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Intraspecific differences in belowground associations and seedling root morphology for the biofuel crop, Panicum virgatum.

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INTRASPECIFIC DIFFERENCES IN BELOWGROUND ASSOCIATIONS AND

SEEDLING ROOT MORPHOLOGY FOR THE BIOFUEL CROP,

PANICUM VIRGATUM

By

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ABSTRACT

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The perennial C_4 grass *Panicum virgatum* (switchgrass) is a promising bioenergy feedstock. Switchgrass is symbiotic with arbuscular mycorrhizal fungi (AMF). I examined 12 varieties of switchgrass grouped into 2 ecotypes (upland, lowland) to see if they differed in AMF abundance and root colonization using the GLBRC Variety Trials experiment. There was higher root colonization of lowland ecotype.

A greenhouse experiment evaluated the role of live soil organisms in seedling establishment of 11 varieties. Live soil increased seedling survival, growth and influenced root morphology. Ecotypes differed in root

architecture. Lowland developed a higher specific root length (SRL), a trait associated with productivity.

The field experiment provided evidence that lowland ecotypes had higher root colonization, while the greenhouse study found no differences in dependence on live soil microbiota. The greenhouse study does, however support the idea that AMF could be important in the seedling establishment stage.

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CHAPTER ONE: BIOFUEL HISTORY AND

THE GREAT LAKES BIOENERGY RESEARCH CENTER

<u>Biofuel History</u>

Scientists are increasingly aware of the impacts of climate change
on ecosystems. These environmental impacts include species extinction,
species range shifts that result in community compositional change, and
spread of invasive species (Walther, 2002). Climate change is also a threat
to agroecosystems due to changes in temperature fluctuations,
precipitation cycles, mismatch between pollinators and flowering
phenology, and increased pests (Piao, 2010). The main cause of climate
change is the release of CO_2 through the burning of fossil fuels (Marland,
2003). In order to avoid the most negative of these impacts on
ecosystems and the biota, society needs to look for alternative energy
sources outside of fossil fuels.

A biofuel is an energy source that comes from living biota instead
of being sourced from fossil fuels. Some examples of biofuel feedstocks
include algae, corn, sugarcane, miscanthus, beets, and switchgrass.
Biofuels have been produced commercially since the 1970s, most notably

from Brazilian sugarcane (Goldemberg, 2007). Since biofuel crops

capture carbon as a part of photosynthesis they have the potential to be

carbon neutral (Schmer, et al., 2008). Bioenergy crops have gained

attention as possible ways to enhance agroecosystem function while

diversifying a farmer's portfolio (Manatt et al., 2013). Biofuels represent

potential stabilization of sourcing fuels since they can be produced locally

instead of depending on a global market (Goldemberg, 2007). While wind,

solar, and nuclear power sources will become important as sustainable

forms of energy, they don't have the same potential to fill the need to

support non-stationary engines such as vehicles (Wyman, 2008).

C_4 grasses are being developed as biofuel feedstocks more than C_3

plants because the C_4 photosynthetic pathway is more efficient than the

C_3 process in the warmer, drier climes that climate change scenarios

predict (van der Weijde et al., 2013). Among the C_4 grasses considered as

potential biofuel feedstocks are corn, sugarcane, miscanthus, and

switchgrass. Each of these plants has their own unique advantages.

Biofuel production in the past has focused on high sugar, low lignin plants

such as corn and sugar cane. Interest has shifted to perennial non-food

crops such as miscanthus and switchgrass because they can be grown in

more marginal lands with fewer inputs of fertilizers and pesticides

(Heaton, et al., 2004). Perennial grasses may enhance ecosystem services

by increasing soil organic matter with their deep rooting systems and

controlling topsoil erosion because they don't need to be tilled and

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seeded yearly. There is a tradeoff, however, of increased ecosystem services for decreased biomass and less ease of processing. High-lignin grasses like miscanthus and switchgrass require a thermal pre-treatment of their biomass to encourage the conversion of ligno-cellulosic fiber to polysaccharides (Sarip, et al., 2016). The advantage of perennial grasses such as *Panicum virgatum* (switchgrass) is due to their longevity and minimal management requirements (Hartman, et al., 2011; van der Weijde et al., 2013).

Switchgrass is of particular interest in North America because it is native to most of the eastern and central contiguous U.S.A. Switchgrass is associated with increased ecosystem function belowground and maintaining avian species richness aboveground (Robertson, et al., 2011; Werling et al., 2014). Switchgrass has deep roots, which help to stabilize soil preventing erosion and reducing mineral runoff (Blanco-Canqui, et al., 2004; Lee, et al., 2000). Switchgrass has long been grown for use as fodder. Its flexibility in being able to be grown in marginal lands and increased uses of erosion control, wildlife habitat, and as a bioenergy source make switchgrass an attractive and low cost crop for farmers to diversify their portfolios.

Great Lakes Bioenergy Research Center

In 2007, the Great Lakes Bioenergy Research Center (GLBRC) was established by the Michigan State University and the University of Wisconsin-Madison to research sustainable sources of energy, after receiving funds from the U.S. Department of Energy ("Great Lakes Bioenergy Research Center," 2016). In 2009, GLBRC partnered with the W.K. Kellogg Biological Station at Michigan State University to establish a series of field experiments designed to examine the potential of different energy crops, including switchgrass ("W.K. Kellogg Biological Station," 2017). One of these experiments, in which twelve different varieties of switchgrass were grown in a replicated common garden setting, is the focus of Chapter Two.

CHAPTER TWO: SWITCHGRASS ASSOCIATIONS WITH ARBUSCULAR

MYCORRHIZAL FUNGI IN AN AGRICULTURAL SETTING

<u>Introduction</u>

Belowground organisms play important roles in ecosystem function (Altieri, 1999). Arbuscular mycorrhizal fungi (AMF) are ubiquitous soil organisms that influence soil aggregation, which in turn plays a role in nutrient cycling, water regulation, and soil sediment retention (Auge, et al., 2001; Daynes, et al., 2013; Zhang et al., 2013). Mycorrhizal activity also contributes to soil organic matter and carbon storage belowground through its network of hyphal growth (Treseder & Allen, 2000). Mycorrhizae source minerals such as phosphorus and nitrogen as well as water in exchange for carbon from the plants. AMF have extensive hyphal networks throughout the soil that can access minerals that are outside of the rhizosphere. AMF will form arbuscules inside plant root cortical cells in order to transfer minerals in exchange for sugar.

Around 90% of terrestrial plants associate with mycorrhizae to some degree (Latef, et al., 2016). However, some plants are much more

dependent on mycorrhizae than others. Some species are obligate symbionts, while others have a more facultative and opportunistic association (Holste, et al., 2016). For example, plants from late successional systems are more responsive to mycorrhizae than early successional plants (Koziol & Bever, 2015). Within a species, mycorrhizal associations can vary with life stage (Wang, et al., 2013). For example, the effects of mycorrhizal symbioses varied among plant species in tallgrass prairie communities with stronger and weaker effects at seedling, juvenile, and adult stages depending on the species (Holste, et al., 2016). However, it is unclear if mycorrhizal associations also vary among individual plants in a single population independent of their age, across different populations within a species, or across subspecies or varieties. As plant traits such as height, reproduction, and drought tolerance vary within species, it is expected that associations with symbionts are also varied, though little work has examined this in field conditions (Wang, et al., 2003).

Agricultural systems offer a unique opportunity to study how belowground symbiotic associations vary intra-specifically, as plant breeding has resulted in a large amount of intraspecific diversity in the form of registered crop varieties (Tester & Langridge, 2010). For example, wheat has been cultivated for 10,000 years and as a consequence of many generations of selection varieties exist that thrive across a wide latitudinal range from Russia to Argentina as well as high

and low elevations (Shewry, 2009). We also know that mycorrhizal associations with crops are important for water and nutrient uptake, and may partially explain wide range adaptations in crops (Jeffries, et al., 2003). Understanding how AMF associations vary within a crop species across different varieties and genotypes will help growers make better predictions about soil health, land management, and plant performance.

Switchgrass (*Panicum virgatum*) has been bred into a number of different commercially available varieties for a diversity of uses including fodder, erosion control, prairie restoration, wildlife nesting, and more recently biofuel production (Vandevender, 2001), and so displays a wide range of intraspecific trait variation (Hartman, et al., 2012). Switchgrass varieties are often divided into two ecotypes: upland and lowland. Upland varieties are typically found in cooler, northern regions or higher elevations. They typically produce lower biomass, but are more cold stress tolerant. Lowland varieties are found in more southern climes and lower elevations. They generally produce higher biomass and experience more drought stress in their native habitats (Parrish & Fike, 2005). The varied abilities of these two ecotypes to tolerate cold and drought stress may be due to differences in associations with soil symbionts. Switchgrass in native systems is strongly dependent on AMF, and so all varieties of switchgrass are expected to have healthy AMF communities when grown in agricultural settings (Hartnett, et al., 1994). In a field experiment using native populations of switchgrass, inoculation with

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mycorrhizae increased aboveground biomass (Entry, et al., 1999). Mycorrhizae may also ameliorate switchgrass drought and nutrient stress (Finlay, 2004; Reynolds, et al., 2005). However, it is unknown whether different varieties of switchgrass differ in their root colonization and abundance of AMF.

To explore intraspecific variation in AMF abundance and its potential effects on soil structure, I ask 1) Does switchgrass vary intraspecifically in AMF abundance at the variety or ecotype level? And 2) How does soil organic matter develop in fields planted with different varieties of switchgrass, as one measure of ecosystem function often associated with AMF? I expected to find that lowland ecotypes would have higher root colonization by AMF than upland ecotypes as this mutualistic relationship can be more important in drought stress conditions. However, as a C_4 grass, upland ecotypes would still have high rates of AMF colonization. I expected that Southlow, an upland variety sourced from southern Michigan with minimal bred improvements, would have higher colonization by AMF than other varieties as it is represents the most locally sourced genotype.

Methods and Materials

Site Description

This field study is a part of the Long-Term Ecological Research (LTER) at the W.K. Kellogg Biological Station (KBS) and the Great Lakes Bioenergy Research Center (GLBRC) in southern Michigan (42.4 °N 85.4 °W). KBS gets an average rainfall of 89 cm and 157 cm of snow and lies in planting zone 6a. Each year has an average growing season of 140 to 160 days ("U.S. Climate Data," 2015). The soil is mixed active, mesic Typic Hapludalfs ranging from coarse to fine loamy texture (Fry, 2015).

Experiment Design

In May of 2009, 10 varieties of switchgrass were planted in a common garden experiment in the GLBRC. The following year, two varieties were replanted due to failed establishment and two new varieties were added for a total of 12 varieties. Each of the 12 varieties were planted in 4.6 m x 12.2 m tangential plots in a random order and replicated in 4 blocks ("KBS LTER: Kellogg Biological Station Long-Term Ecological Research," 2015)(Figure 1). Nine were commonly available varieties: Southlow, Cave-in-rock, Trailblazer, Blackwell, Dakota, Alamo, Kanlow, NE 28, and Shelter, and three were experimental varieties developed by Ceres, Inc. especially for biofuel production: EG 2101 (improved Cave-in-rock), EG 1101 (improved Alamo), and EG 1102 (improved Kanlow). Eight are considered upland ecotype: Southlow,

Cave-in-Rock, Trailblazer, Blackwell, Dakota, Shelter, NE 28, and EG 2101. The remaining four are lowland ecotypes: Alamo, Kanlow, EG 1101, and EG 1102.

Low nitrogen fertilizer (78 kg ha^{-1}) was applied at the start of every growing season in May. Pre-switchgrass emergence weeds were controlled with Quinclorac (Drive®, 1.1 kg ha^{-1}) and atrazine (0.6 kg ha^{-1}). Glyphosate, 2,4-D or dicamba were used for post-emergence weeds as needed. The biomass was harvested yearly at the end of the growing season in November following senescence.

Soil Sampling

In May of 2015 fifteen 15cm x 2cm soil cores were collected near the base of haphazardly chosen plants in each plot and combined for analysis of soil organic matter and for use as soil inoculum for the Greenhouse experiment (see Ch. 3). In July of 2015, fifteen 15cm x 2cm soil cores were collected in each plot for analysis of extra-radical hyphae (ERH) and root colonization by AMF. Soil samples were all sieved to 4mm for coarse homogenization of the replicate cores and to remove rocks larger than 4mm. July samples were then dried at 60°C for 72 h to preserve samples before analysis.

Mycorrhizal Analysis

For root colonization, dried soil samples were rehydrated and wet sieved to pick out 1-2mm diameter fine root fragments. Using the method described in Vierheilig et al. (1998), root samples were cleared in 10% KOH for 60 min and stained in 5% ink-vinegar solution (Schaeffer Black ink) for 30 min. From each sample, ten root fragments of 1 cm length were mounted to a microscopic slide to be analyzed for AMF colonization. Ten fields of view per root fragment at 200x magnification were scored based on the presence or absence of AMF colonization (including arbuscules, intra-radical hyphae, or intra-radical spores), for a total of 100 fields of view per sample. Due to variation in root reactions to clearing, staining, and de-staining, there were some fields of view that were unable to be read, resulting in fewer than 100 fields of view for some samples. Colonization percentages were calculated by dividing the number of fields where presence of AMF was noted by the total number of fields of view for that root sample, multiplied by 100.

Twenty grams of soil were subsampled from each composited plot soil sample for ERH analysis. ERH samples were separated from soil particles by suspending in water and wet sieving to 212μm and dyed with 4% Trypan Blue solution for a minimum of 60 min. After rinsing, 20mL samples were suspended in water then vacuum mounted onto a 0.45μm mesh nylon filter paper (Staddon, et al., 1999). ERH samples were read at 100x magnification. Twenty-five fields of view from each filter paper

were viewed using the grid line intersection method (McGonigle, et al.,

1990). ERH was calculated as mm ERH g^{-1} soil by dividing the number

ERH intersections by the 25 fields of view. The quotient was then

multiplied the 0.1mm length of the grid and the area of the filter paper.

The product was then divided by the 20g volume of soil.

Soil Organic Matter Analysis

Soil Organic Matter (SOM) was determined by combustion (Ryan,

et al., 1990). After sieving soils collected in May, 15g of fresh soil were air-

dried in paper bags for ~10 days until dry at ambient room temperature

(~21°C). Once air-dried, samples were weighed again and placed in a

muffle furnace at 475°C for 4.5 hrs. Samples were allowed to cool

overnight and then were weighed to determine the mass lost from SOM

combustion in the soil sample. The percentage of ash-free dry mass

(AFDM) as an estimate of SOM was calculated by subtracting the final

mass from the mass weighed after air-drying. The difference was then

divided by the air-dried mass and multiplied by 100 to obtain a

percentage.

Statistical Analysis

To answer the questions of whether AMF abundance and

associated SOM varied by switchgrass variety or ecotype a mixed model

analysis was completed using R 3.2.2 (R, 2014). ERH, root colonization,

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and SOM were analyzed using a General Linear Mixed Model (glmer) in the lme4 package with either variety or ecotype as the fixed effect and block included as a random effect. If variety had a significant effect, a Tukey's test in the multcomp package was conducted to correct for multiple comparisons. Residuals were checked to be uniformly dispersed and a Levene's test was performed for each variable to examine homogeneity of variances. To test the relationship between ERH and SOM, a regression of SOM by ERH was conducted using Systat 12 (Systat, 2007).

Results

Mycorrhizal Associations

There were no differences among varieties or ecotypes in ERH (Table 1). ERH abundance ranged from 101.8 mm ERH g^{-1} soil to 145.9 mm ERH g^{-1} soil (Table 2). There was an overall average of 119.5 mm ERH g^{-1} soil

AMF root colonization ranged from 85.5% to 92.9% (Table 2). There were no differences among varieties in root colonization (Table 1). However, ecotypes did show significant differences in root colonization (Table 1). The average infection of upland ecotype was 89.0% and the average infection by AMF for lowland ecotype was 94.5% (Table 2). There were significant block effects for ERH, root colonization, and SOM

(Table 1, Figure 2). However, there is no apparent pattern to the block effects.

Soil Organic Matter

SOM percentage ranged from 3.66% to 4.48% (Table 2, Figure 3). There was no significant difference between the two ecotypes (Table 1). At the variety level, the random block effect was highly significant (Table 1, Figure 2). A Tukey's comparison of the varieties yielded differences between some varieties (Figure 3). The regression between ERH and SOM yielded no significant relationship (Figure 4; p = 0.491).

Discussion

In this study, I found that root colonization was high amongst all 12 varieties and both ecotypes. ERH abundance ranged above 100 mm ERH g^{-1} soil. The lowland ecotypes had higher AMF root colonization than the upland ecotypes. I found little variation in AMF root colonization between varieties of switchgrass. There was a difference in SOM at the variety level but not at the ecotype level.

I had expected differences between ecotypes for root colonization. The lowland ecotype depends on AMF to improve water acquisition in drought conditions (Latef, et al., 2016; Parrish & Fike, 2005). The common garden experiment site is considered an upland habitat. In this experiment lowland varieties may have depended on AMF more strongly

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because the upland habitat represented a novel habitat. Some exotic species have higher AMF root colonization in their non-native range; it may be possible that something similar is happening at a smaller scale of an ecotype having higher colonization outside of its adapted range (Yang, et al., 2012). This may be particularly important if future systems disrupted by climate change experience drier weather patterns such as rain events spaced farther apart (Seager & Vecchi, 2010).

The general lack of differences in AMF colonization and ERH abundance among varieties was surprising. Studies in switchgrass seedling establishment have shown that different varieties vary in their success based on the presence of AMF (Ghimire, et al., 2009). Switchgrass varieties vary widely in their aboveground traits, in some cases with more variability than what is found between different grass species (Stalheber, et al. unpublished data), thus were expected to vary in AMF abundance. Notably, the Southlow variety, which was cultivated from switchgrass populations in southern Michigan and potentially represented a locally adapted selection, did not significantly differ in AMF root colonization or ERH abundance compared with other varieties. Similar results were found in a recent meta-analysis showing that native and non-native plants do not differ significantly in AMF in most systems (Bunn, et al., 2015). As this experiment is situated in a heavily disturbed post-agricultural soil, a locally sourced genotype may not have any

discernible difference because the post-agricultural soil does not represent the native soil community anymore.

For species such as switchgrass that obligately associate with mycorrhizal symbionts, there may not be significant variation. One caveat is that my results are based on a single sampling time in midsummer. AMF are dynamic and may be different at other times of year (Hartnett, et al., 1994). For example seedling survival is related to AMF (Brejda, et al., 1998) and so early-season AMF activity may be more important than mid-season activity.

While varieties did not differ in AMF root colonization or ERH abundance, I did find differences in soil organic matter under the different varieties. There are many factors contributing to soil organic matter, such as plant litter inputs, root activity, soil microbes, etc. (Bolinder, et al., 1999), with AMF only playing a small direct role. The regression between ERH and SOM was not significant indicating the differences in SOM between varieties are not being directly driven by the AMF abundance in the soil. However, AMF can affect plant root architecture, which could represent a larger indirect contribution to differences in SOM (Miller, et al., 1995). Switchgrass shows intraspecific differences in aboveground traits including aboveground mass, height, density, transpiration, flowering, and leaf chlorophyll (Hartman, et al., 2012; Stahlheber, et al., unpublished data). It may be that varieties show as much variability in root traits belowground as they show aboveground,

and that this affects soil organic matter. As plant roots are a major input to soil organic matter, variation in root traits that are associated with a faster decomposition rate, including high specific root length and low specific root volume may explain the variation seen in SOM across varieties (Liang, et al., 2016). These root traits will be examined further in the next chapter of my thesis.

KBS GLBRC Switchgrass Variety Experiment
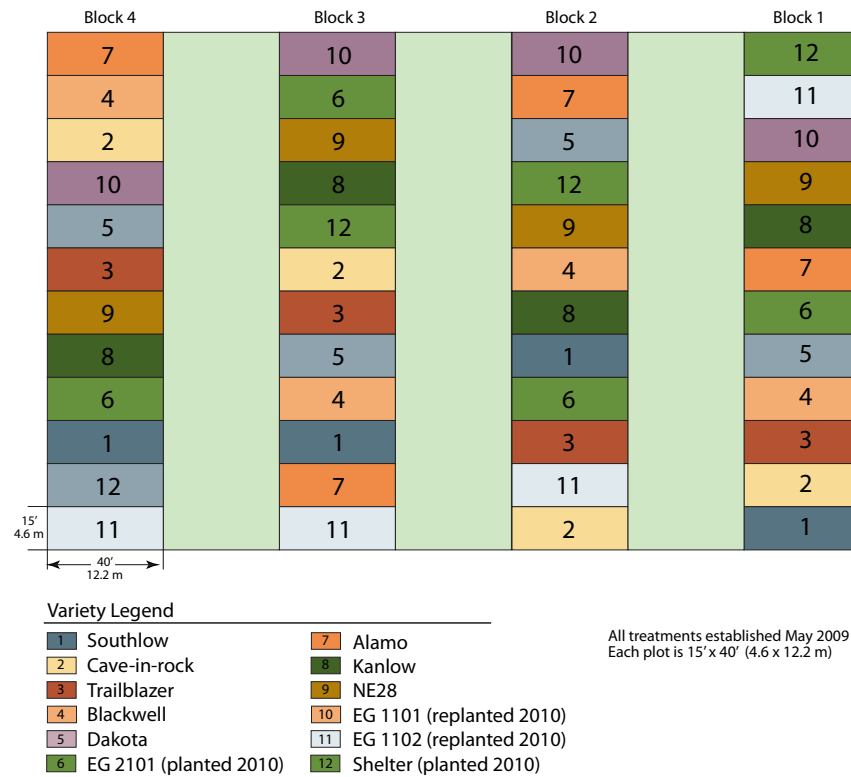
Figure 1: Kellogg Biological Station Great Lakes Bioenergy Research Center Switchgrass Variety Long-term Ecological Research Experiment Layout Map. Map copied from KBS GLBRC (2017).

Table 1: ANOVA results from Variety Trials field experiment for two different models (Variety or Ecotype and random effect of Block). Significant differences are in bold (p < 0.05).

Factor	df	Extra-radical hyphae (ERH)		AMF Root Colonization		Soil Organic Matter (SOM)	
		F	p-value	F	p-value	F	p-value
Variety	11	0.55	0.681	0.903	0.364	1.292	**0.003**
Block	11		**0.042**		**0.004**		**0.017**
Factor							
Ecotype	1	0.077	0.766	7.322	**0.007**	0.03	0.827
Block	1		**0.038**		0.076		**0.016**

Table 2: Means and standard error for ERH, Root Colonization, and Soil Organic Matter by switchgrass variety and ecotype.

	ERH (mm g^{-1})		SOM (%)		AMF Root Colonization (%)	
Variety	mean	SE	mean	SE	mean	SE
Southlow	121.675	13.062	3.948	0.203	91.750	3.705
Cave-in-rock	145.932	26.920	3.740	0.167	90.962	3.510
Trailblazer	113.197	11.440	3.952	0.311	88.453	6.531
Blackwell	104.719	12.238	4.210	0.219	88.767	2.666
Dakota	133.529	18.526	4.057	0.355	89.000	3.028
EG2101	114.218	10.879	4.033	0.203	85.500	5.188
Alamo	116.730	24.763	3.882	0.201	94.650	2.873
Kanlow	137.297	30.154	4.183	0.288	92.240	2.087
NE 28	113.040	4.252	4.484	0.227	89.558	2.460
EG 1101	118.692	19.816	4.431	0.067	98.250	1.031
EG1102	112.883	13.062	3.660	0.185	92.935	0.646
Shelter	101.815	6.944	4.083	0.239	87.913	4.844
Ecotype						
Upland	118.515	5.176	4.063	0.085	88.988	1.347
Lowland	121.400	10.497	4.039	0.118	94.519	1.032

a.

b.

c.
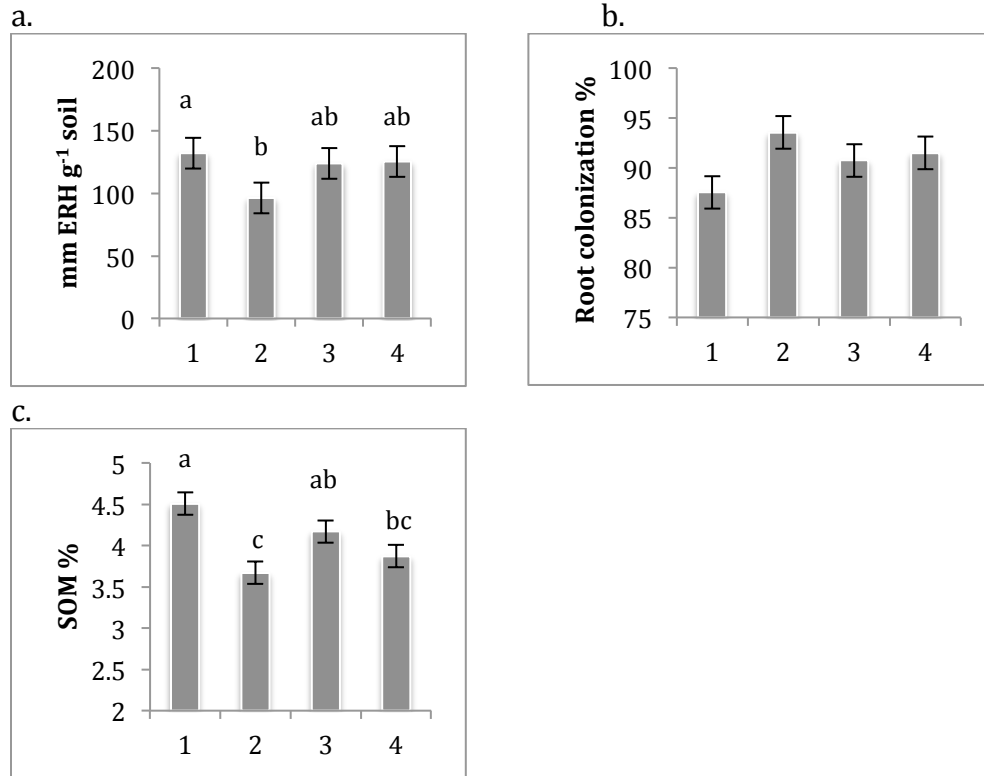
Figure 2: a.) ERH by block. b.) Root colonization by block. c.) Soil organic matter by block. Values are arithmetic means. Bars represent +/- 1 SE. Letters on graphs indicate significant Tukey-corrected pairwise comparisons ($p < 0.05$).
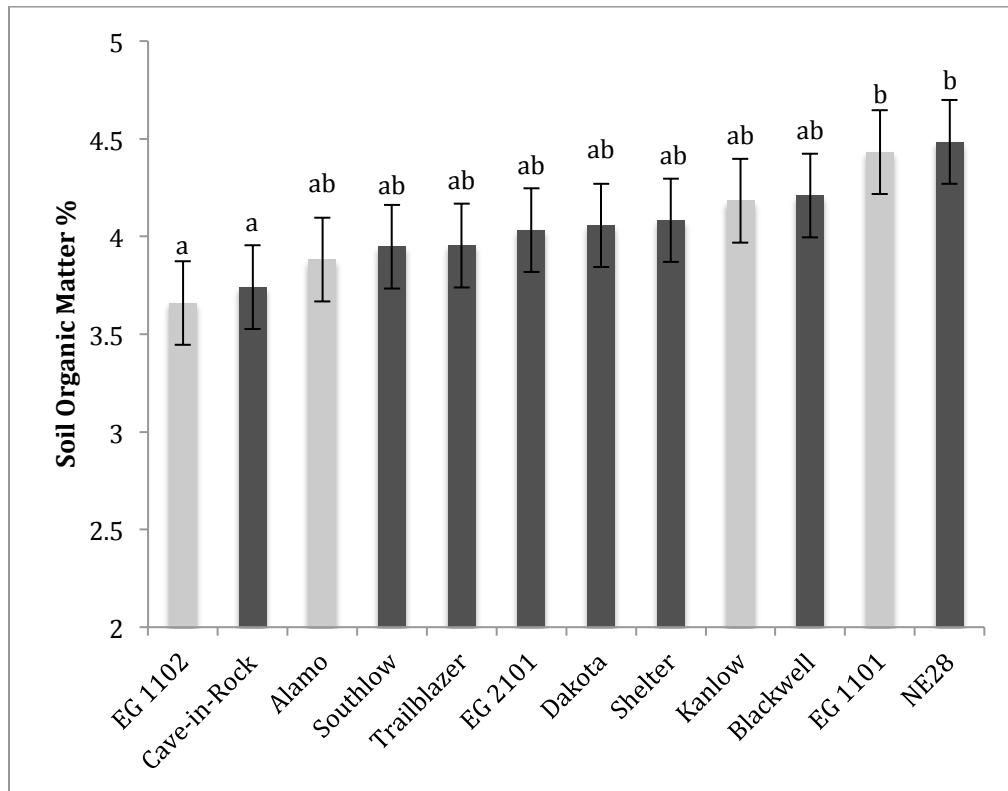
Figure 3: Soil organic matter by switchgrass variety. Values are least squares means correcting for random block effect. Dark grey represent upland varieties, Light grey represents lowland varieties. Bars represent +/- 1 SE. Letters on graphs indicate significant Tukey-corrected pairwise comparisons (p < 0.05).

Figure 4: Regression between soil organic matter and ERH. Red line represents estimate. Blue lines represent upper and lower confidence limits. Brown lines represent upper and lower predictions. r^2 = 0.010; p-value = 0.491.

CHAPTER THREE: DIFFERENCES IN SWITCHGRASS SEEDLING ROOT

MORPHOLOGY AND DEPENDENCE ON LIVE SOIL MICROBIOTA

<u>Introduction</u>

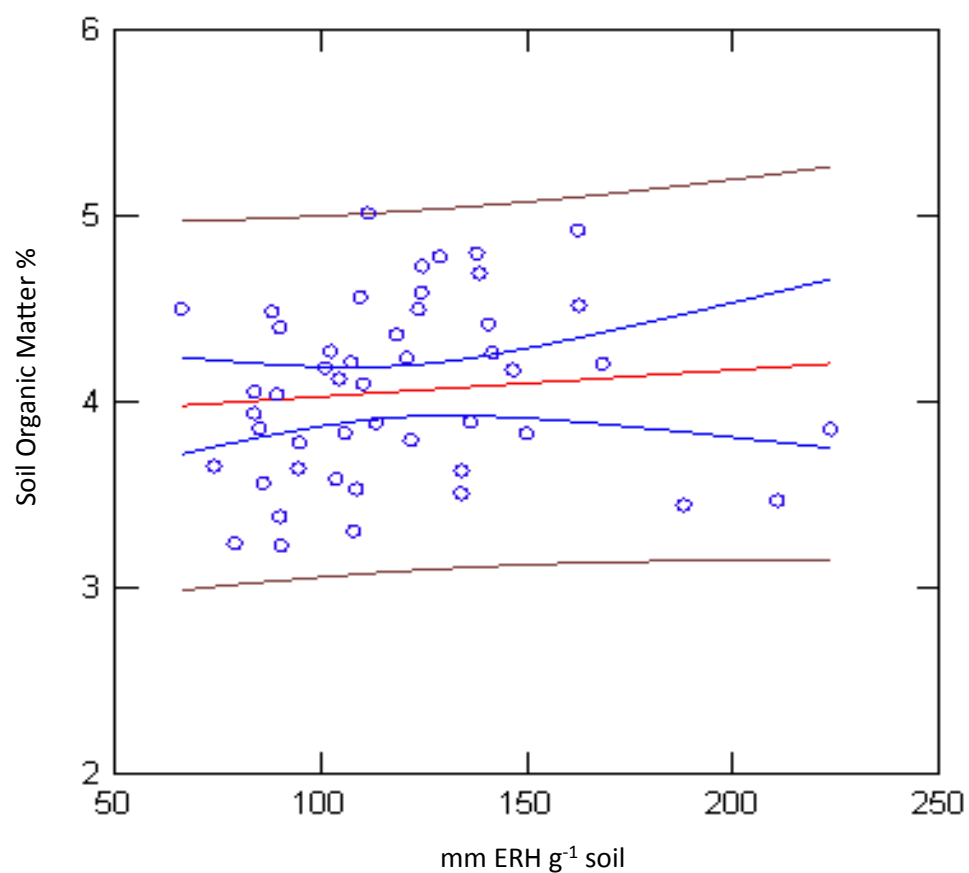Seedling establishment is a key demographic stage for plants, and can be the most important factor in predicting plant population growth (Buckley, et al., 2010). It is well known that both abiotic and biotic factors strongly influence seedling establishment, with consequences for long-term plant population success (Maestre, et al., 2003). For example, abiotic factors such as soil acidity can limit seedling establishment (Luizao, et al., 2007). Climate and edaphic factors can interact to regulate soil moisture, which for some plants is the most predictive factor for seedling survival (Maschinski, et al., 2004). Biotic factors can encourage establishment, such as micro-climate control from adult plants acting as nurse plants and belowground associations (such as AMF) enhancing resource acquisition (Ghimire, et al., 2009; Maun, 1994). However, the general roles of these biotic interactions are less well understood.

One group of soil biota that may strongly influence seedling success is arbuscular mycorrhizal fungi (AMF). While many plant species are strongly dependent on AMF as adults, there is more uncertainty over the role that AMF play in seedling success. Recently, biotic associations with AMF and other soil organisms have been shown to play an important role in the establishment of seedlings (Ghimire, et al., 1999). For example, AMF can reduce seedling mortality in big sacaton in prairie systems (Davidson, et al., 2016; Richter & Stutz, 2002). AMF can better regulate water for seedlings during vulnerable early development. Additionally, AMF can provide essential phosphorus to seedlings with limited root structures (van der Heijden, 2004). Conversely, seedlings can also be parasitized by AMF when resources are low (Johnson, et al., 1997). Different plant species vary in their reliance on AMF as adults, and this may also be the case for seedling stages (Hartnett, et al., 1994).

Seedling establishment is a key stage in agricultural systems in particular, where growers must pay for seed and maximize germination rates (Aiken, et al., 1995). In intensive annual row crop agricultural systems, AMF are known to enhance seedling establishment by providing nutrients to seedlings (Liu, et al., 2014). AMF can also help agricultural crops overcome abiotic stress such as heat that can result in seedling mortality (Zak, et al., 1998). Even when crops are less dependent on AMF as adults due to external inputs of nutrients, AMF may play key roles in crop success by altering initial root establishment.

Switchgrass (*Panicum virgatum*) is a relatively new perennial crop targeted for use in bioenergy production in marginally productive lands (Lemus & Lal, 2005). Switchgrass monocultures can be difficult to establish due to weak seedling vigor, seed dormancy, and how deep seeds are sown even though switchgrass is hardy and resilient once established (Berti & Johnson, 2013; Kimura, et al., 2015). There is variability in switchgrass establishment rates across varieties and ecotypes (Aiken & Springer, 1995), which may be due to differences in seedling root development. Additionally, establishment rates may vary in AMF dependence. While switchgrass in general is known to be strongly mycorrhizal (Brejda, et al., 1993), it is unknown how much variability there is in dependence on AMF for establishment of different switchgrass varieties and ecotypes. In this study I ask 1) whether switchgrass varieties and ecotypes differ in their seedling establishment rates and seedling root development, and 2) Does the presence of belowground microbial communities which include AMF affect these traits?

Methods and Materials

Experiment Design

A two-factor (switchgrass variety x soil inoculum) greenhouse experiment was set up in the summer of 2015. Seeds from eleven switchgrass varieties grouped into two ecotypes were used in this study. Upland ecotype varieties included Blackwell, Carthage NC, Cave-in-rock,

Shelter, and Trailblazer. Lowland ecotype varieties included Alamo, BoMaster, Colony, Kanlow, Performer, and Timber.

Each switchgrass variety was planted in one of two soil treatments: live soil inoculum "LIVE" or sterile soil inoculum "STERILE". LIVE soil inoculum was obtained from the KBS GLBRC Variety Trials experiment (see Ch. 2) in May 2015, by coarse homogenization 150 mL of soil collected from each field plot. The collected soil was divided with half used in the LIVE soil treatment while the other half was sterilized by heating in an autoclave at 121°C for 60 minutes twice for use in the STERILE soil treatment.

Twenty switchgrass seeds per each variety and soil treatment were planted in replicates of 8 in 983 mL conetainers, 6.9 cm x 35.6 cm, filled with SAKRETE© commercial play sand and 20 mL of either LIVE or STERILE soil inoculum stirred into the top two inches of sand. Each conetainer was thinned to one seedling as germination commenced. The seedlings were well watered every other day with city tap water to prevent water stress. The pots were rotated weekly to reduce the effects of environmental variability inside the greenhouse on results. The experiment ran for 62 days.

Harvest and Data Collection

Seedling mortality was recorded throughout the experiment to calculate the total survival time for each plant. Seedlings were harvested

upon death, unless they survived until the end of the experiment when they were then harvested. Harvest consisted of clipping and drying aboveground biomass at 60°C for 48 h for dry shoot mass. Root systems were carefully rinsed and scanned on an Epson Perfection V700 Photo scanner using WinRHIZO v2009c (WinRHIZO, 2010). For each image, a suite of measurements was quantified including root length, tips, forks, volume, and surface area (SA). Additionally, wet root mass was obtained and root: shoot (RS) ratio, specific root length (SRL, cm g^{-1}), and specific root volume (SRV, cm^3 g^{-1}) were calculated. These are morphology traits that are often altered by symbiotic AMF interactions or are important productivity predictors.

Statistical Analysis

Statistical analyses were performed for all but seedling survival using Systat 12 (Systat 2007). A Pearson correlation analysis was performed to examine relationships among root traits including root mass, length, tips, forks, SA, and volume. Root length, tips, forks, and SA were highly correlated (r>0.90) with root mass in these analyses and were not analyzed further. Root mass stands as proxy for the correlated morphologies of root length, tips, forks, and SA. Two-factor Gaussian ANOVAs were then conducted using root and shoot mass, RS ratio, root volume, SRL, and SRV as response variables, and soil treatment and either variety or ecotype as independent variables as well as the interaction

term between soil treatment and either ecotype or variety. As seedlings varied in survival time, seedling age was added as a covariate for all ANOVA analyses. For significant variety results in the above characteristics, a Tukey's pairwise comparison was conducted.

Seedling survival was analyzed using a binomial two factor logistic model (glm, family = binomial) with two independent factors of soil treatment and either variety or ecotype and the interaction between soil treatment and variety or ecotype. This analysis was conducted using R 3.2.2 (R, 2014).

Results

Switchgrass variety/ecotype differences

There was a trend toward higher survival of seedlings for the upland compared to lowland ecotype (75.9% vs. 58.9%), though this was not statistically significant (Table 3). Varieties likewise showed some variability in survival though this was not statistically significant (Table 3).

The Lowland ecotype had a significantly higher root volume (Table 3), although root volume did not differ among varieties. RS ratio was not significant for either variety or ecotype. SRL significantly differed among ecotypes and varieties (Table 3). The Lowland ecotype had higher SRL (23,424 cm g^{-1}, vs. 12,777 cm g^{-1}). Blackwell had the lowest SRL at 9,427c g^{-1}, while the highest SRL was Performer at 35,371 cm g^{-1} (Figure

5). SRV significantly differed amongst the varieties. Performer had the highest SRV at 445 cm^3 g^{-1} with Blackwell at the lowest 91 cm^3 g^{-1} (Figure 5). Neither ecotype nor variety showed significant differences in shoot mass or root mass (Table 3).

Soil biota effects

Seedling survival was increased significantly by the presence of LIVE soil (78.8% vs. 56.0%; Table 3, Figure 6). Seedling root and shoot biomass also increased in LIVE soil treatments (Table 3, Figure 7). Soil treatment was not significant for root volume, or SRL. There were no interactions between soil treatment and either variety or ecotype for any of the analyses (Table 3).

Discussion

Do switchgrass varieties and ecotypes differ in their seedling establishment rates and seedling root development?

This is some of the first work comparing initial stages of seedling establishment and root development across switchgrass varieties. Neither varieties nor ecotypes varied in overall seedling survival. However, there were differences detected in root architecture as measured by SRL across both ecotypes and varieties, and for root volume between ecotypes. SRL is often correlated with higher productivity in C_4 grasses (Craine, et al., 2002). Lowland varieties tend to be more

productive than upland varieties (Parrish & Fike, 2005) and results here suggest that at least at the seedling stage, lowland varieties have a higher SRL than upland. While ecotypes did not differ in root or shoot mass, it may be that this higher SRL in the seedling stage in lowland varieties allows them to achieve higher productivity as an adult.

Do belowground microbial communities affect seedling establishment and root development?

The presence of live soil biota increased survival of switchgrass seedlings and had an effect on several root architectural responses. While this greenhouse experiment treated the LIVE soil inoculum as a black box, and so specific causes of increased survival can't be pinpointed, the biggest benefactor to the seedlings was likely AMF, although soil bacteria cannot be discounted (Barea, et al., 2005). AMF have widely been shown to increase seedling survival (Davidson, et al., 2016). Greenhouse conditions were also fairly stressful, with temperatures often exceeding 38°C, which may have enhanced benefits of belowground associations. For example, AMF play an important role in water acquisition for a wide variety of species (Finlay, 2004). In field conditions, AMF increased seedling survival of *Hypericum perforatum* by mitigating abiotic stress (Moora & Zobel, 1998).

Additionally, seedling roots had altered characteristics in the presence of LIVE soil. The presence of mycorrhizae can encourage plants

to put fewer resources into roots, corresponding to decreasing SRL, root hairs, and fine root production and increased RS ratio (Hetrick, 1991). However, LIVE soil treated seedlings had an increase in root tips, root forks, and root SA. This may be explained by overall increased growth in the presence of soil biota as root mass and shoot mass were both increased under the LIVE soil treatment. It is somewhat surprising that not all traits responded to the live soil treatment (e.g. RS ratio, root volume, SRV, and SRL). It is possible that these traits are more important for the survival of juvenile plants rather than seedling establishment

There was no interaction between switchgrass variety or ecotype and soil treatment, which was surprising. The lack of significant interaction between variety/ecotype and soil treatment means that the there is no difference in how these varieties/ecotypes depend on live soil microbiota at this seedling stage. Grass species are known to vary in their dependence on AMF (Hartnett, et al., 1994) and switchgrass varieties show wide variability in aboveground traits, sometimes equal or greater to those found among different grass species growing in similar habitats (Stahlheber, et al., unpublished data) Considering the soil inoculum came from the soils collected from KBS in Michigan, an upland habitat, it is surprising that the upland ecotype did not receive more benefit. Similarly, Southlow, the native variety to southern Michigan did not appear to benefit either. As this experiment only focused on seedling development,

it is still possible that the different varieties and ecotypes rely differently on soil biota in later stages.

It is clear that seedling vigor was improved by the presence of LIVE soil inoculum. For management purposes, encouraging AMF in early seedling stages may improve seedling vigor and seedling survival in new switchgrass fields. While there were some differences between varieties and ecotypes in root morphology, it is unclear whether this would be of import to a newly established switchgrass in an agroecosystem.

Table 3: ANOVA results for the two different models (Variety or Ecotype x Soil Treatment (Soil)), for response variables including Seedling Survival, Shoot Mass, Root Mass, Root Volume, Specific Root Length (SRL), and Specific Root Volume (SRV). Significant effects are in bold ($p<0.05$).

	Survival			Shoot Mass			Root Mass		
Factor	**df**	**F**	**p**	**df**	**F**	**p**	**df**	**F**	**p**
Variety	10		0.723	10	0.439	0.925	10	0.459	0.914
Soil	1		**0.001**	1	9.311	**0.003**	1	10.015	**0.002**
Variety x Soil	10		0.995	10	0.819	0.611	10	0.748	0.679
Factor									
Ecotype	1		0.116	1	0.038	0.845	1	0.048	0.826
Soil	1		**0.001**	1	9.592	**0.002**	1	10.403	**0.002**
Ecotype x Soil	1		0.376	1	0.012	0.913	1	0.303	0.583

	RS Ratio			Root Volume			SRV		
Factor	**df**	**F**	**p**	**df**	**F**	**p**	**df**	**F**	**p**
Variety	10	0.840	0.591	10	1.503	0.144	10	3.379	**0.001**
Soil	1	1.286	0.259	1	0.214	0.644	1	1.456	0.230
Variety x Soil	10	0.783	0.645	10	1.089	0.375	10	1.075	0.385
Factor									
Ecotype	1	0.600	0.440	1	11.539	**0.001**	1	17.451	**0.000**
Soil	1	1.096	0.297	1	0.372	0.543	1	0.977	0.324
Ecotype x Soil	1	0.248	0.619	1	0.094	0.759	1	1.06	0.305

	SRL		
Factor	**df**	**F**	**p**
Variety	10	3.525	**0.000**
Soil	1	3.347	0.069
Variety x Soil	10	0.744	0.682
Factor			
Ecotype	1	15.051	**0.000**
Soil	1	2.221	0.138
Ecotype x Soil	1	1.183	0.278

a.
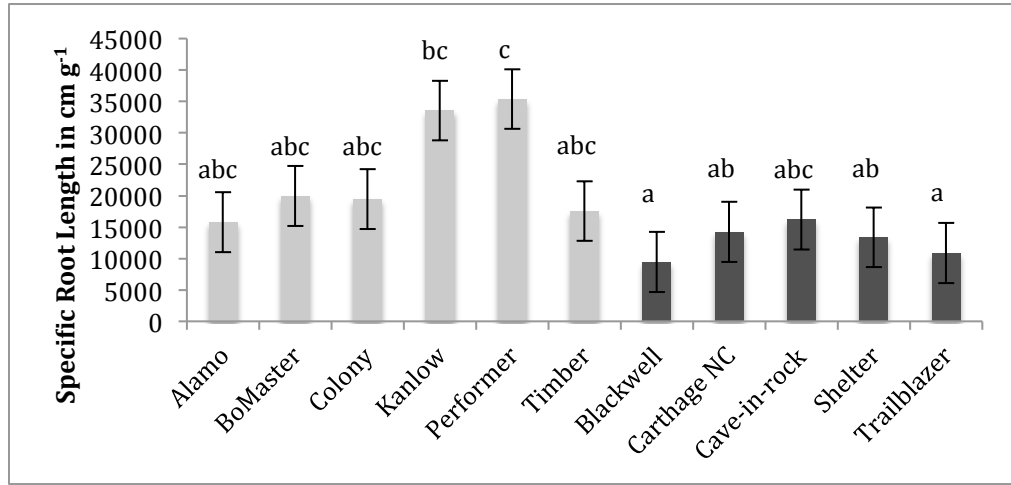
b.
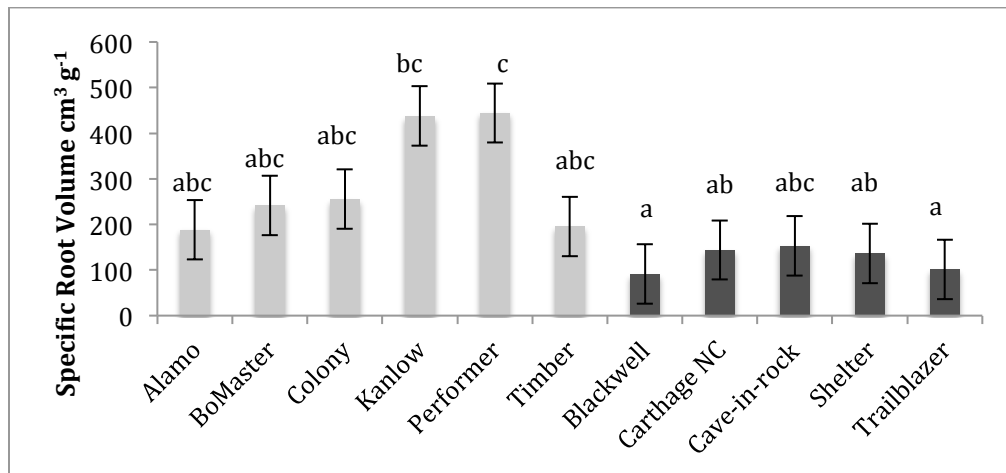
Figure 5: a.) Specific Root Length by switchgrass variety. b.) Specific Root Volume by switchgrass variety. Values are least square means to correct for age. Light grey bars represent lowland varieties and dark grey represents upland varieties. Bars represent +/- 1 SE. Letters on graphs indicate significant Tukey-corrected pairwise comparisons ($p < 0.05$).

Figure 6: Seedling Survival by Soil Treatment. Values are Least Square

Means correcting for age. Bars represent +/- 1 SE.

a.

b.

Figure 7: a.) Shoot mass by soil treatment. b.) Root mass by soil treatment. Values are arithmetic means. Bars represent +/- 1 SE.
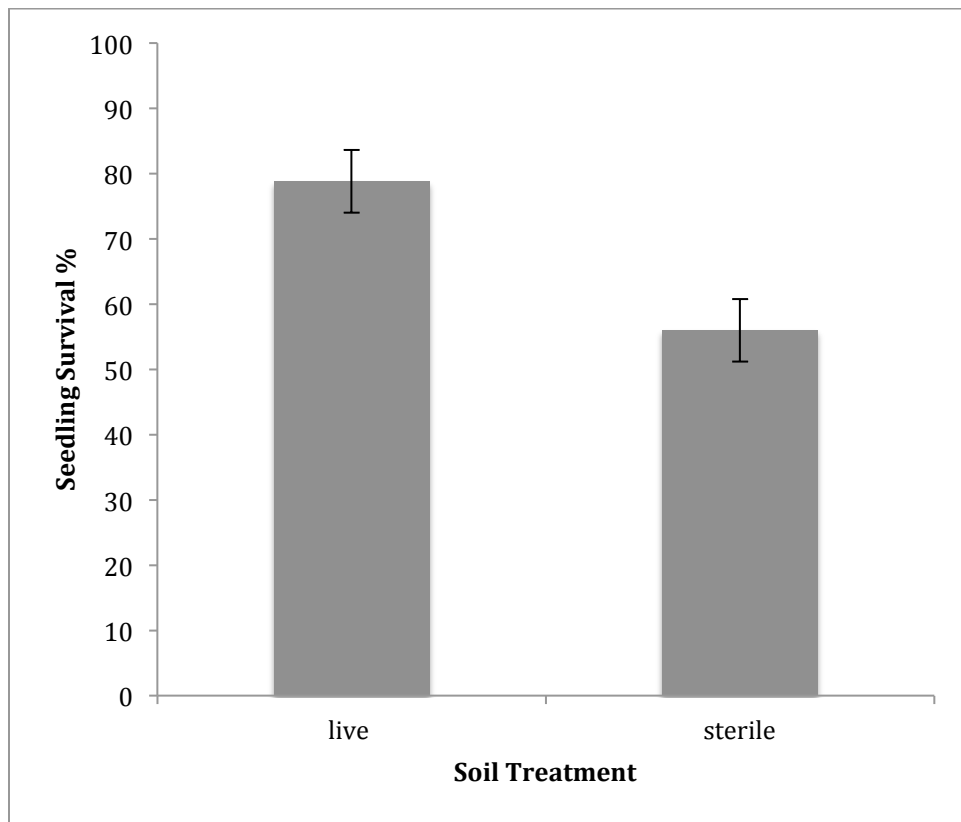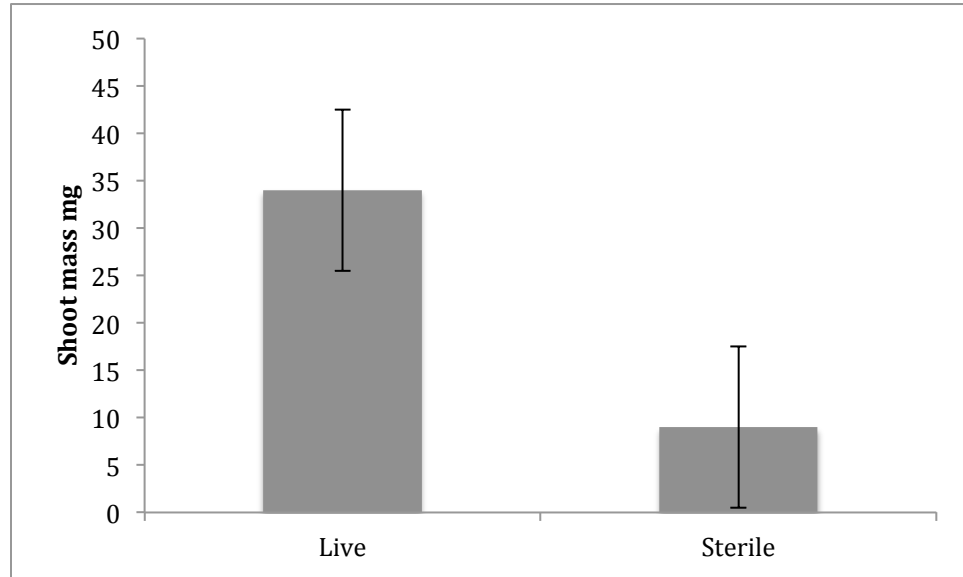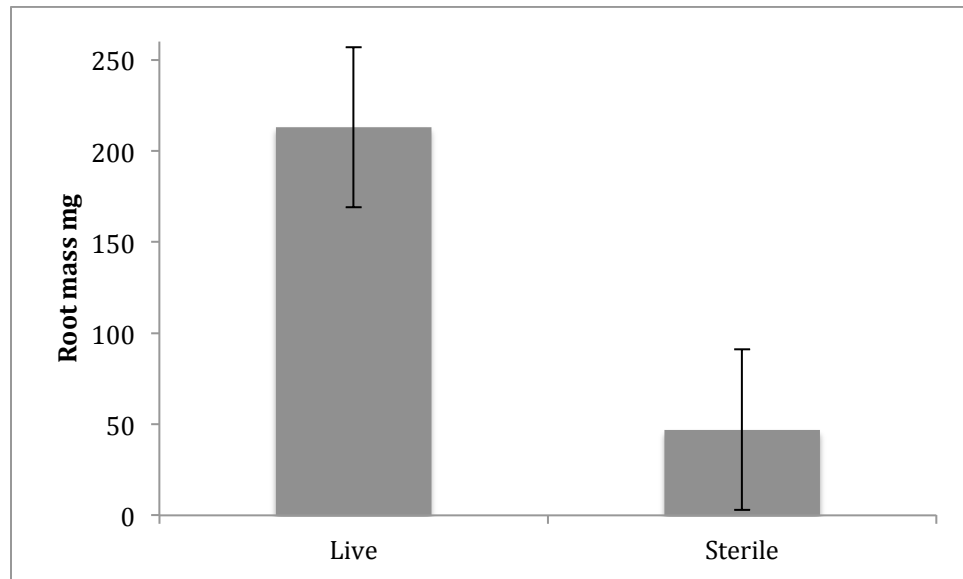
CHAPTER FOUR: SUMMARY AND FUTURE DIRECTIONS

Summary

The field experiment provided some evidence that the two switchgrass ecotypes have differences in root colonization by AMF, while the greenhouse study did not find that the ecotypes relied differently on live soil biota at the seedling stage. AMF root colonization and abundance was high amongst all varieties and ecotypes, which is in keeping with evidence that Switchgrass is obligately associated with AMF. SOM was not different at the ecotype level but did have some differences amongst varieties. However, the differences in SOM do not seem to be driven by the AMF activity in the soil.

The greenhouse study found that LIVE soil, likely driven by the presence of AMF, could be important in the seedling establishment stage of a new agroecosystem. Finally, the greenhouse study reveals differences between ecotypes in root morphology of root volume, SRL, and SRV at the seedling stage, which may be part of the reason that lowland ecotypes are more productive than upland ecotypes in adult stages. High SRL is sometimes associated with high SOM as a main root decomposition is a

major contributor to SOM. While the switchgrass varieties used in the field experiment do not entirely overlap with the varieties in the greenhouse experiment, there were some varieties in common. However, there doesn't seem to be any relationship between higher SRL in seedlings and a higher SOM in the field.

For management purposes these studies found that AMF is important for switchgrass both at the seedling stage and at the adult stage. It may be that the yearly nitrogen fertilization is supplanting switchgrass' dependence on AMF to some degree. There may be significant differences in switchgrass dependence on AMF and associated ERH abundance in an unfertilized system. While this study did find some differences at both the variety level and ecotype level, it seems that the intraspecific differences belowground in similar agroecosystems are not large enough to warrant different management approaches.

Future Directions

AMF Activity Throughout the Growing Season

In the field study in Ch. 2, all AMF activity was measured midseason in July. Upland varieties are more cold hardy and this leads to upland sometimes emerging earlier than lowland in early springtime. This may stimulate a higher AMF abundance among upland varieties in the early spring due to plant activity. Lowland varieties often have a longer growing season, which may lead to a later senescence in the fall.

This may encourage more AMF abundance in lowland varieties later in the season. For future work, I would recommend evaluating AMF abundance in spring and fall, as well as summer, to get a more complete picture of how switchgrass varieties vary in their associations with AMF.

Microbial Wash in Greenhouse Experiment

In the greenhouse experiment in Ch. 3, there were only two soil treatments: LIVE and STERILE. The limitation of a study like that is that the LIVE soil is treated like a black box. Adding a microbial wash along with a LIVE soil treatment and a sterile soil treatment will be able to isolate the separate roles of soil bacteria and AMF for switchgrass seedlings.

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