Soil mycorrhizal and nematode diversity vary in response to bioenergy crop identity and fertilization

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Abstract

The mandate by the Energy Independence and Security Act of 2007 to increase renewable fuel production in the USA has resulted in extensive research into the sustainability of perennial bioenergy crops such as switchgrass (*Panicum virgatum*) and miscanthus (*Miscanthus× giganteus*). Perennial grassland crops have been shown to support greater aboveground biodiversity and ecosystem function than annual crops. However, management considerations, such as what crop to plant or whether to use fertilizer, may alter belowground diversity and ecosystem functioning associated with these grasslands as well. In this study, we compared crop type (switchgrass or miscanthus) and nitrogen fertilization effects on arbuscular mycorrhizal fungal (AMF) and soil nematode abundance, activity, and diversity in a long-term experiment. We quantified AMF root colonization, AMF extra-radical hyphal length, soil glomalin concentrations, AMF richness and diversity, plant-parasitic nematode abundance, and nematode family richness and diversity in each treatment. Mycorrhizal activity and diversity were higher with switchgrass than with miscanthus, leading to higher potential soil carbon contributions via increased hyphal growth and glomalin production. Plant-parasitic nematode (PPN) abundance was 2.3 × higher in miscanthus plots compared to switchgrass, mostly due to increases in dagger nematodes (*Xiphinema*). The higher PPN abundance in miscanthus may be a consequence of lower AMF in this species, as AMF can provide protection against PPN through a variety of mechanisms. Nitrogen fertilization had minor negative effects on AMF and nematode diversity associated with these crops. Overall, we found that crop type and fertilizer application associated with perennial bioenergy cropping systems can have detectable effects on the diversity and composition of soil communities, which may have important consequences for the ecosystem services provided by these systems.

Keywords: carbon, cave-in-rock, Great Lakes Bioenergy Research Center, MiSeq sequencing, Bradford reactive soil proteins, NINJA, nitrogen

Introduction

The mandate by the Energy Independence and Security Act of 2007 to increase renewable fuel production in the USA has resulted in extensive research into the economic viability and sustainability of bioenergy crops. While current bioenergy production relies heavily on corn grain and residues (Wallander *et al.*, 2011) there is growing interest in developing lignocellulosic biofuels produced from dedicated perennial energy crops because of their potential to enhance sustainability of bioenergy systems. Warm season grasses including switchgrass (*Panicum virgatum*) and miscanthus

(*Miscanthus× giganteus*) are leading candidates for bioenergy feedstock due to their perenniality and capacity for multiple harvests after planting (10+ years of production), and efficient nutrient and water use (Parrish & Fike, 2005; Hamilton *et al.*, 2015). While lower in biomass production compared to corn, these perennial energy crops have been shown to provide a number of ecosystem services that make them more desirable in terms of energy return. For example, cultivation of perennial feedstocks produces half of the greenhouse gas emissions compared to annual grain feedstock (Robertson *et al.*, 2011). These grasses can be grown on marginal lands which are not amenable to grain production (Gelfand *et al.*, 2013). Perennial grassland crops have also been shown to have greater soil carbon storage and methane consumption (Werling *et al.*, 2014), and they are associated with higher aboveground biodiversity than annual cropping systems (Robertson *et al.*,

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2012; Landis *et al.*, in press). However management practices to enhance productivity of perennial cropping systems, such as fertilizer or pesticide applications, may eliminate or reduce many of the benefits provided by these perennial grasslands.

Inorganic fertilizer is often recommended to increase yields of biofuel crops on marginal lands (Tulbure *et al.*, 2012). While nitrogen (N) fertilization has been shown to increase yields of switchgrass (Sanderson & Reed, 2000; Heaton *et al.*, 2004) and miscanthus (Arundale *et al.*, 2014b; Stepien *et al.*, 2014), the use of synthetic N fertilizers for bioenergy crop production comes with economic costs to growers ($37 ha^{-1} estimated by Hallam *et al.*, 2001), and does not always deliver higher yields (e.g., Dierking *et al.*, 2016; Duran *et al.*, 2016). Use of synthetic fertilizers also can compromise the climate benefits of planting perennial cellulosic biofeedstocks by increasing nitrate leaching and nitrous oxide emissions (Ruan *et al.*, 2016). Additionally, research from annual row-cropping systems has shown that synthetic fertilizers can have negative effects on soil biodiversity, which can reduce ecosystem functioning and services (Thiele-Bruhn *et al.*, 2012).

Arbuscular mycorrhizal fungi (AMF) are soil organisms that provide important ecosystem functions in agricultural systems (Douds & Millner, 1999). AMF are root symbionts and are known to form beneficial associations with plants, particularly C4 grasses (Wilson & Hartnett, 1998; Gosling *et al.*, 2006). They have been shown to have direct benefits for many agricultural food crops through mechanisms such as nutrient acquisition and drought tolerance (Douds & Millner, 1999; Kivlin *et al.*, 2013). AMF also can provide benefits to crop plants through indirect mechanisms such as increasing resistance to pests and pathogens, including plant-parasitic nematodes, via increased plant defenses (Schouteden *et al.*, 2015). In addition, AMF contribute to soil carbon storage through the decomposition and turnover of extra-radical hyphae (ERH) which increases concentrations of glomalin, a recalcitrant glycoprotein found in fungal cell walls (Wright & Upadhyaya, 1996). It is estimated that glomalin contributes 4%–8% of soil organic carbon in natural ecosystems (Treseder & Turner, 2007) and 2–4% of soil organic carbon in agricultural systems (Borie *et al.*, 2006).

AMF diversity, abundance, and activity vary greatly among sites, and much of this variation can be attributed to differences in soil fertility and management (Johnson *et al.*, 1997; Hoeksema *et al.*, 2010). For example, AMF diversity decreased in California grasslands across an N-deposition gradient (Egerton-Warburton & Allen, 2000). Similarly, a study in grain producing cropland in England found AMF diversity decreased with conventional fertilizer application (Van Der Gast *et al.*,

2011). High soil nitrogen (N) conditions can also result in negative plant-AMF interactions (Hoeksema *et al.*, 2010; Kivlin *et al.*, 2013). AMF associations also vary among plant hosts (e.g., Eom *et al.*, 2000). A comparison of bioenergy systems in Wisconsin and Michigan USA found that switchgrass had over twice the amount of AMF biomass compared to corn (Jesus *et al.*, 2016), which may be do to the combination of species and management differences. However, very little is known about how AMF associations vary in different perennial biofuel crops or how they will respond to fertilization of these systems.

Soil nematodes are also an important component of belowground biodiversity. While plant-parasitic nematodes (PPN) are the most studied functional group of nematodes due to their ability to inflict crop damage, nematodes occupy multiple trophic levels in soil food webs (bacterivores, fungivores, plant parasites, and predators), and their abundance and diversity can reflect overall soil ecosystem health (Yeates, 2003; Liu *et al.*, 2016). A recent meta-analysis found that inorganic N addition generally reduces soil nematode abundance and diversity in row-crop agricultural systems (Liu *et al.*, 2016), though it is unknown whether this holds true for perennial biofuel systems.

Both switchgrass and miscanthus are C4 grasses and are known to form associations with AMF (Eom *et al.*, 2000; Stewart *et al.*, 2009). Additionally, parasitic nematodes are reported to be potential pests of both switchgrass and miscanthus (Mekete *et al.*, 2011). Although several studies have evaluated belowground organisms associated with one or the other of these grasses in comparison to annual crops (e.g, corn, wheat; see Jesus *et al.*, 2016; McCormack *et al.*, 2013), to our knowledge, no study has directly compared AMF and nematode activity and diversity in switchgrass and miscanthus grown in an agricultural setting; though see Oates *et al.* (2016) for a comparison of AMF biomass across crops. As these two crops are leading contenders as perennial bioenergy feedstocks, it is important to know if they differ in belowground diversity and ecosystem functioning in order to make informed management decisions regarding sustainable production practices.

In this study, we compare mycorrhizal and nematode diversity and activity in replicate fields of switchgrass and miscanthus that are managed for bioenergy production as part of a large-scale, long-term experiment associated with the Great Lakes Bioenergy Research Center (GLBRC). The experiment includes plots that have been either fertilized annually or left unfertilized since planting in 2009 allowing us to compare the belowground communities of these two important prospective bioenergy crops under different levels of management.

Specifically, we ask: (1) Do AMF activity and diversity differ between switchgrass and miscanthus? (2) Do belowground nematode abundance and diversity differ between switchgrass and miscanthus? And (3) Does fertilization alter these belowground relationships?

Materials and methods

Site description and soil sampling

This research was conducted at the Great Lakes Bioenergy Research Center's (GLBRC) Biofuel Cropping Systems Experiment (BCSE) located at the W.K. Kellogg Biological Station of Michigan State University in southwest Michigan USA (KBS, 42°23′47″N, 85°22′26″W). Initiated in 2008, this experiment includes seven candidate bioenergy cropping systems planted in 28 × 40 m plots in a randomized complete block design with five replicates per system. Monoculture plantings of miscanthus (*Miscanthus× giganteus*, 'Illinois clone') and switchgrass (*Panicum virgatum* var. Cave-in-rock) are two of the systems in this experiment. Additional experiment details (planting densities, weed control, harvest practices, etc.) for these crops are reported in Sanford *et al.* (2016). Miscanthus and switchgrass plots were fertilized with urea yearly in the spring at a rate of 56 kg N ha^{-1}. Each plot also included a 2 × 40 m subplot that was never fertilized, allowing us to compare AMF responses to fertilization for both crops. Crop biomass yield data from this experiment are available in Sanford *et al.* (2016) and are not the focus of this study. In May 2014 and May, July, and October 2015, we collected soil cores (2 × 15 cm; 10 per plot) from near the bases of haphazardly selected plants in each replicate plot and unfertilized subplot. Cores for each plot were pooled, sieved through a 4-mm sieve to remove rocks and large roots, and then stored at 4 °C until processing.

AMF activity

To determine the extent of AMF colonization in plant roots, we extracted fine roots from 100 ml subsamples taken from the pooled soil cores per plot in May 2014, July and October 2015 using a wet-sieve process (500-μm sieve; Milchunas, 2012). Roots were cleared with 10% KOH and stained using a 5% vinegar-ink solution using methods modified after Vierheilig *et al.* (1998). Visual estimation of percent root length colonization was made using 100 fields of view per sample under 200× magnification.

To estimate AMF activity, extra-radical hyphae (ERH) were extracted from 20 ml subsamples taken from the pooled cores collected in May 2014, July and October 2015 for each plot. Soil subsamples were suspended in water, then stained and vacuum filtered through a 45-μm filter, following methods described in Staddon *et al.* (1999). Hyphal length was estimated using the gridline-intercept method (McGonigle *et al.*, 1990) at 100× magnification.

Total soil glomalin was determined from 1 g of air-dried soil subsampled from pooled cores collected in May 2014 and May 2015 from each plot, as this recalcitrant protein was not expected to vary substantially throughout the growing season, and we were interested in long-term, not seasonal, changes. We used the 0.8 mM sodium citrate buffer and autoclaving method described in Janos *et al.* (2008) to extract protein and quantified the Bradford reactive fraction (Bio Rad, Hercules, CA, USA) using bovine serum as a standard (Koide & Peoples, 2013). Total soil glomalin has several extractable fractions (Cornejo *et al.*, 2008), and Bradford reactive soil protein (BRSP) has been shown to consistently represent the largest fraction of total soil glomalin (approximately 90% by volume; Koide & Peoples, 2013). Therefore we used BRSP to operationally define glomalin for this work.

AMF community composition and diversity

To estimate the diversity and composition of the AMF community, we extracted DNA from 0.25 g of fresh soil subsampled from the pooled soil core samples collected in July 2015. We used Powersoil DNA Extraction kits to isolate DNA (MOBIO Laboratories, Carlsbad, CA, USA) following the manufacturer's instructions. The 28s region of rRNA was targeted using AMF-specific fusion primers. PCR and MiSeq Illumina paired-end sequencing (San Diego, CA, USA) was conducted by the Research Technology Support Facility Genomics Care at Michigan State University, East Lansing, Michigan. Briefly, forward primer, FLR3 (5′-TTG AAA GGG AAA CGA TTG AAG T-3′). and reverse primer, FLR4 (5′-TAC GTC AAC ATC CTT AAC GAA-3′) (Gollotte *et al.*, 2004) with a Fluidigm CS1 and CS2 fused to their 5′ ends were used for primary PCR. Secondary PCR to add dual indexed, Illumina compatible adapter sequences was performed with primers targeting the Fluidigm CS1 and CS2 oligos at the ends of the primary PCR products. Final PCR reaction products were normalized using Invitrogen (Carlsbad, CA, USA) SequalPrep DNA Normalization plates, normalized outputs were pooled, and the pooled products were cleaned up with AmpureXP magnetic beads. The pool was quality controlled and quantified using a combination of Qubit dsDNA HS, Caliper LabChipGX HS DNA, and Kapa Illumina Library Quantification qPCR assays. Sequencing was performed using an Illumina MISEQ v2 standard flow cell and 500 cycle reagent cartridge, 2 × 250-bp paired-end format. The primary PCR fusion primers CS1-FLR3/CS2-FLR4 were added to the appropriate wells of the reagent cartridge to serve as primers for read 1 and read 2, respectively. Base calling was performed by Illumina Real Time Analysis (RTA) v1.18.64, and output of RTA was demultiplexed and converted to FastQ format with Illumina BCL2FASTQ v1.8.4.

Reads were assembled using fastq_mergepairs and quality filtered using fastq_filter removing sequences with E scores > 1 and sequences >300 bp in USEARCH8 (http://drive5.com/usearch/). Sequences were dereplicated using fastx_uniques, and then clustered chimera were checked, filtered de novo, and clustered into unique operational taxonomic units (OTUs, i.e., DNA sequences or amplicon types) based on 97% identity using the default settings in UPARSE implemented in UEARCH9 (Edgar, 2013, 2016). USEARCH quality filtering, chimera checking using UCHIME, and OTU clustering lead to 260 OTUs and 1 902 178 reads. Representative sequences were then

classified using the RDP naïve Bayesian classifier against the Fungal LSU training set 11 (Wang et al., 2007; Cole et al., 2014). Any sequences with bootstrap values below 60% of falling within Glomeromycota were removed from the dataset because we expected that these were from non-AM fungi. Taxonomic filtering for AMF-specific sequences resulted in 138 OTUs and 1 562 891 reads. We transformed OTU tables using variance stabilizing transformation (VST) in the DESEQ2 package (Love et al., 2014) in R (R Core Team, 2016) to control for biases in PCR amplification and to avoid biases due to rarefaction (McMurdie & Holmes, 2014). We used the VEGAN package in R to calculate Shannon Diversity (H′) and Chao1 richness based on OTUs.

Nematode abundance and diversity

Nematodes were extracted from fresh 100-ml subsamples of soil from the pooled soil cores from each plot collected in July and October 2015 using the centrifugal flotation method (Jenkins, 1964) and used this to estimate the abundance of plant-parasitic nematodes (PPN) and other nematode functional groups. Nematodes were relaxed and fixed in hot 3% formalin for long-term storage and identification (Nickle, 1991). Nematodes in each sample were counted under a dissecting microscope ($50\times$) based on 20 fields of view, and 50 individuals from each sample were identified to family under $100–1000\times$ magnification and classified into feeding group based on Yeates et al. (1993). Family richness and family Shannon diversity (H′) were calculated for each plot based on these data.

Data analyses

We compared AMF activity and diversity and nematode abundance and diversity across cropping systems and fertilization treatments using split-plot, 2-factor general linear models. Crop identity was the between-plots fixed factor and fertilizer level was the within-plots fixed factor. The specific response variables were: AMF % root colonization, AMF-ERH (mm g^{-1} soil), soil glomalin (BRSP mg g^{-1} soil), AMF richness (Chao1 index) and AMF diversity (Shannon Index; H′) based on OTUs, plant-parasitic nematode abundance (individuals ml^{-1} soil), and nematode family diversity (H′). A repeated-measures model was used for measures of AMF activity only, as these were the only responses that were consistently measured across seasons. Glomalin data were square-root transformed, nematode

abundance and ERH data were ln transformed, and AMF root colonization data were arcsine-square-root transformed to better meet model assumptions for analyses. These analyses were performed using SYSTAT v.12 (SYSTAT Software Inc, 2007).

We used a two-factor blocked PERMANOVA (Anderson, 2001), to examine overall differences in AMF and nematode community composition due to crop identity and fertilization treatments. To visualize differences in AMF and nematode community structure due to crop identity and fertilization, we performed non-metric multidimensional scaling (NMS) ordinations (McCune et al., 2002) with Bray–Curtis dissimilarity measures based on square-root-transformed AMF OTU and nematode abundance data. We used a blocked indicator species analysis (ISA) (McCune et al., 2002) to evaluate which individual AMF OTUs and nematode groups were associated with the different crop and fertilization treatments, with Monte Carlo randomizations to test for indicator value significance. PERMANOVA and NMS analyses were performed using PRIMER v. 6 (Anderson et al., 2008), and ISAs were performed using PC-ORD v.6.08 (McCune & Mefford, 1999). Nematode feeding group composition, community maturity, and food web condition were analyzed using the NINJA program (Ferris & Bongers, 2009; Sieriebriennikov et al., 2014).

Results

AMF activity

Fertilizer and crop identity did not have consistent effects on AMF root colonization, with significant crop × fertilizer interactions in all sampling dates. Switchgrass had on average 24% greater root colonization by mycorrhizae compared to unfertilized miscanthus in May, while unfertilized switchgrass had double the root colonization of miscanthus in October (Table 1, Fig. 1a). In July samples, fertilizer increased AMF root colonization in switchgrass, but decreased AMF root colonization in miscanthus (Table 1, Fig. 1a). Averaged together, switchgrass tended to have ~25% more AMF root colonization than miscanthus with no consistent effects of fertilizer, though trends varied across sampling dates. Switchgrass soil samples also had 84% greater ERH activity compared to miscanthus (Table 1, Fig. 1b). Fertilizer did not have significant direct or

Table 1 Results of split-plot repeated measures (df 3, 4; AMF root colonization, ERH length, glomalin) and general linear models (df 1, 8; OTU richness, OTU diversity) examining the effects of crop species (switchgrass or miscanthus) and fertilizer (added or not) on measures of AMF activity and diversity. Significant effects are in bold

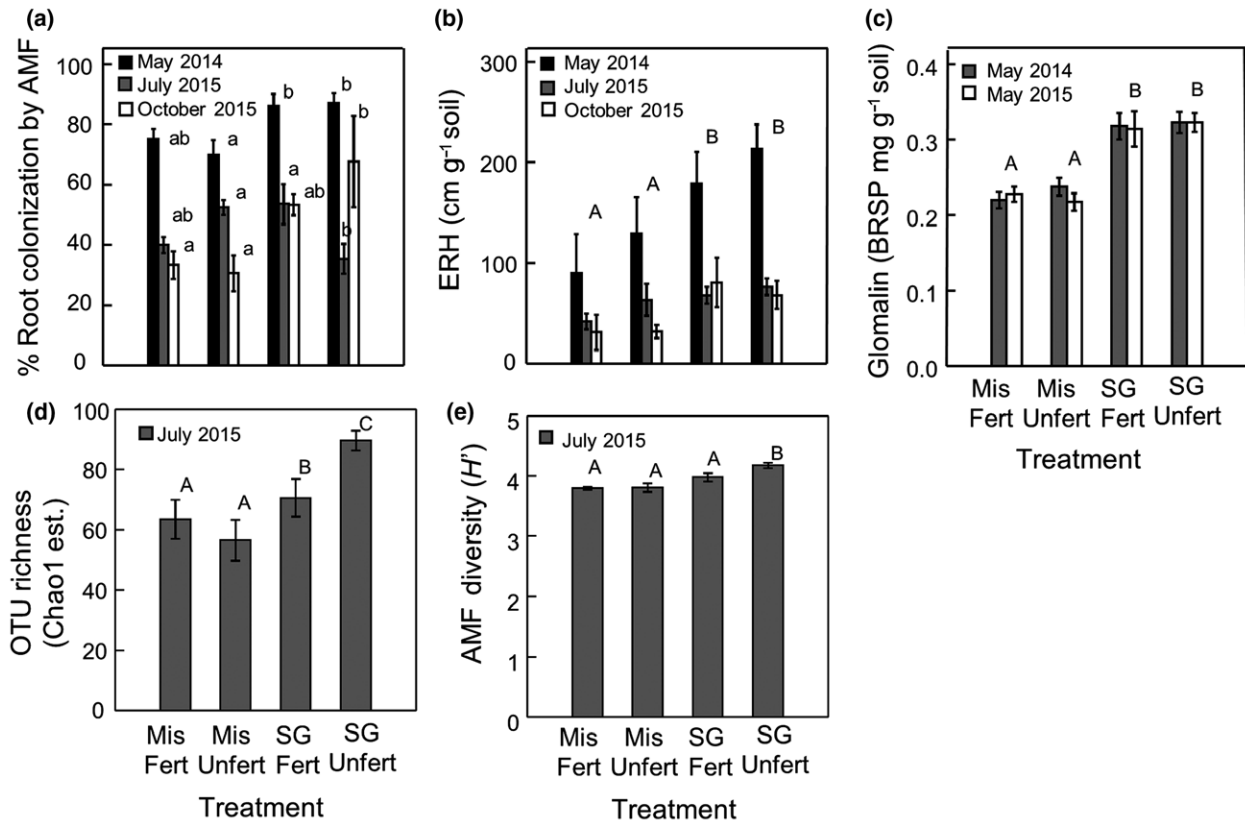| Factor	AMF root colonization		AMF-ERH length		Soil glomalin		AMF OTU Chao1 richness		AMF OTU diversity (H′)	
	F	P	F	P	F	P	F	P	F	P
Crop species	**8.30**	**0.034**	**6.68**	**0.049**	**15.81**	**0.003**	**9.28**	**0.016**	**19.19**	**0.002**
Fertilizer	0.15	0.927	4.97	0.079	2.15	0.187	3.94	0.082	**12.08**	**0.008**
Crop x fert	**10.64**	**0.022**	1.67	0.310	1.27	0.339	**18.05**	**0.003**	**9.88**	**0.014**
Sampling date	**39.1**	**<0.001**	**21.6**	**<0.001**	0.85	0.373	n/a	n/a	n/a	n/a

Fig. 1 Differences in AMF activity and diversity among Miscanthus (Mis) and Switchgrass (SG) fertilized (Fert) and unfertilized (Unfert) treatments: (a) % AMF root colonization, (b) Extra-radical hyphal length, (c) Glomalin (BRSP), (d) AMF OTU richness (Chao1) and (e) AMF OTU diversity (H′). Bars are means ± 1 SE. Letters on graphs indicate significant Tukey-corrected pairwise comparisons ($P < 0.05$), with lowercase indicating differences *within* sampling dates, and uppercase for consistent effects across all sampling dates.

interactive effects on ERH in either species. Glomalin, measured as BRSP, was 42% greater on average in switchgrass plots compared to miscanthus plots (Table 1, Fig. 1c); however, again fertilizer had no significant direct or interactive effects on soil glomalin.

AMF diversity

The estimated AMF OTU richness in soil samples differed among crops and fertilizer treatments with unfertilized switchgrass having 27-58% more OTUs than the other treatments (Table 1; Fig 1d). There was also a significant, but small, difference in AMF OTU diversity (H′) among crops and fertilizer treatments, with unfertilized switchgrass being 5–10% more diverse than other treatments (Table 1, Fig. 1e).

The composition of the AMF community varied with crop and fertilizer treatment, although the fertilizer effect was relatively minor (Fig. 2). The indicator species analysis revealed that switchgrass was associated with 19 OTUs; 17 of these matched *Rhizophagus*

sequences and 2 matched *Septoglomus*. In contrast, miscanthus was associated with 5 OTUs corresponding to *Septoglomus* and only 1 OTU matching *Rhizophagus* (Table S1). The fertilizer effects on community composition were reflected by 2 *Septoglomus* OTUs and 3 unknown Glomeraceae OTUs that were associated with unfertilized plots, while only 1 unknown Glomeraceae OTU was significantly associated with fertilized plots (Table S1).

Nematode abundance and diversity

Soils collected from near miscanthus roots had, on average, 2.3 times more plant-parasitic nematodes (PPN) compared with switchgrass (Table 2, Fig. 3). However, fertilizer had no significant direct or interactive effects on abundance of PPN. There were no significant crop effects on nematode family richness or Shannon Diversity (H′) (Table 2). However, nematode diversity was 16% higher in unfertilized compared to fertilized plots in July samples. The nematode community composition
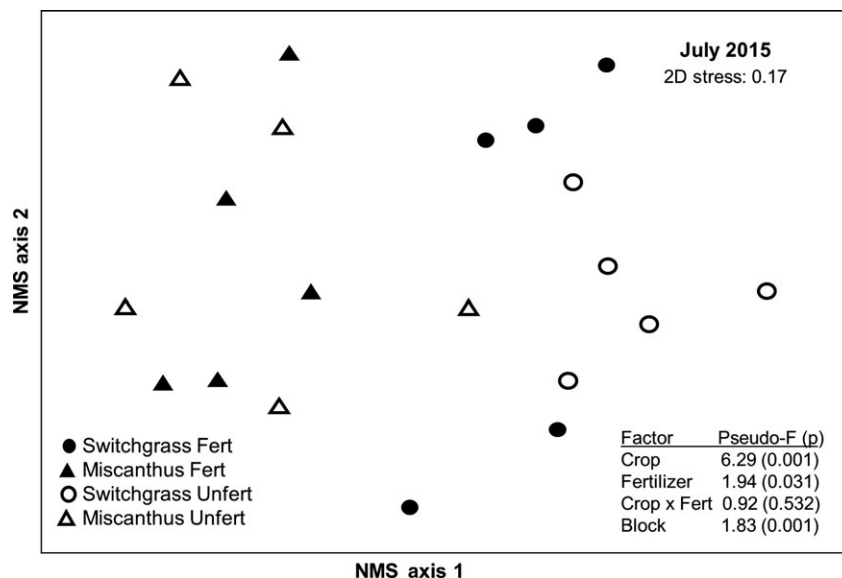
Fig. 2 PERMANOVA statistical results and non-metric multidimensional scaling (NMS) ordination of AMF communities in July 2015 showing significant divergence among crops and fertilizer treatments in AMF community composition (model $r^2 = 0.323$).

Table 2 Results of split-plot general linear models examining the effects of crop species (switchgrass or miscanthus) and fertilizer (added or not) on soil plant-parasitic nematode (PPN) abundance and diversity (measured as both family richness and family Shannon diversity, H') in July and October. Significant effects are in bold

Factor	df	July PPN abundance		Oct. PPN abundance		July nematode family richness		Oct. nematode family richness		July nematode diversity (H')		Oct. nematode diversity (H')	
		F	P	F	P	F	P	F	P	F	P	F	P
Crop species	1, 8	**5.36**	**0.049**	**6.94**	**0.039**	2.49	0.153	1.30	0.297	3.43	0.101	0.95	0.368
Fertilizer	1, 8	1.08	0.328	0.26	0.628	**6.77**	**0.032**	0.17	0.699	**12.25**	**0.008**	0.01	0.944
Crop x fert	1, 8	1.05	0.335	0.07	0.796	0.90	0.372	0.03	0.873	2.21	0.175	1.36	0.287

significantly varied with both crop and fertilizer regime (Fig. 4). The ISA revealed that switchgrass was associated with bacteria-feeding nematodes in the family Cephalobidae and minor PPN in the family Tylenchidae, while miscanthus was associated with economically important PPN in the family Longidoridae and fungivores in the family Aphelenchidae (Table 3). This was confirmed with the NINJA feeding group analysis, where miscanthus had higher relative abundance of herbivores while switchgrass had higher relative abundances of other feeding groups (Fig. 5). This corresponded to a higher Plant Parasitic Index in miscanthus compared to switchgrass (Table 4). There were no differences in community maturity or food web condition between crops, however (Table 4). Fertilized plots were associated with bacterivore and omnivore families (Alaimidae, Qudsianematidae) in the ISA, while

unfertilized plots were associated with fungivorous families (Diphtherophoridae, Leptonchidae). The NINJA feeding group analyses also showed increased relative abundance of fungivores in the unfertilized plots (Fig. 5). This corresponded to higher community maturity and structure indices in unfertilized plots, while fertilization was associated with a higher nutrient enrichment index (Table 4).

Discussion

Do AMF activity and diversity differ between switchgrass and miscanthus?

Perennial cellulosic bioenergy crops have been shown to reduce carbon emissions compared to annual crops, and to support a number of below ground ecosystem
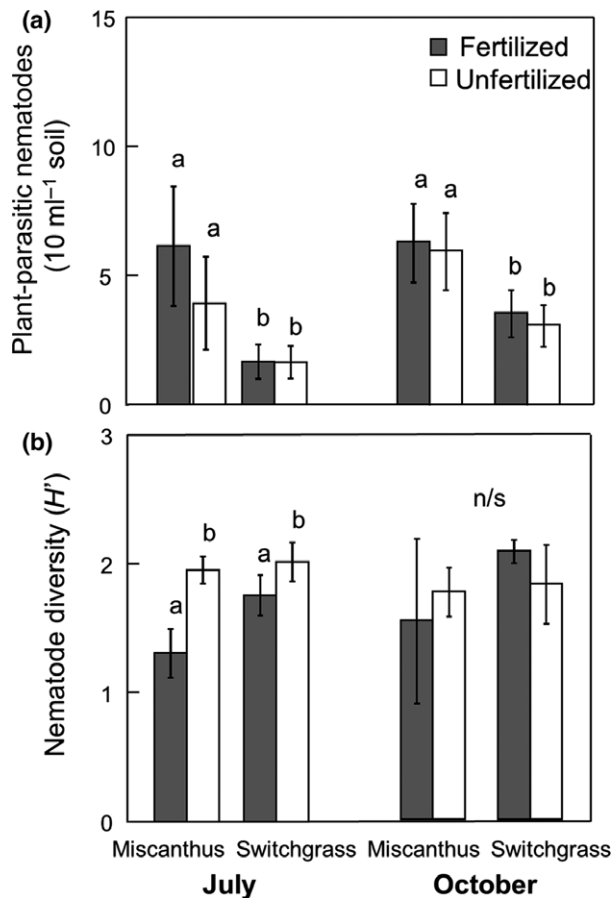
Fig. 3 Differences among crop and fertilizer treatments for plant-parasitic nematode abundance and diversity in different seasons. Bars are means ± 1 SE. Letters on graphs indicate significant Tukey-corrected pairwise comparisons ($P < 0.05$), with lowercase indicating differences within sampling dates.

services and functions that can make these crops more sustainable (Robertson *et al.*, 2011; Jesus *et al.*, 2016). Our research has shown that the selection of crop type can impact soil communities in perennial feedstocks that may have important consequences for sustainability of these systems. For example, differences in AMF associations among crops have implications for soil carbon storage and related ecosystem functions. While crop root AMF colonization rates were not consistent across treatments, we found that mycorrhizal diversity and activity as measured by ERH were generally higher with switchgrass than with miscanthus, and that this contributed to higher potential soil carbon via ERH and glomalin production. ERH are important factors in soil aggregate formation (Wright & Upadhyaya, 1996), and previous work in this system has shown that large soil aggregates (>1 mm) are more abundant in switchgrass than miscanthus (Tiemann & Grandy, 2015). Our results suggest that switchgrass plantings are more likely to increase

AMF-associated ecosystem functions compared to miscanthus. Switchgrass root exudates are known to enhance colonization of some species of AMF (Mao *et al.*, 2014), and a study in a similar system found enhanced AMF biomass in switchgrass compared to other biofuel crops including miscanthus (Oates *et al.*, 2016). Notably, previous work in this GLBRC experiment found that switchgrass and miscanthus plots did not differ in soil organic matter, total soil carbon, active carbon pools, or slow carbon pools (Sprunger, 2015; Tiemann & Grandy, 2015), though these studies were conducted just three years after experiment initiation, and so may not reflect development of long-term differences among crops.

While not a major focus of our study, we did find variation across sampling dates in AMF activity measures, with May samples often quite high in ERH length and % root colonization compared to the other sampling dates. While this could have been simply due to technician variability, general patterns across treatments were consistent in all sampling periods for ERH. AMF activity is known to fluctuate through time and between species and root colonization and ERH abundance can both be highest at the beginning of the growing season, often tracking soil moisture (e.g., Lutgen *et al.*, 2003). However, more work is needed to more explicitly quantify seasonal variation in AMF activity in bioenergy systems.

Our results with regards to AMF diversity responses across crops showed that *Rhizophagus* sequences were mostly associated with switchgrass plots in our study, while *Septoglomus* sequences were mostly associated with miscanthus. Both groups can be characterized as ruderal genera associated with agricultural fields and early successional systems, demonstrating fast growth and hyphal turnover rates (Chagnon *et al.*, 2013). However, understanding of the functional differences across AMF taxa is still in preliminary stages, and more work is needed to understand and predict how individual groups will respond to biotic and abiotic factors (Chagnon *et al.*, 2013)

Do belowground nematode abundance and diversity differ between switchgrass and miscanthus?

Soil nematodes occupy multiple trophic levels in soils and serve as important and sensitive indicators of soil health (Yeates, 2003). Additionally, plant-parasitic nematodes often dominate soil communities in grasslands and may limit crop production (Yeates & Bongers, 1999). The lower abundance of plant-parasitic nematodes (PPN) and lower Plant-Parasitic Index associated with switchgrass was somewhat unexpected, as switchgrass produces twice the amount of fine roots as miscanthus in this system (Sprunger, 2015). The increased AMF root colonization of
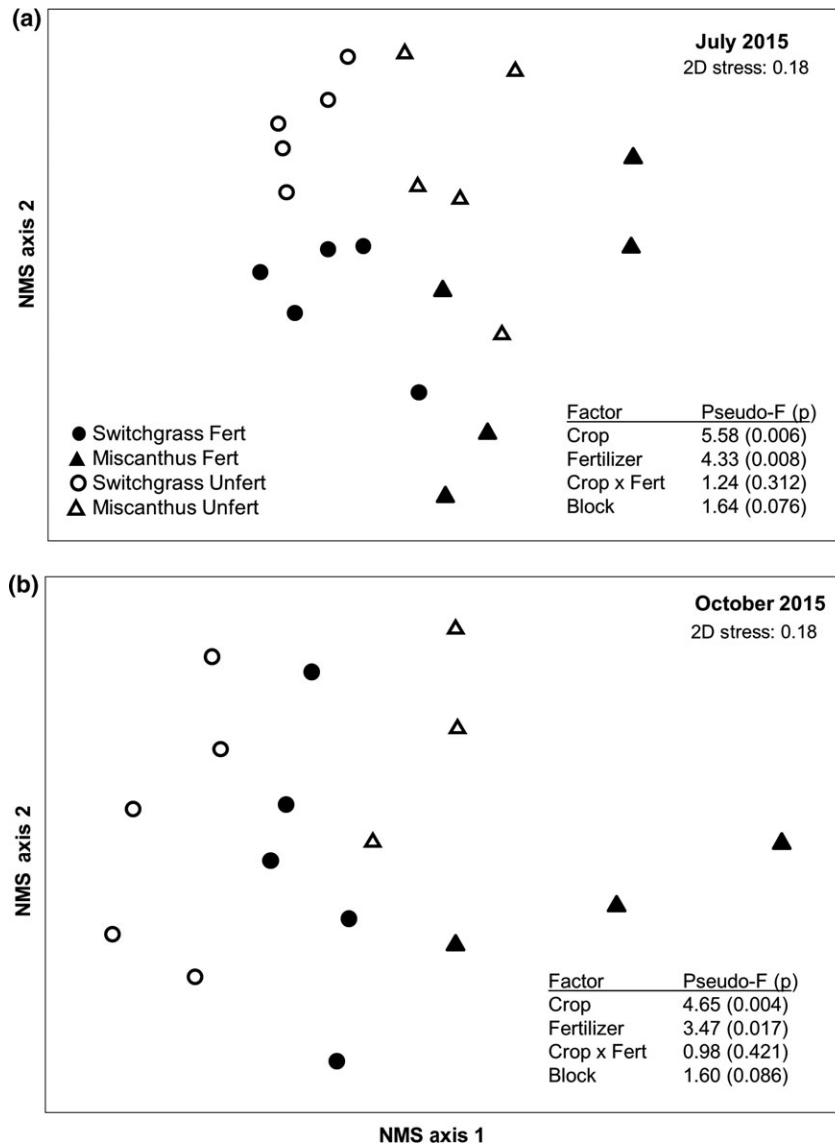
Fig. 4 PERMANOVA statistical results and non-metric multidimensional scaling (NMS) ordination of nematode communities in (a) July 2015 (model r^2 = 0.499) and (b) October 2015 (model r^2 = 0.52) showing significant divergence among crops and fertilizer treatments in nematode community composition.

switchgrass may explain this reduction in PPN, as AMF are known to decrease PPN through a variety of mechanisms, including induced systemic resistance, direct competition, enhanced tolerance, and altered rhizosphere interactions (Schouteden *et al.*, 2015). Additionally, the AMF groups most closely associated with both crops are known to be particularly efficient at protecting plant hosts from root pathogens (Chagnon *et al.*, 2013). It may also be that switchgrass is less susceptible to PPN than miscanthus due to differences in root exudates or root architecture, though very little is known about host suitability or damage thresholds for these species in field conditions (Mekete *et al.*, 2011).

While nematode diversity (richness and H′) and community maturity indices did not differ among switchgrass and miscanthus, our results revealed significant differences in nematode community structure among these crops. Switchgrass plots were associated with a variety of nematodes, while miscanthus was associated with plant parasites in the family Longidoridae (specifically *Xiphinema*, the dagger nematodes). Members of this family are ectoparasites and known to cause considerable damage to corn and other annual crops, as well as transmit plant nepoviruses (Brown *et al.*, 1995). A previous survey of grasslands in three Midwestern US states showed that *Xiphinema spp.* were more abundant in miscanthus fields than switchgrass fields, causing

Table 3 Results from blocked Indicator Species Analysis for nematode families associated with crop or fertilizer treatment. *P*-values are calculated based on 1000 randomizations in Monte Carlo simulations

Nematode family	Feeding group	Indicator group(s)	Indicator value (*P*-value)
Alaimidae	Bacterivore	Fertilized (Oct.)	63.8 (0.054)
Aphelenchidae	Fungivore	Miscanthus (Oct.)	63.5 (0.026)
Aphelenchoididae	Fungivore	n/s	n/s
Aporcelaimidae	Omnivore	n/s	n/s
Cephalobidae	Bacterivore	Switchgrass (July)	75.5 (0.005)
Criconematidae	Herbivore	n/s	n/s
Diphtherophoridae	Fungivore	Unfertilized (July)	59.7 (0.028)
Discolaimidae	Predator	n/s	n/s
Dolichodoridae	Herbivore	n/s	n/s
Leptolaimidae	Bacterivore	n/s	n/s
Leptonchidae	Fungivore	Unfertilized (July)	67.4 (0.033)
Longidoridae	Herbivore	Miscanthus (July)	88.4 (<0.001)
		Miscanthus (Oct.)	73.6 (0.003)
Mononchidae	Predator	n/s	n/s
Nordiidae	Omnivore	n/s	n/s
Nygolaimidae	Predator	n/s	n/s
Plectidae	Bacterivore	n/s	n/s
Pratylenchidae	Herbivore	n/s	n/s
Prismatolaimidae	Bacterivore	n/s	n/s
Psilenchidae	Herbivore	n/s	n/s
Qudsianematidae	Omnivore	Fertilized (Oct.)	63.7 (0.029)
Rhabditidae	Bacterivore	n/s	n/s
Thornenematidae	Omnivore	n/s	n/s
Tripylidae	Predator	n/s	n/s
Tylenchidae	Herbivore	Switchgrass (July)	56.6 (0.013)

stunting of lateral roots and destroying fibrous roots of miscanthus (Mekete *et al.*, 2009). Buildup of PPN in soils may also explain the characteristic decrease in biomass yields of perennial cellulosic biomass crops over time, especially for miscanthus (Arundale *et al.*, 2014a).

Does fertilization alter these belowground relationships?

Perennial bioenergy crops such as switchgrass and miscanthus may be good options to increase marginal land use, though some studies suggest that fertilization will be needed to improve economic viability (Qin *et al.*, 2015). However, fertilization does not always increase yield (e.g., Dierking *et al.*, 2016), and can actively reduce ecosystem services associated with perennial crops even when yields increase (Duran *et al.*, 2016). Our study showed that nitrogen fertilizer shifted AMF community composition in both crops, reduced diversity in switchgrass, but not miscanthus, and had little overall effect on AMF activity. While AMF are generally most sensitive to soil phosphorus (Treseder, 2004), soil N addition has been documented to reduce AMF diversity, root colonization, and hyphal growth and biomass in natural grasslands and agricultural systems (e.g., Egerton-Warburton & Allen, 2000; Gosling *et al.*, 2006; Oates *et al.*,

2016), though this can be dependent on background soil N : P (Johnson *et al.*, 2003) and the form and amount of N added (Valentine *et al.*, 2002). Some studies have shown that N addition shifts AMF community composition without altering AMF root colonization rates (Jumpponen *et al.*, 2005). The effects we saw in our study were fairly small, with only a few AMF taxa varying in response to fertilizer. Fertilizer was applied at relatively low rates in this system (56 kg N ha^{-1}), which may not be enough to alter AMF activity. However, similar application rates have been used in other studies (Arundale *et al.*, 2014b). While there is no consensus on optimal N application rates for bioenergy systems due to variation in climate and soil characteristics, fertilization rates in this system were based on published recommendations for the upper Midwest (Laboski *et al.*, 2012). It is also possible that plots suffered from excessive edge effects in the experimental design which compromised the fertilization treatment, though we still saw evidence for significant overall fertilizer effects on AMF diversity and community composition.

N fertilizer also reduced nematode diversity and community maturity and altered soil nematode community composition. Such a reduction in diversity matches findings from a recent meta-analysis of nitrogen fertilizer
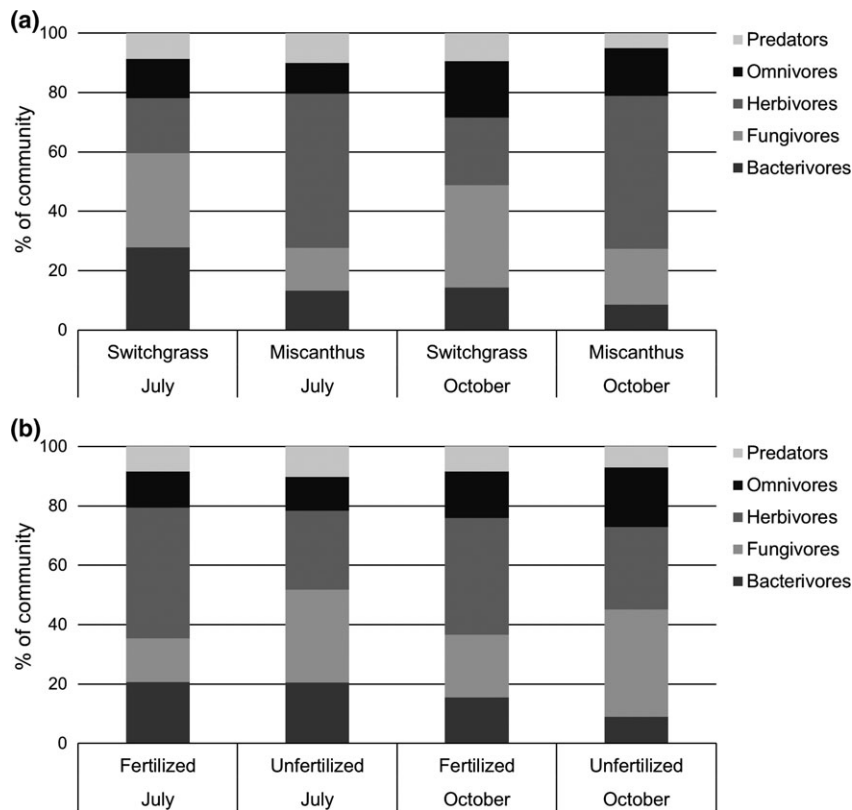
Fig. 5 Differences in nematode feeding group composition between (a) crop and (b) fertilizer treatments.

Table 4 Nematode community maturity and food web condition indices (means and standard deviations) for different crop and fertilizer treatments. Significant differences between treatments ($P < 0.05$) are in bold. See Ferris & Bongers (2009) for details on index calculations

Community index	July		October		July		October	
	Switchgrass	Miscanthus	Switchgrass	Miscanthus	Fertilized	Unfertilized	Fertilized	Unfertilized
Maturity index (MI)	3.48 (0.28)	3.64 (0.28)	3.72 (0.33)	3.67 (0.47)	3.49 (0.3)	3.63 (0.27)	**3.47 (0.32)**	**3.93 (0.26)**
Enrichment index (EI)	1.4 (4.52)	2.8 (8.33)	18.2 (28.2)	34.9 (22.3)	4.4 (9.07)	0.0 (0.0)	**36.8 (27.7)**	**11.3 (18.4)**
Structure index (SI)	91.2 (5.27)	93.5 (5.31)	95.6 (3.34)	93.2 (5.52)	90.9 (5.47)	93.8 (4.94)	**92.2 (4.29)**	**97.2 (2.57)**
Plant parasitic index (PPI)	**2.93 (0.86)**	**4.67 (0.31)**	**2.86 (0.89)**	**4.17 (0.62)**	4.23 (0.95)	3.66 (1.23)	3.72 (0.96)	2.98 (0.99)

effects on nematode communities (Liu *et al.*, 2016). Fertilized plots were associated with bacterivores and omnivores, while unfertilized plots were associated with fungivores. In general, shifts from fungivore- to bacterivore-dominated communities are associated with disturbance and N addition which increase growth rates of bacteria (Neher, 2010; Liu *et al.*, 2016). However, results from some studies have found N additions to increase fungivorous nematodes (e.g., Eisenhauer *et al.*, 2012), possibly due to increases in plant root and fungal activity. While not statistically significant, in our study AMF hyphae tended to decrease in fertilized plots (Table 1, Fig. 1b) which may further account for the

observed lower abundances of fungivores. In our study, fertilized plots had a higher enrichment index than unfertilized plots, which corresponds to an increase in opportunistic nematode groups often associated with agricultural systems (Ferris *et al.*, 2001). Unfertilized plots had significantly higher maturity and food web structure indices than fertilized plots, corresponding to a more stable nematode community (Ferris & Bongers, 2009), though the food web structure indices for all treatments indicated presence of relatively structured, undisturbed nematode communities. Fertilization had only minor effects on PPN, as unfertilized plots had a lower proportion of PPN in their communities but total

numbers of PPN and Plant-Parasitic Indices were not affected. These results were somewhat surprising as inorganic N fertilizers, especially urea, have been shown to be toxic to soil nematodes in other systems, though often only at very high concentrations (e.g., urea applied in excess of $300 \, \mathrm{mg \, kg^{-1}}$ soil; Rodriguez-Kabana, 1986). The relatively low levels of N fertilizer applied in our system appear to shift community structure without reducing PPN abundance.

In conclusion, perennial cellulosic bioenergy crops offer growers a way to more sustainably manage soils. However, management choices such as which crop to plant or whether to fertilize can influence belowground organisms and their associated soil ecosystem services. In this study, mycorrhizal activity and diversity were higher with switchgrass than with miscanthus, leading to higher potential soil carbon contributions via increased hyphal growth and glomalin production. Plant-parasitic nematode (PPN) abundance was $2.3\times$ higher in miscanthus plots compared to switchgrass, mostly due to increases in dagger nematodes (*Xiphinema*). Nitrogen fertilization had minor effects on AMF and nematode communities associated with these crops, possibly due to the relatively low levels of nitrogen added in this experiment. Overall, we found detectable effects on the diversity and composition of soil communities associated with different perennial bioenergy cropping systems which may have important consequences for the ecosystem services provided by these systems.

Acknowledgements

Data accessibility

All raw sequence data from this study are available through the NCBI Sequence Read Archive under accession SAMN06447199.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Table S1 Results of AMF Indicator Species Analysis.