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Possibilities & Potential of Perennial Wheat: A Comparison of Arbuscular Mycorrhizal Fungi Diversity and Abundance between Winter Wheat and Kernza

Senior Project Submitted to
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by
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This project is in dedication to my mother, Wynne Noble, whose life and legacy taught me to learn something new every day, and to never let go of a dream. It is the memory of her passion, diligence and love that made me the person I am today.

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Abstract

In an effort to harness the benefits of perenniality on soil health, a perennial wheatgrass called Kernza (*Thinopyrum intermedium*) has been developed for commercial use at the Land Institute in Salina, Kansas. The effects of a cultivar of Kernza intended for the Hudson Valley, NY were examined in this study using Arbuscular Mycorrhizal fungi (AMF) as an indicator of soil health. AMF are an obligate symbiotic fungi known to provide physical and biological benefits to soil and plant systems. Fields containing one and four year old Kernza, as well as a field of an annual winter wheat were sampled to assess how time since planting affects relative abundance and diversity of AMF. ITS metagenomics were used for data collection and analysis. Results showed the highest relative abundance of the phylum *Glomeromycota* (members of which are classified as AMF) in the four year old Kernza plot, followed by the one year old Kernza, and the lowest relative abundance in the winter wheat. The highest levels of diversity were also found in the four year old Kernza plot, followed by the one year old Kernza, and the lowest levels of diversity were found in the winter wheat. Sample site (field) was found to have a statistically significant effect on diversity (Shannon; F=3.6297, p=0.04868, Observed; F= 32.225, p=1.645e-06). Though there were limitations in data collection and analysis, these results point to the significance that time without disturbance, year round ground cover, and the deep rooted nature of perennial crops has on AMF community composition and abundance.

Chapter 1: Introduction

1.1 **Introduction**

The publication of *Tree Crops: a Permanent Agriculture* by Joseph Russell Smith in 1929 began as a reaction to the rapid industrialization of crop farming in the early 20th century. Smith, born in 1876, proposed in his book what he believed to be a solution to the vast environmental destruction he had been witnessing across the US; a redesigning of American cropping systems in which trees are the major crop. In his book he proposes the 'reengineering' of trees to yield crops, thus allowing farmers to take advantage of trees' deep root system and erosion protection. Smith's book voices a concern for the loss of suitable soil for farming, a concern that has been shared in literature many times over since.

Fifty years later, Wes Jackson, who proposed the creation of agroecosystems that mimic the prairie in his 1980 book '*New Roots for Agriculture*', offered the idea of the breeding of perennial grains, pulses, and oilseeds in an effort to transform the farmscape to a soil benefitting system, as opposed to the soil degrading system of most commercial agriculture today. Jackson's vision is being explored through the research and development of perennial crops at his research institution, the Land Institute, in Salina, Kansas. The first perennial grain developed there was Kernza, a variety of intermediate wheatgrass bred for commercial use as an annual wheat replacement.

The breeding of perennial grains is not entirely new. In the 1930s Soviet researchers attempted to breed perennial wheat through hybridization processes (Armstrong 1936), however, by the 1960s the project was abandoned due to a lack of success. Similarly, in California in the mid-20th century perennial wheat efforts were picked up and abandoned due to low yields as compared to annuals (Krug 2019). Aside from select individuals and research, perennial farming has remained a fringe concept over the last couple hundred years, separated almost entirely from mainstream agricultural practices. Interest in perennial farming systems stems primarily from the benefits perenniality provides to soils; with deeper and larger roots systems than annuals, no tillage required, and rapid growth in the spring to outcompete weeds (Sprunger et al. 2018, Crews et al. 2016, Crews et al. 2018, Kruger 2019), perennials have been shown to have positive effects on soil ecosystems as compared to annuals (De Olivera et al. 2018, Crews et al. 2016, Kane et al. 2016). However, perennial cropping systems are not fully developed or understood as of today.

A perennial farmscape is a far reach from the farming systems employed today to produce most of the world's food. Conventional farming practices include the use of pesticides, chemical fertilizers, monocropping systems and annual plants, and these practices have dominated most of the western world's food systems for at least a hundred years. Concern has risen for the negative human health and environmental effects of conventional practices such as health complications as a result of pesticide use (EPA 2017), the creation of dead zones in waterways (Adams and Clemens 2017, Dartmouth 2012) and other negative ecological consequences of this kind of land use, in tandem with rising concern for soil viability and future food production (Glover et al. 2010, Crews et al 2016). Additionally, as research is becoming more focused on the impact of soil biology (in addition to soil chemistry and physiology) on soil health, the pivotal role soil biological communities play in farming sustainability is becoming clearer. Healthy bacterial and fungal communities are a critical component of healthy soil, yet recent research has shown that conventional farming practices have a negative impact on soil microbiota, including arbuscular mycorrhizae fungi (AMF) communities (Schmidt et al. 2019, Helgason et al. 2010, Stover et al. 2018, Rillig 2004a).

AMF dominate almost all vegetation, grassland, and desert biomes, have been shown to form mutualistic associations with 80% of land plants, and are known to be prevalent in semi-arid grasslands (Goss et al. 2017, Bardgett 2005). AMF have also been shown to influence plant community structure, biodiversity, primary production, and ecosystem dynamics (Rillig 2004a, Otgonsuren et al. 2010, Hontoria et al. 2019). AMF's role in plant nutrient acquisition makes them a primary point in nutrient cycling, meaning that AMF can be considered 'keystone mutualists' (Rillig 2004a). AMF can be viewed as indicators of soil microbial community health, as well as as indicators of general soil and plant health.

Perennial plant communities have been shown to positively impact AMF (Culman et al. 2013, Crews et al. 2016), creating temporally consistent C access that AMF rely on and allowing for long periods without soil disturbance (Oehl et al. 2009, Schneider et al 2015, Crews et al. 2018), meaning that belowground AMF networks can remain intact. The long term effects of breeding commercially feasible perennial cash crops could be enormously positive on soil and soil microbiota health. Crops, such as the perennial wheat Kernza, have the potential to create strong, diverse AMF communities, but the dynamics of this relationship have yet to be explored.

1.1.1 *Kernza*

Kerza is an intermediate wheatgrass (*Thinopyrum intermedium*), a perennial relative of annual wheat, which has been bred as a commercially viable wheat variety. Developed at The Land Institute in Salina, Kansas, Kerza at its current breeding stage produces long and slender seed heads which contain more seeds than annual varieties, however, the seeds produced are 1/5th of the size of the annual's. Kerza's yield has been shown to vary with location and plot size, so diversified breeding efforts on over 2,000 acres of US are being employed to create a more commercially viable crop (Dehaan 2020, The Land Institute 2020).

Being a perennial, Kernza soils are covered by vegetation year round, providing consistent C access for microbial communities belowground. Kernza trials in Michigan, USA showed soil food webs in Kernza plot to be more complex and established as compared to annual wheat (under organic, high and low N regimes) four years after establishment (Crews et al 2016). Eddy covariance measurements have shown that total carbon fixed over Kernza is ~50% higher than C lost via respiration from soil organic matter decomposition, demonstrating that over short time spans Kernza can operate as a carbon sink (dependent on the amount of Kernza harvested per year) (Crews et al 2016), furthering the investigation into the capacity for perennial crops like Kernza to fight climate change.

The development of Kernza has been a slow process of trying to detect changes of SOM and soil regeneration. The long term effects of a transition to a perennial system from an annual cropping system have shown soil carbon accumulation rates between 0.33-1.88 t ha-1 yr-1, however, these rates were only shown to continue for one to two decades, then to gradually decline until no net carbon was sequestered after half a century (Crews et al 2016). Perennial grains are expected to require more water per unit of food as compared to annual grains due to their year round growing season, however, the continued work on improvement through breeding might be able to improve water productivity. Perennial crops being developed will also likely experience increased nutrient acquisition with minimal nutrient rich run-off (De Oliveria et al. 2019, Crews et al 2016).

Kernza has been shown to maintain a high water-use efficiency (WUE) throughout the growing season, thereby mitigating water stress and having the capacity to survive under unfavorable weather conditions. It has also displayed higher evapotranspiration and net carbon uptake rates as compared to annual counterparts, making it a viable option for the environmental stresses that will present themselves in future climates (De Oliveira et al 2018).

While the current understanding of Kernza and its benefits provides a general understanding of WUE, C flux rates, nutrient cycling and NUE, the future of this perennial cash crop is still somewhat unknown. There is a major gap in understanding the relationship Kernza has to soil microbiota. The relationship Kernza has to AMF, for example, is still relatively unexplored. Based on previous research on AMF colonization of perennial plants, as well as the general scientific understanding of the life strategies and influences plant physiology and perenniality has on AMF, we can assume that there is likely to be a strong relationship between AMF and Kernza, but ultimately there is little data to prove it.

1.2 **Goals and hypotheses**

The aim of the following research is to examine the temporal dynamics of the relationship between AMF species richness and the perennial wheat *Thinopyrum intermedium*, henceforth referred to as Kernza. Soil samples from 0-5cm depth in plots of one year old Kernza, four year old Kernza, and freshly planted annual wheat (acting as the control, representing the youngest Kernza) at the FarmHub in Kingston, NY were analyzed using ITS metagenomics techniques. I hypothesize that soil samples from the oldest Kernza plots will have the greatest species richness and abundance of AMF, and that there will be statistically significant differences found in AMF communities in the three plots examined. The following chapters explore this relationship. In Chapter two, I examine the literature regarding the conditions that promote AMF as well as the role of perenniality and distrudence regimes in supporting soil health. In particular, I present the current state of the science on AMF abundance and species diversity in the rhizosphere of perennial grains which led to the development of my hypothesis. The methods of my data collection and analysis are presented in Chapter 3. In Chapter 4 I present the results and provide a discussion of the implications of the findings in future farming practices and research. I close with conclusions about the project, and an appendix regarding optimizing DNA extraction.

Chapter 2: Literature Review

2.1 Introduction to AMF

It has long been known that arbuscular mycorrhizal fungi (AMF) are critical in plant phosphorus (P) acquisition. AMF produce phosphatase enzymes that bind P to C in organic matter and produce low molecular weight organic acids that allow AMF to pull P out of the soil solution. By creating fungal networks that are deeper and more expansive in soil than the plant roots alone, AMF are able to access and transfer P to their host plant. Much of the research surrounding AMF from the 20th century focused on the fungus' capacity to enhance nutrient acquisition in plants, and much of AMF research was limited to topics surrounding P uptake in plants. AMF were given their due credit for their influence over plant P acquisition, however their recognition as important soil biota ended there.

As research continued, the impact of AMF on host plants' and soil's physical and biological traits began to be more fully recognized. AMF were discovered to aid in soil N transportation and uptake (as well as uptake of micronutrients such as Zn, Fe, and Mn) for plants, to have an effect on soil aggregation and carbon sequestration, and to play a role in determining plant diversity (Rillig 2004b, Bardgett 2005). Presence of AMF has been shown to improve water and inorganic nutrient uptake (especially of P), and to improve tolerance to environmental stresses such as nutrient deficiency, disease, drought, and salinity (likely from increased nutrient acquisition) (Otgonsuren et al. 2010). Even with research about AMF expanding, the full extent to which AMF influences soil and plant systems is still being discovered.

Challenges to understanding AMF community structure and influence on plant and soil properties are due in part to the nature of AMF. AMF are endomycorrhizae comprised of a intraradical mycelium made of the external hyphal network and highly branched arbuscules within root cortical cells (which functions as the site of exchange between the fungus and the plant thanks to the large surface area of the arbuscule interface), as well as the extraradical mycelium which produces the spores and absorbs nutrients for transfer (Bardgett 2005, Hart 2001). In addition to nutrient acquisition, the role of AMF in determining soil physiology is critically important in the contexts of soil health, C cycling and water relations. Soil aggregation, which is essential for stable C storage, is greatly aided by the creation of glomalin by AMF, a substance which binds soil particles together to create aggregates (Crews et al. 2018, Rillig 2004b). Aggregation allows organic C to be safely stored in soil, protected from microbial decomposition and creating a physical soil composition that is favorable to other soil biota (Goss et al. 2017, Rillig 2004b). Mycorrhizal fungi are often the largest C sinks for primary productivity, and the necessity of mycorrhizal fungi is especially high in conditions where nutrient availability is limited (Goss et al. 2017, Rillig 2004a).

However, in the past without a stain for AMF hyphae, much of the previous research on AMF was done via morphological trait analysis on spores and arbuscules on roots, making it time consuming and challenging (Rillig 2004b, Bardgett 2005). In recent years AMF quantification and diversity has been able to be done with PCR and ITS metagenomic analysis, decreasing the time required and increased accuracy (Bardgett 2005, Utobo et al. 2011, Martin-Sanchez et al. 2018).

Facilitating plant nutrient access and improving soil carbon storage are generally recognized as the primary ecosystem services of AMF in cropping systems. Providing nutrient uptake is the general framework of AMF acknowledgement in ecology, however the large scale function and importance of AMF in ecosystems is often overlooked. AMF are often excluded from ecosystem modeling and soil carbon modeling (Rillig 2004a) despite their influence on ecosystem processes. Furthermore, the effects of land use and management practices on AMF abundance and diversity is often overlooked in agricultural contexts.

In systems (such as grasslands, forests and even agroecosystems) where AMF are the main components of mycorrhizal associations (Bardgett 2005, Goss et al. 2017) a large gap is left in our big picture understanding of the role of AMF. We know that AMF require stability in the soil matrix and consistent C access in order to survive as obligate symbionts (Rillig 2004a, Bardgett 2005, Goss et al. 2017, Hart et al. 2001), yet modern land management practices that rely heavily on soil disturbance and seasonality of ground cover are not conducive to strong AMF communities (Schmidt et al. 2019, Crews et al. 2016, Crews et al. 2018). Obtaining a fuller understanding of AMF and AMF's response to disturbance is crucial to creating more sustainable food systems that will provide higher yields, increased soil health and potentially increased sequestered C. Research on the effects of alternate, non-conventional farm management practices point to the critical role AMF play and show the potential for perennial systems (as opposed to only farm management alterations) to create a farmscape more equipped to support AMF communities.

2.2 Physical Disturbance

Ecological succession, which describes changes in biological communities and composition over time, is influenced by disturbance. Primary succession, when soil parent materials are first colonized by microorganisms, transitions into secondary succession after a disturbance event of any kind such as fire, flood, drought, or, in the case of agroecosystems, tillage. Following a disturbance event there is usually a predictable transition from annual to perennial species occupying the space as the disturbance has allowed the room for perennials to come in and conquer (Crews et al 2016). Disturbances restart the successive process, forcing microorganisms (such as endomycorrhizae like AMF) to begin colonization again and create entirely new hyphal networks. Tillage removes root systems from the ground, and without plant roots in place to provide them with C access, the distribution, diversity and richness of obligate microbes is compromised.

Conventional farming systems have relied on tillage for over 2 thousand years, however, criticism of physical disturbance of farming systems has been extensive. Rationale for no- or low-till practices covers a range of topics, including arguments specific to AMF. Tillage practice significantly affects C and N dynamics (Schmidt et al. 2019), and highly disturbed ecosystems are also known to 'leak' nutrients into other ecosystems, as compared to undisturbed ecosystems which develop sucessionally and are able to maintain regulated nutrient levels (Crews et al. 2016).

Natural grasslands maintain a steady midsuccesional state, sometimes acting as C sinks and sometimes as C sources when natural disturbances occur (Crews et al 2016). Following the plowing of land to turn a grassland, for example, into cropland there is immediate decline in SOM creating what Crews et al. determine to be a 'retro-successional' period (Crews et al 2016). In breaking apart soil aggregates and exposing previously protected C to microbial attack, loss of organic C is inevitable. In addition, the typically lower rates of NPP in agroecosystems compared with native ecosystems diminishes C inputs to soil, further altering organic C access for microbiota. SOM, being a fluctuating soil property (representing the inputs of new organic compounds from plant photosynthesis and plant, animal and microbial decomposition, as well as the outputs of microbial metabolizing organic matter and respiring CO2), is altered with ecosystem conversion to cropland. Annuals plants usually are only able to thrive in ecosystems which suffer frequent and extreme disturbance. Though cover cropping, no-till practices, organic management, and/or compost or manure application can increase SOM contents, the SOM of an annual cropping system will not achieve the SOM levels of native grassland even with these non-conventional management practices (Crews et al. 2016).

When soil is disturbed for the conversion of natural ecosystems to agroecosystems via plowing or tillage, mycorrhizal fungi communities are degraded and soil aggregates are broken, exposing the SOM that had previously been physically bound and protected. Tillage leads to an immediate influx of microbial activity, leading to the loss of 20-70% of SOM (Rillig 2004a). Soil disturbance in the form of tillage also destroys aggregates and macro-pores that have been naturally created over time, compressing the soil and compromising its capacity for infiltration down the line. Research into plant-microbe interactions has exposed the challenges soil disturbance poses to AMF communities, showing that tillage breaks the physical link between AMF and the host vegetation which AMF relies on for carbon. Annually tilled systems force AMF to recreate their hyphal networks every year. There is also evidence that in no-till systems plant roots may follow previous root channels, leading them to encounter more AMF propagules than plants growing in tilled land (Kabir 2004).

AMF have been found to consistently display reduction in sheer quantity, root colonization, and diversity in tilled farming systems (Schmidt et al 2019, Helgason et al 2010). Disturbance has been shown to have a negative influence over AMF biomass in soil, with conventional tillage practices having the capacity to reduce fungal diversity by 40% as compared to no-till (Brito et al. 2012). Extraradical hyphae are the main source of inocula in soil, however, they are not as resilient as other types of fungal propagules such as spores. Additionally, AMF are most abundant in topsoil, so deep disturbance from plowing can dilute propagules of AMF throughout soil depths, diminishing the quantity of AMF propagules in the topsoil that can infect plant roots (Kabir 2004), further translating to alterations in soil aggregation and changes in net primary productivity rates (NPP) as well as nutrient uptake capacity for plants (Rillig 2004a).

Tillage practices, which disturb AMF hyphae, have been shown to negatively affect some species of AMF more than others (Kabir 2004, Bowles et al. 2017). Higher levels of AMF species riches in no-till soils as compared to tilled soils has been found in multiple studies (Kabir 2004, Bowles et al. 2017, Brito et al. 2012), suggesting that conventional tillage practices may only allow for survival of AMF with certain characteristics regarding the speed of growth and nutrient uptake rate. Soil disturbance has also been found to alter AMF communities in soil, allowing non-native species to invade and establish dominance. In a 2018 study AMF species richness was shown to be positively correlated with plant species richness, diversity and native plant cover (Stover et al 2018).

No-till systems have been found to be the optimal systems to increase AMF colonization, followed by other alternative tillage systems (shallow inversion and ridge tillage), as compared to conventional tillage (Bowles et al. 2017). Low intensity tillage systems have displayed increased AMF taxa richness by 11% as compared to conventional tilled systems, likely as a result of increased niche-space in the rhizosphere and root zone to support disturbance sensitive AMF taxa (Bowles et al. 2017).

AMF in annual agricultural systems do not have the benefit of consistent C access when vegetation is removed from the system and ground is left bare. Timing of tillage has been found to be of importance to AMF hyphal survival, with fall tillage being more harmful to AMF hyphal networks than spring tillage (Kabir 2004); immediate regrowth after tillage allows for consistency in C inputs to AMF networks.

2.2.1 Perennial crops

There is a strong argument in scientific communities for a reimagined farmscape; a perennialized future in which major cash crops such as wheat, corn, soy and oilseeds are perennial crops. Beneficial changes in soil and water quality have been shown to be greater in systems converted to perennial wheat cropping from annual as compared to systems with changes only made to land management practices (Culman et al 2013). Designing agroecosystems that mimic diverse natural ecosystems (prior to the introduction of annual crops) may provide a path to more sustainable grain production via the breeding of perennial crops and altered management practices for higher diversity.

As agro-ecological interest in AMF grows, interest in the use of AMF to aid crop growth has also expanded. There has been an effort in recent years to innoculate seedlings with AMF to observe the influence AMF has, and to demonstrate the capacity for inoculation to be used for increased crop yields or even increased wild grassland resilience. In a 2010 study in Mongolia increased growth in inoculated wheatgrass seedlings was observed after three weeks of growth. After six months inoculated seedlings were found to be 175% taller and have roots 191% longer than the control. N, P, potassium, calcium, sodium and magnesium concentrations in roots, stems and leaves of inoculated samples were also found to be significantly higher than the control (Otgonsuren et al 2010), providing a clear display of the benefit AMF had on the wheatgrass. Mycorrhizal influence was also found to be stronger in aerial biomass than in root biomass, likely because the allocation of carbohydrates to the shoot of the plant was greater than that given to the roots after AMF colonization (Otgonsuren et al 2010).

As evidence of the benefits of perenniality, AMF, and AMF's relationship to perenniality increases with scientific research, there has been a movement to reimagine the global farmscape as perennial dominated systems. Natural perennial grasslands, even those which have been harvested for hay for over 75 years such as those in Kansas, USA, have been shown to maintain higher levels of soil organic carbon, total nitrogen and water stable aggregates to depths of 60cm as compared to adjacent high input annual croplands. Microbial biomass has also been found to be significantly higher in perennial natural grasslands as compared to annual wheat plots (Culman et al 2010). The knowledge of the influence of AMF on soil and plant health has become an incentive for breeding efforts to create perennial alternatives for the world's major cash crops, including a perennial wheat. Breeding for commercially viable perennial crops will be inherently challenging; annual plants, which allocate most of their energy and nutrient resources to aboveground growth, naturally produce larger, stronger seeds than perennial plants which prioritize belowground growth (Weih et al. 2011, Crews et al. 2018). Modern farming values wheat crops which produce high yields and high levels of shatter resistance, traits that are beneficial to annual plants that rely on seed growth in previous seasons to scatter and survive until next season (Roumet et al. 2006). While perennial wheatgrass does not naturally provide these traits, efforts to create usable perennial wheatgrass still persists.

2.3 Temporal Consistency

With continuous annual cropping systems, SOM is in a constant state of decline. Diminished organic C storage in farmland not only affects crops, but also the obligate symbionts that rely on host plants for C (such as AMF). Annual cropping systems pose a significant problem for microbial communities in soil; with bare ground for a large portion of the year, in addition the disturbance annual cropping systems are often paired with, there is a lack of temporal consistency in carbon access for microbes. Plant roots, being the most significant pathways for the introduction of SOM to the soil profile, are removed every year and, in restarting the growth cycle every year, annual plants are at constant risk of being outcompeted for sunlight and soil resources.

Having consistent access to carbon is crucial to the health of AMF communities. As obligate symbiotes, AMF benefit greatly from the undisturbed, deep root systems of perennial plants. Perennials, which allocate a higher portion of available resources to belowground growth rather than aboveground growth (like most annuals do) are well equipped for long-term survivorship with their conserved resources. The perennial root systems which exist belowground year round are also able to foster more structured and diverse soil biota communities as compared to their annual counterparts. The depth of the roots of perennial grasses allows for the maintenance of strong soil food webs at lower depths than what exists below annual wheat. C access at deeper soil levels also has a beneficial impact on belowground microbial community structure, subsequently benefitting AMF communities.

Perennial ecosystems are also able to increase their uptake of nutrients via increased transpiration of precipitation as compared to annuals. Scientific literature has displayed greater N-use efficiency from larger root systems, also leading to greater C stabilization from root C storage (which persists for a longer period of time than shoot C storage) (Sprunger et al 2019). Deep rooted crops aid in subsoil C deposition, maintaining soil C levels and mitigating CO2 additions to the atmosphere by storing organic C in stabilized forms that are slow to be microbially metabolized. In perennial grasses 50-67% of fixed carbon is allocated towards and stored in root tissues belowground, whereas in annual species (such as wheat, corn and sunflowers) only 15-30% of carbon is used for belowground production (Crews et al 2018). Perennial crops have been found to protect even relatively small C amounts that were deposited in deep soil layers down to 3.6m (Peixoto et al 2020).

In the removal of perennials from the landscape to make way for annual crops, soil is susceptible to erosion from wind, rain and even the effects of gravity. Soil degradation has been estimated to be a loss of 1.52mm of soil per year in tilled agricultural systems and 0.065mm per year in non-tilled agricultural systems, giving a rate of soil loss that is 360 and 16 times the rate of global soil formation, respectively (Crews et al. 2018). Soil erosion, along with loss of SOM and nutrient imbalance, are often considered some of the most significant threats to soil function, however, working perennials into landscapes could significantly positively alter soil C accumulation (Sprunger et al 2019).

Long term perennial systems have a multitude of benefits, and these benefits become stronger and more clear the more seasons any given perennial is in the land. The time since planting has been previously shown to have an effect on nutrient retention and nitrogen use efficiency (NUE). In a 2018 study, differences in soil C between annual and perennial intermediate wheatgrass were not shown until after the first 3 years of growth, despite intermediate wheatgrass having a statistically significant larger root biomass (Sprunger et al 2018). In the same study intermediate wheatgrass was shown to aid in enhanced NUE and greater N retention as compared to annual wheat. Perennial intermediate wheatgrass was shown to produce 8 times as much root biomass as compared to annual wheat, a trait providing ecosystem services including increased N cycling and accumulation, allowing for greater NUE overall (Sprunger et al. 2018). Deep rooted perennial systems, while maintaining protected C, are simultaneously maintaining levels of nutrient cycling equal to that of annual plants (Crews et al 2016). Thanks to these traits, as succession progresses in perennials, belowground microbiota communities (including AMF communities) become more stable and complex (Crews et al. 2016).

When provided with undisturbed root systems, the life-history strategies of AMF can be examined. Previous studies have found that these strategies may be a determining factor of AMF persistence over time. The Hart et al. Driver/Passenger hypothesis describes two mechanisms by which AMF succession may be influenced; ‘driver’, in which AMF communities are responsible for plant community changes, and ‘passenger’ in which AMF community changes are a product of plant community changes (Hart et al 2001). Being asexual obligate biotrophs, AMF hyphal networks have the capacity to colonize multiple plants at once, making their lifespan as individuals harder to define. In addition to being challenging to separate when multiple species colonize a singular host plant, AMF behavior may be largely influenced by host and environmental conditions. AMF may be a consequence or a cause of plant dynamics, posing the question of how land-use history affects AMF, and how AMF affect vegetation. If ‘driving’ plant dynamics, AMF could potentially be aiding or inhibiting plant growth, thereby diminishing the power a farmer (for example) has over the land they work.

2.3.1 Cover Crops

Some practices, such as no or minimal tillage, the use of cover crops, and implementing polyculture strategies have been employed in an effort to maintain soil physical and biological health. While staying short of transitioning to an entirely perennial system, these management practices have shown a generally positive effect on AMF communities (Hegason et al 2010, Hontoria et al 2019, Crews et al 2016).

The use of cover crops in maintaining groundcover and increasing N fixation is a practice that can influence C access for soil microbial communities, and prevents against some exposure that soil biota might have otherwise endured under bareground. In stimulating AMF, cover crops enhance soil health and promote agroecosystem sustainability.

One study examining different cover crop treatments on maize fields in central Spain found that cover crop treatments had a significant effect on AMF community composition, with barley cover cropping resulting in greater colonization of maize roots as compared to winter fallow cover cropping. AMF communities that were promoted from barley were then inherited by the subsequent maize crop, a result which differed from previous studies (Hontoria et al 2019). The ability to pass on AMF communities from cover crop to cash crop appeared to be greater with grass cover crops (such as barley) than with legumes (such as vetch). Differences in root morphology, root growth or mycorrhizal dependency could cause this (Hontoria et al 2019).

Meta-analysis found that, though some studies found changes in diversity as a response to cover cropping, these changes were inconsistent over the studies examined (Bowles et al. 2017). Cover crops were found to increase AMF formation with or without tillage use, displaying that root associations for AMF are potentially of equal importance to AMF colonization as the disturbance regime. AMF in fields that used cover crops (especially legumes) were shown to be able to survive some tillage (Bowles et al. 2017).

2.4 Conclusions

Previous research on the role of AMF in soil health has shown that type of cover crop and type of alternative tillage have been found to be the key factors influencing change in AMF colonization, as well as that managing AMF community composition may be site-specific and oriented around farming technique and environmental conditions (Bowles et al. 2017). Though alterations in farm management away from conventional systems can be beneficial to AMF communities, the complexity and species richness of AMF in perennial systems cannot be obtained in an annual system (organic, no-till, polyculture, or otherwise) due to the 'chronically disturbed' nature of agricultural soils supporting annual crops.

AMF community composition influence can be considered on temporal scales ranging from seasonal to successional, and increased time without disturbance to create and maintain hyphal networks within root systems allows for the creation of stronger and more diverse AMF networks. Plant and microbe communities in disturbed land are placed in a constant state of existence somewhere between primary and secondary succession. A transition to a perennialized farmscape has the potential to greatly impact the microbial communities of agricultural soils. Kernza, as a perennial wheatgrass, may be a key player in producing commercially viable perennial cash crops.

Though there is previous research that points to the potential of perennials to benefit AMF communities in farmland, there is also an abundance of questions remaining about the effects of perennial crops on soil microbial health, and about AMF communities specifically.

As farmers and agricultural communities begin to shift their focus to the potential for farmscapes to be more sustainable and to create stronger soil systems, all while maintaining high levels of yield for our ever growing world, the possibility of a perennial future becomes more palpable. Ecological intensification, a key process by which ecosystem services are harnessed to create sustainable food production systems, will require below-ground biological activity that facilitates resource utilization and efficiency. The relationship between new perennial crops and soil microbial communities should continue to be explored through scientific research, and the aim of this paper is to follow through with this goal.

Chapter 3: Methods

3.1 Sampling

Samples were taken on November 6, 2020 at the Hudson Valley Farm Hub in Hurley, NY from two plots of Kernza and one plot of an annual hard red winter wheat (Rouge de Bordeaux). Field 8 consisted of Kernza plots that had just completed their fourth year of growth, field 3 consisted of Kernza plots that had just completed their first year of growth, and field 20 consisted of the winter wheat which had been planted in late September of 2020. Sampling locations at each field were determined by sampling units from Cornell Soil Health Test results from 2018, creating sub-sections of each field based on soil type, crop history, and history of management.

Sampling was conducted with a soil auger with a soil probe 15” in length and ¾” in diameter. 30 samples were collected as soil cores from each of the three sampling fields from 0-5cm belowground. All equipment used during sampling was cleaned between samples with ethanol wipes, and samples were stored in sterile Whirl-Paks. During fieldwork, samples were kept in a cooler over ice, and after fieldwork samples were transported back to Bard College and frozen (-20°C) until processing.

Each sample was defrosted for homogenization and then refrozen (-20°C) until DNA extraction. Sub-samples were created with three samples from each field (combining equidistant samples with one another; for example samples number 1, 11, and 21 were combined). Samples were homogenized using a 2mm sieve, and any larger root, leaf, or other large biological material in the samples were discarded. All materials used for sub-sample preparation were cleaned with a brush, washed with deionized water, and soaked in 70% ethanol between each sub-sample homogenization.

3.2 DNA Extraction and Metagenomics

DNA extraction was performed with the ZYMO *Quick*-DNA Fecal/Soil Microbe Miniprep Kit (17062 Murphy Ave. Irvine, CA 92614, U.S.A.) using 25 μg aliquots according to the manufacturer instructions. One modification was made to the processing; samples were shaken for 20 minutes as opposed to the instructed 5 minutes in an effort to encourage the extraction of DNA from AMF spores (Schneider et al. 2015). DNA was extracted from all 30 subsamples and stored frozen (-20°C) until samples were sent for metagenomic processing for raw ITS rRNA reads (Wright Labs; Juniata College, Huntingdon, PA).

3.3 Statistical Analysis

ITS rRNA sequence data was processed using a DADA2 pipeline developed from the DADA2 Pipeline Tutorial 1.6 found at https://benjjneb.github.io/dada2/tutorial_1_2.html (Callahan et al. 2016, Perron 2021 [unpublished]) using the UNITE database and sequence management environment (Kõljalg 2020). DADA2 displays microbial communities using amplicon sequence variants (ASVs) (instead of operational taxonomic units (OTUs) used in other pipelines). Data was then processed into a phyloseq object. Samples with below 5,000 reads were removed from the data. Alpha diversity measurements were taken using the phyloseq packages in R for Observed and Shannon diversity indexes.

Figures and tables were made with the ggplot2, phagorn, DECIPHER, magrittr, ShortRead, BioStrings, readr, and statistical packages of R. Analysis of variance tables were created in R using data frames made from the pruned phyloseq object.

Chapter 4: Results and Discussion

4.1 Results

The three sites in this study were field 3 (the one year old Kernza plot), field 8 (the four year old Kernza plot), and field 20 (the six week old annual winter wheat plot containing Rouge de Bordeaux). These three plots are the three treatments examined in the data.

It should be noted that of the 10 subsamples from each field, five were removed from the data from field 3, two were removed from the data from field 8, and two were removed from the data from field 20 due to having read counts below 5,000 in the raw metagenomic data.

4.1.1 Diversity Indexes

Two diversity indexes were used to analyze relative abundance of the phylum *Glomeromycota*; the Shannon Diversity Index (*fig. 1*) and the Observed Diversity Index (indicating the count of unique OTUs found at each site, *fig. 2*). Members of the phylum *Glomeromycota* are known to be arbuscular mycorrhizas, and for the purpose of this study the entire phylum is considered to be AMF.

The highest average Shannon diversity was from field 3 (3.0982), followed by field 8 (3.0322), and the lowest was from field 20 (2.5530). Sample site was shown to have a statistically significant effect on Shannon diversity of *Glomeromycota* (F=3.6297, p=0.04868).

The average Observed diversity was highest in field 8 (76.14), followed by that of field 3 (65.00), and the lowest from field 20 (20.88). Sample site was also shown to have a statistically significant effect on Observed diversity of *Glomeromycota* (F= 32.225, p=1.645e-06).

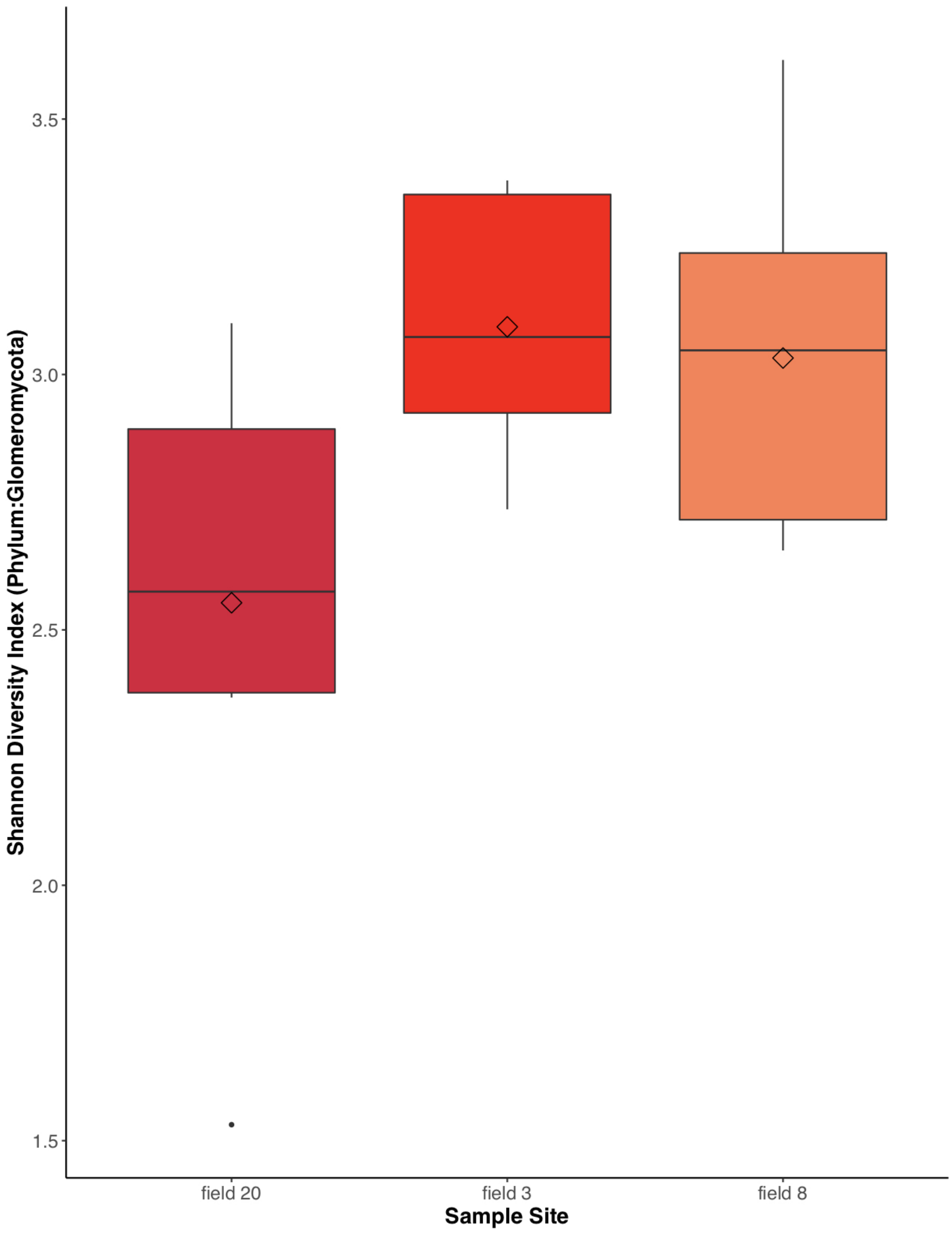

Figure 1: Shannon diversity index of the phylum Glomeromycota by sample site (F=3.6297, p=0.04868)

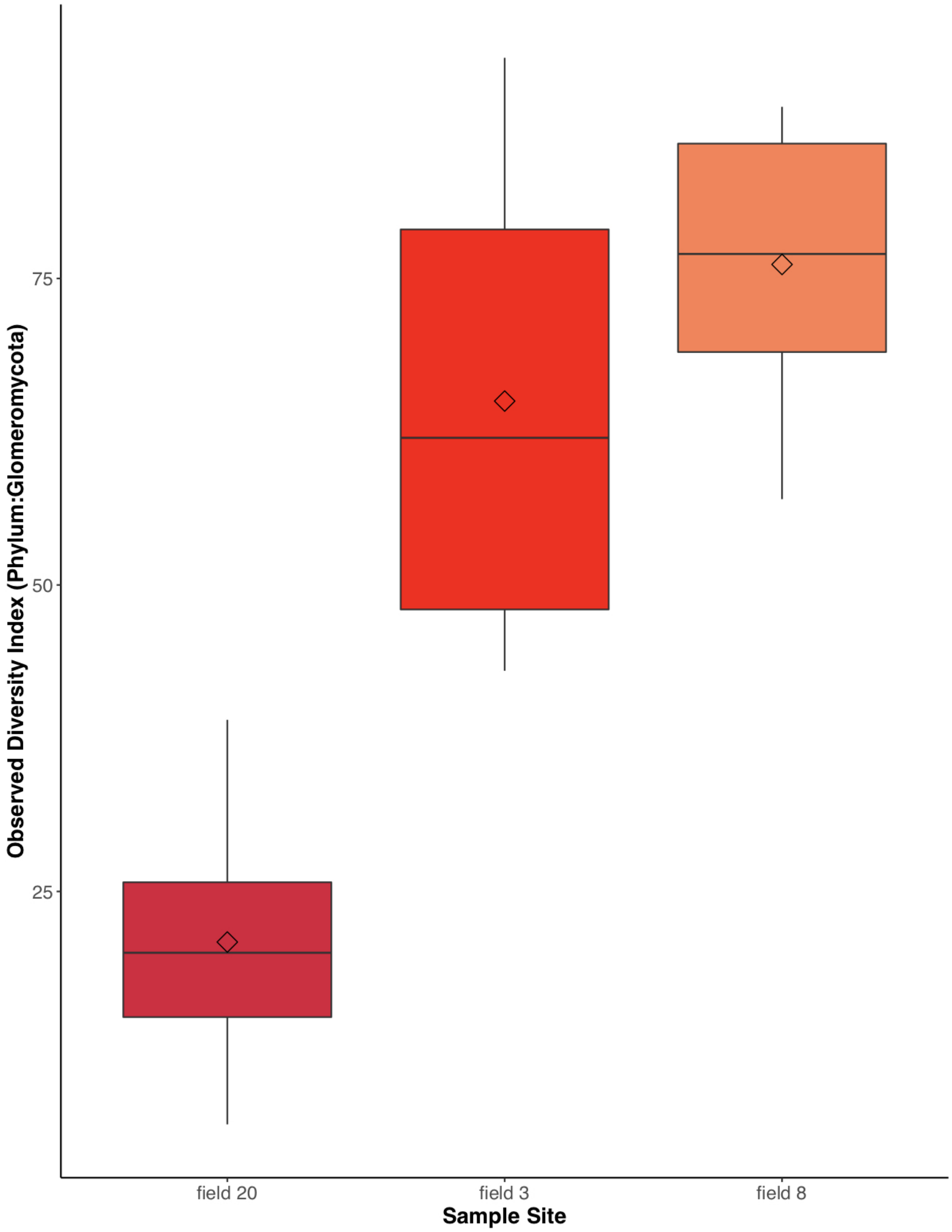

Figure 2: Observed diversity index of the phylum Glomeromycota by sample site (F= 32.225, p=1.645e-06).

4.1.2 Relative Abundance of AMF

Relative abundance measurements of the phylum *Glomeromycota* by field (*fig. 3*) displayed highest relative abundance in field 8 (40434), followed by field 3 (17128), and the lowest relative abundance in field 20 (3897).

Within the phylum *Glomeromycota*, the highest relative abundance of the families within the orders *Diverisporales*, *Glomeralies*, and *Paraglomerales* were found in field 8, and the highest relative abundance of the families within the order *Gigasporales* was found in field 3 (*fig. 4*), reflecting the Shannon diversity and Observed diversity measurements found in *fig. 1* and *fig. 2*.

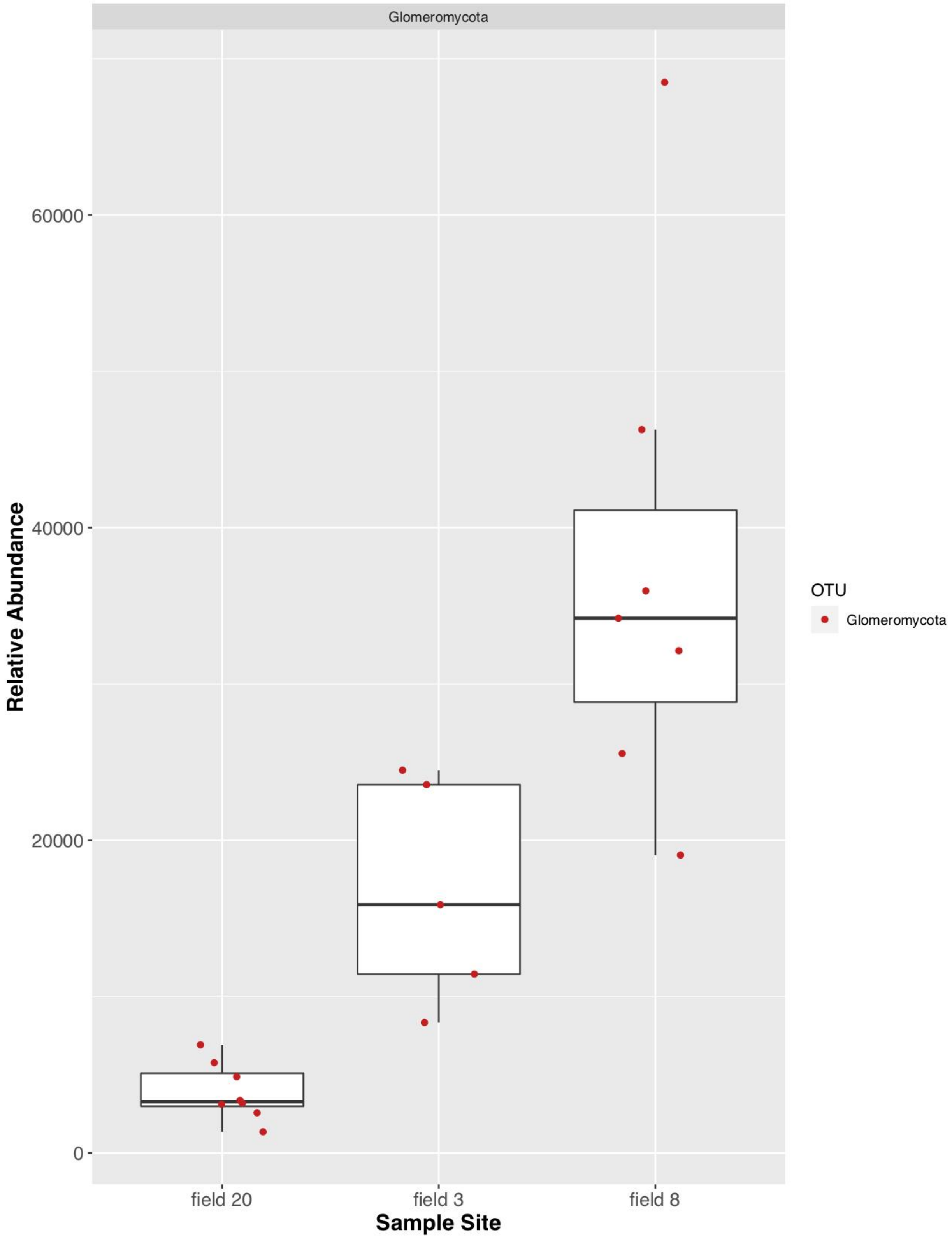

Figure 3: Relative abundance of the phylum Glomeromycota by sample site.

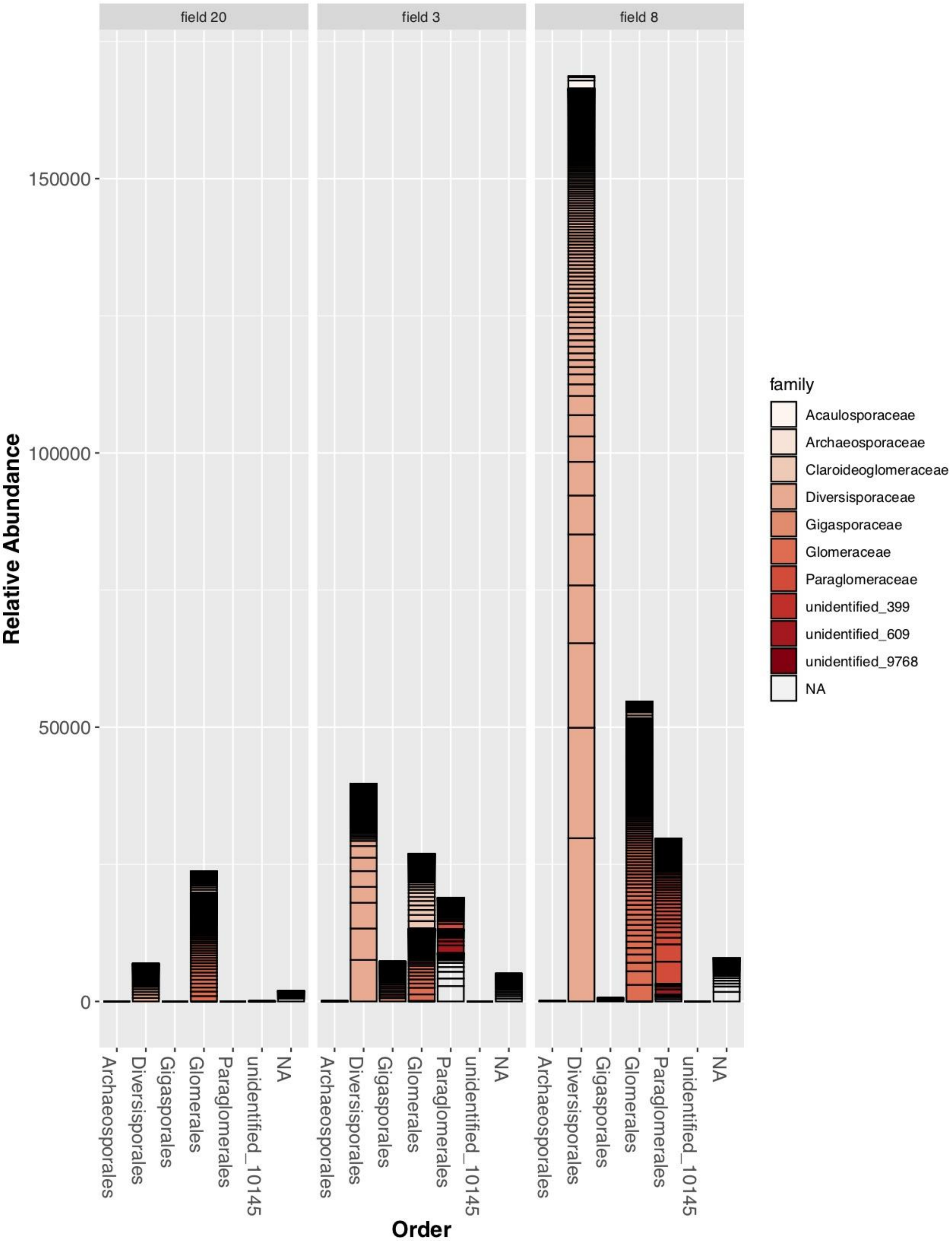

Figure 4: Relative abundance of each family within each order of the phylum Glomeromycota, split by sample site.

4.2 Discussion

Results of this study aligned with the hypothesis that field 8 (containing the oldest Kernza, which had been growing on field 8 for five years) would have the highest levels of diversity and the highest relative abundance of AMF (fungal phylum *Glomeromycota*). This may be due to relative lack of disturbance of field 8, however, due to inconsistencies in land use history and plant communities existing on the three fields in which samples were collected, the reason for this result cannot be confirmed.

There are currently three recognized classes (*Archaeosporomycetes*, *Glomeromycetes*, and *Paraglomeromycetes*), five orders (*Archaeosporales, Diversisporales*, *Gigasporales*, *Glomerales* and *Paraglomerales*), 14 families, 29 genera, and approximately 230 species within the phylum *Glomeromycota* (Oehl et al. 2011). Five definitive orders and 10 definitive families were found in the data from this study. The functional implications of the AMF community found in this data is not well understood; it is clear from previous studies that there are key differences in nutrient cycling (such as P uptake per unit of hyphal length), colonization strategies, sporation, and other characteristics between different taxa of AMF (Van Der Heijden et al. 2007), however, many questions still remain about the exact traits of each member of the AMF phylum, *Glomeromycota*.

The operational and strategic differences that exist within the phylum *Glomeromycota* are well displayed between the members of the *Glomerales* and *Gigasporales* orders of AMF, for example. Major differences are found in root colonization rates, storage of lipids, and external mycelium lengths between these two orders (Van Der Heijden et al. 2007), and they provide a strong example of how, while both being AMF, the two orders utilize different life strategies.

Differences that exist between members of the class *Glomeromycetes* suggests that a higher diversity of orders, families and genera of AMF also results in high functional diversity (Van Der Heijden et al. 2007).

The species *Geosiphon Pyriformis* are a notable exception within the phylum of *Glomeromycota*; though considered to be AMF, this species does not form arbuscules in the same form that other AMF do (Malar et al. 2021). This data is not suitable to accurately assess the relative abundance of *G. Pyriformis* within the AMF taxa data, so for the purposes of this study *G. Pyriformis* is grouped together with all other AMF.

Differences found in the data from each site display the highest relative abundance of all orders of AMF in the oldest field of Kernza, field 8. The highest levels of any accurately identifiable taxa were of the family *Diversisporaccae*, within the order *Diversispora*, in field 8. This family is known to have significant differences in spore wall phenotype as compared to other families of AMF, and is also known to germinate without the use of a germination shield (Walker et al. 2004). The functional implications of the unique spore and colonization development of this family is not well understood.

According to Hart's (2001) Driver/Passenger Hypothesis, a more intact hyphal network leads to an increased proportion of AMF species with persistence traits and a lower proportion of AMF species with colonization traits (Hart 2001). It is not yet well understood which lower taxa of AMF display which traits. However, the high abundance of the family *Diversisporaccae* found in field 8 suggests that this family of AMF might have a high proportion of persistence traits (Hart 2001) as the abundance of this taxa rises in fields that have experienced longer periods of time without disturbance. Higher levels of diversity in the oldest Kernza field, which has remained undisturbed for four years, displays the impact long periods without disturbance have on AMF community composition within this dataset.

Additionally, the year round ground cover that perennial crops such as Kernza provide may have impacted the AMF diversity and abundance found in this data (Crews et al. 2016). Providing consistent C access to soil microbiota (Peixoto et al 2020), the AMF communities in the Kernza plots examined in this study were likely provided a belowground environment that supported their colonization and abundance. Though all samples were taken from 0-5cm belowground, it can be inferred that the deep root systems of the older Kernza plots supported a similar abundant and diverse AMF community represented in this data at deeper depths than in the younger Kernza or the annual winter wheat (Culman et al 2010).

Plant response to AMF communities can differ by plant species, and plant community composition may also affect AMF community composition (Van Der Heijden et al. 2007, Hart 2001). AMF community structure has been previously shown to be a result of both preferred root association and disturbance regime (Bowles et al. 2017), thus the land use history and plant communities on the examined plots may have influenced the AMF community composition found. It should therefore be noted that red clover was interseeded with the Kernza in field 8 in 2019, a year prior to when the samples were collected. Red clover was also interseeded in the planting of Kernza in field 3, and volunteer vetch and wild mustard also exist in field 3.

AMF taxa data from fields 3 and 8, both of which contain Kernza of different ages, may be the best representation of the difference that time since planting has on AMF community composition. Field 20, though being reminiscent of the early stages of Kernza growth, did not contain Kernza and instead contained a winter wheat that had been in the ground for six weeks.

Differences in community composition of AMF were likely due at least in part to the more recent soil disturbance in field 20, however, the exact impact that plant species differences between fields 20, 3, and 8 had on the AMF taxa data is inconclusive due to there not being an accessible field of exclusively Kernza, and therefore no exact control.

Limitations within this study primarily existed due to the availability of Kernza plots. Working with what existed in the Hudson Valley, this study was limited to sampling in sites that had other plants growing in Kernza plots, and no there was no available access to a freshly planted Kernza plot (hence the use of a annual winter wheat, Rouge de Bordeaux, in field 20 to represent the early stages of Kernza growth). Data was likely also influenced by the differing land histories at each sampling site.

4.2.1 The Use of ITS

The ITS rRNA gene, which was used in this study to examine fungal taxa in the samples, is known to be a variable region and has been previously considered insufficient for examining closely related fungal species (Krüger et al. 2012). Though ITS rRNA barcoding has become the official fungal DNA barcode (Krüger et al. 2012), ITS phylotypes cannot be accurately affiliated with species level identification of AMF, and therefore statistical analysis of the results of this study do not go beyond the family taxa level.

4.3 Future Research

Future studies examining AMF using metagenomic data should consider using the nuclear large subunit (LSU) rRNA gene for AMF detection (Krüger et al. 2012). Examining the levels of aggregate stability as they compare to relative abundance of AMF could also be of use in future studies, as aggregate levels should theoretically rise with increasing abundance of AMF (Crews et al. 2018, Rillig 2004b). Additionally, examining glomalin levels (via a controlled, likely ex situ, study) in soils with perennial crops such as Kernza could point to AMF community abundance and/or traits of species that associate with Kernza (Crews et al. 2018, Rillig 2004b).

The functional significance of different AMF traits, such as the amount of root colonization and spore production, still remains unclear in research (Van Der Heijden et al. 2007). Future research into the different functional traits of AMF diversity and community composition would be beneficial in creating a stronger understanding of the significance of a diverse AMF community, especially as it relates to the future and capacity of perennial crops to facilitate strong and diverse microbiota communities in soil.

Conclusion

The conventional practices that are employed to produce much of the U.S.' (as well as the world's) food prioritizes anything but soil health; with an emphasis on yield, speed and money making, annual cash cropping systems rely heavily on physical disturbance of soil, monocropping, and often periods of time with bare ground. These practices result in ever diminishing levels of SOM, aggregation, and microbiota diversity (Crews et al. 2016, Rillig 2004a, Kabir 2004). With an interest in sustainability in our climate change informed world, non-conventional practices that recruit ideals of ecological intensification and place importance on soil health have led to the development and use of perennial crops as an alternative to annual systems.

The potential for perennial crops to answer some of these calls for sustainability and soil health has risen over the past two decades as research into the benefits of perennial systems has grown and perennial crops have been developed (Culman et al 2013, Otgonsuren et al. 2010, Weih et al. 2011, Crews et al. 2018). While scientific and agricultural communities have plenty of evidence for the capacity of perennials to increase NUE, soil aggregation, P uptake, water quality, and C storage and use (Culman et al 2013, Crews et al. 2018), there is far less understood about the impact a perennial cropping systems may have on soil microbial communities.

An enormous amount of effort has been put into the development of perennial cash crops such as Kernza, however, to see the full impact perennial farmscapes might have on agricultural systems there needs to be a continuation of research in this arena. Examining the microbial communities associated with crops such as Kernza aids in creating a more full picture of these new cultivars, and might even be a path to a greater understanding of the function and properties of soil microbes such as AMF. The importance of AMF has long been understood, however, the capacity to harness the benefits of AMF in agricultural systems has not yet been fully realized. Studies like this one, which examines the relationship between perenniality, time, disturbance and AMF, will lead to smarter, more sustainable, and ecologically sound farming systems.

The efforts of institutions such as the Land Institute to develop perennial crops has opened a door to the possibility of a new farmscape; one in which no-till, low input practices are inherently built into the system. The necessity for these types of projects is imperative in re-imagining and realizing food and agricultural systems which adapt to our greater understanding of agroecology and biological soil processes.

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Appendix: DNA Extraction Optimization

Due to a lack of access to DNA extraction kits intended for soil samples, an effort was made to optimize IBI Genomic DNA Mini Kit (IBI Scientific, 7445 Chavenelle Road, Dubuque, Iowa, 52002, U.S.A.) intended for blood/cultured cell samples for soil samples instead. This appendix describes the alterations that were made to the IBI Genomic DNA Mini Kit manufacturer's protocol as a part of this effort.

Soil samples of varying sizes (SS1E1: 250mg, SS1E2: 200mg, and SS1E3: 150mg) were tested to determine which sample size would yield best results based on NANODrop nucleic acid concentration measurements. Soil samples were added to beaded tubes from the ZYMO *Quick*-DNA Fecal/Soil Microbe Miniprep Kit (17062 Murphy Ave. Irvine, CA 92614, U.S.A.), along with much RBC lysis buffer as could fit in the tubes. According to manufacturer instructions, 750 microliters RBC lysis buffer was intended to be used for each sample, however it did not fit in the tubes along with the soil sample, resulting in the 250mg samples using 600microL, 200mg samples using 700microL, and 150mg samples using 750microL RBC lysis buffer. All samples were there then incubated at 60C for 12 mins (following suggestion from Schnieder et al. 2015), and put in a bead beater on the 'vortex' setting one for 10 minutes. All samples were then centrifuged at 10,000xg for one minute. As much supernatant as possible was then pipetted off and added to a microcentrifuge tube (375µl from SS1E1, 425µl from SS1E2 and 475µl from SS1E3).

After extraction of the supernatant, the manufacturer's instructions were followed exactly from "Genomic DNA mini Kit (blood/cultured cell) yeast/fungus protocol'. The manufacturer's instructions were intended to yield 50µg eluted DNA, and concentrations of DNA were examined using the NANODrop 8000 (Thermofisher Scientific, Waltham MA, U.S.A.) from 1µl of each sample. All samples were inverted three times prior to taking the NANODrop measurement.

NANODrop measurements showed that the 150mg sample (SS1E3) produced the highest concentration of DNA (19.5ng/µL), followed by the 200mg sample (SS1E2, 17.6 ng/µL). The least DNA was extracted from the 250mg sample (SS1E1, 1.7ng/µL). These results were likely due to the lack of space in the beaded tubes during incubation and vortexing in the larger soil samples. With more space in the tube for beads to break up the samples, the smaller 200mg and 150mg soil samples were able to extract higher concentrations of DNA, resulting in more usable DNA for analytical processes. The following table indicates the procedure and results of each sample.

Sample Name	Amount of soil	Amount of RBC lysis buffer	Incubation	Bead beater process	Amount pipetted off	NANOdrop measurement (ng/µL)
SS1E1	0.25g	600microL	60C, 12min	‘Vortex’ level 1, 10 mins	375	1.7
SS1E2	0.2g	700microL	60C, 12min	‘Vortex’ level 1, 10 mins	425	17.6
SS1E3	0.15g	750microL	60C, 12min	‘Vortex’ level 1, 10 mins	475	19.5