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Research Article

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RESEARCH ARTICLE

Impacts of nitrogen addition on switchgrass root-associated diazotrophic community structure and function

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One sentence summary: Distinct communities of nitrogen-fixing bacteria from three field soils assembled into consistent communities in association with switchgrass roots under long-term and short-term N additions.

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ABSTRACT

Cellulosic bioenergy crops, like switchgrass (*Panicum virgatum*), have potential for growth on lands unsuitable for food production coupled with potential for climate mitigation. Sustainability of these systems lies in identifying conditions that promote high biomass yields on marginal lands under low-input agricultural practices. Associative nitrogen fixation (ANF) is a potentially important nitrogen (N) source for these crops, yet ANF contributions to plant N, especially under fertilizer N addition are unclear. In this study, we assess structure (*nifH*) and function (ANF) of switchgrass root-associated diazotrophic communities to long-term and short-term N additions using soil from three marginal land sites. ANF rates were variable and often unexpectedly high, sometimes 10× greater than reported in the literature, and did not respond in repeatable ways to long-term or short-term N. We found few impacts of N addition on root-associated diazotrophic community structure or membership. Instead, we found a very consistent root-associated diazotrophic community even though switchgrass seeds were germinated in soil from field sites with distinct diazotrophic communities. Ultimately, this work demonstrates that root-associated diazotrophic communities have the potential to contribute to switchgrass N demands, independent of N addition, and this may be driven by selection of the diazotrophic community by switchgrass roots.

Keywords: switchgrass; diazotroph; microbial community composition; *nifH*; associative nitrogen fixation

INTRODUCTION

Cellulosic bioenergy crops, including the C4 perennial grass switchgrass (*Panicum virgatum*), represent a renewable fuel source and a potential alternative to fuel production from food crops like maize (*Zea mays*; Robertson *et al.* 2017). These perennial grasses have high climate mitigation potentials through carbon (C) capture and belowground C allocation, resulting in soil C accrual and mitigation of ~9.5 Mg CO_2-eq ha^{-1} yr^{-1} (Robertson *et al.* 2017). However, the challenge of such cropping systems is

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in enabling sustainable plant growth on lands not suitable for food production (i.e. marginal lands) under low inputs of nutrients and water (Gelfand *et al.* 2013; Robertson *et al.* 2017). Switchgrass is a particularly promising bioenergy crop, producing high biomass yields when grown on marginal lands even with minimal inputs of fertilizer nitrogen (N) or water (Gelfand *et al.* 2013; Mehmood *et al.* 2017; Robertson *et al.* 2017). In fact, switchgrass productivity on marginal lands is often unresponsive to fertilizer N additions; similar yields have been observed at N levels both above and below plant N demands (Ruan *et al.* 2016; Wang *et al.* 2019). Furthermore, yields remain consistently high despite N removal via yearly harvest (Fike *et al.* 2006). An N mass balance suggests that switchgrass is accessing an unaccounted-for N source at rates of 35–58 kg N ha^{-1} yr^{-1} (Roley *et al.* 2018). Recent evidence points toward associative nitrogen fixation (ANF) as this unaccounted-for N source (Roley *et al.* 2019; Smercina *et al.* 2019a).

ANF, here defined as N-fixation occurring in (endosphere), on (rhizoplane) or near roots (rhizosphere) without formation of specialized structures such as legume nodules, is increasingly recognized for its potential to increase bioenergy crop sustainability by providing an alternative N source in lieu of fertilizer N additions (Reed, Cleveland and Townsend 2011; Bloch *et al.* 2020). The energy-intensive process of ANF transforms dinitrogen (N_2) gas to biologically available ammonia under dynamic conditions and is carried out by a diverse community of bacteria living in soils and in the rhizosphere, rhizoplane and endosphere (Smercina *et al.* 2019a). ANF readily occurs in association with the roots of many grasses, including maize (Chalk 2016; Kuan *et al.* 2016) and switchgrass (Roley *et al.* 2018; Smercina *et al.* 2019b), where roots exude easily accessible C that can support ANF activity. ANF occurring in association with these grasses has the potential to contribute significantly to plant N demands (Bormann *et al.* 1993; Reed, Cleveland and Townsend 2011; Ladha *et al.* 2016). However, in order to harness these N contributions, we need a better understanding of the controls on ANF and the diazotrophic organisms that carry out this process.

Nitrogen has been shown to be a major control on ANF (Hobbs and Schimel 1984; Patra *et al.* 2007; Reed, Cleveland and Townsend 2011; Kox *et al.* 2016; Smercina *et al.* 2019b). Diazotrophs are not strictly reliant on fixed N to meet their N demands and can access external N sources, including organic and inorganic N (Reed, Cleveland and Townsend 2011; Norman and Friesen 2017). Because ANF is energy intensive, it is often more energetically favorable to use external N than to fix N from the atmosphere (Reed, Cleveland and Townsend 2011; Smercina *et al.* 2019b). Consequently, high N availability generally downregulates ANF and low N availability, such as what might be observed near switchgrass roots often promotes ANF (Reed, Cleveland and Townsend 2011; Smercina *et al.* 2019b). Thus, N fertilization of switchgrass cropping systems is likely to impact the potential contributions of N from ANF.

The magnitude of this response in ANF to N is likely to be driven by the composition of the diazotrophic community. The switchgrass root-associated diazotrophic community is very diverse, found to contain members of several different Phyla including Alpha, Beta, Gamma, and Deltaproteobacteria and Firmicutes (Bahulikar *et al.* 2014; Roley *et al.* 2019; White *et al. in review*). These organisms represent diverse life histories and metabolic strategies, ranging from oligotrophs to copiotrophs and from strict anaerobes to aerobes, and produce multiple forms of nitrogenase, the enzyme involved in N-fixation, all of which influence a given organism's ANF activity (Smercina *et al.* 2019b). Diazotrophs also differ in their response to N availability. Some diazotrophs, such as *Azospirillum brasilense*, can regulate the function of nitrogenase post-translationally thereby shutting off ANF activity when external N becomes available (Dixon and Kahn 2004). However, many diazotrophs only regulate nitrogenase at the level of transcription; therefore, ANF activity via already synthesized nitrogenase may occur in these organisms even when external N is available (Dixon and Kahn 2004). Overall, the relative representation of different metabolic strategies as well as enzyme forms and regulation present within the diazotrophic community is likely to influence ANF activity at the level of the root.

Composition of the diazotrophic community and relative representation of different growth strategies is likely to be impacted by N fertilization. Long-term N fertilization, on order of 20 years of application, has been shown to cause evolutionary shifts in symbiotic N-fixers, resulting in declining mutualisms between rhizobia and their hosts (Klinger, Lau and Heath 2016), though not necessarily reducing N-fixation function (Schmidt, Weese and Lau 2017). Similarly, N fertilization for 7 to upward of 30 years has also been observed to cause shifts in diazotrophic communities (Wang *et al.* 2016; Feng *et al.* 2018; Fan *et al.* 2019; Roley *et al.* 2019). Under pressures of long-term N fertilization, the diversity of diazotrophic communities may decrease (Feng *et al.* 2018), potentially shifting toward those organisms that can rapidly regulate ANF activity and effectively compete for externally available N through rapid growth (Fan *et al.* 2019; Smercina *et al.* 2019b). Such shifts in community composition will also likely influence the response of ANF and diazotrophic community composition to short-term N availability.

In this study, we explore the impact of long-term and short-term N additions on function (ANF rates) and structure (*nifH*) of the switchgrass root-associated (rhizosphere, rhizoplane and endosphere) diazotrophic community across marginal land sites. We posit three hypotheses: (i) Increasing N availability (long-term and short-term), moderated by site, will reduce ANF rates and diazotrophic community diversity; (ii) The magnitude of response to N for ANF rates and diazotrophic community composition will be more pronounced under short-term N additions as compared with long-term effects; and (iii) Diazotrophic community structure will be linked to measured ANF rates such that we will observe distinct community composition and presence of specific diazotrophs where we measure greater ANF.

METHODS

Experimental setup

We explored the response of the switchgrass (*P. virgatum*) root-associated diazotrophic community, including composition (i.e. *nifH* amplicon sequencing) and activity (i.e. ANF), to long-term and short-term N addition treatments using a reciprocal treatment experimental design (Fig. 1A). To represent long-term N treatments, field soils were collected from fertilized and unfertilized plots at three different field sites each with four field blocks (described below). Short-term N treatments were created in the greenhouse where plants received a single addition of either high N or low N (described below) at the planting. Overall, 48 experimental units were represented (i.e. 3 soil sites × 4 field blocks × 2 long-term N × 2 short-term N) each with six analytical replicates for a total of 288 greenhouse pots. Six analytical replicate pots were included to account for potential losses due to lack of plant growth or plant death.

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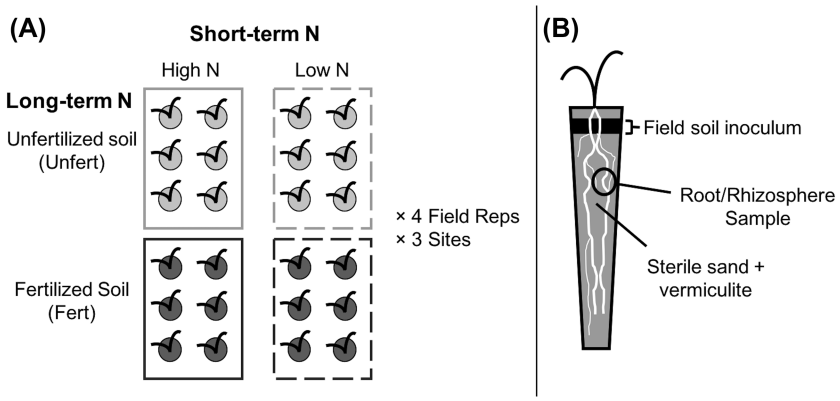

Figure 1. Experimental design of **(A)** N treatments and **(B)** planting. There were six analytical replicate pots per long-term × short-term N treatment combination. This was repeated for each field block (4 per site) and each field site (3—Escanaba, Lake City and Lux Arbor) for a total of 288 analytical replicate pots and 48 experimental units. Field soils were used to inoculate pots by adding a thin layer of soil near the surface of each pot.

In the greenhouse, switchgrass (var. Cave-in-Rock) plants were grown individually in large deepots (Stuewe and Sons, Tangent, OR, USA) filled with a 50:50 (v/v) mixture of autoclave sterilized sand and vermiculite. Each pot also received ∼2.5 cm of field soil as an inoculum at a depth of ∼1–2 cm from the top of the pot (Fig. 1B). The field soil was then covered with 50:50 sand–vermiculite mix, filling the remainder of the pot. Field soils were collected from three Michigan field sites, Lux Arbor (LUX; 42.476365, −85.451887), Lake City (LC; 44.296098, −85.199612) and Escanaba (ESC; 45.7627, −87.1877), with distinct climate and soil characteristics (Table 1; Kasmerchak and Schaetzl 2018). These field sites are maintained as part of the Great Lakes Bioenergy Research Center's Marginal Land Experiment (GLBRC MLE; https://www.glbrc.org/). Each site has four replicate split-plots of switchgrass (var. Cave-in-Rock) monoculture that have been fertilized annually since 2013. Main plots are divided into fertilized (+56 kg urea-N ha^{-1} yr^{-1}) and unfertilized (no added N) split-plots. Field soils were collected using a standard soil probe to a depth of 10 cm from both fertilized and unfertilized split-plots to represent our long-term N treatments. Soils were stored in a cooler until returning to the lab, and then stored at 4°C until further use. Each soil sample was sieved (4 mm) and homogenized before addition to the greenhouse pots. A subset of each field soil sample (24 in total with 3 sites × 4 field blocks × 2 long-term N treatments) was frozen at −80°C for determination of initial diazotrophic community composition (described below).

Planting and growth

Prior to planting, switchgrass seeds were scarified, stratified and sterilized as follows. Approximately 500 seeds were acid scarified by shaking in 50 ml of 8 M sulfuric acid for 15 min. Seeds were then washed at least three times with distilled water by shaking for 5 min each time. Seeds were then stratified by plating onto a petri dish containing an autoclave-sterilized Whatman #1 filter paper (GE Healthcare Life Sciences, Chicago, IL, USA). Seeds were covered with a second sterile filter paper and then received 5 ml of autoclaved 0.2% (m/v) potassium nitrate. The petri dish was sealed with parafilm and then stored at 4°C in the dark for at least 4 days and no more than 2 weeks. After stratification, seeds were bleach and vapor-phase sterilized. For bleach sterilization, seeds were transferred to a sterile specimen cup and shaken with 5% sodium hypochlorite for 15 min and then washed at least three times with autoclaved nanopure. Seeds were then plated onto a sterile petri dish, spread into a single layer and then placed in a desiccator for vapor-phase sterilization. Vapor-phase sterilization uses chlorine gas and all work was carefully carried out in a fume hood. To generate chlorine gas, 3 ml of 8.25% sodium hypochlorite (household bleach) was added to 100 ml of concentrated hydrochloric acid in a beaker contained within a desiccator. Immediately following addition of the bleach, the desiccator was sealed for 4 h. After 4 h, seeds were removed and the petri dish was quickly sealed. Seeds were stored in the dark at 4°C until planting.

Sterility of seeds was confirmed by plating a subset on Luria-Bertani (LB) agar prior to planting. Sterile seeds were planted directly into the field soil, thus allowing the seedling and its roots to be inoculated with the soil microbial community. At planting, pots received 50 ml of autoclave sterilized $\frac{1}{2}$ Hoagland's solution (2.5 mM KCl, 2.5 mM $CaCl_2$, 0.5 mM KH_2PO_4, 1.0 mM $MgSO_4$, 0.024 mM H_3BO_3, 0.004 mM $MnCl_2{\cdot}4H_2O$, 0.102 μM $CuSO_4{\cdot}5H_2O$, 0.382 μM $ZnSO_4{\cdot}7H_2O$, 0.248 μM $Na_2MoO_4{\cdot}2H_2O$, 5.4 μM NaFeEDTA) with either high (28.3 mM; equivalent to 125 kg N ha^{-1}) or low (5.7 mM; equivalent to 25 kg N ha^{-1}) additions of N as urea according to their associated short-term N treatment. Plants were grown in the greenhouse under mist irrigation for four months then were harvested for determination of aboveground biomass, root-associated ANF and root-associated diazotrophic community composition as described below. At harvest, 18 plants of the 288 analytical replicates had died leaving 270 plants for harvest with plant losses distributed randomly across treatments and no experimental unit having fewer than four analytical replicates.

Aboveground biomass

Plants were carefully removed from their pots onto aluminum foil sterilized with 70% ethanol. All aboveground biomass for each plant was clipped from the roots, dried at 60°C for 48 h and then weighed.

Root-associated nitrogen fixation

Root samples representing rhizosphere, rhizoplane and endosphere were collected by first shaking roots to remove any loose sand and vermiculite. Any sand or vermiculite still adhering to the roots was considered part of the rhizosphere. Portions of root with adhering field soil were intentionally avoided for this assay.

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Table 1. Site characteristics, including soil properties, climate and land use history for three field sites, Lux Arbor (LUX), Lake City (LC) and Escanaba (ESC).

Site	Soil taxonomy	Texture	% Sand	% Silt	% Clay	pH	Total N (%)	Total C (%)	Inorg. P (ppm)	30-yr avg. precipitation (mm)	30-yr avg. temp. (°C)
LUX	Typic Hapludalfs (Alfisol)	Loam	51.1	31.7	17.2	5.8	0.06	0.77	12	842.01	9.0
LC	Oxyaquic Haplorthod (Spodosol)	Loamy sand	84.7	7.8	7.5	7.3	0.06	0.92	24	812.29	6.5
ESC	Inceptic Hapludalf (Alfisol)	Sandy loam	57.1	27.7	15.2	7.0	0.15	1.73	14	728.22	5.3

Note: Data provided by Great Lakes Bioenergy Research Center (GLBRC) marginal land experiment (https://lter.kbs.msu.edu/research/long-term-experiments/marginal-land-experiment/) and Kasmerchak and Schaetzl (2018).

This sampling method allowed us to capture N-fixation occurring inside roots, on root surfaces and in the surrounding growth media collectively capturing all ANF.

A subset of root material (ranging from 0.1–1.8 g depending on root system size) from each analytical replicate was clipped and placed in a 20-ml gas vial for measurement of ANF via $^{15}N_2$ incorporation. An additional root subset was collected from one analytical replicate for each of the 48 experimental units as an unlabeled reference for ^{15}N analysis. Vials were immediately placed in a cooler until further analysis. All root material in each vial was weighed and then samples were stored at room temperature to equilibrate for 24 h prior to analysis. Following methods described by Smercina *et al.* (2019a), we added a 4-carbon source cocktail containing glucose, sucrose, citrate and malate at a ratio of 1 ml solution per g root/rhizosphere material. After C addition, vials were sealed and evacuated. Vial headspace was replaced with 1 ml of acid-washed $^{15}N_2$ (Sigma-Aldrich, Inc., St Louis, MO, USA), 10% ultra high purity (UHP) oxygen and balanced with UHP Helium. Reference samples received UHP-N_2 in place of $^{15}N_2$. Vials were incubated for three days at room temperature and then uncapped and dried at 60°C for 48 h before grinding and weighing for ^{15}N analysis. Samples were analyzed following standard procedures at University of California Davis's Stable Isotope Facility (Davis, CA). Root-associated ANF rates were calculated in μg N fixed g^{-1} root/rhizosphere day^{-1} as

$$\frac{AE_i \times TN_i}{AE_{atm} \times t}$$

where AE_i represents atom % excess of sample against an unenriched reference sample, TN_i represents total nitrogen content in sample, AE_{atm} represents atom % excess in the vial atmosphere (98 atom% in our case) and t is incubation time in days (Warembourg 1993; Roley *et al.* 2018).

Root-associated diazotrophic community composition

A subset of root/rhizosphere material from each analytical replicate was collected as described above, immediately frozen on liquid N_2 and then transferred to a −80°C freezer until DNA extraction. Roots with field soil adhering were intentionally avoided for this analysis. Root-associated microbial DNA was extracted from ∼0.5 g of root/rhizosphere material per sample using the Qiagen DNeasy PowerSoil kit (QIAGEN, Germantown, MD, USA). The initial lysis step via bead beating with glass beads was extended by 10 min to ensure adequate disruption root/rhizosphere material. Field soil samples (0.5 g soil per sample) was also extracted for sequencing to determine initial diazotrophic community composition.

Diazotrophic community composition was determined by sequencing the *nifH* functional gene amplified using the IGK3 (5′–GCIWTHTAYGGIAARGGIGGIATHGGIAA–3′) forward primer and DVV (5′–ATIGCRAAICCICCRCAIACIACRTC–3′) reverse primer, an optimal primer pair for capturing the widest diversity of diazotrophs (Gaby and Buckley 2012). PCR amplification of *nifH* was carried out in 25 μl reactions with 2 μl of DNA extract, 1× AmpliTaq Gold 360 Master Mix (Applied Biosciences, Foster City, CA), 0.027 μg T4 gene 32 Protein (New England Biolabs, Ipswich, MA, USA) and 500 nM concentrations of both the forward and reverse primers. The PCR program was as follows: 95°C start for 10 min, 34 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 30 s, extension at 72°C for 30 s and final extension at 72°C for 7 min. Amplification of target gene was confirmed via gel electrophoresis (1.5% gel agar, 90 V, 45 min). Despite several attempts at amplification, only 200 of the 270 root-associated analytical replicates successfully amplified while 17 of the 24 field soil samples successfully amplified. All samples with successful amplification were cleaned and sequenced as described below.

PCR products were cleaned to remove potential primer dimers in the <100 bp band using the QIAquick PCR purification kit (QIAGEN, Germantown, MD, USA) and then quantified via Quant-iT PicoGreen dsDNA Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Samples were normalized to a 1–6 ng μl^{-1} DNA concentration before library preparation. Samples were submitted to the Michigan State University RTSF Genomics Core Facility (East Lansing, MI) for library preparation and 2 × 250 bp paired-end read sequencing on the MiSeq platform (Illumina, San Diego, CA, USA) using MiSeq standard reagent kit v.2.

Sequence processing was performed following a modified version of the NifMAP pipeline (Angel *et al.* 2018). Sequence data were received as demultiplexed fastq files. Forward and reverse reads were merged via USEARCH v. 10.0.240 fastq_mergepairs and then quality and length filtered to maximum expected errors of 1 and minimum length of 300 bp via USEARCH v. 10.0.240 fastq_filter. Sequences were then filtered for non-*nifH* reads using four Hidden-Markov Models (HMM) as described in Angel *et al.* (2018). Sequences were then dereplicated using USEARCH v. 10.0.240 fastx_uniques and clustered using USEARCH v. 10.0.240 cluster_otus resulting in 6896 representative OTUs. The cluster_otus command also filters chimeras. Sequences were then mapped back to reference OTUs using USEARCH

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v. 10.0.240 usearch_global at 97% similarity. Ninety-six percent of sequences (4 820 591 of 5 019 796 sequences) successfully mapped to reference OTUs. Sequences were then frameshift corrected and translated to protein sequences using Framebot (Wang *et al.* 2013) and then filtered for homologs using HMM as described in Angel *et al.* (2018) before generating a final OTU table. At the end, 3025 OTUs were identified and used for phylogenetic tree construction and taxonomic classification (GenBank: KELG00000000). A phylogenetic tree was constructed using an amino acid reference alignment (Angel *et al.* 2018). Sequences were aligned to the reference using MAFFT v. 7.305 and then used to generate a tree via FastTree v. 2.1.9. Finally, taxonomy was assigned to sequences using Blast+ v. 2.7.1 tblastn command that allows querying of protein sequences against a nucleotide database. We used Gaby and Buckley's *nifH* sequence database as the taxonomic reference database for our sequences (Gaby and Buckley 2014). Taxonomy was assigned according to % similarity using empirically derived cutoffs of 75% similarity for family, 88.1% for genus and 91.9% for species (Gaby *et al.* 2018). All other taxonomic assignments matching at <75% similarity were only assigned at the order level (Gaby *et al.* 2018).

Statistical analysis

Data from all analytical replicates per experimental unit ($n = 48$) were averaged prior to statistical analysis. Median OTU counts across all analytical replicates per experimental unit were used as representative values for each experimental unit.

Results from aboveground biomass and root-associated ANF rates were analyzed by a split–split plot with RCBD ANOVA followed by Tukey's post hoc test with site as the main plot, long-term N as the split-plot and short-term N as the split–split plot using the R *agricolae* package (R Core Team 2018). Differences between treatment groups (site∗long-term∗short-term) were considered significant at $\alpha \leq 0.05$.

OTU counts were first rarefied to an even sampling depth of 800 using *rarefy_even_depth* in the R *phyloseq* package. *Rarefy_even_depth* was used to randomly resample OTU counts without replacement from each experimental unit. This sampling depth was chosen based on the rarefaction curves generated using *rarecurve* in the R *vegan* package. Curves indicated that most of the diversity was captured within a sample size of ~1000, evidenced by a leveling off of species counts with increasing sampling size. A sampling depth of 800 allowed us to capture a reasonable sampling of the measured diversity while limiting loss of experimental units due to low OTU counts. Four of the 48 experimental units were removed from downstream data analysis because they had too few OTU counts (i.e. <800 sample size). These units were distributed across sites (two from ESC, one from LUX and one from LC) and N treatments (two long-term and two short-term N). All downstream analyses are based on the rarefied OTU counts.

To evaluate beta-diversity of field soil and root-associated samples, we used Bray–Curtis dissimilarity to generate distance matrices using the distance function in R *phyloseq* and then ordinated via principal coordinates analysis (PCoA) using the *ordinate* function. We used *adonis* in R vegan to conduct PERMANOVA of the soil and root-associated distance matrices by site∗long-term N (field soils) and site∗long-term N∗short-term N (root associated). Differences between treatment groups were considered significant at $\alpha \leq 0.05$.

We also explored differences in the relative abundance of diazotrophic classes after first normalizing using arcsine transformation. Differences across treatments for root-associated samples were analyzed using a split–split-plot ANOVA with Tukey's post hoc as described above. Differences across treatments for soil samples were analyzed using a split-plot with RCBD ANOVA followed by Tukey's post hoc test with site as the main plot and field treatment as the split-plot.

Lastly, we explored diazotrophic community data for OTUs potentially important to predicting ANF. First, ANF data was binned as follows: 0–1, 1–2 and >2 μg N fixed g^{-1} root/rhizosphere day^{-1}. We then used *indicspieces* package in R to identify OTUs whose relative abundance was associated with each N-fixation bin, with a focus on OTUs associated with ANF rate bins >1.

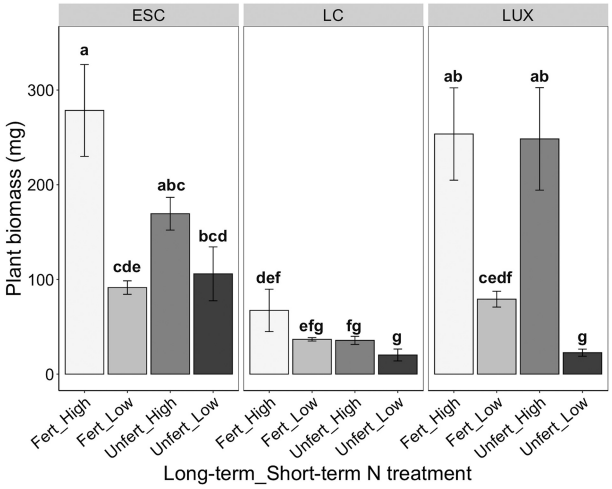

Figure 2. Plant aboveground biomass (mg) by site (ESC = Escanaba, LC = Lake City, LUX = Lux Arbor) and long-term by short-term N treatments (Fert_High = Fertilized field + High N addition in greenhouse, Fert_Low = Fertilized field + Low N greenhouse, Unfert_High = Unfertilized field + High N greenhouse, Unfert_Low = Unfertilized field + Low N addition). Each bar is representative of $n = 4$. Letters indicate significant differences where $P < 0.05$.

RESULTS

Plant metrics

Aboveground plant biomass differed significantly by site and N treatment (Fig. 2). Short-term N additions of high N tended to result in greater aboveground biomass across all sites, while responses to long-term N varied by site. We were unable to assess total belowground biomass in this study.

Root-associated diazotrophic community composition

Initial diazotrophic community composition, those organisms present in the field soil inoculum, differed significantly by site ($F = 9.207$, $P = 0.001$; Fig. 3A). Two groups appeared to drive these observed differences in community composition: Actinobacteria and Alphaproteobacteria (Fig. 4 and Table 2). Actinobacteria had greater relative abundance in ESC soils than in LC or LUX soils, while Alphaproteobacteria were most abundant in LUX soils relative to ESC and LC soils. Long-term N had no impact on overall composition of the field soil diazotrophic community ($F = 0.0397$, $P = 0.204$) and had minimal impacts on the relative abundance of community members, with only the Methanococci class showing any significant response (Figure S1, Supporting

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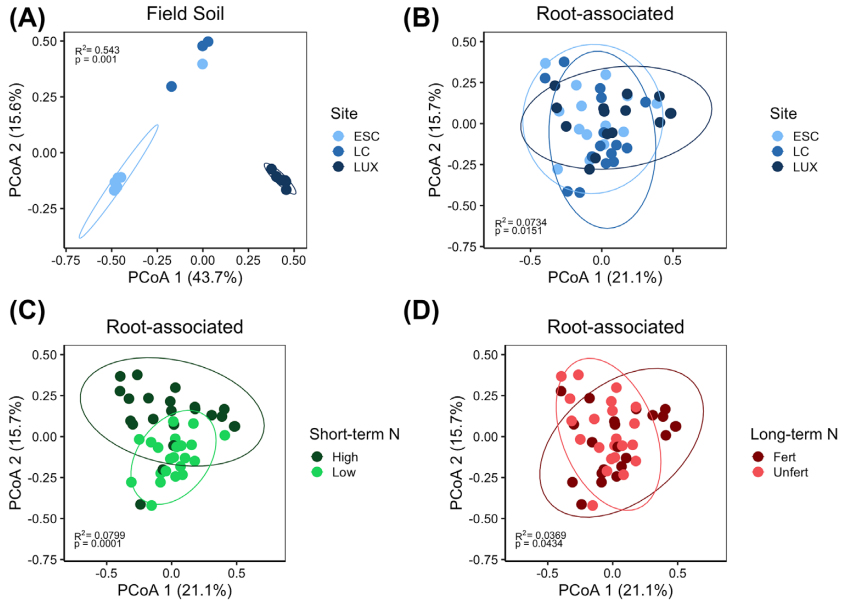

Figure 3. PCoA of **(A)** field soil and **(B)** root-associated samples colored by site, **(C)** by short-term N treatment and by **(D)** long-term N treatment. Points are based on Bray–Curtis distance matrix of the relative abundance of rarefied OTU counts. Each point represents one replicate. Ellipses represent 95% confidence ellipse of the centroid. Note that LC field soil does not have an ellipse because only three soil samples (of the eight total) amplified for *nifH* and were sequenced.

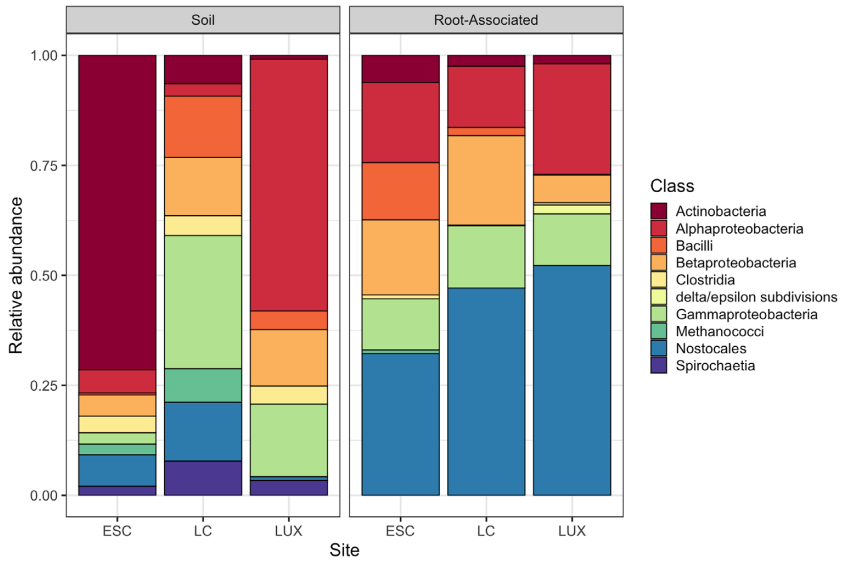

Figure 4. Average relative abundance of diazotroph classes by site (ESC = Escanaba, LC = Lake City, LUX = Lux Arbor) for soil (those soils used to inoculate the greenhouse pots) and root-associated samples. Bars represent the average of 8 samples for soil (only 3 for LC) and 16 samples for roots. Full results are presented in Table 2.

Information). Clear differences in overall diazotrophic community composition across sampling sites were no longer apparent in the greenhouse root-associated communities (Fig. 3B). However, root-associated diazotrophic community composition clustered by N treatment (Fig. 3C and D). This was particularly notable for short-term N additions, where root-associated communities under low N tended to cluster more tightly than those under high N (Fig. 3C).

Examining relative abundance of root-associated diazotrophic community members by class across sites revealed strikingly similar community composition, with only Actinobacteria abundance showing a site response (Fig. 4; Table 2). These differences in Actinobacteria relative abundance mirrored those of the field soil communities, with greatest Actinobacteria abundance at ESC (Table 2). However, Actinobacteria relative abundance was more similar across sites in the root samples than in the field soil. Additionally, several diazotrophic groups whose relative abundance differed by site in the field soil were no longer significantly different in the root-associated communities (Table 2).

Root-associated diazotrophic communities did not differ significantly between long-term N treatments (Fig. 5). However, short-term N additions altered the relative abundance of several diazotrophic classes. We observed greater relative abundance of Actinobacteria ($F = 6.392$, $P = 0.0241$), Clostridia ($F = 11.21$, $P = 0.00478$), Delta/Epsilon ($F = 1724.9$, $P < 0.0001$) and Nostocales (F

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Table 2. Average relative abundance and standard errors of diazotroph classes by sample type and site (presented visually in Fig. 3). Asterisks indicate significant differences between sample type within each site where $P < 0.05$. Lowercase letters indicate significant difference across sites within each sample type where $P < 0.05$. Relative abundances with significant differences by sample type or across sites are bolded for ease of reading.

	Soil			Root-associated		
Class	ESC	LC	LUX	ESC	LC	LUX
Actinobacteria	0.715 ± 0.096^{a*}	0.064 ± 0.039^{b*}	0.009 ± 0.003^{b}	0.062 ± 0.012^{a}	0.024 ± 0.012^{ab}	0.019 ± 0.005^{b}
Alphaproteobacteria	0.053 ± 0.021^{b}	0.028 ± 0.007^{b}	0.572 ± 0.074^{a}	0.182 ± 0.025*	0.139 ± 0.02	0.252 ± 0.041
Bacilli	0.004 ± 0.004^{b}	0.139 ± 0.103^{a*}	0.042 ± 0.021^{b*}	0.13 ± 0.124	0.019 ± 0.019	0.002 ± 0.002
Betaproteobacteria	0.048 ± 0.018	0.132 ± 0.077	0.128 ± 0.048	0.171 ± 0.097	0.203 ± 0.101	0.063 ± 0.022
Clostridia	0.038 ± 0.011*	0.045 ± 0.023*	0.041 ± 0.011*	0.009 ± 0.003	0.001 ± 0.001	0.005 ± 0.002
Delta/epsilon subdivisions	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.02 ± 0.02
Gammaproteobacteria	0.026 ± 0.011^{b}	0.302 ± 0.163^{a*}	0.165 ± 0.053^{b}	0.116 ± 0.048	0.142 ± 0.058	0.117 ± 0.028
Methanococci	0.024 ± 0.016	0.077 ± 0.05*	0 ± 0	0.008 ± 0.008	0 ± 0	0 ± 0
Nostocales	0.072 ± 0.05	0.133 ± 0.052	0.009 ± 0.007	0.322 ± 0.051*	0.471 ± 0.101*	0.523 ± 0.133*
Spirochaetia	0.021 ± 0.015	0.078 ± 0.06	0.034 ± 0.019	0 ± 0	0 ± 0	0 ± 0

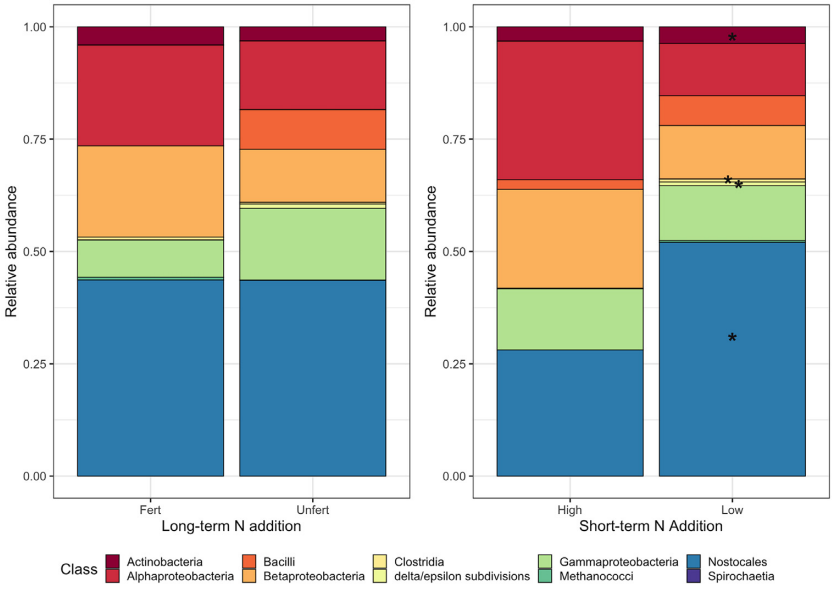

Figure 5. Average relative abundance of diazotroph classes by long-term N (Fert = Fertilized, Unfert = Unfertilized) and short-term N additions (High = High N addition, Low = Low N addition) for root-associated samples. Asterisks indicate significant difference in diazotroph class by N treatment at $P < 0.05$ with asterisk placed on the bar with greater relative abundance. Each bar represents the average of 24 samples.

= 28.26, $P = 0.0001$) under low N compared with high N. Interactions between short-term N and long-term N were not significant for any diazotroph community members.

Indicator species

We identified five diazotroph OTUs whose relative abundance in root-associated communities was associated with ANF rates measured at greater than 1 μg N fixed g^{-1} root/rhizosphere day^{-1} (Table 3). These included four Alphaproteobacteria and one Actinobacteria. All of these OTUs were identified as organisms known to commonly fix N in association with plants, though typically thought of as symbionts, and consisted of a member of the *Frankiaceae* family and four *Bradyrhizobium species*.

Root-associated nitrogen fixation rates

ANF rates, measured in association with switchgrass roots, were not significantly impacted by long-term ($F = 1.208$, $P = 0.300$) or short-term N ($F = 1.277$, $P = 0.273$). Interactions between short-term N and long-term N were not significant ($F = 0.154$, $P = 0.699$). However, there was a marginally significant difference in ANF rates across sites ($F = 4.7565$, $P = 0.0579$) with greater ANF measured at ESC and LUX relative to LC (Figure S2, Supporting Information). Rates were highly variable across N treatments (Fig. 6), even within sites, ranging from below detection to over 10 μg N fixed g^{-1} root/rhizosphere day^{-1}. Coefficients of variation (CV) of ANF rates across sites, long-term N treatments and short-term N treatments were large ranging from 109.4% to upward of 164.8% with an overall CV of 136.9%. Variation in ANF between analytical replicates for the same treatment was partic-

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Table 3. Table of OTUs whose relative abundance is associated with ANF rate bins above 1 μg N fixed g^{-1} root/rhizosphere day^{-1} as identified by *indicspecies*.

ANF rate bin	OTU #	Kingdom	Phylum	Class	Order	Family	Genus	Species	Specificity	Fidelity	P-value
>1	162	Bacteria	Actinobacteria	Actinobacteria	Frankiales	Frankiaceae	Unidentified	Unidentified	0.91	0.43	0.042
>1	265	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Bradyrhizobiaceae	*Bradyrhizobium*	*Bradyrhizobium japonicum*	0.85	0.86	0.032
>1	2343	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Bradyrhizobiaceae	*Bradyrhizobium*	*Bradyrhizobium japonicum*	0.85	0.71	0.012
1–2	2566	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Bradyrhizobiaceae	*Bradyrhizobium*	*Bradyrhizobium arachidis*	0.90	0.4	0.042
>2	777	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Bradyrhizobiaceae	*Bradyrhizobium*	*Bradyrhizobium japonicum*	1	0.33	0.034

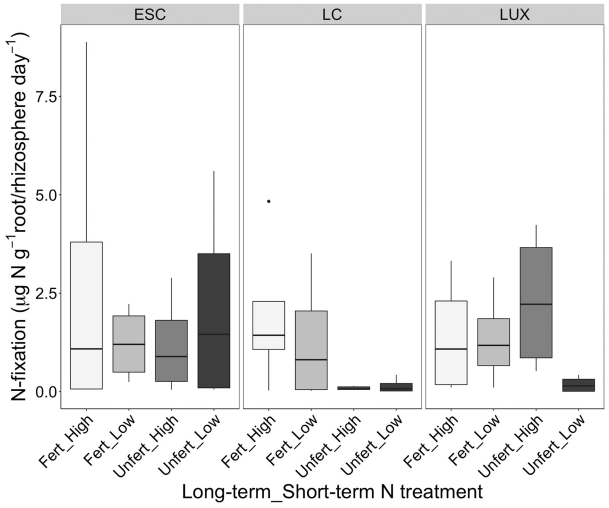

Figure 6. Root-associated nitrogen fixation rates for each site (ESC = Escanaba, LC = Lake City, LUX = Lux Arbor) by the long-term and short-term N treatments (F_High = Fertilized field + High N addition in greenhouse, F_Low = Fertilized field + Low N greenhouse, U_High = Unfertilized field + High N greenhouse, U_Low = Unfertilized field + Low N addition). Boxplots show full range of N-fixation values for each treatment with solid black horizontal bars representing average N-fixation ($n = 4$ per bar).

ularly large with an overall CV of 299.7% (Figure S3, Supporting Information). ANF rates did not correlate significantly with any diazotrophic groups or specific community members.

DISCUSSION

This work aimed to improve our understanding of ANF and its potential to contribute plant-available N to switchgrass cropping systems. We aimed to evaluate the impact of long-term and short-term N additions on ANF through measurement of ANF rates and characterization of the switchgrass root-associated diazotrophic community. We found that diazotrophic community composition of field soil was significantly different across sites, but was not influenced by long-term N additions. These initial differences in diazotrophic community structure across sites were no longer evident in root-associated communities at plant harvest. ANF rates were highly variable with no clear relationships with N additions, site or overall root-associated diazotrophic community structure.

Diazotrophic community composition in response to nitrogen availability

Perhaps the most intriguing finding of this study is the strong selective pressure that switchgrass roots exerted on the root-associated diazotrophic community. Despite soils being collected from different field sites, and displaying different initial diazotrophic communities, root samples (representing rhizosphere, rhizoplane and endosphere) collected at the end of the greenhouse experiment showed little evidence of this historical effect. Rather, there was a strong homogenization in the composition of the root-associated diazotrophic community driven by shifts in the relative abundance of specific community members.

Unlike diazotrophic communities from the field soil, which did not vary according to N treatment, short-term N treatment

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was a driver of root-associated diazotrophic community composition. Specifically, we observed that root-associated communities under the low short-term N treatment tended to cluster tighter than those under the high short-term N treatment, suggesting a greater role for selection in the assembly of these communities. Interestingly, samples under low short-term N were also found to have greater alpha diversity than those under high short-term N (Figure S4, Supporting Information). This higher diversity is not immediately consistent with greater selection, but could result from potentially larger overall population densities under low N that would lead to greater opportunities for diverse communities to assemble. The resulting community differences suggest that composition of the root-associated diazotrophic community was primarily driven by changes in switchgrass root exudation rates or root exudate chemistry and indirectly by N availability. Indeed, both total exuded C and C chemistry of switchgrass root exudates has been observed to shift in response to N availability (Smercina *et al.* 2020).

We similarly find that the abundance of some specific community members varied with both long-term and short-term N. These findings partially support our first hypothesis, demonstrating that N treatment is a driver of diazotrophic community composition. These results also support our second hypothesis that short-term N addition would have a greater impact than long-term N. Previous work has demonstrated alteration of diazotrophic community composition under long-term N fertilization (Wang *et al.* 2016; Feng *et al.* 2018; Roley *et al.* 2019). However, in our study, long-term N had negligible effects relative to short-term N additions. Overall, responses of the root-associated diazotrophic community to N treatment were limited in comparison to effects of switchgrass roots. This suggests N availability itself may be an indirect driver of root-associated diazotrophic community structure by altering attributes of the plant or other aspects of the microbial environment. Rather, direct root effects may be dominant, which are influenced by N availability.

Previous studies have observed a similar selective pressure of the roots on community composition where rhizosphere and root-associated communities were distinct from bulk soil communities (Costa *et al.* 2006; Singh *et al.* 2007; Roley *et al.* 2019). However, the role of plant species is not clear with some studies finding different plant species have distinct rhizosphere communities (Costa *et al.* 2006; Garbeva, Van Elsas and Van Veen 2008) and others finding plant species is a small or non-significant driver (Singh *et al.* 2007; Jesus *et al.* 2010). Switchgrass has specifically been observed to exert selective pressure on soil communities, cultivating a root-associated community distinct from bulk soil and other plant species (Chaudhary *et al.* 2012), with variation in the rhizosphere microbiome between switchgrass cultivars (Rodrigues *et al.* 2017). Our results do not allow us to state that the observed root-associated diazotrophic community is specifically selected by switchgrass, because we did not include a no-plant control to measure greenhouse effects. However, coupled with previous work, our study supports the concept that roots shape diazotrophic community composition. The design of our system with a sand/vermiculite dominated growth media ensured that diazotrophs surviving in rhizosphere, rhizoplane or endosphere were strictly reliant on C supplied by switchgrass roots. Thus, it is likely that the observed diazotrophic community is indicative of those organisms that can survive in the rhizosphere, rhizoplane or endosphere through metabolism of switchgrass root exudates.

In addition to selective pressures from switchgrass roots, we also acknowledge the possibility that some of the selection effect on root-associated diazotrophic communities may be due to growth conditions during the study. In particular, the sandy texture of our growth media may have selected for community members common in sandy soils, like those found at LC. Soil texture at LC was distinct from LUX and ESC, being predominately sand (~84%), while soils at LUX and ESC had a more even distribution of sand, silt and clay (Kasmerchak and Schaetzl 2018). We observed that diazotrophic communities from ESC and LUX field soil, when associated with switchgrass roots, tended to shift toward a composition similar to that observed in LC field soil (Figure S5, Supporting Information). We also found LC field soils and LC root-associated communities to be closely clustered, while soil communities clustered separately from root-associated communities for LUX and ESC field soils (Figure S5, Supporting Information). Thus, the sandy composition of our growth media may have mimicked conditions most like those at LC. Other work has shown that soil type is a major driver of bacterial and fungal communities (Singh *et al.* 2007; Garbeva, Van Elsas and Van Veen 2008; Jesus *et al.* 2010).

In addition to soil texture, greenhouse conditions could have played some role in the homogenization of the root-associated diazotrophic community. All plants were grown in the greenhouse under consistent and controlled conditions of ideal moisture, light and temperature. This homogenization of environmental conditions could have exerted selective pressure on the root-associated diazotrophic community, which would ordinarily experience dynamic and heterogeneous growth conditions in association with plants in the field (Parkin 1993). Collectively, our results suggest that switchgrass roots have the potential to select a specific and consistent diazotrophic community and that this selection is likely moderated by N availability and potentially by soil texture.

ANF in response to long-term and short-term nitrogen

ANF rates were highly variable across all sites and N treatments, ranging from below detection to over 10 μg N fixed g^{-1} root/rhizosphere day^{-1}, rates ~2–10× greater than those typically reported for switchgrass roots and associated soils (Roley *et al.* 2019; Smercina *et al.* 2019a). These rates even exceed those typically observed in other grassland systems. ANF of temperate grasslands is estimated at an average of 4.7 kg N ha^{-1} yr^{-1} with an upper limit of 20 kg N ha^{-1} yr^{-1} (Reed, Cleveland and Townsend 2011). Our highest measured rates would be roughly equivalent to 13.1 kg N ha^{-1} yr^{-1} (assuming 700 g rhizosphere m^{-2} to 15 cm depth; Roley *et al.* 2019), near the upper estimate for temperate grasslands and well above the average ANF rates for these systems. This discrepancy may be, in part, because we measured ANF rates in the root-associated material rather than the soil as a whole, and higher amounts of C proximal to roots could fuel higher ANF rates. Further, our ANF rates represent a potential as we assayed ANF under ideal conditions (i.e. C, moisture, temperature, etc.).

Neither long-term nor short-term N addition treatments predicted ANF rates in our study, countering our first hypothesis that increasing N availability would reduce ANF rates. This was surprising as N availability is thought to be a major control on ANF (Reed, Cleveland and Townsend 2011; Smercina *et al.* 2019b). However, other studies measuring ANF in association with switchgrass roots and rhizosphere have similarly observed no response of ANF to fertilizer N additions (Roley *et al.* 2018), suggesting complex N dynamics near roots.

Overall, no factors measured in this study explained the large sample variability observed (average CV = 136.9%) in ANF rates.

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Sporadic and episodic data have been observed in other measurements of switchgrass-associated ANF rates (Roley *et al.* 2018) and *nifH* transcripts (Bahulikar *et al.* 2014), suggesting that controls on ANF may also be variable or sporadic. We hypothesize that the high variability in ANF rates observed in this study may relate to heterogeneity in the distribution of diazotrophs, N availability and/or C availability. We know that unexpectedly high microbial process rates can link to spatial and temporal heterogeneity in microbes and nutrients—so-called 'hot spots' and 'hot moments' (Groffman *et al.* 2009; Kuzyakov and Blagodatskaya 2015 and references therein). Roots used in our study to measure ANF rates were chosen at random and therefore were likely heterogeneous in size/volume, diazotroph colonization and mycorrhizal associations. Additionally, root samples may have included dead and decaying roots. Differences in root size and volume, presence of dead/decaying roots and abundance of mycorrhizal associates could influence the availability of C in the rhizosphere (Jones, Nguyen and Finlay 2009; Nie *et al.* 2013). Diazotrophs are dependent on readily available C to support ANF activity, thus C is likely a major control on ANF (Smercina *et al.* 2019a). Root colonization is also likely heterogeneous, the controls of which are not well understood; thus, it is likely that capture of a highly active, root-associated diazotrophic community would occur at random.

Surprisingly, we find that site and N availability are not strong drivers of root-associated ANF rates. Further, we find no evidence that differences in timing of N availability, long-term versus short-term, impact measured ANF. Rather, our results seem to suggest that N availability, as measured at the plant to ecosystem-scale, may not accurately predict N availability at the diazotroph scale. Alternatively, these results may indicate that N availability is not a major driver of ANF, as expected from past work. More so, our results point toward C availability as the dominant driver of ANF in association with roots. This leaves large questions about whether N availability drives ANF and if/how we can predict ANF from environmental conditions.

Linking diazotrophic community composition to ANF rates

In contrast to our third hypothesis, we did not find direct evidence for an association between overall composition of the root-associated diazotrophic community and ANF rates. As with many previous studies, we were unable to establish a direct link between functional community structure and measured process rates. Shifts in relative abundance or physiology of diazotrophs and other functional guilds are difficult to assess, but are likely to be where the link between community and function or process rates resides (Jansson and Hofmockel 2018; Jansson and Hofmockel 2020 and references therein). Other studies have similarly struggled to link *nifH* diversity or *nifH*-based community structure and N-fixation function (Fürnkranz *et al.* 2008; Knief *et al.* 2012). Despite no observable link between community and function, the presence of some diazotrophs may be associated with greater ANF. Surprisingly, all OTUs indicative of greater ANF were identified as diazotrophs typical of symbiotic N-fixation including members of the *Frankiaceae* and *Bradyrhizobiaceae* families.

Bradyrhizobium species are among the most well-studied N-fixing symbionts, but their contribution to ANF is not well understood. Many *Bradyrhizobium* ecotypes are found in soils as non-symbiotic or free-living organisms (VanInsberghe *et al.* 2015) and have been shown to associate with many non-legume plants as root endophytes (Schneijderberg *et al.* 2018). They have also been previously identified in association with switchgrass roots (Bahulikar *et al.* 2014; Roley *et al.* 2019), including as root endophytes (Bahulikar *et al.* 2020), and are abundant in natural grassland systems (Delmont *et al.* 2012). It has been suggested that these free-living ecotypes lack or are diminished in their N-fixation capacity (VanInsberghe *et al.* 2015; Schneijderberg *et al.* 2018), and may even cheat plant associates (Sachs, Ehinger and Simms 2010), although they do represent the only known group of rhizobia that are able to fix N outside of symbiosis with a legume host (Regensburger *et al.* 1986; Boyd and Peters 2013; Wongdee *et al.* 2016). However, the association between *Bradyrhizobium* presence and ANF rates in our study would suggest they are actively fixing N in association with switchgrass roots. Indeed, a study of ANF associated with energy sorghum (*Sorghum bicolor*) cultivars found that those cultivars with highest root N-fixation harbored a high abundance of *Bradyrhizobium* species (Hara *et al.* 2019). This highlights a potentially important role of diazotrophs typically studied for their symbiotic N-fixing capacity to contribute N to plants when living associatively rather than symbiotically.

CONCLUSIONS

This work demonstrates that ANF has the potential to contribute to switchgrass N demands independent of N fertilization and that switchgrass roots exert selective pressure on the associated diazotrophic community. Though the switchgrass root-associated diazotrophic composition was altered under different N fertilization conditions, particularly short-term N, this did not impact measured ANF rates. Rather, this seems to suggest that switchgrass roots, likely through root exudate chemistry changes in response to N availability, exert selective pressure on diazotrophic communities. This selective pressure is evidenced by a clear root effect on diazotrophic community composition whereby effects of site on field soil diazotrophic communities were almost completely masked in the root-associated communities. Further, our results lend support to the idea that ANF is a 'hot spot/hot moment' process, occurring in discrete locations or times when conditions are optimal for microbial growth and function, as has been suggested in other work (Roley *et al.* 2018). Our findings also highlight a need to examine more closely functional groups such as diazotrophs whose role in supporting N demands of bioenergy crops, like switchgrass, has been relatively unexplored.

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DATA AND MATERIAL AVAILABILITY

The sequence data underlying this article are available in the GenBank Nucleotide Database at [https://www.ncbi.nlm.nih.gov/nucleotide/], and can be accessed with accession number KELG00000000. - also added accession number to methods.

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CODE AVAILABILITY

Code used in processing of amplicon sequence data is available from the corresponding author upon request.

SUPPLEMENTARY DATA

Supplementary data are available at *FEMSEC* online.

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Conflict of Interest. None declared.

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