2021

Effects of biofertilizer on soil nutrient cycling and the productivity of bioproduct crops in marginal soils

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Effects of biofertilizer on soil nutrient cycling and the productivity of bioproduct
crops in marginal soils

Kieran B. Liseski

Thesis submitted
to the Davis College of Agriculture, Natural Resources, and Design
at West Virginia University

in partial fulfillment of the requirements for the degree of
Master of Science in
Applied and Environmental Microbiology

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2021

Keywords: biofertilizer, bioproduct crop, marginal lands, microbial amendment

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ABSTRACT

Effects of biofertilizer on soil nutrient cycling and the productivity of bioproduct crops in
marginal soils

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Recently there has been increased interest in the use of marginal lands in order to grow bioproduct crops. Utilizing marginal lands to grow bioproduct crops such as *Miscanthus x giganteus* and *Panicum virgatum* could not only improve soil quality and minimize negative environmental impacts on the surrounding ecosystems but could also offer economic benefits to growers in rural areas. Because marginal soils tend to reduce crop yields compared to prime farmlands even when growing bioproduct crops, which tend to be more robust than traditional food crops, augmenting the soil with biofertilizers (i.e., the direct addition of viable microbial cells) could provide plants with better access to nutrients and lead to increased crop yields. We hypothesized that biofertilizers will increase plant growth and microbial activity as well as augment the relative abundance of the added microorganisms in marginal soils. Additionally, we hypothesized that biofertilizers will have the same effect on *Panicum virgatum* L. (switchgrass) and *Miscanthus x giganteus* growth and soil carbon and nitrogen availability. In this study, we inoculated pots containing either *Miscanthus*, *Panicum*, or no plant with either a commercial biofertilizer or a biofertilizer made by isolating the microbial and fungal community from soil from an established *Miscanthus* stand. We found positive belowground biomass effects of the commercial biofertilizer, as well as differences in microbial function driven by plant species. Biofertilizers did not appear to affect microbial activity as we had predicted, nor did they appear to increase the relative abundance of the added microorganisms present in the commercial biofertilizer. Our results suggest that Switchgrass and *Miscanthus* select for a taxonomically and functionally distinct microbial community and that biofertilizers have limited impact on microbiome composition and function.

ACKNOWLEDGMENTS

I greatly appreciate all of the assistance I received from members of Dr. Morrissey's laboratory including Chansotheary Dang, Jeth Walkup, Kinsey Reed, Jenni Kane, and the wonderful undergraduates who assisted at the end of my project. I also thank my committee, Drs. Freedman, Morrissey, and Panaccione, for their investment in this project and subsequently me as a scientist. I also want to thank my parents who encouraged me to pursue a career in science and have been incredibly supportive throughout my education.

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1. Introduction

1.1 Sustainable Agriculture

Agriculture has, and continues, to transform landscapes globally. According to the Food and Agriculture Organization of the United Nations (FAO), one-third (approximately five billion hectares) of ice-free terrestrial land area is used for agricultural production (FAO 2019). Management practices (such as crop rotation, tillage and irrigation practices) as well as anthropogenic inputs (such as chemical fertilizers and pesticides) to these agroecosystems are often major influences on the ecological processes in these systems (Stinner and House 1989). For example, the cycling of nutrient elements such as N or C increases with anthropogenic inputs of fertilizers while choosing an appropriate crop or cultivar can help improve resistance to abiotic stresses like N deficiency and drought conditions (Wezel et al. 2014). While the primary goal of many agroecosystems is to maximize yields, this land-use type can lead to negative effects on the ecosystem like soil erosion and contamination of surface and groundwater caused by runoff of agricultural chemical amendments. This has led to a shift away from intensive agriculture to more sustainable agroecosystems.

The management of sustainable agroecosystems generally focuses on finding a balance between optimizing production and limiting environmental impact to reduce some of the undesirable effects seen in intensively managed agricultural systems. The use of many sustainable production practices, such as crop rotations and biological pest control, have been dated as far back as 2,000 years ago and were widely used even before being labeled as sustainable practices (Francis and Porter 2011; Wezel et al. 2014). There are three general stages which are used when transitioning into a sustainable agroecosystem: efficiency increase, substitution, and redesign. Efficiency increase refers to practices which seek to decrease the amount of inputs into a system (Wezel et al. 2014). For example, optimizing the use of chemical

fertilizers can maximize the economic and ecological benefit of an agroecosystem by decreasing the risk of nutrient leaching. Nutrient losses through leaching would not only likely reduce available nutrients for plants but it could also have harmful effects on the surrounding environment (Francis and Porter 2011). Substitution refers to the practice of changing an input or a practice for a more sustainable alternative (Wezel et al. 2014). Often this involves substituting a product like a chemical pesticide for a natural pesticide, or a chemical fertilizer for an organic fertilizer such as manure or compost (Wezel et al. 2014). Perhaps the most complicated of the three stages, redesign refers to the reconsideration of the whole farming system (Wezel et al. 2014). This often involves incorporating several sustainable changes together in order to maximize potential benefits (Francis and Porter 2011). For example, a system could incorporate a different crop and cultivar to help mitigate the effects of nitrogen and water deficiencies, and increase resistance to pathogens and diseases (Tilman et al. 2002). Of particular interest are plant growth promoting bacteria (PGPB), which are bacteria that can enhance plant growth and protect plants from a variety of diseases and abiotic stresses. Some cropping practices, such as tillage and the addition of organic amendments, can affect the effectiveness of plant growth promoting bacteria present in the soil, which could then feed back on plant and soil health (Wezel et al. 2014). For example, biofertilizers (or microbial inoculants meant to increase plant growth and nutrient uptake) have the potential to increase agricultural productivity by decreasing the need for chemical nutrient amendments (Wezel et al. 2014). In this way, biofertilizers have promise in sustainable agriculture by improving some of the consequences associated with the use of traditional fertilizers such as soil degradation, leaching of harmful chemicals, and reduced disease resistance in plants (Singh et al. 2016; Chakraborty and Akhtar 2021).

1.2 Surface Mining and Restoration

Surface mining is one of the most common mining practices today. In fact, over 80% of all resources obtained from the mining industry come from surface mining methods (Ramani 2012). Surface mining is the practice of removing soil or rock to access ore deposits below the surface (Lima et al. 2016). Although mining practices are often regionally specific, in general surface mining occurs by first removing soil and rock from above or between a coal seam, also known as the overburden. The coal seam is then removed with different heavy machinery depending on the mining practice being utilized and the scale of the operation (Skousen and Zipper 2021). There are several different practices that constitute surface mining including strip mining, open-pit mining, and mountaintop-removal mining. These methods often share similar operations in which some form of drilling, blasting, loading, and hauling occur in order to carry out the mining process (Ramani 2012). The common factor between all surface mining practices however is that they all disturb the landscape and can result in impact ecosystem functions (Lima et al. 2016). Common legacies of surface mining often include loss of soil fertility, altered hydrology, and leaching of contaminants such as arsenic associated with mine pits (Isosaari and Sillanpää 2010; Ramani 2012; Evans et al. 2015).

Notable changes to surface mining practices in the U.S. occurred due to the Surface Mining Control and Reclamation Act (SMCRA) in 1977 (Evans et al. 2015). This act requires the rehabilitation, reclamation, and restoration of land disturbed by surface mining, though the outcomes of this effort have been mixed both in the ability to successfully restore land to pre-mining conditions and in restoring soil health (Lima et al. 2016). Under SMCRA regulations, either the overburden must be replaced to rebuild the landscape to an approximate original contour or the area can be reclaimed to support post-mining land uses that are similar or better to the conditions prior to mining (Skousen and Zipper 2021). The most common method of

satisfying these requirements is to convert the land use type of the mining effected area into grassland or pasture in the hopes that natural succession will eventually return the land to their previous ecological state (Gilland and Mccarthy 2014; Kane et al. 2020). However, it has been speculated that increased soil compaction as a result of the use of heavy machinery and erosion prevention could contribute to a lack of recovery to natural ecological conditions (Holl and Cairns 1994).

The reclamation process actually starts during the mining process when topsoil is removed and sequestered for later use (Feng et al. 2019). This topsoil however—due to processes such as excavation, transport, and dumping—does not retain its original structure or properties (Feng et al. 2019). These soils are referred to as reclaimed minesoils, or RMS (Guzman et al. 2019). Proper reclamation techniques and good mining practices can restore these degraded soils, but without these measures RMS could take anywhere from 50 to 100 years to recover (Bradshaw 2000; Shrestha and Lal 2010). If these soils are left unreclaimed for an extended period of time they could continue to degrade and could contribute to increased rates of leaching and erosion (Guzman et al. 2019). Disturbances associated with surface mining negatively impact the environment around them, and more work needs to be done to combat the legacy effects of surface mining globally. For example, in the Appalachian region alone, of the 1.13 million ha of land disturbed by surface mining only around 5% has been fully reclaimed (Guzman et al. 2019).

1.3 Marginal land

Marginal lands can be hard to define, and are therefore hard to quantify (Kang et al. 2013). Some estimates suggest around 13% of the total land area in the U.S. can be considered marginal while some estimates suggest the global total of marginal land area is anywhere from

1,100 to 6,650 million hectares (Cai et al. 2011; Milbrandt et al. 2014; Quinn et al. 2015) .

Marginal lands are typically characterized by low productivity and reduced economic return (Kang et al. 2013). Accordingly, they are also generally unsuitable for agricultural use. Lands may be marginal due to low pH, highly erodible soil, low soil organic carbon (SOC), and the slope of the land among other factors. These limitations make growing traditional food crops on the land difficult, and crop yields are often less profitable than the cost of the land (Kang et al. 2013). While these limitations are not necessarily a result of human activity, marginal lands can arise from human activities such as mining and industrial pollution (Blanco-Canqui 2016). In fact, human activity can often exacerbate marginal lands. Through intensive agricultural practices and large-scale land clearances on land already at risk of becoming marginal (e.g. hillslopes), human activity has historically increased the amount of marginal lands (Vandam et al. 2019). The soil in these lands are often marked by erosion, salinization, or low carbon contents which cause the soil to become nutrient poor (Guzman et al. 2019). Erosion, in particular, is a good indicator of the impacts of human activity on land marginality (Vandam et al. 2019).

1.4 Bioproduct crops (Miscanthus and Switchgrass)

Bioproduct crops can be used for both energy applications, such as biofuels (Scordia et al. 2018), as well as for non-energy applications (for example, biopolymers; (Shahi et al. 2020)). Recent research has shifted from using first generation bioproduct crops to the use of lignocellulosic perennial grasses like switchgrass and *Miscanthus* (Jiang et al. 2019). Perennial grass production offers a variety of potential end products including insulating material, mulch and other biodegradable products for garden use, as well as green chemistry products like biopolymers (Scordia et al. 2018).

Miscanthus x giganteus (hereafter referred to as *Miscanthus*) is a sterile, highly productive perennial grass. It was first cultivated in Japan in 1935, but has since spread throughout Europe and North America mainly for use as a bioproduct crop (Lewandowski et al. 2000). *Miscanthus* uses the C₄ photosynthetic pathway and has been found to remain highly productive across many environmental conditions including those in marginal soils (Anderson et al. 2011). This is partially because C₄ plants tend to have higher water use efficiencies and nitrogen use efficiencies than their C₃ counterparts (Kubien et al. 2003). *Miscanthus* has also been shown to aid carbon sequestration in the soil which could help to restore degraded soils with low soil C (Blanco-Canqui 2016). This increase in SOC is notable as it can help to reduce loss of soil moisture and provide a long-term nutrient stock for plants (Mi et al. 2014). Many of the factors which can improve C sequestration, e.g. increasing input rates of organic matter and decreasing the decomposability of organic matter inputs, have been shown to occur when land is converted to grow perennial grasses (Post and Kwon 2000; Schneckenberger and Kuzyakov 2007).

Plants are associated with an incredible amount of diverse microorganisms in a system referred to as a microbiome (Uroz et al. 2019). Some of the microbes in this plant-associated microbiome can be symbiotic with the plant (e.g. plant-growth promoting bacteria), wherein both the plant and the microbe benefit from interactions (Martin et al. 2017). Recent research has suggested that these symbioses might drive the composition of the plant-associated microbiome (Uroz et al. 2019). *Miscanthus* microbiomes can harbor some symbiotic plant growth promoting microbes, including N-fixing bacteria from the families Bradyrhizobiaceae and Hyphomicrobiaceae among others (Liu and Ludewig 2019). A recent study indicated that age and fertilization have an effect on microbial community structure in *Miscanthus* soils, with older stands generally having less variation in their community structure. In younger fertilized stands,

there were notable increases in phyla which have previously been shown to be associated with N cycling (Ma et al. 2021). Another study suggested that cultivation of *Miscanthus* increases the abundance of several PGPB, including several genera (e.g. *Bradyrhizobium*, and *Rhizobium*) previously shown to be n-fixers (Fischer 1994; Chen et al. 2020). These studies highlight that there seems to be consistent microbial communities associated with *Miscanthus*, although how the *Miscanthus* microbiome may facilitate plant productivity and improve soil conditions in marginal soils is unknown (Chen et al. 2020; Ma et al. 2021).

Likewise, *Panicum virgatum* L., or switchgrass, is a perennial C₄ grass. Switchgrass is widely distributed across North America, and is commonly used for biofuel production (Wolf and Fiske 2009). Like *Miscanthus*, switchgrass has also been shown to be effective in increasing soil quality and sequestering carbon, especially in marginal soils (Qin et al. 2006). Switchgrass could be ideal for growth on marginal lands since it can tolerate a wide variety of environmental conditions and could improve the quality of the soil. The structure of the switchgrass microbiome is driven by many factors including site conditions, climate, the location of the microbes (e.g. plant tissue, roots, or rhizosphere soil), and season (Hestrin et al. 2021). The microbiome has also been shown to be able to significantly influence plant growth through a number of mechanisms due to the wide range of life strategies and metabolic capabilities represented (Ma et al. 2021). Some of these potential plant function influencing mechanisms include nitrogen fixation driven by N-fixing bacteria, as well as plant growth promotion and improved nutrient acquisition due to both PGPB and mycorrhizal fungi (Brejda et al. 1998; Ker et al. 2012; Roley et al. 2018).

1.5 Using bioproduct crops as a sustainable use of marginal land

The increasing global population has led to a need to cultivate marginal lands for food production. When marginal lands are utilized for food production, agricultural yields are often low while potential degradation risks, such as increased erosion, are increased (Wood et al. 2000). One plausible economically and ecologically favorable marginal land use are bioproduct crop production systems due to their high biomass potentials as well as potential benefits for the soil (Jiang et al. 2019). The use of marginal lands for growing dedicated bioenergy crops provides an attractive option for reducing competition for land between energy and food production. The first generation bioproduct crops, e.g. corn and sugarcane, have contributed most of the biofuel production in the U.S. to date, but have also given rise to concerns about the feasibility of replacing petrol-oil products due to high production costs of bioenergy (Petersen 2008; Jiang et al. 2019; Energy Information Administration 2020). Second generation bioproduct perennial crops, which include grasses such as switchgrass and *Miscanthus*, are an attractive solution for some of the problems presented by first generation bioproduct crops (Jiang et al. 2019). Growing perennial bioenergy crops—which are often better suited to growing in marginal soils than traditional food crops—could also help restore degraded soils, fix soil carbon and support nutrient cycling activities, and benefit the environment (Kang et al. 2013). While promising, several questions remain on how to grow dedicated bioenergy crops on marginal lands, including how these crops will perform on different types of marginal lands, and whether these sites can produce abundant biomass while maintaining and potentially improving ecosystem services. Even though these perennial bioenergy crops are more robust than annual crops, their production could still be constrained by environmental conditions present in marginal lands. Therefore, evaluating the suitability of marginal land to support and sustain biomass production while maintaining or improving ecosystem services is needed.

1.6 Biofertilizers

Plant growth promoting bacteria (PGPB) can be free-living, form symbiotic partnerships with plants, or endophytic and live within certain tissues of a plant, but they all utilize the same mechanisms (Singh 2018). PGPB facilitate plant growth either directly by regulating plant hormone levels or by facilitating the acquisition of nutrients, or indirectly by mitigating the effects of pathogens on plant growth and development (Glick 2012). Nutrient acquisition is perhaps the best studied of the direct mechanisms (Glick 2012). Microbes that aid in nutrient acquisition can employ several different mechanisms including nitrogen fixation, phosphate solubilization, and siderophore production among others to aid in providing plants with nutrients (Pii et al. 2015; Backer et al. 2018). This suggests that it could be beneficial to manipulate microbial activity or target certain microbes, such as those we know to be PGPB, in order to stimulate crop growth in challenging soils like those found in marginal lands.

Plant growth promoting fungi (PGPF) also influence plant growth through both direct and indirect mechanisms (Hossain et al. 2017). Well known PGPF include members of the genera *Trichoderma*, *Aspergillus*, and *Penicillium* as well as arbuscular mycorrhizal symbionts in the order Glomerales (Verma et al. 2019). PGPF facilitate plant growth similarly to PGPB, though less is known about PGPF (Hossain et al. 2017; Verma et al. 2019). They mainly work by aiding in nutrient acquisition through mechanisms like more extensive root systems (Hossain et al. 2017), and by acting as biological control agents (e.g. *Trichoderma*; (Waghunde et al. 2016)) to protect the plant from pathogens. Like PGPB, it could be useful to utilize specific PGPF to stimulate crop growth in marginal soils, particularly soils with well diagnosed challenges (e.g. abundant phytopathogens).

Biofertilizers are microbial inocula which when added to soil can promote plant growth by stimulating the transformation of important nutrient elements like nitrogen and carbon, and by utilizing plant hormones like auxins (Mahanty et al. 2017) and are increasingly researched as a potential sustainable method to increase crop yields. There are several different categories of biofertilizer, and they often grouped based on the nutrient they are targeting (commonly N, P, or K) and the mode of action they use to acquire those nutrients or they are grouped more broadly into the category of PGPB (Nosheen et al. 2021). For example, a common microbial inoculant is N-fixing bacteria which can fix N_2 from the air into ammonia which is more readily available for the plant to uptake (Nosheen et al. 2021). Other functional groups of microbes that commonly serve as biofertilizers have also been explored (e.g., phosphate or potassium solubilizing bacteria; Bünemann et al. 2006; Bahadur et al. 2014). Additionally, some biofertilizers take advantage of symbiotic relationships between mycorrhizal fungi (commonly arbuscular mycorrhizal fungi or AMF) and plants. These biofertilizers work by introducing or bolstering AMF populations in the soil to aid in plant nutrient uptake, particularly N, P, and K (Garcia and Zimmermann 2014; Igiehon and Babalola 2017). Biofertilizers containing N-fixing bacteria and other PGPB have increased yields of bioproduct crops such as *Miscanthus × giganteus* and *Panicum virgatum* L. in both field and greenhouse conditions by utilizing several mechanisms associated with increased nutrient acquisition. These include releasing organic acids to increase the amount of available phosphate and increasing root hair production (Fei et al. 2017, Fei et al. 2019). The positive effects of biofertilizers, however, can often be short lived (Kim et al. 1997). Additionally, the increased yield seen following treatment with biofertilizers could be due to the native population of microbes feeding on the biofertilizer (Bashan 1998). The addition of microbial amendments in the form of biofertilizers could help improve the yields of bioproduct

crops in marginal soils, but additional research is needed to determine the efficacy and persistence of biofertilizers in these soils in order to ascertain the feasibility of this potential agricultural practice.

Another way to manipulate microbiomes is through the use of microbiome transplants. Biofertilizers are often specialized to only include certain microbes that are known to be beneficial to plant health. Alternatively, some researchers have attempted to transplant whole microbiomes into new environments, with varied success (Howard et al. 2017). Researchers have tested a variety of methods of microbiome transfer, with the most common being direct soil transfer and the soil wash. Direct transfers are also generally considered the most feasible at large scales so would likely be the most suitable option for field trials (Trexler and Bell 2019). These transfer methods have their limitations; direct soil transfers transplant soil compounds like nutrients in addition to microbes, and soil washes may alter microbiome structure by favoring microbes that are less attached to the soil (Howard et al. 2017). Though most studies agree that microbiome transfer does result in a change to the preexisting microbiome in nonsterile soils, there have been suggestions that the survival of transplanted microorganisms is influenced by the presence of other soil organisms and/or the concentration of soil compounds that are transferred in addition to the microbiome (Calderón et al. 2017; Howard et al. 2017). While it is possible to transplant a microbiome, it is less known whether a transplanted microbiome can improve plant and soil quality.

1.7 Competitive Exclusion and Biofertilizers

Competitive exclusion is an ecological principle which theorizes that two species competing for the same limiting resource cannot coexist. In other words, one species will always outcompete the other for that limiting resource leading to the other species dying out or adapting

to fill a different niche (Hardin 1960). Accordingly, it is plausible that microbes added to soil as biofertilizer may fail to persist due to competition with the native microbiota (Bünemann et al. 2006). While some of the added microbes could fill empty niches present in the environment, in nutrient limited environments the chance of competition between added microbes and native microbes for niche space increases (Calderón et al. 2017). It is plausible that this competition inhibits the positive effects of biofertilizers, however, the extent to which competitive exclusion effects biofertilizer persistence is unknown.

Though there is growing interest in using marginal lands to grow bioproduct crops, marginal soils still tend to produce relatively low crop yields even when growing robust bioproduct crops. Augmenting the soil with appropriate biofertilizers could provide plants with improved access to bioavailable nutrients and may lead to increased yields. The effects of biofertilizers, however, can be short-lived (Kim et al. 1997). Highly disturbed communities, particularly ones in which the microbiome is significantly altered due to the disturbance (e.g. decreased bacterial diversity or abundance), could be particularly susceptible to colonization by added microbes (Calderón et al. 2017). This could be due to resource-based niche theory which suggests the establishment of introduced microbes is directly related to available resources and the characteristics of the original microbiome (Tilman 2004). However more research is needed into the establishment and efficacy of introduced microorganisms in marginal soils and whether introduced microorganisms can improve plant and soil health.

Additionally, commercial biofertilizers are typically made using a more general blend of PGPB (such as *Azospirillum*, *Rhizobacterium*, and diazotrophic bacteria) which have been shown to increase the amount of plant available carbon and nitrogen and plant biomass in multiple bioproduct crops such as *Miscanthus × giganteus* and *Panicum virgatum* L. (Fei et al. 2017;

Mahanty et al. 2017; Fei et al. 2019). Plant-associated microbiomes, however, have a relatively high degree of plant specificity (Berg et al. 2016) so a targeted biofertilizer (e.g. a transplanted microbiome) of native microorganisms may be more effective. Consequently, it is important to examine the species specificity of the effects of the biofertilizer made from the microbial community of a particular plant species on other plant species.

In this study, our first research objective was to investigate the efficacy of biofertilizers for bioproduct crop production systems in marginal soil. We hypothesized that biofertilizers will increase plant growth and microbial activity as well as augment the relative abundance of the added microorganisms in marginal soils. Our second research objective was to determine if the benefits of biofertilizer is specific to a particular plant species and its associated microbiome. We hypothesized that biofertilizers will have the same effect on *Panicum virgatum* L. (switchgrass) and *Miscanthus x giganteus* growth and soil carbon and nitrogen availability. To accomplish this, we performed a greenhouse experiment with two different forms of biofertilizers, then after a growth cycle of four months, we harvested the plants and soil to determine the microbial community composition and activity based on the treatment and plant type present.

2. Methods

2.1 Site Description and Sample Collection

Soil was collected from 5 random 1x1 meter plots in both the West Virginia University Animal Science and Agronomy Farms for the greenhouse experiment in May, 2020. The sites were tilled to 10 cm due to the presence of a dense rock layer at the Agronomy Farm. This soil was then collected and transported on ice to West Virginia University where it was homogenized separately by collection plot. Large roots and rocks were removed before the soil was distributed into 90 one-gallon pots, which had five 1.5 cm holes on the bottom to allow for drainage of

excess water. Pots were filled with 18 cm of soil leaving 2 cm excess at the top of the pot. 30 control pots that contained no plants were made using either Agronomy or Animal Science farm soil (15 of each).

Miscanthus x giganteus rhizomes were obtained from The Issachar Group (Mifflintown, PA). Rhizomes were first surface cleaned by removing as much soil as possible, then rinsing the rhizomes with water to remove excess soil. Rhizomes were sanitized following a procedure modified from Kim et al. (2012) by being sprayed with 70% ethanol and then rinsed thoroughly twice with DI water (Kim et al. 2012). The rhizomes were then dried with paper towels and returned to the lab where they were stored at 4°C until planting. Prior to planting, rhizomes were trimmed to 6 cm. Two rhizomes were initially planted in each of 30 one-gallon pots containing either Animal Science (15 pots) or Agronomy Farm soil (15 pots). Once growth was established, excess spouted rhizomes were removed and transplanted to pots with soil collected from the same farm which had no established growth if necessary, leaving only one *Miscanthus* plant in each pot before the time of inoculation. Plants were grown under natural light conditions in the greenhouse and temperature was controlled to be 21°C to reflect conditions at the beginning of the growing season (June 2020).

Switchgrass seeds (Cave-in-Rock cultivar) were surface sterilized following the same method as the *Miscanthus* rhizomes. Following surface sterilization, switchgrass seeds were immediately placed on a water-soaked paper towel to promote germination. Once switchgrass seeds had germinated, they were carefully transplanted into the 30 one-gallon pots containing soil from either the Animal Science (15) or Agronomy sites (15) (Wolf and Fiske 2009). Five seedlings were transplanted into each pot to ensure growth. Once growth was established, excess plants were transplanted into pots with soil collected from the same farm site which had no

established growth. Upon initiation of the experiment, one established switchgrass seed remained in each one gallon pot.

2.2 Biofertilizer Sample Collection

Two types of biofertilizers were used in this experiment. The first is a commercial biofertilizer, (Supre Myco Tea (0-0-6); Supreme Growers, Chattanooga, TN). This biofertilizer was suspended in water following the manufacturer's instructions before inoculation. Prior to inoculation, approximate concentrations (122 CFUs/ml) of biofertilizer following manufacturers recommendations was determined using the plate count technique in order to have a baseline concentration that could be adjusted based on the approximate concentration of the transplant treatment (Buck and Cleverdon 1960). Serial dilutions were performed and the dilutions (1:10-1:100000) were plated onto nutrient agar plates. Plates were allowed to incubate at room temperature for 48 hours before being counted using the standard plate count method (Taylor et al. 1983).

The second biofertilizer was a microbial transplant made from soil from an established (*i.e.*, 11 year) *Miscanthus* stand in Alton, WV. The *Miscanthus* stand at Alton is located in Upshur County, WV on a previously surface mined area and was established in 2010. This site was mined for the Upper, Middle, and Lower Kittanning coal seams. The 30 ha of land selected for *Miscanthus* production was reclaimed in 1985 (Scagline-Mellor et al. 2018). Soil was collected from the site by randomly selecting 10 locations within the *Miscanthus* stand, then sampling soil from around the base of a *Miscanthus* plant to an approximate depth of 15 cm using a shovel. Large rocks and pieces of rhizomes were removed by hand and the soil was hand homogenized in the field before transporting to the lab on ice. Upon return to the lab the soil was

stored at 4°C. Soil was again homogenized and large objects such as rocks, roots, and insects were removed before the soil was used to make the transplant slurry.

The transplant solution was made following the methods of Lindahl (1996) by suspending 200 g of soil into 1000 mL of a 50 mM Sodium Pyrophosphate (NaPP) solution to create a 1:5 ratio of soil to NaPP. This solution shook for one hour at 150 RPM. The sediment then settled without shaking for 24 hours. The supernatant was extracted and stored at 4°C until inoculation. A serial dilution was also performed using the supernatant, and the dilutions were plated in order to determine approximate concentration of heterotrophic bacteria (1.35×10^4 CFUs/ml). This approximate concentration was then used to adjust the concentration of the commercial biofertilizer so that the final concentration of both biofertilizer treatments were standardized for inoculation (1.35×10^4 CFUs/ml).

Each pot received 80 ml of the appropriate biofertilizer treatment after 45 days (from planting). These treatments consisted of either a mixture of live and heat killed commercial or transplanted biofertilizer, or a control treatment which contained heat killed versions of both biofertilizer. The heat killed versions of the biofertilizers were made by separately autoclaving live versions of both biofertilizers for 1 hour. The treatments were then mixed so that each bottle contained either live commercial biofertilizer and heat killed transplant, live transplant and heat killed biofertilizer, or heat killed transplant and biofertilizer (Fig. 1). The 30 pots of each plant type were inoculated so that both the Agronomy farm and the Animal Science farm soils would have 5 replicates of each biofertilizer treatment type (Fig. 1).

After 120 days of growth, the experiment was destructively sampled. Rhizosphere soil was collected by removing the plant from its pot, and shaking the roots. The soil that stuck to the roots was operationally defined as rhizosphere soil and was analyzed separately (Smiley 1974).

Bulk soil was collected from the remaining soil in the pot. Soil was then stored at 4°C until further use.

2.3 Soil Characteristics

Soil pH was measured with a 1:5 soil:CaCl₂ suspension of air dried soil on an Accumet AE150 pH meter (Fisher Scientific, Hampton, NH, USA) (Rayment and Higginson 1992). Soil moisture was measured gravimetrically from soil samples after drying for 24 hours at 100 °C. The percent of soil organic matter was estimated from mass loss on ignition at 500 °C for 5.5 hours (Ball 1964). Soil N was extracted from bulk soil in 1 M KCl by shaking at 100 RPM for 90 minutes followed by filtration through a Whatman #42 filter (Whatman, Maidstone, UK). Extracts were then stored at -20 °C until nitrate concentrations were measured colorimetrically (Miranda et al. 2001) using a Biotek Synergy HTX Multi-Mode MicroPlate Reader (Biotek Instruments Inc., Winooski, VT).

2.4 Microbial Respiration and Biomass

Soil was preincubated for 48 hours to ensure an active microbial community by wetting soil to 60% water holding capacity. This soil was then used for both microbial respiration estimates as well as microbial biomass estimates. The dry weight equivalent of 10 g soil was measured into a Mason jar. Microbial respiration was estimated according to Walkup et al. 2020. Samples were incubated in sealed jars and 10 mL of headspace was sampled after 0, 24, and 48 hours on a LI-COR 6400XT (LI-COR Biosciences, Lincoln, Nebraska) (Walkup et al. 2020).

Microbial biomass was estimated using the substrate induced respiration (SIR) method modified from West and Sparling, 1986 (West and Sparling 1986; Dang et al. 2021). Approximately 7 g of preincubated soil was measured into a serum bottle. 7 mL of a 1X yeast solution was added to the serum bottle, and bottles were capped, sealed, and shaken on an orbital

shaker for 4 hours. Gas samples (10 mL) were taken from the headspace at 0, 2, and 4 hours on a LI-COR 6400XT (LI-COR Biosciences, Lincoln, Nebraska).

2.5 $^{15}\text{N}_2$ Free Living Nitrogen Fixation

Free living nitrogen fixation (or FLNF) methods were measured following the protocol adjusted from Smercina et al. (2019). Soil (3g) was collected and placed into 16 x 125mm Hungate tubes. Because previous studies suggested providing a C source to optimize N-fixing conditions but favorable C sources vary depending on the microbial community composition (Smercina and Friesen 2019), a carbon cocktail containing glucose, sucrose, malic acid, and citric acid adjusted to a pH of 7 using potassium hydroxide was added to bring each sample to 60% water holding capacity. C sources were added at a concentration of 4 mg C g⁻¹ dry soil with each C source in the cocktail providing 1 mg C g⁻¹ dry soil. Vials were then capped and evacuated. Immediately after evacuation, vials received 1 mL of 98 atom% acid-washed $^{15}\text{N}_2$ gas (Sigma-Aldrich, Inc., St. Louis, MO), and reference vials received $^{14}\text{N}_2$ (Matheson Tri-Gas, Irving, TX). Prior to addition, $^{15}\text{N}_2$ gas was acid washed using 5% sulfuric acid to ensure no contamination of $^{15}\text{N-NH}_3$ and other gases. After N_2 addition, O_2 (Grainger, Lake Forest, IL) was added to the vials at a 10% concentration. Vial atmospheres were balanced using He (Grainger, Lake Forest, IL). Samples were incubated for three days, then vials were uncapped and samples were placed in a drying oven for 48 hours. After drying, the samples were ground into a fine powder, weighed into tin capsules, and then sent to the University of Maryland Center for Environmental Science Appalachian Laboratory Central Appalachians Stable Isotope Facility to be analyzed. The samples were analyzed using Thermo Fisher Delta V+ isotope ratio mass spectrometer and the GasBench II.

2.6 Potential Nitrification

Potential nitrification was estimated according to Hart et al. 1994. Briefly, 7.5 g of soil was suspended in 50 mL of 0.75 mM $(\text{NH}_4)_2\text{SO}_4$, 0.3 mM KH_2PO_4 , 0.7 mM K_2HPO_4 solution and allowed to shake on an orbital shaker at room temperature for 6 hours. Samples of the solution were collected every 2 hours by allowing soil to partially settle and pipetting 2 mL into a microcentrifuge tube. NO_3^- concentration was measured colorimetrically (Miranda et al. 2001) on a Biotek Synergy HTX Multi-Mode MicroPlate Reader (Biotek Instruments Inc., Winooski, VT).

2.7 Molecular Analysis

DNA was extracted from homogenized soil samples using the DNeasy PowerSoil Pro Kit (Qiagen, Frederick, MD) following manufacturer protocol. DNA was quality checked and quantified using NanoDrop and PicoGreen dsDNA assay, respectively. Extracts were stored at -20 °C. Prokaryotic 16S rRNA genes were amplified using primers 515F and 806R (Kozich et al. 2013). Fungal ITS region amplicons were amplified using primers ITS1f and ITS 2 (Gardes and Bruns 1993, White et al. 1990). The resulting amplicons were used to build libraries and sequenced at the University of Minnesota Genomics Center using the MiSeq platform (V3 chemistry; Illumina, San Diego, CA, USA) and a dual-indexed approach (Gohl et al. 2016).

2.8 DNA Sequence Processing and Quality Control

DNA sequence processing was performed in QIIME 2 (version 2021.2; (Bolyen et al. 2019)). First, 16S rRNA gene forward reads were demultiplexed and quality filtered using a quality score of 20 and the q2-demux plugin. The resulting sequences were then denoised and assigned amplicon sequence variants (ASVs) using Deblur (via q2-deblur; Amnon et al. 2017) and a trim length of 257. Taxonomy was assigned to the resulting prokaryotic ASV table using

the QIIME2 feature classifier (classify-sklearn; (Bokulich et al. 2018)). Taxonomic classification was performed by a trained classifier (Silva v138; (Quast et al. 2013; Yilmaz et al. 2014)).

For ITS forward and reverse reads were demultiplexed and merged using VSEARCH to produce paired-end reads (Rognes et al. 2016). The paired sequences were then quality filtered using a quality score of 20 and ASVs were assigned using DADA2 (Callahan et al. 2016).

Taxonomic assignment of the fungal ASVs was performed using the QIIME2 feature classifier (classify-sklearn; (Bokulich et al. 2018)) against the UNITE reference database (version 8; (Nilsson et al. 2019; Kõljalg et al. 2020)).

To determine the relative abundance of bacterial taxa associated with N fixation, ASVs of the families Frankiaceae, Beijerinckiaceae, Hyphomicrobiaceae, Labraceae, Rhizobiaceae, and Xanthobacteraceae were further analyzed (Carvalho et al. 2008; Borken et al. 2016; Xun et al. 2018; Zhu et al. 2018; Smercina et al. 2020). When possible, these ASVs were probed to the genus level to confirm N-fixing capabilities. For example, only one genus of the family Frankiaceae were included, *Frankia* (Zhu et al. 2018), because not all members of this family are able to fix N.

2.9 Statistical Analysis

Microbial respiration rates were calculated using a standard curve of known CO₂ and CH₄ concentration. Microbial biomass was determined from the SIR measurements using the following conversion: Biomass-C (μg C·g-OM⁻¹) = 433 · SIR + 59.2 (West and Sparling 1986). Microbial respiration rates were log₁₀ transformed to achieve normality (McCune and Grace 2002).

All statistical analyses were performed with RStudio (RStudio Core Team 2016) running R 3.6.3 (R Core Team 2020) using the Vegan package (Oksanen et al. 2019). Data was first

subset by plant type. Subsetted data was then tested for normality using the Shapiro-Wilk test (Miranda et al. 2001). Differences in soil characteristics (pH and OM), NO_3^- concentrations, and *Miscanthus* and switchgrass above and belowground biomass in response to treatments were analyzed using a two-way Analysis of Variance (ANOVA) with soil origin (i.e., soil from each research farm), treatment, and interactions as factors with an α -value of 0.05 (Rasch and Verdooren 2020). Differences in microbial respiration, microbial biomass, potential nitrification, free living nitrogen fixation rates, biofertilizer and N-fixing bacteria abundances in response to treatments were then analyzed using a three-way Analysis of Variance (ANOVA) with soil origin, soil compartment (i.e. bulk or rhizosphere), treatment, and interactions as factors with an α -value of 0.05 (Rasch and Verdooren 2020). Post-hoc differences of means were analyzed using Tukey's Honestly Significant Difference (R Core Team, 2016).

A principal component analysis (PCA) was performed to find correlations between the different plant types and microbial functional measurements (e.g. microbial respiration) and to analyze clustering for different variables (e.g. soil origin, soil compartment). Statistical significance of this PCA was determined with a Multivariate Analysis of Variance (MANOVA) with plant type, soil origin, and soil compartment as factors. Similarly, a PCA was performed to determine correlations between plant types and environmental measurements (e.g. pH). Linear regressions were used to assess relationships between plant biomass, microbial activity, and the relative abundance of microbial groups. All analysis was performed in RStudio (RStudio Core Team 2016) running R 3.6.3 (R Core Team 2020).

3. Results

3.1 Soil Chemical Properties

The addition of biofertilizer treatments had no effect on *Miscanthus* or switchgrass soil chemical properties (reported as mean $\pm$ standard error, all $p>0.05$). Soil chemical properties were influenced by both soil origin (Animal Sciences farm vs Agronomy farm) and vegetation. The pre-planting soil pH was relatively acidic in both Agronomy (5.71 ± 0.25) and Animal Science farm soils (6.43 ± 0.03), though the Animal Science farm soils were less acidic ($p<0.01$) than Agronomy farm soils. The pH of *Miscanthus* soils differed by soil origin (Table 1), though both the Animal Science (6.77 ± 0.04) and Agronomy farm soil pH (6.02 ± 0.04) were comparatively less acidic than pre-planting soil pH. The Animal Science farm soil had lower percent organic matter (9.12 ± 0.20 %, $p<0.01$) in comparison to Agronomy farm soil (11.52 ± 0.29 %) under *Miscanthus* ($p<0.01$, Table 1)

In switchgrass soils, nitrate concentration and percent organic matter differed by soil origin but pH was not significantly influenced by any factor (Table 1). As with the *Miscanthus* soils, the switchgrass Animal Science soils had significantly lower ($p<0.01$) percent organic matter (8.62 ± 0.20 %) than the Agronomy farm soils (10.53 ± 0.28 %). Nitrate concentration was also lower in the Animal Science soils (6.88 ± 1.04 ug NO₃ g soil⁻¹, $p=0.05$) than in Agronomy farm soils (15.45 ± 4.07 ug NO₃ g soil⁻¹).

In control soils, nitrate concentrations and moisture were not affected by any factor (all $p>0.05$). As with both *Miscanthus* and switchgrass soils, the control Animal Science soils had significantly lower ($p<0.01$) percent organic matter (9.61 ± 0.52 %) than the Agronomy farm soils (11.91 ± 0.25 %). In control soils, pH was also influenced by soil origin ($p<0.01$). Soils were more acidic in Agronomy farm soils (5.46 ± 0.08) compared to Animal Science farm soils (6.3 ± 0.06).

Additionally, vegetation type influenced environmental conditions (MANOVA, $p < 0.01$) as visualized on a PCA (Fig. 2). The overlap in ordination space of the control and switchgrass samples demonstrate that *Panicum virgatum* L. (switchgrass) did not have as large of an effect on environmental parameters. In contrast, *Miscanthus* altered the soil environment as seen by the separate cluster in the PCA plot (Fig. 2). *Miscanthus* soils were associated with higher average pH (6.40 ± 0.06) than switchgrass (6.03 ± 0.04). Alternatively, switchgrass pots were associated with higher average nitrate concentrations ($11.32 \pm 2.29 \text{ ug NO}_3 \text{ g soil}^{-1}$) than *Miscanthus* ($2.89 \pm 1.60 \text{ ug NO}_3 \text{ g soil}^{-1}$).

3.2 Plant Biomass

Miscanthus above-ground biomass differed by soil origin (Table 1), as aboveground biomass was higher in Agronomy farm soils ($17.05 \pm 0.52 \text{ g}$) as compared to Animal Science soils ($14.04 \pm 0.41 \text{ g}$). The biofertilizer treatments did not affect *Miscanthus* aboveground biomass ($p > 0.05$). Unlike aboveground biomass, the addition of biofertilizer treatments did have a significant effect on *Miscanthus* belowground biomass (Table 1). Belowground biomass significantly increased ($p = 0.02$) in the commercial biofertilizer treatment (Fig. 3; $29.16 \pm 2.71 \text{ g}$) in comparison to the heat killed treatment ($18.46 \pm 0.71 \text{ g}$). In contrast, switchgrass above and belowground biomass did not differ in response to either soil origin or biofertilizer treatments (root avg = $4.28 \pm 0.28 \text{ g}$, shoot avg = $1.50 \pm 0.11 \text{ g}$, Table 1).

3.3 Soil Microbial Function and Biomass

Microbial functional measurements consisted of potential nitrification rates, FLNF rates, as well as microbial respiration. Under *Miscanthus*, soil origin influenced potential nitrification rates (Table 2) as well as rates of FLNF by N-fixing bacteria (ANOVA, $p = 0.01$). Potential

nitrification rates were higher in Animal Science farm soils ($0.47 \pm 0.08 \text{ ug NO}_3 \text{ hr}^{-1} \text{ g soil}^{-1}$, $p < 0.01$) than in Agronomy soils ($0.11 \pm 0.02 \text{ ug NO}_3 \text{ hr}^{-1} \text{ g soil}^{-1}$). Agronomy farm soils had significantly higher FLNF rates ($0.78 \pm 0.12 \text{ ng } ^{15}\text{N g soil}^{-1} \text{ day}^{-1}$, $p = 0.01$) than in Animal Science farm soils ($0.49 \pm 0.03 \text{ ng } ^{15}\text{N g soil}^{-1} \text{ day}^{-1}$). FLNF rates were also influenced by soil compartment (Table 2), with the highest rates of FLNF occurring in rhizosphere soil ($p = 0.02$; Table 3). Microbial respiration rates also varied by soil compartment, with rhizosphere soils containing the more active microbial communities ($p < 0.01$; Table 3) compared to bulk soil. Unlike the previous microbial measurements, microbial biomass did not differ significantly (Table 2) in response to soil origin or soil compartment. Additionally, no measurements of microbial function or biomass were affected by the addition of the biofertilizer treatments (all $p > 0.05$).

In contrast to *Miscanthus*, microbial functional measurements in switchgrass soils did not differ by soil origin. However, differences in soil compartment were observed across all functional measurements (Table 2). Again, rhizosphere soil had the highest rates of FLNF ($p < 0.01$, Table 3), potential nitrification ($p < 0.01$, Table 3), and microbial respiration ($p < 0.01$, Table 3). As with *Miscanthus*, the addition of biofertilizer treatments showed no effects on soil microbial function or biomass in switchgrass pots.

In the control soils, both FLNF and microbial biomass were influenced by soil origin ($p = 0.01$ and $p = 0.04$ respectively). In contrast to *Miscanthus* soils, FLNF rates were higher in Agronomy farm soils than in Animal Science farm soils (Table 4, $p < 0.01$). Microbial biomass was higher in Animal Science farm soils ($1023.68 \pm 16.67 \text{ ug-C g}^{-1} \text{ soil}$, $p = 0.04$) than in Agronomy farm soils ($961.99 \pm 20.01 \text{ ug-C g}^{-1} \text{ soil}$). Unlike the previous microbial measurements, microbial respiration and potential nitrification did not differ significantly (both

$p > 0.05$) in response to soil origin or soil compartment. Additionally, no measurements of microbial function or biomass were affected by the addition of the biofertilizer treatments (all $p > 0.05$).

To explore connections between below ground biomass and microbial respiration rates, microbial respiration was regressed against the belowground biomass of both *Miscanthus* and switchgrass. Microbial respiration was positively correlated with *Miscanthus* belowground biomass, but no correlation was seen for switchgrass (Fig. 4).

As demonstrated previously with the environmental measurements, microbial functional measurements when considered together, were influenced by vegetation type (Fig. 5; MANOVA, $p < 0.01$). *Miscanthus* had a stronger influence on microbial function than switchgrass producing a shift on the PCA (Fig. 5). *Miscanthus* increased soil respiration rates ($\mu\text{g CO}_2\text{-C day}^{-1} \text{ dry soil}^{-1}$) (56.59 ± 2.57) in comparison to switchgrass (24.81 ± 1.50). Additionally, FLNF rates ($\text{ng } ^{15}\text{N g soil}^{-1} \text{ day}^{-1}$) were also higher in *Miscanthus* soils (0.63 ± 0.06) than in switchgrass soils (0.40 ± 0.05). In contrast, switchgrass soils were associated with increased microbial biomass ($1031.80 \pm 9.11 \mu\text{g-C g}^{-1} \text{ soil}$) in comparison to *Miscanthus* pots ($923.12 \pm 6.85 \mu\text{g-C g}^{-1} \text{ soil}$).

3.4 Commercial *Bacillus* and *Trichoderma* Relative Abundance

The commercial biofertilizer was primarily comprised of 10 ASVs which were all members of the genus *Bacillus*. These ASVs dominated in the commercial biofertilizer, accounting for 96% of 16S rRNA genes, and will hereafter be collectively referred to as ‘commercial *Bacillus*’. In the microbiome transplant treatment, the most abundant prokaryotic ASVs included members of the genera *Alkaliphilus* (12%), *Rickettsiella* (6%), *Enterococcus* (4%), and *Candidatus Nitrocosmicus* (1%), among others.

Fungal communities in the commercial biofertilizer were primarily composed of 3 ASVs assigned to the genus *Trichoderma*. These ASVs made up 82% of the fungal ITS gene sequences in the commercial biofertilizer and will be hereafter referred to as ‘commercial *Trichoderma*’. In the microbiome transplant, the most abundant fungal ASVs were members of the genera *Tolypocladium* (26%), the sub-phylum Rozellomycotina (12%), and the genera *Mortierella* (1%).

The relative abundance of the commercial *Bacillus* was influenced by soil compartment in both *Miscanthus* and switchgrass pots (Table 2). Commercial *Bacillus* relative abundance was highest in switchgrass bulk soil (Fig. 6; Table 3). The biofertilizer treatments did not have a significant effect on commercial *Bacillus* abundance (Table 2). In order to investigate the relationship between *Miscanthus* belowground biomass and the commercial biofertilizer, *Miscanthus* belowground biomass was regressed against commercial *Bacillus* relative abundance with soil origin as a factor. In the *Miscanthus* rhizosphere soil, commercial *Bacillus* abundance positively correlated to *Miscanthus* belowground biomass, but only in Agronomy farm soils (Fig. 7). No correlation in Animal Science soil was observed (Fig. 7). The relative abundance of the commercial *Bacillus* was not influenced by any factor in the control pots (all $p > 0.05$).

The fungal component of the commercial biofertilizer, composed mainly of commercial *Trichoderma*, was only influenced by one factor in switchgrass pots (Fig. 8). Soil origin influenced commercial *Trichoderma* abundance (Table 2), where *Trichoderma* were more abundant in Agronomy farm soil as compared to Animal Science soil. The relative abundance of the commercial *Bacillus* under switchgrass was also greater in Agronomy farm soil (0.13 ± 0.03 %) as compared to Animal Science soils (0.04 ± 0.01 %). Unlike with the commercial *Bacillus*

abundance, no factors significantly influenced the relative abundance of the commercial *Trichoderma* in *Miscanthus* or control soils (all $p > 0.05$).

3.5 Nitrogen Fixer Abundance

The relative abundance of putative N-fixing taxa (% 16S rRNA gene sequences) was influenced by soil origin in *Miscanthus* and control soils ($p < 0.01$), while in switchgrass soils n-fixer abundance was influenced both by soil origin and soil compartment (Table 2). Average relative abundance of N-fixing bacteria was highest in Agronomy soils for *Miscanthus* (9.33 ± 0.14 %) and switchgrass (8.45 ± 0.16 %). The Animal Science farm soils, though statistically different, were not much lower in *Miscanthus* (6.11 ± 0.14 %) or switchgrass (5.83 ± 0.17 %). N-fixer abundance was highest in switchgrass rhizosphere soil ($p < 0.01$, Table 3). In the control pots, Agronomy farm soils had higher average relative abundance of N-fixing bacteria than in Animal Science farm soils (Table 4). Additionally, the relationship between the relative abundance of N-fixing bacteria and FLNF rates in *Miscanthus* pots was determined using a linear regression. The abundance of N-fixing taxa in *Miscanthus* soils positively correlated with FLNF rates, but only in Agronomy farm soils (Fig. 9).

4. Discussion

In this study we investigated the efficacy of biofertilizers in marginal soils, as well as the plant specificity of those biofertilizers. We found positive belowground biomass effects of biofertilizer on *Miscanthus*, as well as differences in microbial function driven by plant species and soil origin. Differences in these microbial functional measurements driven by soil origin demonstrate that plant effects may not mask differences in the initial soil quality within one

growing season. Lastly, biofertilizers showed potential to increase root biomass, but contrary to our hypothesis they did not affect microbial activity.

4.1 Differences in Plant Type and Microbial Amendment Composition Drive Belowground Biomass Increases

The commercial biofertilizer increased belowground biomass in *Miscanthus*. This is consistent with a previous study of the use of biofertilizers in *Miscanthus*, which determined plant biomass was increased with the addition of the PGPB *Bacillus altitudinis* (Pranaw et al. 2020). *Bacillus altitudinis* possesses many plant growth promoting traits, including P solubilization which can aid in nutrient acquisition, indole-3-acetic acid (or IAA) production which can stimulate root development, and hydrogen cyanide (HCN) production which can inhibit the growth of certain fungi (Pranaw et al. 2020). The production of auxins like IAA and other plant hormones (e.g. ethylene) are important plant growth promoting attributes that many successful biofertilizers possess, including biofertilizers that positively affect *Miscanthus* growth (Straub et al. 2013; Lally et al. 2017; Schmidt et al. 2018). Plant hormones like IAA work through several mechanisms, but often result in increased root production (Pacifici et al. 2015; Cox et al. 2018; Qin et al. 2019). *Trichoderma* spp. have also been shown to increase belowground biomass through the production of plant hormones such as IAA (Gravel et al. 2007). *Trichoderma* can also indirectly increase belowground biomass by acting as a biocontrol agent by preventing phytopathogens from damaging the plant and by helping bioremediate soils contaminated with heavy metals (Lo and Lin 2002; Fiorentino et al. 2013; Hyder et al. 2017). The effect of biofertilizers containing *Trichoderma* on *Miscanthus* biomass has not been previously investigated. Our results, however, suggest that the commercial *Bacillus* was responsible for the increase in belowground biomass, as commercial *Bacillus* relative abundance and *Miscanthus* belowground biomass were positively correlated (Fig. 7).

Recent research into the use of biofertilizers containing microbial consortia has shown promising results (Zayadan et al. 2014; Anderson 2017; Herrera Paredes et al. 2018; Trexler and Bell 2019; Behera et al. 2021), but questions remain of the feasibility of microbial consortia in larger scale field trials (Awasthi 2019). When examining the relative abundance of ASVs present in both the commercial and transplant biofertilizer treatments, the composition of the commercial biofertilizer was dominated by a single taxonomic group (composed of several ASVs), both for bacteria and fungi (e.g. *Bacillus* and *Trichoderma*). In contrast, the transplant inoculum was much more diverse with the most dominant genera being represented at relatively lower abundances. Since the goal of most transplant studies is to transfer whole microbiomes to a new environment (Howard et al. 2017), the high ASV diversity in the transplant treatment suggests the transplant treatment consisted of at least a partial microbiome from the established *Miscanthus* stand. However, since only the commercial biofertilizer treatment resulted in increased belowground biomass in *Miscanthus*, this could suggest that a more specific biofertilizer formulation (e.g. a microbial consortium consisting of known PGPB) may be the most beneficial for use with *Miscanthus* (Fig. 2). Additionally, the *Miscanthus* microbiome has previously been shown to become less diverse and more stable as the stand ages, which may suggest that younger *Miscanthus* microbiomes are more susceptible to the establishment of introduced microbes (Calderón et al. 2017; Ma et al. 2021). These results suggest that the composition and ecology of a microbial consortium should be carefully considered to maximize its benefit in soil (Nannipieri et al. 2003; Maherali and Klironomos 2007; Berg et al. 2016; Malusà et al. 2016; Singh et al. 2016).

No biofertilizer treatment effect was seen in switchgrass soils, which is consistent with several other studies of biofertilizer effects on switchgrass (Allaire et al. 2015; Shanta et al.

2016; Arunachalam et al. 2018). This suggests that switchgrass may require a different assortment of microorganisms than were provided in the commercial biofertilizer or that the introduced microbes were not able to establish as well in switchgrass soils. Indeed, some biofertilizer effects have been seen in switchgrass when the microbial consortium consisted of endophytic PGPB which were not present in either of our biofertilizer treatments (Ker et al. 2012; Ker et al. 2014). These previous studies both utilized microbial consortia which consisted of P solubilizing bacteria and N-fixing bacteria which could aid in nutrient acquisition, and bacteria capable of producing auxins (particularly IAA) which could help stimulate root growth (Ker et al. 2012; Ker et al. 2014). While the commercial biofertilizer added in our study was also capable of producing auxins, we did not see any effect of the commercial *Bacillus* on switchgrass belowground biomass. This suggests that non-endophytic PGPB struggle to colonize the switchgrass microbiome, therefore mitigating the potential benefit the commercial biofertilizer used in our study. Evidence that *Burkholderia phytofirmans*, a well-studied endophytic PGPB, can colonize switchgrass (Kim et al. 2012) further emphasizes that endophytic bacteria are likely key to successful biofertilizer inoculation of switchgrass. Additionally, the transplant treatment was made from soil collected from an established *Miscanthus* stand. This could explain why the transplant did not affect switchgrass soils, as the microbes associated with mature *Miscanthus* stands likely do not benefit switchgrass.

4.2 Belowground Biomass Positively Correlates to Commercial *Bacillus* Relative Abundance

Commercial *Bacillus* abundance positively correlated with root biomass in *Miscanthus* Agronomy farm rhizosphere soils (Fig. 7), suggesting that the increased belowground biomass is being driven by increased commercial *Bacillus* presence in the soil. Many *Bacillus* species have previously been identified as PGPB, working in a variety of mechanisms to stimulate plant

growth including acting as a biocontrol agent and directly releasing plant hormones like auxins which can regulate root development (Borriss 2015; Bokhari et al. 2019; Wagi and Ahmed 2019; Masood et al. 2020). There are several methods of biocontrol exhibited by *Bacillus* spp. including the production of antibiotics (Ajilogba et al. 2013; Sunar et al. 2015) and antifungals (Torres et al. 2019; Pranaw et al. 2020), and the production of siderophores which reduce Fe availability for pathogens (Schmidt et al. 2018). *Bacillus* spp. which have previously been shown to increase *Miscanthus* belowground biomass have demonstrated both antifungal properties as well as siderophore production (Schmidt et al. 2018; Pranaw et al. 2020). These mechanisms could help explain the increase in belowground biomass shown in *Miscanthus*.

In addition to directly releasing plant hormones such as IAA, *Bacillus* spp. can directly affect root growth through mechanisms which promote nutrient acquisition (Miljakovic et al. 2020). These methods commonly include either P solubilization (García-López and Delgado 2016) or N fixation (Szilagyi-Zecchin et al. 2014) which both work by providing bioavailable forms of the nutrient for easier absorption by the plant (Miljakovic et al. 2020). P solubilization has been shown to increase *Miscanthus* belowground biomass in studies using *Bacillus* spp. as inoculum (Pranaw et al. 2020).

Bacillus spp. can also aid in the bioremediation of contaminated soils (Hassan et al. 2017) which can also indirectly increase root growth. Reclaimed mine soils can be contaminated by heavy metals (e.g. As, Cu, Zn) which can damage the quality of the soil and have potential harmful effects on humans (Wang et al. 2013; Ashraf et al. 2019). *Bacillus* spp. have been shown to positively affect belowground biomass in these contaminated soils, for example *Miscanthus* belowground biomass increased in heavy metal contaminated soils in response to treatment with *Bacillus altitudinis* (Hassan et al. 2017). This might explain why the commercial *Bacillus*

abundance and belowground biomass only correlated in the Agronomy farm soils and not Animal Science farm soils. The Agronomy farm soils (which can be considered low quality soil compared to Animal Science soils due to lower pH and soil conductivity) could be contaminated with heavy metals, as this site has a history of surface mining, and therefore the commercial *Bacillus* is having a greater effect on belowground biomass when combined with the other plant growth promoting mechanisms discussed above.

This correlation with relative abundance also suggests that the establishment and growth of a microbial inoculum (like a commercial biofertilizer) may be driving the root growth in *Miscanthus*. Indeed, previous research has concluded that the success of an inoculum is related to its ability to establish and persist in the new environment (Lupwayi et al. 2006; Mitter et al. 2021). Other factors may influence the success of a biofertilizer, including inoculum formulation (e.g. liquid, peat, etc. (Stephens and Rask 2000)) and the microbial composition which can affect a biofertilizer's compatibility with a plant host (Vargas et al. 2012; Ehinger et al. 2014), as well as its establishment and survival in soil (Mitter et al. 2021). This is a common problem for many modern biofertilizer formulations, as there is a trade-off between traits that determine persistence and desirable PGP traits which can making choosing the correct microbes to include in a biofertilizer particularly challenging (Hart et al. 2018; Kaminsky et al. 2019). *Bacillus* species are very promising candidates for use in biofertilizers, partially due to their ability to withstand harsh environmental conditions by producing endospores (Bradshaw 2000; Nicholson et al. 2000; Toyota 2015; Torres et al. 2019). Studies examining the persistence of microbial inoculants in soil have revealed large variations (anywhere from between 6 days to several months) in the amount of time an inoculum can survive in the soil (Gu et al. 2019). One factor that determines the success of an inoculum is the amount of niche overlap the inoculum has with

the native soil microbes (Wei et al. 2015; Mawarda et al. 2020). The less competition for limiting resources an inoculum faces from the native microbes, the higher the likelihood for the persistence of the inoculum in the new environment (Mallon et al. 2015; Wei et al. 2015). Once established, bacteria that contain traits that make them competitively superior to the native microbes can help an increase the persistence of an inoculum (Mawarda et al. 2020). As such, spore forming bacteria like *Bacillus* have a large advantage over other non-spore forming bacteria (Toyota 2015). Additionally, *Bacillus* species are capable of colonizing a wide variety of soil and root environments and have not been shown to be plant specific (Gabra et al. 2019). These traits could help to explain why the commercial *Bacillus* was able to persist in *Miscanthus* soils despite the evidence that many other biofertilizers struggle to survive in their introduced environment (Calderón et al. 2017).

4.3 Differences in Plant and Soil Origin Drive Soil Microbial Functional Measurements

Miscanthus soils were associated with increased microbial respiration and FLNF rates while switchgrass soils were associated with increased microbial biomass (Fig 5). Additionally, switchgrass and control soils had increased nitrate concentrations (Fig 3) compared to *Miscanthus*. While FLNF rates were low in all samples compared to other studies reporting FLNF rates in *Miscanthus* (Davis et al. 2010; Keymer and Kent 2014), this could help to explain the increase in FLNF rates associated with *Miscanthus*, as less bioavailable nitrogen in the form of nitrate in the soil could stimulate the N-fixing microbes present in the soil (Duval et al. 2012; Smercina et al. 2019). N-fixing bacteria are more abundant in low N environments, and evidence suggests that available N rates are inversely related to FLNF (Cusack et al. 2009; Reed et al. 2011). This is because many N-fixing bacteria will switch off the energetically expensive n-fixation process when nitrogen is readily available in the environment (Reed et al. 2011).

Conversely, the increased nitrate concentrations in switchgrass soils could explain why FLNF rates were comparatively lower in switchgrass than in *Miscanthus*. Additionally, the increased rates of microbial respiration in *Miscanthus* could also play a role in the increased FLNF rates. Increased levels of O₂ have been shown to inhibit nitrogenase, which is the enzyme that catalyzes n-fixation (Reed et al. 2011; Smercina et al. 2019). High microbial respiration rates therefore could help mitigate the effects oxygen has on n-fixation by helping to decrease the amount of O₂ present in the soil (Robson and Postgate 1980; Reed et al. 2011).

In both *Miscanthus* and switchgrass soils, respiration was higher in rhizosphere soils which is consistent with expectations, as microbes in the rhizosphere tend to be more active than in bulk soil (Li et al. 2014). Soil origin determined many differences in microbial functional measurements (e.g. microbial respiration, potential nitrification, and FLNF rates) in *Miscanthus* soils. This could suggest that soil microbiome function under *Miscanthus* is influenced by soil physicochemical properties. Interestingly, soil origin did not have an effect on microbial functional measurements in switchgrass soils. Soil conditions have previously been shown to influence switchgrass microbial community structure (Hestrin et al. 2021) but our results may suggest that the differences seen in community structure might not influence microbial functional measurements under switchgrass.

4.4 Linking N-fixing bacterial abundance with increased free living nitrogen fixation in Miscanthus soils

Linking microbial community structure and microbial processes has always been challenging, and while developments in sequencing technology have helped researchers be able to identify the community structure of almost any environmental sample, there remains much work to be done to truly understand the link between microbial community and function (Bier et

al. 2015). However, by targeting N-fixing bacteria, our study was able to assess links between the functional measurement of FLNF to the microbial community structure in *Miscanthus*. N-fixing bacterial abundance correlated to increased FLNF rates in *Miscanthus* soils, though only in Agronomy (low quality) farm soils. The Animal Science farm soils had lower relative abundance of N-fixing bacteria as well as lower FLNF rates compared to Agronomy farm soils (Fig. 9) which could suggest that the Animal Science farm has more available N and therefore does not require high abundances of N-fixing bacteria or higher rates of FLNF to supply plants with bioavailable N. FLNF rates have previously been correlated to N-fixing bacterial abundance in switchgrass (Smercina et al. 2021), but no such correlation has been made with *Miscanthus*. Previous research has suggested that *Miscanthus* at least partially relies on FLNF to meet its nitrogen needs (Davis et al. 2010). In first year *Miscanthus* stands, it was estimated that 16% of plant nitrogen was derived by nitrogen fixation, though this measurement includes both FLNF and symbiotic nitrogen fixation (Keymer and Kent 2014). Understanding this microbial community-function relationship could help researchers maximize benefits when utilizing N-fixing bacteria as microbial inoculants in *Miscanthus* soils.

4.5 Conclusions

Taken together, our results partially support the hypothesis that biofertilizers will increase plant growth and microbial activity as well as augment the relative abundance of the added microorganisms in marginal soils. The commercial biofertilizer treatment increased belowground biomass in *Miscanthus*, but did not increase the relative abundance of commercial *Bacillus* in either plant. Biofertilizer treatments also had no effect on microbial activity. Differences in microbial activity were driven by plant type. This supports the recent shift towards biofertilizer formulations which focus on microbes with known mechanisms for stimulating plant growth

rather than trying to target microbes which may have more of an effect on microbial activity (Nosheen et al. 2021). Furthermore, our results do not support the hypothesis that biofertilizers will have the same effect on *Panicum virgatum* L. (switchgrass) and *Miscanthus x giganteus* growth and soil carbon and nitrogen availability. The commercial biofertilizer only increased belowground biomass in *Miscanthus* and not in switchgrass. This could suggest that more research is required to develop species specific biofertilizers, or alternatively to find PGPB that are true generalists. Biofertilizers also did not affect soil carbon and nitrogen availability, instead, differences were again driven by plant type. Switchgrass had increased nitrate concentrations and potential nitrification rates compared to *Miscanthus*, while *Miscanthus* had comparatively larger percent organic matter. These findings suggest that in order to optimize the growth of bioproduct crops on marginal lands, a balance between the type of vegetation and microbial inoculum used must be achieved.

5. Figures

Fig. 1 Experimental Design. Pots were filled with either agronomy or animal farm science soil, then were assigned to a plant type. After plant was established, pots were inoculated with one of three biofertilizer treatments. Each soil origin had 5 replicates of each biofertilizer treatment

Fig. 2 Principal Components Analysis (PCA) of environmental factors (arrows), samples are designated by soil origin (shape) and plant type (color). Plant clusters are shown as 95% concentration ellipses, assuming bivariate normal distributions.

Fig. 3 Average belowground biomass in *Miscanthus* samples. Lowercase letters indicate significant differences between treatment. Bars represent means + SE

Fig. 4 Linear regression between soil microbial respiration and belowground biomass of *Miscanthus* and *Switchgrass* rhizosphere soils. Regression lines and corresponding statistical analyses are colored by plant type

Fig. 5 Principal Components Analysis (PCA) of microbial functional measurements (arrows), samples are designated by soil origin (shape) and plant type (color). Plant clusters are shown as 95% concentration ellipses, assuming bivariate normal distributions.

Fig. 6 Average relative abundance of commercial *Bacillus* species. Bars represent means + SE

Fig. 7 Linear regression between commercial *Bacillus* relative abundance and *Miscanthus* belowground biomass in rhizosphere soils. Regression lines and corresponding statistical analyses are colored by soil origin.

Fig. 8 Average relative abundance of commercial *Trichoderma*. Bars represent means + SE

Fig. 9 Linear regression between N-fixing bacteria relative abundance and Miscanthus soil FLNF rates. Regression lines and corresponding statistical analyses colored by soil origin

6. Tables

Table 1 Summary of two-way ANOVA tables testing soil origin, treatment, and soil origin by treatment for each plant species separately.

	Miscanthus						Switchgrass					
	Soil Origin		Treatment		Soil Origin:Treatment		Soil Origin		Treatment		Soil Origin:Treatment	
	F	P	F	P	F	P	F	P	F	P	F	P
Belowground biomass (g)	0.36	0.55	4.16	0.03 *	2.63	0.09 .	0.10	0.75	1.47	0.24	1.04	0.36
Aboveground biomass (g)	8.99	<0.01 **	0.15	0.86	0.14	0.87	0.46	0.50	0.07	0.94	0.01	0.99
pH	101.86	<0.01 ***	0.47	0.63	0.87	0.43	1.15	0.30	0.03	0.97	1.05	0.37
Nitrate (ug NO₃ g soil⁻¹)	3.49	0.07 .	1.14	0.34	1.16	0.33	4.50	0.05 *	3.12	0.07 .	0.84	0.45
% Organic Matter	20.55	<0.01 ***	0.23	0.79	0.19	0.83	12.48	<0.01 **	0.11	0.90	0.50	0.62

.P<0.10

*P<0.05

**P<0.01

***P<0.001

Table 2 Summary of three-way ANOVA tables testing soil origin, soil compartment, treatment, soil origin by soil compartment, soil origin by treatment, and soil origin by soil compartment by treatment for each plant species separately.

	Soil Origin		Soil Compartment		Treatment		Soil Origin:Soil Compartment		Soil Compartment: Treatment		Soil Origin: Treatment		Soil Origin: Soil Compartment: Treatment	
	F	P	F	P	F	P	F	P	F	P	F	P	F	P
Miscanthus														
Soil Respiration (ug CO ₂ -C Day ⁻¹ Dry Soil ⁻¹)	0.68	0.41	10.17	<0.01 **	0.32	0.73	2.88	0.10 .	0.43	0.65	0.85	0.43	0.01	0.99
Microbial Biomass (ug g ⁻¹ soil)	0.12	0.73	0.46	0.50	1.26	0.29	0.85	0.36	0.29	0.75	0.57	0.57	0.47	0.63
Nitrogen Fixation (ng ¹⁵ N g soil ⁻¹ day ⁻¹)	7.16	0.01 *	6.63	0.01 *	0.38	0.68	11.54	<0.01 **	0.46	0.64	0.46	0.64	0.43	0.65
Potential Nitrification (ug NO ₃ hr ⁻¹ g soil ⁻¹)	18.67	<0.01 ***	3.10	0.08 .	0.50	0.61	0.26	0.61	0.52	0.60	0.14	0.87	0.70	0.50
Bacillus Relative Abundance (%)	1.34	0.25	6.02	0.02 *	0.29	0.75	3.56	0.07 .	0.66	0.52	2.34	0.11	1.11	0.34
Nitrogen Fixer Relative Abundance (%)	286.11	<0.01 ***	0.28	0.60	1.56	0.22	8.17	<0.01 **	0.06	0.94	0.04	0.96	1.66	0.20
Commercial Trichoderma Relative Abundance (%)	3.41	0.07 .	2.92	0.09 .	0.87	0.43	0.37	0.55	0.25	0.78	0.89	0.42	0.09	0.91
Switchgrass														
Soil Respiration (ug CO ₂ -C Day ⁻¹ Dry Soil ⁻¹)	0.43	0.51	11.02	<0.01 **	1.47	0.24	1.51	0.22	0.20	0.82	0.12	0.89	0.10	0.91
Microbial Biomass (ug g ⁻¹ soil)	2.16	0.15	1.75	0.19	0.14	0.87	0.96	0.33	0.08	0.92	0.86	0.43	0.16	0.85
Nitrogen Fixation (ng ¹⁵ N g soil ⁻¹ day ⁻¹)	2.67	0.11	17.67	<0.01 ***	0.88	0.42	6.63	0.01 *	1.00	0.37	0.83	0.44	1.13	0.33
Potential Nitrification (ug NO ₃ hr ⁻¹ g soil ⁻¹)	3.48	0.07 .	12.14	<0.01 ***	1.49	0.23	0.22	0.64	1.94	0.15	0.22	0.80	0.36	0.70
Bacillus Relative Abundance (%)	2.23	0.14	17.14	<0.01 ***	1.05	0.36	4.33	0.04 *	0.71	0.50	1.28	0.29	0.47	0.63
Nitrogen Fixer Relative Abundance (%)	150.23	<0.01 ***	12.31	<0.01 **	0.33	0.72	1.69	0.20	0.18	0.84	0.10	0.90	1.19	0.32
Commercial Trichoderma Relative Abundance (%)	6.77	0.01 *	0.98	0.33	0.52	0.60	0.16	0.69	0.35	0.70	0.60	0.55	0.13	0.88

.P<0.10

*P<0.05

**P<0.01

***P<0.001

Table 3 Microbial functional measurements and relative abundances (mean $\pm$ SE) of commercial *Bacillus* and N-fixing bacteria by soil compartment for each plant in soils originating from the Animal Sciences and Agronomy Farms.

	Miscanthus				Switchgrass			
	Animal Science		Agronomy		Animal Science		Agronomy	
	Bulk	Rhizosphere	Bulk	Rhizosphere	Bulk	Rhizosphere	Bulk	Rhizosphere
Soil Respiration ($\mu\text{g CO}_2\text{-C Day}^{-1}\text{ Dry Soil}^{-1}$)	46.97 $\pm$ 2.83	70.04 $\pm$ 5.99	50.91 $\pm$ 3.54	58.59 $\pm$ 5.80	18.22 $\pm$ 1.67	33.15 $\pm$ 3.63	19.69 $\pm$ 1.76	28.31 $\pm$ 2.77
Nitrogen Fixation ($\text{ng } ^{15}\text{N g soil}^{-1}\text{ day}^{-1}$)	0.53 $\pm$ 0.06	0.45 $\pm$ 0.02	0.46 $\pm$ 0.10	1.11 $\pm$ 0.17	0.24 $\pm$ 0.03	0.60 $\pm$ 0.16	0.18 $\pm$ 0.03	0.59 $\pm$ 0.11
Potential Nitrification ($\mu\text{g NO}_3\text{ hr}^{-1}\text{ g soil}^{-1}$)	0.57 $\pm$ 0.12	0.38 $\pm$ 0.09	0.16 $\pm$ 0.03	0.06 $\pm$ 0.03	4.5 $\pm$ 1.09	1.37 $\pm$ 0.15	2.87 $\pm$ 0.77	0.52 $\pm$ 0.09
<i>Bacillus</i> Relative Abundance (%)	5.64 $\pm$ 0.84	4.48 $\pm$ 1.06	4.09 $\pm$ 0.25	5.19 $\pm$ 1.12	14.53 $\pm$ 2.92	4.76 $\pm$ 0.63	8.92 $\pm$ 0.68	5.68 $\pm$ 0.54
Nitrogen Fixer Relative Abundance (%)	6.33 $\pm$ 0.25	5.89 $\pm$ 0.12	9.00 $\pm$ 0.15	9.65 $\pm$ 0.21	5.59 $\pm$ 0.27	6.06 $\pm$ 0.19	7.95 $\pm$ 0.14	9.00 $\pm$ 0.20

Table 4 Microbial functional measurements and relative abundances (mean $\pm$ SE) of commercial *Bacillus* and N-fixing bacteria for control pots in soils originating from the Animal Sciences and Agronomy Farms.

	Control	
	Animal Science	Agronomy
Soil Respiration ($\mu\text{g CO}_2\text{-C Day}^{-1} \text{ Dry Soil}^{-1}$)	26.38 $\pm$ 1.85	27.58 $\pm$ 2.60
Nitrogen Fixation ($\text{ng } ^{15}\text{N g soil}^{-1} \text{ day}^{-1}$)	0.10 $\pm$ 0.01	0.13 $\pm$ 0.00
Potential Nitrification ($\mu\text{g NO}_3 \text{ hr}^{-1} \text{ g soil}^{-1}$)	6.17 $\pm$ 1.46	3.36 $\pm$ 1.01
Bacillus Relative Abundance (%)	7.93 $\pm$ 0.77	8.29 $\pm$ 0.84
Nitrogen Fixer Relative Abundance (%)	6.36 $\pm$ 0.20	8.94 $\pm$ 0.19
Commercial Trichoderma Relative Abundance (%)	0.08 $\pm$ 0.02	0.17 $\pm$ 0.08

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