THE UBIQUITOUS UNSEEN:

NITROGEN AMENDMENT OF SWITCHGRASS HAS SITE-SPECIFIC EFFECTS ON ARBUSCULAR MYCORRHIZAL DIVERSITY AND RELATIVE ABUNDANCE

by

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Abstract

Switchgrass (*Panicum virgatum* L.) is a perennial warm-season grass endemic to the tallgrass prairies of North America and is cultivated as a bioenergy feedstock and forage crop. Nitrogen amendment has variable effects on switchgrass growth. Given that switchgrass is highly dependent on symbiotic microorganisms for nutrient acquisition, we investigated the effects of nitrogen addition on arbuscular mycorrhizal fungi (AMF) in the switchgrass rhizosphere. We sampled soil from paired N-amended and control switchgrass stands at three agricultural research stations in Wisconsin. DNA was extracted from the bulk soil, amplified with the AMF-specific primers *wSSUmCf* and *wLSUmBr*, and sequenced with PacBio Sequel. The resulting amplicons were *c*. 1.5-kb long and included the partial SSU, full ITS, and partial LSU tracts of the rDNA operon. We analyzed the AMF community at the level of amplicon sequence variant for a high-resolution investigation of changes in AMF genotypes stemming from nitrogen amendment across sites. The effects of nitrogen addition (56 kg ha^{-1} yr^{-1}) were highly context dependent. Nitrogen enhanced switchgrass yield only at the northernmost site (Rhinelander). AMF diversity generally decreased with nitrogen amendment, but this was not consistent across all three sites. AMF diversity and soil parameters were differentially related to switchgrass yield, sometimes explaining up to 99.8% of the variation in yield. The relative abundance of amplicon sequence variants changed with nitrogen addition – principally ones belonging to the species *Paraglomus brasilianum* (Spain & J. Miranda) J.B. Morton & D. Redecker – indicating further investigation into intraspecific differences is warranted. Understanding the site-specific relationships among soil parameters, AMF diversity, and switchgrass yield might be important to maximize switchgrass productivity and ecosystem services on marginal lands.

Introduction

Arbuscular mycorrhizal fungi (AMF) are ubiquitous soil-dwelling organisms in Phylum Glomeromycota that associate with upwards of 80% of vascular plant families in symbiotic relationships called mycorrhizae (Schüßler *et al.*, 2001). AMF can confer many benefits to their host including pathogen protection, drought tolerance, and water acquisition, but principal among them is the transfer of nutrients such as phosphorus and nitrogen. The AMF mycelium mines the soil volume for mineral nutrients and transfers these to the host via intraradical structures called arbuscules in exchange for photosynthetically fixed carbon (Smith & Read, 2008). In recent years, global interest in sustainable agricultural intensification has brought AMF to the forefront as natural biofertilizers (Rillig *et al*., 2016). However, vast knowledge gaps remain concerning the ability of AMF to efficiently transfer nutrients to the host while maintaining high yields (Hart *et al.*, 2017; Hoeksema *et al.*, 2010; Thirkell *et al*., 2017).

Nitrogen is an especially important nutrient to manage in agricultural systems because it is widely applied to crops and is associated with negative impacts on the environment and human health (Robertson & Vitousek, 2009). Once applied to a landscape, synthetic nitrogen fertilizer can be converted to nitrous oxide – a potent greenhouse gas (Jenkinson, 2001). Nitrate leaching into groundwater is a major public health concern as contaminated drinking water can cause methaemoglobinaemia, or blue baby syndrome (Knobeloch *et al.*, 2013; Knobeloch & Proctor, 2001). Furthermore, downstream pollution can lead to the collapse of aquatic ecosystems as a result of eutrophication and dead zones (Cameron *et al.*, 2013). AMF reduce nitrogen leaching by taking up and transferring substantial amounts to the host plant, but these benefits are dependent on the particular AMF species involved and edaphic properties of a given area (Asghari & Cavagnaro, 2012; Bender *et al.*, 2015; Leigh *et al.*, 2009; van der Heijden,

2010). Consequently, tailored management of AMF communities to achieve those most efficient at the uptake and transfer of nitrogen to plant hosts could be a means to better deliver, recycle, and retain nitrogen within ecosystems. However, more research is required to understand the effects of nitrogen on complex AMF communities in the field (Bender & Van der Heijden, 2015; Cotton, 2018).

To investigate the impacts of nitrogen on AMF diversity, we studied the mycorrhizal communities associating with switchgrass (*Panicum virgatum* L.) in nitrogen-amended and unamended stands throughout Wisconsin. Switchgrass – a warm-season plant endemic to the tallgrass prairies of North America – is cultivated as a cellulosic biofuel feedstock and forage crop for cattle (Parrish & Fike, 2005). This crop provides a number of important ecosystem services such as biodiversity habitat for grassland birds and soil carbon sequestration (Robertson *et al.*, 2017). However, switchgrass cultivation is hampered – in part – by the unpredictable relationship between nitrogen and plant growth. In a two-year agronomic trial, nitrogen amendment did not increase switchgrass biomass and resulted in triple the quantity of nitrous oxide emissions compared to unamended plots (Duran *et al.*, 2016). Similar results were found by Jach-Smith & Jackson (2015) and Ruan *et al.* (2016). On the other hand, a global meta-analysis reported that nitrogen fertilization significantly increased switchgrass growth (Heaton, *et al.*, 2004). Duran *et al*. (2016) hypothesized that this variation in switchgrass response is caused by the different rates at which microbial organisms immobilize nitrogen. Since switchgrass is highly mycorrhizal-dependent (Hetrick *et al.*, 1988), AMF are likely an important mediator of nitrogen uptake in switchgrass cropping systems.

While AMF facilitate nitrogen acquisition for switchgrass (Leigh *et al.*, 2009; Schroeder-Moreno *et al.*, 2012), exogenous nitrogen inputs can decrease the abundance and functioning of

AMF (Jach-Smith & Jackson, 2019; Oates *et al.*, 2016), potentially negating their symbiotic benefits. Jach-Smith & Jackson (2018) found that nitrogen decreased AMF root colonization and allocation of resources to nutrient-transfer structures. In a follow-up study, Jach-Smith & Jackson (2019) showed that nitrogen addition effectively replaced nitrogen that otherwise would have been supplied to switchgrass by AMF. Conversely, in a microcosm experiment, elevated nitrogen did not influence mycorrhizal-mediated nitrogen uptake and transfer to switchgrass (Schroeder-Moreno *et al.*, 2012). Moreover, Emery *et al.* (2017) found that nitrogen amendment did not affect AMF root colonization or extraradical hyphae, but it decreased AMF diversity. In summary, the effects of nitrogen on AMF are context-dependent and vary from study to study making it difficult to discern a general pattern.

We hypothesized that the uncertainty about nitrogen's effects on AMF and switchgrass productivity could stem from the limited taxonomic resolution of traditional methodologies. For example, the use of staining procedures to measure root colonization can fail to detect certain groups – namely, the Paraglomerales – with weakly staining or completely non-staining tissues (Błaszkowski *et al.*, 2017; Wheeler, 2017). Additionally, the 16:1ω5*cis* fatty acid biomarker commonly used to measure AMF abundance in soils (Joergensen & Wichern, 2008; Oates *et al.,* 2017) is only present at miniscule quantities or is completely absent from certain groups of AMF (Graham *et al.*, 1995). Moreover, fatty acid indicators for Paraglomeraceae and Gigasporaceae (e.g., 16:1ω7*cis*, 18:1ω9*cis*, 20:1ω9*cis*) are typically treated as biomarkers for saprotrophic fungi and gram-negative bacteria, conflating the abundance of multiple guilds (Bentivenga & Morton, 1994; Graham *et al.*, 1995; Ngosong *et al.,* 2012). Finally, the conventional use of the SSU gene region with high-throughput, short-read sequencing platforms for AMF metabarcoding does not discriminate AMF to the species level (Cotton, 2018; Schlaeppi *et al.,* 2016; Tedersoo *et al.*,

2018). This problem is compounded when operational taxonomic units are clustered at 97% similarity – a highly conservative threshold and arbitrary proxy for species-level delimitation of filamentous fungi (Callahan *et al.*, 2017; Egan *et al*., 2018; Vu *et al*., 2019).

Higher taxonomic resolution may improve our understanding of AMF responses to nitrogen management and their relationships to plant growth. Phylum Glomeromycota has evolved extensive genotypic and physiological diversity over 500 million years, which is likely masked by methodologies with coarse taxonomic resolution (Lee *et al.*, 2013). For example, abundance of AMF nutrient-transfer structures has no inherent meaning for plant performance given that AMF species exist along a symbiotic spectrum ranging from mutualism to parasitism (Johnson & Graham, 2013). In fact, alternative strains *within* a given AMF species can result in completely different outcomes for plant performance (Ehinger *et al.*, 2012; Mensah *et al.*, 2015). Resolving AMF at the species level or below should help elucidate AMF functional diversity and its effects on plant growth and broader ecosystem functioning (Powell & Rillig, 2018).

In the context of a long-term, replicated bioenergy cropping systems experiment, we characterized AMF communities to the level of amplicon sequence variant (ASV) for a high-resolution investigation of the impacts of nitrogen on AMF. ASVs are exact, error-free sequences and thus serve as biologically meaningful representatives of AMF genotypes (Callahan *et al.,* 2017). Next-generation sequencing platforms like PacBio Sequel offer comparatively long sequence reads and an unprecedented opportunity to investigate AMF at this fine taxonomic resolution, revealing intraspecific dynamics and strains of interest for bioprospecting. Specifically, we asked: (1) Does nitrogen amendment affect AMF ASV diversity? (2) Does AMF ASV diversity correlate with switchgrass yield? And (3) Are some ASVs more sensitive than others to the addition of nitrogen? We hypothesized that nitrogen

amendment would decrease AMF diversity and that AMF diversity would positively correlate with switchgrass yield. Furthermore, we hypothesized that ASVs would change in relative abundance in response to nitrogen addition.

Methods

Sites and experimental design

The Great Lakes Bioenergy Research Center designed the Marginal Land Experiment (MLE) to test the efficacy of growing perennial biofuel feedstock crops on abandoned agricultural fields and land deemed unsuitable for high-productivity agriculture. Three sites were established each in Wisconsin and Michigan. This study focused on the Wisconsin sites only: Rhinelander in northern Wisconsin, Hancock in the central part of the state, and Oregon in the south (Fig. 1).[1] Mean soil physicochemical properties of the three sites are presented in Table 1. For a comprehensive assessment of the soils at each site, see Kasmerchak & Schaetzl (2018).

Monocultural plots of switchgrass (*Panicum virgatum* variety "Cave-in-Rock") measuring 19.5 x 12.2 m were planted in 2013. In 2015, our independent variable – nitrogen treatment – was randomly applied to the plots in a paired experimental design with one half receiving no nitrogen amendment (control) and the other half receiving 56 kg N ha^{-1} (amended) applied annually as granular ammonium nitrate. Thus, the experimental units in this study were the paired half plots (19.5 x 6.1 m). Rhinelander and Oregon each consisted of four replicates per treatment while Hancock consisted of three for a total sample size of 11. See Figure 1 for a map of the sites and diagram of the paired experimental units.

Agronomic and soil data

[1] For information on the ecology and land-use history of each site, see https://lter.kbs.msu.edu/research/long-term-experiments/marginal-land-experiment/

Switchgrass yields were determined in November 2018 by harvesting switchgrass with a combine, leaving 15.25 cm residual stubble. Following switchgrass harvest, soil cores from each experimental unit were taken to a depth of 25 cm and analyzed for pH, phosphorus quantity (ppm), and cation exchange capacity (milli equivalents per 100 g soil) by the University of Wisconsin Soil and Forage Laboratory. Soil samples from August (see below for sampling procedure) were analyzed with a Flash EA 1112 Flash Combustion Analyzer for total carbon and nitrogen determination.

Soil sampling and processing for sequencing

In August 2018, a sliding hammer soil corer fitted with a five-cm diameter head was used to extract soil cores to a depth of 15 cm. Three soil cores spaced 1.8 meters from the edge of the plot and one meter from each other were collected in each experimental unit (Fig. 1). The cores were placed into Whirlpak bags and transported in an iced cooler. Within 8 h, soil samples were transferred to a -80˚C freezer for storage prior to sieving. Soil samples were manually disintegrated through a 2-mm sieve. Sieved soil was returned to the Whirlpak bags and preserved at -80˚C until DNA extraction.

DNA extraction and sequencing

DNA was extracted from soil samples with DNeasy PowerSoil Kit from Qiagen (Qiagen catalog #12888-100) according to the manufacturer's instructions. DNA concentration was measured with a Thermo Scientific NanoDrop 1000 Spectrophotometer to determine dilution ratios for PCR. Extracts were amplified using the AMF-specific wobble primers *wSSUmCf* (5'-TAT YGY TCT TNA ACG AGG AAT C-3') and *wLSUmBr* (5'-AAC ACT CGC AYA YAT GYT AGA-3'), which span an rDNA fragment consisting of the partial small subunit (pSSU), whole internal transcribed spacer (ITS), and partial large subunit (pLSU; Krüger *et al.*, 2009;

Schlaeppi *et al.*, 2016). Dried, 12-nmol RxnReady® Primer Pools (forward and reverse primers premixed) were manufactured by Integrated DNA Technologies according to PacBio Sequel specifications, namely the addition of a 5' PacBio universal sequence and HPLC purification.[2]

PCR reactions were prepared using the Phusion High-Fidelity PCR Kit from New England Biolabs (NEB catalog #E0553L) in 25 µL reactions: 1 µL of template DNA at a concentration of 0.1-40 ng μL^{-1}, 2.5 µL 10 µM primers, 0.5 µL 10 mM dNTPs, 0.25 µL Phusion DNA Polymerase, 5 µL 5X Phusion HF Buffer, and 15.75 µL water. PCR reactions were run on an Eppendorf Mastercycler® pro S thermal cycler using the parameters specified by Schlaeppi *et al.* (2016): 2 min initial denaturation at 98°C, 40 cycles of 10 s denaturation at 98°C, 30 s annealing at 60°C, and 1 min elongation at 72°C, with a final elongation of 10 min at 72˚C. Of the 66 soil samples, 48 (at least two samples per experimental unit) were selected for sequencing. Samples were submitted to University of Wisconsin Biotechnology Center and purified using AMPure XP beads, ligated with universal hairpin adapters for circular sequencing, and barcoded with sample-specific primers. The 48 samples were pooled based on molarity and sequenced on one PacBio Sequel SMRT Cell with Sequel Sequencing Kit 3.0 chemistry.

Bioinformatics

PacBio Sequel subreads were demultiplexed with SMRT Analysis 6.0.0.47841 (*lima* 1.7.0) and assembled into circular consensus sequences (CCS) with *pbccs* 3.4.1. Sequences with fewer than five passes and a minimum predicted consensus accuracy less than the PacBio default (~ 95%) were removed, resulting in a pre-filtering error rate comparable to Illumina MiSeq (Schlaeppi *et al.,* 2016). CCS BAM files were converted to FASTQ files and their quality scores

[2] For more information on the primer-specific requirements for successful PacBio sequencing, see https://www.pacb.com/wp-content/uploads/2015/09/Procedure-and-Checklist-Preparing-SMRTbell-Libraries-PacB-Barcoded-Universal-Primers.pdf

reformatted to the conventional 0-41 system using the bash script reformat.sh from *BBMap* 38.50 (Bushnell, 2019). CSS FASTQ files were processed with *DADA2* 1.12.1 (Callahan *et al.,* 2016, 2019) according to the workflow and recommendations of the "*DADA2* + PacBio" tutorial (Callahan *et al.*, 2019)[3] on a MacBook Pro (early 2011 model) in about one hour using R 3.6.0 (R Core Team, 2019). Taxonomy was assigned to ASVs using a custom FASTA database consisting of representative NCBI pSSU-ITS-pLSU sequences from 11 of the 12 taxonomic families (excluding Geosiphonaceae, a monotypic and non-mycorrhizal family) and 31 of the 37 AMF genera present in Phylum Glomeromycota (Schüßler, 2019).

Data analysis

Data analysis and statistical tests were conducted in R 3.6.0 (R Core Team, 2019). All response variables were summed or averaged by experimental unit. The ASV community composition matrix was visualized using nonmetric multidimensional scaling (NMDS) with *phyloseq* 1.28.0 (McMurdie & Holmes, 2013), excluding rare ASVs with fewer than five representatives. Alpha diversity metrics (richness, Shannon index, and Simpson's index) were calculated using *vegan* 2.5.5 (Oksanen *et al.,* 2019). For each site separately, we tested the effect of nitrogen treatment on the dependent variables (switchgrass yield, AMF diversity, and soil parameters) using random-intercept linear mixed-effects models with *lme4* 1.1.21 (Bates *et al.*, 2015). Nitrogen was included as a fixed effect and plot as a random effect. To test for relationships between AMF diversity and switchgrass yield, we specified yield as the response variable and added AMF diversity metrics as fixed effects to models containing nitrogen and plot. We calculated p-values using likelihood ratio tests of the full model with the fixed effect in question against the reduced model that excluded the fixed effect of interest. We calculated

[3] The online tutorial is available at https://benjjneb.github.io/LRASManuscript/LRASms_fecal.html

conditional and marginal coefficients of determination for each linear mixed-effects model with *piecewiseSEM* 2.0.2 (Lefcheck, 2016). Parameter coefficients are reported as the model estimate ± one standard error. Finally, to investigate the response of individual ASVs to nitrogen addition, we followed the recommendations of McMurdie & Holmes (2014). We did not rarefy our ASV matrix and instead analyzed differential abundance using a Wald test with a negative binomial distribution in *phyloseq* and *DESeq2* 1.24.0 (Love, Huber, & Anders, 2014). Assumptions of normally distributed errors and homogeneity of variance for linear models were visually checked with QQ-plots and residual plots.

Results

Sequencing results

PacBio Sequel produced a total of 373,811 circular consensus sequences. The *DADA2* software filtered approximately 72.8% of these resulting in a total of 101,773 non-chimeric sequences. See Table 2 for a full breakdown of the number of sequences remaining after each filtering step. The non-chimeric sequences clustered into 412 ASVs spread across four orders, eight families, and 19 genera in Glomeromycota (Fig. 2). NMDS was an accurate depiction of the community dissimilarity matrix (stress = 0.059) and revealed strong clustering of ASV communities by site but no pattern of clustering by nitrogen treatment. There was no consistent direction in which communities moved in ordination space with the addition of nitrogen relative to the paired control (Fig. 3).

Effects of nitrogen addition on switchgrass yield, AMF diversity, and soil parameters

At Rhinelander, nitrogen amendment affected yield, AMF richness, and Shannon index, with marginally significant effects on soil C:N ratio and phosphorus. Nitrogen amendment increased yield ($\chi^2_{(1)} = 13.20$, $p = 0.0003$) by about 1.7 ± 0.2 Mg ha^{-1}. AMF richness and

Shannon index both decreased with nitrogen addition ($\chi^2_{(1)} = 4.50$, $p = 0.03$; $\chi^2_{(1)} = 5.82$, $p = 0.012$) by about 4 ± 1.5 ASVs and 0.4 ± 0.1 index units, respectively (Table 3, Figure 4).

Unlike at Rhinelander, switchgrass yield did not increase with nitrogen amendment at Hancock ($\chi^2_{(1)} = 2.37$, $p = 0.12$). However, AMF richness, Shannon index, and C:N ratio all decreased with the addition of nitrogen. Richness decreased by about 14 ± 6 ASVs ($\chi^2_{(1)} = 4.31$, $p = 0.04$) and Shannon index decreased by about 0.5 ± 0.2 index units ($\chi^2_{(1)} = 4.40$, $p = 0.04$; Figure 4). C:N ratio responded strongly to nitrogen, decreasing by 0.76 ± 0.12 ($\chi^2_{(1)} = 12.12$, $p = 0.0005$; Table 3).

Finally, at Oregon, switchgrass yield *decreased* by 0.8 Mg ha^{-1} with nitrogen addition, although this effect was not significant ($\chi^2_{(1)} = 2.12$, $p = 0.15$). Soil percent carbon was the only parameter that was significantly affected by nitrogen amendment ($\chi^2_{(1)} = 6.29$, $p = 0.01$), increasing by about 0.2 ± 0.05 percentage points (Table 3).

In summary, switchgrass yield had a variable response to nitrogen amendment with significant increases at Rhinelander only. AMF richness and Shannon index generally decreased with nitrogen amendment, but this was not consistent across all three sites (Figure 4). With a few exceptions, soil parameters were not significantly affected by nitrogen amendment.

Relationship between switchgrass yield and AMF diversity and soil parameters

Switchgrass yield was related to various soil parameters and AMF diversity metrics, but the direction and magnitude of the relationship differed depending on the site. At Rhinelander, switchgrass yield was negatively associated with AMF richness ($\chi^2_{(1)} = 5.47$, $p = 0.02$), decreasing by about 0.05 ± 0.01 Mg ha^{-1} for every additional AMF ASV, holding nitrogen treatment constant. At Oregon, this relationship was the opposite with switchgrass yield increasing by 0.08 ± 0.03 Mg ha^{-1} with every additional AMF ASV ($\chi^2_{(1)} = 4.92$, $p = 0.03$). At

Hancock, switchgrass yield and AMF richness were not significantly related ($\chi^2_{(1)} = 0.50$, p = 0.48). Shannon index showed a different pattern with a positive relationship with switchgrass yield at Rhinelander ($\chi^2_{(1)} = 8.44$, p = 0.004, -0.7 ± 0.1) but nonsignificant relationships at Hancock and Oregon ($\chi^2_{(1)} = 2.96$, p = 0.09, 0.8 ± 0.4; $\chi^2_{(1)} = 2.24$, p = 0.13, 0.9 ± 0.5; Table 4).

At all three sites there was a positive relationship between soil percent N and switchgrass yield. This relationship was strongest at Hancock where an increase of one percentage point in soil N was associated with an increase in yield of 26.33 Mg ha^{-1}, holding nitrogen treatment constant ($\chi^2_{(1)} = 9.70$, p = 0.002). At Rhinelander and Oregon, a similar increase in soil percent N was associated with an increase in yield of 19.63 and 11.36 Mg ha^{-1}, respectively ($\chi^2_{(1)} = 6.74$, p = 0.009, $\chi^2_{(1)} = 5.30$, p = 0.02). Other notable soil parameters included phosphorus, which had a positive relationship with switchgrass yield at Hancock; cation exchange capacity, which also showed a positive relationship to switchgrass yield at Hancock as well as Rhinelander; and C:N ratio, which had a negative relationship with switchgrass yield at both Rhinelander and Oregon. See Table 4 for a complete breakdown of the regression models relating soil and AMF diversity parameters to switchgrass yield.

In addition to its association with switchgrass yield, soil pH was strongly associated with AMF diversity metrics. At Rhinelander, one unit change in pH corresponded to an increase in 24 ± 2.8 ASVs ($\chi^2_{(1)} = 10.39$, p = 0.001), 1.97 ± 0.21 Shannon index units ($\chi^2_{(1)} = 17.71$, p = 0.0003), and 0.46 ± 0.12 Simpson's index units ($\chi^2_{(1)} = 7.31$, p = 0.007). In contrast, at Oregon there was a negative relationship between pH and AMF diversity. Holding nitrogen treatment constant, one unit change in pH was associated with a loss of 17.6 ± 6.5 ASVs ($\chi^2_{(1)} = 5.23$, p = 0.02), 1.52 ± 0.34 Shannon index units ($\chi^2_{(1)} = 9.11$, p = 0.003), and 0.22 ± 0.05 Simpsons index units ($\chi^2_{(1)} = 8.52$, p = 0.004). Soil pH was not significantly associated with AMF diversity at

Hancock ($p > 0.15$). Instead, phosphorus content was positively associated with AMF richness ($\chi^2_{(1)} = 2.74$, $p = 0.098$), Shannon index ($\chi^2_{(1)} = 7.62$, $p = 0.006$), and Simpson's index ($\chi^2_{(1)} = 7.02$, $p = 0.008$).

In summary, at all three sites one or more metrics of AMF diversity were significantly associated with switchgrass yield. Soil parameters were also correlated with yield at all three sites. Almost all of the variation in switchgrass yield could be explained by our predictors, especially soil parameters. For example, at Rhinelander, 98.2% of the variation in switchgrass yield could be explained by a linear mixed-effects model containing nitrogen, soil C:N ratio, and plot (Table 4). At Hancock, 99.3% of the variation in switchgrass yield could be explained by soil phosphorus content as the fixed effect in addition to nitrogen treatment. Finally, at Oregon, 63.5% of the variation in switchgrass yield was explained when soil pH was included in the model. AMF diversity was significantly associated with soil parameters, especially pH, but the direction of this relationship differed depending on site.

Effects of nitrogen addition on ASV relative abundance

The negative binomial Wald test identified 11 ASVs that changed in relative abundance as a result of nitrogen addition (Table 5, Fig. 6). Three ASVs responded positively, all in the genus *Paraglomus* (order Paraglomerales, family Paraglomeraceae). A manual BLAST search of the NCBI GenBank database identified these ASVs as *Paraglomus brasilianum* (Spain & J. Miranda) J.B. Morton & D. Redecker (query coverage = 100%, percent identical > 98.9% to *P. brasilianum* clone pCK084-12, GenBank Accession FR750048). Eight ASVs had a negative response to nitrogen amendment. Seven of these belonged to *Paraglomus* and one to *Dominikia* (order Glomerales, family Glomeraceae). A manual BLAST search identified five of the seven *Paraglomus* ASVs as *P. brasilianum* with high percent identity to the same pCK084-12 clone

(query coverage = 100%, percent identical; > 99.8%). The three remaining ASVs could not be confidently identified to species according to BLAST results.

Discussion

Nitrogen amendment of switchgrass negatively affected AMF diversity, although this was not a universal pattern across the three sites. As indicated by NMDS, each site consisted of communities that were more similar to each other than to other sites. Along with differences in soil parameters and climatic variables, a consortium of variables appeared to interact in unknown ways resulting in large context dependency in relationships between nitrogen, switchgrass yield, soil parameters, and AMF community composition. While our limited number of sites did not allow us to test the relationship between environmental gradients and response variables across Wisconsin, given the high taxonomic resolution of AMF communities achieved with PacBio we could generate hypotheses according to AMF natural history.

At Rhinelander, nitrogen amendment resulted in a complete loss of *Dentiscutata* and *Glomus* spp. ASVs. At Hancock, nitrogen amendment coincided with the loss of ASVs belonging to *Claroideoglomeraceae* and the appearance of *Acaulosporaceae* ASVs. At Oregon, nitrogen amendment also resulted in the appearance of genera belong to *Acaulosporaceae*. According to Chagnon *et al.* (2013), AMF species in the *Acaulosporaceae* family embody characteristic of a stress-tolerant life history strategy. *Acaulosporaceae* tend to be more common in adverse conditions that limit plant carbon fixation. It is interesting to note that sites in which nitrogen treatment coincided with the appearance of *Acaulosporaceae* ASVs were the same sites where switchgrass yield did not respond to nitrogen amendment. This might indicate that increased nitrogen deposition caused heightened plant stress at Hancock and Oregon, potentially limiting growth and shifting the AMF community towards stress-tolerant species.

AMF diversity was significantly associated with switchgrass yield, particularly at Rhinelander and Oregon. Analysis of edaphic properties revealed soil parameters that may be important mediators of the switchgrass-AMF symbiosis. For example, soil pH was associated with both AMF diversity and switchgrass yield at Rhinelander and Oregon. At both sites, yield tended to increase in more acidic conditions. AMF richness had orthogonal responses to soil pH, showing a positive relationship at Rhinelander and negative relationship at Oregon. In other words, at Oregon greater acidity was associated with more yield and more AMF richness. At Rhinelander, greater acidity was associated with more yield but less AMF richness. Soil pH could affect AMF community composition and the benefits conferred by the mycorrhizal community, resulting in the negative relationship between pH and yield. In a study measuring the relative benefits of AMF associating with alpine strawberry, Hayman & Tavares (1985) found that AMF species differed in the pH at which the authors observed the most enhanced plant growth. Species in *Acaulosporaceae* and *Gigasporaceae* are known to prefer acidic soil and may enhance mineral nutrient acquisition at low pH (Clark, 1997). Lower pH may have coincided with a shift in AMF communities towards more *Acaulosporaceae* and *Gigasporaceae* ASVs with subsequent improvements in plant-acquisition of mineral nutrients. The differential changes in AMF richness associated with soil pH requires further studies into the competitive dynamics of the communities present at these different sites under varying pH regimens.

Certain ASVs changed in relative abundance in response to nitrogen addition, especially in the species *Paraglomus brasilianum,* which stands out for producing extensive mycelial networks (Wheeler, 2019). According to the functional equilibrium model, plants invest less carbon in their mycorrhizal partners when amended with nitrogen because they no longer need AMF to acquire it for them (Johnson, 2010). The mycelium of *P. brasilianum* comes at a large

carbon cost to the host and thus should be selected against in high nitrogen environments (Chagnon *et al.*, 2013; Treseder *et al.,* 2018). The fact that different ASVs of *P. brasilianum* responded in opposite directions to nitrogen suggests that there could be intraspecific differences in their physiological tolerance to nitrogen or relative benefit to switchgrass. A paucity of ecological studies on species in the order Paraglomerales makes further interpretations difficult. In our study, the notable abundance of *P. brasilianum* in the switchgrass rhizosphere and its dynamic response to nitrogen warrants further research into the relative benefits that this species confers to switchgrass.

This study is the first to employ PacBio Sequel with amplicon sequence variants in the analysis of AMF communities and one of only a handful to use PacBio for AMF metabarcoding in general (Banerjee *et al*., 2018; Bender *et al.*, 2019; Schlaeppi *et al*., 2016; Symanczik *et al.*, 2017). PacBio is criticized for a low sequencing depth and high error rate, which may deter its broader use in the field (Kennedy *et al.,* 2018). While PacBio Sequel has a lower sequencing depth than Illumina MiSeq and therefore may not fully detect microbial communities, this is not a problem for low-diversity groups like Glomeromycota (Tedersoo *et al.,* 2018; Vasar *et al*., 2017). Furthermore, sequencing depth may be a moot point with increased access to PacBio Sequel 2, which generates up to 4 million reads compared to Sequel's 500,000. With regard to a high error rate, the circularization and multiple sequencing passes of individual DNA molecules results in an error rate comparable to – or even less than – that of leading platforms (Schlaeppi *et al.*, 2016). In conjunction with the use of error-learning bioinformatics algorithms like *DADA2*, the average number of erroneous bases is less than one for every 2000 nucleotides, resulting in single-nucleotide resolution for medium-length amplicons (Callahan *et al*., 2019). The fine taxonomic resolution achieved with PacBio Sequel and amplicon sequence variants would be

complemented by traditional methodologies like lipid analysis and root colonization measurements if such methodologies could better detect obscure groups like the Paraglomerales. Incidentally, in our study Paraglomerales was the most represented order in switchgrass cropping systems. We hope this encourages others to develop methods to explore the relative benefits that *Paraglomus* spp. and other understudied AMF species confer to switchgrass growth.

In conclusion, improved switchgrass cultivation could benefit from greater context-specific knowledge. Consistent with previous research, our study found that switchgrass yield was enhanced by nitrogen amendment at some locations but not at others. The context dependency of relationships between AMF diversity, switchgrass yield, and soil parameters indicates that researchers, extension agents, and land managers should treat generalizations of AMF ecology as starting points for hypotheses rather than universal patterns for decision making. Furthermore, the negative correlation between yield and ASV richness at Rhinelander was contrary to the popular conception that greater AMF richness is inherently better for plant performance. Given the limitations of our current understanding of the species-level diversity of AMF and their functional ecology, soil properties like pH – and not AMF community – might be useful factors to consider when selecting a location to cultivate switchgrass.

Data Availability

Reproducible bioinformatics scripts, statistical analyses, and R markdown documents are available at https://github.com/aldendirks/amf_metabarcoding.

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Tables

Table 1. Site descriptions with Ap horizon soil properties (Kasmerchak & Schaetzl, 2018).

Site			Soil properties						
Name	Latitude	Longitude	Texture	C:N	pH	Sand [%]	Silt [%]	Clay [%]	Bulk density [g cm^{-3}]
Rhinelander	45° 39' 56.16" N	89° 13' 4.8" N	Sandy loam	15.9	5.7	59.1	25.8	15.1	1.35 ± 0.20
Hancock	44° 7' 9.84" N	89° 32' 1.68" W	Loamy sand	12.7	6.3	87.6	5.9	6.5	1.44 ± 0.03
Oregon	42° 57' 57.96" N	89° 21' 21.96" W	Silt loam	10.2	6.9	9.1	74.8	16.1	1.11 ± 0.37

Table 2. Sequences retained through each of the steps of the *DADA2* bioinformatics pipeline. "CCS" (circular consensus sequences) is the number of sequences that were generated with a minimum of five passes during PacBio sequencing. "Primers" is the number of sequences that contained the AMF-specific pSSU-ITS-pLSU primer sequences with a maximum of two mismatches. "Filtered" is the number of sequences that had a quality score greater than or equal to three, expected error less than or equal to two, and sequence length between 1000 and 1600 base pairs. "Denoised" is the number of sequences that were inferred as ASVs according to the PacBio error-learning algorithm of *DADA2*. "Non-chimeric" is the number of sequences remaining after removing chimeric ones. Finally, the last column is the percent of sequences retained at the end of the pipeline (non-chimeric sequences divided by circular consensus sequences). The bottom two rows of the table show the averages and sums of each column.

Site	Block	Subsample	Nitrogen treatment	CCS	Primers	Filtered	Denoised	Non-chimeric	Retained (%)
Rhinelander	R1	B	amended	7625	4831	2847	1991	1991	0.261115
Rhinelander	R1	C	amended	9580	5826	3908	3200	3200	0.334029
Rhinelander	R1	E	control	7971	5182	2952	1250	1250	0.156818
Rhinelander	R1	F	control	4284	3253	1239	896	896	0.20915
Rhinelander	R1	G	control	4735	3284	1593	1040	1040	0.219641
Rhinelander	R2	A	amended	7443	5044	1676	128	128	0.017197
Rhinelander	R2	B	amended	8115	4925	2866	1896	1896	0.233641
Rhinelander	R2	E	control	9348	5804	2782	1560	1560	0.166881
Rhinelander	R2	F	control	9673	5991	2920	2044	2044	0.21131
Rhinelander	R2	G	control	1760	1298	437	67	67	0.038068
Rhinelander	R3	A	amended	6316	4062	1678	993	993	0.15722
Rhinelander	R3	B	amended	9902	6326	2783	1398	1398	0.141184
Rhinelander	R3	C	amended	10029	6077	3566	2810	2810	0.280187
Rhinelander	R3	E	control	3183	2171	1125	706	706	0.221803
Rhinelander	R3	G	control	8523	5321	3554	3323	3038	0.356447
Rhinelander	R4	B	amended	8769	5423	3636	2909	1811	0.206523
Rhinelander	R4	C	amended	9470	5818	3396	2493	2493	0.263252
Rhinelander	R4	E	control	9121	6126	3146	2644	2644	0.289881
Rhinelander	R4	G	control	3950	3154	952	649	649	0.164304
Hancock	R2	A	amended	8132	5147	3209	2888	2888	0.35514
Hancock	R2	C	amended	7178	4908	3177	2983	2751	0.383254
Hancock	R2	E	control	6446	5139	2333	1853	1845	0.286224
Hancock	R2	G	control	6444	4650	2569	2112	2104	0.326505
Hancock	R3	A	amended	2947	2327	936	851	786	0.266712
Hancock	R3	C	amended	6515	4355	2025	1576	1493	0.229163
Hancock	R3	E	control	10195	6521	3282	2582	2514	0.246591
Hancock	R3	G	control	9905	6106	3445	2308	2169	0.21898
Hancock	R4	A	amended	8902	5389	3565	3084	2926	0.32869
Hancock	R4	B	amended	10559	6427	2398	1886	1813	0.171702
Hancock	R4	C	amended	2314	1705	992	862	788	0.340536
Hancock	R4	E	control	10105	6556	3870	2611	2442	0.241663
Hancock	R4	G	control	10458	6046	4283	3954	3648	0.348824

Oregon	R1	A	amended	9277	5655	3248	2594	2585	0.278646
Oregon	R1	B	amended	12384	7141	5321	4790	4659	0.376211
Oregon	R1	E	control	190	165	68	38	38	0.2
Oregon	R1	G	control	11320	6654	4065	3281	3043	0.268816
Oregon	R2	A	amended	11048	7024	5452	5379	5346	0.483888
Oregon	R2	B	amended	11959	6965	4899	4705	3880	0.324442
Oregon	R2	E	control	10351	6200	4610	4336	3548	0.342769
Oregon	R2	G	control	9263	5931	4022	3416	3354	0.362086
Oregon	R3	A	amended	8433	4975	3306	2756	2631	0.311989
Oregon	R3	B	amended	6795	4844	2629	2139	1975	0.290655
Oregon	R3	E	control	9754	6673	4083	3700	2977	0.305208
Oregon	R3	G	control	5337	4038	1480	1099	1099	0.205921
Oregon	R4	A	amended	10589	6518	4108	3284	3223	0.304372
Oregon	R4	C	amended	10295	6111	3504	2695	2495	0.242351
Oregon	R4	E	control	6785	4790	2681	2275	2128	0.313633
Oregon	R4	F	control	134	126	32	11	11	0.08209
AVERAGE				**7788**	**4979**	**2847**	**2251**	**2120**	**25.76**
SUM				**373811**	**239002**	**136648**	**108045**	**101773**	**–**

Table 3. Results of likelihood ratio tests comparing the effects of nitrogen addition on the specified response variable compared to a null model without nitrogen treatment. Conditional R^2 (proportion of variance explained by both the fixed and random effects) and marginal R^2 (proportion of variance explained by the fixed effects alone) are also reported. Rows highlighted in green represent dependent variables that were significantly affected by nitrogen addition ($p \leq 0.05$). Dependent variables in rows highlighted in orange were only marginally significantly affected by nitrogen addition ($0.05 < p \leq 0.1$)

Site	Dependent variable	$\chi^2_{(1)}$	P value	Avg. change	Standard error	Conditional R^2	Marginal R^2
Rhinelander	Yield	13.2	0.0003	1.7	0.2	0.922	0.768
	Richness	4.5	0.034	-4.3	1.5	0.939	0.073
	Shannon index	5.8	0.012	-0.4	0.1	0.957	0.081
	Simpson's index	2.6	0.11	1.7	0.2	0.818	0.093
	pH	1.005	0.3	0.05	0.05	0.958	0.007
	C (%)	0.04	0.85	-0.01	0.07	0.569	0.002
	N (%)	2.01	0.16	0.014	0.009	0.246	0.246
	C:N ratio	3.4	0.06	-1.45	0.7	0.381	0.381
	Phosphorus (ppm)	3.6	0.06	-11	4.6	0.967	0.028
	Cation exchange capacity	0.51	0.48	-0.4	0.5	0.822	0.014
Hancock	Yield	2.4	0.12	0.3	0.2	0.979	1.49E-02
	Richness	4.3	0.038	-14.3	5.7	0.558	5.58E-01
	Shannon index	4.4	0.036	-0.5	0.17	0.690	5.37E-01
	Simpson's index	3	0.08	0.3	0.17	0.632	3.77E-01
	pH	0	1	0	0.05	0.956	0.00E+00
	C (%)	1.3	0.26	0.1	0.09	0.958	1.30E-02
	N (%)	1.84	0.18	0.01	0.009	0.946	2.72E-02
	C:N ratio	12.12	0.0005	-0.76	0.12	0.887	8.87E-01
	Phosphorus (ppm)	2.4	0.12	27	14.2	0.869	9.57E-02
	Cation exchange capacity	0.01	0.91	-0.06	0.47	0.976	6.82E-05
Oregon	Yield	2.1	0.15	-0.8	0.5	0.345	0.251
	Richness	0.77	0.38	4	4.4	0.104	0.104
	Shannon index	0.03	0.86	0.04	0.23	0.475	0.002
	Simpson's index	0.31	0.56	-0.8	0.5	0.669	0.015
	pH	0.09	0.76	-0.03	0.08	0.780	0.003
	C (%)	6.29	0.012	0.2	0.05	0.845	0.337
	N (%)	0.48	0.49	0.01	0.02	0.750	0.018
	C:N ratio	2.7	0.1	0.55	0.28	0.896	0.058
	Phosphorus (ppm)	1.43	0.23	-1.5	1.15	0.924	0.019
	Cation exchange capacity	0.13	0.71	-0.22	0.6	0.883	0.002

Table 4. Results of likelihood ratio tests comparing the relationship between switchgrass yield (response) and AMF diversity and soil parameters (predictors). Conditional R^2 (proportion of variance explained by both the fixed and random effects) and marginal R^2 (proportion of variance explained by the fixed effects alone) are also reported. Significant predictors of switchgrass yield are highlighted in green; marginally significant ones are highlighted in orange.

Site	Fixed effect	$\chi^2_{(1)}$	P value	Slope estimate	Standard error	Conditional R^2	Marginal R^2
Rhinelander	Richness	5.47	0.019	-0.049	0.014	0.914	0.914
	Shannon index	8.44	0.004	-0.67	0.14	0.941	0.941
	Simpson's index	3.81	0.051	-2.15	0.71	0.894	0.894
	pH	7.30	0.007	-1.33	0.32	0.939	0.931
	C (%)	1.81	0.18	1.43	0.99	0.925	0.831
	N (%)	6.74	0.009	19.63	5.69	0.976	0.863
	C:N ratio	4.57	0.03	-0.24	0.07	0.982	0.766
	Phosphorus (ppm)	0.99	0.32	-0.006	0.005	0.914	0.819
	Cation exchange capacity	5.45	0.02	0.25	0.089	0.964	0.885
Hancock	Richness	0.50	0.48	0.01	0.02	0.981	0.019
	Shannon index	2.96	0.086	0.78	0.38	0.989	0.045
	Simpson's index	8.78	0.003	7.99	1.23	0.998	0.066
	pH	2.65	0.10	-2.56	1.14	0.995	0.212
	C (%)	10.09	0.001	2.38	0.33	0.988	0.943
	N (%)	9.70	0.002	26.33	3.59	0.986	0.942
	C:N ratio	0.31	0.58	-0.42	0.75	0.981	0.017
	Phosphorus (ppm)	7.55	0.006	0.01	0.003	0.993	0.320
	Cation exchange capacity	14.32	0.0001	0.38	0.02	0.985	0.985
Oregon	Richness	4.92	0.03	0.076	0.029	0.700	0.587
	Shannon index	2.24	0.13	0.85	0.52	0.453	0.453
	Simpson's index	2.17	0.14	5.88	3.67	0.448	0.448
	pH	2.38	0.12	-1.76	1.02	0.635	0.483
	C (%)	0.39	0.53	1.14	1.79	0.354	0.293
	N (%)	5.30	0.02	11.36	4.11	0.636	0.636
	C:N ratio	4.77	0.03	-0.43	0.17	0.609	0.609
	Phosphorus (ppm)	0.19	0.66	0.02	0.05	0.356	0.273
	Cation exchange capacity	2.65	0.10	-0.16	0.09	0.482	0.482

Table 5. BLAST results for ASVs significantly affected by the addition of nitrogen in switchgrass cropping systems.

Negative Binomial Model			BLAST Results					
ASV	Log_2 fold change	P value	Species	Clone/isolate	Max score	Query coverage (%)	Percent identical (%)	Accession
9	-27.14234	3.730556e-12	*P. brasilianum*	pCK084-12	2678	100	99.46	FR750048
12	22.25124	1.460792e-08	*P. brasilianum*	pCK084-12	2658	100	99.19	FR750048
14	-30.00000	2.921384e-14	*P. brasilianum*	pCK084-12	2643	100	99.05	FR750048
18	-26.86851	4.803967e-12	*P. brasilianum*	pCK084-12	2699	100	99.66	FR750048
21	-28.84183	3.498068e-13	*P. brasilianum*	pCK084-12	2715	100	99.86	FR750048
26	-11.54232	7.937559e-03	*P. brasilianum*	pCK084-12	2663	100	99.25	FR750048
38	-17.48532	1.222052e-05	*P. laccatum*	Isolate 26	2636	100	98.28	KY630228
45	21.99423	1.939161e-08	*P. brasilianum*	pCK084-12	2647	100	99.05	FR750048
85	20.69746	1.723358e-07	*P. brasilianum*	pCK084-12	2636	100	98.92	FR750048
124	-25.27850	1.408645e-10	*Paraglomus* sp.	uncultured	2712	100	98.45	KY242693
176	-23.49408	1.838072e-09	Glomeraceae	uncultured	2630	100	98.47	JX276891

Figures

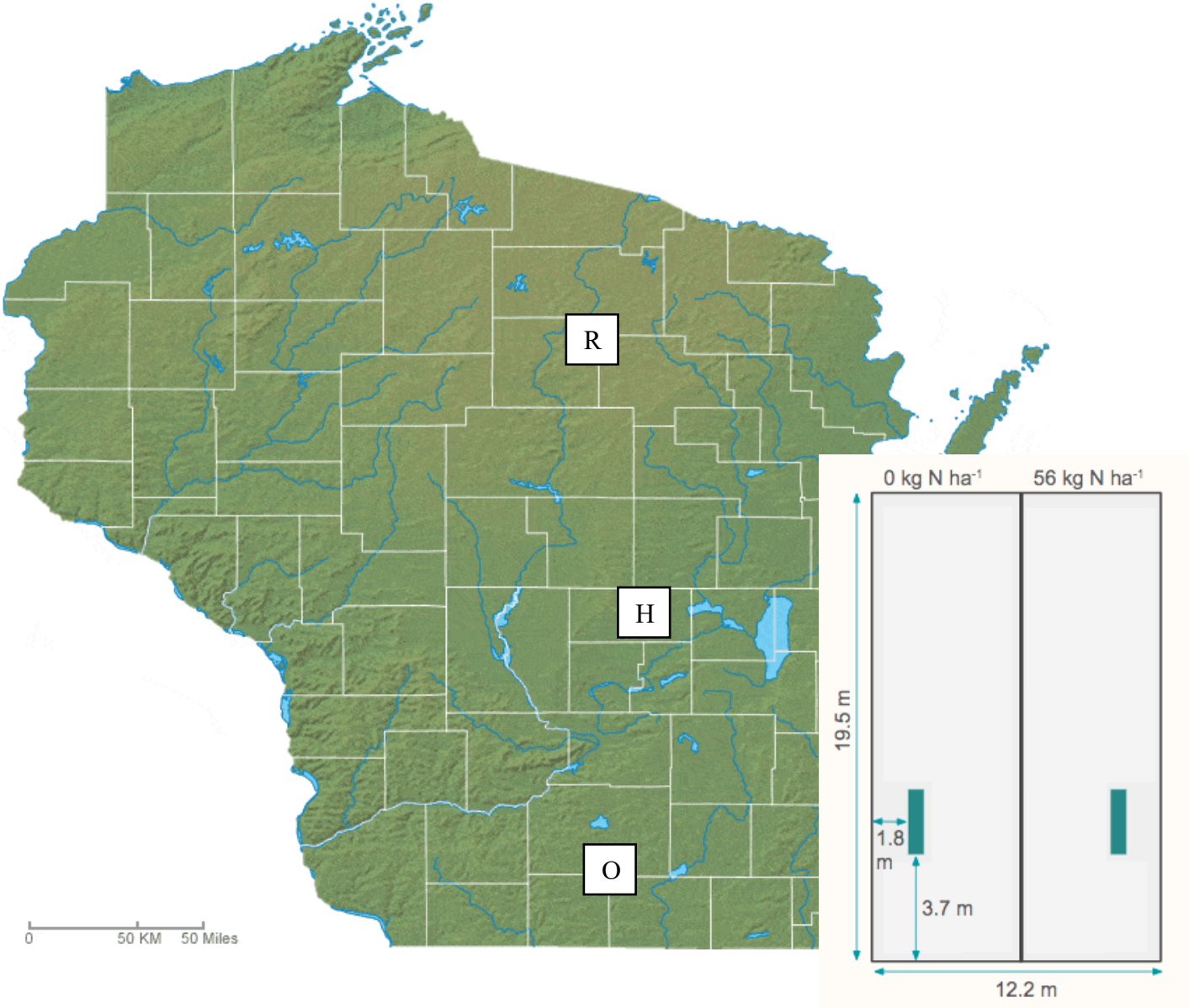

Fig. 1. Map of Wisconsin labelled with the three Great Lakes Bioenergy Research Center Marginal Land Experiment sites and a representative diagram of the experimental units. “R” stands for Rhinelander, “H” for Hancock, and “O” for Oregon. The inset shows a plot of switchgrass divided into two halves (experimental units), one of which received no nitrogen amendment and the other 56 kg N/ha annually. Rhinelander and Oregon both consisted of four replicates of each nitrogen treatment, whereas Hancock consisted of three. Soil samples for percent carbon and nitrogen analysis and PacBio sequencing were collected from the areas indicated by the teal rectangles.

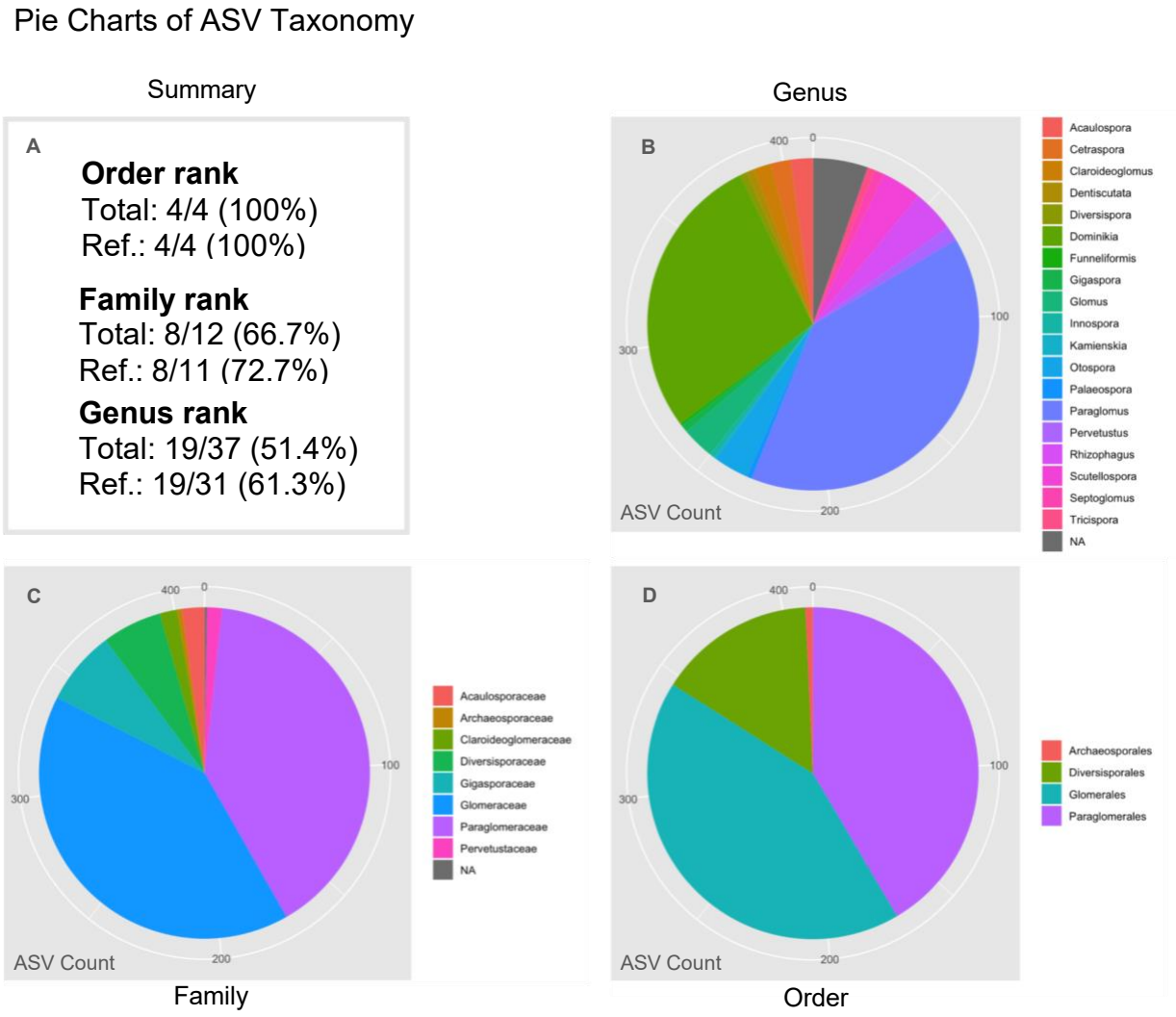

Figure 2. Taxonomic assignments of ASVs according to *DADA2* and a custom pSSU-ITS-pLSU AMF reference database. A) Summary of ASV taxonomy relative to the total number of described genera, families, and orders for Glomeromycota and the total number of these ranks included in the custom reference (Schüßler, 2019). B) Pie chart of ASV assignment to AMF genera. C) Pie chart of ASV assignment to AMF families. D) Pie chart of ASV assignment to AMF orders.

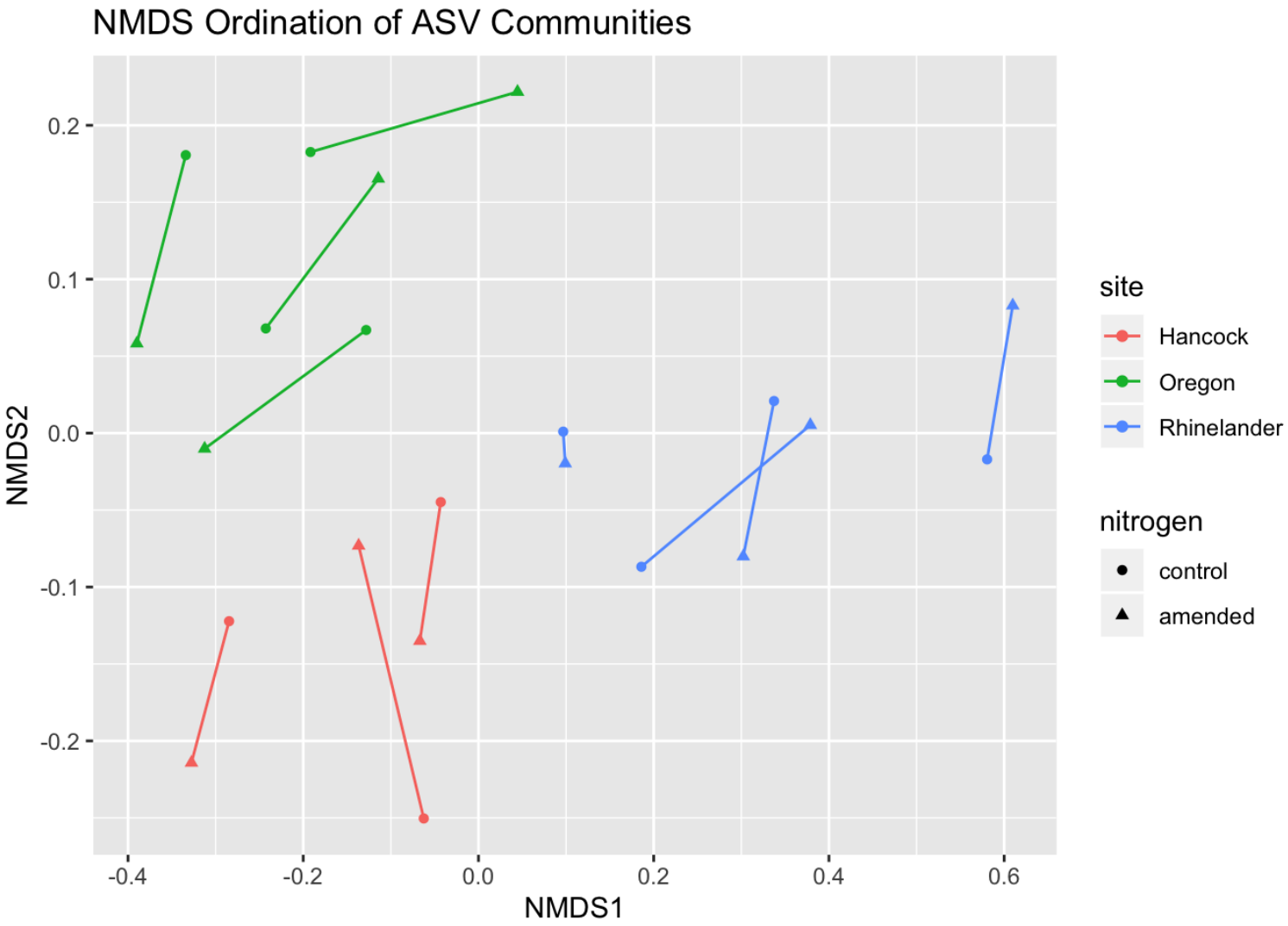

Figure 3. Non-metric multidimensional scaling ordination of ASV community matrix. Each point on the graph refers to an experimental unit. Lines connect paired control and nitrogen-amended experimental units. Points are colored by site and nitrogen treatment (stress = 0.059).

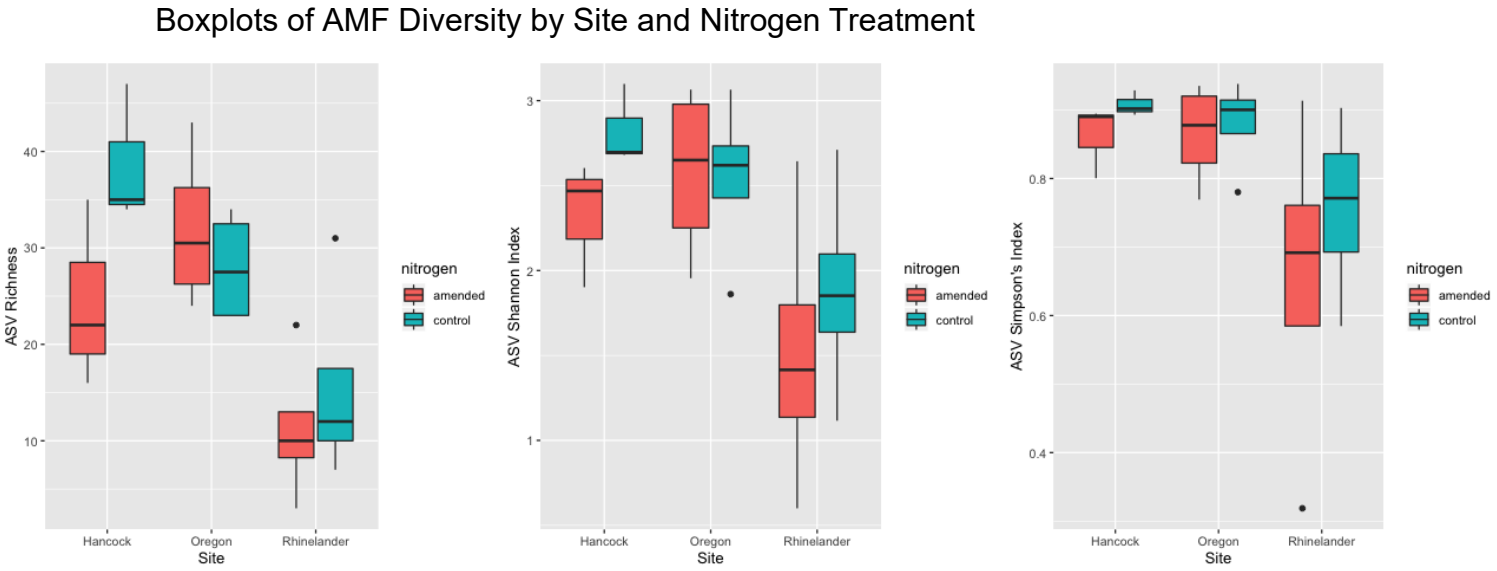

Figure 4. Boxplots of AMF diversity by site and nitrogen treatment. The differently colored boxes indicate nitrogen treatment: orange refers to nitrogen-amended experimental units and blue refers to control experimental units.

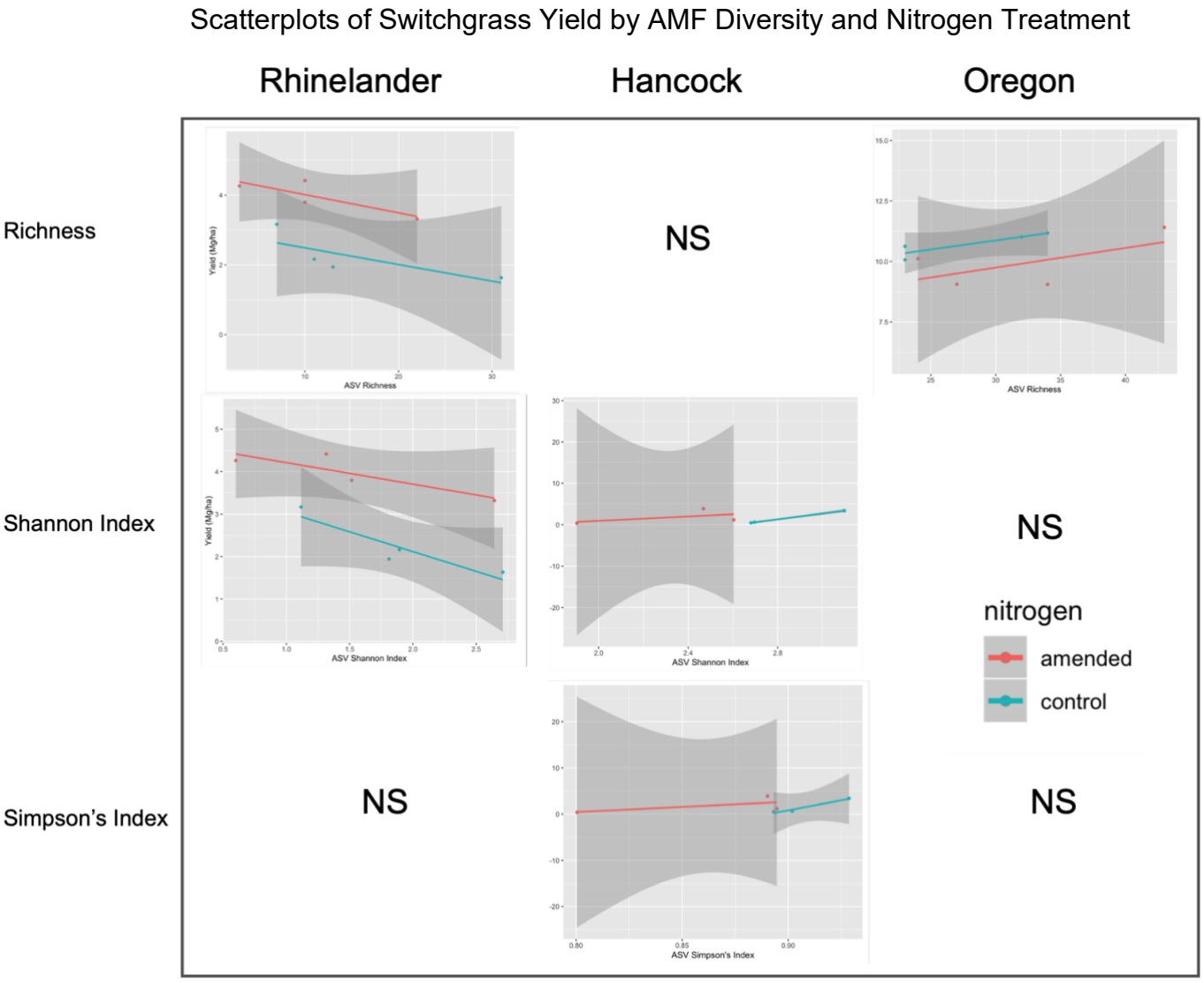

Figure 5. Scatterplots of switchgrass yield (Mg ha^{-1}) as a function of nitrogen treatment and AMF alpha diversity metrics. Columns correspond to different sites and rows to different diversity metrics. Cells with "NS" indicate nonsignificant relationships whose graphs were not displayed.

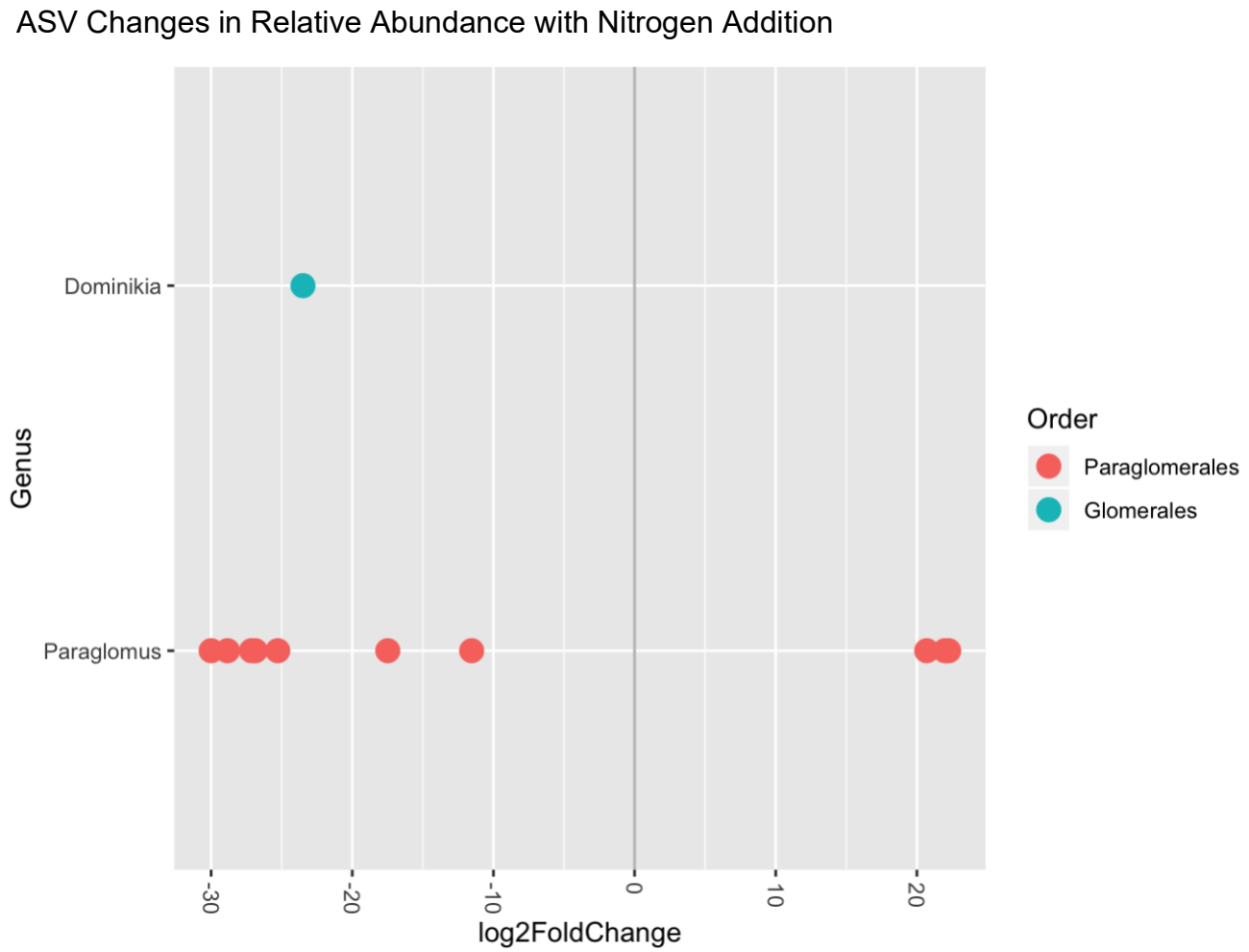

Figure 6. Log_2 fold change in relative abundance of ASVs with the addition of nitrogen according to a negative binomial distribution Wald Test.