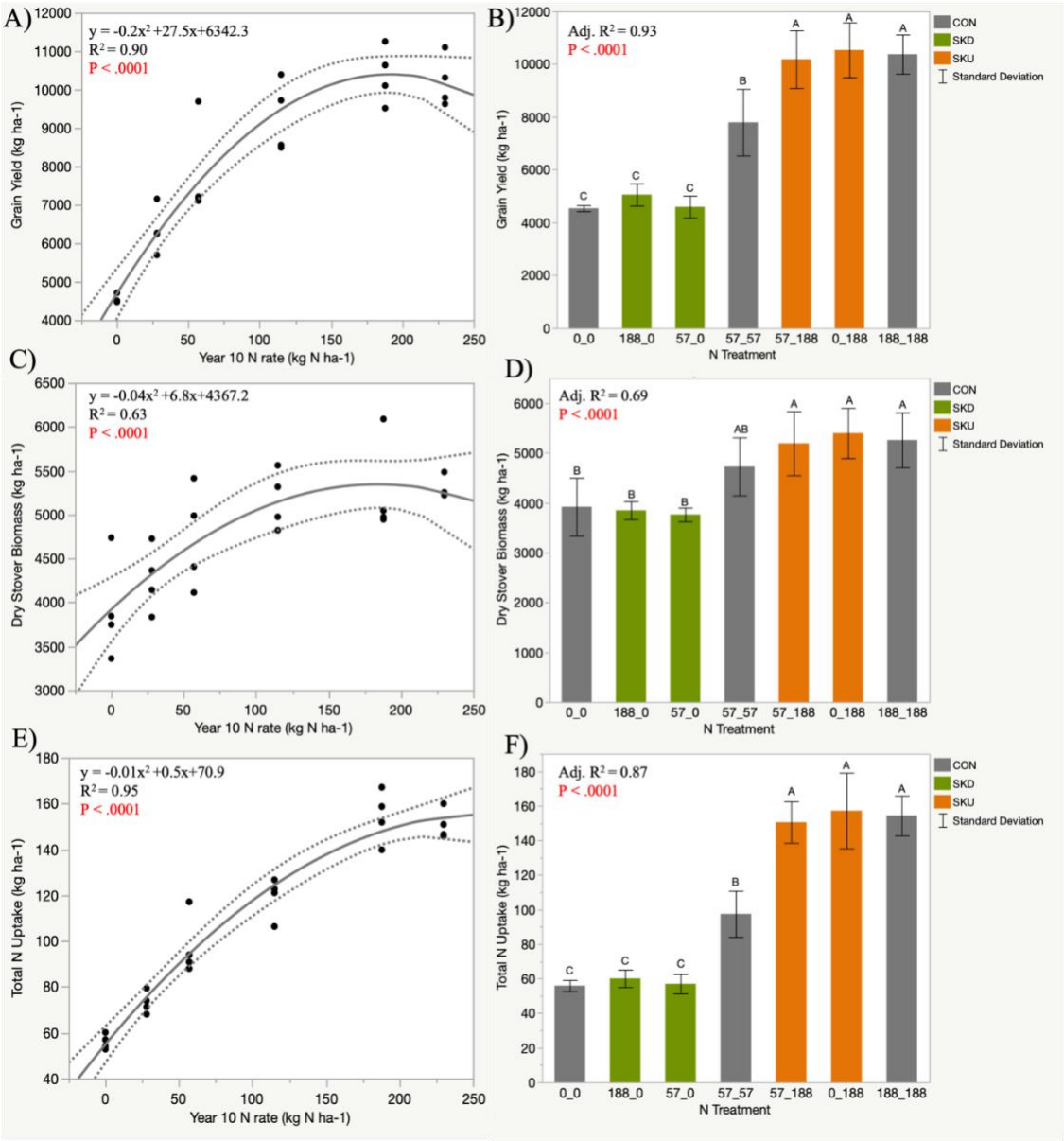
Previous *Glomus* subgroups	**Current *Glomus* genera**	**Functional and physical attributes**	**Environmental attributes**	**Literature**
	Paraglomus	Three-layer spore walls and spores do spores do not float in water (unusual). Subtending hyphae. Infection units sporadically distributed throughout host roots.	Many details about mycorrhizal architecture and behavior remain unknown. Widely distributed throughout the world. One of the most aggressive invaders in acid soils (forest communities) and pot cultures.	INVAM

Figure 2.1. Layout of experimental field site established in 2009 and soil sampled in 2018. Treatments include six 'continuous' (CON) N fertilization rates (0, 28, 57, 115, 188, and 230 kg N ha^{-1}) as well as the four 'shock' (SKD & SKU) treatments in 2018.



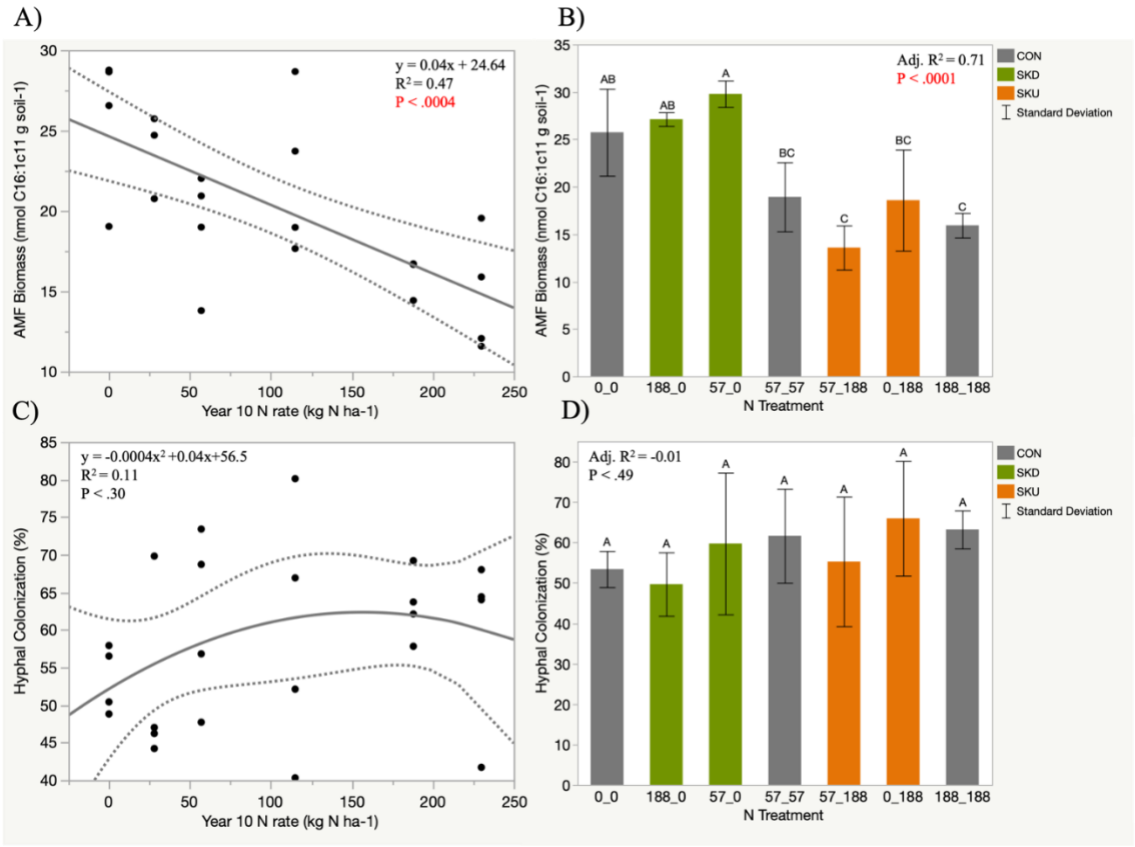
Cumulative N inputs (Σ N kg ha^{-1}) are summed across all 10 years of the experiment. Total N inputs include yearly 30 kg N ha^{-1} incorporated as starter fertilizer (NPK) and a baseline application of 57 kg N ha^{-1} in all treatments in 2009.

Figure 2.2. Agronomic performance of maize from 2018 for continuous (CON) and shocked up (SKU) and down (SKD) N application rates.



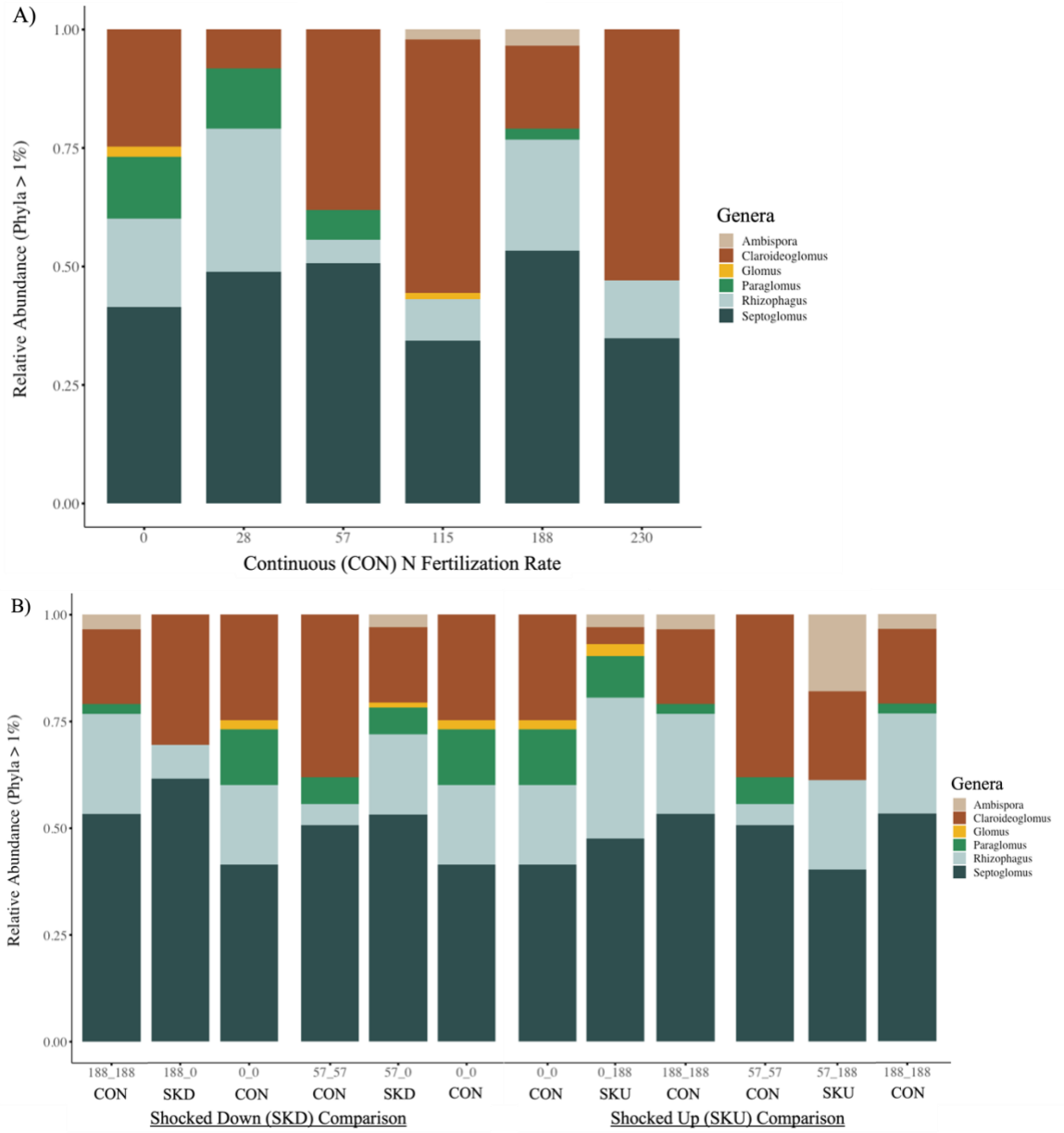
Agronomic variables response to N fertilization treatments include grain yield, dry stover biomass, and total N uptake, significant differences were detected between treatments (alpha=0.05). Soils were sampled on August 7, 2018, in Ontario, Canada. Nitrogen fertilization treatments are grouped by the continuous (CON) and the shocked (SKD & SKU) application rates. The CON N fertilization rates include: 0, 28, 57, 115, 188, & 230 kg N ha^{-1}), SKD includes 188 or 57 kg N ha^{-1} to zero N, and SKU (0 or 57 kg N ha^{-1} to 188 kg N ha^{-1} N).

Figure 2.3. AMF biomass of soil (A, B) and percent (%) hyphal colonization (C, D) of maize roots for continuous (CON) and shocked up (SKU) and down (SKD) N application rates.



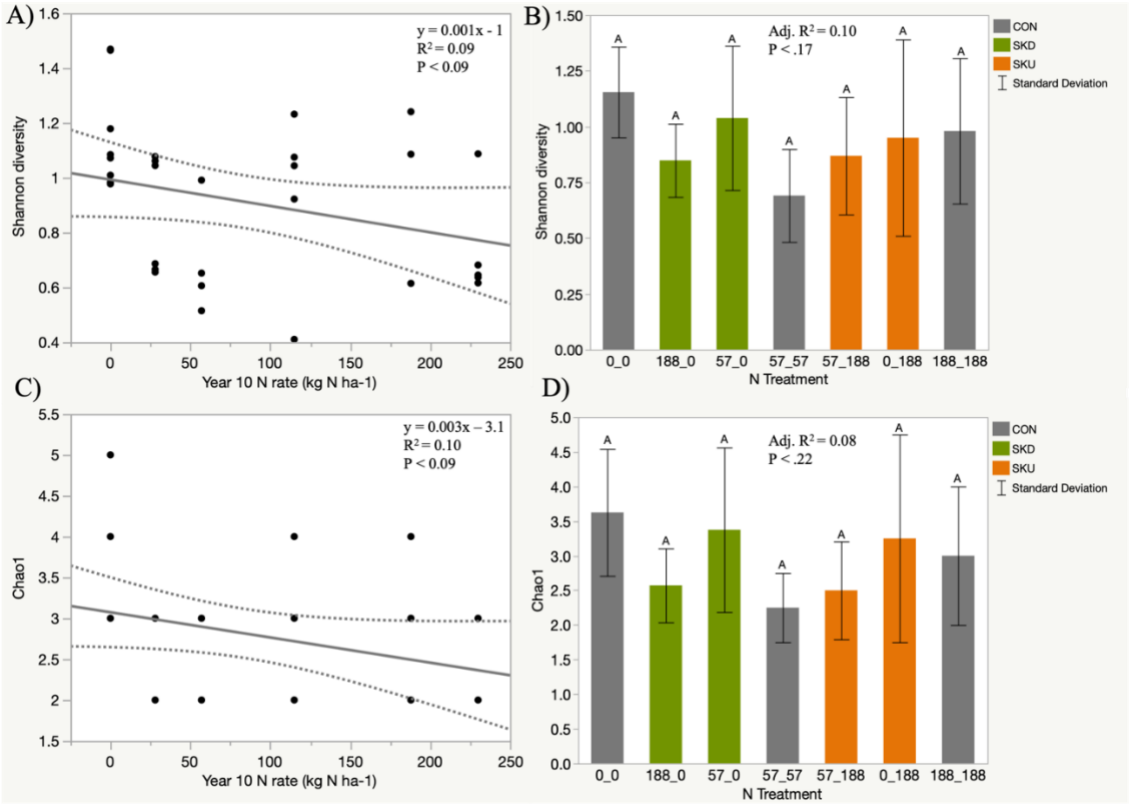
Arbuscular mycorrhizal fungi (AMF) biomass in soils and hyphal colonization of roots response to N fertilization treatments. Significant differences were detected between treatments for AMF biomass in soils (alpha=0.05). Soils were sampled on August 7, 2018, in Ontario, Canada. Nitrogen fertilization treatments are grouped by the continuous (CON) and the shocked (SKD & SKU) application rates. The CON N fertilization rates include: 0, 28, 57, 115, 188, & 230 kg N ha^{-1}), SKD includes 188 or 57 kg N ha^{-1} to zero N, and SKU (0 or 57 kg N ha^{-1} to 188 kg N ha^{-1} N).

Figure 2.4. Changes in relative abundance of AMF genera for (A) continuous (CON) and (B) shocked up (SKU) and down (SKD) N application rates as a percentage of total reads.



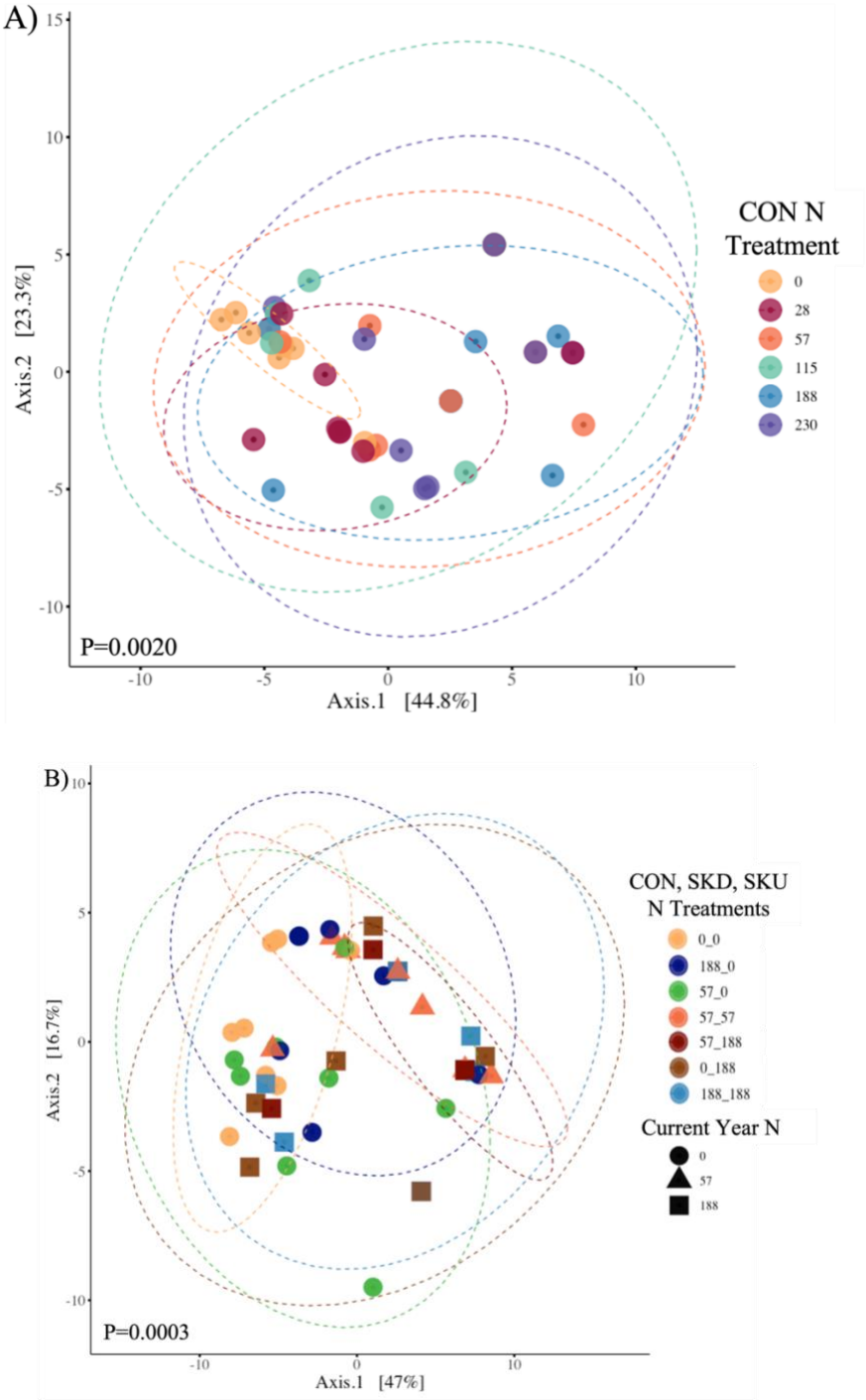
Stacked bar charts for year 2018 showing relative abundance of AMF genera across N fertilization treatments. Soils were sampled on August 7, 2018, in Ontario, Canada. Nitrogen fertilization treatments are grouped by the continuous (CON) and the shocked (SKD & SKU) application rates. The fertilization rates in kg N ha^{-1} include CON 0_0, 28_28, 57_57, 115_115, 188_188, & 230_230; shocked down SKD 188_0 & 57_0 and shocked up SKU 0_188 & 57_188. For sequencing, n=80; 40 soil samples x 2 extractions.

Figure 2.5 Alpha diversity of AMF communities by Shannon (A, B) and Chao1 (C, D) diversity indices based on 18S rRNA sequencing results.



Alpha diversity richness and evenness indices (average ± standard deviation) for the AMF community in soil at maize reproduction. From top to bottom this figure shows Shannon diversity and Chao1 richness indices. Soils were sampled on August 7, 2018, in Ontario, Canada. Nitrogen fertilization treatments are grouped by the continuous (CON) and the shocked (SKD & SKU) application rates. The fertilization rates in kg N ha^{-1} include CON 0_0, 28_28, 57_57, 115_115, 188_188, & 230_230; shocked down SKD 188_0 & 57_0 and shocked up SKU 0_188 & 57_188. For sequencing, n=80; 40 soil samples x 2 extractions.

Figure 2.6. Principal coordinate analysis of AMF community composition for (A) all continuous (CON: 0_0, 28_28, 57_57, 115_115, 188_188, and 230_230 kg N ha-1) fertilization treatments, and (B) for shocked (SKD: 188_0, 57_0 and SKU: 57_188, 0_188) and paired CON (0_0, 57_57, 188_188 kg N ha-1) fertilization treatments. For sequencing, n=80; 40 soil samples x 2 extractions.



2.8. Supplemental Figures and Tables

Supplemental Table S2.1. Relative abundance (as a percentage) of all genera present in the continuous (CON) and shocked (SKD & SKU) N fertilization treatments.

N Treatment	***Septo-glomus***	***Rhizo-phagus***	***Claroideo-glomus***	***Para-glomus***	***Ambispora***	***Glomus***
CON 0_0	41.53	18.60	24.79	13.02	0.00	2.07
CON 28_28	48.84	30.23	8.14	12.79	0.00	0.00
CON 57_57	50.70	4.93	38.03	6.34	0.00	0.00
CON 115_115	34.31	8.79	53.56	0.00	2.09	1.26
CON 188_188	53.40	23.30	17.48	2.43	3.40	0.00
CON 230_230	34.92	12.17	52.91	0.00	0.00	0.00
N Treatment	***Septo-glomus***	***Rhizo-phagus***	***Claroideo-glomus***	***Para-glomus***	***Ambispora***	***Glomus***
CON 0_0	41.53	18.60	24.79	13.02	0.00	2.07
SKD 188_0	61.54	8.01	30.45	0.00	0.00	0.00
SKD 57_0	53.17	18.82	17.72	6.35	2.84	1.09
CON 57_57	50.70	4.93	38.03	6.34	0.00	0.00
SKU 57_188	40.30	20.90	20.90	0.00	17.91	0.00
SKU 0_188	47.56	32.93	4.07	9.76	2.85	2.85
CON 188_188	53.40	23.30	17.48	2.43	3.40	0.00

Of the 16 unique ASVs corresponding to six genera within Glomerales in soil at maize reproduction, we identified four as *Septoglomus*, two as *Rhizophagus*, four as *Claroideoglomus*, one as *Paraglomus*, two as *Ambispora*, and three as *Glomus*.

Supplemental Table S2.2. Global test (MANOVA) of N fertilization history, current year N fertilization rate, and the interaction between the two on AMF community richness and evenness (Chao1 richness, Pielou's evenness, and Shannon Diversity).

Chao1 Richness	**Df**	**SumsOfSqs**	**MeansSqs**	**F**	**P-value**
N History	5	15.341	3.0681	2.7241	0.0295
Current year N	2	17.086	8.5430	7.5851	0.0013
N History x Current year N	2	13.379	6.6895	5.9394	0.0048
Residuals	51	57.44	1.1263		
Pielou's Evenness	**Df**	**SumsOfSqs**	**MeansSqs**	**F**	**P-value**
N History	5	0.9662	0.1932	2.8247	0.0251
Current year N	2	1.0566	0.5283	7.7223	0.0012
N History x Current year N	2	0.5634	0.2817	4.1176	0.0220
Residuals	51	3.4889	0.0684		
Shannon Diversity	**Df**	**SumsOfSqs**	**MeansSqs**	**F**	**P-value**
N History	5	1.4394	0.2879	2.6131	0.0353
Current year N	2	1.7477	0.8738	7.9321	0.0010
N History x Current year N	2	1.1515	0.5757	5.2261	0.0086
Residuals	51	5.6184	0.1102		

Df: degrees of freedom, SumsOfSqs: sums of squares, MeanSqs: mean squares. Analyses carried out with function adonis in R package 'vegan'.

Supplemental Table S2.3. Alpha diversity and measures of evenness of the AMF community in soil at maize reproduction.

N Treatment	Shannon Diversity	Chao1 Richness	Pielou's Evenness
CON 0_0	1.15 ± 0.2 a	3.63 ± 0.9 a	0.92 ± 0.04 a,b
CON 28_28	0.84 ± 0.2 a,c	2.43 ± 0.5 b,c	0.97 ± 0.1 a,c
CON 57_57	0.69 ± 0.2 b,c	2.25 ± 0.5 b,c	0.86 ± 0.1 b
CON 115_115	0.94 ± 0.3 a,c	3.00 ± 0.7 a,c	0.85 ± 0.2 a,b
CON 188_188	0.98 ± 0.3 a,c	3.00 ± 1.0 b,c	0.92 ± 0.1 a,b
CON 230_230	0.73 ± 0.2 b,c	2.20 ± 0.4 b,c	0.94 ± 0.04 a,b
N Treatment	***Shannon Diversity***	***Chao1 Richness***	***Pielou's Evenness***
CON 0_0	1.15 ± 0.2 a	3.63 ± 0.9 a	0.92 ± 0.04 a
SKD 188_0	0.85 ± 0.2 b,c	2.57 ± 0.5 b,d	0.93 ± 0.1 a
SKD 57_0	1.03 ± 0.3 a,c	3.38 ± 1.2 a,b	0.89 ± 0.1 a
CON 57_57	0.69 ± 0.2 b,d	2.25 ± 0.5 c,d	0.86 ± 0.1 a
SKU 57_188	0.87 ± 0.3 b,d	2.50 ± 0.7 c	0.97 ± 0.02 a
SKU 0_188	0.95 ± 0.4 a,c,d	3.25 ± 1.5 a,b,c,d	0.86 ± 0.04 a
CON 188_188	0.98 ± 0.3 a,c,d	3.00 ± 1.0 b,c	0.92 ± 0.1 a

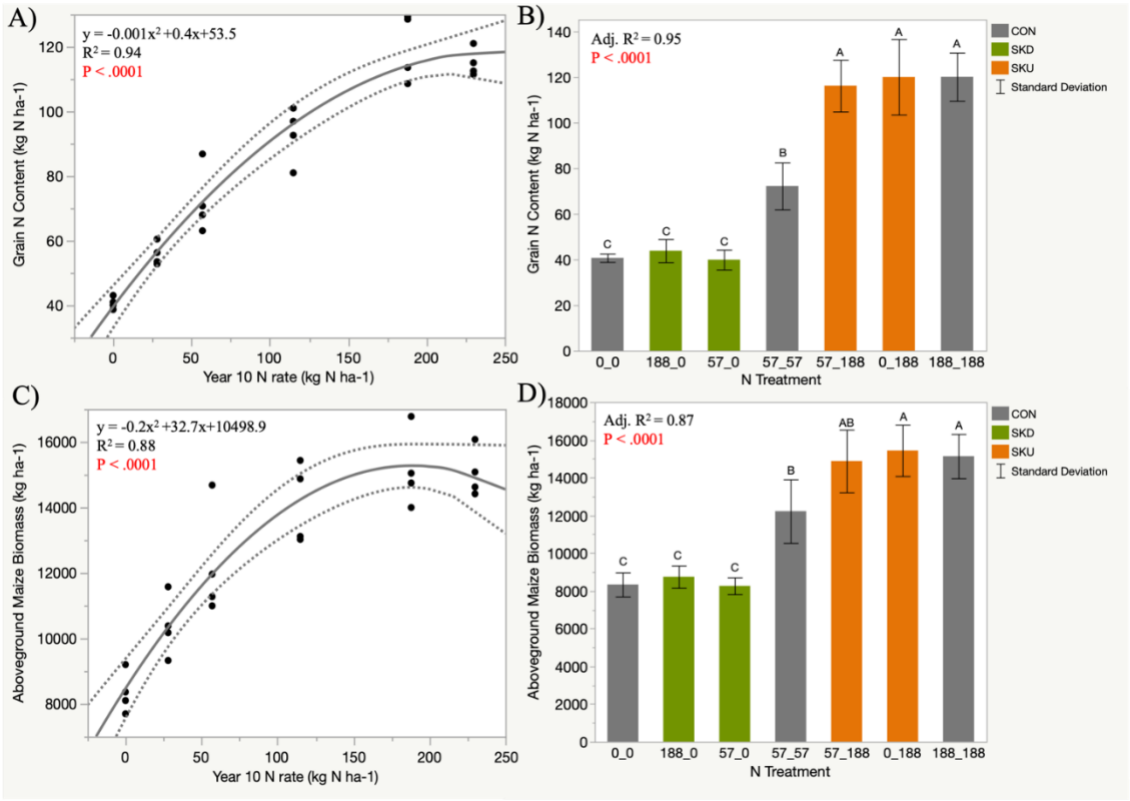
Alpha diversity richness and evenness indices (average ± standard deviation) for the AMF community in soil at maize reproduction. From left to right: Shannon diversity index, Chao1 richness, and Pielou's evenness. Soils were sampled on August 7, 2018, in Ontario, Canada. Nitrogen fertilization treatments are grouped by the continuous (CON) and the shocked (SKD & SKU) application rates. The fertilization rates in kg N ha^{-1} include CON 0_0, 28_28, 57_57, 115_115, 188_188, & 230_230; shocked down SKD 188_0 & 57_0 and shocked up SKU 0_188 & 57_188. For sequencing, n=80; 40 soil samples x 2 extractions. Levels not connected by the same letter are significantly different, Tukey's adjustment at alpha=0.05.

Supplemental Table S2.4. PERMANOVA of N fertilization history, current year N fertilization rate, and the interaction between the two for AMF community composition for Beta diversity.

CON Fertilization	**Df**	**SumsOfSqs**	**R^2**	**F**	**P-value**
N History	5	448.62	0.2288	2.4927	0.0020
Residuals	42	1511.79	0.7712		
Total	47	1960.41	1		
SK & CON Fertilization	**Df**	**SumsOfSqs**	**R^2**	**F**	**P-value**
N History	2	193	0.0604	1.9632	0.0683
Current year N	2	473.5	0.1481	4.8163	0.0003
N History x Current year N	2	122.8	0.0384	1.2495	0.2690
Residuals	49	2408.5	0.7532		
Total	55	3197.8	1		

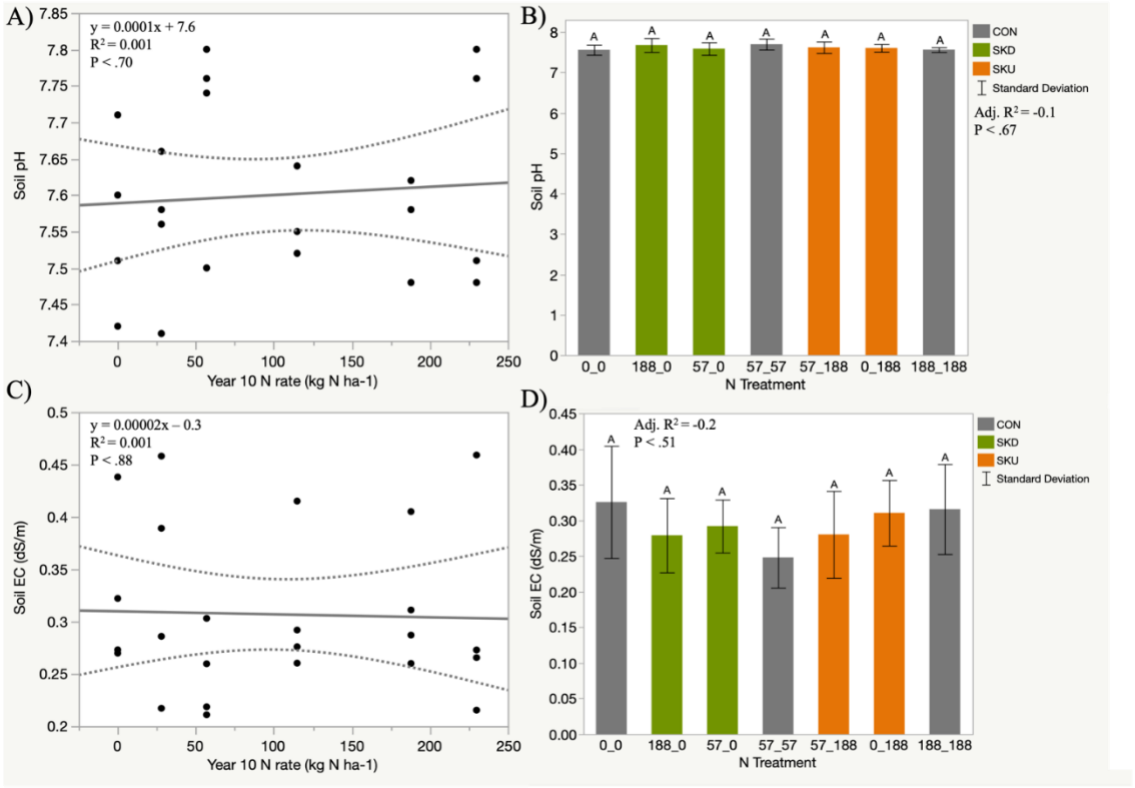
Continuous (CON) N fertilization has 6 N rates (0, 28, 57, 115, 188, 230 kg N ha^{-1}), Shocked (SK) fertilization has 3 N rates (0, 57 and 188 kg N ha^{-1}). Df: degrees of freedom, SumsOfSqs: sums of squares, MeanSqs: mean squares. Analyses carried out with function adonis in R package 'vegan' using 2999 permutations.

Supplemental Figure S2.1. Grain N content (A, B) and above ground maize biomass (C, D) measured at maize harvest in 2018.



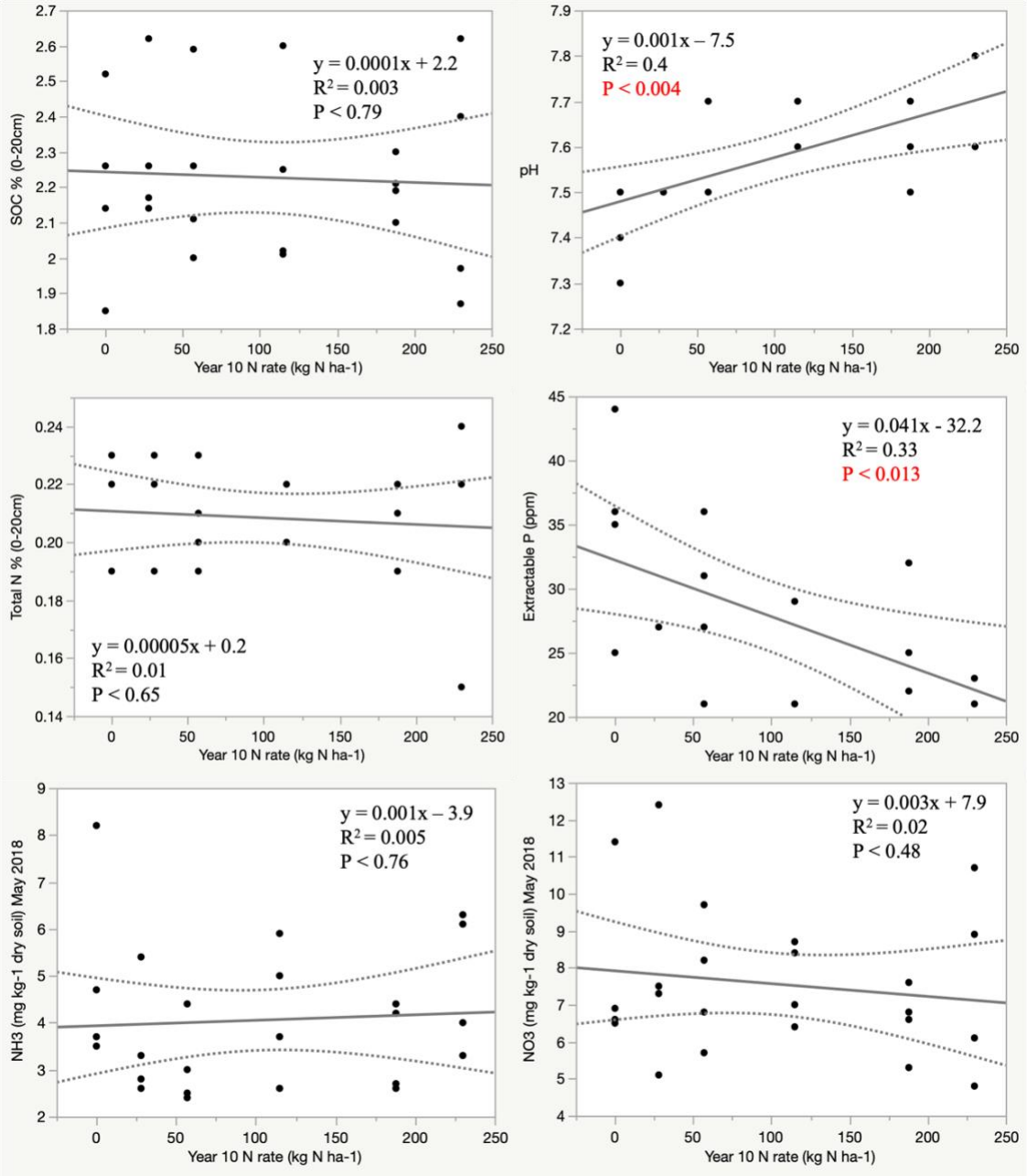
Agronomic variables response to N fertilization treatments include grain N content and aboveground maize biomass, significant differences were detected between treatments (alpha=0.05). Soils were sampled on August 7, 2018, in Ontario, Canada. Nitrogen fertilization treatments are grouped by the continuous (CON) and the shocked (SKD & SKU) application rates. The fertilization rates in kg N ha^{-1} include CON 0_0, 28_28, 57_57, 115_115, 188_188, & 230_230; shocked down SKD 188_0 & 57_0 and shocked up SKU 0_188 & 57_188.

Supplemental Figure S2.2. Soil pH (A, B) and soil EC (C, D) measured in 2021.



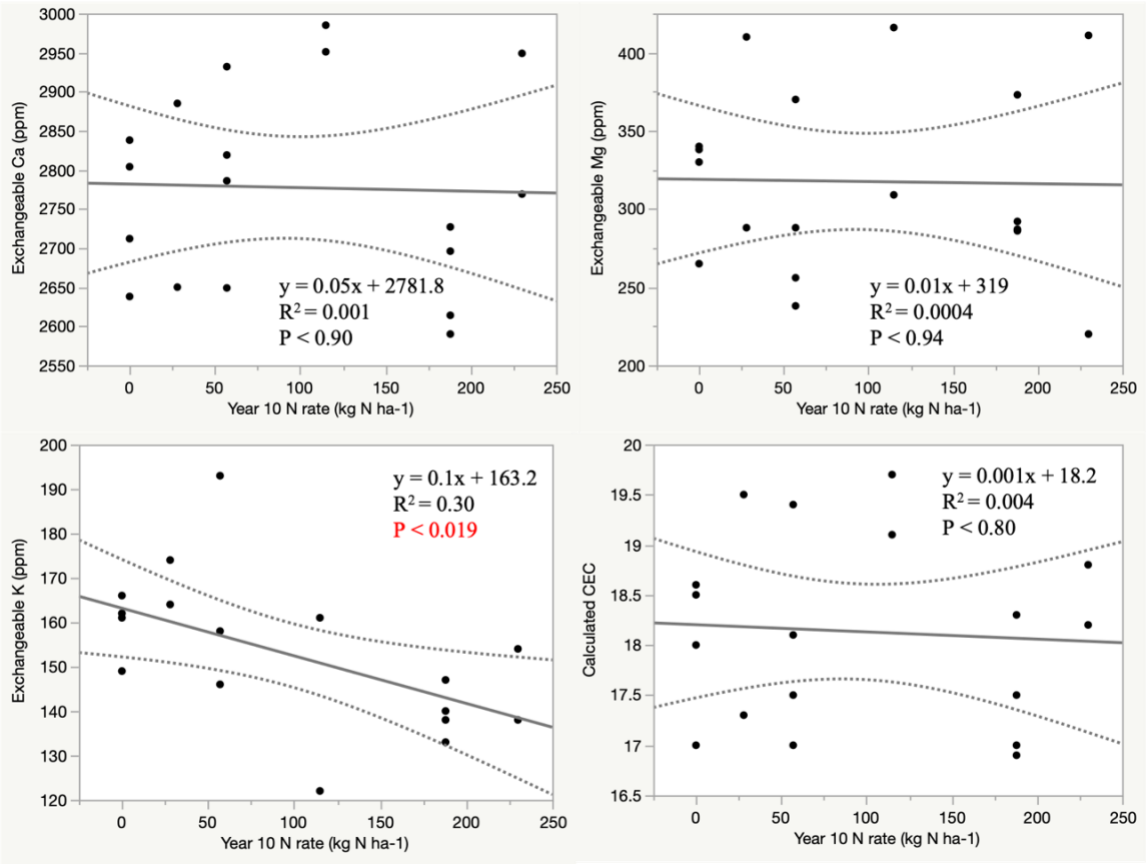
Soil pH and EC measurements' response to N fertilization, no significant differences were detected between treatments (alpha=0.05). Soils were sampled on August 7, 2018, in Ontario, Canada, frozen, and measurements were taken in 2021. Nitrogen fertilization treatments are grouped by the continuous (CON) and the shocked (SKD & SKU) application rates. The fertilization rates in kg N ha^{-1} include CON 0_0, 28_28, 57_57, 115_115, 188_188, & 230_230; shocked down SKD 188_0 & 57_0 and shocked up SKU 0_188 & 57_188.

Supplemental Figure S2.3. Soil properties measured on the 0-20 cm depth in May 2018 and May 2019.



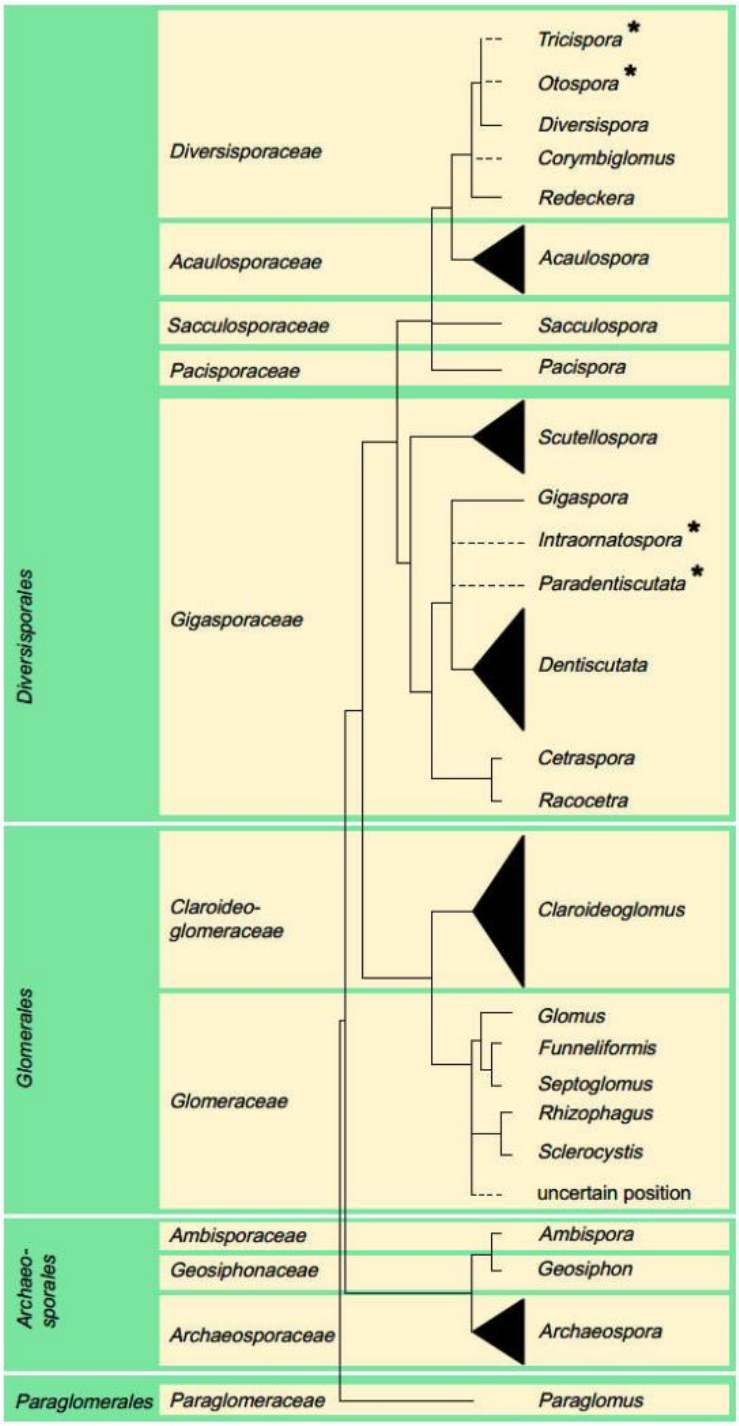
Additional soil variables' response to N fertilization. Nitrogen fertilization treatments are grouped by the continuous (CON) and the shocked (SKD & SKU) application rates. The fertilization rates in kg N ha^{-1} include CON 0_0, 28_28, 57_57, 115_115, 188_188, & 230_230; shocked down SKD 188_0 & 57_0 and shocked up SKU 0_188 & 57_188.

Supplemental Figure S2.4. Soil exchangeable cations measured on the 0-20 cm depth in May 2019.



Additional soil variables' response to N fertilization. Nitrogen fertilization treatments are grouped by the continuous (CON) and the shocked (SKD & SKU) application rates. The fertilization rates in kg N ha^{-1} include CON 0_0, 28_28, 57_57, 115_115, 188_188, & 230_230; shocked down SKD 188_0 & 57_0 and shocked up SKU 0_188 & 57_188.

Supplemental Figure S2.5. Classification of *Glomeromycota* modified from Redecker et al. (2013) from http://www.amf-phylogeny.com/. Genera marked by asterisks are questionable with respect to data used for description and/or with respect to phylogenetic position.



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Chapter 3: Long-term N fertilization and diverse crop rotations influence arbuscular mycorrhizal biomass and community structure in maize cropping systems in Eastern Nebraska

3.1. Abstract

Arbuscular mycorrhizal fungi (AMF) are important plant symbionts that benefit the host plant by enhancing nutrient and water acquisition in exchange for photosynthetically fixed carbon. Although phosphorus acquisition has long been the main focus of AMF research, nitrogen is gaining recognition as a key component of the symbiosis, particularly in agronomic systems receiving high inputs of N fertilizer. Our recent work demonstrated that AMF colonization of maize roots was independent of N fertilizer rate (Tian et al., 2013); however, there was a strong inverse relationship between N fertilization rate and the abundance of extramatrical AMF (Jeske et al., 2018). Understanding these interactions are vital, not only for the success of agronomically important crops but for soil organic carbon stabilization, soil aggregate formation, and carbon sequestration, processes fostered by AMF abundance in soil. To build on our prior research, we further explored the role of crop rotational diversity and N fertilization rate on AMF biomass and community diversity in a long-term, dryland maize cropping system. In collaboration with the USDA and previous work conducted in our lab, this field site brings over 40 years of crop and nitrogen management history and enables us to draw meaningful connections between an evolutionarily important plant symbiont and core ecological processes related to carbon and nitrogen cycling. Results from this work include AMF ERM biomass in the soil as well as amplicon sequencing of AMF to characterize the effect of management history on taxonomic diversity. Outcomes from

this research will help elucidate critical management strategies to optimize the AMF-maize partnership to the benefit of both crop productivity and soil health.

Abbreviations

AMF, arbuscular mycorrhizal fungi; FAMEs, fatty acid methyl esters; CCCC, continuous corn; CSCS, corn-soybean-corn-soybean; CSGO, corn-soybean-sorghum-oats/cover; COGS, corn-oats/clover-sorghum-soybean; ASVs, amplicon sequence variants

Keywords

Arbuscular mycorrhizal fungi (AMF), nitrogen fertilization, crop rotation, AMF extramatrical biomass, long-term field site, FAMEs, 18S rRNA sequencing

3.2. Introduction

Creating more sustainable agronomic systems requires implementation of management strategies that maintain, stabilize, and enhance soil organic carbon (SOC). Soil organic carbon stabilization and carbon (C) sequestration are vital to cultivating agroecosystems that can withstand extreme weather events, which are the reality with continuing climate change (Beillouin et al., 2022; Dignac & Rumpel, 2013; Lal, 2004; Schmer et al., 2020). Arbuscular mycorrhizal fungi (AMF) also contribute to the stabilization of SOM as well as nutrient cycling in agroecosystems, which leads to a larger buffer of the soil system to withstand extreme climate events (Frey, 2019; Johnson, 2010). The AMF contribution to SOM formation and stabilization occurs through

mycorrhizal exudates, necromass accrual, and distribution of plant-derived C throughout the soil matrix for deposition within soil pores and onto mineral surfaces (Frey, 2019). Agricultural management strategies that can enhance SOC in agroecosystems include diverse crop rotations, N fertilizer application in various forms, cover crops, and incorporating residue inputs (Cong et al., 2015; Dias et al., 2015; King & Blesh, 2018; Schmer et al., 2020; Tiemann et al., 2015). Crop rotation practices alleviate and disrupt plant and insect pathogen lifecycles, provide more diverse inputs into the soil system, and enhance soil chemical, biological, and physical properties (Karlen et al., 2006; Katsvairo et al., 2002). These crop rotation practices, along with understanding the intricate dynamics of arbuscular mycorrhizal fungi (AMF), can lead to improved yields in agronomic systems by enhancing SOC and create a more stable aboveground system (Sindelar et al., 2016). Despite this importance, there is limited understanding of how long-term management histories shape AMF community structure and diversity in agronomic soils.

Crop rotation practices are important for crop production and yield stability (Sindelar et al., 2016). The rotation of crops can contribute to more enhanced soil chemical, physical, and biological properties by diversifying inputs into the soil system and ultimately sustaining more biodiverse agroecosystems (Alhameid et al., 2020; Bowles et al., 2020). Guzman et al., (2021) found that increasing crop diversity enriched the AMF community in soils, which mitigated the effects of agricultural intensification (e.g., long-term monoculture, intensive tillage, excessive N fertilization application). Magurno et al., (2015) evaluated AMF communities in four types of crop rotation (maize

monocrop, maize-alfalfa, maize-wheat, and maize-spring barley-peas-wheat) and found that spore abundance and root colonization were significantly different between the rotations. This is likely due to host-specific associations, wherein AMF community structure shifts with the current year crop and contributes to unique community assemblages with higher species richness and overall diversity under more diverse cropping histories (Guzman et al., 2021). The contribution of AMF to agronomic outcomes, including crop biomass and yields has been well-documented, showing that with higher AMF diversity and abundance there were significant increases in plant nutrition, stress resistance, and photosynthesis (Wu et al., 2022). Additionally, AMF contribute substantially to the necromass pool in soils due to the high turnover rate of mycorrhizal tissues, which is a major factor in SOM formation and stabilization (Cotrufo et al., 2013; Frey, 2019; Schmidt et al., 2011). Thus, investigating the role of rotation diversity in supporting AMF community biomass and diversity is important for understanding how crop productivity and soil health may be promoted in sustainable ways.

In addition to increasing plant diversity via crop rotation, N fertilization applications can increase plant crop yields and productivity (Schmidt et al., 2011; Tiemann et al., 2015), however, these practices are not sustainable long-term. Throughout the Western Corn Belt, maize is an intensively grown and produced crop that contributes to grain production, worldwide economics, soil C sequestration, and ultimately food security (Cassman et al., 1999; Duvick & Cassman, 1999; Ren et al., 2018; Sacks and Kucharik, 2011). A main management practice in maize production throughout the

Western Corn Belt is large quantities of N fertilization (Liebig et al., 2002; Tenorio et al., 2021). Nitrogen fertilization practices increase maize yields; however, excess N is lost to the surrounding environment, leading to nitrate leaching and pollution across ecosystems (Nasielski et al., 2020; Zhang et al., 2015). It has been well documented that AMF biomass in the soil decreases as N fertilization increases (Han et al., 2020; Jeske et al., 2018; Tian et al., 2013; Zhang et al., 2020). In addition to decreases in AMF biomass, there are also losses of AMF diversity, through decreases in species present in the AMF community (Zhang et al., 2020; Zhu et al., 2016). Alpha diversity measurements from multiple studies saw a decrease in Shannon diversity, the ACE index, and Chao1 indices of the AMF community as more N fertilizer was applied (Borriello et al., 2013; Zhang et al., 2020; Zhu et al., 2016). Thus, we are interested in how the history of N fertilization application may have long-term consequences on AMF biomass, diversity associated with maize crops, and overall AMF community structure. Deepening our understanding of how crop diversity, including monoculture maize and more diverse crop rotations, along with N fertilization influences AMF development, biomass, and diversity in the surrounding soil will give insight into how to cultivate a more resilient and healthier agroecosystem using multiple management practices.

Previous work from this long-term, rainfed, no-till cropping system showed that monoculture corn, in a two- or four-year rotation, maintained yield stability (Sindelar et al., 2016), and examined how crop diversification, crop sequence, and N management history influence belowground habitat and soil microbial communities. However, with more diverse crop residue and plant C and N exudate inputs into the soil environment via

more rotational diversity, we enhance not only AMF development and subsequent ERM, but the entirety of the soil microbiome. The diverse inputs support more biodiversity in the soil by increasing the plant functional groups and compounds introduced into the soil system, and ultimately plant host diversity shifts the AMF community into a more diverse and rich grouping (Guzman et al., 2021). Thus, we hypothesize long-term N fertilization and increasing rotational diversity support a more biodiverse soil AMF community. We also hypothesize that stabilization of the soil environment molded AMF extraradical mycelium (ERM) biomass and diversity responses within the growing season, as the AMF were able to draw upon larger pools of nutrients and soil organic matter. This field experiment evaluates a monocrop system compared with more diverse crop rotations and a range of N fertilization treatments to evaluate how these management practices can increase SOC stocks throughout the soil profile (Schmer et al., 2020) and examine if crop rotation can offset yield loss with more diverse crop rotations. Ultimately this research will provide important insight into how diverse crop rotation, along with N management history, influence AMF community diversity and structure. Lastly, further understanding how these agronomic management practices influence AMF communities will give more insight into how SOC pools may be stabilized through mycorrhizal necromass and crop production may be enhanced through symbiotic associations in order to promote more sustainable practices.

3.3. Materials and methods

3.3.1. Experimental location and description

This long-term, experimental field site was established in 1972 and later modified in 1983. Additional details regarding these changes can be found in Sindelar et al., 2016. The rainfed field site is located near Ithica, Nebraska (31° 10' N, 96° 25' W) with soils classified as Yutan silty clay loam-Tomek silt loam complex (fine-silty, mixed, superactive, mesic Mollic Hapludalfs, smectitic, mesic Pachich Arguidolls, respectively). This study collected soil samples over two years, 2014 and 2015 (a snapshot of the field design can be found in Supplemental Figure S3.1). The mean annual precipitation and temperature over 30 years (1985-2015) are 78.3 cm and 10.3°C (High Plains Regional Climate Center, Station ID Mead 6S, http://climod.unl.edu/). The monthly average maximum and minimum temperatures, as well as total precipitation amounts from 2014 to 2015 were similar to the 30-year averages. In addition, the total precipitation between the two years was similar, except that precipitation was greater pre-season (March and April) and at corn planting (May) in 2015 compared to 2014.

The experimental design of this field site was a randomized complete block design arranged in split plots with five replicates. Within this experimental design, the main plot factor was crop rotation and the split plot factor was N fertilization. The split plots were 9 m wide (12 rows, 76 cm between rows) and 10 m long. Crop within this study include corn (*Zea mays* L.), soybean [*Glycine max* (L.) Merr.], grain sorghum [*Sorghum bicolor* (L.) Moench], and an oats [*Avena sativa* (L.)]/clover [80 *Melilotus officinalis* Lam. + 20 *Trifolium pretense* L.] mixture. The samples from this two-year

experiment were collected in the corn phase of the rotation (Figure 3.1). The rotations are as follows: continuous corn (CCCC), a two-year corn-soybean (CSCS) rotation, a four-year corn-soybean-sorghum-oats/clover (CSGO) rotation, and another four-year corn-oats/clover-sorghum-soybean (COGS).

3.3.2. Nitrogen application, planting, crop productivity, and soil sampling

Nitrogen fertilizer was surface broadcast annually as ammonium nitrate (34-0-0) before 2007, and as urea (46-0-0) since 2007. The application rates varied with crop as follows: 0 (none), 90 (low), and 180 (high) kg N ha^{-1} for corn and grain sorghum, and 0 (none), 34 (low), and 69 (high) kg N ha^{-1} for soybean and oats/clover. Nitrogen fertilizer was broadcast to corn on May 30, 2014, and June 2, 2015. The study was disked twice annually between 1983 and 2006, and in 2007 the study was converted to no-till. Additional information can be found at Ramirez II (2020), and Sindelar et al. (2016).

Corn was planted on May 5th, 2014, and May 13th, 2015, at a population of approximately 47,000 seeds ha^{-1} using a six-row planter. Harvest dates were on September 25th, 2014, and October 1st, 2015. Corn hybrids expressed transgenic resistance to European corn borer (*Ostrinia nubilalis*) and glyphosate [potassium N-(phosphonomethyl)glycine]. Early group III, glyphosate resistant soybean was planted at approximately 370,000 seeds ha^{-1} in 76 cm rows. Grain sorghum was planted at approximately 173,000 seeds ha^{-1} in 76 cm rows, and the oats/clover (*Rhizobium*-inoculated clover) was planted at 100 and 18 kg ha^{-1} respectively in 19 cm rows using a no-till grain drill.

Crop productivity was previously reported in Sindelar et al. (2016). To summarize, corn, soybean, and sorghum aboveground biomass dry matter samples were collected at physiological maturity by sampling 5 m of a row. The reproductive corn ears and sorghum heads were removed from the stakes and the rest of the plant matter was dried at 60˚C to a constant mass and weighed. The separated corn ears and sorghum heads were also dried to a constant mass at 60˚C, threshed, and then weighed so the grain weights could be used as aboveground biomass. The dried corn cobs, sorghum panicles, and grain were weighed and added to the aboveground biomass calculations. Next, soybean was harvested as a whole plant and dried to a constant mass at 60˚C, and weights were used to calculate aboveground biomass. The grain from the aboveground biomass were weighed to determine total amounts of aboveground biomass. Corn, soybean, and sorghum grain yields were measured by combine-harvesting three rows of the plot and adjusting to a moisture content of 155, 130, and 130 g kg^{-1}, respectively.

Soil samples were collected at multiple time points in both collection years. In 2014, these time points included 10 days post fertilization (June 10th 2014) at the V6 corn growth stage for baseline soil properties and at V9/10 (July 10th 2014), VT/R1 (August 13th 2014), and R5/6 (October 8th 2014) for seasonal soil properties, fatty acid methyl esters (FAMEs) and potential extracellular enzyme activity (EEA). In 2015, the collection dates were as follows: V9/10 (July 1st 2015), VT/R1 (July 21st 2015), and R5/6 (September 3rd 2015). For soil sampling, 15 soil cores were collected using step-down probes (approximately 2cm DIA) to a depth of 20 cm and were then composited by plot after removing the corn stover from the soil surface. Of the 15 soil cores, 10 were

sampled between rows and 5 within rows to represent different soil microsites. The composite soil sample was homogenized in the field and split into two subsamples, one subsample was sieved (4 mm) fresh to remove visible debris and subsequently frozen at -20˚C for FAMEs extraction. The second subsample was air dried for potential EEA analysis and soil chemical analysis (WARD Laboratories, Inc., Kearney, NE).

3.3.3. Seasonal and baseline soil chemical properties

Soils were collected for baseline and seasonal soil chemical properties at the V6 corn growth stage (June 10th, 2014), 10 days post fertilization with broadcast urea. Analyses followed the recommended chemical soil test procedures for the North Central Region (Ward Laboratories, Inc., Kearney, NE). To summarize, soil pH was measured using a 1:1 soil:deionized water extract and a Ross Sure-Flow reference electrode standardized with buffer solution. Soluble salts (1:1) were determined by measuring electrical conductivity (EC) expressed as mmho cm^{-1}. Soil organic matter (SOM) was measured by loss on ignition expressed as a percentage. Nitrate-N was extracted using a 500-ppm calcium phosphate solution and determined by cadmium reduction coupled with sulfanilamide color development measured at 520 nm by a Lachat QuickChem 8500. Exchangeable soil cations potassium (K), calcium (Ca), magnesium (Mg), and sodium (Na), were extracted using 1N ammonium acetate (NH_4OAc) and analyzed by an Inductively Coupled Argon Cooled Plasma Spectrometer (ICAP). Soil cation exchange capacity (CEC) is the sum of cations and was calculated using % base saturation from the exchangeable basic cations from the NH_4OAc extraction along with pH, when applicable.

Soil phosphorous (P) was extracted with Mehlich III and determined by ammonium molybdate and L-ascorbic acid color development measured by a Lachat QuickChem 8500 at 800 nm.

During the 2014 and 2015 corn growing seasons, soil properties were measured at all three dates on the same soil samples collected for soil microbial properties. Soil pH, EC, and OM were determined as described for the above baseline soil properties. Gravimetric water content (% moisture) was determined by oven drying samples at 105˚ C until they reached a constant weight and by then dividing the difference between wet and dry masses by the mass of the dry sample. Water-extractable organic C (WEOC, ppm C) and N (WETN, ppm N) were determined by shaking 4 g of dry soil with 40 mL of deionized water for 10 minutes on a mechanical shaker. Next, samples were centrifuged for 5 minutes at 3500 rpm, filtered through Whatman 2 V filter paper, and analyzed using a Torch Combustion TOC/TN analyzer (Teledyne Tekmar). Water extractable organic nitrogen (WEON) was calculated by subtracting the inorganic N content, which is the summation of NH_4-N and NO_3-N, from WETN. The Haney, Haney, Hossner, and Arnold (H_3A) extractant was used to extract total P (H_3A TP) measured by ICP (Thermo Fisher 6500 Series). This extractant is designed to simulate root exudates and is made up of lithium citrate plus citric acid, malic acid, and oxalic acid (Haney et al., 2006; 2010). Inorganic P was determined on the same extract by ammonium molybdate and L-ascorbic acid color development on a Lachat QuickChem 8500 at 800 nm. H_3A organic P (H_3A OP) was calculated by subtracting inorganic P from H_3A TP. More information on soil chemical properties is expanded upon in Ramirez II (2020).

3.3.4. Quantification of AMF biomass in soil

The AMF-specific fatty acid biomarker, C16:1*cis*11, was used to quantify AMF biomass in the soil (Olsson, 1999). Five grams of soil was extracted with 0.2 M KOH in methanol according to the method of Jeske et al., (2018). The resulting fatty acid methyl esters (FAMEs) were quantified on an Agilent 7890 gas chromatograph fitted with an Ultra 2 HP (Agilent) capillary column (50 m 0.2 mm I.D., 0.33 µm film thickness) using helium as the carrier gas. The injector was maintained at 280ºC and the flame ionization detector at 300 ºC. The oven temperatures were held at 50ºC for 2 minutes, then ramped up by 40ºC min^{-1} to 160ºC for 2 minutes, then ramped up again by 3ºC min^{-1} to 300ºC for 30 minutes. Sample masses of individual FAMEs were calculated from peak areas relative to the internal standard methyl nonadecanoic acid and reported as nmol FAME g^{-1} dry soil or relative abundance (nmol). The identity of C16:1*cis*11 was confirmed by gas chromatography mass spectrometry on an Agilent 7890 GC with a 5975 mass-selective detector using the same column as described above.

3.3.5. DNA extraction and sequencing

DNA extractions were conducted using the DNeasy PowerSoil™ kit (Qiagen, Valencia, CA, USA). Following the manufacturer's instructions, DNA was extracted in duplicate from approximately 0.25 g soil and quantified using a DS-11 Series Spectrophotometer/Fluorometer NanoDrop (DeNovix, Wilmington, DE, USA). Duplicate extractions of all samples (n=360 for 2014; n=360 for 2015) were sequenced at the

University of Minnesota Genomics Center (UMGC) using high throughput 2x250 base pair sequencing on the Illumina MiSeq sequencing platform (Gohl et al., 2016). The 18S (V9) region of the ribosomal DNA was amplified using the '18S_V9_1391_F_Nextera' (GTACACACCGCCCGTC) and '18S_V9_EukBr_R_Nextera' (TGATCCTTCTGCAGGTTCACCTAC) primers (Banos et al., 2018; Berruti et al., 2017; Hadziavdic et al., 2014; Hart et al., 2015; Öpik, M., et al. 2010; and Stockinger et al., 2010). The UMGC staff performed amplification, library preparation, and sequencing.

3.3.6. Data analysis, bioinformatics, and statistical analyses

Raw sequencing data was subjected to a quality control pipeline for downstream analyses. UNL's local Holland Computing Center (HCC) and DADA2 (Callahan et al., 2016a, b) were used to demultiplex, denoise, filter, trim, and merge the demultiplexed paired end reads and ultimately generate amplicon sequence variants (ASVs). Throughout this process, we found that reverse read quality was poor, which prevented reliable matching with forward reads due to the lack of overlap between the forward and reverse reads. Due to this, only the forward reads were used for further processing. All reads less than 250 bp were discarded and chimeric sequences were removed using the 'removeChimeraDenovo' function. For 18S rRNA of soil from 2014 we obtained a total of 10,918,499 quality filtered and trimmed reads, and 7,929,147 for CRS 2015. Next, ASVs were aligned to construct a phylogenetic tree, which would be used in taxonomy assignment. For the 18S rRNA gene, the SILVA reference library was used at 99%

similarity for taxonomic assignment (Oksansen et al., 2007; Quast et al., 2012; Yilmaz et al., 2014). Sequence tables and taxonomy files were then used to create phyloseq objects using the phyloseq R package (McMurdie and Holmes, 2013). The ASV table was then subject to community composition, alpha diversity, and beta diversity analyses using R (R Core Team, 2021). Arbuscular mycorrhizal fungi phyloseq objects were cleaned independently leading to 49 genera out of 360 samples in CRS 2014, and 65 genera out of 360 in 2015 CRS samples.

3.3.7. Statistics

Arbuscular mycorrhizal biomass in soil was analyzed using repeated measures ANOVA in JMP (JMP) and previously analyzed in R, which can be expanded upon in Ramirez II, 2020. For alpha diversity, we used MANOVA to look at historical N application rate, current year N application rate, as well as the interaction for each of the alpha diversity indices measured. We also ran pairwise comparison within each alpha diversity index. For beta diversity, PERMANOVA was used to evaluate CLR transformed data and associated Manhattan distances.

3.4. Results

3.4.1. Climate conditions and corn productivity in 2014 and 2015

The monthly average low and high temperatures in 2014 and 2015 approximated the 30-year average throughout the maize growing season, which ran from February to September in Eastern Nebraska. May 2014 had less precipitation (16.46 cm) compared to May 2015 (19.81 cm), and then this trend flipped with more precipitation in June 2014

compared to June 2015 (21.16 cm and 15.34 cm, respectively). In terms of climate, the two years examined in this study were quite different. These results were previously described in Ramirez II (2020) and can be found in Supplemental Table S3.6.

Maize productivity was higher in 2015 even though the crop was planted a week later compared to May 2014 (May 5th, 2014 versus May 13th, 2015). In addition to the higher maize productivity in 2015, there was also more precipitation (19.81 cm) compared to May 2014 (16.46 cm). The higher amount of precipitation may have contributed to higher maize productivity (Ramirez II, 2020).

3.4.2. AMF biomass in the soil

Arbuscular mycorrhizal fungi biomass showed a clear inverse relationship with N fertilization rate. In 2014, AMF biomass in the soil was significantly different (Table 3.1A and Figure 3.2A) across the maize growth stage ($P<0.0001$), the N fertilization treatments ($P<0.0001$), and crop rotation ($P=0.0086$). There were also significant differences in the interaction terms of growth stage by rotation ($P=0.0021$) and growth stage by N fertilization ($P=0.0023$), and no significance in the three-way interaction of growth stage by N fertilization by crop rotation (Table 3.1A).

In 2015, AMF biomass in the soil presented the same trends as in 2014 (Table 3.1B and Figure 3.2B). There were significant differences in AMF biomass in the soil across the maize growth stage ($P<0.0001$), the N fertilization treatments ($P<0.0001$), and crop rotation ($P=0.0020$). There were also significant differences in the interaction terms of growth stage by rotation ($P<0.0001$) and growth stage by N fertilization ($P=0.0029$),

and no significance in the three-way interaction of growth stage by N fertilization by crop rotation (Table 3.1B).

3.4.3. AMF community composition

In 2014, we detected 49 unique amplicon sequence variants (ASVs) belonging to seven genera within five families, *Claroideoglomeraceae, Glomeraceae, Gigasporaceae, Sacculosporaceae,* and *Paraglomeraceae,* in soil at maize reproduction (Supplemental Table S3.1A and Figure 3.3A). Of the 49 unique ASV's we identified three as *Claroideoglomus,* ten as *Gigaspora,* fourteen as *Glomus,* nine as *Paraglomus,* seven as *Rhizophagus,* one as *Sacculospora,* and five as *Septoglomus.* In 2015, we detected 65 unique ASV's belonging to seven genera within five families, *Claroideoglomeraceae, Glomeraceae, Gigasporaceae, Sacculosporaceae,* and *Paraglomeraceae,* in soil at maize reproduction (Supplemental Table S3.1B and Figure 3.3B). Of the 65 unique ASV's we identified five as *Claroideoglomus,* ten as *Gigaspora,* twenty as *Glomus,* twelve as *Paraglomus,* ten as *Rhizophagus,* one as *Sacculospora,* and seven as *Septoglomus.* In 2014 and 2015, No sequences were found from the orders *Diversisporales* or *Archaeosporales*.

To explore general trends in AMF community composition across growth stages of both years, and then within each treatment and growth stage across both years, we calculated relative abundance as a percentage of all genera. In year 2014, *Septoglomus* (~35-53%) was the most dominant genus across growth stages (inclusive of N fertilization treatment and crop rotation) with *Glomus* following behind (~26-28%). The

more minor genera present included *Paraglomus* (~10-19%), *Rhizophagus* (~8-16%), *Gigaspora* (<1-3%), *Claroideoglomus* (~1-1.4%), and *Sacculospora* (only present at V1012 growth stage at 0.06%). In 2015, *Paraglomus* was the most dominant genus (~28-48%) with *Septoglomus* (~20-26%) and *Glomus* (~11-24%) being the next most abundant genera. The more minor genera included *Rhizophagus* (~11-18%), *Gigaspora* (~2-7%), *Claroideoglomus* (~1-2%), and *Sacculospora* (only present at V89 growth stage at 0.03%). Across both years, the main genera that dominated the AMF community composition were *Septoglomus, Paraglomus, and Glomus*.

To explore these trends in the relative abundance of AMF genera more deeply, we calculated relative abundance as a percentage by each treatment, within each growth stage (Supplemental Table S3.2 for 2014 and S3.3 for 2015). In the 2014 early (V1012) maize growth stage (Supplemental Table S3.2A), we found that three main genera, *Septoglomus, Paraglomus, and Glomus*, were most abundant in most treatments including CCCC (zero, low, and high N), CSCS (zero, low, and high N), CSGO (zero, low, and high N), and lastly all COGS (zero, low and high N) treatments. This trend was present throughout the other two maize growth stages (VTR1 and R6) of 2014 (Supplemental Table S3.2B, C). More minor genera present throughout the growth stages, crop rotations, and N fertilization treatments of 2014 included *Rhizophagus, Claroideoglomus, Gigaspora, and Sacculospora. Rhizophagus* was more abundant in the CSCS crop rotation throughout each maize growth stage, while also having relatively high abundances in the COGS crop rotation (~6-32%) across all growth stages. *Claroideoglomus* was low across all growth stages (~0-8%) and we saw more abundance

in the CSGO crop rotation in each growth stage. *Gigaspora* did not present any obvious trends in relative abundance, other than it was most prevalent in the CCCC crop rotation. Lastly, *Sacculospora* was the rarest genus showing up only in one treatment, CSGO_zero at 0.98% (Supplemental Table S3.2).

In the 2015 early (V89) maize growth stage, we found that three main genera, *Septoglomus, Paraglomus, and Glomus*, with *Rhizophagus* trending towards the top three most abundant genera mentioned previously (Supplemental Table S3.3A). These four genera were the highest in abundance in most treatments including CCCC (zero, low, and high N), CSCS (zero, low, and high N), CSGO (zero, low, and high N), and lastly all COGS (zero, low and high N) treatments. This trend was present throughout the other two maize growth stages (VT and R5) of 2015 and *Rhizophagus* was present in higher amounts in all crop rotations and all N fertilization treatments throughout each maize growth stage, while showing relatively high abundances in the COGS crop rotation (~8-30%) across all growth stages, similar to 2014 (Supplemental Table S3.3). More minor genera present throughout the growth stages, crop rotations, and N fertilization treatments of 2014 included *Claroideoglomus, Gigaspora, and Sacculospora. Claroideoglomus* was low across all growth stages (~0-12%) and we saw more abundance in the CSGO crop rotation in each growth stage. *Gigaspora* did not present any obvious trends in relative abundance. Lastly, *Sacculospora* was the rarest genus showing up only in one treatment, COGS_low at 0.61% (Supplemental Table S3.3).

3.4.4. Alpha diversity

We measured three microbial alpha diversity indices for 2014 and 2015, specifically Shannon for overall diversity, Chao1 for species richness, and Pielou's index for species evenness (Table 3.2, 3.3, and Figure 3.4). In 2014 sequencing data, we ran a global MANOVA and saw no significance for N fertilization (Shannon, P=0.8743; Chao1, P=0.2668; Pielou's, P=0.0554), yet we did see significant differences in crop rotation for each diversity index (Shannon, P=0.0027; Chao1, P=0.0022; Pielou's, P=0.0337), and significance in maize growth stage (Shannon, P<0.0001; Chao1, P<0.0001; Pielou's, P=0.0037). We saw no significant 2-way or 3-way interactions across the alpha diversity measurements, except for in Pielou's evenness of the AMF community (Crop rotation x N fertilization; P<0.0001; crop rotation x maize growth stage, P=0.0221). We saw no block effect in any of the measured alpha diversity indices (Table 3.2).

In 2015 sequencing data, nitrogen fertilization was significant for each diversity index (Shannon, P=0.0012; Chao1, P=0.0059; Pielou's, P=0.0006), as well as significant differences in crop rotation for Shannon diversity (P=0.0397) and Chao1 (P<0.0001), with no significance for Pielou's evenness (P=0.1058). We also saw significance in maize growth stage for Chao1 (Chao1, P=0.0385) but not for Pielou's or Shannon diversity (Pielou's, P=0.7153; Shannon, P=0.1361) and we found no significant 2-way or 3-way interactions. We also saw no block effect in any of the measured alpha diversity indices (Table 3.3).

3.4.5. Beta diversity

To visualize relationships among N fertilization treatments and crop rotations within each growth stage in 2014 and 2015, we used principal coordinates analysis (PCoA). Differences among AMF communities were evaluated using PERMANOVA of CLR transformed data with the adonis2 function in the vegan package in R. Community dissimilarity was calculated using the Manhattan distance metric (Table 3.4) for AMF communities under three N fertilization treatments and four crop rotations shown in Figure 3.5A, B, and C for 2014 and Figure 3.5D, E, and F for 2015. For 2014, using a global MANOVA test (Table 3.4) we show that AMF community structure differed at $P<0.001$ for N fertilization treatment, crop rotation, and maize growth stage. We also found a significant interaction between crop rotation and N fertilization treatment ($P=0.012$). For 2015, using a global MANOVA test (Table 3.4) we show that AMF community structure differed again at $P<0.001$ for N fertilization treatment, crop rotation, and maize growth stage. We also found a significant interaction between crop rotation and N fertilization treatment ($P<0.001$) as well as growth stage by N fertilization ($P<0.001$). The three-way interaction, growth stage by crop rotation by N fertilization treatment, was only slightly significant at $P=0.042$ for the 2015 AMF community. These significant two-way and three-way interactions provide insight into how environmental factors, such as precipitation, can shape the AMF community structure from year to year. As we have previously found, AMF are sensitive to N fertilization application, and we are now able to say they are also sensitive to crop rotations, ranging from monoculture maize to more diverse 4-crop rotations. These findings were also

significant across the maize growing season. To further explore where these significant differences in AMF community came from, we ran a pairwise adonis2 test to examine pairwise interactions between the crop rotations and found significance across every pairwise comparison ($P<0.01$ for all pairwise comparisons, data not shown).

3.5. Discussion

In this long-term agricultural field experiment, we examined the response of AMF communities to a historical and current year N fertilization treatment in four crop rotations, ranging from monoculture maize to two, four-crop rotations, under no-till, rainfed management. In this complex agroecosystem, we hypothesized that long-term N fertilization and increasing rotational diversity support a more biodiverse soil AMF community. We also hypothesized that stabilization of the soil environment molded AMF extraradical mycelium (ERM) biomass and diversity responses within the growing season, as the AMF were able to draw upon larger pools of nutrients and soil organic matter. The following discusses the outcomes of our hypotheses while factoring in agronomic and climate data as explanatory variables.

3.5.1. Field site history and maize productivity are impacted by seasonal precipitation

This long-term, rainfed, no-till field site has demonstrated significant yield stability in the monoculture maize rotation over time (Sindelar et al., 2016). These findings are inclusive of the additional crop rotations that include maize in two or four-

year rotations and have been demonstrated across other maize agricultural systems as well, specifically with crop rotations that include a legume, such as soybean (Gentry et al., 2013; Kaye et al., 2007; Pedersen & Lauer, 2003). As this is a rainfed field site, the yearly and seasonal precipitation is important for maize productivity. The precipitation amounts between 2014 and 2015 were quite different (Supplemental Table S3.6). In May 2014 there was less precipitation (16.46 cm) compared to May 2015 (19.81 cm), and this trend was flipped with more precipitation in June 2014 compared to June 2015 (21.16 cm and 15.34 cm, respectively). Furthermore, the precipitation and overall climate impacts maize productivity and the associated belowground AMF community. Many studies have shown that AMF are highly sensitive to precipitation, with lower quantities of precipitation resulting in lower AMF hyphal length density and overall species diversity (Lu et al., 2020; Zhang et al., 2016). These impacts on AMF community structure over time can also be impacted by the decomposition and incorporation of crop residues into the soil system, which is an important consideration in a no-till, rainfed agronomic system. When the AMF community structure was evaluated by year, inclusive of all growth stages, crop rotations, and N fertilization regimes, soil moisture was a significant ($P=0.0005$; Supplemental Table S3.5) environmental factor in 2015 in shaping the AMF community. These results, along with the precipitation data from each year (Supplemental Table S3.6) ultimately give insight into the formation and persistence of specific AMF genera across this long-term, rainfed agroecosystem. The assembly of the AMF community structure is tied to the stabilization of the aboveground and belowground systems and SOM dynamics, as plants are thought to be important drivers

of AMF community composition due to their symbiotic lifestyle (Tedersoo et al., 2020; Tiemann et al., 2015)

3.5.2. AMF community dominated by two main AMF genera in 2014 and 2015

We detected genera from three of the four main orders of *Glomeromycota*: *Glomerales*, *Diversisporales* and *Paraglomerales*, but none from *Archaeosporales* (Supplemental Figure S3.4). The most dominant genus of the AMF community in 2014 was *Septoglomus* (family *Glomeraceae*), which ranged from ~35-53% of the relative abundance across all N fertilization treatments, crop rotations, and maize growth stages. The next most abundant genus in 2014 was *Glomus* (26-29%), followed by *Paraglomus* (10-19%), *Rhizophagus* (8-16%), *Gigaspora* (0.8-3%), *Claroideoglomus* (0.5-1.4%), and lastly *Sacculospora* (0-0.6%).

There was a switch in the most abundant genus in 2015, which was *Paraglomus* that ranged from 28-48% of the relative abundance across all N fertilization treatments, crop rotations, and maize growth stages (Supplemental Table S3.1). The next most abundant genus in 2014 was *Septoglomus* (20-26%), followed by *Glomus* (11-24%), *Rhizophagus* (11-18%), *Gigaspora* (2-7%), *Claroideoglomus* (1.3-2%), and lastly *Sacculospora* (0-0.3%). Overall, relative abundances of different genera may differ between maize systems due to climate and land use, soil type, host plant(s), variable management practices, seasonality of sampling, and taxonomic reassignment.

3.5.3. AMF community composition response varied across maize growth stages and became more diverse as crop rotations went from 2 to 4 crop rotations

As previously mentioned, the AMF community composition was dominated by two main genera across two years (*Septoglomus* and *Paraglomus)*, yet there were higher amounts of more minor genera present as crop rotational diversity increased. For example, *Claroideoglomus* was more abundant in the CSGO and COGS crop rotations compared to the CSCS and monoculture maize rotations. *Glomus* was present in much higher amounts compared to previous findings from a monoculture maize, rainfed system (Chapter 2). *Gigaspora* had the opposite trend, appearing more frequently in monoculture maize and the CSCS crop rotation. *Rhizophagus* was consistently found in all crop rotations across all growth stages, and *Sacculospora*, the least abundance genus in this study, was only found in the CSGO and COGS crop rotations in both 2014 and 2015, respectively. The appearance of more minor genera in the more diverse crop rotations could be an indication that perhaps crop sequence does not matter as much in AMF community composition, and the fact that there are more plant species present in the agroecosystem has larger impact on the formation of a more resilient AMF community via more diverse exudates and inputs into the soil environment.

3.5.4. Soil moisture and current year precipitation drove AMF community composition and structure

Climate and precipitation are important factors in agronomic productivity and the assembly of the soil microbial communities associated with plants (Lu et al., 2020).

Given the patterns we see across the AMF community composition throughout the maize growing season of both years, there are certain factors, such as precipitation and soil moisture, that weigh more heavily on overall AMF composition and function in these highly productive systems (Supplemental Figure S3.3A, S3.3B & Supplemental Table S3.4, S3.5 for associated statistics). As there was more precipitation in May of 2015 compared to May 2014, perhaps soil moisture drove more of the AMF community assembly and subsequent dynamics. Soil moisture plays a large role in most microbial nutrient facilitated processes, such as N and C cycling, and overall crop productivity; thus, it is an important to take it into consideration when understanding AMF community composition in a rainfed system. These conditions, when compared to an irrigated maize system, can lead to non-optimal soil moisture conditions for more sustained N mineralization synchronized to plant growth. Additional factors in addition to precipitation and soil moisture that impact AMF community composition and diversity include the exact location of soil sampling as there may be AMF ERM 'hotspots', the type of N fertilizer, no-till or other tillage practices, and crop rotation impact the development and subsequent AMF community structure (Guzman et al., 2021; Higo et al., 2020).

These variables have large impacts on overall agroecosystem productivity and function, however, climate and precipitation appeared to be the main drivers of AMF community composition and diversity in this study. These environmental variables, specifically at a rainfed field site, greatly impact other processes such as C cycling, N cycling, and SOM stabilization and destabilization (Frey, 2019; Rillig et al., 2001; Van

Der Heijden et al, 2008). These important ecological processes, in addition to prior cropping rotations and the more diverse residues and inputs, likely influenced the AMF community composition. Some of these more diverse residues and inputs can be described as root exudates and rhizodeposits from the plant host (Bardgett et al., 2005; Wardle et al., 2004). Additionally, there was sampling conducted throughout the maize growing season, which reinforces previous work from our lab and others that has shown changes in AMF biomass, sporulation, and community structure throughout the maize growing season (Alvarado-Herrejón et al., 2019; Gavito & Varela, 1993; Jeske et al., 2018; Tian et al., 2013). Lastly, this is a no-till agroecosystem, thus the hyphal networks have a higher chance of being maintained seasonally, instead of being mechanically broken apart from tillage practices (Higo et al., 2020; Jansa et al., 2002; Jansa et al., 2003). This continuity of the AMF hyphal network(s) may lead to increased amounts of SOM stabilization via increased glomalin production (Frey 2019; Sekaran et al., 2019; Singh et al., 2018). Overall, diversification of the plants aboveground cultivates a more resilient belowground, and subsequent, AMF community.

3.6. Conclusions

Crop rotation and diversification of inputs into this long-term system promotes sustainability of the agroecosystem and of the arbuscular mycorrhizal fungi (AMF) community in the soil. As previously established, AMF have an inverse relationship with N fertilization application across the maize growing season, thus, it is vital to cultivate an agricultural system that can maintain productivity while reducing environmental

pollution. Throughout this study, AMF community composition and diversity were dominated by two to three main genera (*Septoglomus, Paraglomus,* and *Glomus)* from *Glomeromycota* across two years, three N fertilization treatments, and four crop rotations. Our findings show that more diverse crop rotations led to a more diverse AMF community, regardless of the order of plant sequence (CSGO versus COGS). Overall, optimizing the agroecosystem to maintain and promote sustainability requires intimate knowledge on the aboveground and belowground communities, specifically the symbionts of plants, AMF. These aboveground and belowground communities are highly sensitive to the more erratic and extreme climatic events and precipitation, thus, continuing to disentangle these intricacies in the assembly and resiliency of the AMF community in maize systems is of the utmost importance in creating more sustainable production systems.

3.7. Tables and figures

Table 3.1. AMF biomass in the 0-20 cm depth at three corn growth stages during the 2014 (A) and 2015 (B) maize growing season from Ramirez II, 2020.

A)

Stage & rotation			
V9/10 [10 July]	High	Low	None
CCCC	10.4	13.1	18.4
CSCS	9.6	11.0	14.1
CSGO	10.1	10.6	15.5
COGS	11.4	11.4	15.2
VT/R1 [13 Aug.]			
CCCC	17.2	25.4	30.6
CSCS	13.9	18.5	23.8
CSGO	18.7	23.7	28.3
COGS	21.0	21.5	31.5
R5/6 [8 Oct.]			
CCCC	15.2	16.9	23.1
CSCS	12.8	12.6	17.3
CSGO	15.5	16.7	22.7
COGS	15.5	16.2	17.3

Source of Variation	df	Pr>F
Rotation (R)	3	0.0086
R SEM		*0.715*
Nitrogen (N)	2	<0.0001
N SEM		*0.511*
Stage (S)	2	<0.0001
S SEM		*0.602*
N x R	6	NS
S x R	6	0.0021
S x N	4	0.0023
S x N x R	12	NS

B)

Stage & rotation	High	Low	None
V9/10 [1 July]			
CCCC	4.36	6.80	8.56
CSCS	5.46	5.54	7.62
CSGO	5.18	7.36	8.36
COGS	7.10	7.12	10.1
VT/R1 [21 July]			
CCCC	10.7	15.1	18.5
CSCS	11.8	11.4	16.6
CSGO	15.0	19.3	21.4
COGS	13.2	15.5	17.1
R5/6 [3 Sep.]			
CCCC	15.1	21.4	24.3
CSCS	13.8	17.7	21.9
CSGO	18.9	28.0	29.4
COGS	16.4	20.1	23.3

Source of Variation	df	Pr>F
Rotation (R)	3	0.0020
R SEM		*0.644*
Nitrogen (N)	2	<0.0001
N SEM		*0.518*
Stage (S)	2	<0.0001
S SEM		*0.406*
N x R	6	NS
S x R	6	<0.0001
S x N	4	0.0029
S x N x R	12	NS

In corn & grain sorghum, high (180 kg N ha^{-1}), low (90 kg N ha^{-1}), & none (0 kg N ha^{-1});
in soybean & oats/clover, high (69 kg N ha^{-1}), low (34 kg N ha^{-1}), & none (0 kg N ha^{-1})
Continuous corn (CCCC); corn-soybean-corn-soybean (CSCS);
corn-soybean-sorghum-oats/clover (CSGO); corn-oats/clover-sorghum-soybean (COGS)
SEM; stand error of the mean.

Table 3.2. Global test (MANOVA) of the 2014 field block, crop rotation, N fertilization rate, maize growth stage, the then 2-way and one 3-way interaction(s) for AMF community richness and evenness alpha diversity (Shannon Diversity, Chao1 richness, and Pielou's evenness).

Shannon Diversity	**Df**	**SumsOfSqs**	**MeansSqs**	**F**	**P-value**
Block	1	0.0340	0.0339	0.2473	0.6193
Nitrogen	2	0.0370	0.0184	0.1343	0.8743
Rotation	3	1.9810	0.6603	4.8169	0.0027
GrowthStage	2	6.1280	3.0641	22.3520	0.0001
Nitrogen x GrowthStage	4	0.3500	0.0874	0.6375	0.6361
Rotation x Nitrogen	6	0.2620	0.0437	0.3189	0.9269
Rotation x GrowthStage	6	1.3350	0.2225	1.6228	0.1401
Rotation x N x GrowthStage	12	0.9230	0.0770	0.5613	0.8725
Residuals	319	43.730	0.1371		
Chao1 Richness	**Df**	**SumsOfSqs**	**MeansSqs**	**F**	**P-value**
Block	1	0	0.0400	0.0027	0.9586
Nitrogen	2	42.90	21.460	1.3268	0.2668
Rotation	3	240.50	80.170	4.9560	0.0022
GrowthStage	2	974.70	487.330	30.1277	0.0001
Nitrogen x GrowthStage	4	64.60	16.140	0.9977	0.4089
Rotation x Nitrogen	6	121.60	20.270	1.2534	0.2788
Rotation x GrowthStage	6	188.80	31.470	1.9455	0.0732
Rotation x N x GrowthStage	12	42.70	3.560	0.2199	0.9975
Residuals	319	5159.90	16.180	0.0027	0.9586
Pielou's Evenness	**Df**	**SumsOfSqs**	**MeansSqs**	**F**	**P-value**
Block	1	0	0	0.0049	0.9443
Nitrogen	2	0.0212	0.0106	2.9200	0.0554
Rotation	3	0.0319	0.0106	2.9320	0.0337
GrowthStage	2	0.0412	0.0206	5.6935	0.0037
Nitrogen x GrowthStage	4	0.0314	0.0078	2.1651	0.0727
Rotation x Nitrogen	6	0.1265	0.0211	5.8200	0.0001
Rotation x GrowthStage	6	0.0544	0.0091	2.5047	0.0221
Rotation x N x GrowthStage	12	0.0295	0.0025	0.6782	0.7724
Residuals	319	1.1554	0.0036	0.0049	0.9443

Df: degrees of freedom, SumsOfSqs: sums of squares, MeanSqs: mean squares. Analyses carried out with function adonis in R package 'vegan'.

Table 3.3. Global test (MANOVA) of the 2015 field block, crop rotation, N fertilization rate, maize growth stage, the then 2-way and one 3-way interaction(s) for AMF community richness and evenness alpha diversity (Shannon Diversity, Chao1 richness, and Pielou's evenness).

Shannon Diversity	**Df**	**SumsOfSqs**	**MeansSqs**	**F**	**P-value**
Block	1	0.230	0.230	1.6237	0.2035
Nitrogen	2	1.932	0.966	6.8059	0.0012
Rotation	3	1.196	0.399	2.8093	0.0397
GrowthStage	2	0.570	0.285	2.0074	0.1361
Nitrogen x GrowthStage	4	0.767	0.192	1.3505	0.2512
Rotation x Nitrogen	6	1.614	0.269	1.8952	0.0813
Rotation x GrowthStage	6	0.783	0.131	0.9201	0.4806
Rotation x N x GrowthStage	12	1.839	0.153	1.0798	0.3766
Residuals	307	43.569	0.142		
Chao1 Richness	**Df**	**SumsOfSqs**	**MeansSqs**	**F**	**P-value**
Block	1	5.570	5.566	2.3575	0.1257
Nitrogen	2	24.670	12.337	5.2249	0.0059
Rotation	3	54.070	18.023	7.6331	0.0001
GrowthStage	2	15.550	7.774	3.2926	0.0385
Nitrogen x GrowthStage	4	13.260	3.314	1.4037	0.2326
Rotation x Nitrogen	6	29.000	4.833	2.0468	0.0594
Rotation x GrowthStage	6	19.570	3.262	1.3816	0.2216
Rotation x N x GrowthStage	12	18.290	1.524	0.6454	0.8026
Residuals	307	724.870	2.361	2.3575	0.1257
Pielou's Evenness	**Df**	**SumsOfSqs**	**MeansSqs**	**F**	**P-value**
Block	1	0.005	0.004	0.217	0.6417
Nitrogen	2	0.316	0.158	7.616	0.0006
Rotation	3	0.128	0.043	2.0577	0.1058
GrowthStage	2	0.014	0.007	0.3355	0.7153
Nitrogen x GrowthStage	4	0.038	0.009	0.4576	0.7668
Rotation x Nitrogen	6	0.154	0.026	1.2408	0.2851
Rotation x GrowthStage	6	0.103	0.017	0.8259	0.5506
Rotation x N x GrowthStage	12	0.404	0.034	1.6252	0.0835
Residuals	307	6.362	0.021	0.217	0.6417

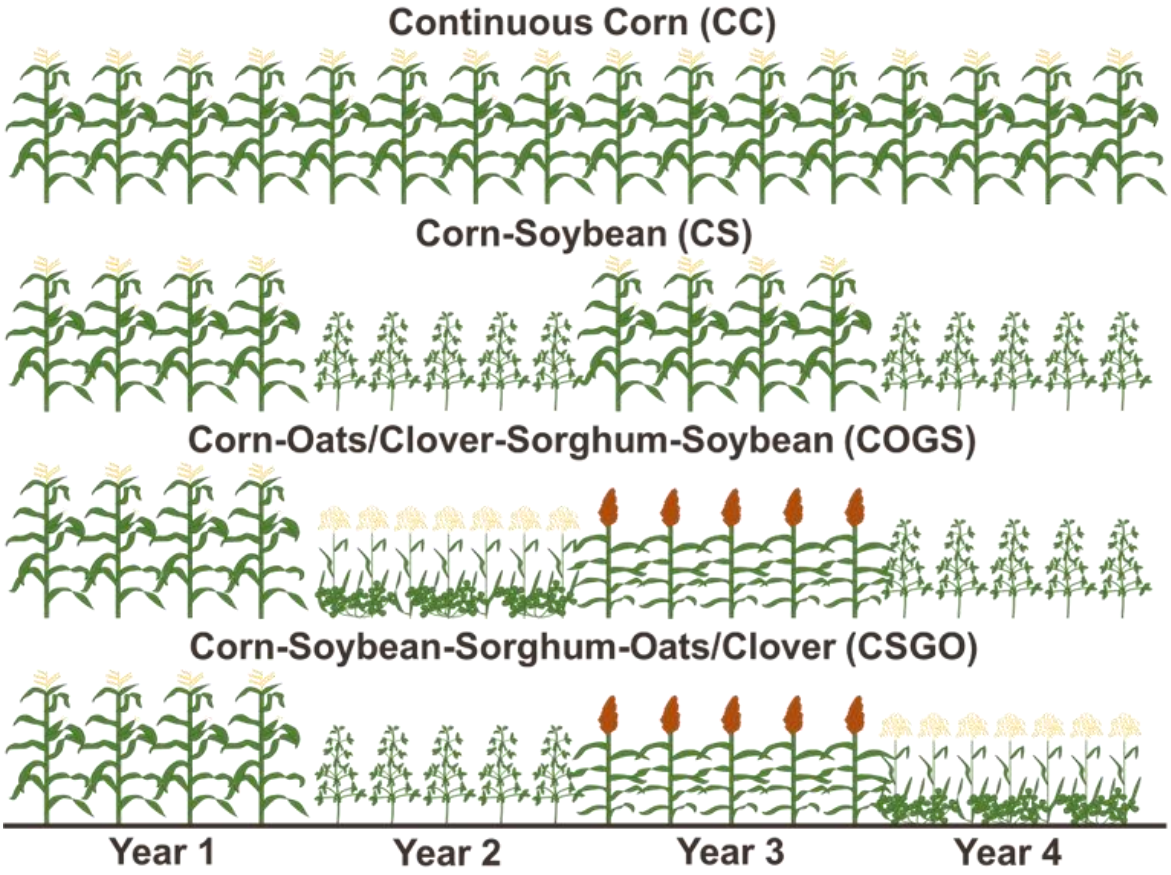
Df: degrees of freedom, SumsOfSqs: sums of squares, MeanSqs: mean squares. Analyses carried out with function adonis in R package 'vegan'.

Table 3.4. Global test (MANOVA) for beta diversity of the 2014 and 2015 field block, crop rotation, N fertilization rate, maize growth stage, the then 2-way and one 3-way interaction(s) for AMF community.

2014 Global MANOVA	**Df**	**SumOfSqs**	**R2**	**F**	**Pr(>F)**
Nitrogen	2	13.64	0.039	7.628	0.001
Rotation	3	12.07	0.035	4.501	0.001
GrowthStage	2	5.64	0.016	3.158	0.001
Rotation x Nitrogen	6	8.75	0.025	1.632	0.012
GrowthStage x Rotation	6	3.96	0.011	0.739	0.900
GrowthStage x Nitrogen	4	3.03	0.009	0.847	0.704
GrowthStage x Rotation x N	12	9.3	0.027	0.867	0.779
Residual	324	289.6	0.837		
Total	359	345.99	1		
2015 Global MANOVA	**Df**	**SumOfSqs**	**R2**	**F**	**Pr(>F)**
Nitrogen	2	38.14	0.077	16.872	0.001
Rotation	3	27.57	0.056	8.132	0.001
GrowthStage	2	10.32	0.021	4.568	0.001
Rotation x Nitrogen	6	16.15	0.033	2.382	0.001
GrowthStage x Rotation	6	8.29	0.017	1.222	0.134
GrowthStage x Nitrogen	4	9.08	0.018	2.009	0.001
GrowthStage x Rotation x N	12	16.77	0.034	1.237	0.042
Residual	324	366.18	0.744		
Total	359	492.5	1		

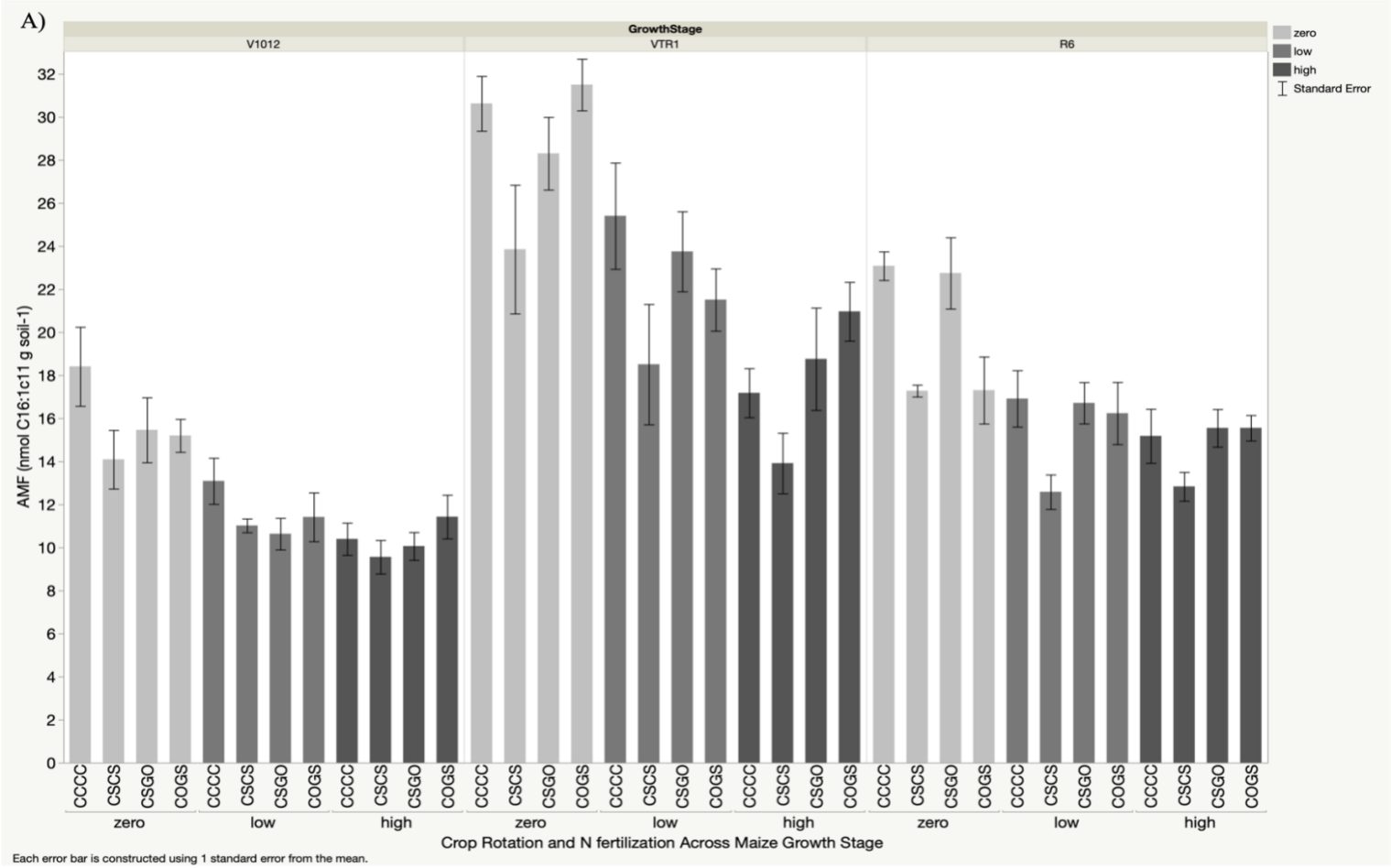
Df: degrees of freedom, SumsOfSqs: sums of squares, MeanSqs: mean squares. Analyses carried out with function adonis in R package ‘vegan’ using 2999 permutations.

Figure 3.1. Summary of the four crop rotations (CCCC, CSCS, COGS, and CSGO) where in each rotation over 2014 and 2015, sampling took place in the corn rotation. Figure from Ramirez II, 2020.

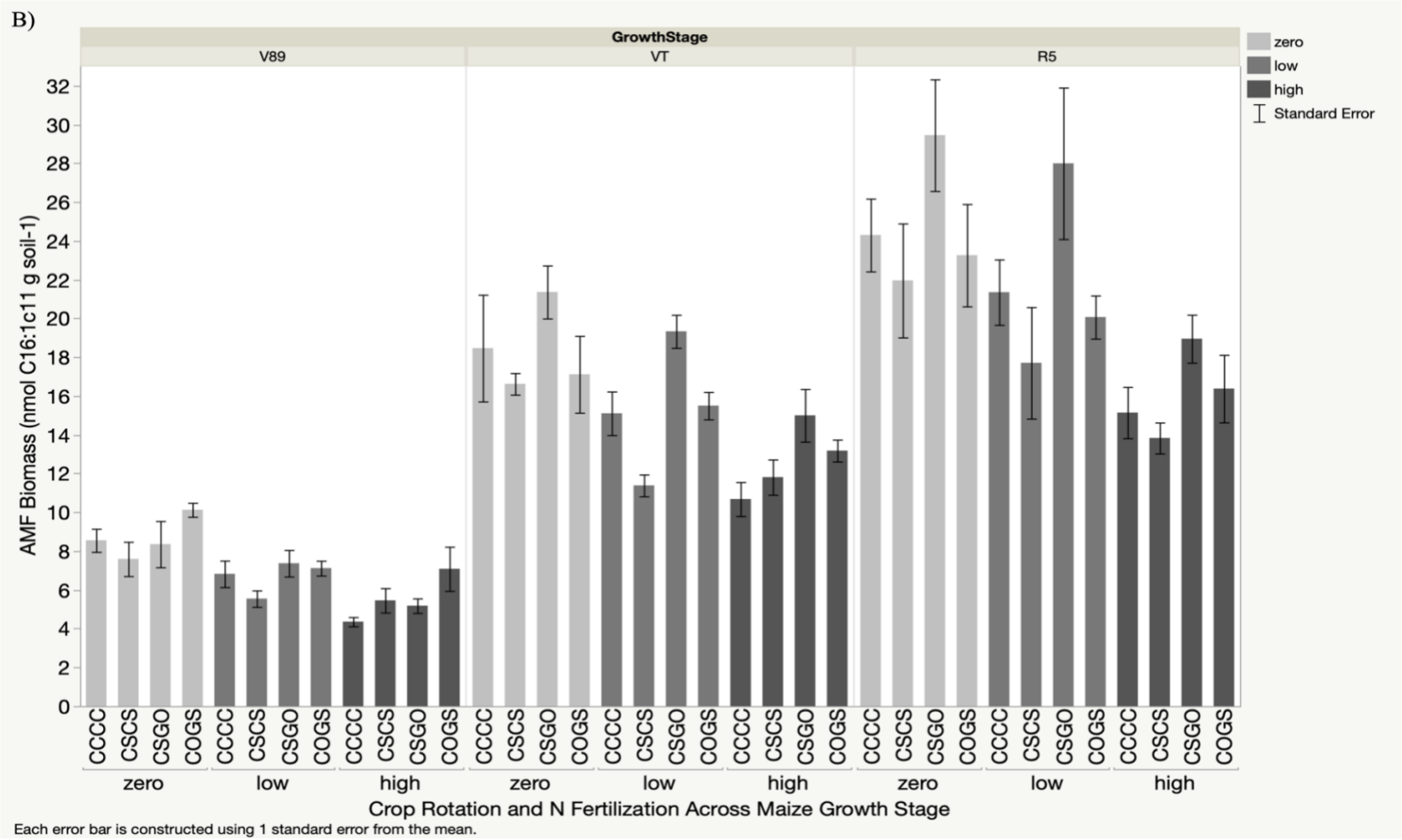


Visual representation displaying the 4 crop rotations of continuous corn (CCCC), corn-soy-corn-soy (CSCS), corn-soy-sorghum-oats/clover (CSGO), and corn-oats/clover-sorghum-soybean (COGS).

Figure 3.2. AMF biomass in soils from 2014 (A) and 2015 (B) by crop rotation and N fertilization application across growth stages.

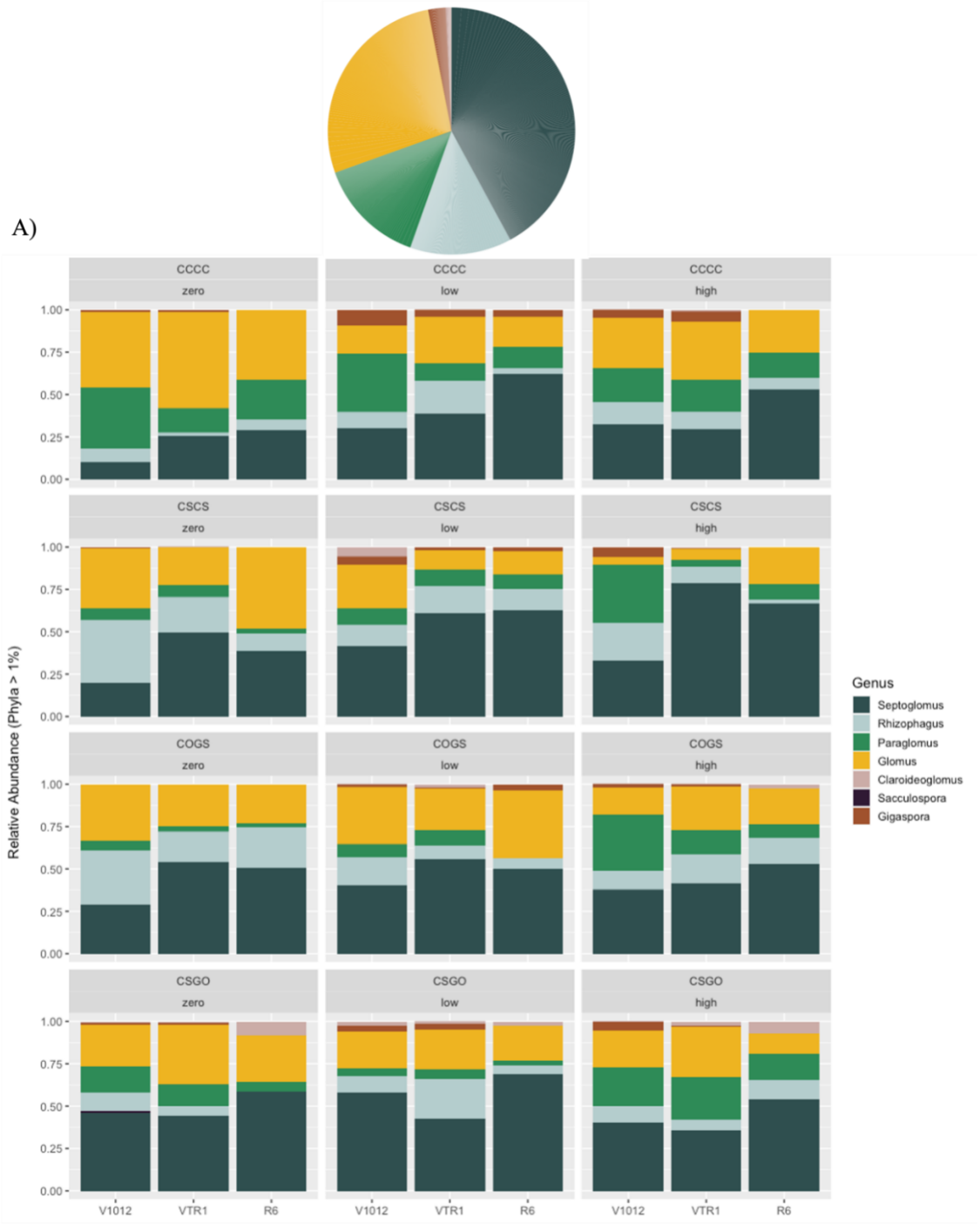


Bar graphs displaying AMF biomass from the soil for year 2014 across crop rotation (CCCC, CSCS, CSGO, COGS), maize growth stage (V1012, VTR1, and R6) and N fertilization treatment (in corn & grain sorghum, high (180 kg N ha^{-1}), low (90 kg N ha^{-1}), & zero (0 kg N ha^{-1}); in soybean & oats/clover, high (69 kg N ha^{-1}), low (34 kg N ha^{-1}), & zero (0 kg N ha^{-1}). Standard error bar constructed using 1 standard error from the mean.

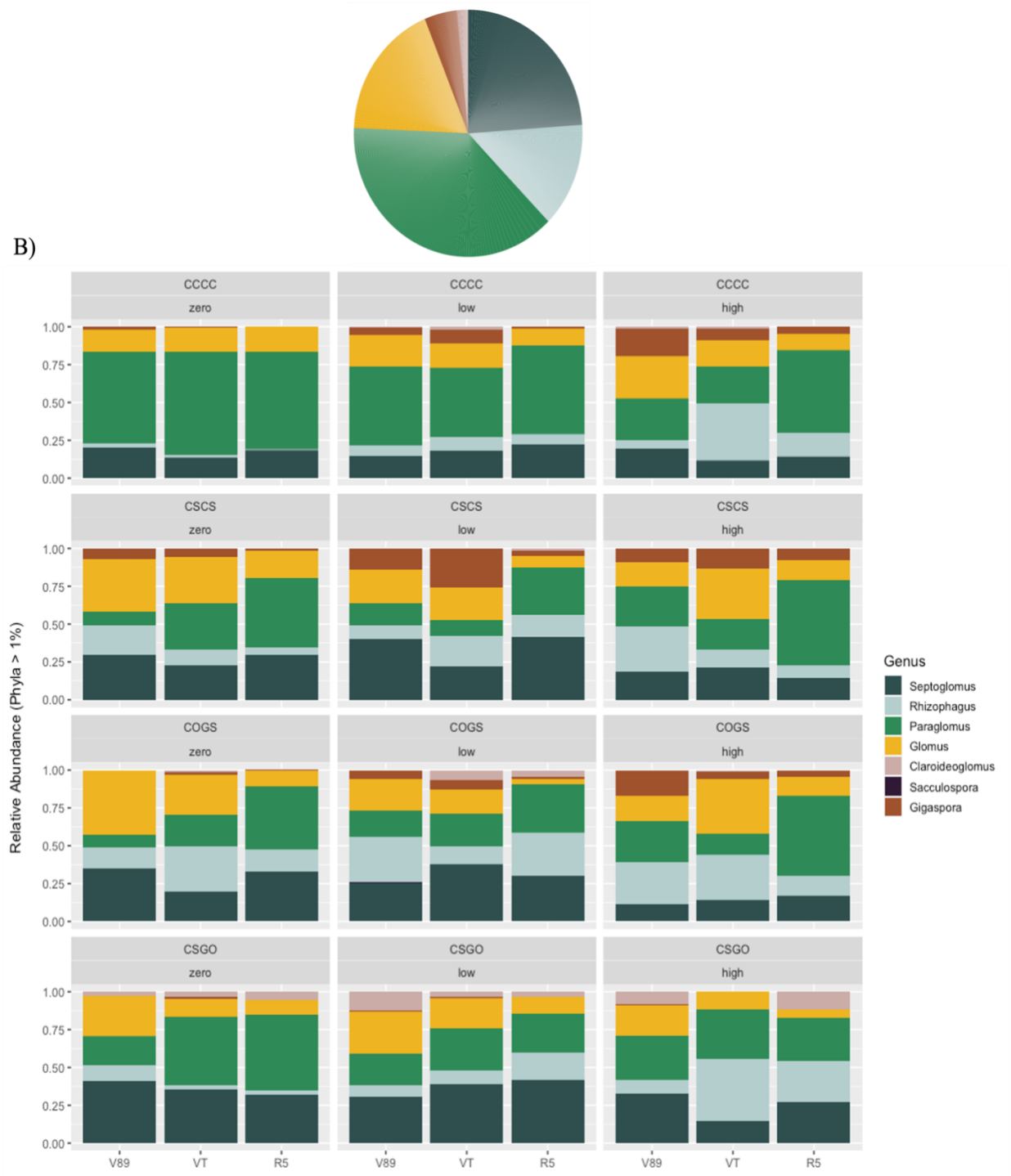


Bar graphs displaying AMF biomass from the soil for year 2014 across crop rotation (CCCC, CSCS, CSGO, COGS), maize growth stage (V1012, VTR1, and R6) and N fertilization treatment (in corn & grain sorghum, high (180 kg N ha^{-1}), low (90 kg N ha^{-1}), & zero (0 kg N ha^{-1}); in soybean & oats/clover, high (69 kg N ha^{-1}), low (34 kg N ha^{-1}), & zero (0 kg N ha^{-1}). Standard error bar constructed using 1 standard error from the mean.

Figure 3.3. Changes in relative abundance of AMF genera 2014 (A) and 2015 (B). Taxa bar plots show the relative abundance as a percentage of total reads.

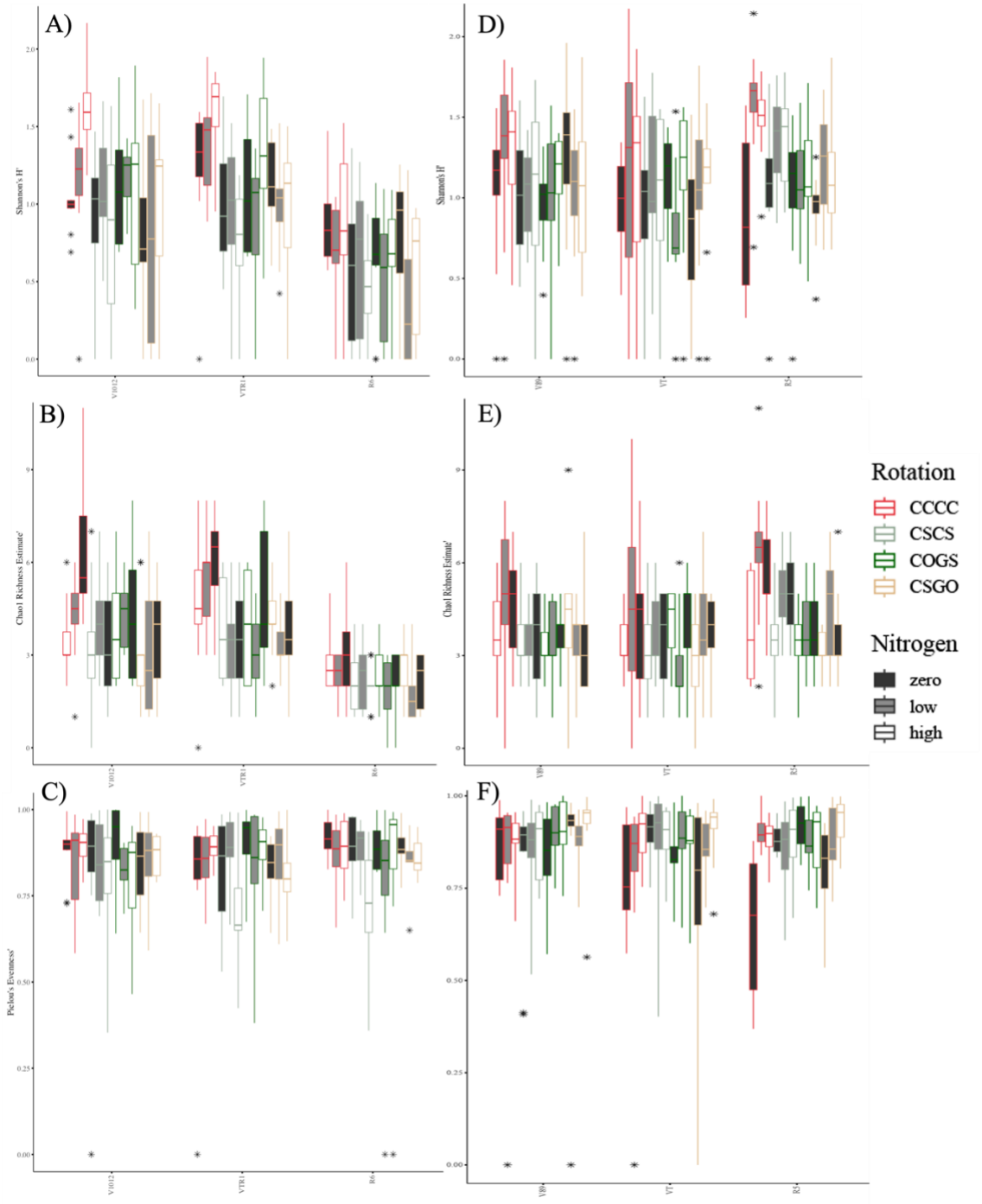


Pie chart shows distribution of AMF genera across all treatments. Stacked bar charts for year 2014 show relative abundance of AMF genera across crop rotation (CCCC, CSCS, CSGO, COGS), maize growth stage (V1012, VTR1, and R6) and N fertilization treatment (in corn & grain sorghum, high (180 kg N ha^{-1}), low (90 kg N ha^{-1}), & zero (0 kg N ha^{-1}); in soybean & oats/clover, high (69 kg N ha^{-1}), low (34 kg N ha^{-1}), & zero (0 kg N ha^{-1})



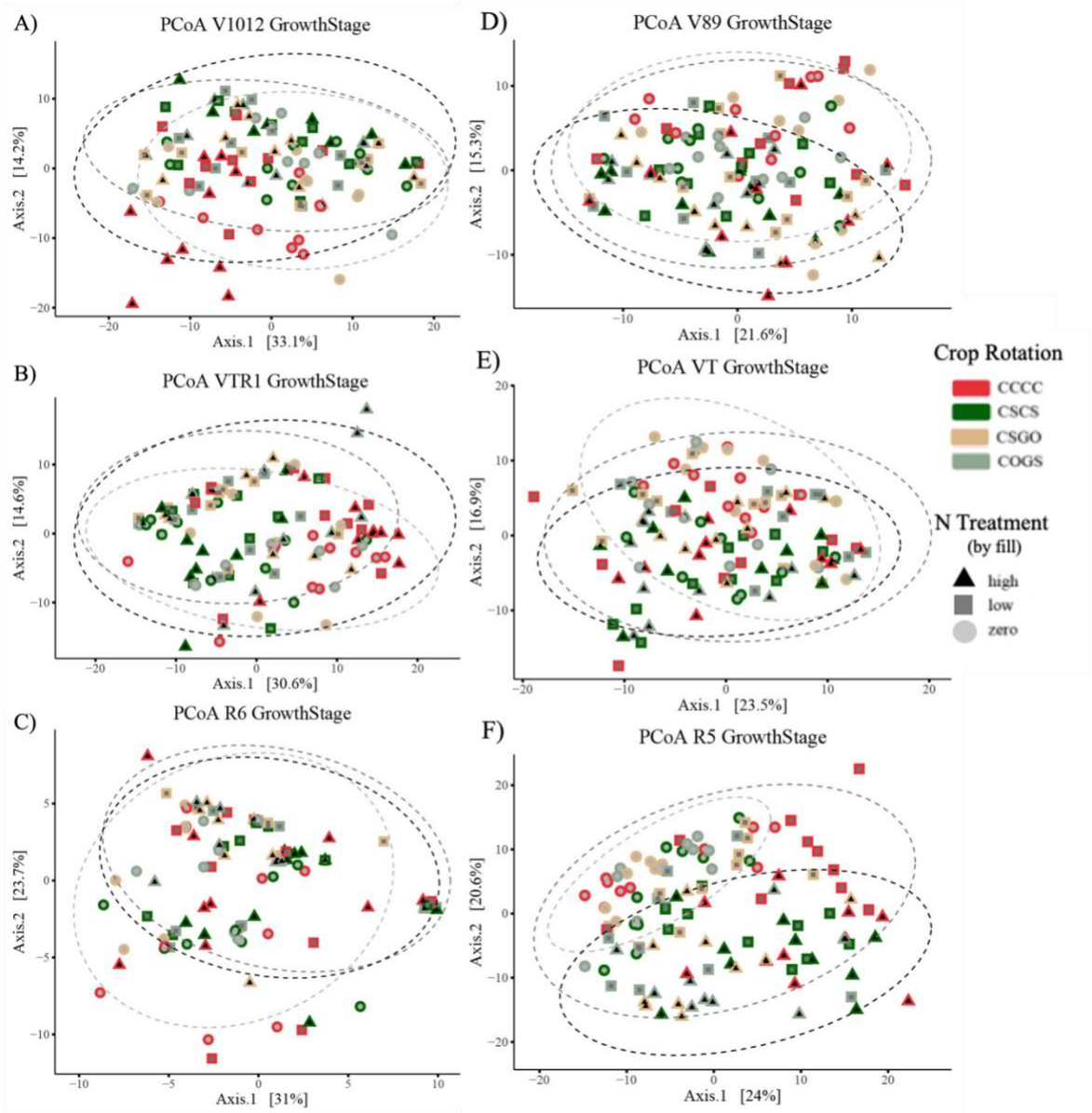
Pie chart shows distribution of AMF genera across all treatments. Stacked bar charts for year 2015 show relative abundance of AMF genera across crop rotation (CCCC, CSCS, CSGO, COGS), maize growth stage (V1012, VTR1, and R6) and N fertilization treatment (in corn & grain sorghum, high (180 kg N ha^{-1}), low (90 kg N ha^{-1}), & zero (0 kg N ha^{-1}); in soybean & oats/clover, high (69 kg N ha^{-1}), low (34 kg N ha^{-1}), & zero (0 kg N ha^{-1}).

Figure 3.4. Alpha diversity measurements across maize growth stages from 2014 (A, B, C) and 2015 (D, E, F), inclusive of Shannon diversity (A, D), Chao1 richness (B, E), and Pielou's evenness (C, F).



Alpha diversity (Shannon's H', Pielou's evenness, and Chao1 richness) indices by crop rotation (CCCC, CSCS, CSGO, COGS), maize growth stage (V1012, VTR1, and R6) and N fertilization treatment (in corn & grain sorghum, high (180 kg N ha^{-1}), low (90 kg N ha^{-1}), & zero (0 kg N ha^{-1}); in soybean & oats/clover, high (69 kg N ha^{-1}), low (34 kg N ha^{-1}), & zero (0 kg N ha^{-1}).

Figure 3.5. Beta diversity of AMF community structure by crop rotation and N fertilization treatment by the three growth stages in 2014 (A, B, C) and 2015 (D, E, F).



PCoA of all by maize growth stage for 2014 and 2015 of all crop rotations (CCCC, CSCS, CSGO, COGS), and N fertilization treatment (in corn & grain sorghum, high (180 kg N ha^{-1}), low (90 kg N ha^{-1}), & zero (0 kg N ha^{-1}); in soybean & oats/clover, high (69 kg N ha^{-1}), low (34 kg N ha^{-1}), & zero (0 kg N ha^{-1}). For each PCoA, axes 1 and 2 explain the amount of variance in AMF community structure. For sequencing, n=360

3.8. Supplemental figures and tables

Supplemental Table S3.1. Relative abundance (as a percentage) of all genera present in the 2014 (A) and 2015 (B) CRS experiment by growth stage, inclusive of crop rotation and N fertilization treatment.

A)

Growth Stage	***Claroideoglomus***	***Gigaspora***	***Glomus***	***Paraglomus***	***Rhizophagus***	***Sacculospora***	***Septoglomus***
V1012	0.78	3.34	26.50	19.05	15.70	0.06	34.58
VTR1	0.56	1.90	28.55	11.55	12.88	0.00	44.56
R6	1.44	0.80	26.05	10.03	8.39	0.00	53.29

Of the 49 unique ASVs corresponding to seven genera within five families *Claroideoglomeraceae, Glomeraceae, Gigasporaceae, Sacculosporaceae,* and *Paraglomeraceae* in soil at maize reproduction, we identified three as *Claroideoglomus,* ten as *Gigaspora*, fourteen as *Glomus,* nine as *Paraglomus,* seven as *Rhizophagus*, one as *Sacculospora,* and five as *Septoglomus*.

B)

Growth Stage	***Claroideoglomus***	***Gigaspora***	***Glomus***	***Paraglomus***	***Rhizophagus***	***Sacculospora***	***Septoglomus***
V89	1.97	6.95	24.02	28.38	12.70	0.03	25.96
VT	1.30	5.66	21.51	33.52	17.53	0.00	20.48
R5	1.60	2.18	11.39	48.27	11.17	0.00	25.40

Of the 65 unique ASVs corresponding to seven genera within five families, *Claroideoglomeraceae, Glomeraceae, Gigasporaceae, Sacculosporaceae,* and *Paraglomeraceae* in soil at maize reproduction, we identified five as *Claroideoglomus,* ten as *Gigaspora*, twenty as *Glomus,* twelve as *Paraglomus,* ten as *Rhizophagus*, one as *Sacculospora,* and seven as *Septoglomus*.

Supplemental Table S3.2. Relative abundance (as a percentage) of all genera present in the 2014 CRS experiment by each maize growth stage V1012 (A), VTR1 (B), and R6 (C) and crop rotation, and N fertilization treatment.

A) V1012 Rotation & N Fert.	***Claroideoglomus***	***Gigaspora***	***Glomus***	***Paraglomus***	***Rhizophagus***	***Sacculospora***	***Septoglomus***
CCCC_zero	0.00	1.47	44.33	36.28	7.62	0.00	10.30
CCCC_low	0.00	9.39	16.40	34.63	9.55	0.00	30.02
CCCC_high	0.00	4.85	29.52	20.21	12.99	0.00	32.43
CSCS_zero	0.00	0.53	35.89	6.62	37.35	0.00	19.60
CSCS_low	5.73	4.66	25.72	9.86	12.72	0.00	41.31
CSCS_high	0.00	5.89	4.39	34.41	22.51	0.00	32.80
CSGO_zero	1.11	0.86	24.45	15.85	10.69	0.98	46.07
CSGO_low	2.53	3.26	21.89	4.53	9.58	0.00	58.21
CSGO_high	0.53	5.10	21.79	22.85	9.25	0.00	40.49
COGS_zero	0.00	0.00	33.50	5.49	32.04	0.00	28.97
COGS_low	0.51	1.36	33.67	7.27	16.70	0.00	40.49
COGS_high	0.00	1.69	16.34	32.86	11.37	0.00	37.74

B) VTR1 Rotation & N Fert.	***Claroideoglomus***	***Gigaspora***	***Glomus***	***Paraglomus***	***Rhizophagus***	***Sacculospora***	***Septoglomus***
CCCC_zero	0.00	0.99	57.23	14.34	2.02	0.00	25.42
CCCC_low	0.00	4.23	27.25	10.55	19.38	0.00	38.59
CCCC_high	1.09	5.79	34.30	19.01	10.21	0.00	29.60
CSCS_zero	0.00	0.27	22.44	6.92	20.64	0.00	49.73
CSCS_low	0.00	2.10	11.31	9.73	15.78	0.00	61.09
CSCS_high	0.80	0.37	6.42	4.19	9.38	0.00	78.84
CSGO_zero	0.78	1.04	35.48	12.96	5.48	0.00	44.26
CSGO_low	1.57	3.44	23.07	5.64	23.59	0.00	42.69
CSGO_high	2.47	0.90	29.69	24.92	6.41	0.00	35.61
COGS_zero	0.00	0.00	24.65	3.39	18.13	0.00	53.83
COGS_low	1.97	0.52	24.46	9.12	8.39	0.00	55.54
COGS_high	0.00	1.53	25.54	14.49	16.93	0.00	41.51

C) R6 Rotation & N Fert.	***Claroideoglomus***	***Gigaspora***	***Glomus***	***Paraglomus***	***Rhizophagus***	***Sacculospora***	***Septoglomus***
CCCC_zero	0.00	0.00	41.21	23.28	6.55	0.00	28.97
CCCC_low	0.00	4.34	17.35	12.80	3.25	0.00	62.26
CCCC_high	0.00	0.00	25.13	15.20	6.91	0.00	52.76
CSCS_zero	0.00	0.00	48.46	2.78	9.88	0.00	38.89
CSCS_low	0.00	2.37	13.79	8.84	12.28	0.00	62.72
CSCS_high	0.00	0.00	21.62	9.68	2.25	0.00	66.44
CSGO_zero	8.19	0.00	27.80	5.60	0.00	0.00	58.41
CSGO_low	2.38	0.00	20.75	3.06	4.76	0.00	69.05
CSGO_high	6.90	0.00	12.36	15.52	11.21	0.00	54.02
COGS_zero	0.00	0.00	22.82	2.70	23.65	0.00	50.83
COGS_low	0.00	3.38	40.00	0.00	6.48	0.00	50.14
COGS_high	2.35	0.00	21.47	7.94	15.29	0.00	52.94

Of the 49 unique ASVs corresponding to seven genera within Glomerales in soil at maize reproduction, we identified three as *Claroideoglomus,* ten as *Gigaspora,* fourteen as *Glomus,* nine as *Paraglomus,* seven as *Rhizophagus,* one as *Sacculospora,* and five as *Septoglomus*.

Supplemental Table S3.3. Relative abundance (as a percentage) of all genera present in the 2015 CRS experiment by each maize growth stage V89 (A), VT (B), and R5 (C) and crop rotation, and N fertilization treatment.

A) V89 Rotation & N Fert.	***Claroideoglomus***	***Gigaspora***	***Glomus***	***Paraglomus***	***Rhizophagus***	***Sacculospora***	***Septoglomus***
CCCC_zero	0.00	1.96	14.85	59.88	2.82	0.00	20.49
CCCC_low	0.85	4.67	20.97	52.21	6.54	0.00	14.77
CCCC_high	1.00	18.68	27.87	27.17	5.89	0.00	19.38
CSCS_zero	0.00	7.14	34.40	9.33	19.53	0.00	29.59
CSCS_low	0.00	13.85	22.58	14.40	9.00	0.00	40.17
CSCS_high	0.00	9.43	15.48	26.51	29.89	0.00	18.68
CSGO_zero	2.53	0.00	27.04	19.10	10.36	0.00	40.97
CSGO_low	12.22	1.11	27.62	21.11	7.14	0.00	30.79
CSGO_high	8.39	0.66	20.07	29.11	9.21	0.00	32.57
COGS_zero	0.00	0.00	42.68	8.54	14.02	0.00	34.76
COGS_low	0.00	5.88	20.89	17.65	29.61	0.61	25.35
COGS_high	0.00	17.06	16.89	26.52	28.04	0.00	11.49

B) VT Rotation & N Fert.	***Claroideoglomus***	***Gigaspora***	***Glomus***	***Paraglomus***	***Rhizophagus***	***Sacculospora***	***Septoglomus***
CCCC_zero	0.00	0.62	16.05	68.00	1.98	0.00	13.34
CCCC_low	1.92	9.05	16.45	45.61	9.14	0.00	17.82
CCCC_high	1.47	7.18	17.40	24.78	37.76	0.00	11.41
CSCS_zero	0.00	5.43	30.50	31.09	10.41	0.00	22.58
CSCS_low	0.00	25.82	21.73	9.97	20.59	0.00	21.90
CSCS_high	0.00	13.46	33.30	20.11	11.67	0.00	21.45
CSGO_zero	3.15	1.29	12.30	44.78	2.72	0.00	35.77
CSGO_low	3.65	0.85	19.81	27.83	8.99	0.00	38.88
CSGO_high	0.00	0.00	11.96	32.51	41.23	0.00	14.30
COGS_zero	1.52	1.52	26.62	21.01	29.75	0.00	19.58
COGS_low	6.61	5.95	16.52	21.37	11.89	0.00	37.67
COGS_high	1.11	4.68	36.30	13.59	30.29	0.00	14.03

C) R5 Rotation & N Fert.	***Claroideoglomus***	***Gigaspora***	***Glomus***	***Paraglomus***	***Rhizophagus***	***Sacculospora***	***Septoglomus***
CCCC_zero	0.00	4.69	12.17	52.79	13.20	0.00	17.16
CCCC_low	0.00	0.00	16.27	64.81	0.77	0.00	18.15
CCCC_high	0.00	1.45	10.81	58.96	6.36	0.00	22.42
CSCS_zero	11.69	0.00	5.58	28.31	27.14	0.00	27.27
CSCS_low	0.00	1.53	18.19	45.42	4.96	0.00	29.90
CSCS_high	1.31	3.94	7.40	31.31	14.38	0.00	41.66
CSGO_zero	1.60	2.18	11.39	48.27	11.17	0.00	25.40
CSGO_low	5.61	0.00	9.85	49.52	3.01	0.00	32.01
CSGO_high	3.33	0.00	10.88	26.26	17.90	0.00	41.64
COGS_zero	0.00	7.66	13.44	56.00	8.39	0.00	14.52
COGS_low	0.00	0.65	10.41	41.46	14.47	0.00	33.01
COGS_high	4.25	1.57	3.46	32.28	28.03	0.00	30.39

Of the 65 unique ASVs corresponding to seven genera within Glomerales in soil at maize reproduction, we identified five as *Claroideoglomus*, ten as *Gigaspora*, twenty as *Glomus*, twelve as *Paraglomus*, ten as *Rhizophagus*, one as *Sacculospora*, and seven as *Septoglomus*.

Supplemental Table S3.4. Evaluation of environmental variables from 2014 that help shape the AMF (18S) microbiome.

Environmental Variable(s)	**NMDS1**	**NMDS2**	**r2**	**Pr(>r)**
Total Microbial Biomass	0.9359	-0.3523	0.0207	0.0325
AMF	0.9417	-0.3365	0.0244	0.0100
OM	0.5598	-0.8287	0.0404	0.0030
Inorganic P	0.4210	0.9071	0.0185	0.0450
Arysulfatase	0.4099	-0.9121	0.0192	0.0435
Soil Moisture	-0.6246	0.7809	0.0065	0.3170
pH	-0.9013	0.4333	0.0002	0.9600
Organic N	-0.2395	-0.9709	0.0058	0.3475
Organic C	0.5421	-0.8404	0.0033	0.5660
Nitrate	-0.4163	0.9093	0.0020	0.6995
Ammonium	0.8527	0.5224	0.0047	0.4405
Organic P	1.0000	-0.0079	0.0124	0.1180
K	-0.4902	-0.8716	0.0041	0.4735
Ca	0.0142	0.9999	0.0014	0.7840
Al	-0.8775	0.4796	0.0040	0.4915
Fe	-0.9786	0.2058	0.0026	0.6230
Bglucosidase	-0.1134	-0.9936	0.0153	0.0650
Bglucosaminidase	-0.8042	-0.5944	0.0126	0.0930
Acidphosphatase	-0.4336	-0.9011	0.0094	0.1865
Alkalinephosphatase	0.6523	-0.7580	0.0063	0.3275
Phenoloxidase	0.6671	-0.7450	0.0009	0.8550
Peroxidase	0.6189	-0.7855	0.0009	0.8590
Cellulase	0.6446	0.7645	0.0125	0.1160

Analyses carried out using global multidimensional scaling using monoMDS and Manhattan distances and 1999 permutations; Some environmental variables have been removed; Stress = 0.2428533.

Supplemental Table S3.5. Evaluation of environmental variables from 2015 that help shape the AMF (18S) microbiome.

Environmental Variable(s)	**NMDS1**	**NMDS2**	**r2**	**Pr(>r)**
Soil Moisture	-0.193	0.981	0.047	0.0005
Total Microbial Biomass	-0.971	0.238	0.038	0.0015
Fungi	-0.986	0.166	0.026	0.0115
Bacteria	0.350	0.937	0.018	0.0355
AMF	-0.994	0.111	0.087	0.0005
F:B Ratio	-0.936	-0.353	0.052	0.0005
Organic C	-0.372	0.928	0.029	0.0065
Arysulfatase	-0.747	-0.665	0.026	0.0090
Bglucosidase	0.970	0.243	0.020	0.0285
Bglucosaminidase	0.988	0.155	0.060	0.0005
Acidphosphatase	0.830	0.558	0.032	0.0030
Alkalinephosphatase	-0.819	-0.573	0.122	0.0005
Phenoloxidase	-0.651	-0.759	0.090	0.0005
Peroxidase	0.898	0.441	0.180	0.0005
Cellulase	0.983	0.183	0.091	0.0005
pH	0.390	-0.921	0.000	0.9750
OM	-0.767	0.642	0.012	0.1225
Organic N	-0.175	0.985	0.012	0.1100
Nitrate	0.581	0.814	0.000	0.9690
Ammonium	-1.000	0.019	0.002	0.6940
Inorganic P	-0.598	-0.802	0.005	0.3830
Organic P	-0.910	0.414	0.012	0.1180
K	-0.827	0.563	0.009	0.2195
Ca	-0.988	0.152	0.003	0.6185
Al	-0.576	0.818	0.007	0.2855
Fe	-0.570	0.821	0.012	0.1205

Analyses carried out using global multidimensional scaling using monoMDS and Manhattan distances and 1999 permutations; Some environmental variables have been removed; Stress = 0.1837656.

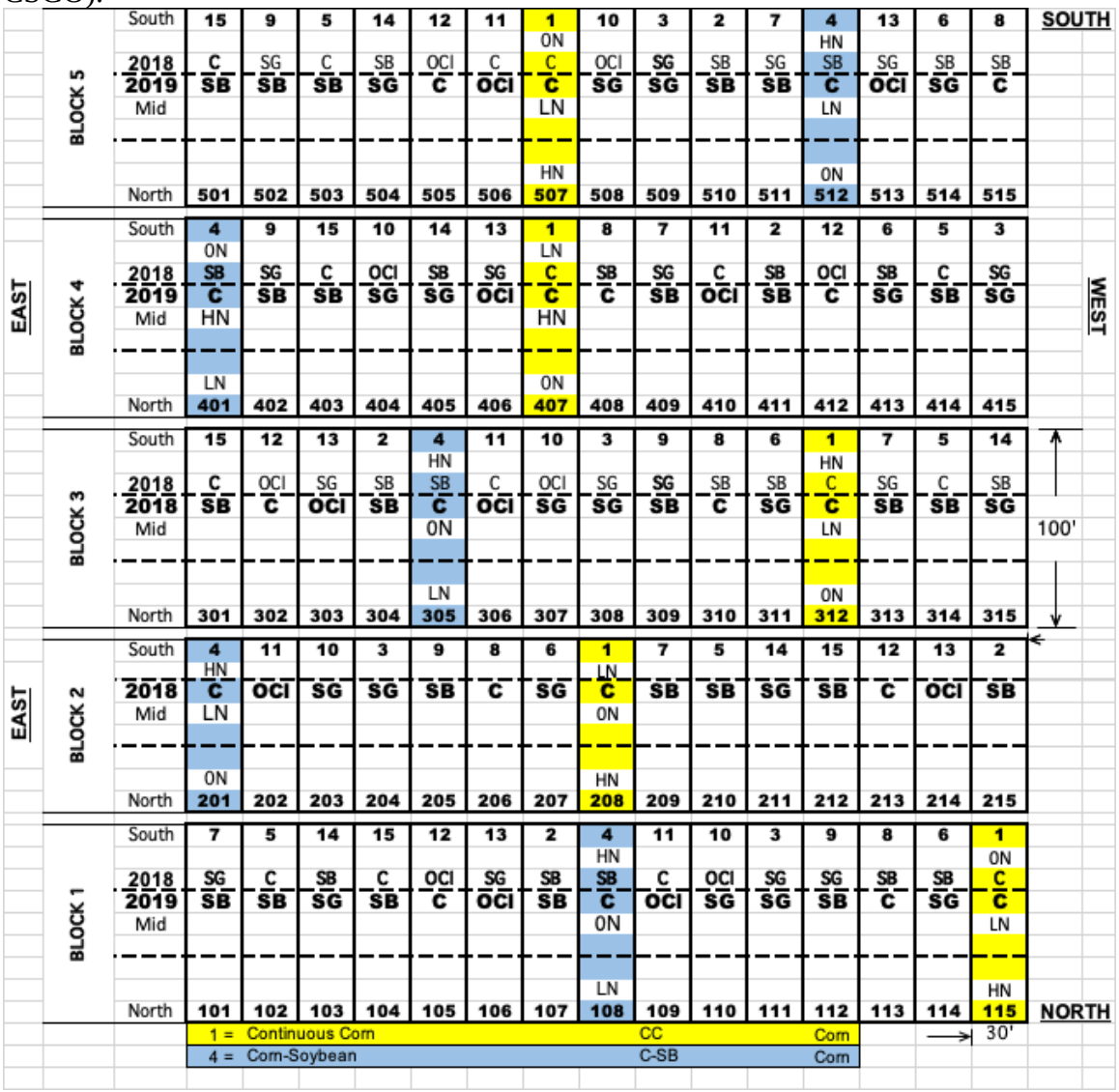
Supplemental Table S3.6. Monthly average max and minimum temperature (T) and precipitation (Precip) before and during throughout the corn growing season (February-September) in Eastern Nebraska from Ramirez II, 2020.

	Feb.		Mar.		Apr.		May		June		July		Aug.		Sept.	
	T-high	T-low	T-high	T-low	T-high	T-low	T-high	T-low	T-high	T-low	T-high	T-low	T-high	T-low	T-high	T-low
Year	Celsius															
2014	0.06	-12.7	9.67	-6.94	17.9	2.06	23.3	9.22	27.9	15.4	28.7	14.4	28.4	17.2	24.3	10.6
2015	-0.56	-13.3	14.0	-3.61	17.9	4.44	20.9	9.50	27.4	15.6	29.2	17.1	27.6	15.1	27.4	14.4
30-yr avg.	2.94	-10.0	10.7	-3.89	17.4	4.43	22.8	11.7	28.3	18.9	30.4	22.8	29.2	21.1	25.6	14.4

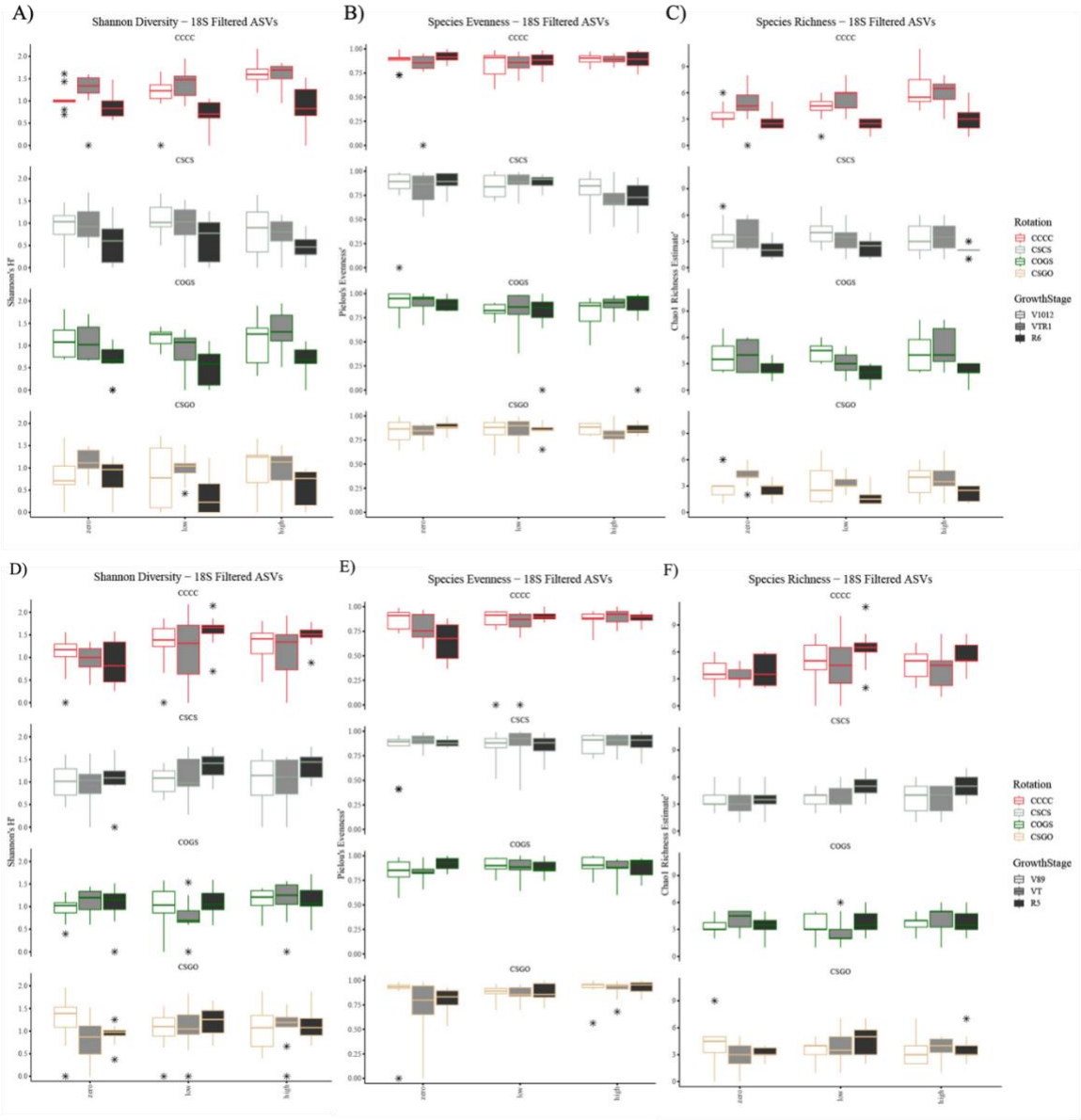
	Precip	Precip	Precip	Precip	Precip	Precip	Precip	Precip
Year	cm							
2014	1.17	0.53	8.18	16.46	21.16	1.40	17.70	8.38
2015	3.27	2.03	9.17	19.81	15.34	8.99	19.53	1.30
30-yr avg.	1.85	3.86	7.42	11.68	11.79	8.61	9.65	7.95

Monthly average high temperatures and low temperatures (1985-2015) were from weather station Mead, NE, 6S (source: www.climod.unl.edu)

Supplemental Figure S3.1. Summary of the field plot where soil samples were collected over 2 years. This plot depicts 2018 and 2019 however our soil samples are form 2014 and 2015 because of the 4-year crop rotation. There are 5 blocks, 3 nitrogen fertilization treatments (0 N, Low N, and High N), and 4 crop rotations (CCCC, CSCS, COGS, CSGO).

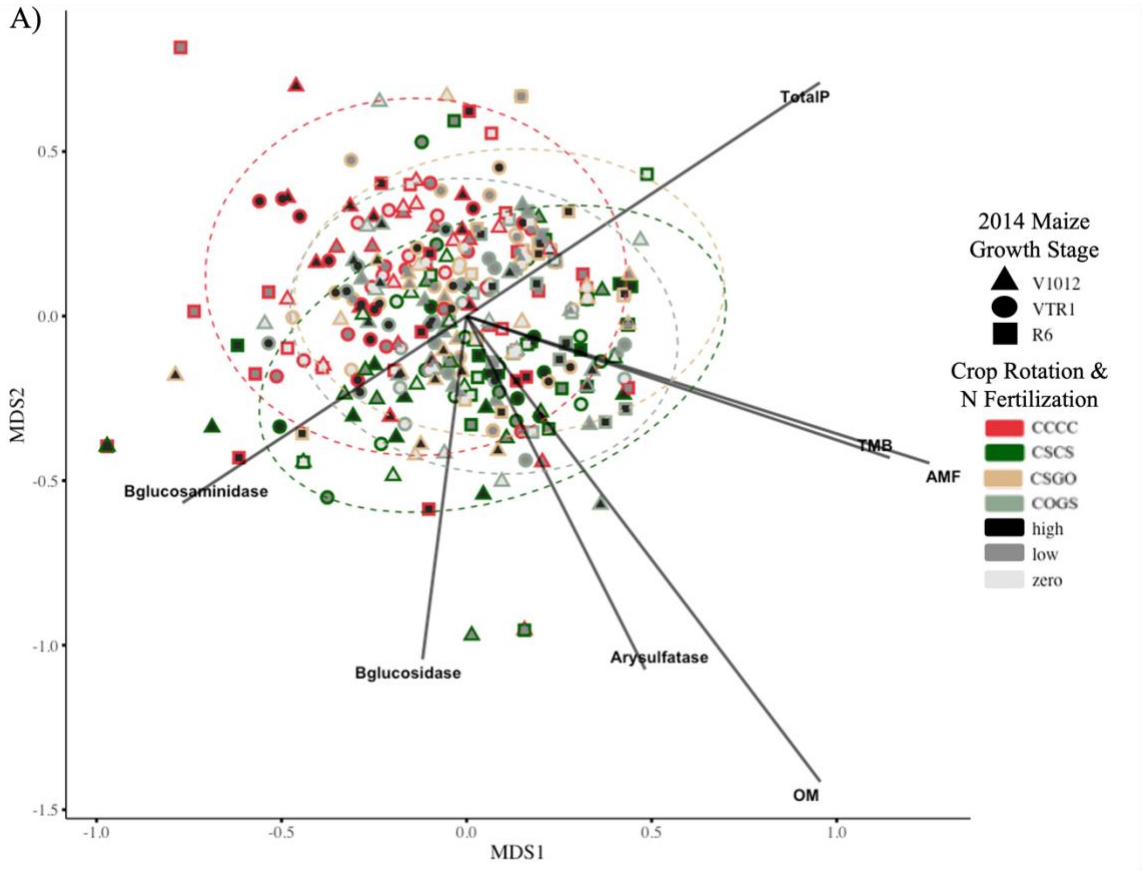


Supplemental Figure S3.2. Alpha diversity measurements of 2014 (A, B, C) and 2015 (D, E, F).

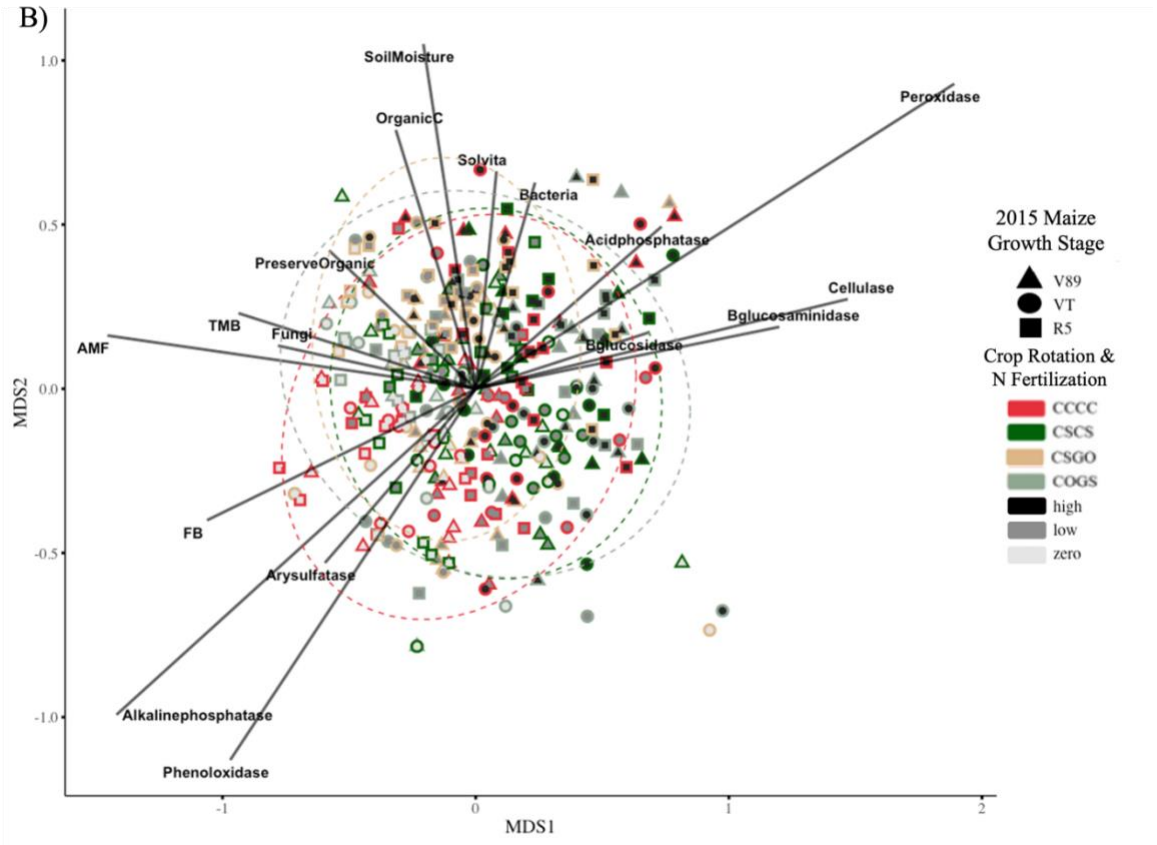


Alpha diversity (Shannon's H', Pielou's evenness, and Chao1 richness) indices by crop rotation (CCCC, CSCS, CSGO, COGS), maize growth stage (V1012, VTR1, and R6) and N fertilization treatment (in corn & grain sorghum, high (180 kg N ha^{-1}), low (90 kg N ha^{-1}), & zero (0 kg N ha^{-1}); in soybean & oats/clover, high (69 kg N ha^{-1}), low (34 kg N ha^{-1}), & zero (0 kg N ha^{-1}).

Supplemental Figure S3.3. Environmental factors shaping the AMF (18S) microbiome composition across all maize growth stages for 2014 (A) and 2015 (B).

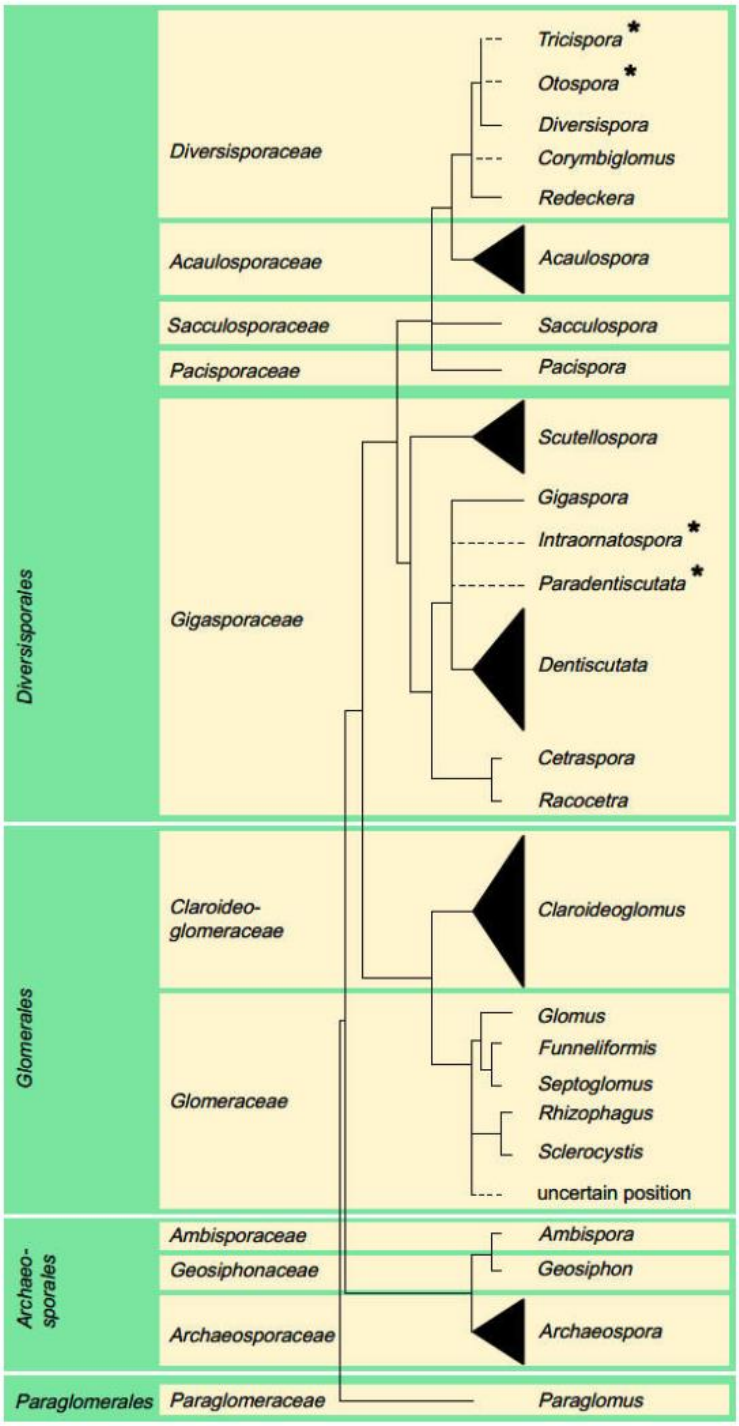


Environmental variables that shape the AMF community in soil from 2014. Vectors are labeled with environmental variables that are significant at alpha = 0.05; stress = 0.2428533. All crop rotations are abbreviated as CCCC, CSCS, CSGO, COGS and N fertilization treatment (in corn & grain sorghum, high (180 kg N ha^{-1}), low (90 kg N ha^{-1}), & zero (0 kg N ha^{-1}); in soybean & oats/clover, high (69 kg N ha^{-1}), low (34 kg N ha^{-1}), & zero (0 kg N ha^{-1}). For sequencing, n=360.



Environmental variables that shape the AMF community in soil from 2015. Vectors are labeled with environmental variables that are significant at alpha = 0.05; stress=0.1837656. All crop rotations are abbreviated as CCCC, CSCS, CSGO, COGS and N fertilization treatment (in corn & grain sorghum, high (180 kg N ha^{-1}), low (90 kg N ha^{-1}), & zero (0 kg N ha^{-1}); in soybean & oats/clover, high (69 kg N ha^{-1}), low (34 kg N ha^{-1}), & zero (0 kg N ha^{-1}). For sequencing, n=360.

Supplemental Figure S3.4. Classification of *Glomeromycota* modified from Redecker et al. (2013) from http://www.amf-phylogeny.com/. Genera marked by asterisks are questionable with respect to data used for description and/or with respect to phylogenetic position.



3.9. Literature cited

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Chapter 4: Synthesis

4.1. Introduction

Microbial life remains one of the largest sources of Earth's diversity and continues to be a dynamic and exciting area of research (Pace, 1997). More specifically, soil microorganisms mediate some of the most intricate and important biogeochemical processes on earth (Pagaling et al., 2014). Due to this, it is necessary to study and further understand how these soil ecosystems continually adapt to their surroundings, especially in midst of increasingly extreme climate events. This chapter synthesizes methodology used to study these systems, variation in microbial community structure in differing soil types, the importance of studying microbial processes long-term, and future research goals. Chapters 2 and 3 assessed the impact of agricultural management practices on AMF community structure and diversity. As AMF are obligate symbionts, it is widely accepted that they are dynamic throughout the life of a plant, more specifically maize in this dissertation. These management practices included N fertilization, tillage and no-till, and crop rotations in rain-fed systems, excluding the data from the greenhouse synthesis (in appendix) which took place in a greenhouse setting. In terms of long-term experiments, CNS was a 10-year experiment and CRS spanned decades, with rotation established in 1972, N fertilization treatment established in 1984, and no-till established in 2007. In both long-term field sites, our results examined not only current year data, which AMF respond more readily to, but also historical inputs and pools within the soil environment. This early-season response was focused on more within the GH experiment, which allowed us to use the same soil resources in a greenhouse setting (soils collected from CRS and mixed with sand for conetainers) while looking at AMF

community structure during the first few weeks of maize's life. Overall, the goal of this dissertation was to further our understanding of how AMF interacts with maize and responds to various agricultural management practices. We aim to create more sustainable and environmentally conscious agroecosystems now and for future generations and disentangling the soil microbiome is an essential step in this process.

4.2. Limitations of methodology

Methodology is core to generating appropriate data for interpretation, and for comparison of results across experimental sites and datasets. Chapters 2, 3, and the greenhouse experiment in the appendix all use methodology that is common in the field of soil microbial ecology such as FAMEs and DNA amplicon sequencing (CNS, CRS, and GH). When interpreting results and scaling up these methods for broader impacts, it is important to remember the massive variability within soils. Throughout FAMEs and DNA extraction protocols, we use anywhere from 0.25 g to 10 g of soil per extraction. When we collect soils from the field, we collect anywhere from 150 g to 500+ g and during processing, the soils are composited, mixed well, and homogenized. This is in attempt to create a uniform sample and reduce some of the massive variability. When working with these samples, can examining soil microbes from such small sample sizes really be representative of a field environment? Are the results we find in our experiments applicable to the field? I think that ultimately scientists are able to draw insightful and impactful conclusions using these methods, and it is important to keep the

limits and best practices of methods in mind when drawing conclusions or providing recommendations for growers.

Another limitation in sequencing methods includes the robustness and diversity of the reference databases. Reference databases are used to assign taxonomy to sequencing data. Once we have worked through a quality control pipeline for amplicon sequencing, we need to use previously identified sequence information to assign taxonomy to our dataset. Of course, databases are maintained and updated, but to what extent? With the speed at which next generation sequencing is moving there are extremely large amounts of new data being generated, which needs to be incorporated into our existing databases. Ultimately, I align with the saying, "we are only as good as our databases."

4.3. Applicability of results to other soil types

Soils and their associated characteristics are dynamic and diverse. The Midwest of the US has highly fertile and deep soils, dominated by Mollisols, Entisols, and Alfisols (Clark et al., 2019). These soils and the climate throughout the Midwest make this environment particularly ideal for food production, providing around 25% of the world's food supply (Swaby et al., 2016). When comparing research across various field sites worldwide, it is good practice to consider the classification of soil(s) in which the microorganisms live and reside.

Additionally, AMF biomass and diversity can change throughout the soil profile. Higo et al., (2013) found that AMF biomass measured from FAMEs substantially decreased in the soil profile, when measured up to 100 cm. However, AMF phylotype

diversity shifted very little throughout the soil profile and responded more to the crop rotations or fallow periods present in this experimental design. Two additional experiments found a similar trend with AMF biomass decreasing through the soil profile at smaller depths, 0-35 cm (Wortmann et al., 2008) and 0-90 cm (Tian et al., 2013). Overall, mycorrhizae are ubiquitous throughout the world and can serve as an important indicator of soil health due to their role in soil structure (aggregation), impacts on plant nutrition and resiliency, and ecological restoration.

4.4. Importance of long-term field sites

Long-term field sites are an effective resource and tool for understanding how soils and their associated microbial communities change throughout time. These field sites are useful for current research and allow future researchers to make comparisons between samples or collections from the past. In agroecosystems, it is especially important to use these valuable resources to examine how management practices shape not only soil microbial communities, such as AMF composition, but also SOM formation and other nutrient cycling processes at a more seasonal, current year scale and across decades.

Throughout the world, there are well known long-term field sites such as Rothamsted long-term experiments in the United Kingdom, which is home to some of the oldest (since 1843) continuing agricultural field experiments (Perryman et al., 2018). To compile the massive amounts of historical and contemporary data, there is an electronic archive that researchers can access. Another invaluable resource is the network of USDA

Long Term Agroecosystem Research (LTAR) field sites (Bean et al., 2021). These research sites provide detailed information about various production systems to experimental manipulations and management practices. Ultimately, networks like this allow researchers to make connections across field sites and throughout time (Bean et al., 2021).

4.5. Environment plays larger role in AMF community structure

Arbuscular mycorrhizae are highly dependent and responsive to a multitude of environmental and management factors, specifically soil moisture and soil chemical composition, mainly soil pH. One example of this is a comparison of AMF community composition, using the same amplicon region and primers (18S rRNA), between an irrigated and rainfed maize system. Via a personal correspondence with Dr. Jeske, we have found that in an irrigated maize system, the relative abundance of the genus *Glomus* increases under increasing N fertilization rate, upwards of 300 kg N ha^{-1}, whereas in the two rainfed field experiments in chapters 2 and 3, the AMF community was dominated by *Septoglomus* and *Paraglomus.* This is potentially an indication that heavily managed maize systems may select for specific genera of AMF, perhaps genera that are more suited to the specified environmental conditions and fit within this ecological niche (Davison et al., 2021). To add to this, perhaps maize is a ‘highly selective’ plant that contributes to AMF community diversity and ultimately plant ecology (Tedersoo et al., 2020). The question remains as to if plants drive mycorrhizal populations, mycorrhizae drive plant populations and dispersal, or if it is a combination of the two. Future research

goals include gaining better insight into the AMF community assembly early in the maize growing season. Once we are able to identify the community composition early in the growing season, we can then make connections to the work conducted in this dissertation and see if the community composition and subsequent function is established early in the maize growing season, or if it fluctuates throughout. Additionally, being able to identify genes that are on or off within the AMF hyphal tips at certain points in the growing season would lead to a deeper understanding of their function, as well as their role in how carbon moves from the plant to the fungus, to the soil environment for sequestration. These functional traits and AMF community assembly are ultimately shaped by the environmental conditions in the soil where the AMF spore germinated. Afterall, AMF have been around for more than 450 million years and will continue to be a vital symbiont of plants as the climate changes. Elucidating how the AMF community composition is assembled, established, and then changes not only seasonally, but over multiple years, will aid in understanding more about the complex biology and ecology of AM fungi in a changing environment.

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Appendix

Chapter 5: AMF community assembly in a greenhouse setting with historical soils treated with variable nitrogen treatments and diverse crop rotational inputs

5.1. Introduction and objectives

Arbuscular mycorrhizae are vital to most terrestrial ecosystems and are especially important contributors to the resiliency and sustainability of agroecosystems worldwide (Duarte et al, 2022). Arbuscular mycorrhizal fungi (AMF) benefit the host plant by enhancing nutrient and water acquisition in exchange for photosynthetically fixed carbon throughout the lifecycle of the plant. Due to the seasonal dynamics of AMF and fluctuations that come with plant growth and climatic variability, it is vital to understand AMF community assembly early in the maize lifecycle, as maize is an important crop worldwide. However, not much is known about assembly of AMF communities in the rhizosphere of maize seedlings. This experiment examines AMF biomass, community composition, and diversity in the early growth stages of maize in a greenhouse environment, supplemented with field soil from the crop rotation study (CRS) in chapter 3 (4 crop rotations & 3 N fertilization treatments).

Although phosphorus acquisition has long been the focus of AMF research, nitrogen is gaining recognition as a key component of the symbiosis, particularly in agronomic systems receiving high inputs of N fertilizer. Previous research in our laboratory demonstrated that AMF colonization of maize roots was independent of N fertilizer rate (Tian et al., 2013); however, there was a strong inverse relationship between N fertilization rate and the abundance of extramatrical AMF (Jeske et al., 2018). Through this greenhouse experiment, we are able to further explore AMF extraradical

mycelium (ERM) development, AMF community assembly, and N cycling in the maize rhizosphere. This ultimately enables us to draw meaningful connections between an evolutionarily important plant symbiont and core ecological processes related to carbon and nitrogen cycling in the agronomically important and highly productive maize systems.

The process of AMF assembly in maize agroecosystems is important to understand how AMF function in the rhizosphere of maize. Previous studies have examined how AMF assembled in maize fields, via diversity and species distribution, and found that AMF community assembly is highly complex and dependent on many niche-related factors (Moebius-Clune et al., 2013). Another variable in AMF community assembly is the diversity of AMF species between the soil and root-associated communities. As AMF integrate themselves into the cortical cells of plant host roots, they are intimately tied into the plant root system. As mentioned previously, the diversity of AMF in roots has been shown to be unaffected by N fertilization (Tian et al., 2013), yet the AMF biomass in the soil is sensitive to N fertilization (Jeske et al., 2018). To add to the N fertilization regimes common in maize agroecosystems, crop rotation and diverse residue inputs may cultivate a more diverse and resilient AMF community. This leads to another key knowledge gap about how prior management practices shape plant soil feedbacks that shape AMF communities.

This chapter evaluates how the community structure of AMF changes early in the growing season with N shock fertilization treatments in a controlled, greenhouse environment. It also evaluates the influence of management history on AMF communities

in soils versus root associated soils. For this experiment, we **hypothesize** that the AMF ERM development pattern is triggered by the current season soil environment, which considers the diverse residues from crop rotational diversity and N fertilization. Briefly, soil was collected from the CRS field site and used in conetainers to examine AMF community recruitment and composition in early development. This experiment directly examined how AMF and maize seedlings determine and maintain a symbiotic relationship using soil from 4 crop rotations of CRS (CCCC, CSCS, CSGO, and COGS) and variable N treatments.

Abbreviations
CCCC, continuous corn; CSCS, corn-soybean-corn-soybean; CSGO, corn-soybean-sorghum-oats/cover; COGS, corn-oats/clover-sorghum-soybean; FAMEs, fatty acid methyl esters; AMF, arbuscular mycorrhizal fungi; CRS, crop rotation study; GH, greenhouse

5.2. Materials and methods

5.2.1. Field soils for greenhouse experiment

We conducted a greenhouse experiment using field soil collected from a long-term, experimental field site in Ithica, Nebraska (31° 10’ N, 96° 25’ W), established in 1972 and later modified in 1983. This field site has been the focus of previous work (Liebig et al., 2002; Schmer et al., 2020; Sindelar et al., 2016; Varvel, 1994) and includes crop rotation as a main factor and nitrogen as a split plot factor across 5-replicated blocks. This rainfed field site is dominated by soils classified as Yutan silty clay loam-Tomek silt loam complex (fine-silty, mixed, superactive, mesic Mollic Hapludalfs, smectitic, mesic Pachich Arguidolls, respectively). The mean annual precipitation and

temperature over 30 years (1985-2015) are 78.3 cm and 10.3°C (High Plains Regional Climate Center, Station ID Mead 6S, http://climod.unl.edu/). We focused on four crop rotations with differing crop diversity and crop sequence. The crops within this study include corn (*Zea mays* L.), soybean [*Glycine max* (L.) Merr.], grain sorghum [*Sorghum bicolor* (L.) Moench], and an oats [*Avena sativa* (L.)]/clover [80% *Melilotus officinalis* Lam. + 20% *Trifolium pretense* L.] mixture. The rotations are as follows: continuous corn (CCCC), a two-year corn-soybean (CSCS) rotation, a four-year corn-soybean-sorghum-oats/clover (CSGO) rotation, and another four-year corn- oats/clover-sorghum-soybean (COGS). Within each rotation, nitrogen fertilization was applied in the spring 2-3 weeks after planting as broadcasted urea. Nitrogen was applied annually at one of three rates: zero N (control of no applied N), low N (90 kg ha^{-1} in corn and sorghum years or 45 kg ha^{-1} in soybean and oat/clover years), and high N (180 kg ha^{-1} in corn and sorghum years or 90 kg ha^{-1} in soybean and oat/clover years). Field soils were collected before planting in the corn-year of the rotation, on May 1, 2018. Composite soil cores were collected for each rotation and each nitrogen fertilization rate (N-rate) across 3 blocks for a total of 21 cores per treatment group. Field soils were transported to the lab sieved to 4 mm, mixed with autoclaved sand in a 2:1 ratio, and placed in 1.5-inch diameter conetainers (volume of 10 cubic inches, height of 8.25 inches).

5.2.2. Greenhouse experimental design Q1: management history

Conetainers prepared with soil:sand mixtures for all 12 management histories (4 crop rotations, each with 3 N-rates) were placed in the greenhouse for 7 days to allow the

soils to stabilize following the disturbance caused by mixing. The Q1 experiment included 4-replicated blocks, with one conetainers per treatment group serving as a bulk soil control and a second conetainer per treatment group serving as the maize plant experimental group (n=96). Including a cone with and without maize plants enabled us to compare transitions in microbial community structure and diversity driven by the presence of early growth stage maize. After 1 week of acclimation, maize (DOW mycogen Pioneer POG21XR)) was planted in half of the conetainers (n=48) and marked day 0 of the experiment. At the same time a collection of 24 baseline cones, 2 per management history, were collected to serve as a baseline of the starting microbial community structure. All maize cones successfully germinated by day 3. Plants were watered daily by adding water until it pooled at the surface of the conetainer, and then allowing the water to drain overnight. Maize plants were monitored daily for changes in host growth stage by collar measurement. Maize plants entered the V1 growth stage on day 6 of the experiment, V2 growth stage on day 9, and all conetainers, including the bulk soil controls, were harvested on day 11 of the experiment after all seedlings reached the V2 growth stage.

5.2.3. Greenhouse experimental design Q2: nitrogen fertilization shock

Concurrently with greenhouse experiment Q1 described above, we conducted a nitrogen shock experiment using conetainers prepared with 2:1 soil:sand mixtures of all 4 crop rotations and the nitrogen extremes (zero vs high N-rate). For all 8 management histories (4 crop rotations, each with 2 N-rates), we included a bulk soil control

conetainer and a maize seedling conetainer (n=64). To test the influence of current year nitrogen application on microbial community structure and diversity, we included a second paired bulk soil control and maize seedling conetainer to each treatment group. This gave us a reciprocal, paired design where-in the current year N-rate either matched the treatment history (continuous) or was different from the treatment history (shock). This experiment was also conducted in 4-replicated blocks (N=128).

Initial set-up of the Q2 experiment is the same as Q1 above. Conetainers were acclimated in the greenhouse for 7 days and watered every other day to allow soil microbial communities to stabilize. Maize seeds were planted after 1 week of acclimation, and all maize successfully germinated by day 3. Maize plants entered the V1 growth stage on day 6 of the experiment and V2 growth stage on day 9. On day 11 of the experiment, N fertilization shock treatments began. N fertilization was applied as a solution (20.5 mg of urea per conetainer) in three equal volumes over 6 days to mimic slow incorporation of urea pellets used under field conditions. For all conetainers receiving nitrogen fertilization (High N continuous; Zero N shock), 15mL of urea solution was applied by dropper. For all conetainers receiving no N-fertilization (Zero N continuous; High N shock), 15mL of DI water was added in order to keep the volume of moisture added to cones the same across all treatment groups. On day 17 of the experiment, after all maize plants reached the V3 growth stage, all conetainers, including bulk soil controls and maize seedling cones were collected.

5.2.4. Conetainer harvesting

For both Q1 and Q2 experiments the following harvesting procedures were followed. On the day of harvesting plant height, leaf length, and chlorophyll content were measured before conetainers were brought to the lab and divided into different fractions. Soil from control cones and soil that did not adhere to the roots from maize cones were collected and stored at -20°C for soil physico-chemical properties, FAMEs and DNA extraction analyses. Above ground biomass was clipped then oven-dried at 75°C for 48 hours to determine aboveground biomass. To reduce disturbance of the microbial communities surrounding and interacting with the rhizosphere and roots of maize plants, a more ecological sampling method was designed and implemented. This method kept root-soil interfaces and fine root hairs intact by gently shaking roots to remove soil to leave only tightly adhered soil particles. This fraction represents root-associated microbial communities that can be described as the "tightly associated" rhizosphere or complete RhizoComplex (RC). The RC was placed directly into a Ziploc bag and stored at -80°C before being freeze-dried and ground in Liquid Nitrogen for FAMEs and DNA extraction.

5.2.5. Quantification of AMF biomass in soil

The AMF-specific fatty acid biomarker, C16:1*cis*11, was used to quantify AMF biomass in the soil (Olsson, 1999). Five grams of soil was extracted with 0.2 M KOH in methanol according to the method of (Jeske et al., 2018). The resulting fatty acid methyl esters (FAMEs) were quantified on an Agilent 7890 gas chromatograph fitted with an

Ultra 2 HP (Agilent) capillary column (50 m 0.2 mm I.D., 0.33 µm film thickness) using helium as the carrier gas. The injector was maintained at 280ºC and the flame ionization detector at 300 ºC. The oven temperatures were held at 50ºC for 2 minutes, then ramped up by 40ºC min^{-1} to 160ºC for 2 minutes, then ramped up again by 3ºC min^{-1} to 300ºC for 30 minutes. Sample masses of individual FAMEs were calculated from peak areas relative to the internal standard methyl nonadecanoic acid and reported as nmol FAME g^{-1} dry soil or relative abundance (nmol%). The identity of C16:1*cis*11 was confirmed by gas chromatography mass spectrometry on an Agilent 7890 GC with a 5975 mass-selective detector using the same column as described above.

5.3. Preliminary results

Preliminary results from this experiment include FAMEs biomass of soil collected from the conetainers with and without maize seedlings at two growth stages. The results of AMF biomass, quantified by the lipid biomarker C16:1c11, indicate an inverse relationship between AMF biomass in the soil and applied N fertilizer, even this early in the lifecycle of the maize plant. More specifically in Figures 5.1 and 5.2, in the V2 maize growth stage, we see significant differences between zero, low, and high N fertilization treatments, between the growth stages, and with and without maize seedlings ($P<.0001$ and $P<.0001$, respectively). When the AMF biomass was measured with maize seedlings at the V2 growth stage, we saw a slight increase in the amount of AMF biomass in the soil present. These findings resonate with overall AMF biology, as indigenous AMF

spores in the soil would begin to germinate and establish themselves in the roots, as well as out into the soil via ERM, which is what we quantified with FAMEs.

In the V3 maize growth stage, we saw a similar trend to the V2 growth stage. The V3 growth stage had an additional N fertilization treatment added to it and examined how AMF community responded to continuous N fertilization and shocked N fertilization (Figures 5.3 and 5.4). In Figure 5.3, when the AMF community was treated with a continuous N fertilization treatment, there were higher amounts of AMF biomass in the soil, compared to when the AMF community was treated with a shocked N fertilization rate, there was a decrease in overall biomass when grown with maize seedlings. Both the continuous and shocked N fertilization rates showed significance across crop rotations and N fertilization treatments ($P<.0001$ for both continuous and shocked). In Figure 5.4 when AMF biomass in the soil was measured without maize seedlings, the response was more variable. Specifically, there was still significance in the continuous N fertilization ($P=.0056$) and the shocked N treatment ($P=.0002$) between crop rotations and N fertilization treatments, yet when grown without maize seedlings the AMF response was not as clear as when grown with maize seedlings.

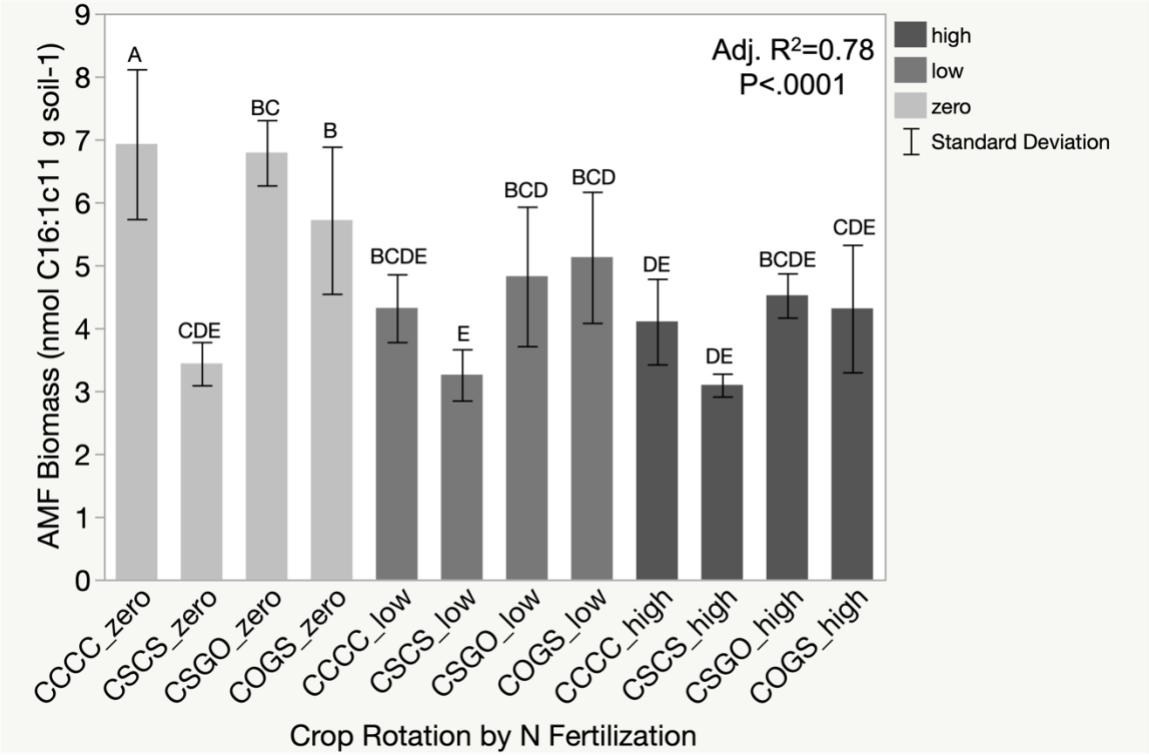
The baseline soils showed more AMF biomass in the historically zero N treated soils compared to the low and high N treatments (Figure 5.5). Overall, there were significant differences between the crop rotations and N fertilization treatments ($P=.0008$), suggesting that the historical soil environment may help shape the AMF community response to current season N treatments.

5.4. Future directions

To build of the FAMEs results that show shifts in AMF biomass in soils in the early growth stages of maize in the greenhouse, we conducted sequencing of the 18S rRNA gene (V9 region) to identify the diversity and community composition of the AMF community early in the maize growing season (growth stage V2 and V3). Based on previous results, we expect to see a more diverse AMF community in low to zero N fertilization environments, however, these results may present differently as this is a greenhouse experiment which leads to a more controlled environment and potentially less selective pressure. Through these results and forthcoming data, we plan to have better insight into the assembly of AMF communities in the maize root system, links to plant soil feedback cycles, and further understand how crop rotation and N management history impact AMF community development. Overall, results from this work will also include amplicon sequencing of AMF in order to characterize the effect of management history on taxonomic diversity and ultimately carbon flow throughout the soil environment. Understanding how AMF communities are assembled early in the maize growing season are vital, not only for the success of agronomically important crops but for soil aggregate formation and carbon sequestration, processes fostered by AMF abundance in soil.

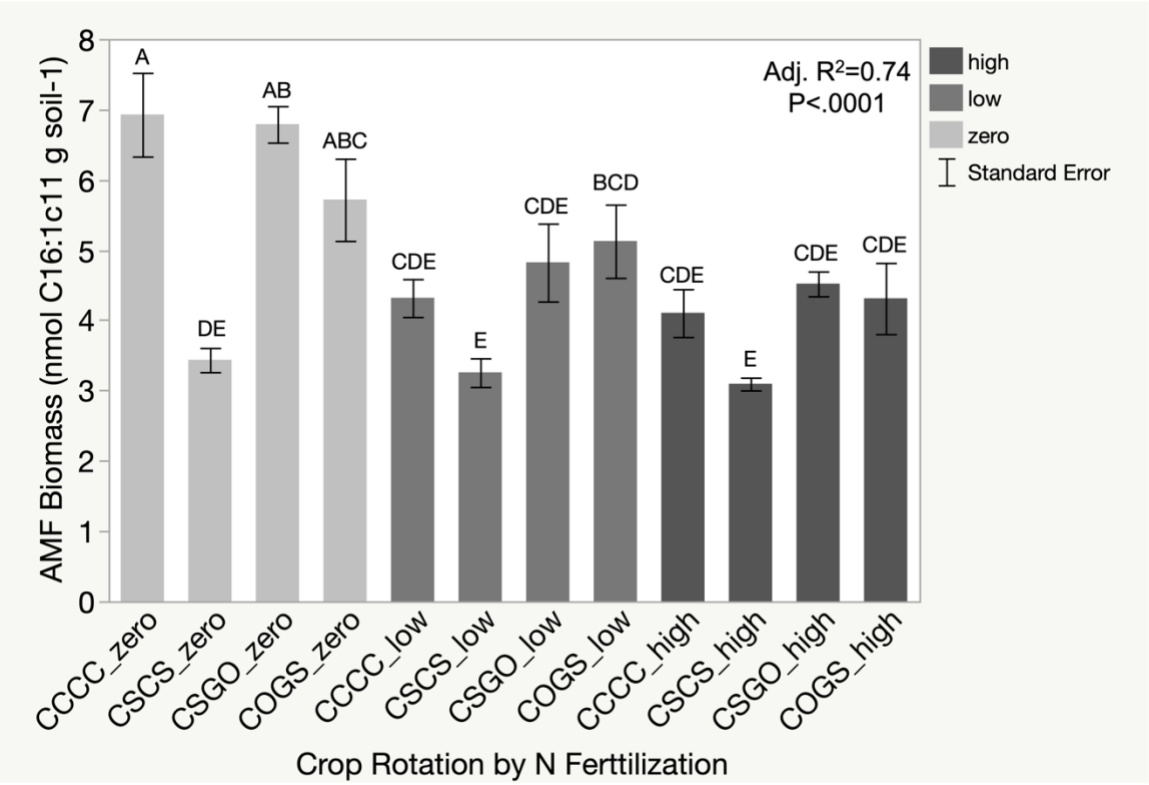
5.5. Figures and tables

Figure 5.1. AMF biomass from the soil by crop rotation and N fertilization history with maize seedlings at the V2 growth stage (Q1).



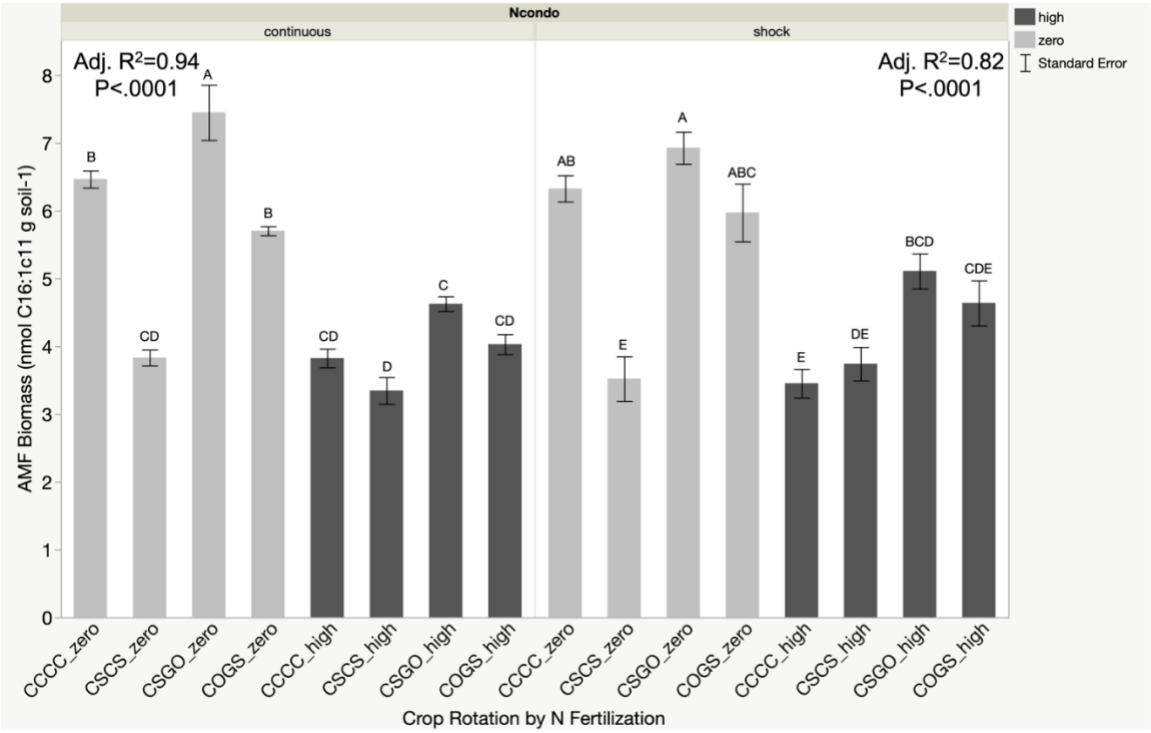
Arbuscular mycorrhizal biomass from the soil from conetainers with maize seedlings collected at V2 and V3 growth stages, quantified by FAMEs. The crop rotations (CCCC, CSCS, CSGO, COGS) and historical N fertilization treatment in the field (in corn & grain sorghum, high (180 kg N ha^{-1}), low (90 kg N ha^{-1}), & zero (0 kg N ha^{-1}); in soybean & oats/clover, high (69 kg N ha^{-1}), low (34 kg N ha^{-1}), & zero (0 kg N ha^{-1}). Standard error bar constructed using 1 standard error from the mean; Tukey's adjustment at alpha=0.05.

Figure 5.2. AMF biomass from the soil by crop rotation and N fertilization history without maize seedlings at the V2 growth stage (Q1).



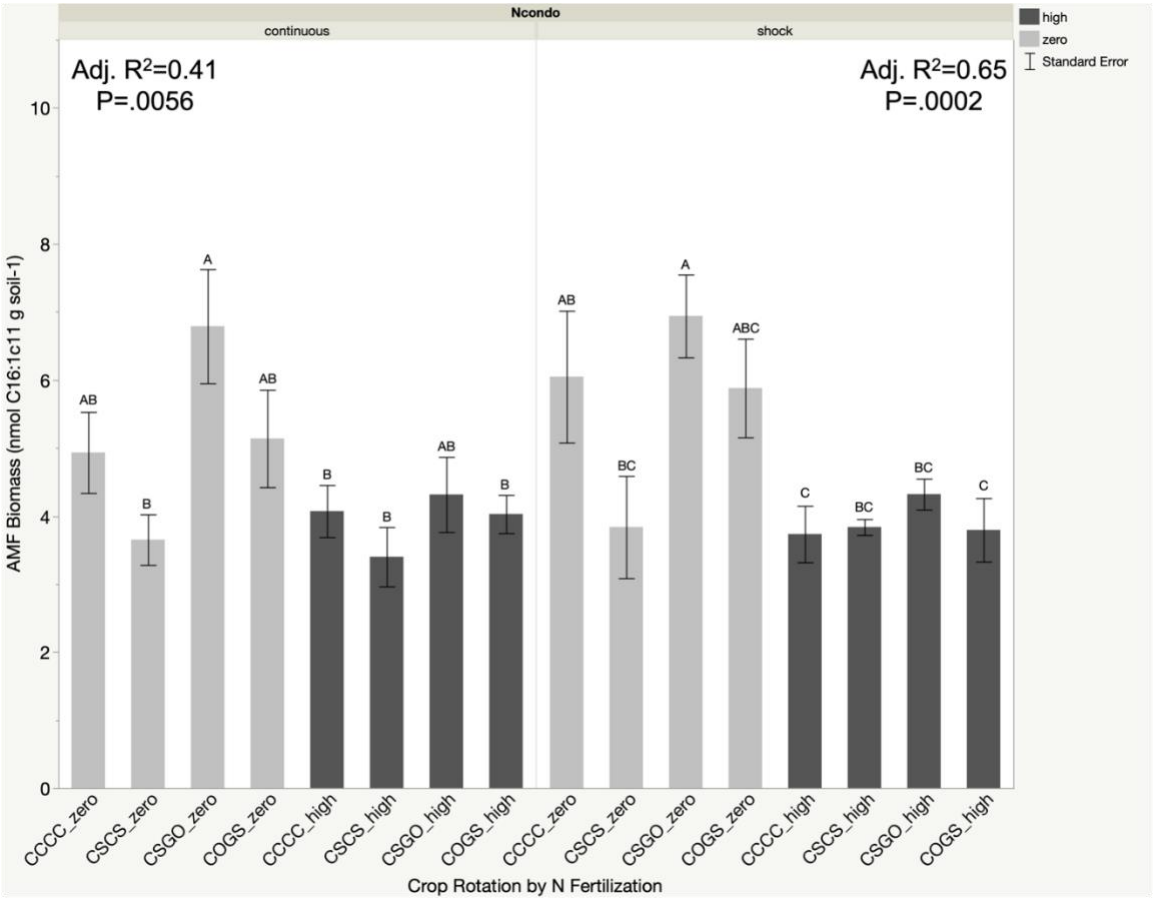
Arbuscular mycorrhizal biomass from the soil from conetainers without maize seedlings collected at V2 and V3 growth stages, quantified by FAMEs. The crop rotations (CCCC, CSCS, CSGO, COGS) and historical N fertilization treatment in the field (in corn & grain sorghum, high (180 kg N ha^{-1}), low (90 kg N ha^{-1}), & zero (0 kg N ha^{-1}); in soybean & oats/clover, high (69 kg N ha^{-1}), low (34 kg N ha^{-1}), & zero (0 kg N ha^{-1}). Standard error bar constructed using 1 standard error from the mean; Tukey's adjustment at alpha=0.05.

Figure 5.3. AMF biomass from the soil by crop rotation and N fertilization history with maize seedlings with the continuous and shocked N treatment at the V3 growth stage (Q2).



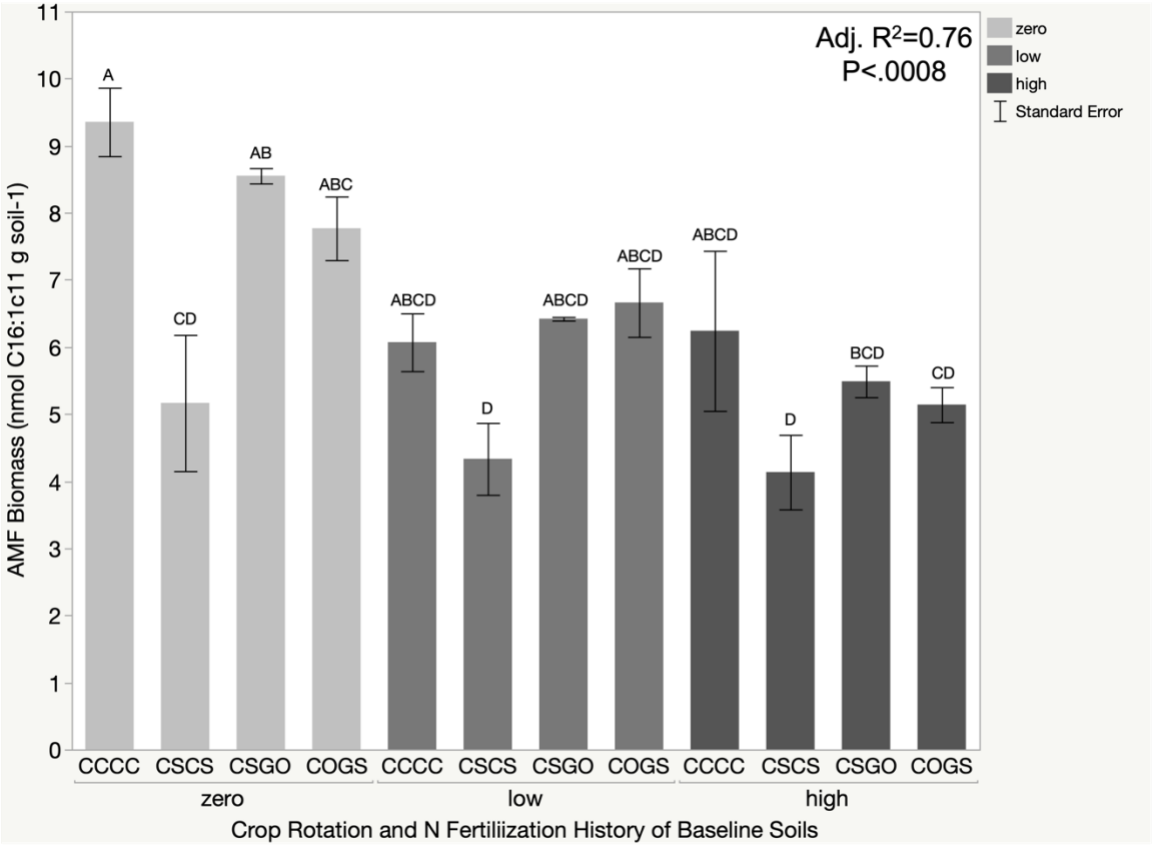
Arbuscular mycorrhizal biomass from the soil from conetainers without maize seedlings collected at V2 and V3 growth stages, quantified by FAMEs. The crop rotations (CCCC, CSCS, CSGO, COGS) and historical N fertilization treatment in the field (in corn & grain sorghum, high (180 kg N ha^{-1}), low (90 kg N ha^{-1}), & zero (0 kg N ha^{-1}); in soybean & oats/clover, high (69 kg N ha^{-1}), low (34 kg N ha^{-1}), & zero (0 kg N ha^{-1}). Standard error bar constructed using 1 standard error from the mean; Tukey's adjustment at alpha=0.05.

Figure 5.4. AMF biomass from the soil by crop rotation and N fertilization history without maize seedlings with the continuous and shocked N treatment at the V3 growth stage (Q2).



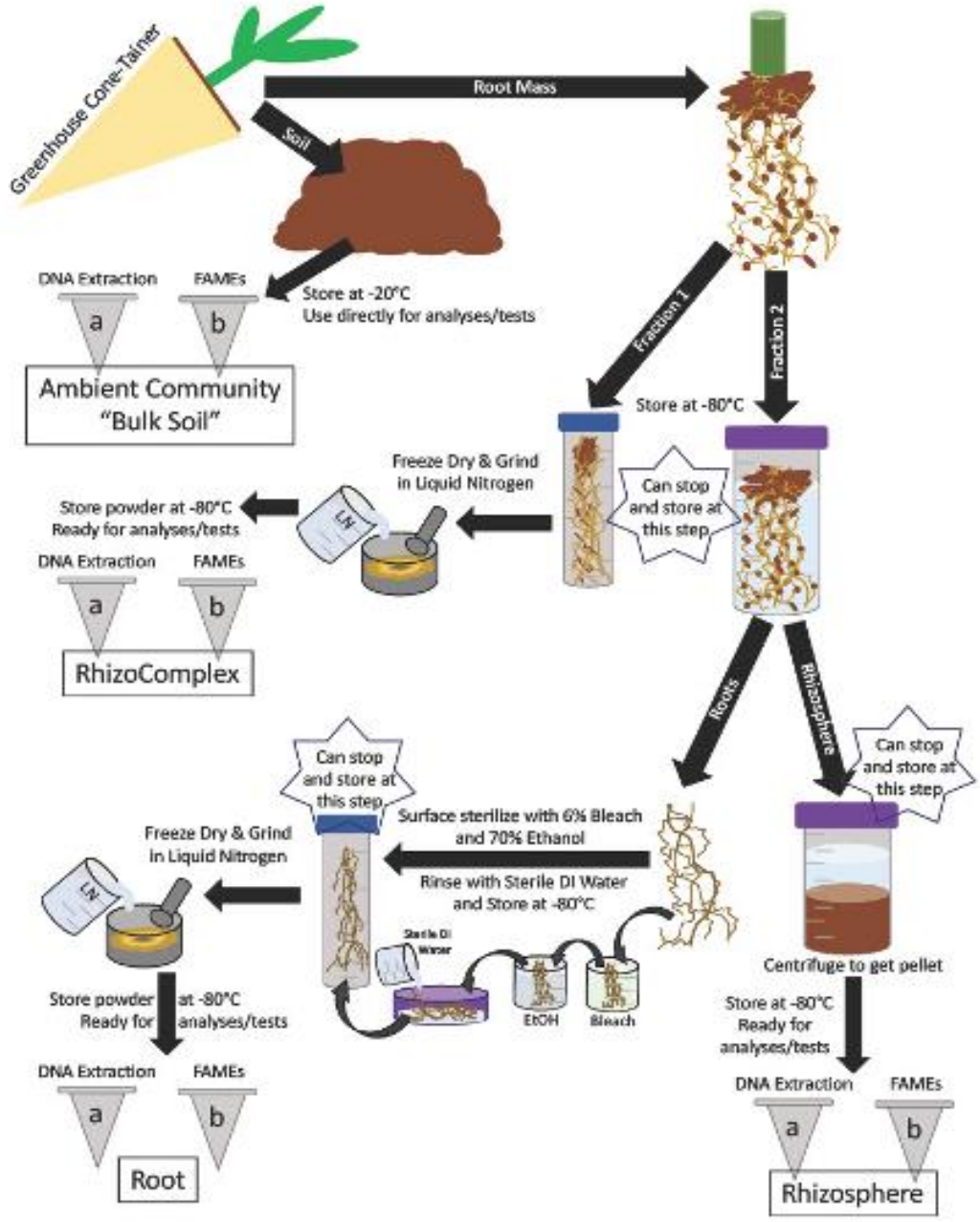
Arbuscular mycorrhizal biomass from the soil from conetainers without maize seedlings collected at V2 and V3 growth stages, quantified by FAMEs. The crop rotations (CCCC, CSCS, CSGO, COGS) and historical N fertilization treatment in the field (in corn & grain sorghum, high (180 kg N ha^{-1}), low (90 kg N ha^{-1}), & zero (0 kg N ha^{-1}); in soybean & oats/clover, high (69 kg N ha^{-1}), low (34 kg N ha^{-1}), & zero (0 kg N ha^{-1}). Standard error bar constructed using 1 standard error from the mean; Tukey's adjustment at alpha=0.05.

Figure 5.5. AMF biomass from the soil by crop rotation and N fertilization history of the baseline soils.



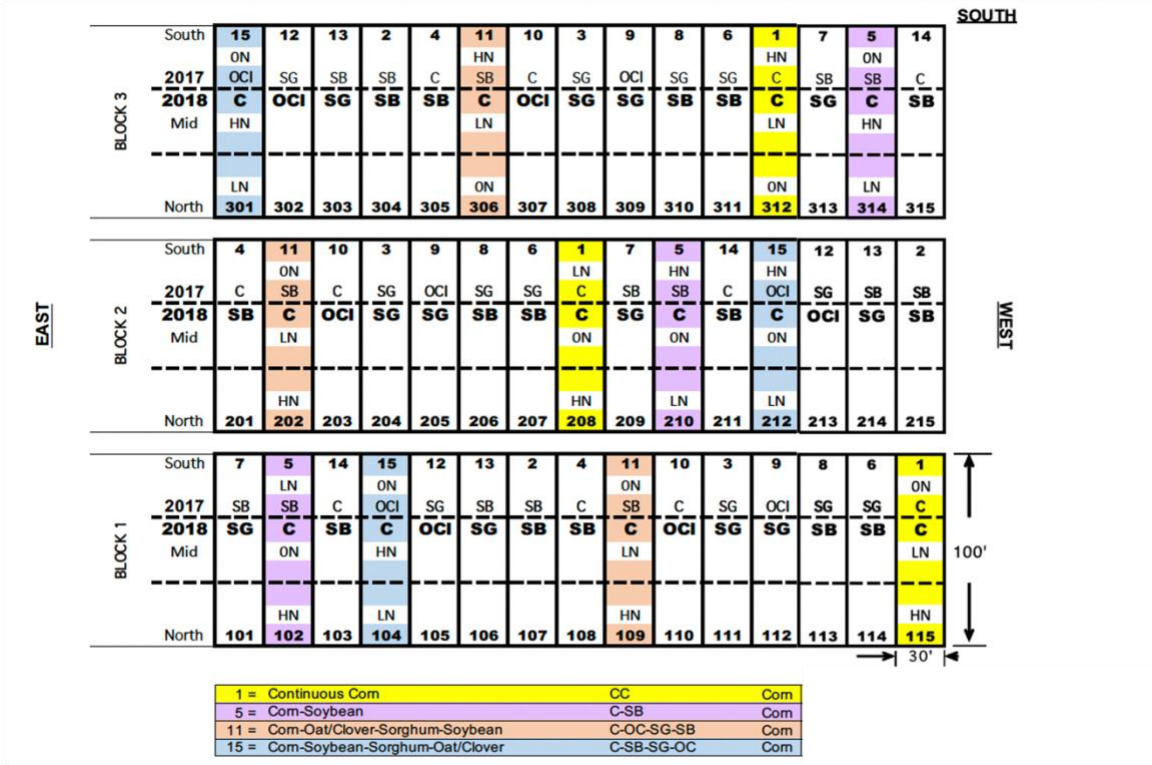
Arbuscular mycorrhizal biomass from the baseline soil from conetainers without maize seedlings, quantified by FAMEs. The crop rotations (CCCC, CSCS, CSGO, COGS) and historical N fertilization treatment in the field (in corn & grain sorghum, high (180 kg N ha^{-1}), low (90 kg N ha^{-1}), & zero (0 kg N ha^{-1}); in soybean & oats/clover, high (69 kg N ha^{-1}), low (34 kg N ha^{-1}), & zero (0 kg N ha^{-1}). Standard error bar constructed using 1 standard error from the mean; Tukey's adjustment at alpha=0.05.

Figure 5.6. Flow diagram demonstrating collection of all sample types and storage in the Greenhouse study.



Various types of samples were collected from Q1 (soil, rhizosphere, and rhizocomplex) and Q2 (soil, rhizosphere, and rhizocomplex). There were 12 treatment histories for Q1 (4 crop rotations x 3 N fertilization rates) and 8 histories for Q2 (4 crop rotations x 2 N fertilization rates, continuous and shocked).

Supplemental Figure S5.1. Field plot layout of where soil samples were collected from the crop rotation experiment (CRS) prior to acclimatization in the greenhouse. There are 3 blocks and 4 crop rotations (CCCC, CSCS, COGS, CSGO).



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