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INTERACTIVE EFFECTS OF MICROBES AND NITROGEN ON *PANICUM VIRGATUM* ROOT FUNCTIONAL TRAITS AND PATTERNS OF PHENOTYPIC SELECTION

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Premise of research. Plants in natural and agricultural systems are influenced in myriad ways by their microbial communities, particularly by providing goods and services that change plant functional traits. Microbes are considered an influential part of the environmental context that change plant trait expression, but often, microbe-mediated effects are contingent on local resources, such as nitrogen. Here, we ask how microbes and nitrogen affect belowground functional traits and patterns of phenotypic selection.

Methodology. We performed a fully factorial greenhouse experiment with switchgrass (*Panicum virgatum*), manipulating microbial community composition and nitrogen availability. We measured plant performance and belowground functional traits and performed 16S amplicon sequencing of the root-associated microbial communities. We looked for correlations between microbial taxa and root functional traits, and we performed phenotypic selection analysis on five belowground functional traits to determine how traits affect plant relative performance across biotic and abiotic contexts.

Pivotal results. All belowground plant functional traits except root tissue density were affected by adding nitrogen. We found that a microbial taxon (amplicon sequence variant [ASV]) in the genus *Micromonospora* correlated with shorter root lengths. We also found strong positive selection for longer roots regardless of the abiotic or biotic environment. In contrast, selection favored lower root-to-shoot ratios in high-nitrogen conditions, and selection on root tissue density was highest in treatments that had high nitrogen levels and perturbed microbial communities.

Conclusions. We did not detect microbial effects on the expression of plant traits (ecological effects); however, patterns of phenotypic selection (evolutionary effects) on root tissue density differed depending on the biotic and abiotic environment. Additionally, we detected strong selection for increased root length across treatments; we also found that one ASV correlated with decreased root length, indicating potential conflict between root microbiome components and plant fitness. Future work would be to include microbial taxa in phenotypic selection analysis and to conduct manipulations of the microbes correlated with functional traits to determine causality.

Keywords: microbiome, extended phenotype, selection analysis, plant-microbe interactions, functional traits, roots, nitrogen.

Online enhancements: supplemental tables and figure.

Introduction

The rhizosphere is an ecosystem characterized by a high diversity of archaeal, bacterial, and fungal microbes (Partida-Martínez and Heil 2011). Rhizosphere communities are shaped by a combination of local environmental conditions and plant genotypic effects (Bulgarelli et al. 2012, 2015; Lundberg et al. 2012; Edwards et al. 2015). In turn, these microbial communities affect plant nutrition, stress tolerance, and health (Berendsen et al. 2012; Nadeem et al. 2014; Petipas et al. 2017; Jack et al. 2019). One way microbes affect plant fecundity is through microbially mediated effects on plant functional traits (Friesen et al. 2011), which are particularly pronounced belowground (Friesen 2013). For example, inoculation with rhizosphere microbes increased root length by 17% in *Zea mays* (Kothari et al 1990). In another

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example, inoculation with ectomycorrhizal fungi increased fine-root diameter by 90% in *Pinus taeda* seedlings (Rousseau et al. 1994). Roots are specialized organs that provide structural support and serve as conduits for nutrient uptake, but the phenotype and function of roots are highly influenced across ecological and evolutionary scales by hidden interactions with a host of beneficial and pathogenic soil microbes. Thus, understanding the ecological and evolutionary implications of interactions between plants and the soil ecosystem is a critical frontier in plant science.

While microbes are often ignored or considered as a part of the environment, they can be more usefully conceptualized as either part of their plant host's extended phenotype (i.e., as traits themselves) or as impacting the nonmicrobial phenotypic attributes of their host (fig. 1*A*). For example, associating with rhizobia and mycorrhizal fungi can provide access to resources otherwise inaccessible to the plant and can thus be thought of as a plant trait (Cornelissen et al. 2003). In contrast, many root-associated bacteria produce phytohormones in the rhizosphere that influence belowground functional traits (Glick 2012) and thereby indirectly affect stress tolerance and resource acquisition. Microbially mediated traits likely play an important role in evolutionary processes. Since natural selection operates whenever there is covariation between trait values and fitness, selection on functional plant traits that are microbially mediated will translate to selection acting on plant phenotypes that promote or reduce the abundances of relevant microbes. For example, if microbes alter host traits in the direction counter to that favored by selection on the host, this would give rise to a fitness conflict between host and microbe and select for plant phenotypes that reduce the abundances of relevant microbes. An important caveat is that roots are habitats for microbial populations, so correlations can be observed between root functional traits and microbial abundances that are unrelated to plant fitness effects. For example, live imaging has revealed that some bacteria prefer to colonize the root elongation zone (Massalha et al. 2017). Thus, microbial populations and root functional traits can impact each other and can independently and jointly impact plant fitness (fig. 1*A*).

Predicting the ecological and evolutionary outcomes of plant-microbe interactions in nature has been hampered by the context dependency of these interactions. Plant-microbial symbioses exist in heterogeneous environments, where strong differences in edaphic conditions occur over small spatial and temporal scales. Abiotic context can impact plant functional traits and/or the population dynamics of relevant microbes directly, thus impacting host fitness. In addition, if the abiotic environment alters patterns of selection on host traits, there could be fluctuating selection on different members of the microbiome depending on the abiotic context. Similarly, the biotic context could shift the relationship between trait values and plant performance if microbes are able to compensate for their host's suboptimal trait values by enhancing resource acquisition or changing stress

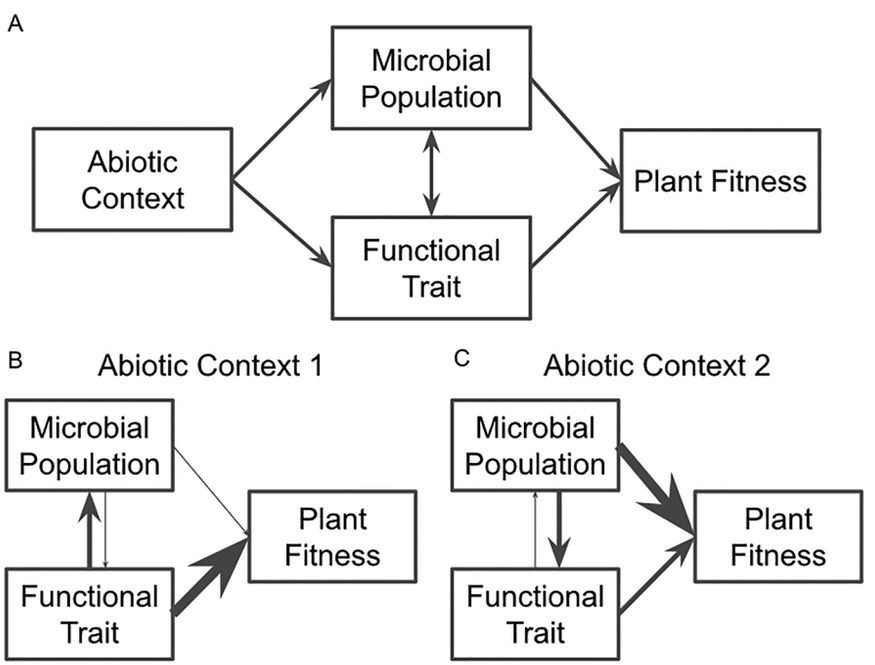

Fig. 1 Potential relationships between abiotic context, plant-associated microbes, belowground functional traits, and plant fitness. *A*, Abiotic context directly influences the microbial populations and/or plant functional traits, which can also influence one another and then both feed into plant fitness. *B*, *C*, Abiotic context alters the relationships between microbes, traits, and plant fitness rather than impacting the population dynamics or trait values themselves. *B*, For example, under high nitrogen levels, root tissue density may have a stronger effect on fitness because the plant is more carbon limited; this trait may also influence microbial populations but is not susceptible to microbial influence. *C*, For example, under low nitrogen levels, bacterial nitrogen fixation could have a greater influence on root functional traits such as root elongation, as well as impact plant fitness directly via resource acquisition.

tolerance (fig. 1*B*, 1*C*). For example, Lau and Lennon (2012) manipulated water availability and microbial community composition to determine how these factors affected plant drought tolerance. They found microbial effects on drought tolerance were more pronounced than plant genotypic effects, and microbe-mediated drought tolerance was facilitated by microbe-mediated changes to flowering time that allowed plants to escape water scarcity.

An important abiotic variable that can have large ecological and evolutionary effects on plants via plant functional traits is nitrogen availability. Soil nitrogen, like microbes, has important effects on aboveground (e.g., Lee et al. 2017; Zheng et al. 2017; Tatarko and Knops 2018) and belowground (e.g., Ostonen et al. 2007; Bonifas and Lindquist 2009; Maire et al. 2009) plant functional traits. In general, when nitrogen is limiting, plants invest more in traits that are related to foraging, such as specific root length (Ostonen et al. 2007), and allocate more biomass to roots versus aboveground organs (Li et al. 2015). Nitrogen availability is a prime example of an abiotic context that can impact plant functional traits directly, affect population dynamics of relevant microbes, or shift the relationships between microbes and traits, thus impacting host fitness (fig. 1).

In this study, we manipulated soil microbial community composition and nitrogen availability to determine the ecological and evolutionary effects on plant functional traits in *Panicum virgatum* (switchgrass). Switchgrass is a perennial bunchgrass that is native to North America. It has been the focus of considerable research because of its potential as a bioenergy crop owing to its drought tolerance and relatively low soil fertility requirements (Mitchell et al. 2014). The observation that switchgrass can produce equivalent biomass across a fertilization gradient (Hong et al. 2014; Ruan et al. 2016) has spurred interest in understanding the switchgrass microbiome, and recent work has demonstrated that bacterial nitrogen fixation contributes to switchgrass nitrogen budgets (Roley et al. 2018, 2019).

We used a factorial greenhouse manipulation to ask three main questions: (1) How do nitrogen and microbial community composition affect belowground plant functional traits? (2) Can we identify microbial taxa that are correlated with belowground plant functional traits? and (3) Do nitrogen and microbial composition affect patterns of phenotypic selection on belowground plant functional traits? We hypothesized that both nitrogen levels and microbial communities would be important for determining trait values and patterns of selection. Clarifying the interactive effects of microbial colonization and soil nitrogen addition is a critical step in understanding how *Panicum* is able to thrive in nutrient-poor conditions and will also elucidate how the relationship between the plant microbiome and plant functional traits is influenced by abiotic conditions, thereby affecting host fitness.

Material and Methods

Soil Inoculum Collection and Preparation

Soils used as microbial inoculum were collected from the Lux Arbor site (G5 plots) at the Great Lakes Bioenergy Research Center (GLBRC), Marginal Land Experiment at Kellogg Biological Station, established in 2013. There are four north-south switchgrass plots at GLBRC, each divided into fertilized and unfertilized sections. In each of the four replicated unfertilized switchgrass monoculture plots, we collected 12 cores, 2.5 cm in diameter and 10 cm deep, to be used as soil microbial inoculum. The soil at these plots has an average pH of 5.01 and 0.11% total nitrogen. Samples were collected and kept in separate bags in a cooler with ice packs and then stored at 4°C. The soil microbial inoculum was either used as is (unperturbed treatment) or autoclaved before use (perturbed treatment). Perturbed inoculum was autoclaved three times for 45 min each at 121°C.

Panicum virgatum *Germination and Growth*

Seeds for the Cave-in-Rock variety of *Panicum virgatum* were sterilized for 6 h using chlorine gas. Cave-in-Rock was used because it is the genotype of *P. virgatum* that is used at GLBRC, where we collected soil to be used as microbial inoculum. Seeds were then placed on moist filter paper in petri dishes at 4°C for 3 wk, then remoistened and moved to a 30°C incubator to elicit germination. Germinated seedlings were planted in August 2016. Because of low germination, additional seedlings were prepared as described above, with the exception that they were not moistened before the 3 wk at 4°C, and these seedlings were planted to complete the full experimental design. Replacement seedlings were added 24 h after the original planting. In order to assess the influence of nitrogen and microbial composition on belowground biomass and plant functional traits, plants were randomly divided into four treatments (high nitrogen with unperturbed microbial community, $N = 22$; low nitrogen with unperturbed microbial community, $N = 22$; high nitrogen with perturbed microbial community, $N = 22$; low nitrogen with perturbed microbial community, $N = 18$).

We filled 0.65-L deepots (D40H; Steuwe and Sons) with sand. To create a depression for microbial inoculum, we added an inverted, sterilized 10-mL test tube cap in the top layer of sand. Both sand and deepots were autoclaved three times for 45 min at 121°C. We used two types of inoculum in this experiment. Liquid soil inoculum was created by adding 500 mL of field-collected soil to 500 mL of milli-Q water and agitating for 10 min. We then let the mixture settle for ~1 h and decanted liquid into an Erlenmeyer flask through a 1-mm filter to remove floating debris. Solid soil inoculum was homogenized field soils added directly to treatment pots. At planting, the test tube cap was removed, and the hollow was filled with 10 mL of either live or autoclaved field soil (solid soil inoculum), or plants received 10 mL of sand, then received 10 mL of either live or autoclaved liquid field soil extract (liquid soil inoculum). After transplanting seedlings, all pots received 10 mL of water. In the greenhouse, supplemental lighting was used to maintain 16-h days, with temperature set to 23°C during the day and 8°C at night. We opted for a greenhouse experiment because microbial manipulations are often complicated by environmental contamination in the field, and patterns of phenotypic selection are easier to detect in experimental settings (compared with observational studies; Caruso et al. 2017). Initially, plants were watered with an automatic misting system for 2 min nine times per day because of high heat. However, this led to fungal growth on the sand surface for many pots. To ameliorate fungal growth, the top layer was replaced with autoclaved sand and a half-inch layer of autoclaved perlite, and watering was reduced to four times per day.

Nitrogen Treatment Preparation

To create our nitrogen treatments, plants received modified half-strength Hoagland's solution. In the first 2 wk after planting (August 9 and August 12), low-nitrogen treatments received the equivalent of 12.5 lb N/acre (~3 g/m^2), and high-nitrogen treatments received the equivalent of 75 lb N/acre (~18.5 g/m^2). Then approximately 2 wk (August 22) and 4 wk (September 8) after the initial application, plants in the high-nitrogen treatment received an additional 25 lb N/acre, while the low-nitrogen plants received only nitrogen-free nutrient solution. In total, the high-nitrogen plants received 200 lb N/acre (49.4 g/m^2), and the low-nitrogen plants received 25 lb N/acre. At the conclusion of the experiment, soil ammonium (NH_4^+) was 43% lower, and nitrate (NO_3^-) was 65% lower in the low-nitrogen treatments (table S1; tables S1–S3 are available online).

Plant Harvesting and Data Collection

Plants were harvested after 8 wk of growth. Shoots were cut at soil level and dried at 50°C for 1 wk before weighing. A soil core 2.5 cm in diameter and 10 cm deep containing a portion of the root system was collected and stored for 4 wk at 4°C for root morphology analysis. The remaining root system was removed from the pots and vigorously shaken to remove nonrhizosphere potting medium (Yanai et al. 2003). For approximately half of our treatment plants (those that received solid soil inoculum), a representative subsample of the root system and rhizosphere soil (i.e., the potting medium still attached to the root system) was collected and snap frozen for DNA extraction. A subsample of the remaining bulk soil was collected on ice for storage at −80°C for nitrogen analysis. Roots from the soil core sample were collected using forceps after washing through a 1-mm sieve and temporarily stored at −20°C until they were scanned. To scan, the roots were placed in a thin layer of water in a transparent tray, spread apart using forceps to minimize overlap, and scanned at 1200 dpi. Scanned root images were assessed for total root length, volume, and mean root diameter using GiA Roots (Galkovskyi et al 2012). The roots were then dried at 50°C for 1 wk before weighing. In total, we measured aboveground and belowground biomass (g) and five functional root traits: root-to-shoot ratio (ratio of belowground biomass to aboveground biomass), root length (cm), mean root diameter (cm), specific root length (cm/g; total length of a given root sample divided by the mass of that sample), and root tissue density (g/cm^3; ratio of dry belowground biomass to root volume).

DNA Extraction and Sequencing

The subsample of snap-frozen roots and attached rhizosphere soil were ground to a fine powder in liquid nitrogen using a mortar and pestle. DNA was extracted from approximately 1 g of the ground samples using the ZR-Duet RNA/DNA Miniprep Plus kit (Zymo Research). Briefly, 800 uL of DNA/RNA lysis buffer was added to each sample. Samples were then vortexed and spun down, and DNA was collected from the supernatant following manufacturer's instructions and eluted in 50 uL of nuclease-free water. All samples were then passed through a OneStep PCR Inhibitor Removal column (Zymo Research) to increase sample purity and remove enzyme inhibitors before library preparation and sequencing. In total, 41 samples were multiplexed (~10 samples per treatment inoculated with solid soil inoculum). We amplified the 16S V4 region using the 515F-806R primer pair (Thompson et al. 2017), barcoded it, and sequenced it on an Illumina MiSeq. Sequencing, demultiplexing, and adapter removing were conducted by the Research Technology Support Facility Genomics Core at Michigan State University.

Microbiome (16S) Data Processing

Microbiome 16S rRNA amplicon denoising and quality filtering were completed using QIIME 2 (Bolyen et al. 2018), with the divisive amplicon denoising algorithm (DADA2) plug-in (Bolyen et al. 2018). In QIIME 2, we utilized the q2-feature-classifier plug-in to improve taxonomic assignment, which uses a pretrained naive Bayes classifier (NBC) machine learning module (Bokulich et al. 2018). QIIME2-DADA2 obtains amplicon sequence variants (ASVs), rather than operational taxonomic units (OTUs), which control errors associated with OTUs (Bokulich et al. 2018). Eukaryotic contaminant sequences related to chloroplast plastid 16S and mitochondria 16S were removed using phyloseq (McMurdie and Holmes 2013). In addition, we rarefied to the lowest number of reads (15,558) in our samples for all analyses.

Statistical Analyses

All analyses were conducted using R version 3.5.2. Figures were created using ggplot2 (Wickham 2016) and cowplot (Wilke 2019). Unless stated otherwise, all models included the main effects of nitrogen level and microbial perturbation. In each model, we tested how presence or absence of fungal growth and inoculum type (liquid soil or solid soil) affected the measured response variables. Fungal growth was an unintended consequence of overwatering early in the experiment so was included to account for possible variation in traits caused by fungal contamination. Fungal growth or inoculum type did not significantly affect any of the measured variables and therefore was not included in our final models. Inoculum source was included as a random effect to account for variation in inoculum composition as a result of using soils from four field plots at GLBRC. When appropriate, we tested the assumptions of linear models using graphical approaches to verify homogeneity of variances and normality of residuals (Zuur et al. 2009), and as necessary, we performed data transformation so data better fit the assumptions of linear regression. We corrected for multiple comparisons using the Benjamini-Hochberg correction (Benjamini and Hochberg 1995). Post hoc tests were done using the emmeans package with P values adjusted using the multivariate t-distribution method (Lenth 2017).

Treatment Effects on Microbial Richness, Community Composition, and Plant Traits

To understand how nitrogen and microbial community perturbation affect microbial community composition in switchgrass roots, we used permutational multivariate analysis of variance (PERMANOVA; Anderson 2001) on rarefied ASV abundances. PERMANOVA was performed using the adonis function in the vegan package (Oksanen et al. 2019) with 999 permutations. PERMANOVA is a nonparametric multivariate statistic that uses dissimilarity matrices to test the hypothesis that treatment groups differ in community composition (Anderson 2001). In this analysis, inoculum source was switched to a fixed effect instead of

used as strata (analogous to block or random effects in a linear model) because adonis cannot accommodate unbalanced strata. Data were visualized using nonparametric multidimensional scaling (NMDS). NMDS was performed using Bray-Curtis dissimilarities in the vegan package. Species (ASV) richness was calculated by tallying the number of ASVs found within a root sample. Similarly, one Shannon diversity value was calculated per plant. Observed species richness and Shannon diversity were calculated using the estimate_richness function in the phyloseq package (McMurdie and Holmes 2013). Microbial alpha diversity (observed richness and Shannon diversity), aboveground biomass, belowground biomass, root length, mean root diameter, root-to-shoot ratio, specific root length, and root tissue density were analyzed using linear mixed models in lme4 (Bates et al. 2015).

Microbial Abundance by Trait Correlations

To identify candidate microbial taxa for trait-by-microbe correlation tests, we generated a list of the 10 most abundant taxa in the perturbed microbial treatment and the 10 most abundant taxa in the unperturbed microbial treatment. To identify the most abundant microbial ASVs, we used the taxa_sums function in the phyloseq package, which sums the individual observations for each ASV. We chose this semitargeted approach because abundant taxa are likely to affect plant phenotype (Winfree et al. 2015). This approach resulted in 13 unique bacterial taxa to test for correlations with plant phenotype (table S2). Perturbed and unperturbed treatments had seven bacterial taxa in common and six bacterial taxa that were uniquely abundant to either perturbed or unperturbed treatments. For these 13 taxa, we tested for correlations with the five measured belowground functional traits using linear mixed models that included trait values as the response variable and both main effects and interactions of ASV abundance, nitrogen level, and perturbation. We corrected for multiple comparisons using the Benjamini-Hochberg correction (Benjamini and Hochberg 1995).

Selection Analysis on Traits

We performed phenotypic selection analysis (Lande and Arnold 1983) to estimate selection differentials on five belowground functional traits: root length, mean root diameter, specific root length, root tissue density, and root-to-shoot ratio. Selection differentials were estimated using univariate analysis that considers single traits individually and measures both direct and indirect (through selection on other correlated traits) contributions to fitness. We calculated relative fitness as absolute fitness of an individual divided by the mean absolute fitness of the population (Etterson 2006), using total shoot mass as a proxy for fitness. Switchgrass is a perennial that sets seed in its first year only under optimal conditions (Vogel et al. 2011), and thus, reproductive measures of fitness were not practical for this experiment. However, previous work has demonstrated that aboveground biomass is a reliable predictor of fecundity (Younginger et al. 2017). We standardized trait values by using the scale function in R, which subtracts the mean of the vector (column) and divides by the standard deviation of the vector (column) for each individual measurement.

Selection differentials were estimated on the full data set and, in each treatment, individually. Accordingly, relative fitness and trait value standardization was conducted across the entire data set and within a treatment, depending on the level of analysis. For example, to estimate selection differentials in the treatment with high nitrogen and perturbed microbial communities, we used relative fitness and standardized trait values calculated within this treatment. Only analysis done on the full data set used relative fitness and standardized trait values calculated using plants from all the treatments. For the selection differential analysis, we constructed mixed models that included relative fitness as a response and the standardized trait value as a predictor variable. To understand how the magnitude and direction of direct and indirect selection changed across treatments, we performed an analysis of covariance (ANCOVA; Wade and Kalisz 1990; Lau and Lennon 2011). The ANCOVA included relative fitness as the response variable and nitrogen level, community perturbation, and the standardized trait value as the predictor variables. A significant interaction between a trait and a main effect of nitrogen level or community perturbation indicated that selection differentials were significantly different between treatments.

Data Deposition and Code Availability

All code for QIIME2-DADA2 processing and workflow, scripts for figure plots, and statistical analysis in phyloseq are listed on Friesenlab GitHub repository (https://www.github.com/friesenlab/MMPRNT_panicum_greenhouse_microbiome). All raw data, processed ASV files, and metadata are on the Open Science Framework (https://osf.io/sta58/).

Results

Treatment Effects on Microbial Richness, Community Composition, and Plant Traits

Nitrogen availability and autoclaving caused significant shifts in the microbial community. Microbial community composition was influenced independently by nitrogen level (PERMANOVA nitrogen level: $F = 2.23$, $P = 0.001$) and perturbation (PERMANOVA perturbation: $F = 3.25$, $P = 0.001$; figs. 2, S1; table S3; fig. S1 is available online). Observed species richness and Shannon diversity were not different between the treatments (observed species richness: $F = 0.15$, $P = 0.7$; Shannon diversity: $F = 2.74$, $P = 0.11$; fig. 3).

Nitrogen levels predominantly affected belowground functional traits. Shoot mass (nitrogen level: $F = 165.90$, $P < 0.001$) and root mass (nitrogen level: $F = 73.89$, $P < 0.001$) were higher, and root-to-shoot ratio decreased by 57% with added nitrogen (nitrogen level: $F = 15.0$, $P < 0.001$). Root length (nitrogen level: $F = 57.46$, $P < 0.001$), mean root diameter (nitrogen level: $F = 10.09$, $P = 0.002$), and specific root length (nitrogen level: $F = 7.58$, $P = 0.007$) were all significantly affected by nitrogen addition (table 1). Root tissue density was the only trait unaffected by nutrient conditions (nitrogen level: $F = 0.34$, $P = 0.56$; fig. 4; table 1).

Microbial Abundance by Trait Correlations

Although we tested five belowground functional traits and 13 abundant microbial ASVs, we found only one microbial taxon that was significantly correlated with belowground functional

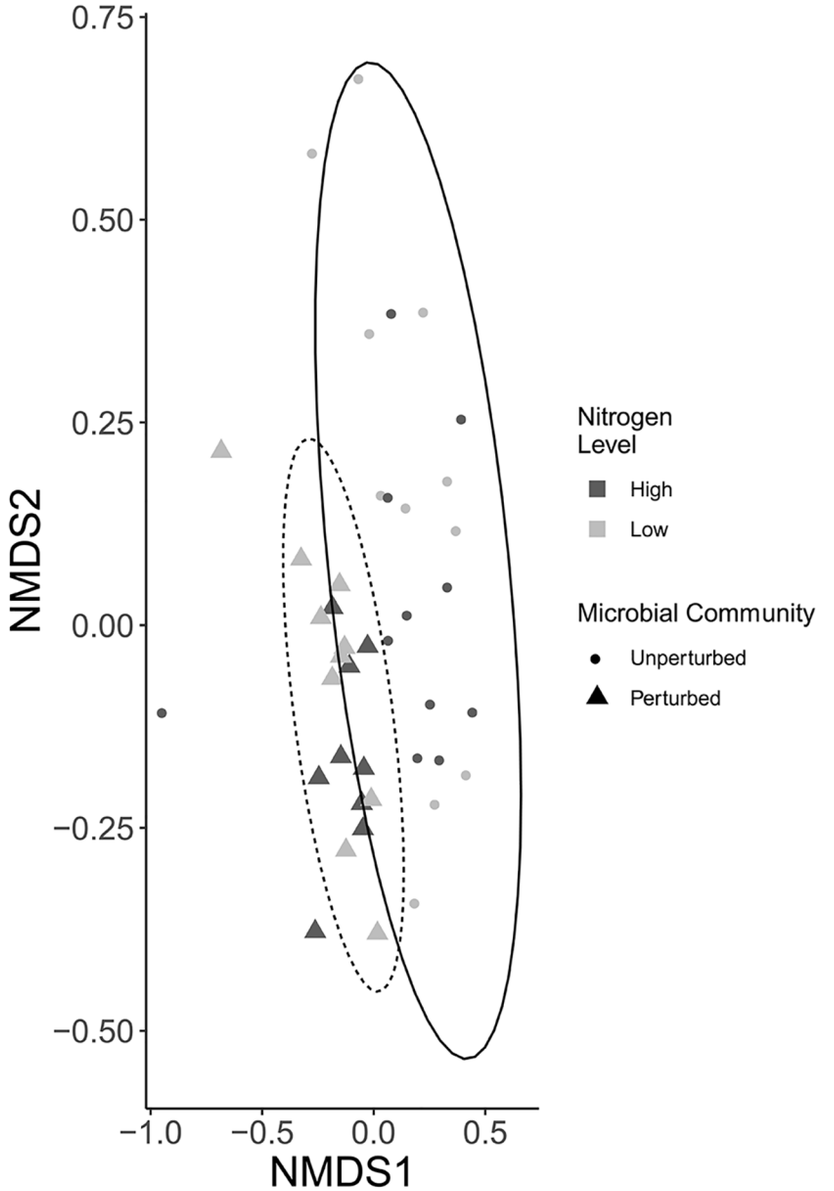

Fig. 2 Nonmetric multidimensional scaling (NMDS; Bray-Curtis; stress = 0.20) ordination of bacterial communities. Dark gray triangles indicate samples that received autoclaved microbial inoculum (perturbed) and high nitrogen, and light gray triangles indicate samples that received autoclaved microbial inoculum (perturbed) and low nitrogen. Dark gray circles indicate samples that received unautoclaved inoculum (unperturbed) and high nitrogen, and light gray circles indicate samples that received unautoclaved inoculum (unperturbed) and low nitrogen. Data ellipses are shown to help delineate the perturbed and unperturbed communities.

traits after correcting for multiple comparisons. ASV 3749 was most closely matched to the genus *Micromonospora* (76% confidence) and was negatively correlated with root length (ASV main effect: $F = 9.72$, $P = 0.004$; fig. 5*A*) in all treatments.

Selection Analysis on Traits

Patterns of phenotypic selection depended on biotic and abiotic environment. Selection favored longer roots across all treatments, as well as within each treatment (full model selection differential = 0.98; fig. 5*B*; table 2). Selection also favored larger root diameters (full model selection differential = 0.25) and lower specific root length across all treatments (full model selection differential = −0.25), but individual selection differentials (within treatment) were not significantly different from zero, indicating weak selection or insufficient sample sizes to detect within-group patterns (table 2). Selection favored lower root-to-shoot ratios, but this depended on nitrogen conditions (fig. 6; table 2). Selection on root tissue density was dependent on nitrogen availability and microbial community composition, such that selection on root

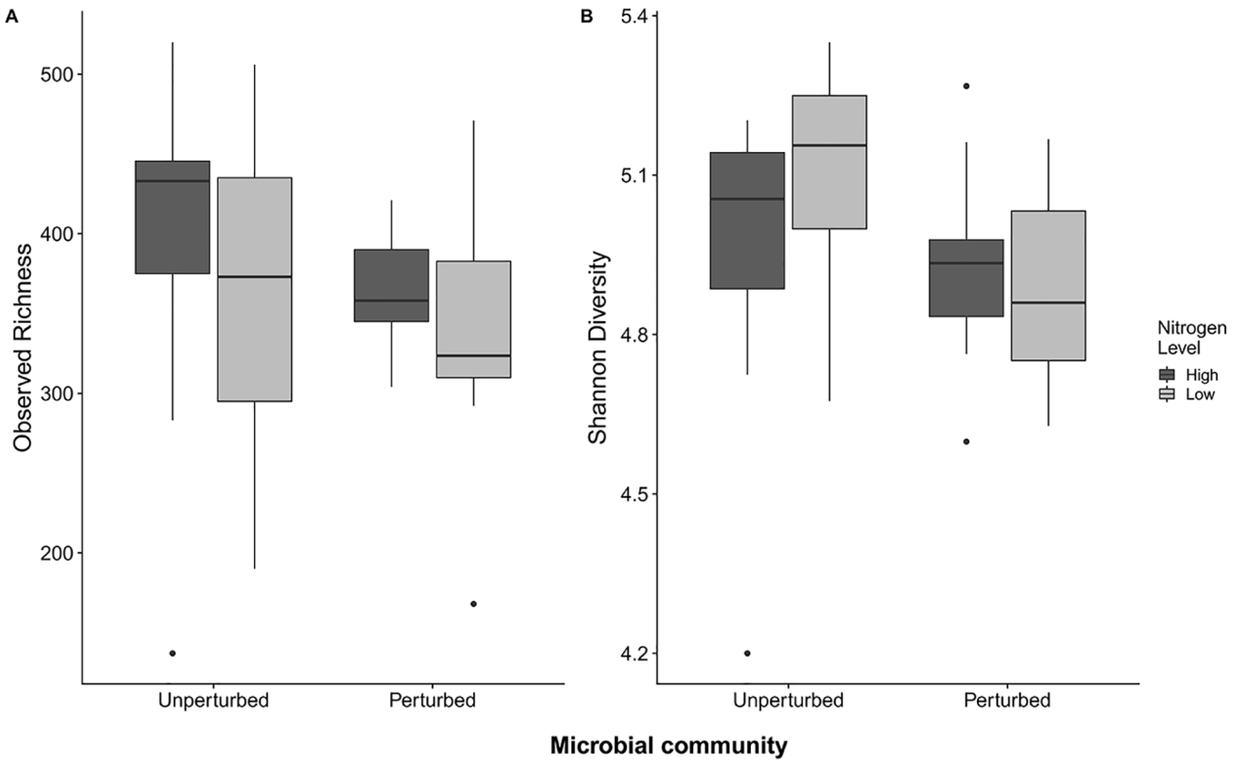

Fig. 3 Observed species richness and Shannon diversity of bacterial taxa in switchgrass roots (*Panicum virgatum*) grown in conditions with either autoclaved (perturbed) or unautoclaved (unperturbed) microbial inoculum and high (dark gray) or low (light gray) nitrogen. Observed richness and Shannon diversity are unaffected by autoclaving and nitrogen addition.

tissue density was most evident in high-nitrogen, perturbed treatments (ANCOVA: $F = 4.26$, $P = 0.04$; fig. 7; table 2).

Discussion

Abiotic Effects on Rhizosphere Microbiome and Belowground Functional Traits

An important frontier in microbiome research is understanding the context dependency of plant-microbe interactions (Greyson-Gaito et al. 2019), of which an important part is understanding shifts in the microbiome composition due to abiotic perturbations. High amounts of nitrogen have been found to shift bacterial communities (Fierer et al. 2012; Ramirez et al. 2012). In our study, we found that nitrogen manipulations accounted for 5% of the variation ($P = 0.001$) in microbial community composition. These effects could be due to the direct effects of nitrogen availability on microbial growth and competition. Alternatively, this could be due to nitrogen stimulating root growth overall or altering root exudation in ways that give particular microbes within the rhizosphere microbiome a stronger competitive advantage. We did not find any effects of nitrogen on species richness or Shannon diversity (a metric that incorporates both abundances and evenness). Previous work has demonstrated that bacterial Shannon diversity is not vulnerable to nitrogen addition, even at long-term nitrogen deposition sites (Freedman et al. 2015).

Although switchgrass is tolerant to low-nitrogen conditions, fertilization sometimes does result in increased yield (Vogel et al. 2011; Mitchell et al. 2014). Consistent with this, in our 8-wk greenhouse study, we found that root and shoot biomass were both enhanced by the addition of nitrogen, but root-to-shoot ratio was reduced, indicative of greater aboveground allocations overall. In addition, the majority of root functional traits that we measured were also nitrogen sensitive, including root length, mean root diameter, and specific root length. Previous work in

Table 1

ANOVA Table Reporting *F*-Statistics from Linear Models Assessing Plant Performance (Shoot Mass and Root Mass) and Plant Functional Traits of Switchgrass (*Panicum virgatum*)

	N	P	N × P
Shoot mass	165.90 (<.001)*	.51 (.48)	.44 (.51)
Root-to-shoot ratio	15.0 (<.001)*	.09 (.77)	.06 (.81)
Root length	57.46 (<.001)*	.001 (.98)	.71 (.40)
Root diameter	10.09 (.002)*	1.21 (.27)	1.42 (.23)
Specific root length	7.58 (.007)*	1.50 (.22)	.08 (.77)
Root tissue density	.34 (.56)	2.00 (.16)	.04 (.85)
Root mass	73.89 (<.001)*	.67 (.41)	.15 (.70)

Note. Plants were grown with microbial inoculum that was autoclaved or unautoclaved (perturbation [P]) in fertilized or unfertilized (N) conditions. *P* values are reported in parentheses.

* Significant after correcting for multiple comparisons.

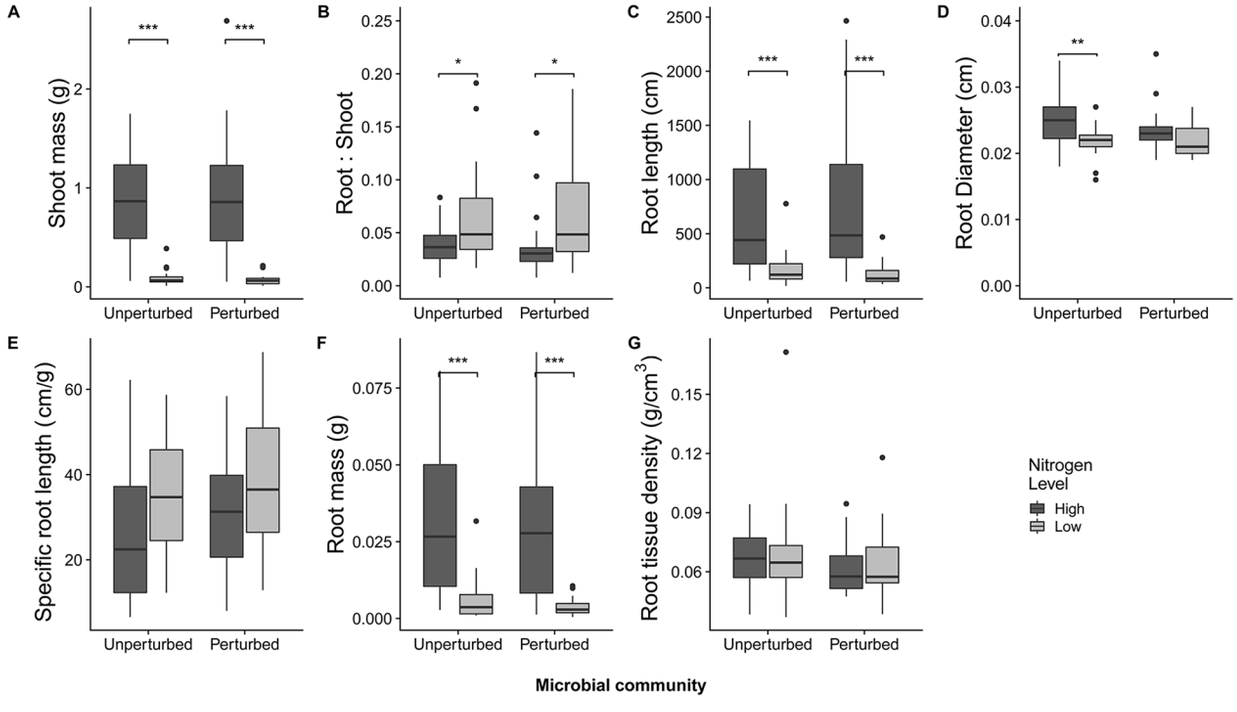

Fig. 4 Plant performance (biomass) and plant functional traits of switchgrass (*Panicum virgatum*) grown with microbial inoculum that was autoclaved (perturbed) or unautoclaved (unperturbed) in fertilized (high-nitrogen) or unfertilized (low-nitrogen) conditions. Shoot mass (*A*), root-to-shoot ratio (*B*), root length (*C*), mean root diameter (*D*), specific root length (*E*), and root mass (*F*) were all affected by nitrogen additions. Root tissue density was not significantly different between treatments (*G*). Asterisks above bars indicate significant post hoc test results. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

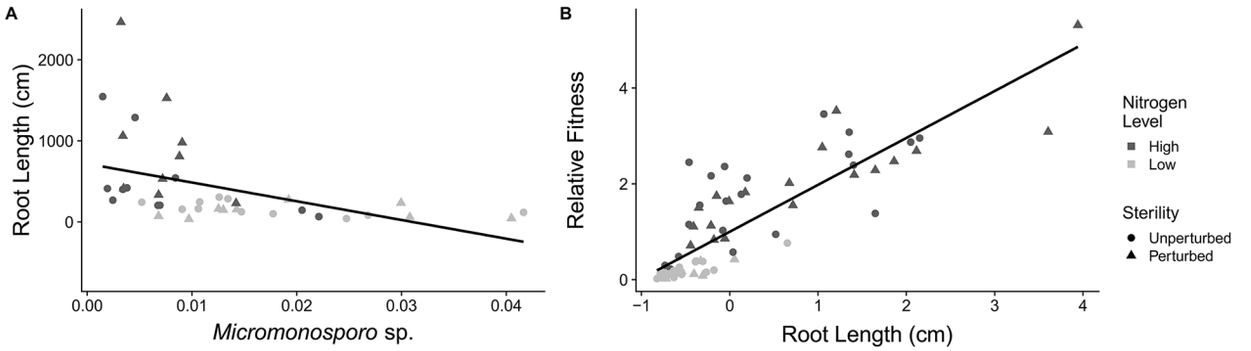

Fig. 5 Root length of *Panicum virgatum* is negatively correlated with the relative abundance of operational taxonomic unit 3749, which matched most closely to a *Micromonospora* sp. (*A*), whereas plant relative fitness is positively correlated with scaled root length within and across all treatments (*B*). Dark gray triangles indicate samples that received autoclaved microbial inoculum (perturbed) and high nitrogen, and light gray triangles indicate samples that received autoclaved microbial inoculum (perturbed) and low nitrogen. Dark gray circles indicate samples that received unautoclaved inoculum (unperturbed) and high nitrogen, and light gray circles indicate samples that received unautoclaved inoculum (unperturbed) and low nitrogen.

a variety of systems suggests that root length (Gruber et al. 2013; Razaq et al. 2017), root diameter (Razaq et al. 2017), and specific root length (Ostonen et al. 2007; Bonifas and Lindquist 2009; Wurzburger and Wright 2015) are all responsive to fertilization. Previous work suggests that root tissue density is sensitive to nitrogen addition also (Wurzburger and Wright 2015; Kramer-Walter et al. 2016); however, in our study, root tissue density was similar in both nitrogen environments. A meta-analysis of trait response to nitrogen fertilization suggests that grassland species might be less responsive along some trait axes to nitrogen fertilization (Li et al. 2015); however, these authors did not explore root tissue density specifically.

Biotic Effects on Belowground Functional Traits

Roots interact with a wide variety of soil microbes that can impact trait values (Ellis et al. 1985; Kothari et al. 1990; Rousseau et al. 1994; Glick et al. 1997; Joner and Leyval 2001; Zhang et al. 2008; Hashem et al. 2016; Henning et al. 2016; Latati et al. 2016; Oliveira et al. 2017; He et al. 2019). However, in our study, belowground functional traits were not affected by perturbing the soil microbial community. There are two nonexclusive potential reasons for these results: (1) The Cave-in-Rock genotype may not be as prone to microbe-mediated trait effects as other switchgrass genotypes. In one study, Cave-in-Rock was not responsive to a known beneficial endophyte, *Burkholderia phytofirmans* strain PsJN (Kim et al. 2012). (2) Additionally, our perturbation treatment (autoclaving) may not have been sufficient to instigate microbe-mediated changes to plant functional traits. We found that autoclaving accounted for 10% of the variation in microbial community composition but did not affect species richness or Shannon diversity, meaning that the same ASVs were present in the perturbed and unperturbed treatment groups but in different abundances.

While we did not experimentally manipulate the abundance of specific microbes and therefore cannot establish causality, we did observe a significant correlation between one belowground functional trait and a microbial ASV. We found that the abundance of ASV 3749, which was assigned to the genus *Micromonospora*, was correlated with shorter root lengths. *Micromonospora*, a gram-positive bacterium in the phylum Actinobacteria, has been found in association with nodules of legumes and actinorhizal plants (Trujillo et al. 2015). They

Table 2

Selection Analysis to Understand How Patterns of Selection on Switchgrass (*Panicum virgatum*) Functional Traits Change with Nitrogen (N) Addition and Microbial Community Perturbations (P)

	Full data set		High N, unperturbed		Low N, unperturbed		High N, perturbed		Low N, perturbed		ANCOVA		
	Diff.	SE	Diff.	SE	Diff.	SE	Diff.	SE	Diff.	SE	P	N	N × P
Root length	.97*	.06*	.32*	.07*	.76*	.09*	.60*	.06*	.57*	.13*	.12	3.05	.03
Root diameter	.25*	.12*	−.09	.10	.02	.19	−.07	.15	−.21	.18	.03	.03	.55
Specific root length	−.25*	.12*	.11	.11	−.16	.19	.08	.14	−.01	.19	.002	.41	1.31
Root tissue density	−.12	.12	−.10	.11	−.17	.19	−.23	.14	−.00	.19	1.16	3.66	4.26*
Root-to-shoot ratio	−.39*	.12*	−.11	.10	−.009	.18	−.18	.14	−.32	.17	.09	4.47*	.01

Note. Selection differentials (diff.) measure the strength of selection on each trait individually (univariate) and do not account for correlations with other measured traits. The analysis of covariance (ANCOVA) estimates how trait effects on relative fitness are affected by microbes and nitrogen (*F*-statistics in italics indicate significance).

* Significant *F*-statistics ($P < 0.05$).

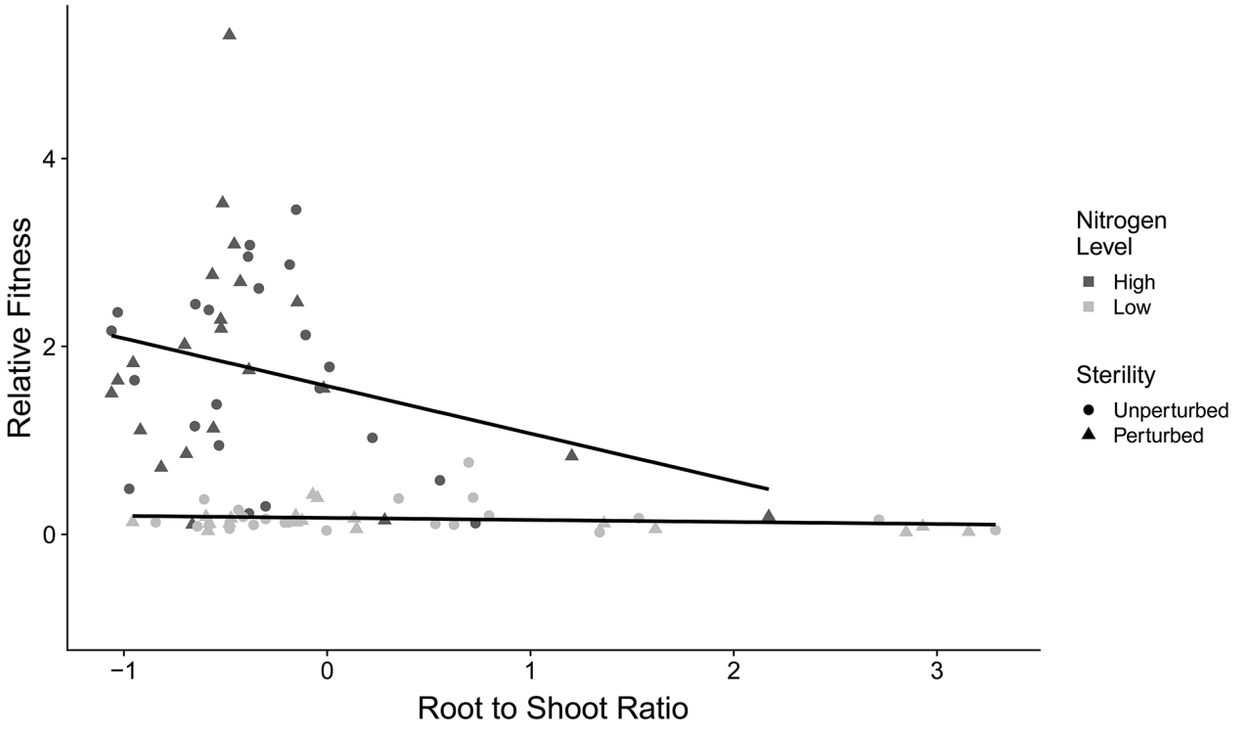

Fig. 6 Plants that have lower root-to-shoot ratios have higher relative fitness in high-nitrogen environments (dark gray circles and dark gray triangles). In low-nitrogen environments (light gray circles and light gray triangles), the relationship between relative fitness and root-to-shoot ratio is not evident.

are often described as growth-promoting bacteria (Sathya et al. 2017); however, we found that *Micromonospora* abundance was negatively correlated with switchgrass root length. Despite the limitations of the present work, which is based on correlations between microbes and traits, the analysis utilized herein is critical to the generation of testable hypotheses. In the future, *Micromonospora* abundance in the rhizosphere could be manipulated to understand cause and effect on host functional traits.

Patterns of Phenotypic Selection on Belowground Functional Traits

Understanding how belowground functional traits are impacted by selection provides a critical link between plant phenotypes and relative fitness in natural and agricultural settings. We found that plants were under selection for longer roots and an allocation shift toward aboveground biomass (smaller root-to-

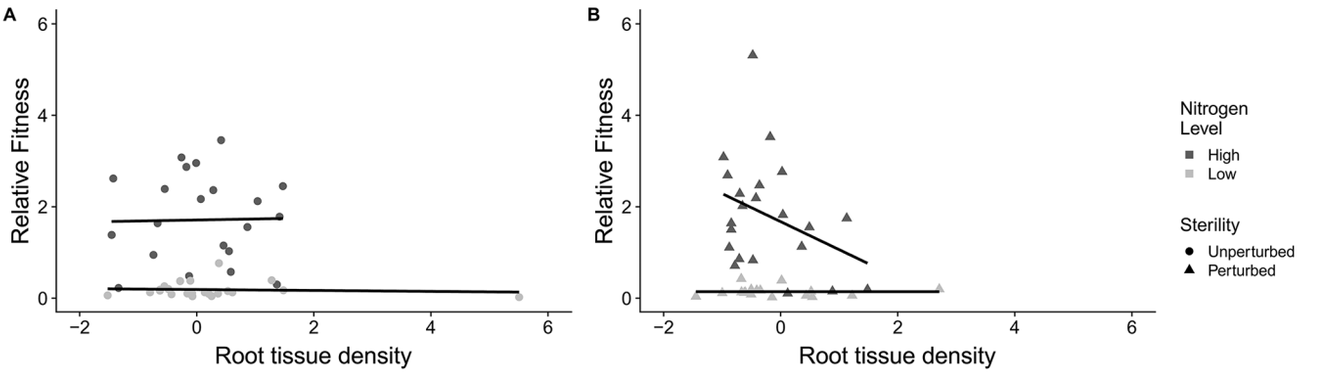

Fig. 7 Selection on root tissue density was dependent on biotic and abiotic conditions. Relative plant fitness was not correlated with higher root tissue densities (*A*) except in the high-nitrogen treatments with perturbed microbial communities (*B*). Dark gray triangles indicate samples that received autoclaved microbial inoculum (perturbed) and high nitrogen, and light gray triangles indicate samples that received autoclaved microbial inoculum (perturbed) and low nitrogen. Dark gray circles indicate samples that received unautoclaved inoculum (unperturbed) and high nitrogen, and light gray circles indicate samples that received unautoclaved inoculum (unperturbed) and low nitrogen.

shoot ratios), but the strength of selection on root-to-shoot ratio was dependent on nitrogen availability. Additionally, we found that plants were under phenotypic selection for lower root tissue density but most notably in treatments with high nitrogen and perturbed microbial communities. Overall, our results are consistent with the finding that abiotic factors have larger effects than biotic factors on phenotypic selection of plant functional traits (Caruso et al. 2020).

Studies that consider phenotypic selection on root functional traits are rare. However, phenotypic selection analysis on wild barley (*Hordeum spontaneum*) indicated that allocation to root biomass was under positive selection under optimal nutrient conditions but that selection was relaxed under nutrient stress (Volis et al. 2004). Although seemingly counterintuitive, the authors argue that allocation to belowground mass is already high in treatments with low nutrients, so additional shifts in allocation do not result in further fitness benefits. In another example, Heschel et al. (2004) found increased allocation to root biomass was adaptive under drought conditions. The only other study to our knowledge to manipulate microbial communities and measure patterns of phenotypic selection was carried out by Lau and Lennon (2011). They found that selection was more intense on plant traits in treatments with simplified microbial communities; similarly, we found more intense selection on root tissue density in perturbed microbial communities, but these effects were dependent on the nitrogen environment.

The process of evolution by natural selection consists of shifts in trait distributions due to differential survival and/or reproduction of organisms coupled with heritable variation in trait values (Geber and Griffen 2003). Here, we measured the first phase to understand which traits are favored in response to the selective environments of varying nitrogen levels and microbial communities. In order for the phenotypic selection that we detected to impact trait evolution, heritability of these belowground traits will need to be established. Work on *Panicum hallii*, a switchgrass relative, suggests that root length is one of the most heritable traits (60%), whereas root tissue density is one of the least heritable traits (40%; Khasanova et al. 2019). A combination of high selection differentials (as we found for root length) and high heritability can lead to rapid evolutionary change. Another important element in determining the response to natural selection is an understanding of selection acting on correlated traits (Geber and Griffen 2003). Selection differentials are a univariate analysis that considers single traits individually and measures both direct and indirect (through selection on other correlated traits) contributions to fitness. Selection gradients are a multivariate phenotypic selection analysis that describes direct effects of a trait on fitness by including correlations with other traits in the analysis as covariates (Lande and Arnold 1983). While the multivariate approach would have been more appropriate for five correlated belowground traits, we did not have sufficient sample sizes to make robust conclusions in this framework.

Future Directions

An exciting future direction in this system will be to disentangle cause and effect relationships between microbes and belowground functional traits. Microbes both act as part of the extended phenotype of plants and affect root functional traits and thereby affect relative fitness, but it is also possible that root functional traits determine the abundance of particular microbial groups. For example, shifts in root morphology could drive differences in host nutrient acquisition but also alter host exudate production with concomitant effects on the microbial community (Zhalnina et al. 2018). An interesting avenue for future work would be to include microbial taxa in phenotypic selection analysis. For example, by including the ASV 3749 (*Micromonospora* sp.) in multivariate selection analysis, we would better understand the relationship between the abundance of this ASV and plant relative fitness and whether effects on plant relative fitness are indirect (mediated through ASV effects on root length) and/or direct (through other antagonistic interactions in the rhizosphere).

Conclusions

Plant roots can both shape and in turn be shaped by the abiotic and biotic conditions belowground; therefore, understanding the ecological and evolutionary implications of these multidimensional interactions is an emerging field of critical importance to food and bioenergy crop production, as well as natural systems. Our study represents an empirical example of how nitrogen and microbial community composition interact to affect the evolutionary ecology of switchgrass—a relevant bioenergy crop—via belowground functional traits. We found that abiotic factors primarily affect switchgrass root functional trait values, but biotic and abiotic factors interact to determine patterns of selection on some root traits.

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ERRATUM

In the January special issue of *IJPS*, "Evolution of Functional Traits in Plants," two of the articles misidentified the editors who were overseeing peer review and editorial decision-making:

The article "Interactive Effects of Microbes and Nitrogen on *Panicum virgatum* Root Functional Traits and Patterns of Phenotypic Selection," by Renee H. Petipas, Alan W. Bowsher, Cody S. Bekkering, Chandra N. Jack, Emily E. McLachlan, Richard Allen White III, Brett S. Younginger, Lisa K. Tiemann, Sarah E. Evans, and Maren L. Friesen (*International Journal of Plant Sciences* 181:20–32), was edited by Christina M. Caruso.

The article "The Scaling of Genome Size and Cell Size Limits Maximum Rates of Photosynthesis with Implications for Ecological Strategies," by Adam B. Roddy, Guillaume Théroux-Rancourt, Tito Abbo, Joseph W. Benedetti, Craig R. Brodersen, Mariana Castro, Silvia Castro, Austin B. Gilbride, Brook Jensen, Guo-Feng Jiang, John A. Perkins, Sally D. Perkins, João Loureiro, Zuhah Syed, R. Alexander Thompson, Sara E. Kuebbing, and Kevin A. Simonin (*International Journal of Plant Sciences* 181: 75–87), was edited by Chase M. Mason.

The publisher regrets these errors.