# INTRA- AND INTERSPECIFIC VARIATION IN RHIZOSPHERE BACTERIAL COMMUNITY COMPOSITION AND METABOLISM AMONG MAIZE AND SUMMER ANNUALS IN AGRICULTURAL FIELDS

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# INTRA- AND INTERSPECIFIC VARIATION IN RHIZOSPHERE BACTERIAL COMMUNITY COMPOSITION AND METABOLISM AMONG MAIZE AND SUMMER ANNUALS IN AGRICULTURAL FIELDS

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Plants interact with diverse microbial communities in the rhizosphere that serve as a critical link mediating soil nutrient cycling and plant nutrient supply. As such, the controls on the composition and activity of this community and, in particular, the role of plant species and genotypes shaping community composition and activity is a subject of ongoing research with important implications for plant breeding and agricultural management. This dissertation seeks to understand the extent of plant genotype driven variation in rhizosphere bacterial community composition within maize (*Zea mays* subsp *mays*) and among species of summer annuals characteristic of agricultural fields. In three field experiments a common garden experimental design is combined with measures of plant growth and nitrogen acquisition, profiling of bacterial community composition (BCC) via 16S rRNA gene amplicon sequencing, and measures of potential extracellular enzyme activity to test hypotheses that: 1) plant variation in rhizosphere BCC is predicted by plant evolutionary history, 2) plant variation in growth and nitrogen economy influences rhizosphere BCC, and 3) historical selection for yield in fertilized production systems has altered maize rhizosphere bacterial community assembly and plant N acquisition efficiencies. We find that plant species differentially select rhizosphere bacterial communities and that the magnitude of variation is related to both plant phylogeny and variation in growth and nitrogen economy. Intraspecific variation in rhizosphere BCC between genotypes

within a plant species is also observed, but these differences are of lower magnitude and not well described by either functional variation or overall genetic distance between genotypes. Secondly, we observe that temporal variation in rhizosphere assembly and activity closely parallels temporal variation in plant growth and nitrogen uptake, which further highlights the link between plant function and plant rhizosphere effects. Finally, our results indicate that breeding has improved nitrogen uptake efficiency of maize hybrids but has not resulted in a parallel change in rhizosphere BCC. The implications of these patterns of variation in rhizosphere BCC are discussed in respect to agroecosystem management and plant breeding.

## BIOGRAPHICAL SKETCH

Bryan Emmett received his Bachelors of Science from the University of Michigan, School of Natural Resources and Environment in 2003. Following graduation he moved to Western Massachusetts to work as Stewardship Manager for the Berkshire Natural Resources Council, Inc. Bryan worked for the Council from 2003 to 2008. It was there, through his interaction with landowners and his work conserving and managing agricultural and forest lands, he developed an interest in agroecology. In 2009, Bryan married Shawnee Alexandra Barnes and moved to Ithaca, NY to begin his graduate program at Cornell Univeristy. In 2012 Bryan completed a Masters of Science in Horticulture, studying root biology and plant pathogen interactions. Following his Masters, Bryan joined Dr. Laurie Drinkwater's lab for his doctoral work to pursue further research in plant-microbe interactions. Bryan is the proud father of Ella Rose Emmett and Wesley David Emmett, who make him laugh to no end and have brought a new dimension of love and joy into Shawnee and his lives. When not in the lab or on the computer, Bryan can be found with his family, in the garden, in the kitchen, or occasionally chasing trout with a fly-rod on a local stream.

Dedicated to Ella Rose and Wesley David:

For the joy you have brought into my life

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## TABLE OF CONTENTS

## LIST OF FIGURES

## LIST OF TABLES

# PREFACE

This dissertation reports research seeking to understand the sources and extent of plant variation in rhizosphere microbial composition and activity. Plants do not live in isolation, but rather grow in close association with microbial organisms colonizing the plant surface and internal structures. The rhizosphere, the zone of interaction between plant roots and soil, is a hotspot of plant-microbial interactions with profound influence on biogeochemical cycling in soils and plant productivity (Philippot *et al.*, 2009; Finzi *et al.*, 2015). Organisms in the rhizosphere perform diverse functions including modulating plant growth, development, plant health, and the critical cycling of limiting plant nutrients. Owing to these myriad interactions, the microbial community surrounding and infecting the root is increasingly recognized as integral to plant function. As such, knowledge of the factors controlling the assembly of this community and the consequences for plant productivity and ecosystem function are critical research challenges.

The rhizosphere is a dynamic environment cooperatively shaped by the soil physiochemical environment and plant root activity (Hinsinger *et al.*, 2005) and as a result both soil and plant factors influence the composition and activity of the rhizosphere bacterial community (Berg & Smalla, 2009). Soil background exerts a high-level of control on rhizosphere assembly through structuring the rhizosphere environment and providing the propagule pool from which the rhizosphere is colonized (Bulgarelli *et al.*, 2015; Leff *et al.*, 2017). Within a soil background, plant species and even plant genotypes have been shown to support distinct rhizosphere communities (Turner *et al.*, 2013; Peiffer *et al.*, 2013; Edwards *et al.*, 2015). Plant genetic driven differentiation of the rhizosphere microbial community has

important implications for natural and managed ecosystems as shifts in the above-ground community that result from disturbance, climate change, invasions or managed rotations could cascade to changes in rhizosphere and soil communities (Maul & Drinkwater, 2010). In agricultural systems, variation in rhizosphere assembly between genotypes further provides an opportunity for selection, breeding and the incorporation of rhizosphere traits into cultivars capable of fostering beneficial interactions to support plant productivity (Drinkwater & Snapp, 2007; Wissuwa *et al.*, 2009).

This importance and potential of rhizosphere processes is particularly relevant to nutrient cycling in soil. Nitrogen (N) is a limiting nutrient in most terrestrial ecosystems. N additions to agricultural systems have greatly increased crop productivity, but gaseous and leaching losses from these systems, which are estimated at near 40% of applied N (Gardner & Drinkwater, 2009), are significant contributors to climate change and nutrient pollution of waterways (Vitousek *et al.*, 1997). The goal of sustainable production systems is to utilize ecosystem processes in place of saturating external inputs and the rhizosphere is a promising point of intervention. Plant root and microbial community activity in the rhizosphere shape N cycling in soil through plant N uptake, microbial N immobilization, associative and symbiotic N-fixation, and through modulating rates of N-mineralization, nitrification and denitrification (Qian *et al.*, 1997; James, 2000; Herman *et al.*, 2006; Laungani & Knops, 2012). Some interactions involve well studied individual organisms involving trading of plant C for microbially provided nutrients, including N in the legume-rhizobia symbiosis and N and P in mycorrhizal symbiosis. Other critical processes are mediated by the broader community and rhizosphere food web. The rhizosphere priming effect is a widely observed increase in decomposition of soil organic matter and increase in N mineralization in the presence of plant roots (Kuzyakov, 2002). Following

microbial death or predation, this N may become available for plant uptake (Clarholm, 1985), and gross N mineralization in the rhizosphere is estimated to be 10 times greater than in bulk soil (Herman *et al.*, 2006). In this way the rhizosphere priming effect may serve plants as a mechanism by which they can increase rates of decomposition and resulting N mineralization in the vicinity of plant roots in order to meet plant nutrient demands (Hamilton & Frank, 2001). From the perspective of crafting sustainable agroecosystems, rhizosphere N flows offer a key advantage in that they occur in close temporal and spatial proximity to the zone of plant N uptake and therefore offer less opportunity for N loss than similar processes occurring in bulk soil or external inputs (Drinkwater & Snapp, 2007). Furthermore, similar to plant controls on rhizosphere microbial community assembly, plant rhizosphere effects on C and N cycling also appear to vary between species and plant genotypes (Cheng *et al.*, 2003; Laungani & Knops, 2012; Pathan *et al.*, 2015). Thus selecting plants and managing soils to foster rhizosphere N flows is an important long term research and management goal.

Despite progress in elucidating the role of plants in shaping the rhizosphere communities and the N transformations they mediate, there is little predictive framework for understanding the diversity of plant impacts on rhizosphere communities, N cycling, or potential feedbacks on plant productivity. For this dissertation three experiments were undertaken to explore the sources and extent of plant variation in rhizosphere community composition and its relation to plant nitrogen acquisition in summer annuals. Our overarching hypothesis is that rhizosphere community assembly and function is linked with plant function. This link could occur via correlated traits that support N acquisition or through the consequences of plant driven C and N fluxes in the rhizosphere. In chapter 1, the alternate, but not exclusive, hypotheses that plant phylogenetic relatedness and plant functional variation explain differences in rhizosphere

bacterial community composition is tested using nested levels of phylogenetic relatedness centered on a diverse collection of maize inbred lines, C4 grasses and dicots. In chapter 2, the functional variation observed in chapter 1 is analyzed to determine how temporal factors interact with plant identity effects in shaping the rhizosphere community. Again, attention is given to how variation in plant growth and N uptake relate to rhizosphere bacterial community assembly. In chapter 3, a historical perspective is taken on the role human selection has played in changing patterns of plant N acquisition and whether there has been indirect selection on the rhizosphere bacterial community. A selection of hybrids representing best-selling and widely adapted corn-belt maize hybrids from 1930s-present are used to assess changes in maize N uptake efficiency and rhizosphere bacterial community assembly.

The results from each experiment are written as stand-alone manuscripts, but share a common research thread, experimental approach and methodology. The experiments all utilize a common garden approach, whereby monocultures of plants are planted in the same environment. By holding environment constant it is possible to assess the role of plant genotype in shaping the plant and rhizosphere phenotypes of interest. Throughout, high-throughput amplicon sequencing of the 16SrRNA gene is used to characterize the taxonomic composition of the bacterial community. This approach benefits from generating large numbers of high quality reads that allow multiplexing of samples for sufficient replication to adapt to our experimental platform and sufficient sequencing depth of individual samples to adequately characterize the community composition (Caporaso *et al.*, 2012). However, 16S based surveys are limited by only providing information on the taxonomic composition of the bacterial community, from which functional inferences are difficult. To compliment this approach, measures of potential extracellular enzyme activity in rhizosphere samples are used to capture functional variation in a key

component of rhizosphere nitrogen cycling, the rate limiting step by which organic macromolecules are broken down (Schimel & Bennett, 2004). Finally, in each experiment our goal is to relate observed patterns of rhizosphere effects to variation in plant function—growth, nitrogen uptake and nitrogen use efficiency. Following these three chapters, a brief conclusion serves to reflect on what has been learned and chart a course forward for further understanding the controls on rhizosphere community assembly and function.

# CHAPTER 1

# PLANT PHYLOGENY AND LIFE HISTORY SHAPE RHIZOSPHERE BACTERIAL MICROBIOME OF SUMMER ANNUALS IN AN AGRICULTURAL FIELD

## ABSTRACT

Rhizosphere microbial communities are critically important for soil nitrogen cycling and plant productivity. There is evidence that plant species and genotypes select distinct rhizosphere communities, however, knowledge of the drivers and extent of this variation remains limited. We grew 11 annual species and 11 maize (*Zea mays subsp. mays*) inbred lines in a common garden experiment to assess the influence of host phylogeny, growth, and nitrogen metabolism on rhizosphere communities. Growth characteristics, bacterial community composition and potential activity of extracellular enzymes were assayed at time of flowering, when plant nitrogen demand is maximal. Bacterial community composition varied significantly between different plant species and genotypes. Rhizosphere beta-diversity was positively correlated with phylogenetic distance between plant species, but not genetic distance within a plant species. In particular, life history traits associated with plant resource acquisition (*e.g.* longer lifespan, high nitrogen use efficiency, and larger seed size) were correlated with variation in bacterial community composition and enzyme activity. These results indicate that plant evolutionary history and life history strategy influence rhizosphere bacterial community composition and activity. Thus, incorporating phylogenetic or functional diversity into crop rotation may be a tool to manipulate plant-microbe interactions in agricultural systems.

## 1.1 INTRODUCTION

The rhizosphere is a hotspot of plant-microbe interactions with profound influence on plant productivity and ecosystem function (Philippot *et al.*, 2013). Shaped by the release of labile carbon (C) from plant roots and root uptake of nutrients and water (Hinsinger *et al.*, 2009), the physiochemical environment of the rhizosphere supports a microbial community compositionally and metabolically distinct from that found in bulk soil (Mendes *et al.*, 2014). The resulting rhizosphere microbiome performs critical functions, modulating plant growth and development (Panke-Buisse *et al.*, 2015), plant health (Mendes *et al.*, 2011; Berendsen *et al.*, 2012), and plant nutrient acquisition (Philippot *et al.*, 2013; Pii *et al.*, 2015).

Nitrogen (N) is a limiting nutrient in most terrestrial ecosystems and plant-microbe interactions in the rhizosphere govern many N transformations in soil. The C-rich and N-limited environment of the rhizosphere is a site of associative N-fixation (James, 2000), and also frequently a site of increased decomposition and subsequent N mineralization of soil organic N pools (Kuzyakov, 2002; Herman *et al.*, 2006). While the details regulating this "rhizosphere priming effect" are still poorly understood, it is broadly thought improved C status of the rhizosphere relieves energetic constraints on microbial activity and production of extracellular enzymes that breakdown SOM (Averill & Finzi, 2011; Dijkstra *et al.*, 2013). The activity of these enzymes is a rate limiting step in decomposition and subsequent N mineralization (Schimel & Bennett, 2004) and increased rates of N cycling that follow can feed back and support plant N acquisition (Hamilton & Frank, 2001; Zhu *et al.*, 2014), particularly when coupled with the turnover or predation of microbial populations (Clarholm, 1985).

The importance of plant-microbial collaborations in plant nutrient acquisition presents an opportunity to modify crop-breeding approaches to select genotypes that foster rhizosphere

microbiomes that can decrease the need for surplus additions of N fertilizer (Drinkwater & Snapp, 2007; Wissuwa *et al.*, 2009). Therefore there is considerable interest in understanding the factors governing the assembly and function of the rhizosphere microbiome. An emerging picture suggests soil background is a dominant force in shaping bacterial community composition (BCC) in the rhizosphere. Within a soil context, plant species and genotypes influence this community (Berg & Smalla, 2009; Peiffer *et al.*, 2013; Edwards *et al.*, 2015; Bulgarelli *et al.*, 2015), which can in turn be modulated by plant developmental stage and plant health status (Zhang *et al.*, 2011; Chaparro *et al.*, 2014; Marques *et al.*, 2014). However, while some studies observe strong plant identity effects, others report no or limited effects (Wagner *et al.*, 2016; Leff *et al.*, 2017), and therefore understanding the sources and extent of plant-driven variation in the composition and function of the rhizosphere bacterial community remains a critical research challenge.

Presumably, variation in rhizosphere community composition and function is most likely driven by the evolutionary and ecological differentiation of host plants. For instance, several host-microbe interactions display a phylogenetic signal, such that closely related species share more similar microbiomes than distantly related species (Ley *et al.*, 2008; Brucker & Bordenstein, 2012a). Such a phylogenetic signal has been observed in the rhizosphere of the Poaceae (Bouffaud et al., 2014), and in the phyllosphere of a broad range of plants, where increasing beta-diversity can be observed at the species, order and division levels (Redford *et al.*, 2010). These patterns can arise from either specific co-evolutionary processes (Brucker & Bordenstein, 2012b) or, as proposed by Bouffaud et al. (2014) from microbiome assembly driven by the ability of phylogenetically conserved plant traits to shape microbial niche space in the rhizosphere.

Conversely, ecological differentiation among closely related hosts may interact with such a phylogenetic signal. For instance, diet is a significant driver of the mammalian gut microbiome and only after controlling for diet is a phylogenetic relationship between mammalian hosts and microbiome composition evident (Ley *et al.*, 2008). Plant uptake of N and release of C are among several factors that shape the rhizosphere physiochemical environment (Bell et al., 2015; Hinsinger et al., 2005), therefore plant traits governing nitrogen and carbon acquisition and use may be strongly linked with plant variation in rhizosphere composition (Zancarini et al., 2013).

Plants adapt to varying levels of N availability through their competitive ability to acquire nitrogen from soil and their nitrogen use efficiency (NUE) defined broadly as the amount of C fixed per unit plant N (Vitousek, 1982). Both strategies, N-acquisition and NUE, may affect rhizosphere communities. The rate of plant N uptake likely shapes plant-microbe competition for N. Correspondingly, differences in rhizosphere BCC have been observed between genotypes or plant species that differ in rates of N uptake (Moreau et al., 2015; Pathan et al., 2015), and these differences extend to indicators of N-cycling and extracellular enzyme activity (Cantarel et al., 2015; Pathan et al., 2015). Conversely, NUE is often associated with improved N retention in plant tissues (Berendse and Aerts, 1987), and plant traits promoting tissue longevity and N retention (*e.g.* increased tissue thickness, lignin content and decreased N content) are associated with decreased rates of decomposition and nutrient-cycling in soils under high NUE plants (Diaz et al., 2004; Orwin et al., 2010). These nutrient-cycling effects may be an indirect consequence of variation in litter quality, but it is also possible that these effects are mediated by direct plant impacts on microbiome composition and function.

To investigate the sources and extent of plant variation in rhizosphere effects we

conducted a common garden experiment with a selection of maize inbred lines and summer annual species commonly found in agricultural systems. We characterized BCC and enzyme activity in plant rhizospheres to test hypotheses that 1) plant rhizosphere effects vary according to the evolutionary history of host species, and 2) that variation in rhizosphere BCC and metabolism is associated with variation in plant growth characteristics and nitrogen economy.

## 1.2 MATERIALS AND METHODS

### 1.2.1 Experimental design

A common garden experiment was conducted at the Musgrave Research Station in Aurora, NY (42°44'11"N 76°39'05"W). The soil at the site is classified as fine-loamy, mesic Oxyaquic Hapludalfs, with a circumneutral pH of 7.65 and consisted of 45.2% sand, 33.5% silt and 21.3% clay. The soil was 1.7 ±0.14% carbon and 0.17 ±0.14% N; inorganic N ($NH_4$ + $NO_3$) content at tillage was 7.1 ±1.3 μg $g^{-1}$. Mehlich extractable P and K concentrations were 19.5 ±1.5 μg $g^{-1}$ and 146 ±16 μg $g^{-1}$ respectively. The field was previously managed as a corn-soy rotation and had been planted to corn in the previous year. Prior to planting the field was moldboard plowed, disked, fit for planting and fertilized with 224 kg $ha^{-1}$ of potassium phosphate (0-15-30).

Plants were selected to encompass a range of intra- and interspecific diversity found in agricultural fields. This included ten founding inbred lines of the maize (*Zea mays subsp mays* L.) Nested Association Mapping (NAM) population, which represents the genetic diversity of improved maize (Yu *et al.*, 2008), as described by Peiffer et al. (2013). Lines were chosen to represent differences in growth, N uptake and yield under fertilized and unfertilized conditions (Meyer, 2006). Additionally, one inbred line (75-062) was included from a public organic

breeding program. We broadened phylogenetic and functional variation by including eight C4 grasses (*Echinochloa crus-galli* (L.) P.Beauv, *Setaria faberi* R.A.W.Herrm., *Eragrostis tef* (Zucc.) Trotter, *Sorghum bicolor subsp. Bicolor* (L.) Moench, *Sorghum x drummondii* (Nees ex Steud.) Millsp. & Chase, and *Eleusine coracana* (L.) Gaertn.), four dicots (*Abutilon theophrasti* Medik., *Amaranthus powellii* S.Watson, *Helianthus annuus* L., *Fagopyrum esculentum* Moench), and a legume (*Glycine max* (L.) Merr.) (Table A1).

Replicated monocultures were planted on June 19$^{th}$ and 21$^{st}$, 2013 in a split-plot randomized complete block design (n = 4). Plots consisted of eight 1.83 m rows spaced at 76 cm, with 23 cm between plants in a row, resulting in a final density of 57,500 plants ha$^{-1}$. Each main plot was split such that half the rows received a nitrogen application of 23.5 kg N ha$^{-1}$ at planting and two side-dress applications (July 11$^{th}$ and August 5$^{th}$) totaling 95 kg N ha$^{-1}$ as $(NH_4)_2SO_4$, while the remaining rows received no N fertilizer. This fertilizer level was chosen to boost plant growth but not provide luxury N conditions. Granular side-dress N was hand applied throughout the plot and incorporated during cultivation. Plots were kept weed free through mechanical cultivation and hand weeding.

### 1.2.2 Plant and rhizosphere sampling

Plants were harvested when at least 50% of the flowers/tassels for that genotype were shedding pollen. Since the phenology of these species are not synchronized this resulted in eight harvests (Table S1). By sampling at a common developmental stage we control for the effects of plant developmental stage on rhizosphere BCC (Chaparro *et al.*, 2014; Marques *et al.*, 2014) and by sampling at anthesis, when plant biomass accumulation and nutrient uptake are maximal, we are able to evaluate rhizosphere composition when it is most relevant for nutrient uptake of each species. Three to four adjacent and representative plants from an interior row of each plot were

clipped at the first nodal roots and dried at 60 °C for dry weight determination. Homogenized and ground tissue was analyzed for tissue C and N content on a PDZ Europa ANCA-GSL elemental analyzer at the University of California Davis Stable Isotope Facility.

At sampling, root systems were loosened from the ground with a spade and soil loosely adhered to the root system was removed by massaging and gentle shaking and discarded. Soil that remained adhered to the roots was considered rhizosphere soil and gently removed with a gloved hand, passed through a 2 mm sieve, and bagged for downstream analysis of inorganic N content and potential extracellular enzyme activity. Additionally, intact roots with adhering rhizospheres were sampled by clipping randomly selected 4 cm segments of root tips and parent 2$^{nd}$ order roots for nucleic acid analysis. On each sampling date, 2 cm diameter by 20 cm deep soil cores were collected from unplanted, weed-free plots to represent bulk/bare soil in downstream analyses. Multiple cores were combined, homogenized, subsampled and passed through a 2 mm sieve. All samples were immediately placed on ice and then stored at 4 °C for downstream analysis of enzymes and inorganic N content and at -40°C for nucleic acid analysis.

### 1.2.3 Extracellular enzyme analysis

Potential activity of enzymes involved in degradation of hemi-cellulose (β-xylosidase (BX)), cellulose (cellobiohydrolase (CB)), protein (leucine aminopeptidase (LAP)) and chitin (β-*N*-acetyl-glucosaminidase (NAG)) were measured using standard fluorometric assays following German *et al.* (2011). Briefly, 2-3 g field moist soil was mixed with 150 ml of 50 mM sodium bicarbonate buffer adjusted to pH 8 for 60 seconds using an immersion blender. 200 µl of soil slurry was added to 8 replicate wells of a 96-well plate containing 50 µl of 200 µM substrate with attached fluorophore. LAP plates were incubated for 2 hrs and BX, CB and NAG incubated for 4 hrs at 30 °C. Fluorescence was measured on a BioTek Synergy HT microplate reader at

365 nm excitation and 450 nm emission. Enzyme activity for each soil was estimated using a standard curve (0-75 μM) prepared from the same homogenate to control for quenching and autofluorescence. Standard curves were made fresh daily. To fix a perceived degradation of our standard over the season, the curves from each date were scaled so the maximum florescence of the 50 μM standard was equal across dates. All enzyme analyses were completed within 48 hrs of sample collection. Subsamples of soil were dried at 60 °C for 48 hrs for soil moisture determination. Enzyme activity is expressed on a soil dry weight basis (nmol g $soil^{-1}$ $hr^{-1}$).

### 1.2.4 Inorganic nitrogen determination

A subset of species and genotypes were chosen to collect sufficient rhizosphere soil for inorganic N determination. Inorganic nitrogen was extracted in duplicate from 8-10 g of rhizosphere or bulk soil in 40 ml of 2 M KCl, shaken for one hour and filtered through pre-rinsed ashless Whatman filter paper. Extracts were analyzed colorimetrically for nitrate and ammonium concentration using a $VCl_3$/Griess method (Miranda et al., 2001) and modified indophenol method (Kandeler and Gerber, 1988), respectively, in a 96-well microplate format following Doanne and Howarth (2003) and Hood-Nowotny *et al.* (2010). Plates were incubated at 37 °C for 2 hrs for nitrate determination and 30min at 21 C for ammonium determination. Absorbance of wells was analyzed on a BioTek Synergy HT microplate reader at 540 nm and 660 nm, respectively. Concentrations were calculated using a standard curve included on each plate and expressed on a soil dry weight basis (μg N g $soil^{-1}$).

### 1.2.5 16S rRNA gene sequence analysis

Root and rhizosphere and bare soil samples stored at -40 °C were lyophilized for 24 hrs on a LabConco FreeZone 2.5 freeze dry system. Roots were chopped to < 1 cm length segments,

mixed, and between 0.01 and 0.05 g of freeze dried roots and adhering soil or 0.15 g of bare soil controls were added directly to each well of a 96-well extraction plate from the MoBio PowerSoil-htp DNA kit (Carlsbad, CA). Rhizosphere samples were added to duplicate wells to adequately capture heterogeneity of the root systems. Samples were homogenized on a BioSpec Mini-Beadbeater-96 (Bartlesville, OK) for 2 min and extractions proceeded according to manufacturer's instructions. The 0.7 mm bead size of the PowerSoil kit does not homogenize root tissue and roots remained largely intact following homogenization (Peiffer et al., 2013). However, there was likely disruption of epidermal and some cortical cells and it is likely that our extracts contained some DNA from root endophytes. Following bead beating, extraction proceeded according to the kit manufacturer's instructions. DNA yields were quantified with the Quant-iT PicoGreen dsDNA assay kit (Invitrogen). Extractions yielded a mean of 375 ± 224 ng DNA template for use in downstream applications.

Dual-barcoded MiSeq libraries of the SSU rRNA V4 region were prepared as in Kozich et al., (2013) using the forward (515F) (Whitman et al., 2016) and reverse primers (806R) adapted from Caporaso et al. (2010). Amplicons were prepared in triplicate reactions. Each reaction included 5 ng of template DNA, 12.5 µl of 2x Q5 High Fidelity, Hot Start PCR Mastermix, 1 µM combined forward and reverse primer, 0.5 µg bovine serum albumin and 0.625 µl of 4x PicoGreen reagent to monitor DNA template production for a total volume of 25 µl. PCR conditions consisted of: 95 °C for 2 min; 30 cycles of 95 °C for 20 sec, 55 °C for 15 sec and 72 °C for 10 sec; final extension 72 °C for 5 min. Pooled triplicate reactions were standardized using the SequalPrep Normalization Plate Kit (Life Technologies). Standardized reactions were pooled then gel purified and extracted using the Wizard SV Gel and PCR Clean-Up System (Promega). The two resulting amplicon libraries were submitted for 2 x 250 bp

paired-end sequencing on the Illumina M-Seq platform with the MiSeq Reagent v2 kit at the Cornell Biotechnology Resource Center Genomics Facility (Ithaca, NY).

Resulting reads were processed in a custom bioinformatics pipeline as in Whitman et al. (2016). Overlapping paired-end reads were merged using PEAR (v0.9.2)(Zhang et al., 2014). Merged reads were de-multiplexed with a custom python script and those that did not match a known barcode were discarded. Remaining reads were filtered to remove sequences with max expected error rates > 1 with USEARCH (Edgar, 2013), ambiguous base calls, ≥8 homopolymers and singletons (unique). Sequences were clustered into operational taxonomic units (OTUs) at a 97% pairwise identity cutoff with USEARCH (Edgar, 2013). Taxonomic assignment of OTUs was performed with Qiime's parallel taxonomy assignment using the uclust consensus taxonomy assignment function (Caporaso et al., 2010) and the Silva reference database (v.111) (Quast et al., 2013). OTUs belonging to chloroplast, mitochondria, eukaryotes, archaea and unassigned sequences were removed. OTUs were aligned using SSU_align and poorly aligned positions masked based on posterior probabilities (Nawrocki, 2009). A phylogenetic tree was created and rooted to *Sulfolobus* (acc. X90478) using FastTree (Price et al., 2009) with default settings. The resulting OTU table contained 11,246 OTUs representing 7,517,735 mapped reads and was combined with phylogenetic tree, taxonomic information and metadata for analysis using the phyloseq package in R (McMurdie and Holmes, 2013). This OTU table was further filtered using a sparsity threshold of greater than three reads in more than three samples in order to remove extremely rare taxa, but retain taxa that may be endemic in the rhizosphere of a particular genotype, which resulted in 4982 OTUs. Sequences and associated metadata were deposited in the NCBI sequence read archive under accession #SRP119673.

### 1.2.6 Phylogeny and genetic distance matrices

Chloroplast rbcL and matK genes were used to construct a phylogeny of the twelve plant species. Representative sequences were downloaded from the GeneBank Nucleotide Database. *Amaranthus powellii* was not represented and sequences from congeneric *A. viridis* were used instead. Sequences were aligned and checked in Unipro UGENE (Okonechnikov *et al.*, 2012) and a tree was constructed using phyloGenerator with *Ginko biloba* used as an out group (Pearse & Purvis, 2013). A distance matrix was derived using the cophenetic.phylo function in the R package "ape" (Paradis et al., 2004). We expected little intraspecific variation in chloroplast rbcL or matK genes and arbitrarily assigned a distance of .0002 to intraspecific comparisons. This approximates intraspecific distances among maize lines found in previous studies using chloroplast markers (Bouffaud et al., 2014). Genetic distance matrices for the ten NAM inbred lines were constructed using GBS markers build 2.7 available at panzea.org. Distance matrices were estimated using TASSEL version 5 (Bradbury *et al.*, 2007).

### 1.2.7 Statistical analysis

Statistical analyses were conducted in R (R Development Core Team, 2012). To compare growth of plants sampled on different days, log-transformed plant biomass, N uptake and NUE (g C g $N^{-1}$) were modeled by days after planting (DAP) (Figure S1) for all species with one representative of maize (cv. B73). Data from an early season biomass cut was included to improve the model of plant growth over the course of the season (Figure S1). Inbred maize lines were modeled separately in order to avoid weighting the model of plant growth. Residuals from the best-fit line were used to estimate variation in plant growth characteristics independent of flowering time.

Univariate tests were conducted in the package "lme4" (Bates et al., 2015) and p-values

estimated with "lmerTest" (Kuznetsova et al., 2016). Sample type, plant genotype, fertilization and interactions were considered fixed effects with the random effects of replicate block and split-N fertilization plots. A similar mixed model was used to test the influence of rhizosphere inorganic N concentration on potential extracellular enzyme activity. Here, plant genotype was included as a random effect to control for influence of plant genotype and date of sampling on enzyme activity. Post-hoc tests were conducted using the glht function in the "multcomp" package (Hothorn et al., 2008).

Bacterial community beta-diversity was analyzed using weighted-UniFrac distance matrices (Lozupone et al., 2011) constructed using an OTU table rarified to 4989 reads per sample in the phyloseq package in R (McMurdie and Holmes, 2013). Treatment effects on beta-diversity were tested using permutational multiple analysis of variance (PERMANOVA) using the "adonis" function in the Vegan package (Oksanen et al., 2012). We tested the effect of phylogenetic distance, maize whole genome genetic distance, and variation in growth characteristics between plant hosts on rhizosphere BCC using a generalized least squares implementation of Clarke's maximum likelihood population effects model (MLPE) (Clarke et al., 2002), using the R function corMLPE (https://github.com/nspope/corMLPE). The MLPE allows correlation between distance matrices by using a random effect parameter to estimate residual covariance of observations sharing a common sample, which would otherwise violate the assumption of independent observations (Clarke et al., 2002). To avoid pseudo-replication in the analysis of phylogenetic and genetic distance, the weighted-UniFrac distance matrix was calculated on OTU tables averaged over each genotype. In the models of phylogenetic and genetic distance, the sampling dates of each pairwise plant comparison was included as a fixed effect to control for variation between sampling dates. These models were evaluated using a

likelihood ratio test against the nested null model of sampling date. Analyses of interspecific variation were conducted using all species with one representative of maize (cv. B73), while intraspecific analyses were conducted using the maize inbred lines. When not explicitly stated, analyses were conducted using all samples. To further explore the role of plant growth characteristics in shaping interspecific variation in rhizosphere BCC we constrained the principle coordinate analysis of weighted UniFrac distances to display only variation that could be explained by plant growth metrics, using the "CAP" method of the "ordinate" function in phyloseq.

The response of individual OTUs to treatments and correlation with covariates was calculated as $\log_2$-fold change using non-rarified OTU table in a negative binomial model within the DESeq2 package (Love et al., 2014) and Benjamini & Hochberg corrected p-values reported. Rhizosphere responders were identified as those OTUs with a significant positive $\log_2$-fold change greater than 0.5 between a genotype's rhizosphere and the bare soil controls sampled on the same date. When testing the role of rhizosphere inorganic N concentration or fertilization on OTU abundance plant genotype was included in the model to control for variation between plants and between sampling dates. All figures were created in the package "ggplot2" (Wickham, 2009) except the circular phylogenetic tree, which was created using the interactive tree of life (iTOL) web server (Letunic and Bork, 2016). Final annotation and formatting of figures was performed in Inkscape. Scripts for bioinformatics pipeline, analysis and figure generation are available at https://github.com/bdemmett/RhizCG.

## 1.3 RESULTS

### 1.3.1 Variation in plant growth and nitrogen economy

Across plant species and genotypes we observed nearly ten-fold variation in biomass accumulation and N uptake, and four-fold variation in NUE at anthesis (Figure A1). This variation derived primarily from differences in flowering time and sampling date. Flowering time ranged from 36 DAP for *F. esculentum* to 88 DAP for *E. coracana*, and from 72 to 88 DAP for short and long season maize lines (Table A1). As a result, DAP captured 69% and 77% of the variation in log-transformed N uptake and NUE among the plant species sampled (91% and 86% among maize inbred lines, respectively) (Figure A1). While this is expected, as longer-lived plants have more time to grow and acquire N from soil, it highlights the variation in resource demand among annual plants. The longer-lived plants had both greater N demand and greater NUE owing to increased effective retention time.

Residuals from the models above were used to evaluate differences in plant growth and N economy, independent of phenology and lifespan. In interspecific comparisons, we observed significant variation in total N uptake ($p < 0.01$), NUE ($p < 0.01$), and corresponding differences in biomass accumulation ($p < 0.01$; Table A2). These results are well illustrated by contrasting *E. crus-galli* and *A. powellii*, which had considerable differences in N uptake despite being harvested on the same date; and are also illustrated by contrasting *E. tef and S. x drummondii*, which had a two-fold difference in N uptake despite their similar phenology (Figure A2). This variation may be partly attributed to seed size. For instance, the extremely small-seeded *E. tef* had relatively low biomass and N uptake residuals. Yet this was not a consistent trend as *S. x drummondii* and *H. annuus* had comparable biomass accumulation and N uptake despite large differences in seed size (Figure A2). In contrast to interspecific comparisons, we did not observe

significant variation in N uptake among maize inbred lines ($p = 0.24$; Table A2). Rather, differences in biomass accumulation between maize genotypes ($p < 0.01$) were associated with differences in NUE ($p < 0.01$; Table A2). As expected, nitrogen fertilizer significantly improved plant growth, N uptake and also lowered plant NUE ($p < 0.05$; Figure A2; Table A2).

### 1.3.2 Extracellular enzyme activity in the rhizosphere

We observed a significant stimulation of hydrolytic enzyme activity in the rhizosphere (Figure 1.1; Table A3). This rhizosphere effect was modulated by nitrogen fertilizer addition, whereby CB, BX and NAG activity increased in the rhizosphere of plants receiving fertilizer, but not in fertilized bare soil plots. In contrast, there was a trend toward increased LAP activity in both bare soil and rhizosphere samples that received fertilizer (Figure 1.1; Table A3). The fertilizer effect exhibited a positive correlation between inorganic N concentration and potential enzyme activity in the rhizosphere ($p < 0.05$; Table A4). Enzyme activity in the rhizosphere also differed between plant genotypes ($p < 0.05$; Table A5), however, this result was only observed when comparing plants with different sampling dates. These differences between dates were not associated with a trend toward increasing or decreasing activity over the growing season (data not shown).

### 1.3.3 Rhizosphere effect on bacterial community composition

In addition to shifts in enzyme activity, we observed a strong differentiation of BCC between bare soil and rhizosphere sample types (Figure 1.2). In a PERMANOVA of weighted-UniFrac distance, sample type was the greatest source of variation (Table 1.1; Figure 1.2a-c). Of the 4982 OTUs, 1502 were significantly enriched in the rhizosphere of at least one plant genotype compared with bare soils (Figure 1.3). Many of the rhizosphere responsive OTUs were

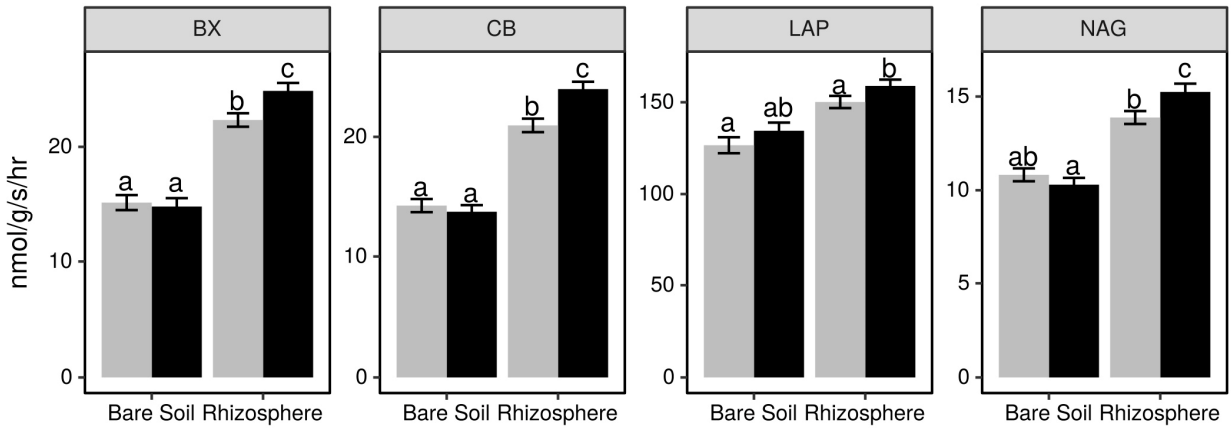


Figure 1.1: Potential activity of extracellular beta-xylosidase (BX), cellobiohydrolase (CB), leucine aminopeptidase (LAP) and N-acetyl-glucosiminidase (NAG) in bare soil and rhizosphere samples from plots receiving 0 kg N $ha^{-1}$ (grey bars) and 95 kg N $ha^{-1}$ (black bars). Letters indicate a significant difference between treatments (tukey HSD $p < 0.05$). Note that scale of y-axis differs among plots.

at low abundance in bulk soil, but obtained high abundance in the rhizosphere, resulting in a dramatic shift in community composition (Figure 1.4a). These rhizosphere responsive taxa included 54 OTUs that were present in rhizosphere samples but not detected in bulk soil (Figure 1.4b), which could result from rhizosphere enrichment of extremely rare taxa or vertical transmission of root endophytes.

Taxa enriched in the rhizosphere belonged to diverse phyla (Figure 1.3; Table A6), but clustered within several groups. The *Proteobacteria* and *Actinobacteria* accounted for 23.6% and 16% of median relative abundance in rhizosphere samples and taxa from the *Bacteroidetes*, *Chloroflexi*, *Verrucomicrobia* and *Firmicutes* also comprised a substantial fraction of the rhizosphere community (Figure 1.3, Table A6). Within these phyla, some families showed a strong rhizosphere preference. The branching depth of clades sharing a phenotype can indicate the degree of phylogenetic conservation of that trait (Martiny *et al.*, 2013). In our dataset, clades with more than 90% of OTUs displaying a rhizosphere response had a deeper average branching depth than expected under a permuted null model (Tau D = 0.02, $p = 0.04$; consenTRAIT), indicating phylogenetic conservation of the rhizosphere response within certain families. This included families within the *Proteobacteria* (*Comamonadaceae*, *Oxalobacteraceae, Caulobacteraceae,* and *Sphingomonadaceae), Actinobacteria (Streptomycetaceae), Firmicutes (Bacillacae* and *Paenibacillaceae), Bacteroidetes (Flavobacteriaceae* and *Chitinophagaceae), Verrucomicrobia (Opitutaceae), and Chloroflexi (Chloroflexaceae).*

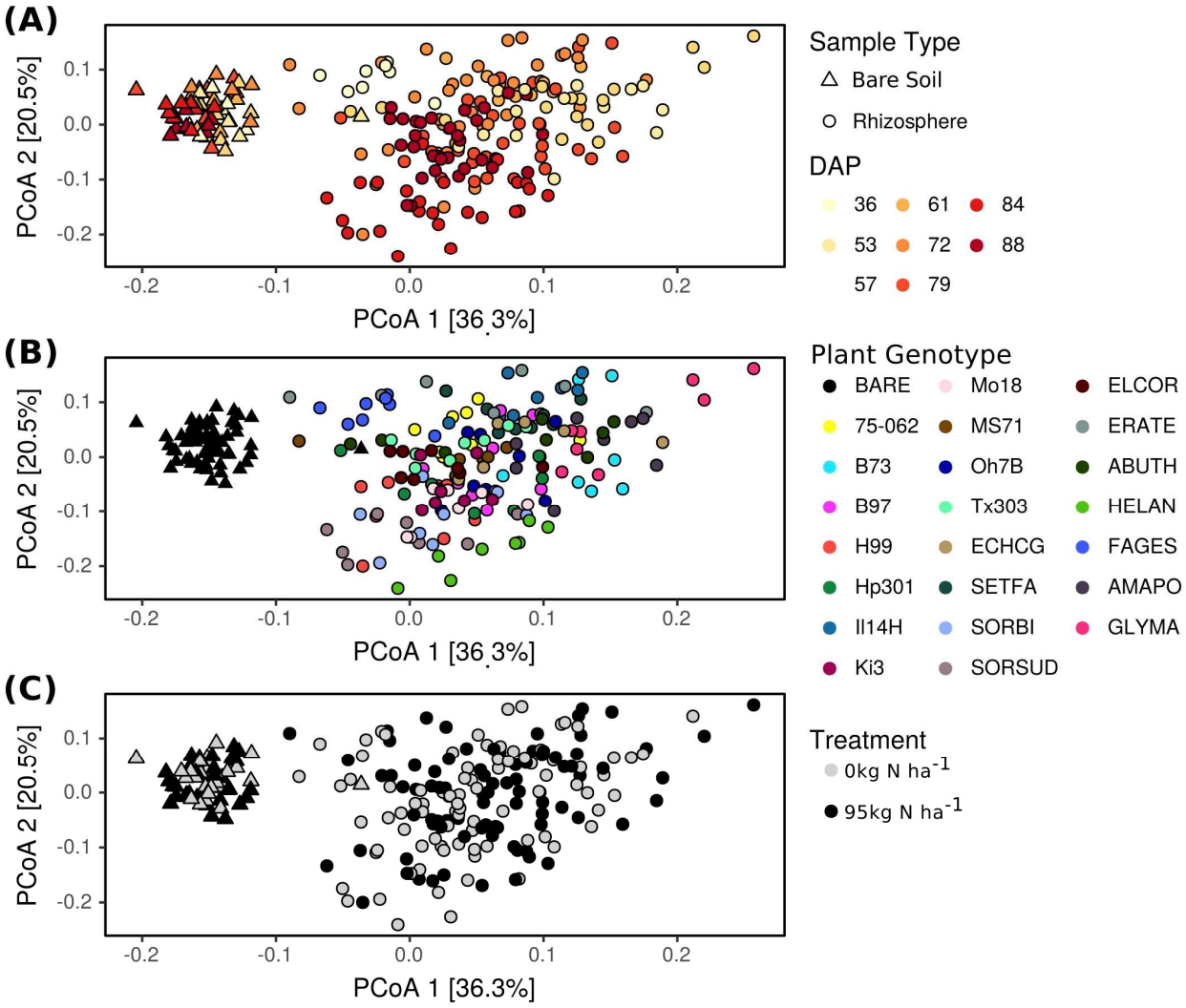


Figure 1.2: Bacterial community composition varies between rhizosphere and bulk soil and with respect to days after planting (A), plant genotype (B), and nitrogen fertilization (C). Changes in bacterial community composition are visualized as a principal coordinate analysis (PCoA) of weighted-UniFrac distances between samples. Genotype codes represent maize inbred lines and species: *E. crus-galli* (ECHCG), *S. faberi* (SETFA), *S. bicolor* (SORBI), *S. x drummondii* (SORSUD), *E. coracana* (ELCOR), *E.s tef* (ERATE), *A. theophrasti* (ABUTH), *H. annuus* (HELAN), *F. esculentum* (FAGES), *A. powellii* (AMAPO), and *G. max* (GLYMA).

Table 1.1: Permutational multiple analysis of variance testing main effects of sample type (rhizosphere vs. bare soil), days after planting (DAP), plant genotype or species identity (genotype), and nitrogen fertilization treatment (0, 95 kg N $ha^{-1}$) on bacterial community beta-diversity (weighted-UniFrac).

| Factor | SS | DF | F | $R^2$ | $p^*$ |
|---|---|---|---|---|---|
| Full dataset | | | | | |
| Sample type | 2.24 | 1 | 141.49 | 0.25 | <0.01 |
| DAP | 1.47 | 7 | 13.20 | 0.17 | <0.01 |
| Genotype | 1.80 | 21 | 5.39 | 0.20 | <0.01 |
| N treatment | 0.04 | 1 | 2.65 | 0.005 | 0.03 |
| Residuals | 3.28 | 207 | | 0.40 | |
| Rhizosphere | | | | | |
| DAP | 1.97 | 7 | 14.87 | 0.33 | <0.01 |
| Genotype | 1.16 | 14 | 4.37 | 0.19 | <0.01 |
| N treatment | 0.06 | 1 | 2.92 | 0.01 | <0.01 |
| Residuals | 2.85 | 151 | | 0.47 | |
| Bare Soil | | | | | |
| DAP | 0.13 | 1 | 2.61 | 0.25 | <0.01 |
| N treatment | 0.009 | 1 | 1.19 | 0.02 | 0.19 |
| Residuals | 0.41 | 61 | | 0.73 | |

* *P*-values based on 999 permutations.

Nitrogen fertilization had a statistically significant, but very small, influence on BCC, accounting for < 1% of the variation in the PERMANOVA of rhizosphere samples, which is not easily perceptible in the ordination (Table 1.1; Figure 1.2c). Nitrogen fertilization led to the enrichment of 118 OTUs and decline of 45 OTUs in relative abundance within plant rhizospheres (Figure A3; log2-fold change $\neq 0$, $p < 0.05$). Many of the OTUs responding positively to N fertilization were from the *Proteobacteria, Actinobacteria and Bacteriodetes*, though these phyla also had representatives that decreased in abundance in response to N fertilization (Figure A3). Several *Nitrospiraceae* and a single *Nitrosomonadaceae* increased in abundance, while several other *Nitrosomonadaceae* decreased in abundance in rhizosphere samples from fertilized plots (Figure A3). In comparison, relatively few OTUs were correlated with inorganic N concentration in the rhizosphere, which suggests that the effects of fertilization on BCC in the rhizosphere are indirect, being driven less by the availability of mineral N and more by changes in plant growth and physiology, which occur in response to fertilizer. This result is not unexpected since plants were sampled at anthesis and not immediately after fertilization. In addition, fertilization did not have a significant impact on BCC in bare soil (Table 1.1; Figure 1.2c), further emphasizing the role of plants in mediating the observed response of BCC to fertilization.

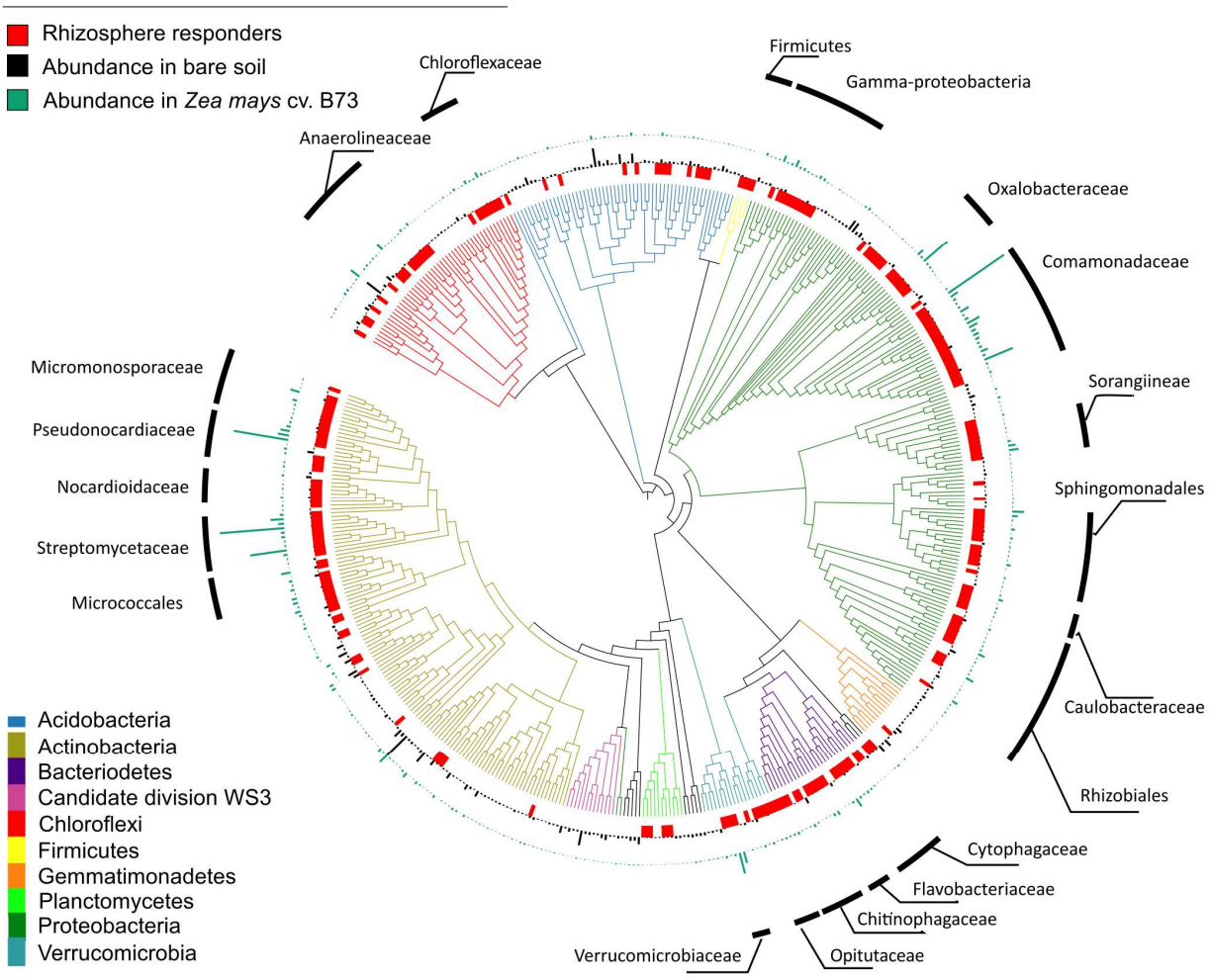


Figure 1.3: Phylogeny of 500 most abundant bacterial taxa in common garden experiment. From inner circle outward: red tiles indicate taxa significantly enriched in the rhizosphere of at least one genotype compared to bare soil controls collected on the same date (DESeq2: $\log_2$-fold change > 0.5; adjusted $p < 0.05$), black bars indicate mean relative abundance of OTUs in bare soil samples, green bars indicate mean relative abundance of OTUs in rhizosphere samples from maize cv. B73, chosen to represent the rhizosphere effect in general. Tree created using the interactive tree of life (iTOL) web server.

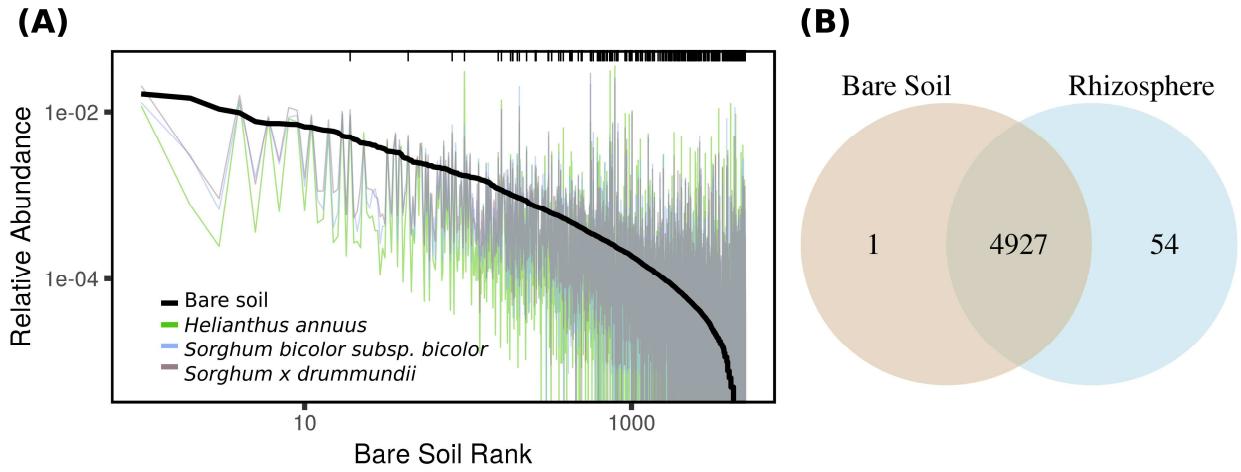


Figure 1.4: (A) Variation in OTU mean relative abundance from bare soil to rhizosphere of three representative species sampled on a single date. Black line indicates rank abundance in bare soil and colored traces indicate shifts in OTU relative abundance in rhizosphere samples. Black ticks indicate positive rhizosphere responders identified on any sampling date (DESeq2: $\log_2$-fold change > 0.5; adjusted $p < 0.05$), highlighting the enrichment in the rhizosphere of taxa at low abundance in bulk soil. (B) OTUs detected in bare soil and rhizosphere samples in full dataset

### 1.3.4 Plant genotype shapes rhizosphere community composition

Plant genotype and flowering time strongly shaped rhizosphere BCC as indicated by PERMANOVA (Table 1.1). Variation between genotypes sampled on different dates accounted for 33% of the variation in rhizosphere BCC (Table 1.1; Figure 1.2a). Sampling date also explained a significant portion of variance in bare soil samples (Table 1.1; Figure 1.2a), however, both the total variance and the proportion of variance explained by sampling date were greater in rhizosphere samples than in bulk soils (Figure 1.2), and rhizosphere BCC varied dramatically from bulk soils (Figure 1.2). Thus, the variation between genotypes sampled on different dates cannot be explained by temporal variation in bulk soils and must be due to either plant genotype effects or unmeasured interactions between plant rhizosphere effects and time.

When added sequentially to the PERMANOVA to control for sampling date, plant genotype accounted for 19% of the variation in rhizosphere samples (Table 1.1). In this model, some plant genotype effects are attributed to sampling date, which provides a conservative estimate of plant genotype influence on the rhizosphere community. It is possible to eliminate sampling date effects on those dates when multiple genotypes were sampled. When assessed within a sampling date, plant genotype explained between 13% ($p = 0.09$) and 43% ($p < 0.01$) of variance in rhizosphere BCC (Table A7). Genotype was also an important predictor of intra-specific variation in rhizosphere BCC. When evaluating just the maize inbred lines, sampling date accounted for 10% of variance ($F_{(2,76)} = 6.44$; $p < 0.01$) and plant genotype explained an additional 26% ($F_{(8, 76)} = 3.95$; $p < 0.01$) of the variance in rhizosphere BCC.

### 1.3.5 Plant phylogeny and growth characteristics explain differences in rhizosphere community composition

To evaluate the influence of plant phylogeny on rhizosphere BCC we constructed an MLPE model using a weighted-UniFrac distance matrix of average OTU relative abundance for each plant genotype. Beta-diversity increased in relation to plant phylogenetic distance through the taxonomic rank of family (Figure 1.5a, c), explaining 8% of the variation between plants ($\chi^2_2$= 19.17, $p < 0.01$). This effect was robust when controlling for variation in BCC attributed to pairwise sampling date comparisons ($\chi^2_2$= 13.50, $p < 0.01$) and when intraspecific comparisons were removed from the dataset ($\chi^2_1$= 7.93, $p < 0.01$). Beta diversity appeared to plateau beyond the rank of family, such as when comparing between grasses and dicots, suggesting that plants with similar characteristics had similar effects on rhizosphere BCC. The phylogenetic signal was evident in overall beta-diversity and at the level of individual OTUs (Figure 1.6). Non-maize species had more differentially abundant OTUs than maize genotypes when compared to maize reference line B73 (DESeq2: log2 fold change $\neq 0$; BH adjusted $p <$ 0.05). In addition, changes in relative abundance for the differentially abundant OTUs were greater for non-maize species than for other maize inbred lines when compared to maize reference line B73 (Figure 1.6; Figure A4).

Flowering time, which explained most variation in plant growth and N economy (Figure A1), was also the best continuous predictor of beta-diversity in plot level data. Rhizosphere beta-diversity increased with time between sampling dates in a polynomial fashion (Figure 1.5b), accounting for approximately 28% of the variation in rhizosphere BCC ($\chi^2_2$ = 77.63, $p < 0.01$). Beta-diversity also increased with differences in plant NUE and seed size ($\chi^2_2$ = 37.08, $p < 0.01$),

but these factors only explained an additional 2% of the variation in rhizosphere BCC. The effect of sampling date could have multiple drivers including differences in physiology of plants with different lifespan, seasonal variation in soil characteristics, and temporal autocorrelation between sampling dates. There was also a relationship between flowering time and plant phylogenetic distance ($r = 0.56$, $p < 0.01$). Maize and many of the C4 grasses flowered later in the season while four of five dicots flowered early in the season. As a result, temporal variation in the relative abundance of OTUs will be driven both by plant species specific rhizosphere effects and by temporal variation in background soils. We highlight two *Streptomyces* OTUs to illustrate these patterns (Figure 1.5d). One is responsive to maize and related crop plants in the subfamily Andropogoneae (*S. bicolor* and *Sorghum x drummondii*). This maize responsive OTU is found in highest relative abundance during anthesis for maize and *Sorghum*, but it remains in low abundance in the rhizospheres of other plants sampled on these same dates (Figure 1.5d, top panel). In contrast, a second OTU from *Streptomyces* increases in abundance less specifically, responding to a range of plant genotypes including both C4 grasses and *H. annuus* (Figure 1.5d, bottom panel).

Intraspecific variation in rhizosphere BCC was not correlated with genetic distance ($\chi^2_1 = 1.51$, $p = 0.22$). Nor was beta-diversity correlated with variation in functional measures including flowering time, plant NUE or N uptake ($p > 0.05$).

### 1.3.6 Changes in bacterial community composition and activity associated with plant resource acquisition and use strategies

To further explore the role of plant growth characteristics in shaping variation in rhizosphere BCC between species, a principal coordinate ordination of weighted UniFrac distances was constrained by explanatory growth characteristics including: days to flowering,

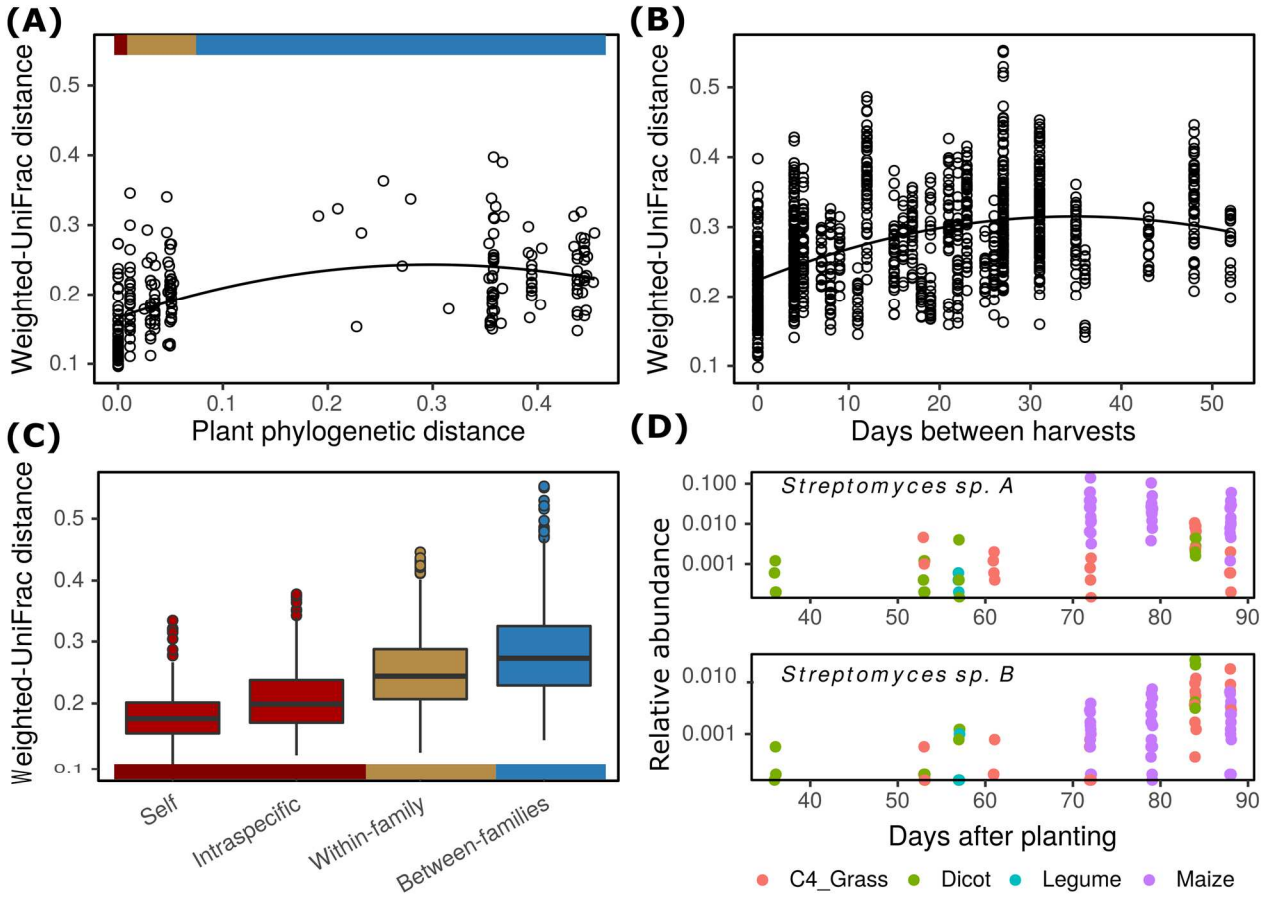


Figure 1.5: Plant phylogeny and flowering time influence rhizosphere bacterial community composition. Rhizosphere bacterial community beta-diversity (weighted-UniFrac distance) is positively correlated with plant host phylogenetic distance (A), days between sampling (B), and increases with plant host taxonomic rank (C). Colored bars indicate correspondence of plant phylogenetic distances and taxonomic rank between panel A and C. Influence of time and plant phylogeny on bacterial taxa abundance is illustrated with two *Streptomyces* OTUs (D). OTUs increase in abundance over time as a result of selective enrichment in maize (top panel) or enrichment over time independent of plant phylogeny (bottom panel) (DESeq2: $\log_2$-fold change per day = 0.08 ± 0.01 and 0.07 ± 0.01 in top and bottom panels respectively; $p < 0.05$). Weighted-UniFrac distances calculated on mean genotype OTU abundances (A) and plot level OTU abundances (B, C).

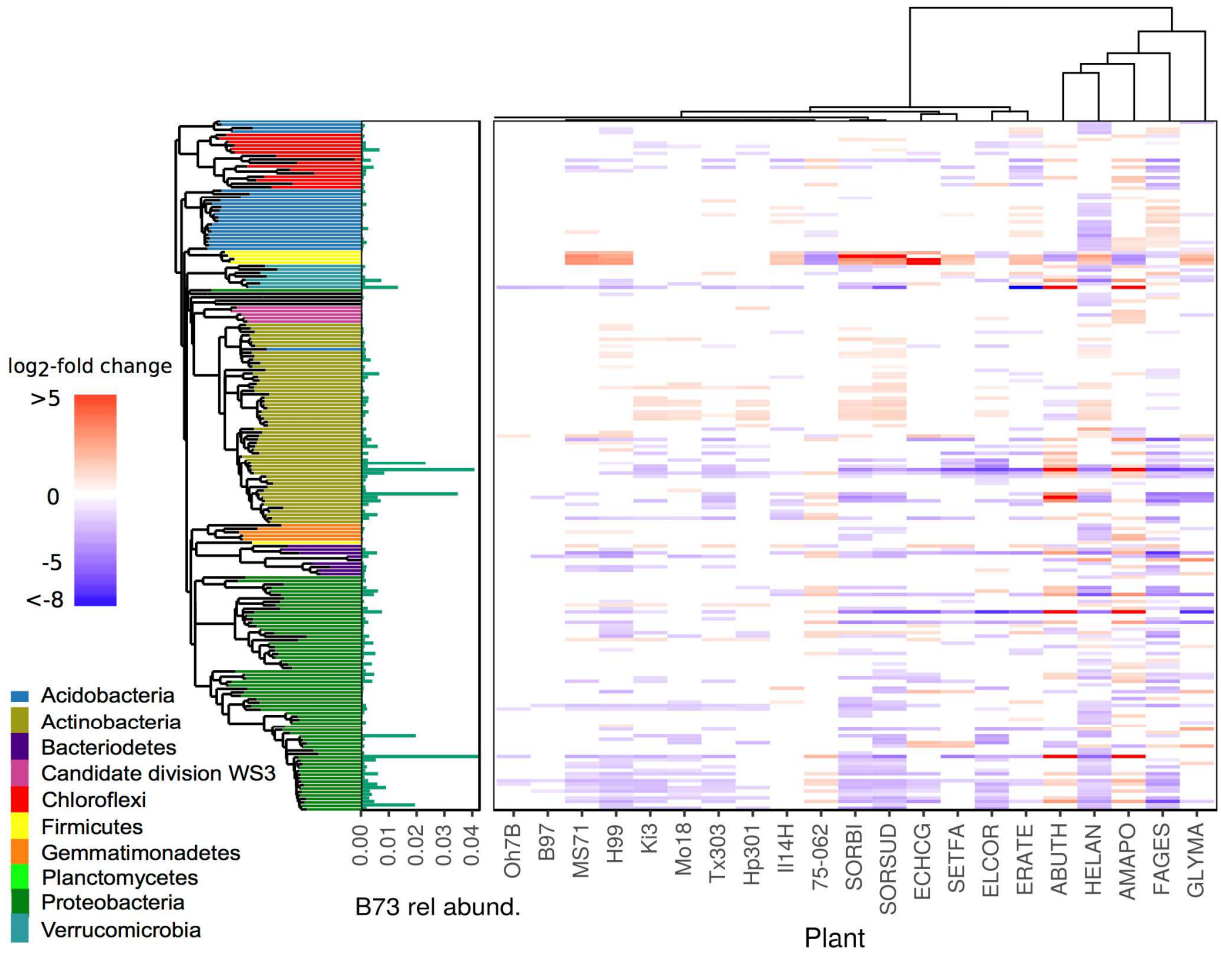


Figure 1.6: Differential abundance between rhizosphere of *Zea mays* cv. B73 and other maize genotypes and annual species. Tiles arranged by plant phylogeny (top tree) and bacterial phylogeny (left tree). Colored tiles indicate significant difference between listed genotype and reference B73 (DESeq2: $\log_2$-fold change $\neq 0$, adjusted $p < 0.05$), color and intensity indicate direction and magnitude to $\log_2$-fold change. Green bars represent mean relative abundance in rhizosphere samples of B73. Genotype codes represent maize inbred lines and species: *E. crus-galli* (ECHCG), *S. faberi* (SETFA), *S. bicolor* (SORBI), *S. x drummondii* (SORSUD), *E. coracana* (ELCOR), *E. tef* (ERATE), *A. theophrasti* (ABUTH), *H. annuus* (HELAN), *F. esculentum* (FAGES), *A. powellii* (AMAPO), and *G. max* (GLYMA).

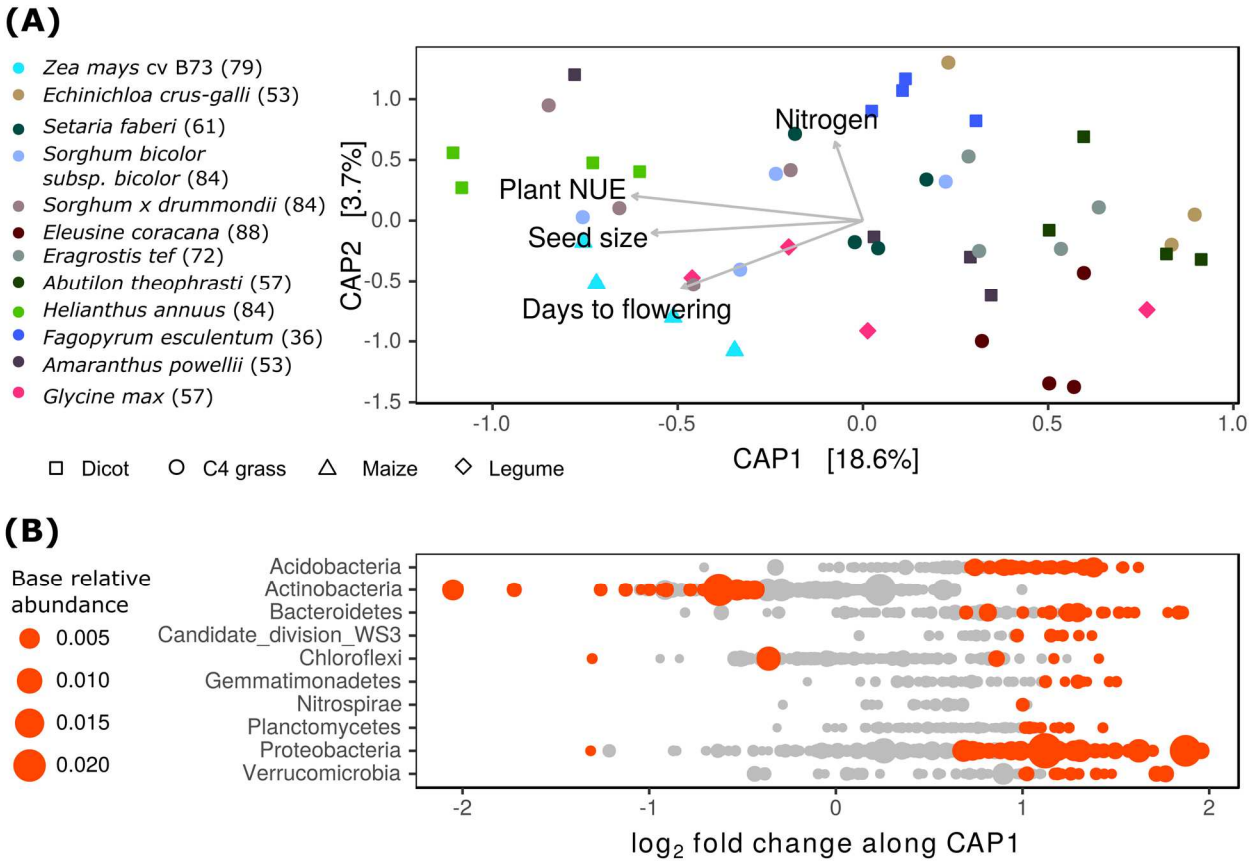


Figure 1.7: Relationship between plant life history strategy and bacterial community composition (BCC) in the rhizosphere. (A) Constrained analysis of principle coordinates (CAP) displaying variation in BCC explained by plant growth characteristics: days to flowering, seed size, plant nitrogen use efficiency (NUE) (g C g $N^{-1}$) and N uptake (g N). Numbers in parentheses alongside species names in figure legend refer to sampling date as days after planting. Nitrogen use efficiency and N uptake are corrected for differences in sampling date by using residuals of model relating growth characteristics to date of harvest. (B) Estimates of $log_2$-fold change in bacterial OTU abundance per unit shift in sample score on CAP1. Points colored in red are OTUs significantly correlated with CAP1 (DESeq2: $log_2$-fold change $\neq 0$; adjusted $p < 0.05$), grey points indicate OTUs not significantly correlated with CAP1 ($p > 0.05$). Point size proportional to relative abundance.

seed size, N uptake and NUE. The constrained ordination explained roughly 22% of the variation in BCC ($p < 0.01$) (Figure 1.7a; Table A8). The primary axis was negatively correlated with traits defining a resource intensive life history: longer lifespan, larger seed size and higher NUE.

Notably, grasses and dicots were intermixed along this axis, with *H. annuus*, maize, and *Sorghum* occupying one end of the spectrum while *E. crus-galli* and *A. theophrasti* occupied the opposite end. Additionally, flowering time was not the sole driver of differentiation (Table A8). Instead, long-lived but low NUE plants such as *E. coracana* and *E. tef* grouped with early season *E. crus-galli* to the exclusion of late season but high NUE plants such as maize and *Sorghum*. This axis captured marked compositional changes in rhizosphere BCC, characterized by the enrichment of many *Actinobacteria* OTUs in association with longer season and higher NUE plants (Figure 1.7b). The second CAP axis represented variation in total plant N uptake independent of flowering time and explained a small portion (3.7%) of variance in rhizosphere BCC. This axis was correlated with several *Bacillus* OTUs as well as a few *Acidobacteria* and *Cyanobacteria*. Neither axis separated the legume, *G. max*, from the other species, possibly indicating the plant's life history traits were more important to its placement on this axis than its ability to fix nitrogen.

The correspondence of rhizosphere BCC with plant growth characteristics coincided with shifts in enzyme activity in the rhizosphere. The potential activity of BX, LAP and NAG were negatively correlated with sample scores on the primary CAP axis, while the secondary axis was positively correlated with the potential activity of BX and CB (Table 1.2). This finding links plant growth characteristics to variation in BCC and enzyme activity such that plants with resource intensive life history traits had higher enzyme activity and differences in BCC

Table 1.2: Correlations between principle coordinates that explain plant life history and bacterial community composition relationships and potential activity of extracellular beta-xylosidase (BX), cellobiohydrolase (CB), leucine amino-peptidase (LAP) and N-acetyl-glucosiminidase (NAG) and inorganic N concentration in the rhizosphere

| | *Pearson correlation coefficients* | | | | |
|---|---|---|---|---|---|
| | BX | CB | LAP | NAG | Inorganic N |
| CAP1* | **-0.38** | -.26 | **-.55** | **-.29** | **.35** |
| CAP2 | **0.51** | **0.53** | 0.10 | -0.14 | -0.11 |

*Constrained analysis of principle coordinates (CAP) from figure 7

Correlations significant at $p < 0.05$ highlighted in bold.

compared to plants with less resource intensive life history traits.

## 1.4 DISCUSSION

In a common garden experiment, we investigated the sources and extent of plant variation in rhizosphere community composition and activity. We observed distinct changes in BCC and enzyme activity, reflecting the different C and N status between rhizosphere and bulk soil. Within this context, we show that rhizosphere BCC and enzyme activity is modulated by plant species and genotype and that this effect is related to plant phylogeny and life history strategy.

### 1.4.1 Rhizosphere effect on bacterial community composition and metabolism

Shifts in BCC and enzyme activity from bare to rhizosphere soils reflect the altered energy status of the rhizosphere environment. Consistent with other studies, rhizosphere samples were dominated by *Proteobacteria* and *Actinobacteria* (Bulgarelli et al., 2012, 2015; Peiffer et al., 2013), which include many bacterial species that grow rapidly in response to the availability of labile carbon substrates (Goldfarb et al., 2011). Additionally, many OTUs enriched in the rhizosphere were phylogenetically clustered and found at low abundance in bare soil, which suggests that rhizosphere competence requires traits that are evolutionarily conserved and which may not be adaptive in bulk soil (Barret et al., 2011; Ofek-Lalzar et al., 2014; Shi et al., 2015).

The potential activity of cellulose, hemi-cellulose, protein and chitin degrading enzymes was consistently greater in the rhizosphere compared to bare soil, which is consistent with studies showing a positive rhizosphere effect on enzyme activity, SOM decomposition, and N mineralization in the rhizosphere (Herman *et al.*, 2006; Zhu *et al.*, 2014). Controls on enzyme production in soil can include nutrient demand, target substrate availability, energetic constraints, and nutrient constraints (Sinsabaugh & Moorhead, 1994; Allison & Vitousek, 2005; Geisseler &

Horwath, 2008). Accordingly, increased enzyme activity in the rhizosphere could reflect substrate flow from plant roots and release of C limitation. Nitrogen fertilizer further increased activity in the rhizosphere for all enzymes assayed, which was not observed in bare soil. These results could indicate that microbes experience greater N limitation in the rhizosphere, or that labile C from the plant roots is necessary to take advantage of the increased nutrient availability (Averill & Finzi, 2011).

A surprising result is that, while fertilizer addition increased plant growth and rhizosphere enzyme activity, its addition explained little variation in BCC relative to the effects of plant species and genotype. There are well documented effects of N fertilization on soil BCC (Ramirez *et al.*, 2012; Leff *et al.*, 2015) and evidence that N fertilization can shift rhizosphere BCC (Zancarini *et al.*, 2012). Inorganic N fertilizer can influence BCC through a variety of mechanisms including: immediate direct responses to inorganic N availability (Verhamme *et al.*, 2011), short term indirect responses caused by the effect of fertilizer on plant growth (Paterson *et al.*, 2006), and long term indirect effects of fertilizer on soil properties such as pH (Hallin *et al.*, 2009). Furthermore, these mechanisms may interact such that short-term effects vary depending on the fertilization history of the site. We propose two explanations for the minimal effect of N fertilization on BCC that we observed. First, temporal decoupling between fertilizer application and sampling may minimize detection of direct fertilization effects on BCC. Second, we propose that long-term use of mineral fertilizer at this site has minimized the responsiveness of BCC to short term fertilization effects. We note that those OTUs that increased in abundance in response to fertilizer were not tightly coupled with inorganic N concentration in soil. This result suggests that the fertilizer effects we did observe on BCC were mediated indirectly by plant response to fertilizer, such as increased root growth and exudation. This would explain why fertilization

enhanced enzyme activity without causing substantial changes in rhizosphere BCC and why fertilizer had little effect on BCC and enzyme activity in bare soils.

### 1.4.2 Plant identity shapes rhizosphere bacterial community

Our finding that 12 – 39% of the variation in beta-diversity on a single sampling date could be attributed to plant species or genotype is consistent with previous reports of variation within a single field (Peiffer *et al.*, 2013; Edwards *et al.*, 2015), yet some studies have reported little or no plant identity effect on rhizosphere BCC (Bulgarelli *et al.*, 2012; Wagner *et al.*, 2016; Leff *et al.*, 2017). To some degree these conflicting reports are expected. Genotype influences are less apparent in analyses where multiple fields or sample types increase total variance of the BCC (Peiffer *et al.*, 2013; Edwards *et al.*, 2015; Bulgarelli *et al.*, 2015). Heritable plant phenotypes that influence rhizosphere communities may also be most influential during specific growth stages (İnceoğlu *et al.*, 2010) or within a particular soil context. For example, Bell *et al.* (2014) observed that willow cultivars grown in contaminated soils selected distinct rhizosphere fungal communities, while those grown in non-contaminated soils did not. By situating our study in a single field it is implicit that the genotype effects we observed may not always emerge. However, reduced environmental variation allows a deeper look at the factors driving plant genotype and species variation in rhizosphere BCC. Here we investigated the strength of two plant factors—plant evolutionary history and variation in growth and N economy—in predicting variation in rhizosphere BCC.

### 1.4.3 Plant phylogeny shapes rhizosphere community

Plant evolutionary history explained a significant portion of variation in rhizosphere BCC. This adds to a growing body of studies detailing a link between host phylogeny and

microbiome composition (Ley *et al.*, 2008; Redford *et al.*, 2010; Brucker & Bordenstein, 2012a). Similar to Bouffaud *et al.* (2014) our experimental design centered around the maize and the Poaceae. Here we demonstrate that a phylogenetic signal is evident in the rhizosphere of field grown plants, whereby increasing phylogenetic distance leads to a more dissimilar bacterial community. This relationship has important implications. First, it suggests the phylogenetic conservation of plant traits that influence BCC. As discussed by Bouffaud *et al.* (2014), several traits that exhibit phylogenetic conservatism are likely to influence rhizosphere communities. For instance, root morphology displays a phylogenetic signal coincident with mycorrhizal association (Brundrett, 2002; Comas *et al.*, 2014). In addition, secondary metabolite pathways, which may serve as signals in host-microbe communication, are often conserved at the family level (Wink, 2003). Furthermore, host immune responses directly influence the composition of the root microbiome and can be conserved phylogenetically (Brucker & Bordenstein, 2012a; Lebeis *et al.*, 2015). A second implication is that the phylogenetic structure of plant communities would be expected to cause long-term changes in soil BCC (Barberán *et al.*, 2015). This may serve as a mechanism underlying the positive relationship between plant phylogenetic diversity and ecosystem functions (Flynn *et al.*, 2011). Introducing phylogenetic diversity to agricultural systems, either through rotations or intercropping, could therefore represent a management tool to influence rhizosphere and soil BCC and ultimately influence nutrient cycling in these soils (Berthrong *et al.*, 2013).

### 1.4.4 Plant life history strategy shapes rhizosphere bacterial community

Beta diversity in rhizosphere BCC varied in response to plant flowering time, seed size and life-span independent variation in NUE. These observations are consistent with the hypothesis that variation in plant growth and N economy influence rhizosphere microbiome

composition. Within annual agricultural fields, where fitness is limited to ruderal plants with high growth rates, life history strategies are primarily differentiated by lifespan, which leads to differences in plant biomass accumulation, N demand, and NUE. These terms, along with seed size, reflect key dimensions of plant form and function (Moles & Westoby, 2006; Díaz *et al.*, 2016) and could be linked with variation in rhizosphere BCC through multiple mechanisms.

Variation in N-uptake between plant species has strong impacts on nitrogen cycling dynamics in soil (Tilman & Wedin, 1991), and may contribute to variation in rhizosphere BCC between plant genotypes (Pathan *et al.*, 2015; Bell *et al.*, 2015; Moreau *et al.*, 2015). In our study, extended N uptake of longer-lived plants may exacerbate N limitation within rhizosphere bacterial communities. It is possible that actinobacterial OTUs, enriched in long-lived and high NUE plants, such as *H. annuus,* maize and *Sorghum*, have adaptations to withstand N limitation in the rhizosphere. *Actinomycetes* produce a range of extracellular enzymes to degrade organic matter in soil (McCarthy & Williams, 1992). This could provide access to soil N pools in an otherwise N limited environment and underlie the increase in putative N-accessing enzymes observed in the rhizopshere of longer-lived, high NUE plants.

Alternately, plant traits correlated with lifespan and NUE may alter BCC. For example, plants classified as nitrogen competitive (high uptake) or conservative (high NUE) have been found to vary in the quantity and composition of their root exudates (Guyonnet et al., 2017; Kaštovská et al., 2015). In turn, species with higher rates of exudation supported increased microbial growth, turnover and high rates of N transformations in the rhizosphere. (Blagodatskaya *et al.*, 2014; Kaštovská *et al.*, 2015). Thus, it is possible that shifts in rhizosphere C flows in long-lived high NUE plants alter the rhizosphere bacterial community. There is a rich literature connecting plant growth strategies to litter quality and subsequent

impacts on nitrogen cycling in soil (Diaz *et al.*, 2004; Hawkes *et al.*, 2005; Orwin *et al.*, 2010). Our findings suggest that plant life history strategy can also have direct impacts on rhizosphere BCC and activity.

By sampling at the onset of flowering, we captured a primary dimension of plant variation while limiting the effects of plant development on BCC. Since the phenology of these species was not synchronized, we cannot rule out that temporal shifts in edaphic factors contributed to our results. Nevertheless, if seasonal effects, rather than endogenous plant effects, are the source of changes in rhizosphere BCC observed here, they remain directly related to realized rhizosphere communities as they impact and interact with plants in the field. Furthermore, the grouping of long-lived, low NUE species with short-lived species in the constrained ordination supports the interpretation of a strong plant life history mediated effect on rhizosphere bacterial communities. Sequential sampling or staggered plantings to synchronize developmental stage (e.g. Wagner et al., 2016) in similar field experiments will be necessary to disentangle the interrelated effects of plant variation in growth, life history, and temporal variation.

Neither genetic relatedness nor growth and N economy successfully described intra-specific variation among maize lines, despite differences in rhizosphere BCC between genotypes. In this regard our results are consistent with previous work where genetic distance, plant height and plant size have not predicted intra-specific variation in rhizosphere BCC (Peiffer *et al.*, 2013; Leff *et al.*, 2017). In contrast, ecophysiological measures related to carbon and nitrogen acquisition did parse variation in rhizosphere BCC between *Medicago* genotypes (Zancarini *et al.*, 2013a). While it seems clear from our data that different plant species have different impacts on rhizosphere BCC, which are associated with differences in life history traits

and rhizosphere function, it is less clear how intraspecific variation in plant traits influences microbiome composition and function.

## 1.5 CONCLUSIONS

We demonstrate that both plant phylogeny and life history traits, including variation in lifespan, growth and N economy, explain significant variation in rhizosphere BCC and enzyme activity. These results suggest that differences in plant functional traits drive variation in BCC and impact resource acquisition from soil, which likely has both short and long-term consequences for soil BCC and N-cycling dynamics. Crop selection, cover cropping and crop rotation are key management interventions in below ground processes in agricultural systems. If the rhizosphere phenotypes observed in this study are repeatable in other fields, then incorporating phylogenetic and functional diversity into crop rotations may provide a mechanism to manipulate plant-microbe interactions over time. Fully understanding the implications of plant-induced shifts in the rhizosphere and soil microbiome will be critical in selecting plants and beneficial rotations for maximal agronomic benefit.[1]

[1] This chapter has been accepted for publication in *Frontiers in Microbiology* as an article under the same title.

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# CHAPTER 2

# PLANT SPECIES AND TEMPORAL VARIATION IN PLANT GROWTH AND NITROGEN UPTAKE SHAPE RHIZOSPHERE BACTERIAL COMMUNITY COMPOSITION AND ACTIVITY IN AN AGRICULTURAL FIELD

## ABSTRACT

Plant-microbial interactions in the rhizosphere are an essential link in soil nitrogen cycling and plant nitrogen supply. Plant species and genotype identity govern microbial community structure and activity in the rhizosphere. However, the rhizosphere environment is shaped by interactions between plant identity, physiology, phenology, and soil nutrient availability. In addition, plant-microbe interactions can result in feedbacks that further influence plant growth and resource acquisition. The relative contributions of plant identity, phenology, and resource availability in shaping plant rhizosphere effects are not well understood. To address this knowledge gap we grew a collection of maize hybrids and four summer annuals in a common garden experiment conducted at two levels of organic nutrient availability. Plant growth, nitrogen uptake, rhizosphere bacterial community composition, and rhizosphere potential extracellular enzyme activity were assessed on three dates corresponding to the vegetative, flowering and grain filling stages of maize. We observed strong coupling between plant nitrogen uptake and protease activity in the rhizosphere. Rhizosphere bacterial community composition varied among plant species and these differences were partly explained by variation in plant growth dynamics and nitrogen uptake. We show that a subset of microbes in the rhizosphere were responsive to variation observed in plant growth and N uptake among plant species and across plant growth stages. Together, these findings indicate that plant species differ

in their effects on rhizosphere bacterial communities, that these effects vary in response to plant phenology, and that they are linked to variation in plant functional traits associated with variation in resource acquisition.

## 2.1 INTRODUCTION

Nitrogen is a limiting nutrient in most terrestrial ecosystems (Vitousek *et al.*, 1997) and plant microbial interactions in the rhizosphere are an essential link in terrestrial nutrient cycling and plant nutrient acquisition (Philippot *et al.*, 2013; Finzi *et al.*, 2015). Plant root activity shapes the rhizosphere environment through release of labile root exudates and rhizodeposits and the simultaneous uptake of nutrients and water. The rhizosphere environment thus relieves the energetic constraints of bulk soil and fosters a distinct microbial community with altered soil N cycling (DeAngelis *et al.*, 2008; Hinsinger *et al.*, 2009; Mendes *et al.*, 2014). Relative to bulk soil, the rhizosphere is associated with increased rates of associative N fixation (Jones *et al.*, 2003) and modulated nitrification and denitrification (Qian *et al.*, 1997; Herman *et al.*, 2006). The improved carbon (C) status and increasing nutrient limitation in the rhizosphere are also thought to lead to an increase in microbial production of extracellular enzymes that degrade soil organic matter and mineralize soil organic N (SON), the widely observed rhizosphere priming effect (Kuzyakov, 2002; Dijkstra *et al.*, 2013). As a result, gross N mineralization rates can be much higher in the rhizosphere compared to bulk soil (Herman *et al.*, 2006; DeAngelis *et al.*, 2009).

When coupled with microbial turnover or trophic interactions, these rhizosphere processes can substantially increase soil N supply to the plant (Clarholm, 1985; Hamilton & Frank, 2001; Zhu *et al.*, 2014). Therefore, plant impacts on the rhizosphere community have substantial feedbacks on plant productivity. For example, Sanchez *et al*. (2002) found that maize increased mineralization of soil N nearly 50% compared to wheat and unplanted controls, while it appears that tropical maize can derive a substantial portion of plant N from associative N-fixation (Montañez *et al.*, 2009). These rhizosphere N-cycling processes have potential to supply

plant N spatially and temporally coupled with plant root N uptake, thereby limiting potential N loss compared to similar processes occurring in bulk soil (Drinkwater *et al.*, 2007). Therefore, there is particular interest in understanding the factors controlling rhizosphere N-cycling as well as the communities that mediate these transformations.

Broadly, soil context influences both the bacterial community colonizing the rhizosphere (Peiffer *et al.*, 2013; Edwards *et al.*, 2015; Bulgarelli *et al.*, 2015) and the rhizosphere effect on C and N cycling (Dijkstra *et al.*, 2006). However, it is also known that plant species and genotypes can influence rhizosphere community composition and nutrient cycling (Grayston *et al.*, 1998; Cheng *et al.*, 2003; Berg & Smalla, 2009; Turner *et al.*, 2013; Kaštovská *et al.*, 2015; Edwards *et al.*, 2015). These differences are likely mediated by specific traits such as the quantity and quality of root exudates, root morphology or immune response (Briones *et al.*, 2002; Bais *et al.*, 2006; Broeckling *et al.*, 2008; Doornbos *et al.*, 2012), but also may be linked more broadly to plant ecophysiology (Zancarini *et al.*, 2013b). For example, plants with high relative growth rates have correspondingly high rates of root exudation, supporting increased growth and turnover of microbial populations in the rhizosphere (Blagodatskaya *et al.*, 2014; Kaštovská *et al.*, 2015). In contrast, high rates of N uptake can exacerbate plant-microbe competition for N in the rhizosphere and lead to N limitation of the microbial community (Blagodatskaya *et al.*, 2014; Kaštovská *et al.*, 2015; Pathan *et al.*, 2015; Moreau *et al.*, 2015). Thus, plant phenotype in relation to growth and N acquisition may be an important source of variation in rhizosphere bacterial community composition (BCC).

Rhizosphere communities also shift markedly over the course of a season, which is coincident with changes in plant development that influence root exudate profiles, rhizosphere C flows and cumulative plant impacts on the soil environment (Chaparro *et al.*, 2014; Bell *et al.*,

2015; Shi *et al.*, 2015). It is relatively unknown how plant identity interacts with temporal variation and plant development in structuring rhizosphere communities, though there is some indication that plant species and genotype effects may be more readily observed at particular developmental stages (İnceoğlu *et al.*, 2010).

Finally, there is considerable evidence that microbial communities respond to N enrichment (Leff *et al.*, 2015) and plant influence on C and N balance in the rhizosphere appears to be a significant force shaping BCC (Pathan *et al.*, 2015; Bell *et al.*, 2015). Thus, it would be expected that soil fertility management would alter plant rhizosphere effects and several authors have observed changes in rhizosphere BCC under N fertilization in greenhouse studies (Zancarini *et al.*, 2012; Li *et al.*, 2016). However, in an earlier field study we found only a limited response of rhizosphere communities to additional N fertilization (Emmett *et al. in review*), so it is unclear whether short-term fertility treatments are a significant driver of rhizosphere assembly in a field setting.

To investigate the factors influencing rhizosphere BCC and activity we undertook an experiment embedded in an organic grain field and sought to test whether: 1) plant identity or temporal effects were dominant in shaping rhizosphere BCC and activity, 2) how rhizosphere BCC and activity shifted in relation to variation in plant growth and N acquisition, and 3) whether nutrient inputs influenced patterns of rhizosphere assembly and metabolism. We hypothesized that plant-microbial interactions were likely to be most relevant to N acquisition during otherwise N-limiting conditions. During these times we expect plant genetic and phenotypic differences to drive changes in rhizosphere community composition and metabolism.

## 2.2 METHODS

### 2.2.1 Experimental Design

A common garden experiment was conducted in a field in Penn Yan, NY (42°40'25.3"N 77°02'24.2"W) that has been under organic management for over 20 years. The field was in a corn-soy-small grain-clover-corn rotation and had been frost seeded to red clover (*Trifolium pretense*) the previous spring. The soil in this field is classified as a Honeoye loam (fine-loamy, mixed, semiactive, mesic Glossic Hapludalfs), with a pH of 5.92 ± 0.19 and consisted of 51.4% sand, 30.4% silt and 18.3% clay. The soil had 1.35 ± 0.06% C and 0.11 ± 0.004% N content, 60 ± 6 g $g^{-1}$ Mehlich 3 extractable phosphorous and 139 ± 16 g $g^{-1}$ Mehlich 3 extractable potassium. On May 14, 2015, a fertility treatment was implemented in the field by cutting aboveground biomass of clover (88 ± 24 kg N $ha^{-1}$), collecting the residue in the mulch bag of a push lawnmower and transferring to adjacent strips (estimated transfer of 55 kg N $ha^{-1}$). On May 21$^{st}$, the field was moldboard-plowed, and fit for planting. One week following tillage, inorganic N concentration in soil was 13.3 ± 1.3 mg $kg^{-1}$ in plots with clover removed and 15.1 ± 1.3 mg $kg^{-1}$ in plots with clover added.

Replicate plots were seeded with thirteen different plant varieties selected to represent intra- and interspecific variation in plant functional traits. Interspecific variation in life history and growth potential is represented in the selection of four summer annuals: *Helianthus annuus* L., *Fagopyrum esculentum* Moench, *Sorghum x drummondii* (Nees ex Steud.) Millsp. & Chase, and *Echinochloa esculenta* (A.Braun) H.Scholz. Nine maize hybrids were chosen to capture interspecific variation. These included five hybrids derived from the founding inbred lines of the maize nested association mapping population (NAM) crossed with B73 (Yu *et al.*, 2008), a commercial hybrid developed in an organic breeding program and three commercial hybrids

released from year 1936, 1953 and 1984 (Table B1).

Seeds were hand planted in replicated plots, split by fertility treatment, in a randomized complete block design (n = 4) on May 21st and 22nd, 2015. Split plots were 3.048m x 3.048m and consisted of four rows spaced at 0.762 m. A buffer row of maize was planted between each + and – clover strip to accommodate any movement of residues during tillage. Weeds were controlled through one mechanical tining on May 27th and one mechanical cultivation on June 19th. Plots were subsequently hand hoed and weeded throughout the growing season. On July 13th plants were thinned to a final density of 57,400 plants $ha^{-1}$.

### 2.2.2 Plant growth and N uptake

Above and below-ground samplings of the four annual species and two of the nine maize hybrids—B73xB97 and B73xTx303—were conducted at 36, 67 and 90 days after planting, when maize was at vegetative stage (six-leaf: V6), flowering stage (silking: R1) and grain filling stage (milk: R3). The sampling during the vegetative stage of maize was coincident with the onset of flowering in *F. esculentum*, while *S x drummondii* and *E. esculenta* had also initiated flowering by the sampling at R1. Pollen shed for *H. annuus* occurred between the sampling at R1 and R3. All biomass clips occurred in the interior two rows of the plot. At V6, two representative plants were sampled by clipping at the soil line. Where possible, the sampling at V6 was used to thin the row to target density, thereby leaving the plot intact for subsequent harvests. At R1 four adjacent and properly spaced plants were sampled by clipping at the soil line. At R3, two adjacent and representative plants were cut from the opposite end of the row harvested at R1. The full set of maize hybrids was also sampled at R1. At R3 buckwheat was senescent and not sampled. All biomass clips were taken from the two interior rows of each plot and left at least two plants as a buffer on row ends. This sampling scheme ensured that we only sampled roots

from the target genotype and limited edge effects. Above ground biomass was bagged, dried to a constant weight at 60 °C and weighed for dry mass determination. Plant samples were ground, homogenized and analyzed for total C and N content on a LECO True Mac elemental analyzer (Saint Joseph, Michigan).

Plant growth metrics were calculated for each plot on an area basis. Relative growth rate was then calculated as:

$$RGR = \frac{\ln(W_2) - \ln(W_1)}{t_2 - t_1}$$

where $W_t$ is the above ground dry mass at the time (t) of sampling. The rate of plant N uptake (kg N $ha^{-1}$ $day^{-1}$) for the period prior to sampling was calculated as:

$$Nup = \frac{W_2 * NF_2 - W_1 * NF_1}{t_2 - t_1}$$

where *NF* is the fractional N content of above ground biomass. Initial seed size and N content were used as $t_0$ for calculating growth metrics. Because of limited seed availability, $t_0$ weights and N content of B73xB97 and B73xTx303 were estimated using seed from crosses of the same female (B73) but different male parent. The assumption that these $t_0$ values reflect the two maize genotypes of interest influences only the relative growth rate and nitrogen uptake rate reported at V6.

### 2.2.3 Rhizosphere and soil sampling

Root and rhizosphere sampling occurred during the sampling at V6, R1 and R3. Samples were collected by loosening the root system from the ground with a spade. Soil within 2 mm of a root and adhering to the root system after massaging and gentle shaking was considered

rhizosphere soil. Rhizosphere soil was collected and passed through a 2 mm sieve by gently removing the soil that had previously remained adhered to the roots with a gloved hand. Additionally, intact rhizospheres, defined as the root with adhering soil, were sampled by clipping 4 cm segments of root tips and 2$^{nd}$ order roots chosen at random. On each sampling date 2 cm diameter by 20 cm deep soil cores were collected from the unplanted, weed-free plots to serve as bare soil controls. Multiple cores were combined, homogenized, subsampled and passed through a 2 mm sieve. Samples were placed on ice in the field. Rhizosphere and soil samples were stored at 4 °C for downstream enzyme analysis and the intact root and rhizosphere samples and soil samples were stored at -40 °C for downstream DNA extraction.

### 2.2.4 Enzyme analyses

Potential activity of the extracellular enzymes β -glucosidase (BG), β-xylosidase (BX), cellobiohydrolase (CB), leucine-aminopeptidase (LAP) and β-*N*-acetyl-glucosaminidase (NAG) were measured using a standard fluorometric assay following German *et al.* (2011). Field moist soil (2.00-2.25 g) was mixed with 150 ml of 50 mM sodium bicarbonate buffer adjusted to pH 7 for 60 sec using an immersion blender. 200 µl of each soil slurry was added to 8 replicate wells of a 96-well plate containing 50 µl of 200 µM substrate with attached fluorophore. Plates were incubated for 2 (BG and LAP) and 4 hrs (BX, CB and NAG) and fluorescence measured on a BioTek Synergy HT microplate reader at 365 nm excitation and 450 nm emission wavelengths. A standard curve (0-75 µM) prepared from the same soil slurry was used to estimate enzyme activity while controlling for quenching and auto-fluorescence. Standard curves were made fresh daily. Subsamples of soil were dried at 60 °C for two days and used to calculate soil moisture content. Enzyme analyses were completed within 48 hrs of sample collection and enzyme activity was calculated on soil dry weight basis (nmol $hr^{-1}$ g $soil^{-1}$).

### 2.2.5 16S rRNA gene sequence analysis

DNA was extracted from intact root and rhizosphere samples as in Peiffer et al (2013). Roots were chopped to < 1 cm length segments, mixed, and approximately 0.1 g of roots and adhering soil or 0.2 g of bare soil controls were added directly to each well of a MoBio PowerMag Microbiome DNA/RNA isolation kit (Carlsbad, CA). Rhizosphere samples were added to duplicate wells to adequately capture heterogeneity of the root systems. Samples were homogenized on a Bio-spec Mini-Beadbeater-96 (Bartlesville, OK) for 1 min. Roots remained intact following homogenization (Peiffer *et al.*, 2013). Following bead beating, extraction proceeded according to the kit manufacturer's instructions on a Hamilton Star automated liquid handling system (Reno, NV).

A dual-barcoded MiSeq library of the 16S rRNA V4 region was prepared as in *Kozich et al.* (2013) using the forward (515F) and reverse primers (806R) adapted from Caporaso *et al.* (2011). Amplicons were prepared in triplicate reactions. Each reaction included approximately 5 ng of template DNA, 12.5 µl of 2x Q5 High Fidelity Hot Start PCR MasterMix (NE Biolabs), 1 µM combined forward and reverse primer, and 0.5 µg bovine serum albumin (NE Biolabs) and 0.625 µl of 4x PicoGreen reagent (Invitrogen) to monitor DNA template production in a total volume of 25 µl. PCR conditions consisted of: 95 °C for 2 min; 30 cycles of 95 °C for 20 sec, 55 °C for 15 sec and 72°C for 10 sec; final extension 72 °C for 5 min. Pooled triplicate reactions were standardized using the SequalPrep Normalization Plate Kit (Life Technologies). Standardized reactions were pooled, purified on a 1.5% low melt agarose gel and the target band size was extracted using the Wizard SV Gel and PCR Clean-Up System (Promega). The resulting amplicon library was submitted for a single run of paired-end sequencing on the Illumina MiSeq platform with the MiSeq Reagent v2 kit at the Cornell Biotechnology Resource

Center Genomics Facility (Ithaca, NY). A mock community control and negative ($H_2O$) controls from each PCR plate were included in the submission to assess library integrity.

Resulting reads were processed in a custom bioinformatics pipeline as in Whitman *et al.* (2016). Overlapping paired-end reads were merged using PEAR (v0.9.2) (Zhang et al., 2014) and de-multiplexed with a custom python script. Reads were filtered to remove sequences with max expected error rates > 2 and ambiguous base calls with USEARCH (Edgar, 2013). Sequences were aligned in mothur (Schloss *et al.*, 2009) using the align.seqs command and the Silva reference database (v.111) (Quast *et al.*, 2013). Sequences that did not align to the SILVA reference alignment, contained ≥8 homopolymers or were singletons (unique) were discarded. Sequences were clustered into operational taxonomic units (OTUs) at a 97% pairwise identity cutoff with the UPARSE algorithm in USEARCH, which also removes chimeric sequences (Edgar, 2013). Taxonomic assignment of OTUs was performed with QIIME's parallel taxonomy assignment using the uclust consensus taxonomy assignment function (Caporaso *et al.*, 2010) and the Silva reference database. The forward and reverse primers are specific to bacteria, therefore OTUs belonging to chloroplast, mitochondria, eukaryotes, archaea and unassigned sequences represent non-specific amplification and these sequences were removed. OTUs were aligned using SSU_align and poorly aligned positions masked based on posterior probabilities (Nawrocki, 2009). A phylogenetic tree was created and rooted to Sulfolobus (acc. X90478) using FastTree (Price *et al.*, 2009) with default settings.

A single run on the Illumina MiSeq instrument generated 4,630,081 merged sequences that matched a known barcode. Following quality control, this left 2,474067 reads clustered into 7,045 OTUs, with an average 13,051 sequences per sample (± 7086 sd). The OTU table was combined with the phylogenetic tree, taxonomic information and metadata for analysis using the

phyloseq package in R (McMurdie & Holmes, 2013). The OTU table was filtered to include only those OTUs with more than three reads in more than three samples, which was chosen to limit very rare and spurious OTUs, but retain those that may be endemic to a single genotype, resulting in 2,550 OTUs.

### 2.2.6 Statistical analysis

Statistical analysis was completed using the R software package (R Development Core Team, 2012). Univariate analyses testing treatment effects on growth, N uptake and extracellular enzyme activity were conducted using mixed models in the R package "lme4" (Bates *et al.*, 2015) and significance tests conducted with "lmerTest" (Kuznetsova *et al.*, 2016). Sample type, plant genotype, clover treatment, sampling date and interactions were considered fixed effects with the random effects of field replicate (blocking factor), clover strip and split-plot. Post-hoc tests were conducted with the "lsmeans" package using Bonferroni correction for multiple comparisons (Lenth, 2016).

Bacterial community analyses were conducted on non-rarified OTU tables (McMurdie & Holmes, 2014). Beta-diversity was estimated with weighted-UniFrac distance matrices (Lozupone *et al.*, 2011) and visualized using principal coordinate analysis (PCoA) in the "phyloseq" package (McMurdie & Holmes, 2013). Beta-diversity variance was partitioned between the effects of replicate, sample type, date of sampling, plant genotype and clover treatment using permutational multiple analysis of variance (PERMANOVA) in the vegan packages in R (Oksanen *et al.*, 2012). The replicate term was included to capture field variation as well as batch effects in DNA extraction and library preparation, which were prepared in a sequence randomized with replicate blocks.

The relationship between growth metrics and rhizosphere BCC was analyzed by

constraining the principal coordinate analysis to the portion of variation that could be described by plant growth measures in a constrained analysis of principal coordinates (CAP), while controlling for replicate. Differential abundance of OTUs between treatments and correlations with covariates were identified using the DESeq2 package (Love *et al.*, 2014). All p-values were corrected for multiple comparisons using Benjamini & Hochberg (BH) correction. Rhizosphere responders were identified as those OTUs that increased in abundance (>1 $\log_2$-fold change (LFC)) in the rhizosphere of any genotype on any of the three sampling dates when compared to the bare soil controls on that date. OTUs differentially abundant between sampling dates in either bare or rhizosphere soil were calculated using the core genotypes (two maize hybrids and four summer annuals) in a model testing the effect of sampling date while controlling for replicate block and genotype. Similarly, plant species' influence on OTU abundance was identified in a negative binomial model controlling for sampling date. OTUs that responded to an interaction between date of sampling and plant species were identified using a likelihood ratio test of a model with the interaction term against a reduced model without the interaction term. All figures were created in the package "ggplot" (Wickham, 2009), except for the phylogenetic tree with associated heatmaps, which were created using the interactive tree of life web server (Letunic & Bork, 2016).

## 2.3 RESULTS

### 2.3.1 Plant growth and N uptake

The timing of growth and maximum N uptake varied between the species (Figure 1). By the first harvest at V6, *H. annuus* had the greatest biomass accumulation, followed by the two maize hybrids, and *F. esculentum*. The small-seeded tillering grasses, *S.* x *drummondii* and

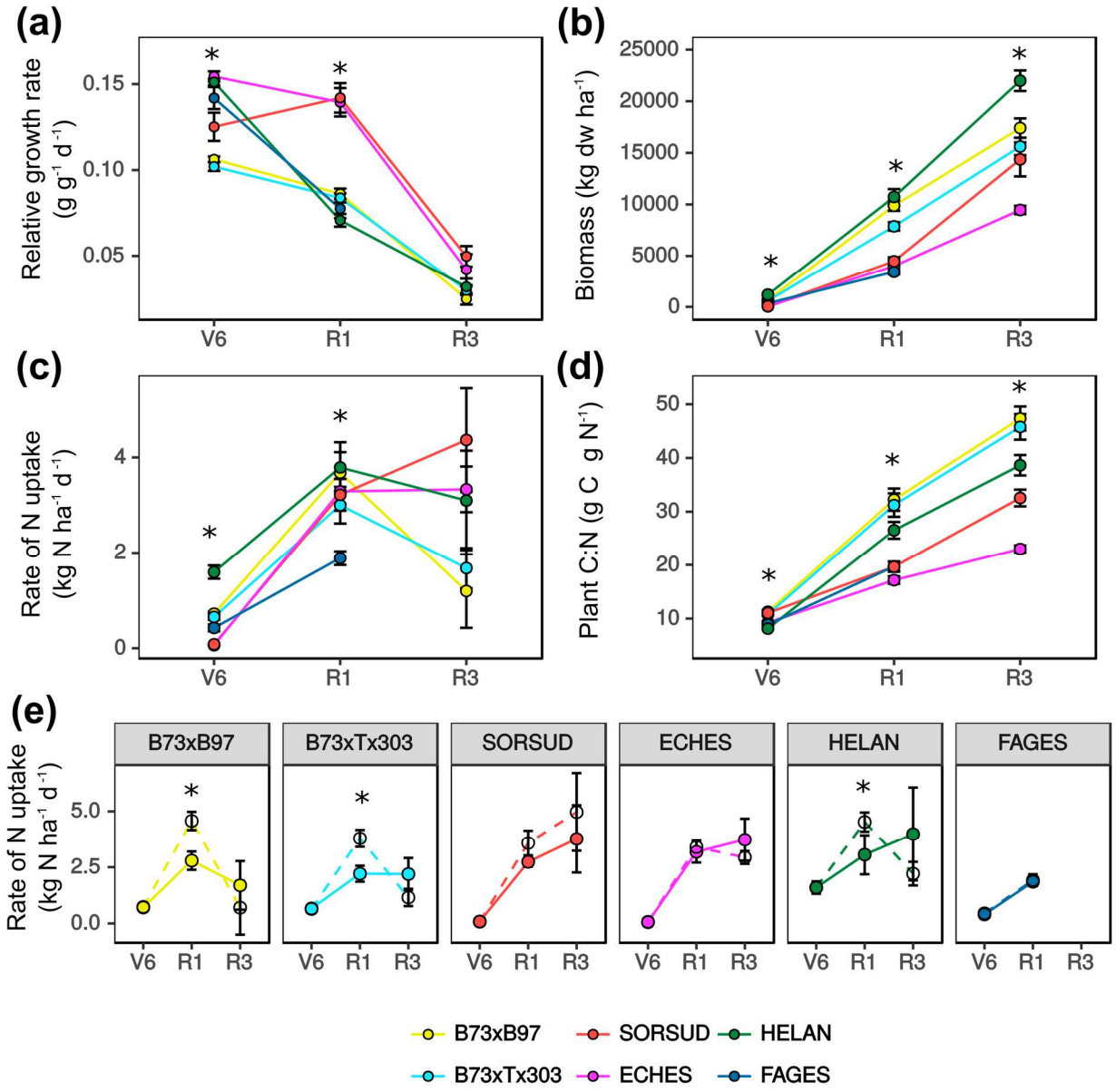



Figure 2.1: Plant growth and nitrogen (N) uptake dynamics of two maize hybrids and four species over time (a,b,c,d) and rate of N uptake in plots with clover removed (closed circles, solid lines) and added (open circles, dashed line) prior to tillage (e). * indicates significant effect of plant genotype on growth metric ($p < 0.05$) (a-d), or difference between genotypes rate of N uptake in + or – clover plots (e). Species and genotype codes: *Zea mays* cv B73xB97 and B73xTx303, *S. x drummondii* (SORSUD), *E. esculenta* (ECHES), *H. annuus* (HELAN) and *F. esculentum* (FAGES)

*E. esculenta* had similar relative growth rates, but because of their smaller seed size they had lower biomass accumulation and N uptake at V6. *S.* x *drummondii* and *E. esculenta* were also damaged during the cultivation and therefore their growth from planting to V6 should be interpreted with caution. At V6, N uptake was limited mainly by plant biomass accumulation and there was no difference in either plant N uptake or biomass accumulation between fertility treatments ($p = 0.84$ and $p = 0.98$ respectively; Table S2). By R1 the rate of N uptake appeared limited by soil N supply (Figure 2.1). Except for *F. esculentum*, which was nearing physiological maturity, the remaining plants all shared a similar rate of N uptake (Figure 2.1c), despite differences in plant size (Figure 2.1b). At this point there was an increase in plant N uptake in response to clover addition ($p = 0.03$; Figure 2.1e, Table B2), but this did not lead to an increase in biomass accumulation across all plant varieties ($p = 0.16$; Table B2). Nor did improved N status at R1 translate to differences in late season growth, as there were no significant differences in biomass accumulation in + or – clover plots at R3 across plant varieties ($p = 0.59$; Table B2). There was a trend toward greater total N uptake in + clover plots by R3, but this difference was not significant ($p = 0.07$; Table S2), a result of a slightly higher rate of N uptake of *H. annuus* and the two maize hybrids in the – clover plots compared to + clover plots between R1 and R3, though again this difference was not statistically significant (Figure 1e). Thus, the clover transfer changed the timing, but not total amount of plant N uptake. The rate of N uptake slowed after anthesis for maize and, to a lesser extent, *H. annuus*. However *S. x drummondii* and *E. esculenta* both maintained high rates of N uptake and higher relative growth rates into the later season.

### 2.3.2 Extracellular enzyme activity

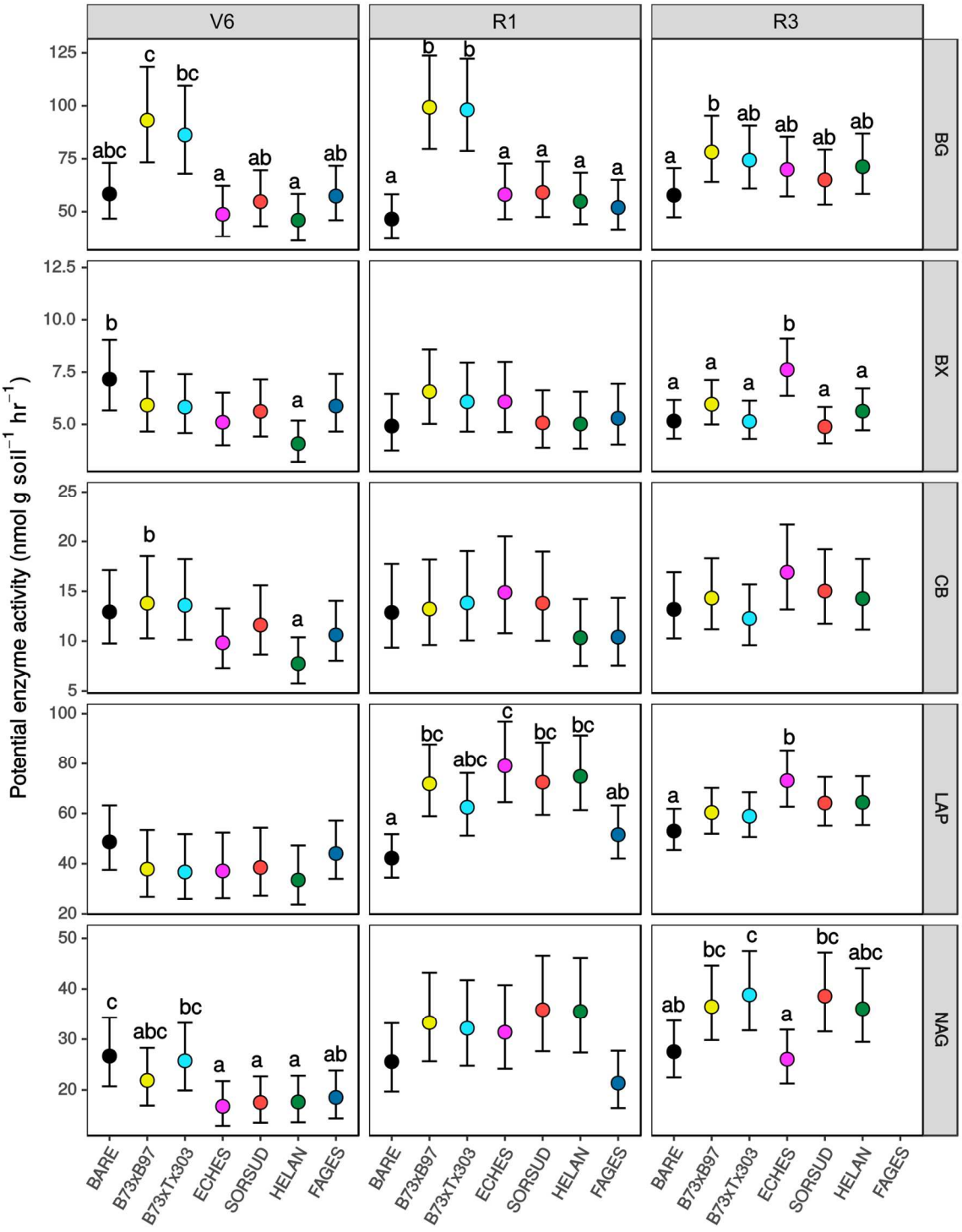
The rhizosphere effect on extracellular enzyme activity varied with respect to sampling

date and enzyme identity. At V6 there was a decrease in the measured activities of BX (-29%; CI [-48, -11]; $F_{(1, 25.85)} = 9.51$, $p = 0.005$) and NAG (-25%; CI[-45,-05]; $F_{(1, 20.64)} = 6.39$, $p = 0.02$) in rhizosphere samples relative to bulk soil, while there was no overall rhizosphere effect on activity of BG, CB and LAP ($p > 0.05$). In contrast, at R1, when plant N uptake dynamics showed evidence of N limitation (Figure 2.1e), only the N-cycling protease (LAP) had a significant increase in measured activity in rhizosphere samples compared to bulk soil controls (58%, CI [39,77]; $F_{(1,22.29)} = 9.87$, $p = 0.005$). NAG, which degrades N containing chitin compounds, followed a similar pattern, but its response was highly variable and not statistically significant ($p = 0.30$). At R3 rhizosphere samples had increased potential activity of BG (28%; CI[4, 52]; $F_{(1, 17.88)} = 7.93$, $p = 0.01$), LAP (23%; CI[8, 37%]; $F_{(1, 18.57)} = 4.39$, $p = 0.05$) and NAG (32%; CI[6,56]; $F_{(1, 17.39)} = 5.00$, $p = 0.04$) compared to bare soil controls, but no change in CB or BX activity ($p > 0.05$).

Plant varieties varied in their effects on rhizosphere enzyme activity and these effects were time dependent. For example, BG activity was higher in the rhizosphere of maize compared to other species (Figure 2.2). In addition, LAP activity was low in the rhizosphere of *F. esculentum* at R1, as it was nearing physiological maturity. In contrast to the variation between species, we did not observe any variation among the maize hybrids in their rhizosphere effect on enzyme activity (Figure B1). Rather, the positive rhizosphere effect on BG observed in B73xB97 and B73xTx303 was also observed across the wider set of maize hybrids evaluated at R1, suggesting a conservation of rhizosphere traits influencing the enzyme profile at the species level (Figure S1).

The fertility treatment did not alter overall rhizosphere effects at any sample date, but did alter the rhizosphere effect of particular species (Figure B2) and these changes broadly mirrored

Figure 2.2: Rhizosphere effect on potential activity of extracellular enzymes shaped is shaped by time and plant species. Points represent least square mean of plant species/genotype (whiskers = 95% CI, n = 8) on each sampling date corresponding to maize six-leaf (V6), flowering (R1) and grain filling (R3). Points sharing the same letters indicate no significant difference in pairwise comparisons, letters removed from points with no significant contrasts for clarity ($p < 0.05$). Enzyme abbreviations: β-glucosidase (BG), β-xylosidase (BX), cellobiohydrolase (CB), leucine-aminopeptidase (LAP) and β-N-acetyl-glucosaminidase (NAG). Species and genotype codes: *Z. mays* cv B73xB97 and B73xTx303, *S. x drummondii* (SORSUD), *E. esculenta* (ECHES), *H. annuus* (HELAN) and *F. esculentum* (FAGES)


V6
R1
R3
BG
BX
CB
LAP
NAG
Potential enzyme activity (nmol g soil$^{-1}$ hr$^{-1}$)
BARE
B73xB97
B73xTx303
ECHES
SORSUD
HELAN
FAGES

differences in rates of N uptake between fertility treatments. At anthesis the maize hybrid B73xB97, which had higher rates of N uptake in the + clover plots, also had a greater rhizosphere effect on activity of BG in these plots (Figure B2). In contrast, at the final harvest, *H. annuus* had greater rhizosphere effect on activity of BG, BX, CB and NAG in the - clover plots compared to those that received clover addition. This reduced rhizosphere effect in + clover plots at the final harvest was also evident in the activity of BX in the rhizosphere of both maize hybrids and for CB in the rhizosphere of *S. x drummondii*.

Enzyme activity was broadly correlated with plant growth dynamics (Table B3). However, this effect was primarily mediated by the increase in potential enzyme activity between sample dates. In an analysis of covariance including sampling date, differences in plant growth and N uptake were not significant predictors of plant rhizosphere effect on activity of most enzymes ($p > 0.05$; Table B4). An important exception was the rhizosphere effect on the N-cycling protease (LAP), for which there was a significant interaction between date of sampling and rate of N uptake ($p = 0.02$; Table B4). LAP activity was linearly and positively correlated with plant rate of N uptake at R1 ($F_{(1,32.4)} = 11.8$, $p = 0.002$; Figure 2.3), but this relationship was not evident at V6 or R3.

### 2.3.3 Rhizosphere bacterial community composition

Bacterial community beta-diversity was influenced by habitat (rhizosphere vs. bare soil), plant species and date of sampling. In a PERMANOVA, the rhizosphere effect captured 19.5% of variation in weighted-UniFrac distances between samples ($p < 0.01$; Table 2.1; Figure 2.4). Of the 2550 taxa that passed our sparsity filter, 197 were enriched in the rhizosphere of at least one plant genotype on one of the three sample dates (LFC > 1, BH adjusted $p < 0.05$). An additional 60 OTUs decreased in abundance in the rhizosphere (LFC < - BCC in both the

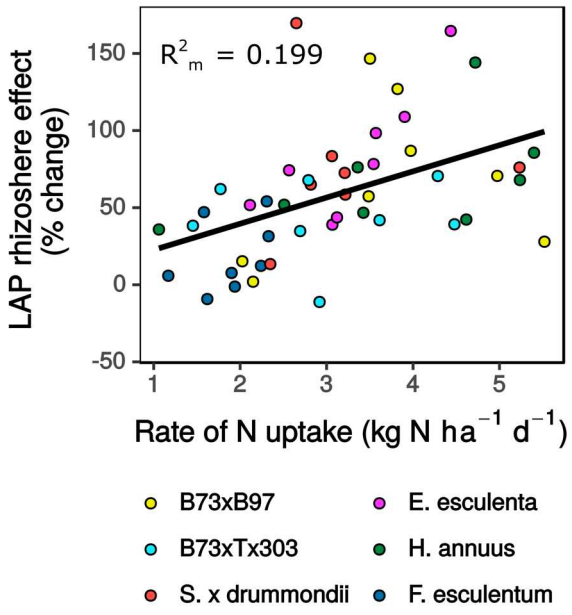



Figure 2. 3: Rhizosphere effect on leucine-aminopeptidase (LAP) activity is linearly correlated with rate of plant N uptake at second harvest (maize R1). Rhizosphere effect plotted as % change from bare soil controls and marginal $R^2$ displayed.

Table 2.1: Variance partitioning of bacterial community beta-diversity (weighted-UniFrac distances) in permutational multiple analysis of variance.

| | *All samples* | | | | *Rhizosphere samples* | | | | *Bare soil samples* | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Factor* | Df | *F* | $R^2$ | *p* | Df | *F* | $R^2$ | *p* | Df | *F* | $R^2$ | |
| Replicate block | 3 | 7.86 | 0.08 | **<0.01** | 3 | 7.55 | 0.11 | **<0.01** | 3 | 3.41 | 0.30 | **<0.01** |
| Habitat[a] | 1 | 58.12 | 0.20 | **<0.01** | - | - | - | - | - | - | - | - |
| Sampling date | 3 | 6.17 | 0.07 | **<0.01** | 2 | 11.25 | 0.11 | **<0.01** | 2 | 2.80 | 0.16 | **<0.01** |
| Genotype[b] | 5 | 8.07 | 0.14 | **<0.01** | 5 | 7.23 | 0.18 | **<0.01** | - | - | - | - |
| Clover swap | 1 | 2.34 | 0.01 | **0.04** | 1 | 2.25 | 0.01 | **0.03** | 1 | 1.45 | 0.04 | 0.13 |
| H x Sd | 2 | 3.86 | 0.03 | **<0.01** | - | - | - | - | - | - | - | - |
| Sd x G | 9 | 2.49 | 0.08 | **<0.01** | 9 | 2.25 | 0.10 | **<0.01** | - | - | - | - |
| Residuals | 123 | | 0.41 | | 95 | | 0.48 | | 17 | | 0.50 | |

*Non-significant interactions removed for simplicity
[a]Habitat: Rhizosphere vs. bare soil samples
[b]Genotype: Includes two maize hybrids (B73xB97, B73xTx303) and four species (*S.* x *drummondii*, *E. esculenta*, *H. annuus*, and *F. esculentum*).
*P*-values significant at $p < 0.05$ highlighted in bold

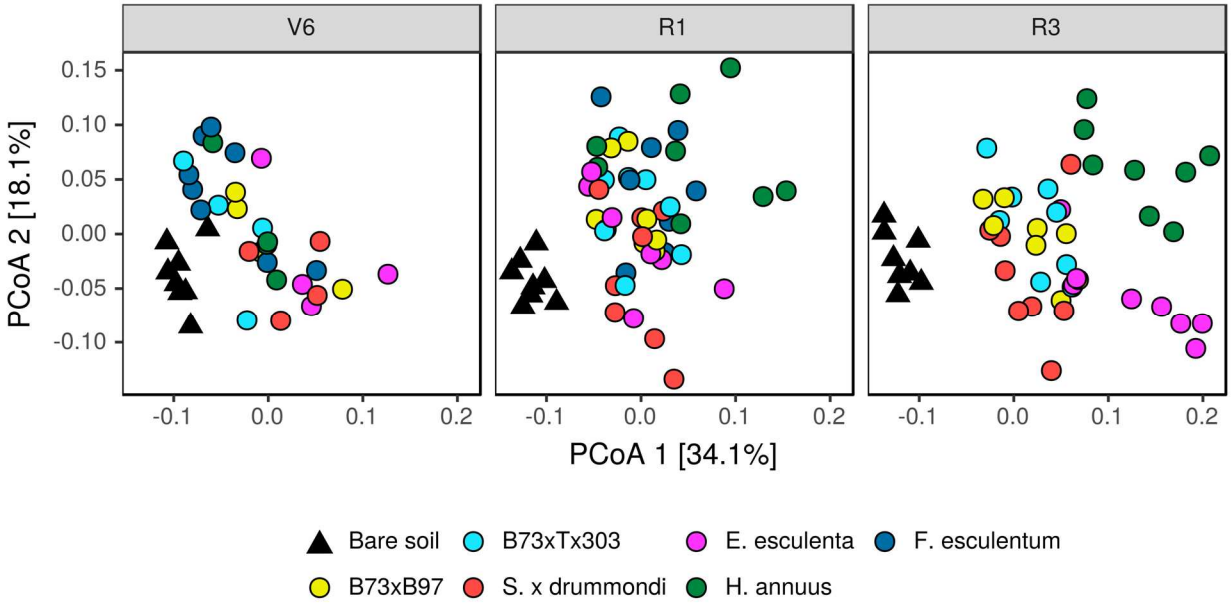



Figure 2. 4: Plant species shapes rhizosphere bacterial community composition and succession as displayed in principal coordinate analysis of weighted-UniFrac distances. Ordination faceted by sampling date (panels share single x and y axis), corresponding to when maize is at vegetative six-leaf (V6), flowering (R1), and grain filling (R3) stages.

rhizosphere and bulk soil shifted over the course of the season, with sampling date accounting for approximately 11% of the variation in rhizosphere samples ($p < 0.01$; Table 1) and 16% of the variation in bare soil plots ($p < 0.01$; Table 2.1). However, temporal changes in rhizosphere BCC were largely uncoupled from changes in the bulk soil community. The variation between bulk and rhizosphere soils increased over time as plants developed (Figure 2.4, Figure 2.5). To determine whether shifts in microbial abundance in bulk soil are amplified during colonization of the rhizosphere we compared the LFC of OTUs in the two habitats over time. Of the 328 OTUs that were differentially abundant between time points in rhizosphere samples, only 5 had statistically significant and parallel shifts in abundance in bare soil samples. A *Massilia sp.* (*Oxalobacteraceae*) OTU illustrates this trend. It was at relatively low abundance in bulk soil at the first sampling (0.04% relative abundance), but highly abundant in rhizosphere samples (3.4% ± 9.6% sd). By R1 this OTU was below detection in 5 of 8 bulk soil samples and accounted for only 0.3% (± 0.4%) of reads in rhizosphere samples. Thus, a decrease in abundance in bulk soil may have limited this OTU's ability to colonize new roots. However, the limited number of OTUs following a similar pattern suggests that changes in abundance in bulk soil are not a primary driver of taxa abundance in rhizosphere samples.

The change in abundance of OTUs over the three sampling dates reflected a strengthening of the rhizosphere effect. Of the 257 OTUs that increased in abundance over time, 152 were positive rhizosphere responders. As a result, rhizosphere responders accounted for an increasing portion of rhizosphere reads, increasing from 22 ± 11% of reads at the first harvest to 38 ±13% of reads at the final harvest. In contrast, the abundance of rhizosphere responders decreased from 6.7 ± 0.8% to 4.9 ±0.4% of reads in bulk soil samples.

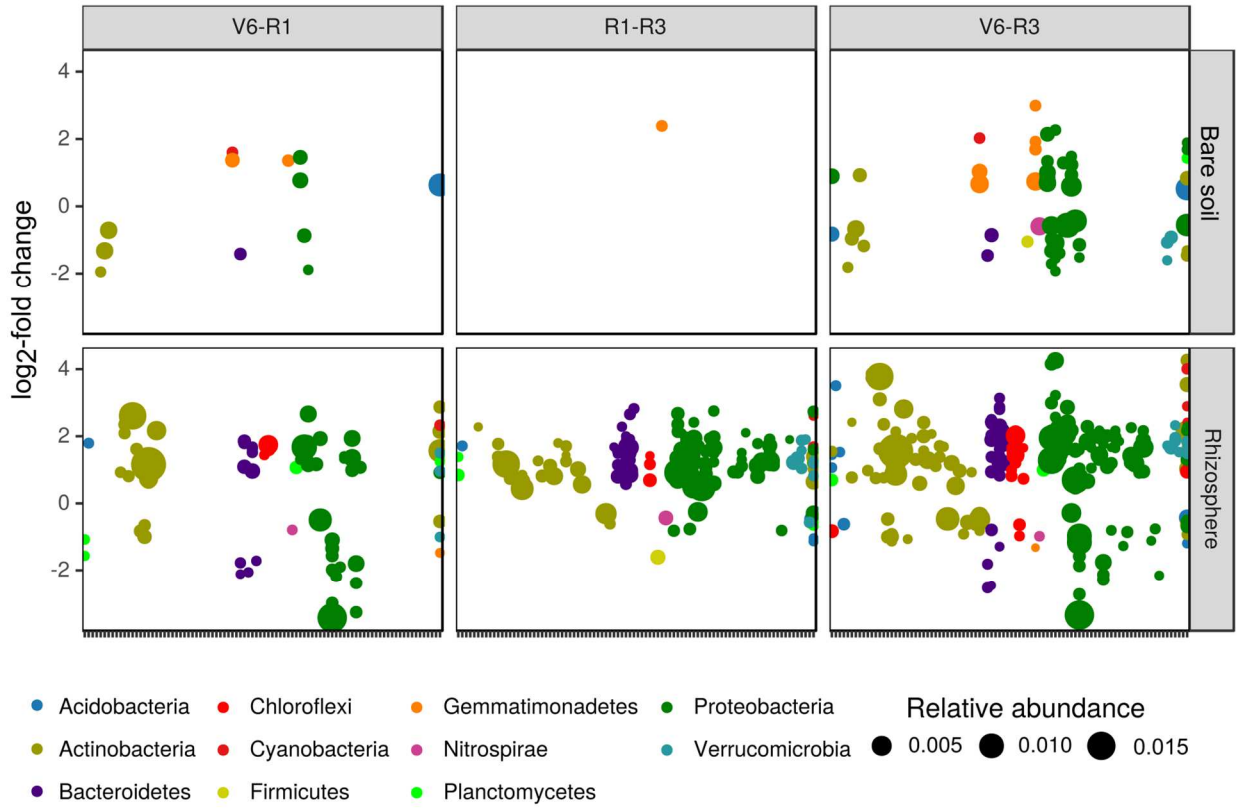



Figure 2. 5: Temporal change in rhizosphere bacterial community in bare soil (top panels) and rhizosphere (bottom panels) are uncoupled, with greater shifts in community composition observed in rhizosphere soils. OTUs with significant differential abundance between sampling dates (log2-fold change $\neq 0$, adjusted $p < 0.05$) colored by phylum.

Table 2.2: Variance partitioning of rhizosphere bacterial community beta-diversity (weighted-UniFrac distances) on three sampling dates in permutational multiple analysis of variance.

| | | Interspecific[a] | | | | | Intraspecific | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Factor | Df | SS | F | R2 | *p* | Df | SS | F | R2 | *p* |
| V6* | | | | | | | | | | | |
| | Rep | 3 | 0.07 | 1.94 | 0.20 | **0.04** | | | | | |
| | Genotype | 5 | 0.11 | 1.75 | 0.30 | **0.03** | | | | | |
| | Residuals | 15 | 0.19 | | 0.51 | | | | | | |
| | | | 0.37 | | | | | | | | |
| R1 | | | | | | | | | | | |
| | Rep | 3 | 0.12 | 6.00 | 0.21 | **<0.001** | 3 | 0.09 | 4.98 | 0.31 | **<0.001** |
| | Genotype | 5 | 0.14 | 4.19 | 0.25 | **<0.001** | 8 | 0.06 | 1.12 | 0.19 | 0.27 |
| | Clover swap | 1 | 0.01 | 1.80 | 0.02 | 0.07 | | | | | |
| | Rep:Genotype | 15 | 0.15 | 1.46 | 0.26 | **0.01** | | | | | |
| | Genotype:Clover | 5 | 0.03 | 0.90 | 0.05 | 0.63 | | | | | |
| | Residuals | 18 | 0.12 | | 0.21 | | 24 | 0.15 | | 0.50 | |
| R3 | | | | | | | | | | | |
| | Rep | 3 | 0.08 | 2.91 | 0.12 | **0.002** | | | | | |
| | Genotype | 4 | 0.26 | 7.39 | 0.39 | **<0.001** | | | | | |
| | Clover swap | 1 | 0.01 | 1.46 | 0.02 | 0.17 | | | | | |
| | Rep:Genotype | 12 | 0.13 | 1.24 | 0.20 | 0.16 | | | | | |
| | Genotype:Clover | 4 | 0.05 | 1.33 | 0.07 | 0.14 | | | | | |
| | Residuals | 15 | 0.13 | | | | | | | | |

*Sampling corresponds to maize at vegetative six-leaf (V6), onset of flowering (R1), and grain filling (R3) stages.

[a] Interspecific comparisons include all species with two representative maize hybrids, while intraspecific comparisons are among nine maize hybrids.

*P*-values < 0.05 highlighted in bold.

### 2.3.4 Plant species influences rhizosphere BCC.

Plant species had a significant influence on rhizosphere beta-diversity, accounting for 18% of the variation in rhizosphere samples ($p < 0.01$; Table 2.1), a greater portion of variation than explained by sampling date. Furthermore, the interaction of genotype and sampling date accounted for an additional 10% of the variation in rhizosphere BCC, indicating that species identity influenced the succession of rhizosphere BCC over time (sampling date x genotype: $p < 0.01$ Table 2.1). This is evident in the ordination (Figure 2.4), where *H. annuus* and *F. esculentum* begin to separate from the grasses at R1, at the same time *E. esculenta* and *S. x drummondii* begin to separate from maize. By R3 each species occupies a nearly non-overlapping space on the ordination (Figure 2.4). In contrast to this variation observed among species, there was not significant intraspecific variation among the maize hybrids in rhizosphere BCC when assessed at R1 (Table 2.2; Figure B1).

The separation of species in the ordination and PERMANOVA reflected broad shifts in OTU abundance among the individual species. The abundance of 374 OTUs was influenced by plant species when controlling for replicate block and date of sampling in a negative binomial model (BH adjusted $p < 0.05$). This included the majority of rhizosphere responders (156 of 197) (Figure 2.6). The wide range of taxa responsive to plant species may indicate compositional effects whereby change in dominance of a subset of organisms alters the perceived relative abundance of other taxa in the community. The differentiation of a subset of OTUs among species was further dependent on the sampling date, indicated by a significant interaction between sampling date and species in the negative binomial model (BH adjusted $p < 0.05$) (Figure 2.6). This is illustrated by several OTUs from the *Actinobacteria* and *Chloroflexi* that increased in abundance in the rhizosphere of *H. annuus* over time (Figure 2.6), while several

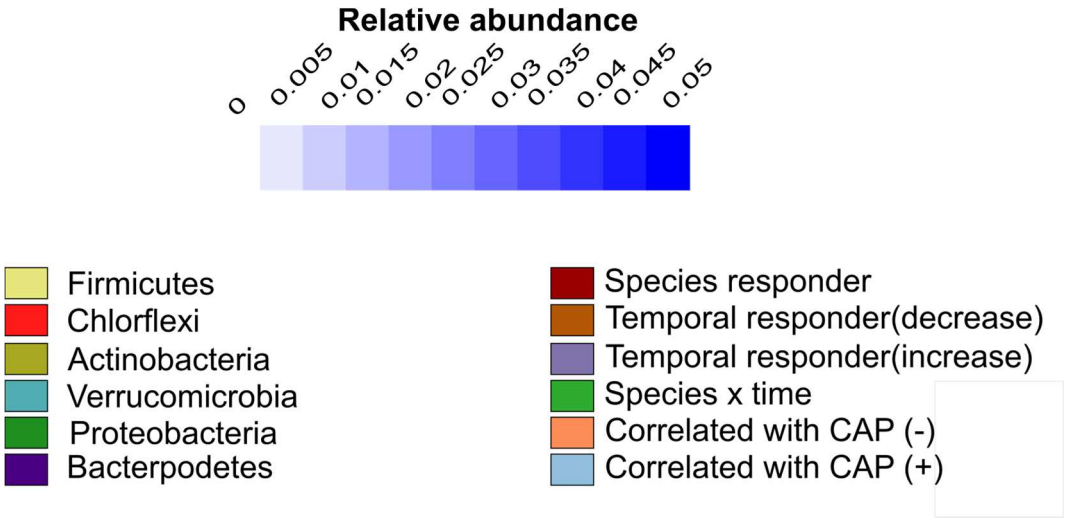



Figure 2. 6: Rhizosphere composition varies through time and among plant species. Heatmap displays OTU relative abundance in the rhizosphere of two maize hybrids and four species on three sampling dates corresponding to maize six-leaf (V6), flowering (R1) and grain filling (R3) stages. Only 100 most abundant rhizosphere responders shown for clarity and arranged by bacterial phylogeny (left tree). Color strips (from left): OTUs with differential abundance among plant species or genotypes (maroon), differential abundance among sampling dates (brown and purple), species specific temporal response (green) and correlation with growth characteristics in constrained ordination (see figure 2.7). Species and genotype codes: *Zea mays* cv B73xB97 and B73xTx303, *S. x drummondii* (SORSUD), *E. esculenta* (ECHES), *H. annuus* (HELAN) and *F. esculentum* (FAGES)


V6
R1
R3
Paenibacillaceae: Paenibacillus
Alicyclobacillaceae: Tumebacillus
Chloroflexaceae: Roseiflexus
Unknown Ktedonobacterales
Ktedonobacteraceae: Unknown
Ktedonobacteraceae: Unknown
Ktedonobacteraceae: Unknown
Ktedonobacteraceae: Unknown
Unknown Streptosporangiales
Actinospicaceae: Actinospica
Streptomycetaceae: Streptomyces
Streptomycetaceae: Streptomyces
Streptomycetaceae: Streptomyces
Streptomycetaceae: Streptomyces
Streptomycetaceae: Streptomyces
Streptomycetaceae: Streptomyces
Unkown Actinobacteria
Micromonosporaceae: Dactylosporangium
Micromonosporaceae: Micromonospora
Micromonosporaceae: Actinoplanes
Micromonosporaceae: Unknown
Micromonosporaceae: Actinoplanes
Pseudonocardiaceae: Unknown
Mycobacteriaceae: Mycobacterium
Pseudonocardiaceae: Unknown
Pseudonocardiaceae: Amycolatopsis
Pseudonocardiaceae: Amycolatopsis
Pseudonocardiaceae: Amycolatopsis
Frankiaceae: Frankia
Microbacteriaceae: Unknown
Micrococcaceae: Arthrobacter
Nocardioidaceae: Aeromicrobium
Nocardioidaceae: Kribbella
Nocardioidaceae: Marmoricola
Opitutaceae: Opitutus
Unknown OPB35_soil_group
Verrucomicrobiaceae: Luteolibacter
Chthoniobacteraceae: Chthoniobacter
Cystobacterineae: Unknown
Sorangiineae: Unknown
Sorangiineae: Unknown
Sphingomonadaceae: Sphingobium
Sphingomonadaceae: Sphingomonas
Sphingomonadaceae: Sphingomonas
Sphingomonadaceae: Sphingomonas
Sphingomonadaceae: Sphingomonas
Bradyrhizobiaceae: Bosea
Caulobacteraceae: Asticcacaulis
Caulobacteraceae: Caulobacter
Phyllobacteriaceae: Unknown
Phyllobacteriaceae: Mesorhizobium
Rhizobiaceae: Rhizobium
Rhizobiaceae: Rhizobium
Rhizobiaceae: Rhizobium
Phyllobacteriaceae: uncultured
Xanthobacteraceae: Labrys
Hyphomicrobiaceae: Devosia
Bradyrhizobiaceae: Bradyrhizobium
Xanthomonadaceae: Dyella
Pseudomonadaceae: Cellvibrio
Sinobacteraceae: Unknown
Sinobacteraceae: Unknown
Oxalobacteraceae: Massilia
Oxalobacteraceae: Unknown
Methylophilaceae: Methylotenera
Burkholderiaceae: Burkholderia
Burkholderiaceae: Burkholderia
Burkholderiaceae: Burkholderia
Oxalobacteraceae: Massilia
Oxalobacteraceae: Massilia
Oxalobacteraceae: Massilia
Oxalobacteraceae: Unknown
Oxalobacteraceae: Massilia
Oxalobacteraceae: Massilia
Oxalobacteraceae: Massilia
Oxalobacteraceae: Massilia
Oxalobacteraceae: Massilia
Comamonadaceae: Unknown
Comamonadaceae: Unknown
Comamonadaceae: Unknown
Comamonadaceae: Unknown
Comamonadaceae: Unknown
Comamonadaceae: Unknown
Comamonadaceae: Unknown
Comamonadaceae: Unknown
Comamonadaceae: Unknown
Comamonadaceae: Pelomonas
Comamonadaceae: Unknown
Comamonadaceae: Comamonas
Cytophagaceae: Flexibacter
Cytophagaceae: Flexibacter
Chitinophagaceae: Chitinophaga
Chitinophagaceae: Flavisolibacter
Chitinophagaceae: Niastella
Chitinophagaceae: Niastella
Sphingobacteriaceae: Mucilaginibacter
Sphingobacteriaceae: Mucilaginibacter
Sphingobacteriaceae: Mucilaginibacter
Sphingobacteriaceae: Mucilaginibacter
B73xB97
B73xTx303
SORSUD
ECHES
HELAN
FAGES
B73xB97
B73xTx303
SORSUD
ECHES
HELAN
FAGES
B73xB97
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SORSUD
ECHES
HELAN

*Proteobacteria, Verrucomicrobia* and *Paenibacillus* (*Firmicutes*) bloomed in the rhizosphere of *S x drummondii* and *E. esculenta* (Figure 2.6).

In contrast to the strength of species and temporal effects shaping rhizosphere BCC, Management-induced shifts in fertility, as influenced by the clover transfer, led to only a small change in the bacterial community composition in both the rhizosphere ($R^2$: 0.01; $p = 0.03$) and bare soil ($R^2$: 0.04; $p = 0.13$). However, this effect was not significant on any sampling date ($p > 0.05$; Table 2.2).

### 2.3.5 Relationships between plant growth dynamics and BCC

To determine whether plant species variation in rhizosphere BCC was linked with plant growth and N uptake dynamics at R1 we constrained the ordination of weighted-UniFrac distances by four plant growth variables (relative growth rate, rate of N uptake, biomass and plant C: N ratio) that represent differences in plant growth strategy and nitrogen use efficiency (g C g $N^{-1}$) (Figure 2.7). We focused on samples from R1 because this is when most plants were at their peak rate of N uptake, when there was considerable variation in relative growth rate, plant C: N and biomass accumulation (Figure 2.1) and when differences in plant growth dynamics and enzyme activity were aligned (Figure 2.3). Furthermore, by focusing on one sample date we avoid spurious correlations between growth dynamics and rhizosphere BCC resulting from the directional change in both measures over the season. The constrained ordination captured a significant portion of the variation in rhizosphere BCC at R1 ($F_{(4,39)} = 4.03$; $p < 0.001$). In particular, the tradeoff between plant relative growth rate and plant size and C:N ratio captured approximately 20% of the variation in rhizosphere BCC, separating the smaller seeded tillering grasses with high relative growth rates (*E. esculenta* and *S. x drummondii*) from maize and *H. annuus* (Figure 2.7). The 20% of variation explained by this axis is a substantial portion of the

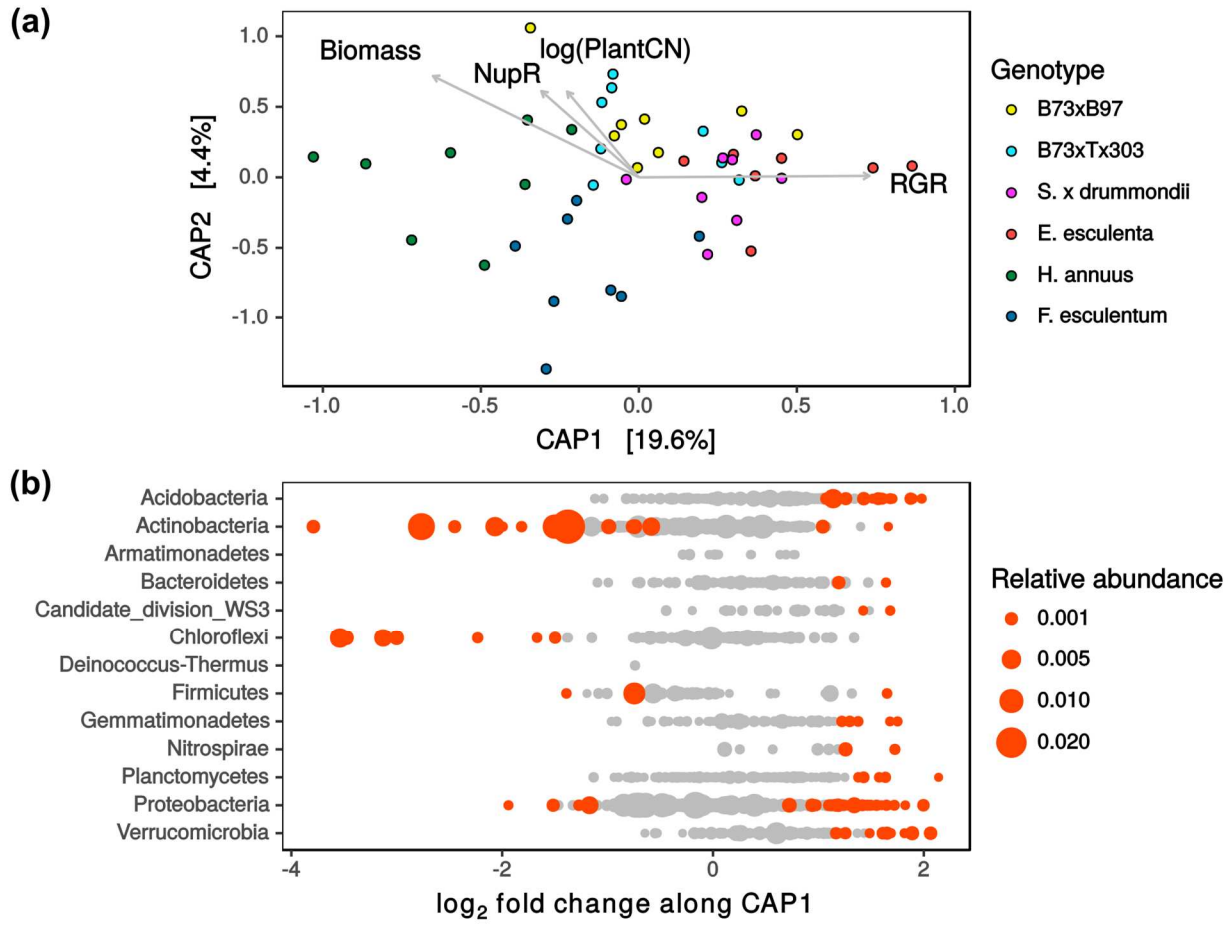


Figure 2. 7: (a) Variation in rhizosphere bacterial community composition is explained by species variation in plant growth and nitrogen uptake dynamics as shown in constrained analysis of principal coordinates (CAP). Loadings of growth characteristics on CAP axes shown by arrows. (b) Taxa correlated with CAP axes are phylogenetically clustered. Points colored in red are OTUs with significant $\log_2$-fold change (DESeq2: BH adjusted $p < 0.05$) per unit of CAP axis 1 when controlling for replicate block and grass vs. dicot contrast. Abbreviations of growth characteristics: relative growth rate (RGR), plant biomass (Biomass), rate of nitrogen uptake (NupR) and plant C:N ratio (Plant CN).

variation explained by the plant species/genotype term in the PERMANOVA at R1 (Table 2.2), lending support for the combined influence of plant genetics and ecophysiology on rhizosphere BCC.

A negative binomial model was used to identify OTUs correlated with the primary axis of the constrained ordination while controlling replicate block and plant functional group (grass vs. dicot) to avoid false positives associated with the grass vs. dicot contrast. The abundances of 30 OTUs were negatively correlated with the primary axis of the ordination (CAP1), and therefore associated with lower relative growth rates and greater total biomass accumulation and N uptake. Among these OTUs, the rhizosphere responders (16 of 30) were predominately *Streptomyces* and *Amycolatopsis* (*Actinobacteria*), *Ktedonobacteraceae* (*Chloroflexi*), and *Paraburkholderia* (*Proteobacteria*) (Figure 2.6; Table B5). The taxa that distinguished species position on the primary CAP axis at R1 were also tested for their relationship to plant relative growth rate and N uptake across rhizosphere samples collected on all dates. The taxa associated with low relative growth rate and high N uptake at R1 were correlated with these same terms over the wider range of variation captured in samples from all three sampling dates (Figure 2.8; Table B5).

In contrast, the many OTUs positively correlated with CAP1 represented diverse phyla and included many non-rhizosphere responders. This may reflect strong compositional effects driven by the change in relative abundance of those OTUs negatively associated with CAP1. The rhizosphere responders positively correlated with CAP1 axis included *Paenibacillus* (*Firmicutes*), *Luteolibacter* and *Opitutus* (*Verrucomicrobia*), and *Sorangiineae* (*Proteobacteria*), (Figure 2.6; Table B5). These OTUs were not correlated, or had poor fit with relative growth rate and N uptake when evaluated across samples from all three dates.

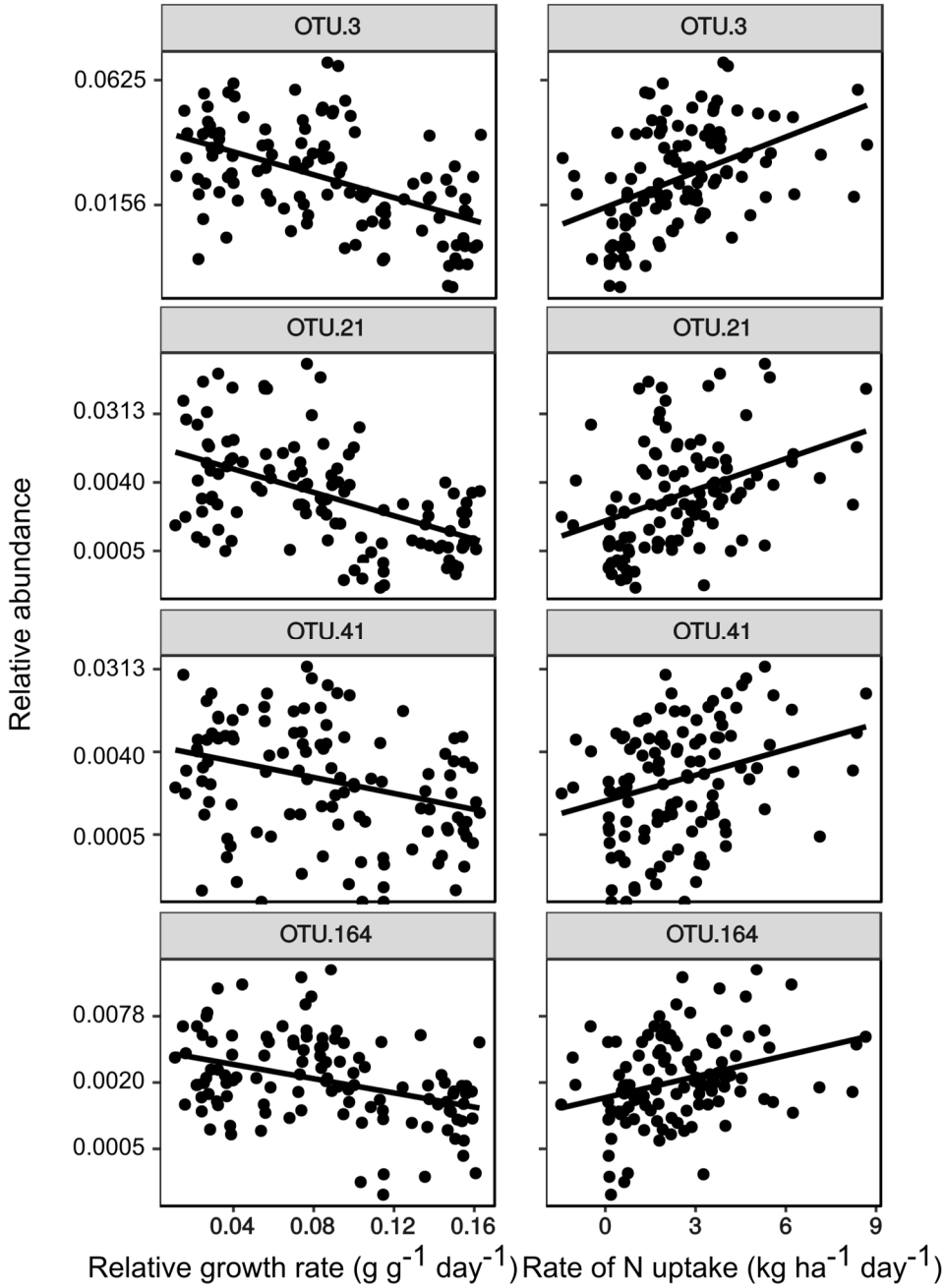


Figure 2.8: Taxa correlated with plant variation in growth characteristics at R1 follow similar trend in samples collected on three sampling dates. Abundance of four representative taxa from *Paraburkholderia* (OTU.164), *Amycolatopsis* (OTU.21), *Streptomyces* (OTU.3) and *Ktedonobacteraceae* (OTU.41) vary in relation to plant relative growth rate and rate of nitrogen (N) uptake.

## 2.4 DISCUSSION

In a rhizosphere study situated in an organically managed field, we demonstrate the interacting effects of plant species and temporal variation in shaping rhizosphere bacterial community composition and activity. Within this context we highlight the role of plant growth and N uptake in structuring rhizosphere communities and their activity.

### 2.4.1 Plant species and temporal effects shape rhizosphere bacterial community

Previous work has detailed compositional changes in the rhizosphere characterized by the increase in abundance of taxa from the *Proteobacteria*, *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, and lineages of the *Verrucomicrobia* and *Chloroflexi* (Turner *et al.*, 2013; Peiffer *et al.*, 2013; Bulgarelli *et al.*, 2015). The enrichment of this rhizosphere community was evident by our first sampling date at 36 days after planting (V6). Over the next two sampling dates the strength of the rhizosphere effect increased with the rhizosphere community becoming progressively more distinct from bulk soil.

Several authors have observed seasonal shifts in rhizosphere BCC, which are postulated to derive from changing exudate profiles that accompany plant development as well as the cumulative impact of plant growth, exudation and N uptake on the rhizosphere physiochemical environment (Chaparro *et al.*, 2014; Bell *et al.*, 2015; Shi *et al.*, 2015). In addition to these plant-mediated factors, exogenous factors may influence rhizosphere assembly through time. Seasonal environmental variation may influence shifts in the bulk soil community and such shifts may alter the propagules pool from which new roots are colonized. In an experimental manipulation, Bakker *et al.* (2015) found that disturbance in the bulk soil community influenced rhizosphere composition of maize. We highlight one *Massilia* sp. OTU to illustrate this potential mechanism.

This OTU was highly abundant in the rhizosphere at the first sampling date, but decreased in abundance by R1. There was a parallel decrease in bulk soil and by the second harvest this OTU was below detection in several bare soil samples. Thus, changing edaphic factors, such as soil moisture or nutrient status influencing the abundance and activity of this OTU in bulk soil may have been amplified in the rhizosphere. *Massilia* is also reported to be an early rhizosphere colonizer that is not competitive with other rhizosphere taxa over a longer time frame (Ofek *et al.*, 2012). Its inability to persist in the rhizosphere and shifts in environmental factors that negatively influence its ability to colonize new roots may both contribute to its competitive exclusion from the rhizosphere at later dates.

However, our finding that very few taxa had significant parallel changes in abundance in bulk and rhizosphere soil suggests an uncoupling of bulk soil and rhizosphere community dynamics and that autogenic or plant mediated factors are more likely shaping seasonal changes in the rhizosphere bacterial community (Chaparro *et al.*, 2014; Bell *et al.*, 2015; Shi *et al.*, 2015; Niu *et al.*, 2017; Hannula *et al.*, 2017). Below we discuss the influence of plant species in shaping succession in the rhizosphere and its relationship with plant growth dynamics.

### 2.4.2 Plant species and growth dynamics shape rhizosphere BCC

The significant role of plant species in shaping bacterial community trajectory over time supports the hypothesis that plant-mediated factors are shaping activity and community succession in the rhizosphere. In our results the variation in rhizosphere BCC attributed to plant species increased over time. Similarly, in a recurrent selection experiment, Tkacz *et al.* (2015) found that plant species diverged in rhizosphere BCC over multiple generations, culminating in stable but distinct communities.

Part of the observed variation in rhizosphere BCC was linked with plant species and

temporal variation in growth and N uptake. These traits may influence rhizosphere BCC in multiple ways. First, high rates of plant N uptake lead to increased plant-microbe competition for N in the rhizosphere and a shift in microbial community composition and metabolism (Pathan *et al.*, 2015; Bell *et al.*, 2015). The increased allocation to the N-cycling enzymes LAP during periods of high plant N demand and in proportion to the rate of plant N uptake is consistent with this hypothesis. Second, N competitive species vs. conservative species have differences in root growth and exudation patterns that accompany different life history strategies. Plants with high relative growth rate have been found to have higher rates of root exudation that maintain increased microbial populations in the rhizosphere (Blagodatskaya *et al.*, 2014; Kaštovská *et al.*, 2015). The higher growth rate observed in *E. esculenta* and *S. x drummondii* may thus maintain a broader set of rhizosphere taxa than *H. annuus* and maize, whose relative growth rate slows more dramatically during the season. A link between above ground relative growth rate and below ground C flows could also explain temporal trends in rhizosphere BCC as relative growth rate declines with plant growth and development. Fu *et al.* (2002) found that root and rhizosphere respiration per unit biomass decreased for several annual species from vegetative through grain filling and physiological maturity. Thus, decreasing C flows to the rhizosphere over time or in species with lower relative growth rates may contribute to compositional shifts observed here.

The taxa linked to decreasing relative growth rate and high rates of N uptake were phylogenetically and morphologically distinct. The majority of these taxa were *Actinomycetes* with a filamentous growth habit, in particular *Streptomyces and Amycolatopsis*. Another cluster of OTUs shared *Ktedonobacter* (87% identity) as their nearest cultured relative. *Ktedonobacter* is a deeply branched lineage loosely grouped with *Chloroflexi* (Cavaletti *et al.*, 2006) that also

has a filamentous growth habit. The one non-filamentous lineage that was positively correlated with high biomass accumulation and N uptake was *Paraburkholderia* (reclassified from *Burkholderia* (Sawana *et al.*, 2014)). *Paraburkholderia* is rich with plant growth promoting bacteria, including several diazatrophs (Estrada-De los Santos *et al.*, 2001), and is a well-known colonizer of the rhizosphere (Tabacchioni *et al.*, 2002). The correlation of these taxa with high N uptake and low relative growth rate over the course of the season suggests that these organisms thrive in the rhizosphere during periods where plant relative growth rate is slowing and high rates of N uptake limit the nutrient status of the rhizosphere. These findings are consistent with a previous field study where several *Actinomycetes*, including *Streptomyces,* were more abundant in the rhizosphere of plants with longer growth periods, greater biomass accumulation, greater N uptake and higher nitrogen use efficiency (g C g $N^{-1}$) (Emmett et al. *In review*). We propose that variation in plant life history that determines growth rate, biomass accumulation and rates of N uptake, is an important source of variation in rhizosphere BCC.

### 2.4.3 Extracellular enzyme activity coupled with plant nitrogen uptake

Similar to rhizosphere BCC, variation in potential enzyme activity in the rhizosphere was coupled with temporal and species variation in plant N uptake. We found that rhizosphere effects on N-cycling protease activity were primarily evident during periods of high plant demand and limited soil N supply, which is broadly consistent with the hypothesis that shifting stoichiometry of the rhizosphere leads to microbial allocation of enzymes to limiting resources and thus supports the depolymerization of soil organic N (Sinsabaugh & Moorhead, 1994; Allison & Vitousek, 2005; Dijkstra *et al.*, 2013). The correlation between rate of plant N uptake at R1 and LAP activity strengthens this inference.

The temporal coupling of rhizosphere activity and plant N demand has important

management implications. In a previous study at this site, soil $NO_3$ peaked within 5-6 weeks following clover incorporation (Han *et al.*, 2017), whereas flowering and peak N uptake in our study did not occur until 9.5 weeks after planting. Priming of soil organic N mineralization may be an important contributor to plant N supply during this time. This experimental field has high short- and medium-term soil organic N pools in the form of microbial biomass and free and occluded particulate organic matter (Berthrong *et al.*, 2013). In the work of Sanchez *et al.* (2002), it was the 70-day N mineralization pool that could be mineralized by maize, but remained stable in unplanted soil. Soil management to increase stocks of medium-term N pools that are accessible through rhizosphere processes could improve synchrony between plant N demand and soil N supply in agricultural systems, a persistent challenge in lower input agroecosystems (Crews & Peoples, 2005).

### 2.4.4 Short term changes in fertility do not alter rhizosphere BCC or enzyme activity

The transfer of aboveground clover residues from – to + clover plots did not alter plant rhizosphere effect on potential enzyme activity or community composition at R1, despite improving plant N uptake. Fertilization treatments have been shown to alter rhizosphere composition in greenhouse settings (Zancarini *et al.*, 2012; Li *et al.*, 2016) and long term fertilization influences the bulk soil microbial community (Ramirez *et al.*, 2012; Leff *et al.*, 2015). In previous work we have inferred that the impact of fertilization on rhizosphere BCC under field conditions is likely mediated through changes in plant growth and root activity rather than directly through changes in inorganic N concentrations (Emmett *et al.* In Review). This is consistent with reports from maize that show altered root growth and exudate profiles with increasing N fertilization (Zhu *et al.*, 2016) Since the additional N uptake observed in + clover plots at R1 was not associated with increased growth and biomass accumulation it may be

expected that it would also not shift the rhizosphere community or associated enzyme activity.

## 2.5 CONCLUSIONS

In a field study we have demonstrated that plant species influence rhizosphere bacterial community composition and succession. We also highlight that temporal and plant species variation in growth and N uptake are linked with rhizosphere effects on community composition and activity. The plant phenotype associated with lower relative growth rate and high rate of N uptake favored a morphologically and phylogenetically distinct subset of the microbial community. Moreover, this selection was consistent with temporal variation in these same traits. These findings help elucidate factors controlling the assembly and succession of the rhizosphere microbiome over critical periods of plant development. Such knowledge is a first step toward selecting plants and managing soils to optimize rhizosphere processes in support of sustainable agroecosystems.

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# CHAPTER 3

# EIGHTY YEARS OF MAIZE BREEDING ALTERS PLANT NITROGEN ACQUISITION BUT NOT RHIZOSPHERE BACTERIAL COMMUNITY COMPOSITION

## ABSTRACT

Rhizosphere microbial communities play a critical role in supply of plant nutrients and there is considerable interest in breeding for genotypes that harness rhizosphere processes. Conversely, evidence from some systems suggests human selection has altered rhizosphere traits, potentially disrupting plant-microbe collaborations and ecosystem services. We evaluated whether modern breeding has altered rhizosphere bacterial community assembly or plant access to endogenous soil nitrogen (N) in maize, a globally important crop species. Twelve best-selling and widely adapted maize hybrids released between 1936 and 2011 were grown in replicated monocultures fertilized with 0, 85 or 170 kg N $ha^{-1}$. Plant growth, N uptake, and potential extracellular enzyme activity of rhizosphere soils were measured during the vegetative stage, anthesis, and grain fill periods and bacterial community composition assessed with 16S rRNA gene amplicon sequencing. Community structure and enzyme activity varied among hybrids, and these differences were most distinct in hybrids from the 1960s and 70s. In contrast, there was a linear increase in grain yield and N uptake with year of release at all fertilization levels. Partitioning of $^{15}N$ fertilizer indicated that plant uptake of soil N was substantial under fertilized conditions and increased with year of hybrid release. These results indicate that breeding for modern production systems has increased plant ability to acquire N from soil reserves, but these

changes were not associated with a corresponding shift in rhizosphere community assembly. The finding that rhizosphere bacterial community composition differed among hybrids developed in a commercial breeding program reveals the potential for future breeding efforts to influence rhizosphere traits.

## 3.1 INTRODUCTION

Nitrogen (N) application supports significant increases in crop productivity, while N losses from agricultural systems have negative environmental impacts and significant financial costs (Vitousek *et al.*, 1997; Sinclair & Rufty, 2012). Improving nitrogen use efficiency (NUE) at the plant and agroecosystem level is therefore essential to address the twin challenges of meeting global food supply and limiting environmental externalities (Cassman *et al.*, 2002). This is often framed in terms of fertilizer use efficiency or the increase in yield resulting from fertilizer addition (Raun & Johnson, 1999; Baligar *et al.*, 2001). However, endogenous soil N contributes substantially to plant N uptake, accounting for approximately 60 – 70% of plant N in wheat and maize and therefore the development of plant cultivars and management systems that maximize these flows are critical components of efforts to improve system NUE (Dawson *et al.*, 2008; Gardner & Drinkwater, 2009).

Microbial communities associated with plant roots in the rhizosphere play a central role in mediating plant N acquisition and carbon (C) and N cycling in soils (Schimel & Bennett, 2004; Philippot *et al.*, 2013) and therefore may be an important link in supporting plant and agroecosystem NUE. Annual plants transfer 3-30% of fixed C to the rhizosphere in the form of exudates, rhizodeposits and transfer to mycorrhizal partners (Lynch & Whipps, 1990). This flux drives changes in the broader microbial community with important consequences for plant N flows. These changes include increased rates of associative N fixation (Jones *et al.*, 2003), changes in rates of nitrification and denitrification (Qian *et al.*, 1997; Herman *et al.*, 2006) and rhizosphere priming of soil organic matter decomposition and N mineralization (Kuzyakov, 2002; Cheng *et al.*, 2014; Zhu *et al.*, 2014). These dynamics are thought to be driven by increased C availability and corresponding increase in nutrient limitation of microorganisms in

the rhizosphere. This shift in stoichiometry promotes an increase in the production of microbial extracellular enzymes that depolymerize soil organic matter, a rate limiting step in N mineralization (Schimel & Bennett, 2004; Herman *et al.*, 2006; DeAngelis *et al.*, 2009; Dijkstra *et al.*, 2013).

There is considerable evidence for plant genetic variation in the assembly of the rhizosphere microbial community, including among cultivars of major crop species such as maize (*Zea mays subsp mays* L.), rice (*Oryza sativa* L.) and wheat (*Triticum aestivum* L.) (Peiffer *et al.*, 2013; Edwards *et al.*, 2015; Mahoney *et al.*, 2017). These findings have increased interest in selecting for plants better able to foster beneficial plant-microbe collaborations in the rhizosphere and maximize internal nutrient supply in agricultural systems (Drinkwater *et al.*, 2007; Wissuwa *et al.*, 2009). In general, breeding programs for grain crops have not specifically targeted belowground traits, however, the agroecological context in which and for which cultivars are bred will determine the selection pressure on these traits. If breeding occurs in a high-input environment that obviates the need for plant or plant-microbe based ecosystem services, then the traits supporting beneficial rhizosphere interactions may be lost during selection (Drinkwater & Snapp, 2007; Pérez-Jaramillo *et al.*, 2015; Schmidt *et al.*, 2016). The reduced ability of modern soybean (*Glycine max* (L.) Merr.) varieties to inhibit non-mutualistic rhizobial symbiosis provides significant support for the hypothesis that breeding for modern production systems has relaxed selection on rhizosphere traits (Kiers *et al.*, 2007). However, results for other symbionts are mixed. For instance, breeding influence on mycorrhizal colonization of maize varied by breeding program (An *et al.*, 2010) and in a meta-analysis by Lehman *et al.* (2012), modern cultivars of grain crops proved to be more responsive to mycorrhizal colonization than their wild relatives.

Beyond specific symbioses, it is an open question whether breeding has altered assembly of the broader rhizosphere microbial community. Several studies have found differences between modern maize and wheat cultivars and their ancient relatives in rhizosphere bacterial community composition (BCC) (Germida & Siciliano, 2001; Szoboszlay *et al.*, 2015), however it is unclear whether this is a consequence of domestication and breeding or an example of widely described species effects on rhizosphere BCC (Grayston *et al.*, 1998; Turner *et al.*, 2013; Dawson *et al.*, 2017). A time course of intermediate genotypes is necessary to reveal a trend or discontinuous changes in rhizosphere assembly patterns resulting from the effect of human selection. For instance, Leff *et al*. (2017) evaluated sunflower (*Helianthus annuus* L.) germplasm representing degrees of domestication and found differences in root and rhizosphere fungal community composition, but not bacterial community composition. Such knowledge of how past breeding efforts influence rhizosphere community assembly, particularly in relation to changes in plant nutrient acquisition under field conditions, will be essential to inform future efforts to harness rhizosphere processes in support of sustainable production systems.

In the context of maize, a globally important crop species, breeding efforts have interacted with profoundly changed management context. Since the widespread planting of hybrids in the 1930s, U.S. maize yields have increased from less than 2 Mg $ha^{-1}$ to approximately 10 Mg $ha^{-1}$ (Duvick, 2005; 'USDA - National Agricultural Statistics Service', 2016). This yield increase has been supported by the application of synthetic N fertilizer, which increased from less than 20 kg N $ha^{-1}$ in the 1950s to over 135 kg $ha^{-1}$ in the 1980s (Duvick, 2005; Sinclair & Rufty, 2012; USDA-ERS, 2015). This rapid increase in fertilizer use led to N surplus in maize production during the 1970s-1990s, while N surplus has re-approached balance under stabilized application rates over the last two decades (Vitousek *et al.*, 2009). It is possible that breeding

under luxury N conditions has relieved selection pressure on rhizosphere traits and adversely influenced maize ability to acquire N from soil pools.

However, other changes in the agro-ecological context may maintain selective pressure on these traits. Maize stand density has increased from less than 30,000 plants $ha^{-1}$ in the 1930s to 80,000 plants $ha^{-1}$ today (Duvick, 2005). Selection for yield under increasing intraspecific competition may have selected for greater competitive ability and more efficient resource acquisition (York *et al.*, 2015). Furthermore, rhizosphere processes appear to contribute substantially to maize N acquisition. For instance, Sanchez *et al.* (2002) found that maize increased N flows from soil N pools by 50% compared to bare soil or wheat. Meanwhile, tropical maize can acquire up to 33% of plant N from associative N fixation (Montañez *et al.*, 2009). Thus, it is an open question whether breeding has altered the ability of maize to acquire N from organic soil pools or maize association with microbial communities in the rhizosphere.

To address this question we undertook a field experiment to examine shifts in plant N economy and rhizosphere effects over an 80-year history of maize improvement. Using a selection of best-selling and widely adapted maize hybrids released over this period, we sought to test the hypotheses that 1) breeding history has altered plant growth and N uptake efficiencies under high or low N conditions, 2) the contribution of soil N sources to maize N uptake has been changed, and 3) breeding has altered assembly and activity of rhizosphere bacterial communities over this period.

## 3.2 METHODS

### 3.2.1 Plant germplasm and growth conditions

Twelve maize hybrids were chosen to represent widely planted and successful corn belt

hybrids released by Pioneer Hi-bred International, Inc. (DuPont Pioneer) between the 1930s and present day (hereafter ERA hybrids) (Table 3.1). The selection of hybrids was recommended by Dr. Mark Cooper as suitable for New York growing conditions.

Seeds were hand planted in replicated monocultures in a field at the Musgrave Research Farm in Aurora, NY (42° 43' 52.25" N, 76° 39' 35.10" W), on May 22$^{nd}$ and 23$^{rd}$ 2015 in a randomized complete block design (n = 4). The field soil consists of Honeoye and Lima silt loams, classified as fine-loamy, mixed, semiactive, mesic Oxyaquic Hapludalfs and mesic Glossic Hapludalfs respectively. The soil had a pH of 7.68 and consisted of 39% sand, 35% silt and 26% clay. Soil C and N was 2.17 ± 0.19% and 0.16 ± %, respectively. The field had been planted to soy in a maize-soy rotation the previous year. Prior to planting the field was disked, fit and received 224 kg ha$^{-1}$ of granular phosphate and potassium (0-15-30). At planting soil inorganic N content was 7.71 ± 0.95 μg g$^{-1}$, with 14.25 ± 0.50 μg g$^{-1}$ and 93 ± 11 μg g$^{-1}$ Mehlich 3 extractable P and K, respectively. Each main plot consisted of twelve 3.048 m rows spaced at 0.762 meters. Main plots were further split by three fertility treatments resulting in three four-row sub-plots. Plots were overplanted at a density of 80,000 seeds ha$^{-1}$ and hand thinned at the second side dress to 60,000 plants ha$^{-1}$. This intermediate density was chosen to avoid lodging of early hybrids but maintain intraspecific competition and N limitation. Plots were kept weed free through a post planting application of Bicep II Magnum with Callisto pre-emergence herbicides (Syngenta) and subsequent hand weeding throughout the growing season.

The available seeds for this series had been treated with a standard commercial seed treatment (Maxim Quattro, Raxil, Cruiser250 and PPCT2012). To assess whether the seed treatment could influence our results, replicated plots (n = 4) were planted from untreated seeds of four hybrids spanning the range of release dates (Table 3.1) and sampled at anthesis and

Table 3.1: ERA maize hybrids evaluated for nitrogen economy and rhizosphere bacterial community composition.

| Year | Type[b] |
|---|---|
| 1936[a] | DX |
| 1939[*] | DX |
| 1942 | DX |
| 1953[*a] | DX |
| 1961 | DX |
| 1971[*] | SX |
| 1975 | SX |
| 1984[*a] | SX |
| 1991 | SX |
| 2001[*] | SX: YG, RR2 |
| 2003[a] | SX: HX1, RR2 |
| 2011[*] | SX: HX1, LL, RR2 |

[*] 85 kg N and 170 kg N plots received 15N tracer

[a] Hybrids with additional untreated seed plots

[b] Double cross(DX), single cross (SX), and transgene technology: Yield Guard (YG), Round Up Ready 2 (RR2), Herculex 1 (HX1) and Liberty Link (LL)

physiological maturity. No influence of seed treatment on growth metrics or rhizosphere measures was detected (see Figure C1, Table C2 and Table C3 for complete details).

### 3.2.2 Fertility treatment and $^{15}N$ tracer

Each split-plot received a nitrogen fertility treatment of 0, 85 or 170 kg N $ha^{-1}$ applied as $(NH_4)_2 SO_4$ to create conditions ranging from severe N limitation to N excess. The nitrogen application was split between two side dress applications on June 16$^{th}$ and July 17$^{th}$ and applied by hand watering the dissolved fertilizer salts between rows 1 and 2 and rows 3 and 4 to deliver an equal amount of fertilizer to each plant and limit N transfer between plots. Six of the hybrids spanning the breeding history received fertilizer that was mixed with $^{15}N$ labeled $(NH_4)_2 SO_4$ (Cambridge Isotope Labs, Tewksbury, MA) to a target of 1 atom % $^{15}N$ in order to partition hybrid uptake of nitrogen from fertilizer or endogenous soil N pools (Table 3.1).

### 3.2.3 Plant and rhizosphere sampling

Plant growth and N uptake were quantified through biomass clippings at 48, 75 and 152 days after planting, which corresponded to maize six-leaf stage (V6), onset of flowering (VT-R1) and physiological maturity (PM). Additionally, hybrids that received $^{15}N$ fertilizer were sampled 101 days after planting during the grain fill period (R3) to quantify late season rhizosphere effects. All biomass clips occurred in the interior two rows of the plot. At V6, two representative plants were sampled by clipping at the soil line. The sampling at V6 was used to thin the row to target density, thereby leaving the plot intact for subsequent harvests. At R1, four adjacent and properly spaced plants were sampled by clipping at the first nodal roots. At R3, two adjacent and representative plants were cut from the opposite end of the row harvested at R1. At physiological maturity approximately 2m of row-length was harvested for final yield and N

partitioning, from the alternate interior row and from the intact portion of the plot. Position of final harvests were placed to minimize adjacent canopy gaps and where inevitable, placed adjacent to the sampling at R3 in order to limit potential growth response of plants adjacent to canopy gaps. All biomass clips left at least two plants as a buffer on row ends. This sampling scheme ensured that we only sampled roots from the target genotype and limited edge effects of fertilizer treatment. At V6, R1 and R3, above ground biomass was bagged, dried at 60deg C to a constant weight and weighed for dry mass determination. At PM ears were separated from stover in the field. Stover was weighed in the field and a subsample collected for moisture and dry weight determination. All ears were bagged, dried, shelled and cobs and grain weighed separately.

Root and rhizosphere sampling occurred during the biomass clips. During the sampling at V6 and R3, we focused our sampling on the genotypes receiving $^{15}N$ fertilizer and sampled at all three fertility levels. At R1, we broadened the sampling to include all genotypes and plots planted with untreated seeds, but limited sampling to only the moderate fertility treatment of 85 kg N $ha^{-1}$. Root and rhizosphere samples were collected by loosening the root system from the ground with a shovel and gently massaging the soil to remove loose and unattached bulk soil. Following gentle shaking, intact roots and adhering rhizosphere soil within 4cm from a tip were clipped and placed in vials on ice. Additional rhizosphere soil was collected by gently massaging roots with a gloved hand and collecting the soil on a 2 mm sieve. On each sampling date 2 cm diameter by 20 cm deep soil cores were collected from unplanted, weed-free plots to serve as bare soil controls. Multiple cores were combined, homogenized, subsampled and passed through a 2 mm sieve and placed on ice. Rhizosphere and bare soil samples were stored at 4°C for downstream analysis of extracellular enzymes and root and rhizosphere samples and bare soil

samples were stored at -40° C for downstream DNA extraction.

### 3.2.4 Plant nitrogen determination and source partitioning

Plant samples were ground, homogenized and analyzed for total C and N content on a LECO True Mac elemental analyzer (Saint Joseph, Michigan). Plant samples from R1 and PM from hybrids receiving $^{15}N$ fertilizer were further ground on a Retsch ball mill, homogenized and analyzed for tissue C and N content and atom % $^{15}N$ isotope on a PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK) at the University of California Davis Stable Isotope Facility. Fraction of N from soil and total uptake of N from soil was calculated using the mixing model:

$$(1)\quad \mathrm{X_{soil}} = \frac{At\%^{15}\mathrm{N}_{sample} - At\%^{15}\mathrm{N}_{fertilizer}}{At\%^{15}\mathrm{N}_{control} - At\%^{15}\mathrm{N}_{fertilizer}}$$

where $X_{soil}$ is the fraction of plant N derived from soil pools, $At\%^{15}N_{sample}$ is the isotopic composition of the sample, $At\%^{15}N_{fertilizer}$ is the isotopic composition of the applied fertilizer (~1 atom% $^{15}N$), and $At\%^{15}N_{control}$ is the isotopic composition of available soil N inferred from plots that did not receive fertilizer addition. Sample N derived from soil pools on an area basis ($N_{soil}$ (kg N ha$^{-1}$)) was then calculated with eq. (2) using the fractional N content of sample biomass ($F_{sample}$).

$$(2)\quad N_{soil} = \mathrm{X}_{soil} * \mathrm{F}_{sample} * \mathrm{dw}_{sample}$$

Random samples of cobs were analyzed to determine that cobs matched stover N content and grain $^{15}N$ isotope composition. The N content of stover, cobs and grain were summed for determination of whole plant N content (kg N ha$^{-1}$). Plant N from fertilizer was calculated using

the $^{15}N$ tracer as the difference between soil N and sample N

$$(3)\ N_{fert-15N} = N_{sample} - N_{soil}$$

and by the difference between plant N uptake in fertilized plots ($N_x$) and the adjacent unfertilized control plots ($N_0$).

$$(5)\ N_{fert-diff} = N_x - N_0$$

### 3.2.5 Enzyme analyses

Potential activity of enzymes involved in the depolymerization of carbon containing macromolecules, cellulose (Beta-glucosidase (BG)) hemi-cellulose (β-xylosidase (BX)), cellulose (cellobiohydrolase (CB)), and carbon and nitrogen containing protein (leucine-aminopeptidase (LAP)) and chitin (β-*N*-acetyl-glucosaminidase (NAG)) were measured using a standard fluorometric assay following German *et al.* (2011). Briefly, 2-2.25 g field moist soil was mixed with 150 ml of 50 mM sodium bicarbonate buffer adjusted to pH 7 for 60 seconds using an immersion blender. A 200 µl sample of soil slurry was added to each of 8 replicate wells of a 96-well plate containing 50 µl of 200 µM substrate with attached fluorophore. BG and LAP plates were incubated for 2 hrs and BX, CB and NAG incubated for 4 hrs at 30°C. Following incubation, fluorescence was measured on a BioTek Synergy HT microplate reader at 365 nm excitation and 450 nm emission. Enzyme activity for each soil was estimated using a standard curve (0-75 µM) prepared from the same homogenate to control for quenching and auto-fluorescence. Standard curves were made fresh daily and enzyme analyses were completed within 48hrs of sample collection. Enzyme activity was calculated on a dry weight basis (nmol $hr^{-1}$ g $soil^{-1}$)

### 3.2.6 16S rRNA gene sequence analysis

Intact root and rhizosphere samples were prepared for extraction following Emmett *et al.* (in submission). Roots were chopped to < 1 cm length segments, mixed, and between 0.05 and 0.1 g of freeze dried roots and adhering soil or 0.2 g of bare soil controls were added directly to each well of a 96-well extraction plate of the MoBio PowerMag Microbiome DNA/RNA isolation kit (Carlsbad, CA). Rhizosphere samples were added to duplicate wells to adequately capture heterogeneity of the root systems. Samples were homogenized on a Bio-spec Mini-Beadbeater-96 (Bartlesville, OK) for one minute and extraction proceeded on a Hamilton Star automated liquid handling system. A dual-barcoded MiSeq library of the 16S rRNA V4 region was prepared as in Kozich et al., (2013) using the forward (515F) and reverse primers (806R) adapted from Caporaso *et al.* (2011). Amplicons were prepared in triplicate reactions containing approximately 5 ng of template DNA, 12.5 µl of 2x Q5 High Fidelity, Hot Start PCR Master Mix (NE Biolabs), 1 µM combined forward and reverse primer, and 0.5 µg bovine serum albumin (NE Biolabs) in a 25 µl volume. PCR conditions consisted of: 95°C for 2 min; 30 cycles of 95°C for 20 sec, 55°C for 15sec and 72°C for 10 sec; final extension 72°C for 5 min. Following amplification, positive and negative controls and 10 randomly selected samples were visualized on a 1% agarose gel to check for amplification and contamination. The final library included a mock community control, negative ($H_2O$) controls from each PCR plate, and a negative control from DNA extraction carried through PCR and library preparation. Triplicate reactions were pooled, standardized on a SequalPrep Normalization Plate Kit (Life Technologies), pooled, gel purified on a 1.5% low-melt agarose gel and extracted using the Wizard SV Gel and PCR Clean-Up System (Promega). The library was submitted for paired-end sequencing on the Illumina MiSeq platform with the MiSeq Reagent v2 kit at the Cornell Biotechnology Resource Center

Genomics Facility (Ithaca, NY).

### 3.2.7 Bio-informatics pipeline

Resulting reads were processed as in Whitman *et al.* (2016). Overlapping paired-end reads were merged using PEAR (v0.9.2) (Zhang et al., 2014), de-multiplexed and filtered to remove sequences that were unique, had ambiguous base calls, max expected error rates > 2, or ≥8 homopolymers. Sequences were aligned in mothur using the align.seqs command and the Silva reference database (v.111); sequences that did not align to the SILVA reference were removed. Sequences were clustered into operational taxonomic units (OTUs) at a 97% pairwise identity cutoff with the UPARSE algorithm in USEARCH, which also removes chimeric sequences (Edgar, 2013). Taxonomic assignment of OTUs was performed with Qiime's parallel taxonomy assignment using the uclust consensus taxonomy assignment function (Caporaso *et al.*, 2010) and the Silva reference database (v.111) (Quast et al., 2013). The forward and reverse primers are specific to bacteria, therefore OTUs belonging to chloroplast, mitochondria, eukaryotes, archaea and unassigned sequences represent non-specific amplification and these sequences were removed. OTUs were aligned using SSU_align, poorly aligned positions masked based on posterior probabilities (Nawrocki, 2009), a phylogenetic tree was created and rooted to *Sulfolobus* (acc. X90478) using FastTree (Price *et al.*, 2009) with default settings. Sequences and associated metadata were deposited in the NCBI sequence read archive under accession #SUB3217952.

A single run on the Illumina Miser instrument generated 14,036,217 reads matching a known barcode among 252 multiplexed experimental samples. Following quality control, this left 9,289,527 reads clustered into 13,919 OTUs, with between 11,799 and 118,274 sequences per sample. The OTU table was combined with the phylogenetic tree, taxonomic information

and metadata for analysis using the phyloseq package in R (McMurdie & Holmes, 2013). To remove very low abundance and spurious OTUs, the table was filtered to include only those OTUs with more than three reads in more than three samples resulting in 4,597 OTUs.

### 3.2.8 Statistical analysis

All statistical analyses were conducted in R (R Development Core Team, 2012). Univariate tests of year of hybrid release on plant growth and enzyme activity were conducted in the package "lme4" (Bates *et al.*, 2015) and *p*-values estimated with "lmerTest" (Kuznetsova *et al.*, 2016). Year of release, fertilization and interactions were considered fixed effects with the random effects of replicate block, hybrid genotype, fertilization strip and split-plot. Post-hoc tests were conducted with the function "lsmeans" (Lenth, 2016).

Microbial community beta-diversity was calculated as Bray-Curtis dissimilarity on square root transformed relative abundances. Permutational multiple analysis of variance (PERMANOVA) was used to partition variance in beta-diversity between treatment effects (Oksanen *et al.*, 2012). The effect of breeding history on beta-diversity was tested using Clarke's maximum likelihood population effects model (MLPE) (Clarke *et al.*, 2002) with the R function corMLPE (https://github.com/nspope/corMLPE), which uses a random effect parameter to estimate residual covariance of observations in distance matrices sharing a common sample (Clarke *et al.*, 2002). To avoid pseudo replication of observations from the same hybrid, we used average relative abundance values from samples of each hybrid on a given sample date.

The response of individual OTUs to treatments and correlation with covariates was calculated as $\log_2$-fold change (LFC) in the DESeq2 package (Love *et al.*, 2014) and p-values corrected for multiple comparisons using Benjamini & Hochberg correction. Rhizosphere responders were identified as those OTUs with a significant positive $\log_2$-fold change greater

than one between a genotype's rhizosphere and the bare soil controls sampled on the same date. At different time points we emphasized collection of rhizosphere samples across fertility treatments (V6 and R3) and across the entire hybrid sequence (R1). Therefore we limit microbial community analyses that compare plots across time to just those genotypes and plots that were sampled at all three time points and refer to this subset as the "core" samples. When addressing treatment effects within a time point we include all genotypes and fertility plots collected on that date. Plots with untreated seeds are not included in any analyses except when directly evaluating the effect of seed treatment on outcome variables (Appendix C). Scripts for bioinformatics pipeline, analysis and figure generation are available at https://github.com/bdemmett/ERA_manuscript_analysis/.

## 3.3 RESULTS

### 3.3.1 Maize growth and nitrogen use efficiency

Heavy rainfall in June (8.0 in, 40-year average: 3.91 in) led to poor growth in saturated soils, high spatial variability and potentially high N loss via leaching and denitrification. Nevertheless, there was adequate growth and yield to differentiate effects of breeding history that were consistent with previous reports of hybrid maize improvement. There was a positive linear relationship between year of hybrid release and grain yield ($p < 0.01$; Figure 3.1; see Table 3.2 for effect tests). Nitrogen fertilization increased grain yield ($p < 0.01$) and increased the rate of yield improvement, with a yearly yield gain of 27 kg $ha^{-1}$ $year^{-1}$ (95%CI [11, 44]) in plots receiving 0 kg N $ha^{-1}$ and 45 kg $ha^{-1}$ $year^{-1}$ at 170 kg N ha-1 (95%CI [30,59]). Total plant N uptake at PM increased with year of hybrid release at a rate of 0.32 kg N $ha^{-1}$ $year^{-}$ (95% CI [0.12, 0.53]). Fertilization increased plant N uptake from 47 kg N $ha^{-1}$ (95%CI [36, 58]) at 0 kg

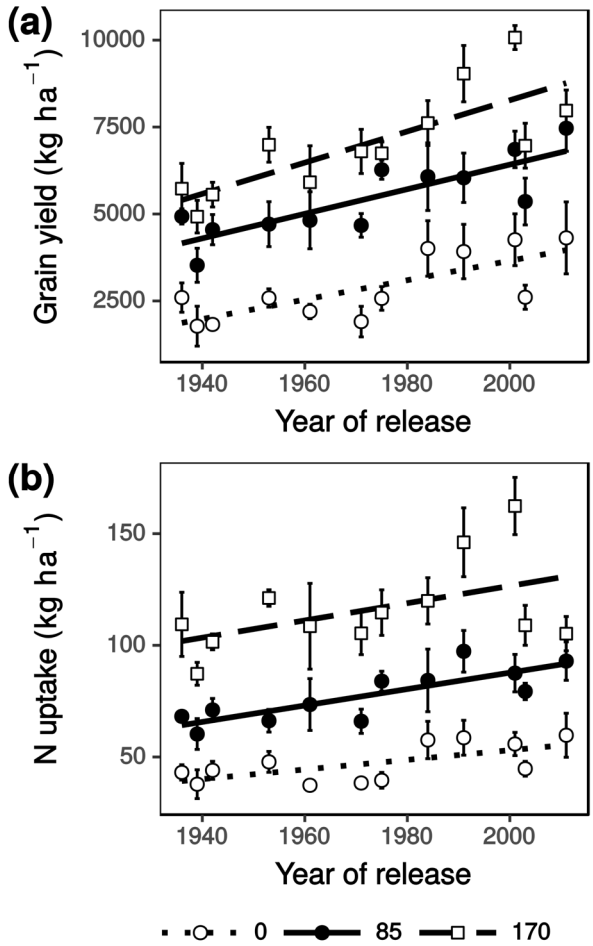


Figure 3. 1: Linear increase in grain yield (a) and plant nitrogen (N) uptake (b) with year of hybrid release at three fertilization levels (0, 85 and 170 kg N ha$^{-1}$). Points represent hybrid means ± 1 s.e.m. for each treatment.

Table 3.2: Analysis of variance F-statistics for fixed effects of year of hybrid release, nitrogen fertilization and interaction on measures of plant growth and N uptake

| | Year | N | YxN |
|---|---|---|---|
| **Growth metrics** | | | |
| Grain yield | $F_{(1,10)} = 22.95$ *** | $F_{(2,28)} = 52.54$ *** | $F_{(2,92)} = 3.05$ $^{+}$ |
| Total N uptake | $F_{(1,10)} = 9.51$ * | $F_{(2,31)} = 61.85$ *** | $F_{(2,91)} = 1.13$ |
| R1 biomass | $F_{(1,10)} = 4.77$ $^{+}$ | $F_{(2,15)} = 11.5$ *** | $F_{(2,90)} = 0.11$ |
| R1 N uptake | $F_{(1,10)} = 3.38$ $^{+}$ | $F_{(2,32)} = 39.54$ *** | $F_{(2,92)} = 1.12$ |
| Post anthesis biomass | $F_{(1,10)} = 8.81$ * | $F_{(2,39)} = 9.81$ *** | $F_{(2,92)} = 1.23$ |
| Post anthesis N uptake | $F_{(1,10)} = 4.58$ $^{+}$ | $F_{(2,31)} = 1.39$ | $F_{(2,91)} = 0.01$ |
| **N partitioning** | | | |
| N uptake from soil | $F_{(1,4)} = 11.09$ * | $F_{(2,31)} = 1.44$ | $F_{(2,41)} = 0.45$ |
| N uptake from fertilizer | $F_{(1,4)} = 5.91^{+}$ | $F_{(1,16)} = 65.58$ *** | $F_{(1,21)} = 0.56$ |
| R1 N uptake from soil | $F_{(1,4)} = 4.06$ | $F_{(2,31)} = 0.22$ | $F_{(2,41)} = 0.11$ |
| R1 N uptake from fertilizer[a] | $F_{(1,4)} = 1.05$ | $F_{(1,16)} = 56.83$ *** | $F_{(1,21)} = 0.38$ |
| N uptake from fertilizer (difference) | $F_{(1,10)} = 2.18$ | $F_{(1,15)} = 31.68$ *** | $F_{(1,45)} = 0.01$ |
| N uptake from fertilizer (difference, 15N subset) | $F_{(1,4)} = 0.9$ | $F_{(1,15)} = 13.56$ ** | $F_{(1,21)} = 0$ |

$^{+}$ $p < 0.1$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

[a] values log-transformed to meet assumptions of equally distributed residuals

N $ha^{-1}$ to 77 kg (95%CI [67,88]) and 117 kg N $ha^{-1}$ (95%CI [105, 126]) in the moderate and high fertility treatments, respectively ($p < 0.01$). However, fertilization did not change the relationship between year of release and improved N uptake (F x Y: $p = 0.33$; Figure 3.1).

Increased N uptake of modern hybrids was a result of combined increase in both pre- and post-anthesis N uptake, though neither term was statistically significant on its own ($p = 0.10$ and $p = 0.06$, respectively; Figure C2). At the intermediate planting density used in this study, lodging was not observed in the field until after R3. The late onset of lodging, combined with manual harvest of whole plants, including root and shoot lodged plants, make it unlikely that differences in standability played a role in driving differences observed in maize N uptake and yield. Influence of year of release and fertilizer treatment on additional plant NUE metrics is reported in supplementary information (Table C4; Figure C2).

Soil N contributed substantially to plant N economy in fertilized plots, accounting for 69% of plant N at 85 kg N $ha^{-1}$ (95%CI [66,72]) and 49% of plant N at 170 kg N $ha^{-1}$ (95%CI [46,52]). The improvement in plant N uptake with year of release was related to increased uptake from soil N. In unfertilized plots there was an increase in N uptake with year of hybrid release of 0.22 kg N $ha^{-1}$ $yr^{-1}$ (95% CI [0.06,0.37], $F_{(1,10)} = 7.06$, $p = 0.02$). Partitioning N uptake between labeled $^{15}N$ fertilizer and endogenous soil N indicated that soil N uptake was not inhibited under fertilized conditions ($p = 0.25$). As a result, there was also a positive relationship between soil N uptake and year of release in fertilized plots ($p = 0.03$) (Figure 3.2). In contrast, there was a weak relationship between year of hybrid release and fertilizer N uptake in the high N plots ($p = 0.33$) (Figure 3.2), but a stronger relationship at 85 kg N $ha^{-1}$ ($p = 0.003$). Overall, the fraction of applied $^{15}N$ labeled fertilizer taken up by the plants was low at 27% of applied N at 85 kg N $ha^{-1}$ (95%CI [0.23,0.32]), but increased to 34% in plots receiving 170 kg N $ha^{-1}$ (95%

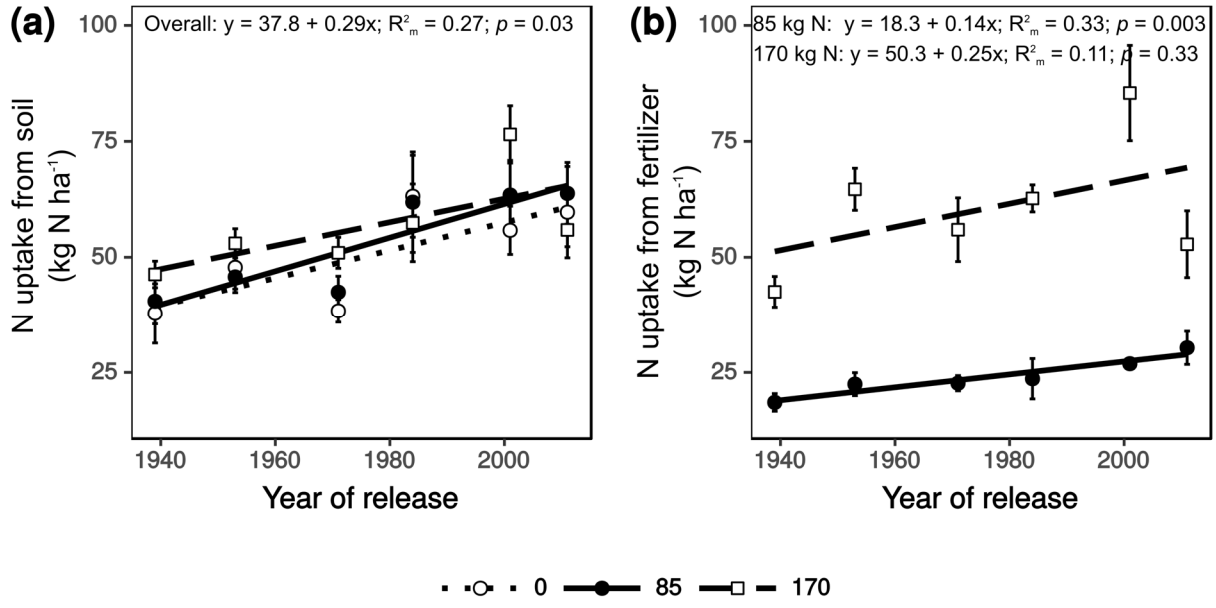


Figure 3. 2: Plant N uptake from soil nitrogen pools increases with year of hybrid release (a). Plant fertilizer N uptake increases with year of hybrid release at 85 kg $N^{-1}$, but improvement is variable at 170 kg N $ha^{-1}$ (b). Points represent hybrid means ± 1 s.e.m. at 0, 85 and 170 kg N $ha^{-1}$. Slope of regression from simplified model shown with marginal $R^2$.

CI[0.29, 0.41]) ($p = 0.03$). Estimates of fertilizer recovery using the $^{15}N$ and difference methods indicated similar trends, but the difference method underestimated $^{15}N$ recovery at the low end of fertilizer response and overestimated fertilizer uptake at the high end of N response (Figure C3).

### 3.3.2 Bacterial community composition

A clear rhizosphere effect was observed on BCC, which explained approximately 9% of the variation in the PERMANOVA of the core samples ($p < 0.05$, Table C5; Figure C4). Within the rhizosphere samples there was both high variation (residuals) and a subtle effect of maize hybrid identity, which explained a significant portion of rhizosphere beta-diversity over the course of the season and on each sampling date ($p < 0.05$; Table 3.3; Figure 3.3). Maize hybrid and sampling date described a similar portion of variation in beta-diversity of core samples (8% and 7%, respectively, Table 3.3). There was not a significant interaction between hybrid and sampling date in the PERMANOVA ($p = 0.76$; Table 3.3), indicating correspondence between hybrid effects on multiple sampling dates. This can be seen in the ordination, where the samples from release year 1971 regularly separate from the samples from year 1939 (Figure 3.3). The effect of fertilizer on rhizosphere bacterial communities was primarily evident in the late season during the sampling at R3, when fertilizer treatment accounted for approximately 5% of the variation in BCC ($p < 0.01$; Table 3.3).

The variation between hybrids in rhizosphere BCC was not consistent with a directional effect of breeding history, where BCC would become more distinct from early releases over the 80-year breeding history. Years between hybrid releases was not correlated with Bray-Curtis dissimilarity in hybrid rhizosphere bacterial community in a MLPE model ($F_{(1,92)} = 0.21$; $p = 0.65$. However, the polynomial of years between hybrid releases was a significant predictor ($F_{(2,92)} = 5.45$; $p < 0.01$) (Figure 3.4). This model reflects the separation of hybrids in the PCoA

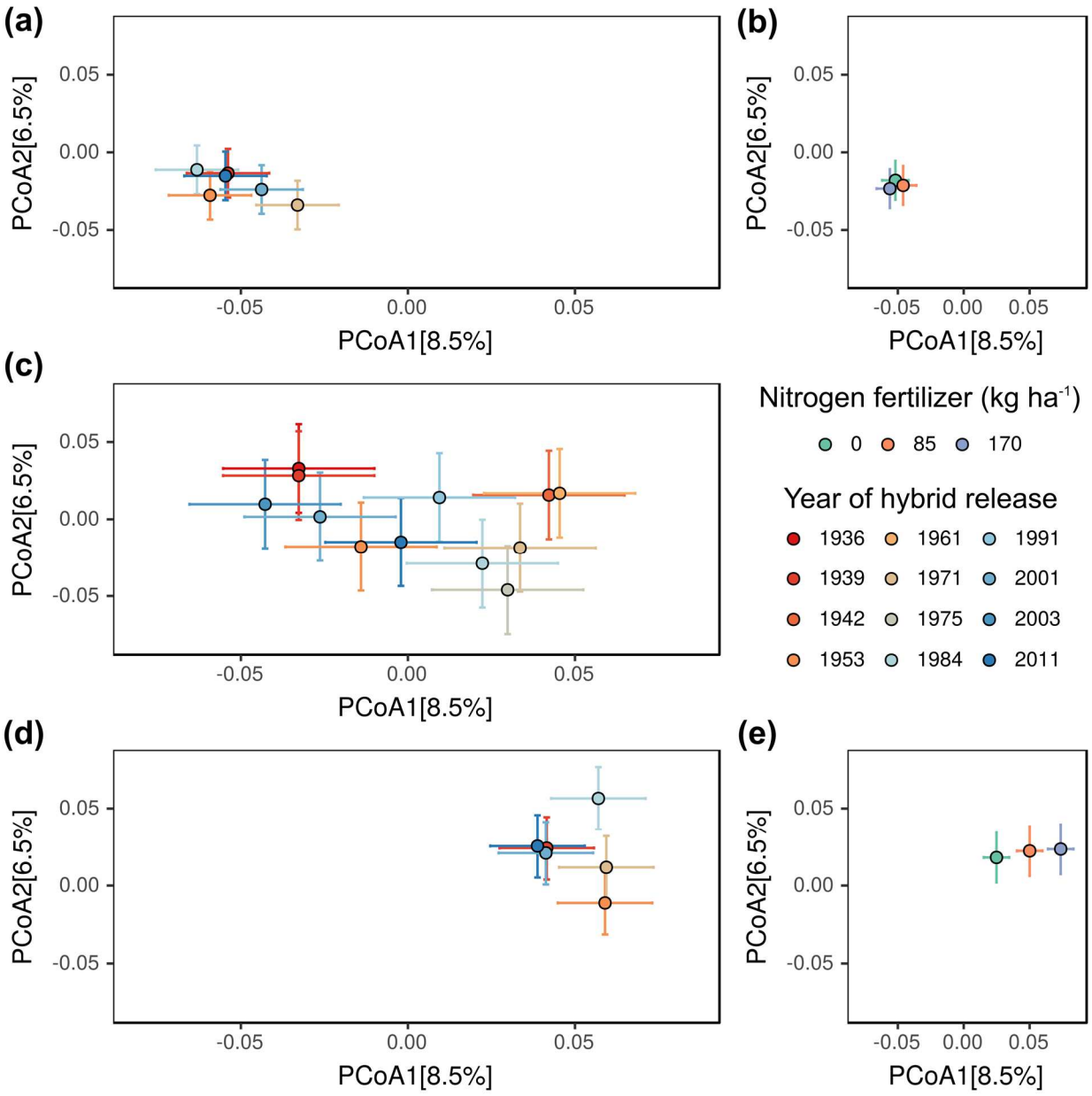


Figure 3. 3: Maize hybrid and nitrogen fertilizer treatment shape rhizosphere bacterial community composition. Panels display results from a single principal coordinate analysis (PCoA) calculated using 16S rRNA gene sequence Bray-Curtis dissimilarity of rhizosphere samples. Panels all share the same axes, but are subdivided by sampling date to indicate variation occurring at six-leaf (a,b), anthesis (c), and grain filling (d,e) stages. Point and line color is varied to indicate year of hybrid release (a,c,d) or level of fertilization (b, e) as indicated in the legend. Points represent least square means ± 1 s.e.m. of sample scores on PCoA.

Table 3.3: Permutational multiple analysis of variance testing replicate block, maize hybrid and fertilization effects on rhizosphere beta-diversity at each sampling date and among core plots sampled over time.

| | SS | DF | *F* | *R2* | p |
|---|---|---|---|---|---|
| V6 | | | | | |
| Rep | 0.25 | 3 | 2.19 | 0.08 | <0.01 |
| Fertilization | 0.07 | 2 | 0.93 | 0.02 | 0.74 |
| Hybrid | 0.22 | 5 | 1.14 | 0.07 | 0.03 |
| F x H | - | - | - | - | |
| Residuals | 2.34 | 61 | | 0.57 | |
| | | | | | |
| R1 | | | | | |
| Rep | 0.24 | 3 | 2.05 | 0.12 | <0.01 |
| Hybrid | 0.47 | 11 | 1.11 | 0.23 | 0.03 |
| Residuals | 1.27 | 33 | | 0.64 | |
| | | | | | |
| R3 | | | | | |
| Rep | 0.28 | 3 | 2.41 | 0.09 | <0.01 |
| Fertilization | 0.14 | 2 | 1.84 | 0.05 | <0.01 |
| Hybrid | 0.25 | 5 | 1.26 | 0.08 | <0.01 |
| F x H | - | - | - | - | |
| Residuals | 2.39 | 61 | | 0.78 | |
| | | | | | |
| Core rhizosphere plots | | | | | |
| Rep | 0.22 | 3 | 1.90 | 0.07 | <0.01 |
| Sampling date | 0.23 | 2 | 2.90 | 0.07 | <0.01 |
| Hybrid | 0.25 | 5 | 1.23 | 0.08 | <0.01 |
| R x S | 0.26 | 6 | 1.06 | 0.08 | 0.162 |
| H x S | 0.38 | 10 | 0.96 | 0.12 | 0.775 |
| Residuals | 1.8 | 45 | | 0.57 | |

*Core rhizosphere plots represent those plots sampled on all three sampling dates, which include six hybrids receiving $^{15}N$ tracer at 85 kg N $ha^{-1}$

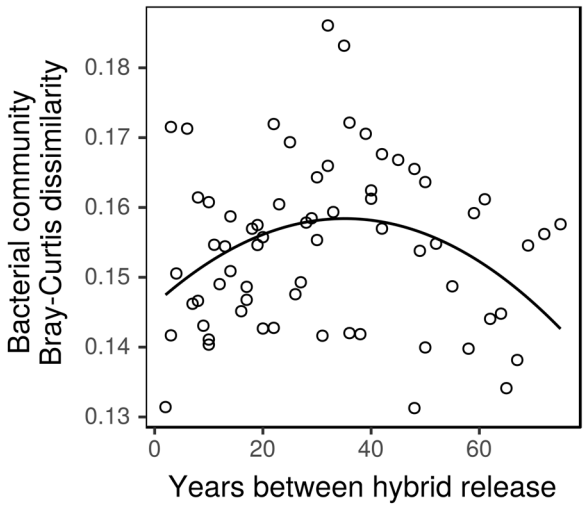


Figure 3. 4: Years between hybrid release influences bacterial community beta-diversity in the rhizosphere in a curvilinear relationship at anthesis. Bray-Curtis dissimilarity calculated using square root transformed mean relative abundance within replicates of a hybrid to avoid pseudo-replication. Line is predicted fit from maximum likelihood population effects model.

at R1, where hybrid centroid shifts with release date along the primary axis from the 1930s through the 70s and 80s, after which the centroids shift back toward the center of the ordination (Figure 3.3c). Together, the sample scores on the PCoA and the MLPE model indicate that BCC in the rhizosphere in early and late release hybrids was more similar compared to middle release hybrids.

### 3.3.3 Abundance of OTUs

We identified 284 OTUs as positive rhizosphere responders (LFC > 1, BH adjusted $p <$ 0.05), with abundant representatives of the bacterial families *Comamonadaceae*, *Oxalobacteraceae*, *Streptomycetaceae*, *Pseudonocardiaceae, Verrucomicrobiaceae*, *Bacillaceae*, and *Sphingomonadaceae* (Table C6 and C7). The resulting rhizosphere community was comprised of a combination of these rhizosphere responders and OTUs previously abundant in bulk soil. Both rhizosphere responders and background soil OTUs were sensitive to hybrid identity. This includes 48 OTUs that were differentially abundant (LFC $\neq$ 0, BH adjusted $p <$ 0.05) between any hybrid and the earliest release reference hybrid (1939 at V6 and R3 and 1936 at R1) (Table C8). The majority of these OTUs were identified at anthesis and were primarily identified in the rhizosphere of hybrids released between 1953 and 1975 (Figure 3.5). The OTUs that differentiated hybrids were at low abundance (median = 0.036% relative abundance in rhizosphere samples), but a few were more abundant members (0.1- 0.7%) of the community, such as taxa from the families *Nocardioidaceae, Streptomycetaceae, Cytophagaceae*, *Oxalobacteraceae, Xiphinematobacteraceae* and *Xanthamonadaceae.*

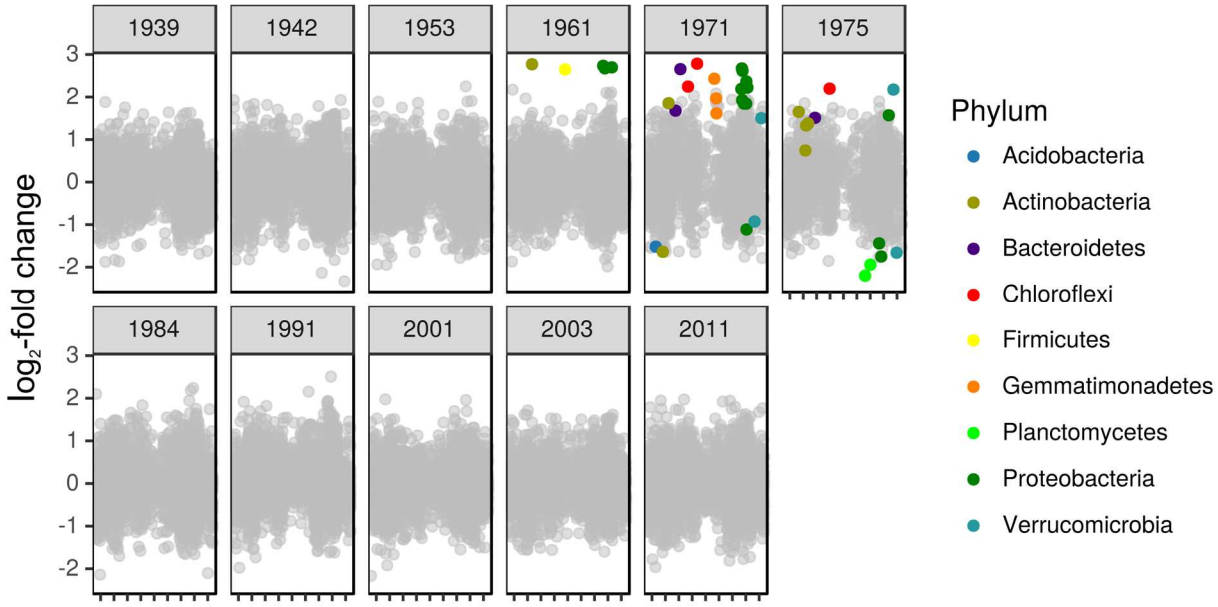


Figure 3. 5: Taxa were differentially abundant between rhizosphere samples of hybrids released in 1960s and 1970s when compared with early release hybrid from 1936 at anthesis. Points display $log_2$-fold change (LFC) between rhizosphere samples of each hybrid and the 1936 release. OTUs significantly different in abundance colored by phylum (LFC $\neq 0$, adjusted $p < 0.05$, n = 4).

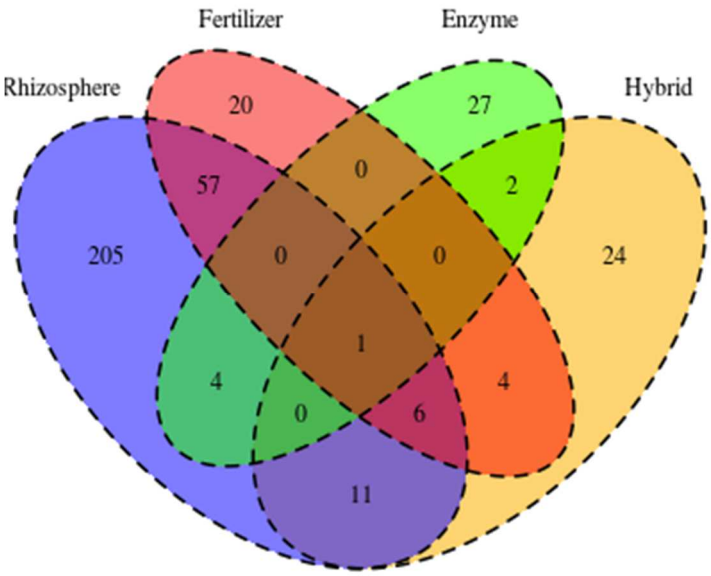


Figure 3. 6: Overlap of OTUs enriched in the rhizosphere compared to bare soil ($log_2$-fold change (LFC) > 1, adjusted $p < 0.05$) and OTUs differentially abundant among hybrids, between fertilization treatments or correlated with enzyme activity in the rhizosphere (LFC ≠ 0, adjusted $p < 0.05$).

At R3 the shifts in composition in response to fertilization mirrored the rhizosphere effect in general. When compared to unfertilized plots, 23 OTUs were enriched in the rhizosphere of plants receiving 85 kg N $ha^{-1}$ and 83 OTUs enriched in the 170 kg N $ha^{-1}$ (LFC $\neq$ 0, BH adjusted $p < 0.05$; Table C9). Of these OTUs, 64 were also identified as positive rhizosphere responders (Figure 3.6). In contrast, only one OTU was depleted in bare soil plots receiving fertilizer and no OTUs were significantly enriched. Of the OTUs enriched in the fertilized rhizosphere, 11 were also differentially abundant among hybrids, nine of which were more abundant in a middle release hybrid compared to the early release reference (Table C9).

### 3.3.4 Extracellular enzyme activity

Similar to measures of rhizosphere BCC, we observed a significant rhizosphere effect on potential activity of extracellular enzymes in the rhizosphere as well as variation among maize hybrids. There was a consistent increase in the activity of the C-accessing enzymes BG and BX as well as the chitinase (NAG) in the rhizosphere at all three time points ($p < 0.05$; Figure C5). The protease, LAP, had higher potential activity in the rhizosphere at V6 ($p < 0.05$), then increased strongly in activity in both bare soil and rhizosphere samples with no discernible rhizosphere effect at later sampling dates ($p > 0.05$; Figure C5).

Maize hybrids varied in their rhizosphere enzyme profile in the principal component ordination of core plots over the course of the season ($p = 0.02$; Table 3.4; Figure C6) and when comparing individual genotypes at R1 ($p = 0.03$; Table 3.4). The hybrid effect at R1 was primarily a result of differentiation of the 1971 release along the primary axis of the PCA, indicating greater overall enzyme activity in the rhizosphere of this hybrid (Figure 3.7). Maize hybrid and fertilization did not significantly influence the enzyme profile at either V6 or R3 ($p > 0.05$; Table 3.4). There was no relationship between extracellular enzyme activity and year of

Table 3.4: Analysis of variance testing year of hybrid release on extracellular enzyme profile principal component scores.

| | Axis 1 | Axis 2 |
|---|---|---|
| Core rhizosphere samples | | |
| Hybrid | $F_{(5, 15)} = 4.11$* | $F_{(5,15)} = 0.60$ |
| Time point | $F_{(2,35)} = 32.28$*** | $F_{(2,35)} = 90.79$*** |
| G x T | $F_{(10,35)} = 0.72$ | $F_{(10,35)} = 0.33$ |
| V6 | | |
| Hybrid | $F_{(5,13)} = 1.08$ | $F_{(5,13)} = 0.92$ |
| Fertilizer | $F_{(2,4)} = 0.38$ | $F_{(2,4)} = 0.26$ |
| H x F | $F_{(10,29)} = 0.77$ | $F_{(10,29)} = 1.02$ |
| R1 | | |
| Hybrid | $F_{(11,33)} = 2.28$* | $F_{(11,33)} = 0.29$ |
| R3 | | |
| Hybrid | $F_{(5,13)} = 0.59$ | $F_{(5,13)} = 1.22$ |
| Fertilizer | $F_{(2,4)} = 3.07$ | $F_{(2,5)} = 1.77$ |
| H x F | $F_{(10,35)} = 0.38$ | $F_{(10,32)} = 0.93$ |

* $p < 0.05$, *** $p < 0.001$

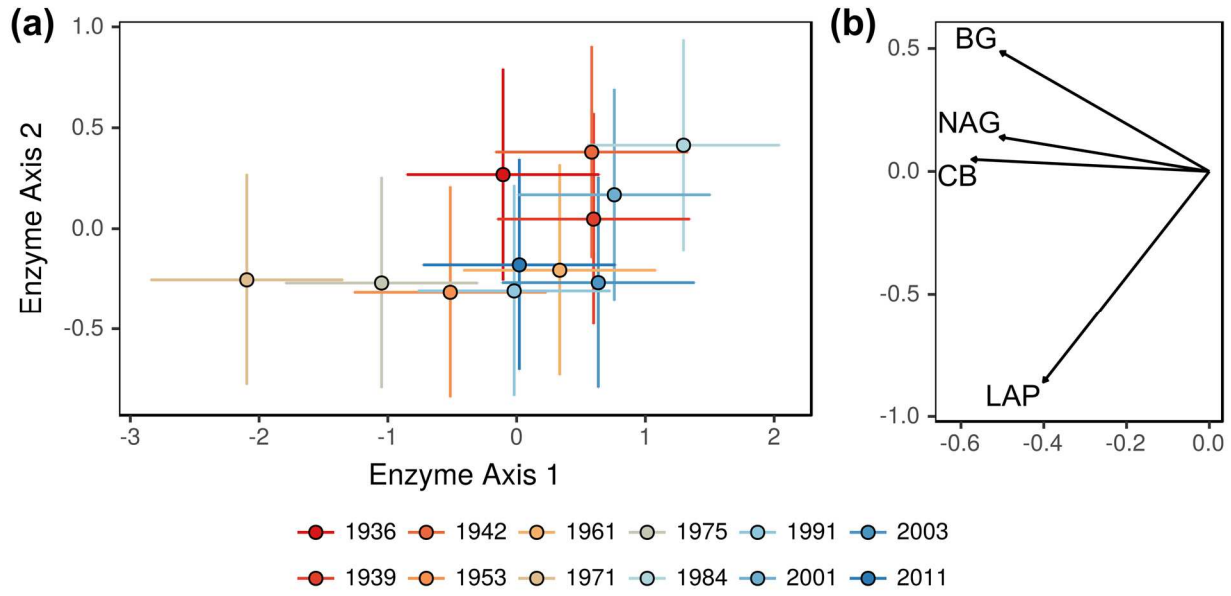


Figure 3. 7: Rhizosphere potential extracellular enzyme profile varies between maize hybrids at anthesis. Points are lsmeans of sample scores ± 1 s.e.m. (a), and substrate activity loadings on principle component axes (b). Enzyme abbreviations: beta-1,4-glucosidase (BG), beta-xylosidase (BX), cellobiohydrolase (CB), leucine-aminopeptidase (LAP), N-acetylglucosaminidase (NAG).

release or its polynomial ($p > 0.05$) on any sampling date.

The abundances of 32 OTUs were correlated with the primary or secondary axes of the enzyme ordination on one of the sampling dates (Table C10). The majority of these OTUs were background soil OTUs, with only 5 being enriched in the rhizosphere (Figure 3.6). The OTUs correlated with enzyme activity were taxonomically diverse, including taxa from the phyla *Acidobacteria*, *Actinobacteria*, *Proteobacteria*, *Verrucomicrobia* and *Chloroflexi*. Of these OTUs, three were also differentially abundant among hybrids—*Iamiacea sp.*, *Altererythrobacter sp.* and *Asticcacaulis sp.* (Figure 3.6). An *Asticcacaulis sp.* was the single OTU that was influenced by fertilization, hybrid identity and was positively correlated with potential extracellular enzyme activity.

## 3.4 DISCUSSION

Modern plant breeding has rarely targeted below-ground traits, but selection for yield and performance in modern production systems has influenced root and rhizosphere traits in multiple systems (Jackson, 1995; Bertholdsson, 2004; Kiers *et al.*, 2007). To the extent that external inputs replace internal plant and ecosystem functions in the breeding and selection process, there may be relaxed selection for plant traits or plant-microbial collaborations to supply required nutrients (Drinkwater & Snapp, 2007; Wissuwa *et al.*, 2009). Historically, maize breeding efforts have interacted with dramatically increasing N inputs to increase on-farm yields (Duvick, 2005; Sinclair & Rufty, 2012). If luxury N conditions during maize breeding have reduced dependence on plant root and rhizosphere traits and/or plant-microbe collaborations that support acquisition of N from soil organic N reserves, then maize lines would be less capable of accessing soil N and plant reliance on fertilizer N sources would be increased (Drinkwater & Snapp, 2007; Schmidt *et al.*, 2016). Conversely, if fertilizer N is unable to replace N acquired

from these soil reserves, maize acquisition of soil N will continue to be essential for yield improvements. To investigate these hypotheses, we evaluated the changes in NUE and rhizosphere bacterial community assembly of a set of twelve widely adapted and best-selling maize hybrids released by Pioneer Hi-bred International between 1936 and 2011 to determine if maize breeding for modern production systems has influenced plant N acquisition and whether rhizosphere BCC and activity has changed in a parallel manner. We demonstrate that improvements in maize nitrogen acquisition among these hybrids reflect increased uptake from both N sources: N fertilizer and soil N reserves. Furthermore, we demonstrate that hybrids vary in their influence on rhizosphere BCC, but were unable to detect parallel changes in the rhizosphere bacterial community that corresponded with the observed increases in N acquisition.

### 3.4.1 Breeding trends in NUE

The ERA collection of Pioneer hybrids studied here and previously by Duvick (2005), Campos *et al*. (2006), and others have provided an important resource to understand changes in maize ecophysiology contributing to yield improvement and adaptation to modern production systems. Consistent with previous results, we observed an increase in hybrid yield at all fertilization levels (Duvick, 2005; DeBruin *et al.*, 2017). This yield improvement was associated with an increase in N uptake at all fertilization levels, including plots receiving no N fertilizer addition. While improved fertilizer recovery in modern hybrids is well established, previous reports have not observed a corresponding increase in N uptake in unfertilized plots (Haegele *et al.*, 2013; Woli *et al.*, 2016). We propose two explanations for this conflict. First, the hybrid series from 1936 to 2011 captures a longer breeding history and therefore greater genetic variability than studies from the 1960s to present day (Haegele *et al.*, 2013; Woli *et al.*, 2016). To our knowledge this is the first report to quantify total plant nitrogen uptake as a component of

plant NUE in a maize hybrid series stretching back to the 1930s and therefore the improvement in plant N uptake under both low and high N conditions represents a novel finding of this dataset. It is also in agreement with a meta-analysis of studies from the 1940s through 2011 that found modern plantings and cultivars have greater N uptake on a per area basis for a given N rate (Ciampitti & Vyn, 2012). Second, water stress (both excess and limitation) may have differentially affected plant growth and biomass accumulation during the growing season with an associated impact on total N uptake (Plénet & Lemaire, 1999). In a review of earlier trials, Duvick (2005) described greater yield of modern hybrids during an exceptionally wet season in central Iowa, indicating that breeding efforts may have indirectly selected for tolerance of saturated soils. Nevertheless, these findings demonstrate that improvement in plant N uptake and yield under fertilized conditions has been accompanied by increased plant ability to acquire soil N under unfertilized conditions.

### 3.4.2 Contribution of soil pools to nitrogen use efficiency under fertilized conditions

The parallel increase in N uptake in both fertilized and unfertilized plots may indicate the mechanisms of plant N acquisition in low N conditions remain relevant in systems receiving fertilizer N. Partitioning of $^{15}N$ fertilizer and soil nitrogen sources indicated substantial uptake of soil N in fertilized plots that was equal to or greater than N uptake from unfertilized plots. This is consistent with a meta-analysis of $^{15}N$ tracer studies showing that fertilizer N accounted for less than 40% of plant N on average in maize (Gardner & Drinkwater, 2009).

In our study, uptake of soil derived N in fertilized plots increased with year of release, whereas the relationship between year of release and fertilizer N recover was more variable. This variability may have resulted from the high rainfall and substantial fertilizer N loss. However, this highlights that the supply of N fertilizer does not necessarily translate to luxury

growth conditions. In the face of environmental variation in N loss there may remain substantial selection pressure for traits that maximize soil N capture.

### 3.4.3 Breeding influence on rhizosphere bacterial community composition

Our finding that maize hybrids did vary in bacterial community composition and extracellular enzyme profiles highlights that a commercial breeding program has deployed hybrids with genetic variation in rhizosphere traits. Several studies have reported genotype effects of maize and other major grain crops on rhizosphere bacterial community composition (Aira *et al.*, 2010; Bouffaud *et al.*, 2012; Peiffer *et al.*, 2013) and our finding that 7-20% of the variation is attributed to plant genotype is in line with the magnitude of these previous reports. While the same genotypes did not separate on every sampling date, the consistency in trajectory over the course of the season indicates that the differences among hybrids observed here are not simply transient phenomena.

Our hypothesis was that plant breeding for a changing agroecological context would lead to greater separation of both plant function and rhizosphere communities over time. However, while N acquisition increased in a linear trend with year of release, hybrid variation in BCC did not follow a parallel pattern. Instead, genotypes separated in a curvilinear pattern over the breeding history, which may be explained by several factors. First, N surplus in corn-belt grain systems peaked during the 1960s to 1980s (Vitousek *et al.*, 2009). In the ordination of BCC, however, there is divergence between the communities in germplasm released by 1941, before the widespread adoption of N fertilizers, so this relationship is not entirely consistent with our *a priori* hypotheses. It is possible that plant breeders were early adopters of plant nutrient management with synthetic fertilizer. Additionally, the contribution of maize genetic groups to ERA germplasm has varied over the decades (Duvick *et al.*, 2004). Our results may therefore

reflect the divergent rhizosphere effects of distinct genetic groups on rhizosphere BCC (Bouffaud *et al.*, 2012). Finally, stand density has more than doubled since the 1940s and avoidance of root and stalk lodging at these densities was a major goal of the Pioneer breeding program (Duvick *et al.*, 2004). Indirect selection over this time has led to changes in root system architecture, which are thought to improve standability and support increased yield potential and N acquisition (Hammer *et al.*, 2009; York *et al.*, 2015) and may also cascade to rhizosphere effects.

The OTUs that were differentially abundant among hybrids were at low abundance, but several lines of evidence suggest they may be functionally important. Dawson *et al.* (2017) similarly found species-specific rhizosphere responders of 19 perennial grassland species were relatively rare, though their enrichment in the rhizosphere suggests they are metabolically active and potentially important contributors to the rhizosphere community. Furthermore, several differentially abundant OTUs were from lineages previously associated with C and N cycling in soil. OTUs enriched in middle release hybrids at anthesis included potential diazatrophs such as the *Rhodobacteraceae* (*Alphaproteobacteria*) and *Dechloromonas* (*Betaproteobacteria*) (Salinero *et al.*, 2009; Li *et al.*, 2014). Differentially abundant OTUs also included taxa previously associated with cellulose degradation in stable isotope probing studies (*Pseudoxanthomonas* (*Gammaproteobacteria*), *Asticcacaulis* (*Alphaproteobactera*), and *Ohtaekwangia* (*Bacteroidetes*)) (Pepe-Ranney *et al.*, 2015), as well as genera with known plant growth promoting rhizobacteria (*Klebsiella* (*Gammaproteobacteria*) and *Hydrogenophaga* (*Betaproteobacteria*)) (Chanway & Holl, 1993; Liu *et al.*, 2016). Also identified were bacterial predators that could indicate changing trophic interactions in the rhizosphere such as *Myxococcales* (*Deltaproteobacteria*) and *Herpetosiphonaceae* (*Chloroflexi*). Turnover of

microbial biomass is a key mechanism by which microbial N becomes available for plant uptake (Clarholm, 1985; Kuzyakov & Xu, 2013) and the potential for genetic variability among maize hybrids in affecting bacterial predator populations and turnover of microbial biomass in the rhizosphere deserves further attention.

The potential of changes in rhizosphere BCC to shift C and N cycling are highlighted by three OTUs that were enriched in the rhizosphere of the 1971 hybrid compared to the 1936 release and were also directly correlated with enzyme activity in the rhizosphere--*Asticcacaulis sp.* (*Caulobacteraceae*)*, Aquihabitans sp.* (*Iamaceae*) and *Erythrobacter sp.* (*Erythrobacteraceae*). The 1971 release also had the highest potential enzyme activity in its rhizosphere when compared to other hybrids. While this hybrid had low N uptake overall, estimates of post-anthesis N uptake were comparatively high (Figure S2), suggesting high root activity in the later season.

### 3.4.4 Assessing human selection and rhizosphere assembly

The likelihood of rhizosphere assembly being influenced by plant breeding hinges on what forces ultimately structure these communities. Kiers and Denison (2008) argue that evolutionarily stable cooperation between plants and microbes requires mechanisms to enforce mutualisms, which becomes less likely in the rhizosphere compared to endophytic compartments. This decreases the likelihood of plant alleles that maintain specific mutualisms that can be acted on by human selection (Kiers & Denison, 2008). If community assembly is driven by physiochemical conditions in the rhizosphere determined by soil type, climate, and root activity (Hinsinger *et al.*, 2005; Nuccio *et al.*, 2016), and general patterns of root activity have been maintained by continuous selection for yield in variable environmental conditions, it may not be surprising that this 80-year history has not led to a divergent community occupying

the rhizosphere. In this regard, positive feedbacks between fertilizer N addition and root proliferation and exudation (Paterson *et al.*, 2006) may provide for an often observed increase in plant uptake of soil N under fertilized conditions (Liu *et al.*, 2017), which would provide a positive selection pressure for such rhizosphere traits. Our finding that fertilization led to an increase in the abundance of many rhizosphere OTUs is consistent with such an overall positive feedback on rhizosphere traits.

Alternately, our methodology may not reveal subtle changes in rhizosphere BCC. Strain level functional variation can be substantial between samples with similar taxonomic composition and can interact with host-genotype with functionally important consequences. For example, wheat cultivars vary in their selection of strains of 2,4-DAPG producing fluorescent pseudomonads in the rhizosphere as well as the establishment of disease suppressive soils (Mazzola & Gu, 2002; Mazzola *et al.*, 2004). Therefore we do not rule out the possibility that maize ability to recruit beneficial strains into the rhizosphere has changed over time but cannot be detected through community profiling using 16S amplicon sequencing and a common 97% nucleotide identity cutoff.

Additionally, management history of a field can alter the composition of soil microbial communities, including the taxonomic and functional composition of plant symbionts, and therefore the propagule pool from which the rhizosphere community is selected (Johnson, 1993; Berthrong *et al.*, 2013). As a result, it is possible that our site, a field with a history of synthetic N inputs, lacked mutualistic strains of rhizosphere organisms that provide maximal plant benefit. However, evidence from a parallel experiment suggests that our findings are robust. Hybrids released in 1936, 1953 and 1984 were included in a separate experiment in a well-studied field with a 20-year history of complex rotations, winter cover crops and leguminous N inputs. The

field has increased reserves of endogenous soil N, mainly in the particulate and occluded organic matter pools, with a distinct microbial community and patterns of C and N cycling compared to a conventionally managed neighboring field (Berthrong *et al.*, 2013). As observed in this study, the most recent release did not differ from early release hybrids in rhizosphere BCC and patterns of plant growth and N economy mirrored those observed in this field study (Figure C7).

## 3.5 CONCLUSION

In summary, our findings indicate that maize breeding has improved plant ability to take up soil N under both fertilized and unfertilized conditions. We propose that the continued importance of soil N pools in supplying plant nitrogen in fertilized systems (Drinkwater *et al.*, 2007; Gardner & Drinkwater, 2009) are an indication that fertilizer has not supplanted reliance on plant traits and plant-microbial collaborations that supply soil N. As such, continuous selection for yield may exert stabilizing selection on rhizosphere traits governing microbial community assembly. This knowledge of past breeding efforts' influence on rhizosphere bacterial community composition, or lack thereof, can inform future efforts to harness the rhizosphere microbiome (Schmidt *et al.*, 2016).

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## SUMMARY

The goal of this research was to understand the sources and extent of plant variation in rhizosphere community assembly and function among maize and summer annuals characteristic of agricultural fields. In two field experiments we repeatedly observed that plant species select for distinct rhizosphere bacterial communities. In two of three experiments we also found that plant genotypes within a species influenced rhizosphere BCC, but note that these effects were less in magnitude and more inconsistently observed than variation among species. This adds to a body of literature that suggests plant species differentially shape the rhizosphere environment and bacterial communities and that similar differentiation can occur among genotypes of the same species. Here we are able to further demonstrate that this differentiation of rhizosphere BCC derives from plant evolutionary history as well as plant functional variation in plant growth and nitrogen economy. As noted in Chapter 1, these relationships may serve as a mechanism by which the incorporation of plant diversity into crop rotations can be used to manage rhizosphere and ultimately soil community composition.

In line with our finding that plant functional variation is linked with plant rhizosphere effects, we found that enzyme activity in the rhizosphere is closely tied with plant N demand. This is observed across time in Chapter 1, where longer lifespan, high NUE plants have greater activity of N-cycling enzymes in the rhizosphere and in Chapter 2, where protease activity peaked during periods of high plant N demand and limited soil N supply. It is further evidenced by the positive correlation with rate of plant N uptake observed in Chapter 2. The correlations between plant N demand and rhizosphere extracellular enzyme activity serve to highlight the temporal coupling of rhizosphere processes and plant N uptake. If the observed activity of these extracellular enzymes is indeed providing for increased N flows to the plant, identifying and

managing the soil nitrogen pools on which they are acting will be an important component of crafting sustainable agroecosystems.

Given the relations between rhizosphere BCC and plant phylogenetic and functional variation, the lower magnitude and inconsistent divergence of rhizosphere BCC among maize genotypes is expected as intraspecific variation in these plant diversity dimensions is reduced compared to interspecific diversity. Nevertheless, differences in rhizosphere BCC were observed among maize inbred lines (Chapter 1) and among maize hybrids (Chapter 3). Thus we conclude that there is genetic variation in traits influencing rhizosphere assembly within maize that future breeding efforts may utilize. Given this potential, the finding that maize breeding has not resulted in a directional shift in rhizosphere BCC may point to the continued relevance of rhizosphere processes in modern production systems.

In the course of these experiments we observed several shifts in the abundance of particular taxa or groups that are worth noting for future investigations. First, plant life history strategy associated with long-lifespan, high plant NUE (g C g $N^{-1}$), high biomass accumulation and high N uptake was repeatedly associated with increased dominance of several *Actinobacteria*, including *Streptomyces* in the rhizosphere. Additionally, in Chapter 2, *Ktedonobacter*, which also shares a similar filamentous morphology with the *Actinobacteria*, was associated with high NUE and high biomass accumulating plants. It is notable that selection for this community was observed in two experiments separated in both time and space. As a repeatedly observed community state, the conditions in the rhizosphere that foster this community and its implications for root and rhizosphere function should be understood.
On the other hand, a group of taxa that were also twice associated with differences among genotypes were bacterial predators including members of the *Myxoccocales*. Bacterial predators

distinguished the rhizosphere of ERA hybrids in Chapter 3 and were associated with low NUE and high relative growth rate typified by *Sorghum x drummondii* and *Echinochloa esculenta* in Chapter 2. This raises several questions for future research, including whether the differential abundance of these bacterial predators indicates changing trophic interactions and its implications of rhizosphere N flows. The traits and mechanisms by which plants may attract or maintain populations of predators in the rhizosphere could be a promising line of research.

These studies, which manipulated above ground plant composition in the monocultures, rely on correlations between plant growth metrics and rhizosphere measures to infer functional relationships. The observation of these patterns in relation to *a priori* hypotheses on the link between plant and rhizosphere function is an important step. However, we can only speculate on the mechanisms that underlie these relationships. Future work that quantifies rhizosphere carbon flow, exudate chemistry and microbial incorporation of plant derived C could elucidate the mechanisms that foster the contrasting communities observed in this research.

Finally, our finding that plant rhizosphere communities diverge over time is intriguing and points to plant-mediated factors influencing rhizosphere succession. Succession dynamics of rhizosphere communities are entirely understudied. In part this is due to the challenges of sampling and assessing the rhizosphere community. In a field setting, rhizosphere communities are observed over periods of new root growth, aging of existing roots, plant growth and development, and changing abiotic factors. The whole plant or plot based experimental unit is poorly matched for this heterogeneity. A full understanding of plant identity effects on rhizosphere assembly and succession will require disentangling these factors and placing the community in the context of plant root growth dynamics, root age, plant age, environmental conditions and the microbe-microbe interactions within the rhizosphere.

# APPENDIX A

## SUPPLEMENTARY MATERIALS FROM CHAPTER 1

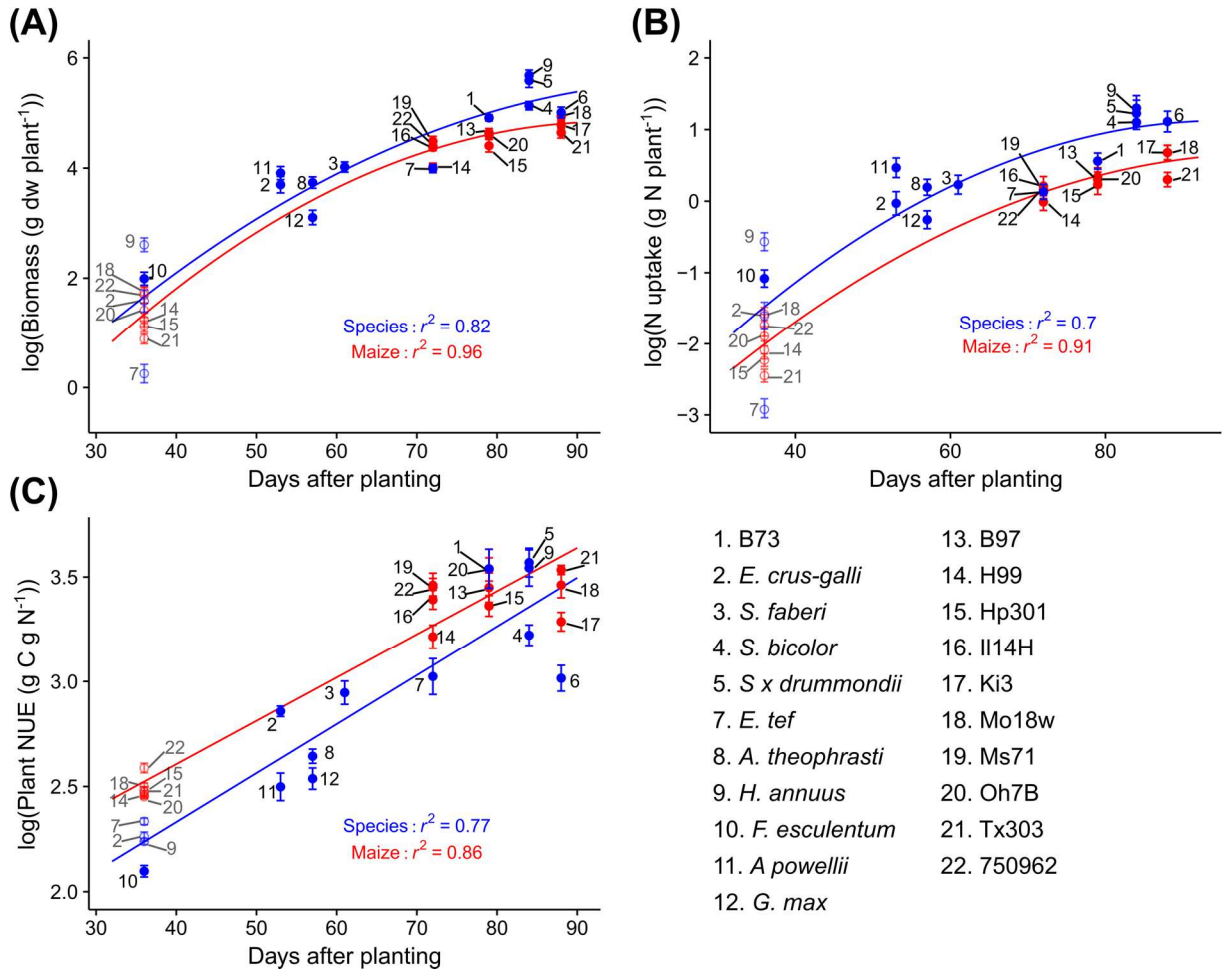


Figure A1: Days after planting vs log transformed plant biomass (A), nitrogen uptake (B) and plant nitrogen use efficiency (NUE) (C). Points are genotype mean (n = 8, ±1 s.e.m.). Line is fit against individual observations for annual species (blue) and maize inbred lines (red) separately using samples from both anthesis (closed circles) and, for a subsample of plants, vegetative stage (open circles).

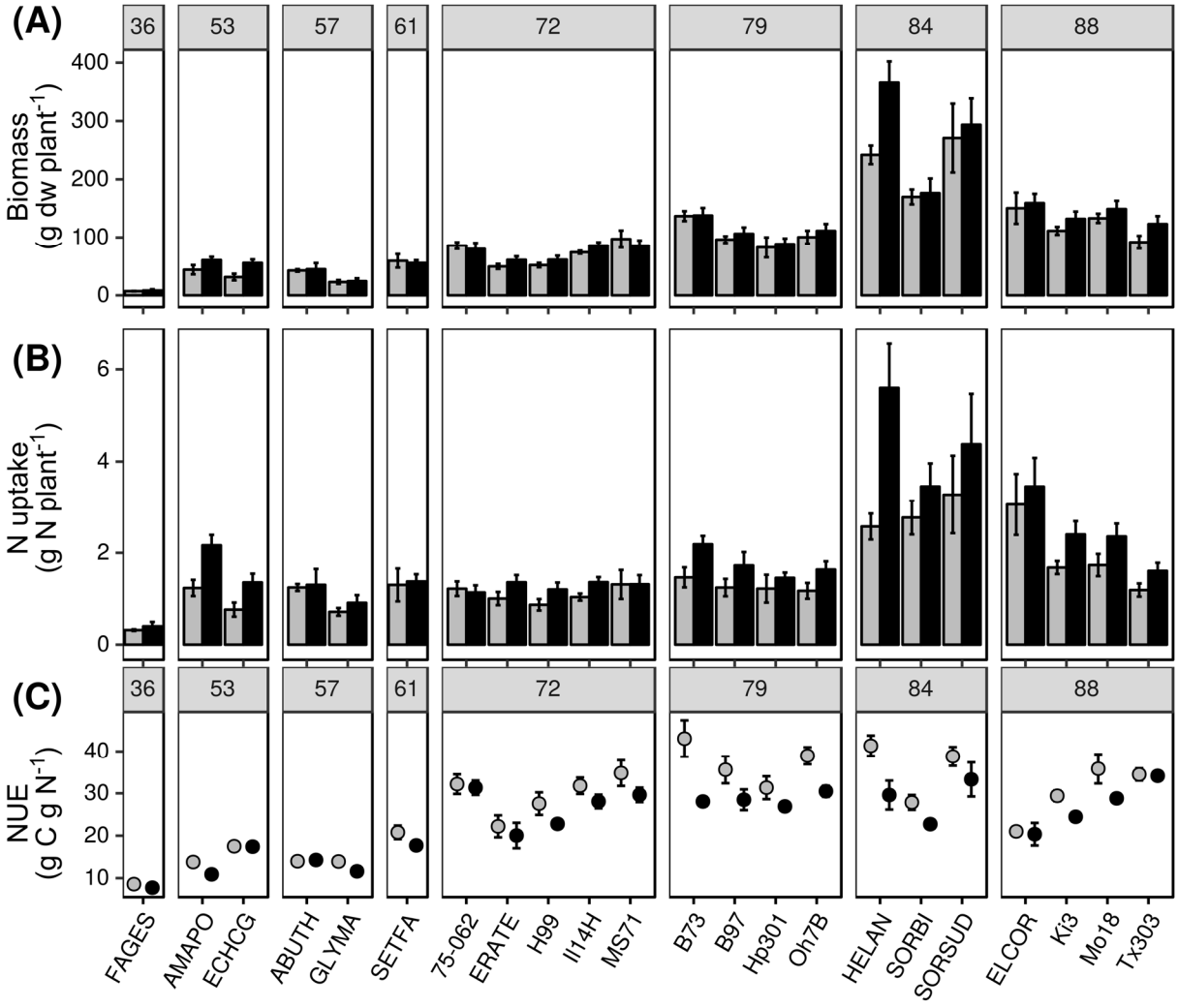


Figure A2: Cultivar and species variation in biomass accumulation, nitrogen (N) uptake, and nitrogen use efficiency (NUE) at anthesis under unfertilized (grey) and fertilized (black) conditions. Panels faceted by sampling date (days after planting). Bars are plant means ± 1 s.e.m. (n = 4). Species codes represent (*E. crus-galli* (ECHCG), *E. tef* (ERATE), *S. faberi* (SETFA), *S. bicolor* (SORBI), *S. x drummondii* (SORSUD), *E. coracana* (ELCOR), *A. theophrasti* (ABUTH), *H. annuus* (HELAN), *F. esculentum* (FAGES), *A. powellii* (AMAPO), and *G. max* (GLYMA); remainder are maize inbred lines.

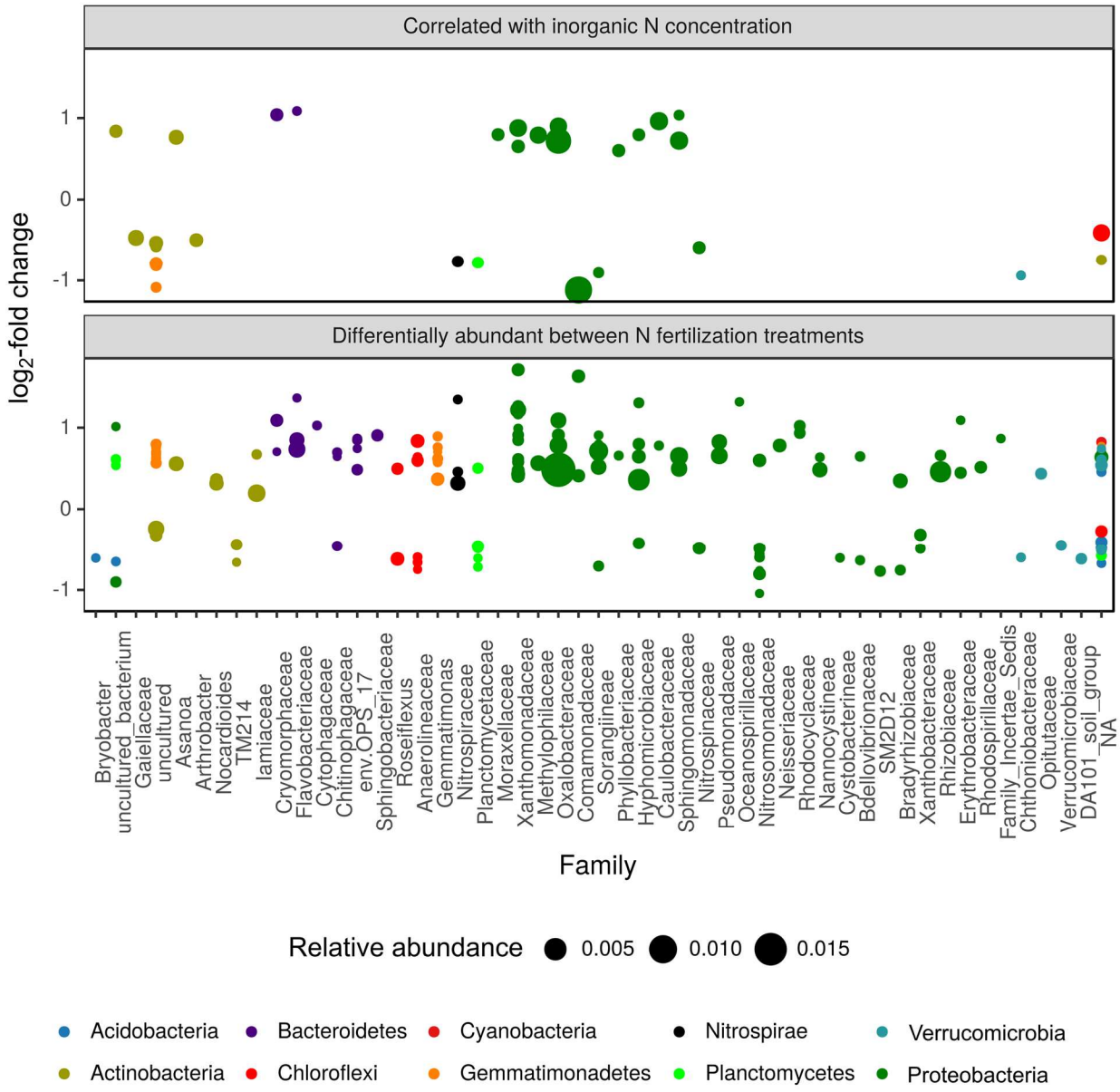


Figure A3: OTUs correlated with inorganic N concentration and differentially abundant between nitrogen fertilization treatments (0, 95 kg N $ha^{-1}$) in rhizosphere samples. $Log_2$-fold change in abundance with log transformed inorganic N concentration (top panel; n = 84), and between fertilized and unfertilized plots (bottom panel; n = 174). Only OTUs with significant differential abundance shown (DESeq2: $log_2$-fold change $\neq 0$, BH adjusted $p < 0.05$).

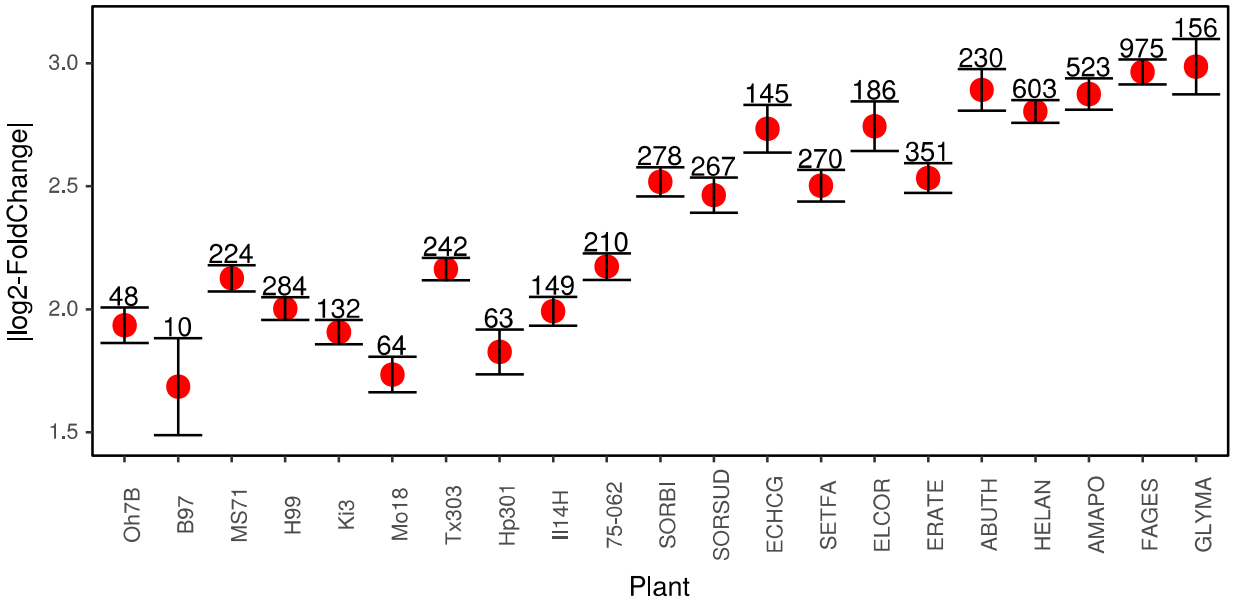


Figure A4: Number of OTUs differentially abundant between *Zea mays subsp mays* cv. B73 and each genotype and average magnitude of $\log_2$-fold change. Red dots indicate mean $\log_2$-fold change ± 1 s.e.m. of differentially abundant OTUs . Number of differentially abundant OTUs indicated above bars (n = 8, $\log_2$-fold change ≠ 0; BH adjusted $p < 0.05$).

Table A1: Plant genotypes grown in common garden experiment, sample types collected and GeneBank accession numbers for chloroplast sequences of species or congeneric representative used to construct plant phylogeny.

| Maize Line | Species | Common Name | Days to flowering | Family | Sub-family | Rhizosphere | | | matK /rbcL GeneBank accession: | Germplasm source |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Enzymes | Inorganic N | Bacterial community | | |
| 75-062 | *Zea mays ssp. mays* | | 72 | Poaceae | Panicoideae/ Andropogoneae | ■ | ■ | ■ | | Courtesy of Margaret Smith |
| B73 | " | | 79 | " | " | ■ | ■ | ■ | X86563.2/ X86563.2 | Courtesy of Ed Buckler |
| B97 | " | | 79 | " | " | ■ | ■ | ■ | | USDA GRIN : PI 564682 |
| H99 | " | | 72 | " | " | ■ | ■ | ■ | | USDA GRIN : PI 587129 |
| Hp301 | " | | 79 | " | " | ■ | ■ | ■ | | USDA GRIN : PI 587131 |
| Il14H | " | | 72 | " | " | ■ | ■ | ■ | | USDA GRIN : Ames 27118 |
| Ki3 | " | | 88 | " | " | ■ | | ■ | | USDA GRIN : Ames 27123 |
| Mo18W | " | | 88 | " | " | ■ | | ■ | | USDA GRIN : PI 550441 |
| Ms71 | " | | 72 | " | " | ■ | ■ | ■ | | USDA GRIN : PI 587137 |
| Oh7B | " | | 79 | " | " | ■ | ■ | ■ | | USDA GRIN : Ames 19323 |
| Tx303 | " | | 88 | " | " | ■ | | ■ | | USDA GRIN : Ames 19327 |

Table A1 cont.: Plant genotypes grown in common garden experiment, sample types collected and GeneBank accession numbers for chloroplast sequences of species or congeneric representative used to construct plant phylogeny.

| Species Code | Species | Common Name | Days to Flowering | Family | Sub-Family | Enzymes | Inorganic N | Bacterial community | Chloroplast matK /rbcL GeneBank accession: | Germplasm source |
|---|---|---|---|---|---|---|---|---|---|---|
| ERATE | *Eragrostis tef* | Teff (Tiffany) | 72 | Poaceae | Chloridoideae/ Eragrostideae | ■ | ■ | ■ | HE586095.1 / HE577859.1 | Hancock Seed Co. |
| ELCOR | *Eleusine coracana* | Finger millet | 88 | Poaceae | Chloridoideae/ Cynodonteae | ■ | | ■ | HQ180864.1/ HQ182427.1 | USDA Grin: PI 427233 |
| SORBI | *Sorghum bicolor ssp bicolor* | Grain sorghum | 84 | Poaceae | Panicoideae/ Andropogoneae | ■ | | ■ | EF115542.1/ AM849341.1 | King's Agri-Seed |
| SORSUD | *Sorghum x drummondii* | Sudangrass | 84 | Poaceae | Panicoideae/ Andropogoneae | ■ | | ■ | | Johnny's Selected Seeds |
| ECHCG | *Echinochloa crus-galli* | Barnyard Grass | 53 | Poaceae | Panicoideae/ Paniceae/ Boivinellinae | ■ | ■ | ■ | 966202092/ AM887871.1 | Aurora, NY |
| SETFA | *Setaria faberi* | Giant foxtail | 61 | Poaceae | Panicoideae/ Paniceae/ Cenchrinae | ■ | ■ | ■ | KF163774.1/ KF163540.1 | Aurora, NY |
| ABUTH | *Abutilon theophrasti* | Velvetleaf | 57 | Malvaceae | Malvoideae | ■ | ■ | ■ | HQ696683.1/ HM849734.1 | Aurora |
| HELAN | *Helianthus annuus* | Sunflower (Mammoth) | 84 | Asteraceae | Asteroideae | □ | | ■ | AY215805.1/ AF097517.1 | Livingston Seed Co. |
| FAGES | *Fagopyrum esculentum* | Buckwheat | 36 | Polygonaceae | Polygonoideae | ■ | ■ | ■ | JF829981.1/ D86285.1 | Lakeview Organic Grain variety not stated |
| AMAPO | *Amaranthus powellii* | Pigweed | 53 | Amaranthaceae | Amaranthoideae | ■ | ■ | ■ | 169146456/ KJ773261.1 | Aurora, NY |
| GLYMA | *Glycine max* | Soybean cv Sheyenne | 57 | Fabaceae | Faboideae | ■ | ■ | ■ | AF142700.1/ Z95552.1 | Lakeview Organic Grain |

Table A2: Analysis of variance testing main effects of plant genotype, nitrogen (N) treatment (0, 95 kg N ha-1) and interaction on plant growth metrics*

| | Interspecific Comparisons | | | | Intraspecific comparisons | | | |
|---|---|---|---|---|---|---|---|---|
| | SS | DF | F | *p* | SS | DF | F | *p* |
| **Biomass** | | | | | | | | |
| Genotype | 6.56 | 11, 33 | 11.24 | **<0.01** | 1.30 | 10, 30 | 3.99 | **<0.01** |
| N treatment | 0.55 | 1, 36 | 10.42 | **<0.01** | 0.17 | 1, 33 | 5.30 | **0.03** |
| Genotype x Treatment | 0.89 | 11, 36 | 1.52 | 0.17 | 0.27 | 10, 33 | 0.85 | 0.58 |
| **N uptake** | | | | | | | | |
| Genotype | 4.00 | 11, 33 | 5.29 | **<0.01** | 0.88 | 10, 30 | 1.38 | 0.24 |
| N treatment | 2.39 | 1, 36 | 33.99 | **<0.01** | 1.54 | 1, 33 | 24.22 | **<0.01** |
| Genotype x Treatment | 1.19 | 11, 36 | 1.57 | 0.15 | 0.42 | 10, 33 | 0.65 | 0.76 |
| **C:N** | | | | | | | | |
| Genotype | 2.63 | 11, 33 | 33 | **<0.01** | 1.40 | 10, 30 | 10.67 | **<0.01** |
| N treatment | 0.64 | 1, 36 | 36 | **<0.01** | 0.66 | 1, 33 | 50.32 | **<0.01** |
| Genotype x Treatment | 0.36 | 11, 36 | 36 | **0.03** | 0.24 | 10, 33 | 1.83 | 0.09 |

*Growth metrics are corrected for harvest date by using residuals from best fit-model of plant growth by days after planting (see figure S2).
P-values < 0.05 are highlighted in bold

Table A3: Analysis of variance testing main effects of sample type (rhizosphere vs bare soil), nitrogen (N) treatment (0, 95 kg N ha-1) and interactions on potential activity of extracellular beta-xylosidase (BX), cellobiohydrolase (CB), leucine aminopeptidase (LAP) and N-acetyl-glucosiminidase (NAG).

| | | SS | DF | F | *p* |
|---|---|---|---|---|---|
| BX | | | | | |
| | Sample Type | 224.5 | 1, 49 | 15.06 | **<0.01** |
| | N Fertilization | 55.8 | 1, 146 | 3.74 | **0.05** |
| | S x N | 96.3 | 1, 146 | 6.46 | **0.01** |
| CB | | | | | |
| | Sample Type | 279.9 | 1, 43 | 20.00 | **<0.01** |
| | N Fertilization | 73.7 | 1, 147 | 5.26 | **0.02** |
| | S x N | 145.9 | 1, 147 | 10.43 | **<0.01** |
| LAP | | | | | |
| | Sample Type | 1958.8 | 1, 47 | 4.65 | **0.04** |
| | N Fertilization | 3248 | 1, 147 | 7.70 | **<0.01** |
| | S x N | 9.3 | 1, 147 | 0.02 | 0.88 |
| NAG | | | | | |
| | Sample Type | 59.6 | 1, 448 | 9.25 | **<0.01** |
| | N Fertilization | 8.3 | 1, 147 | 1.28 | 0.26 |
| | S x N | 41.9 | 1, 147 | 6.50 | **0.01** |

*P*-values < 0.05 are highlighted in bold.

Table A4 Analysis of covariance testing correlation between inorganic nitrogen (N) concentration in the rhizosphere and potential activity of extracellular beta-xylosidase (BX), cellobiohydrolase (CB), leucine aminopeptidase (LAP) and N-acetyl-glucosiminidase (NAG).

| | | SS | DF | F | *p* | $R^2_m$ |
|---|---|---|---|---|---|---|
| BX | | | | | | |
| | Inorganic N[a] | 211 | 1, 77 | 10.45 | **<0.01** | .10 |
| CB | | | | | | |
| | Inorganic N | 225 | 1, 79 | 9.92 | **<0.01** | .12 |
| LAP | | | | | | |
| | Inorganic N | 6155 | 1, 69 | 19.70 | **<0.01** | .11 |
| NAG | | | | | | |
| | Inorganic N | 81 | 1, 77 | 12.96 | **<0.01** | .14 |

***** Marginal $R^2$ from a model testing inorganic N concentration as a fixed effect and plant genotype, rep and main plot as random effects. *P*-values < 0.05 are highlighted in bold.

[a]only samples for which inorganic N measured (Table S1) are included in analysis.

Table A5: Analysis of variance testing fixed effects of plant genotype and nitrogen (N) fertilization (0, 95 kg N ha-1) on potential activity of extracellular beta-xylosidase (BX), cellobiohydrolase (CB), leucine aminopeptidase (LAP) and N-acetyl-glucosiminidase (NAG) in rhizosphere samples.

| | | SS | DF | F | *p* |
|---|---|---|---|---|---|
| BX | | | | | |
| | Genotype | 1.00 | 10, 30 | 3.75 | **<0.01** |
| | N treatment[a] | 0.34 | 1, 32 | 12.68 | **<0.01** |
| | P x N | 0.33 | 10, 31 | 1.24 | 0.31 |
| CB | | | | | |
| | Genotype | 824 | 10, 29 | 4.04 | **<0.01** |
| | N treatment | 288 | 1, 32 | 14.12 | **<0.01** |
| | P x N | 190 | 10, 32 | 0.93 | 0.52 |
| LAP | | | | | |
| | Genotype | 20710 | 10, 30 | 6.02 | **<0.01** |
| | N treatment | 4327 | 1, 32 | 12.58 | **<0.01** |
| | P x N | 5481 | 10, 31 | 1.59 | 0.15 |
| NAG | | | | | |
| | Genotype | 0.98 | 10, 30 | 3.81 | **<0.01** |
| | N treatment | 0.99 | 1, 32 | 38.42 | **<0.01** |
| | P x N | 0.20 | 10, 32 | 0.78 | 0.64 |

[a] Only samples for which inorganic N measured (Table S1) are included in analysis. *P*-values < 0.05 highlighted in bold.

Table A6: Phylum statistics of rhizosphere composition at OTU level and aggregated by phylum.

| | | OTU Relative Abundance | | | Phylum Relative Abundance | | |
|---|---|---|---|---|---|---|---|
| Phylum | # OTU responders* | Min | Median | Max | Min | Median | Max |
| Proteobacteria | 507 | 0 | 7.92E-06 | 1.55E-03 | 1.40E-01 | 2.36E-01 | 4.13E-01 |
| Bacteroidetes | 206 | 0 | 0.00E+00 | 8.05E-04 | 1.62E-02 | 4.07E-02 | 7.40E-02 |
| Actinobacteria | 174 | 0 | 5.53E-05 | 2.95E-03 | 1.00E-01 | 1.60E-01 | 3.19E-01 |
| Chloroflexi | 158 | 0 | 1.83E-05 | 7.83E-04 | 1.75E-02 | 4.47E-02 | 9.11E-02 |
| Planctomycetes | 144 | 0 | 0.00E+00 | 2.92E-04 | 5.08E-03 | 1.17E-02 | 3.62E-02 |
| Verrucomicrobia | 93 | 0 | 8.95E-06 | 9.69E-04 | 1.07E-02 | 3.03E-02 | 4.25E-02 |
| Firmicutes | 58 | 0 | 0.00E+00 | 2.79E-03 | 2.37E-03 | 1.30E-02 | 9.87E-02 |
| Acidobacteria | 56 | 0 | 3.89E-05 | 9.63E-04 | 9.35E-03 | 1.95E-02 | 5.13E-02 |
| Gemmatimonadetes | 38 | 0 | 1.17E-05 | 4.00E-04 | 1.53E-03 | 4.81E-03 | 1.17E-02 |
| Cyanobacteria | 24 | 0 | 0.00E+00 | 6.77E-04 | 7.58E-04 | 2.48E-03 | 1.25E-02 |
| Armatimonadetes | 21 | 0 | 0.00E+00 | 2.72E-04 | 1.27E-03 | 2.10E-03 | 3.68E-03 |
| Fibrobacteres | 8 | 0 | 0.00E+00 | 4.76E-04 | 2.21E-05 | 5.45E-04 | 3.52E-03 |
| Spirochaetes | 5 | 0 | 0.00E+00 | 1.11E-03 | 1.89E-04 | 5.24E-04 | 5.52E-03 |
| Candidate_division_BRC1 | 3 | 0 | 0.00E+00 | 2.98E-04 | 2.32E-05 | 8.57E-05 | 8.63E-04 |
| Elusimicrobia | 3 | 0 | 0.00E+00 | 2.02E-04 | 1.26E-05 | 1.38E-04 | 4.71E-04 |
| Chlorobi | 2 | 0 | 5.79E-06 | 2.23E-04 | 2.53E-05 | 1.04E-04 | 3.17E-04 |
| Nitrospirae | 2 | 0 | 0.00E+00 | 4.63E-04 | 0.00E+00 | 1.27E-04 | 9.27E-04 |
| TM6 | 2 | 0 | 0.00E+00 | 8.77E-05 | 0.00E+00 | 3.17E-05 | 1.73E-04 |
| Candidate_division_OD1 | 1 | 0 | 0.00E+00 | 1.23E-04 | 0.00E+00 | 3.79E-05 | 1.23E-04 |
| Deinococcus-Thermus | 1 | 0 | 0.00E+00 | 3.59E-04 | 0.00E+00 | 4.31E-05 | 3.59E-04 |

* Rhizosphere responders are identified in DESeq2 in comparison of samples from each genotype with bare soil control ($log_2$-fold change > 0.5, BH adjusted $p < 0.05$). Metrics represent minimum, median and max relative abundance in in samples aggregated by genotype.

Table A7: Permutational multiple analysis of variance testing main effects of plant genotype and species identity and nitrogen (N) treatment (0, 95 kg N $ha^{-1}$) on rhizosphere bacterial community beta-diversity (weighted-UniFrac) on each sampling date.

| Factor | SS | DF | F | $R^2$ | $p^*$ | Factor | SS | DF | F | $R^2$ | $p^*$ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 53 DAP | | | | | | 79 DAP | | | | | |
| Genotype | 0.05 | 1 | 1.92 | 0.13 | 0.09 | Genotype | 0.11 | 3 | 1.77 | 0.15 | **<0.01** |
| Treatment | 0.03 | 1 | 1.06 | 0.08 | 0.36 | Treatment | 0.04 | 1 | 0.06 | 0.06 | **0.02** |
| Residuals | 0.26 | 13 | | 0.79 | | Residuals | 0.54 | 27 | | 0.78 | |
| 57 DAP | | | | | | 84 DAP | | | | | |
| Genotype | 0.12 | 1 | 3.46 | 0.20 | **<0.01** | Genotype | 0.14 | 2 | 4.36 | 0.28 | **<0.01** |
| Treatment | 0.03 | 1 | 0.94 | 0.05 | 0.43 | Treatment | 0.03 | 1 | 2.01 | 0.07 | 0.07 |
| Residuals | 0.46 | 13 | | 0.75 | | Residuals | 0.33 | 20 | | 0.65 | |
| 72 DAP | | | | | | 88 DAP | | | | | |
| Genotype | 0.44 | 4 | 6.58 | 0.43 | **<0.01** | Genotype | 0.29 | 3 | 6.53 | 0.41 | **<0.01** |
| Treatment | 0.02 | 1 | 0.92 | 0.02 | 0.38 | Treatment | 0.02 | 1 | 1.56 | 0.03 | 0.14 |
| Residuals | 0.57 | 34 | | 0.55 | | Residuals | 0.40 | 27 | | 0.56 | |

* *p*-values based on 999 permutations; *p*-values < 0.05 highlighted in bold. For reference, genotypes harvested on each date include 53: *E. crus-gali* and *A. powellii*; 57: *A. theophrast* and *G. max*; 72: *E. tef,* 75-062, H99, Ms71, and Il14H; 79: B73, B97, Hp301 and Oh7B; 84: *S. biocolor*, *S. x drummondii* and *H. annuus*; 88: Ki3, Mo18W, Tx303. Data from day 36 and 61 not shown as only one genotype was sampled on each date.

Table A8**:** Permutation tests for marginal effects (Type III) of terms in constrained analysis of principal coordinates relating plant growth characteristics to rhizosphere bacterial community composition.

| Factor | DF | SS | F | *p** |
|---|---|---|---|---|
| Days after planting | 1,41 | 0.13 | 4.71 | **<0.01** |
| Seed size[a] | 1,41 | 0.07 | 2.52 | **0.02** |
| NUE[b] | 1,41 | 0.11 | 3.73 | **<0.01** |
| N uptake[c] | 1,41 | 0.10 | 3.35 | **<0.01** |

***** *P*-values based on 999 permutations

[a] Log-transformed

[b,c] Plant nitrogen use efficiency (NUE: g C g $N^{-1}$) and total nitrogen (N) uptake corrected for days after planting using residuals from fit line in Figure S1.

# APPENDIX B

## SUPPLEMENTARY MATERIAL FROM CHAPTER 2

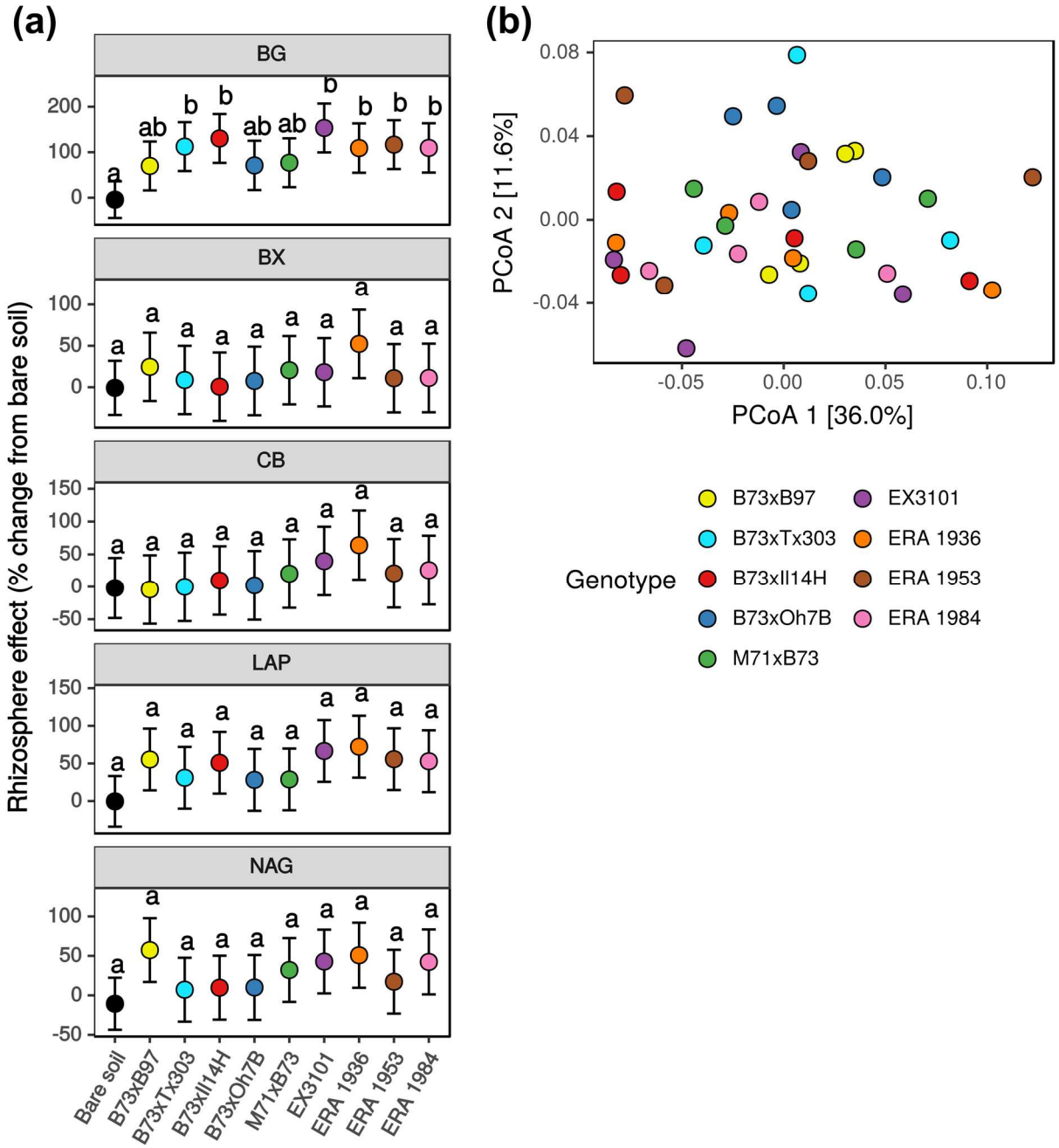


Figure B1: Maize hybrids did not vary in rhizosphere extracellular enzyme activity (a) or rhizosphere beta-diversity (b) when assessed at flowering (R1). Extracellular enzyme activity displayed as rhizosphere effect in relation to bare soil. Points represent least square means (whiskers = 95% confidence interval, n = 4), points sharing same letters are not significantly different at ($p < 0.05$). Rhizosphere beta-diversity calculated as weighted-UniFrac distance between samples and displayed in principal coordinate analysis. Enzyme abbreviations: βglucosidase (BG), β-xylosidase (BX), cellobiohydrolase (CB), leucine-aminopeptidase (LAP) and β-*N*-acetyl-glucosaminidase (NAG).

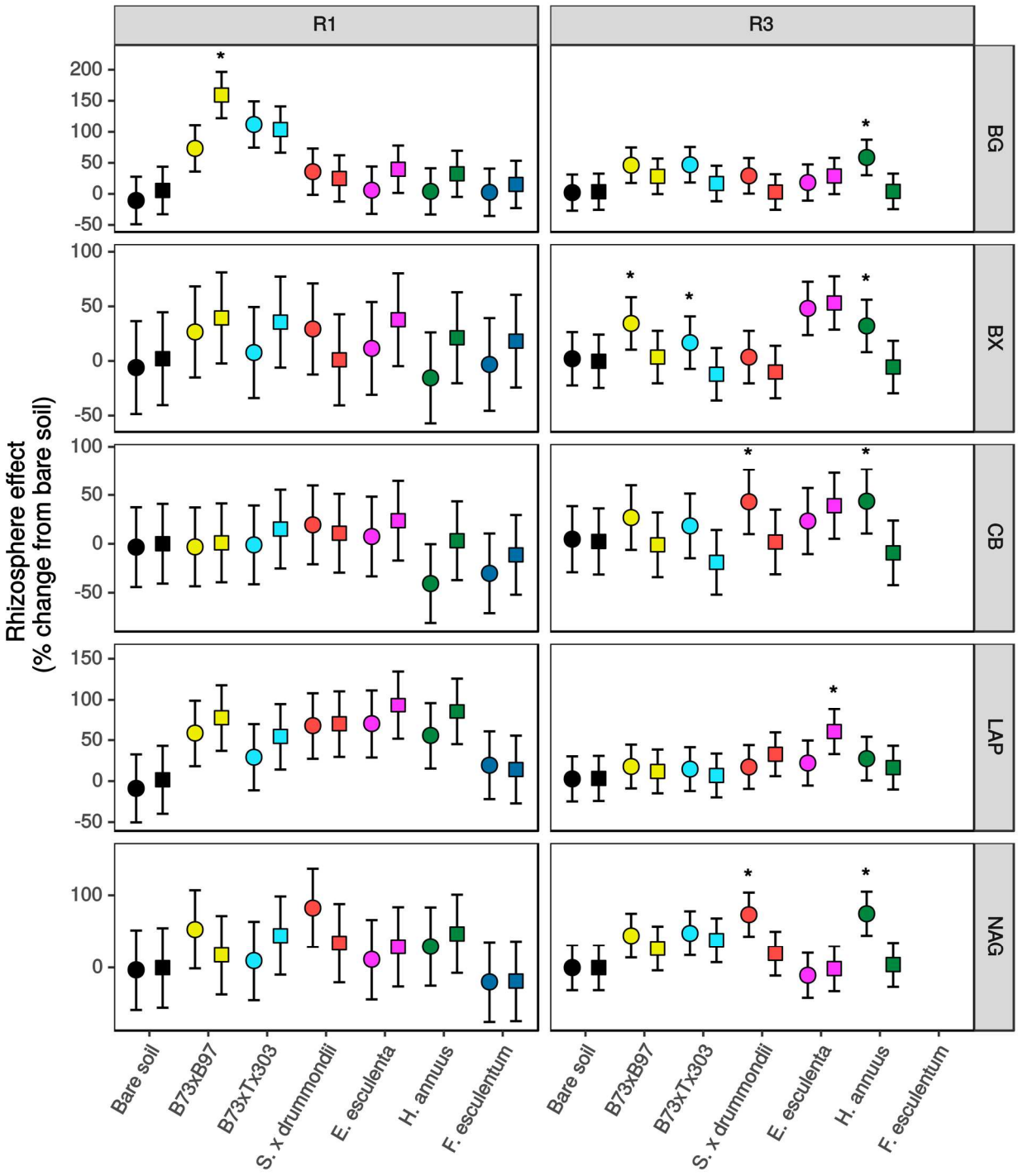


Figure B2: Clover transfer influences rhizosphere effect on potential extracellular enzyme activity in a time and species dependent manner. Points represent least square means (whiskers = 95% CI, n = 4) from plots with clover removed (+- 55kg ha; circles) and added (squares) prior to tillage. Asterisk marks significant differences in enzyme activity between + and – clover plots of a genotype ($p < 0.05$). Enzyme abbreviations: β-glucosidase (BG), β-xylosidase (BX), cellobiohydrolase (CB), leucine-aminopeptidase (LAP) and β-*N*-acetyl-glucosaminidase (NAG).

Table B1: Summary of sample collection from plant species and maize hybrids. Closed squares indicate both – and + clover plots were sampled, open squares indicate only - clover plots sampled.

| | | V6 | | | VT/R1 | | | R3 | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Genotype | Species | Plant Growth | Enzymes | Microbial Community | Plant Growth | Enzymes | Microbial Community | Plant Growth | Enzymes | Microbial Community |
| | *Fagopyrum esculentum* | ■ | ■ | ■ | ■ | ■ | ■ | | | |
| | *Echinochloa esculenta* | ■ | □ | □ | ■ | ■ | ■ | ■ | ■ | ■ |
| | *Sorghum x drummondii* | ■ | □ | □ | ■ | ■ | ■ | ■ | ■ | ■ |
| | *Helianthus annuus cv. Mammoth* | ■ | □ | □ | ■ | ■ | ■ | ■ | ■ | ■ |
| B73 x B97 | *Zea mays subsp. mays* | ■ | □ | □ | ■ | ■ | ■ | ■ | ■ | ■ |
| B73 x Tx303 | *Zea mays subsp. mays* | ■ | □ | □ | ■ | ■ | ■ | ■ | ■ | ■ |
| B73 x Il14H | *Zea mays subsp. mays* | | | | ■ | □ | □ | | | |
| M71 x B73 | *Zea mays subsp. mays* | | | | ■ | □ | □ | | | |
| B73 x Oh7B | *Zea mays subsp. mays* | | | | ■ | □ | □ | | | |
| EX3101 | *Zea mays subsp. mays* | | | | ■ | □ | □ | | | |
| ERA 1936 | *Zea mays subsp. mays* | | | | ■ | □ | □ | | | |
| ERA 1953 | *Zea mays subsp. mays* | | | | ■ | □ | □ | | | |
| ERA 1984 | *Zea mays subsp. mays* | | | | ■ | □ | □ | | | |
| Bare Soil | | | ■ | ■ | | ■ | □ | | ■ | ■ |

Table B2: Effect tests from models testing fixed effects of plant genotypes/species, clover transfer and interaction on plant growth metrics at three time points corresponding to maize six leaf (V6), flowering (R1), and grain fill (R3).

| . | Relative growth rate ($g\ g^{-1}\ d^{-1}$) | Growth rate ($kg\ dw\ ha^{-1}\ d^{-1}$) | N uptake ($kg\ N\ ha^{-1}\ d^{-1}$) | Plant N ($kg\ N\ ha^{-1}$) | Biomass ($kg\ dw\ ha^{-1}$) | NUE ($gC\ gN^{-1}$) |
|---|---|---|---|---|---|---|
| V6 | | | | | | |
| Genotype | $F_{(5,13)} = 18.12$*** | $F_{(5,12)} = 49.81$ *** | $F_{(5,12)} = 49.14$ *** | $F_{(5,12.16)} = 49.43$*** | $F_{(5,12.19)} = 50.56$ *** | $F_{(5,11.96)} = 43.82$ *** |
| Clover swap | $F_{(1,3)} = 0.39$ | $F_{(1,4)} = 0$ | $F_{(1,4)} = 0.04$ | $F_{(1,3.67)} = 0.04$ | $F_{(1,3.83)} = 0$ | $F_{(1,2.87)} = 2.98$ |
| G x C | $F_{(5,15)} = 0.61$ | $F_{(5,14)} = 0.91$ | $F_{(5,14)} = 0.31$ | $F_{(5,14.33)} = 0.31$ | $F_{(5,14.48)} = 0.91$ | $F_{(5,15.81)} = 2.32^{+}$ |
| R1 | | | | | | |
| Genotype | $F_{(5,12)} = 32.96$ *** | $F_{(5,13)} = 18.48$ *** | $F_{(5,13)} = 3.21$ * | $F_{(5,12)} = 12.94$ *** | $F_{(5,12)} = 26.47$ *** | $F_{(5,12.19)} = 30.79$*** |
| Clover swap | $F_{(1,3)} = 0.13$ | $F_{(1,3)} = 3.57$ | $F_{(1,3)} = 17.2$* | $F_{(1,3)} = 17.42$* | $F_{(1,3)} = 3.34$ | $F_{(1,2.96)} = 19.15$* |
| G x C | $F_{(5,15.82)} = 0.94$ | $F_{(5,16)} = 1.87$ | $F_{(5,16)} = 1.6$ | $F_{(5,16)} = 1.34$ | $F_{(5,16)} = 1.73$ | $F_{(5,16.15)} = 2.97$ * |
| R3 | | | | | | |
| Genotype | $F_{(4,9)} = 2.52$ | $F_{(4,10)} = 1.75$ | $F_{(4,10)} = 1.65$ | $F_{(4,10)} = 3.31^{+}$ | $F_{(4,10)} = 9.89$ ** | $F(4,8.9) = 51.1$*** |
| Clover swap | $F_{(1,3)} = 0.06$ | $F_{(1,3)} = 0.27$ | $F_{(1,3)} = 1.32$ | $F_{(1,3)} = 7.25^{+}$ | $F_{(1,3)} = 0.36$ | $F_{(1,3.29)} = 12.73$ * |
| G x C | $F_{(4,13)} = 0.63$ | $F_{(4,13)} = 1.99$ | $F_{(4,13)} = 0.69$ | $F_{(4,13)} = 1.83$ | $F_{(4,13)} = 4.74$ * | $F_{(4,12.72)} = 1.04$ |

$^{+}\ p < 0.1$, * $p < 0.05$, ** $p < 0.01$, ***, $p < 0.001$

Table B3: Pearson correlation coefficients between plant growth dynamics and rhizosphere effect on potential extracellular enzyme activity.

| | BG | BX | CB | LAP | NAG |
|---|---|---|---|---|---|
| RGR | -0.16 | -0.27** | -0.16 | -0.02 | -0.25** |
| Biomass | 0.19* | 0.26** | 0.23* | 0.11 | 0.41** |
| N uptake rate | 0.21* | 0.28** | 0.13 | 0.34** | 0.32** |
| Plant C:N | 0.30** | 0.28** | 0.20* | 0.10 | 0.38** |

* $p < 0.05$, ** $p < 0.01$

Table B4: Effect tests from models testing main effects of plant growth dynamics, sampling date and interactions on extracellular enzyme activity in the rhizosphere.

| Models | BG | BX | CB | LAP | NAG |
|---|---|---|---|---|---|
| Relative growth rate | $F_{(1, 92)} = 0.06$ | $F_{(1, 77)} = 1.13$ | $F_{(1, 44)} = 0.03$ | $F_{(1, 49)} = 0.01$ | $F_{(1, 92)} = 0.49$ |
| Sampling date | $F_{(2, 95)} = 0.37$ | $F_{(2, 96)} = 0.40$ | $F_{(2, 84)} = 2.82$ | $F_{(2, 86)} = 0.18$ | $F_{(2, 97)} = 1.98$ |
| RGR x S | $F_{(2, 101)} = 0.42$ | $F_{(2, 100)} = 0.68$ | $F_{(2, 84)} = 2.57$ | $F_{(2, 87)} = 1.00$ | $F_{(2, 103)} = 0.28$ |
| Biomass | $F_{(1,86)} = 3.23$ | $F_{(1,40)} = 0.10$ | $F_{(1,39)} = 0.09$ | $F_{(1,40)} = 0.36$ | $F_{(1,47)} = 0.64$ |
| Sampling date | $F_{(2,84)} = 0.32$ | $F_{(2,82)} = 6.57$ ** | $F_{(2,81)} = 4.05$* | $F_{(2,82)} = 2.33$ | $F_{(2,82)} = 3.05$ |
| B x S | $F_{(2,72)} = 3.55$ * | $F_{(2,72)} = 0.72$ | $F_{(2,71)} = 0.22$ ; | $F_{(2,72)} = 2.18$ | $F_{(2,75)} = 0.20$ |
| Nitrogen uptake rate | $F_{(1, 91)} = 2.58$ | $F_{(1, 85)} = 0.19$ | $F_{(1, 84)} = 0.89$ | $F_{(1, 63)} = 0.00$ | $F_{(1, 90)} = 0.59$ |
| Sampling date | $F_{(2, 88)} = 2.02$ | $F_{(2, 86)} = 1.39$ | **$F_{(2, 85)} = 3.6$*** | **$F_{(2, 76)} = 4.8$*** | **$F_{(2, 89)} = 6.88$**** |
| N x S | $F_{(2, 91)} = 2.72$ | $F_{(2, 89)} = 1.81$ | $F_{(2, 88)} = 1.17$ | **$F_{(2, 75)} = 4.16$*** | $F_{(2, 93)} = 1.34$ |
| Plant C:N | $F_{(1,96)} = 0.05$ | $F_{(1,62)} = 0.06$ ; | $F_{(1,39)} = 1.15$ | $F_{(1,43)} = 0.10$ | $F_{(1,75)} = 0.06$ |
| Sampling date | $F_{(2,79)} = 0.19$ | $F_{(2,82)} = 0.84$ | $F_{(2,72)} = 1.88$ | $F_{(2,75)} = 1.02$ | $F_{(2,79)} = 1.58$ |
| CN x S | $F_{(2,83)} = 0.00$ | $F_{(2,82)} = 0.38$ | $F_{(2,72)} = 1.36$ | $F_{(2,75)} = 0.02$ | $F_{(2,82)} = 1.41$ |

* $p < 0.05$, ** $p < 0.01$

**Table B5**: Rhizosphere OTUs correlated with growth characteristics at R1 as captured by constrained ordination of principal coordinates

| OTU | log2 Fold Change [a] | Silva Annotation | Phylum; Class; Order | LFC with RGR[b] | LFC with N uptake[b] |
|---|---|---|---|---|---|
| OTU.7197 | -1.99 (-4.01) | Catenulispora | Actinobacteria; ; Catenulisporales | -7.07** | 0.28** |
| OTU.1732 | -2.45 (-4.79) | Amycolatopsis | Actinobacteria; ; Pseudonocardiales | -7.86** | 0.31** |
| OTU.21 | -2.77 (-6.42) | Amycolatopsis | Actinobacteria; ; Pseudonocardiales | -15.86** | 0.23** |
| OTU.4529 | -3.79 (-7.8) | Amycolatopsis | Actinobacteria; ; Pseudonocardiales | -10.69** | 0.21* |
| OTU.1801 | -0.99 (-3.8) | Streptomyces | Actinobacteria; ; Streptomycetales | -10.31** | 0.14** |
| OTU.3910 | -1.5 (-4.13) | Streptomyces | Actinobacteria; ; Streptomycetales | -11.39** | 0.15** |
| OTU.3 | -1.38 (-5.29) | Streptomyces | Actinobacteria; ; Streptomycetales | -9.32** | 0.17** |
| OTU.313 | -2.07 (-6) | Streptomyces | Actinobacteria; ; Streptomycetales | -11.23** | 0.18** |
| OTU.25 | -3.13 (-7.41) | uncultured Ktedonobacteraceae | Chloroflexi; Ktedonobacteria; Ktedonobacterales | -11.04** | 0.23** |
| OTU.2171 | -3 (-6.28) | uncultured Ktedonobacteraceae | Chloroflexi; Ktedonobacteria; Ktedonobacterales | -10.87** | 0.24** |
| OTU.3866 | -3.48 (-7.96) | uncultured Ktedonobacteraceae | Chloroflexi; Ktedonobacteria; Ktedonobacterales | -10.79** | 0.26** |
| OTU.41 | -3.54 (-8.57) | uncultured Ktedonobacteraceae | Chloroflexi; Ktedonobacteria; Ktedonobacterales | -11.76** | 0.26** |
| OTU.625 | 1.65 (3.27) | Paenibacillus | Firmicutes; Bacilli; Bacillales | 6.18** | 0 |
| OTU.164 | -1.17 (-2.97) | Burkholderia | Proteobacteria; Betaproteobacteria; Burkholderiales | -6.66** | 0.16** |
| OTU.137 | -1.52 (-3.82) | Burkholderia | Proteobacteria; Betaproteobacteria; Burkholderiales | -7.59** | -0.02 |
| OTU.7288 | -1.94 (-4.07) | Burkholderia | Proteobacteria; Betaproteobacteria; Burkholderiales | -2.27 | 0.1 |
| OTU.1889 | -1.27 (-3.41) | Candidatus_Nitrotoga | Proteobacteria; Betaproteobacteria; Nitrosomonadales | -4.93* | 0.1 |
| OTU.610 | 1.82 (3.52) | uncultured Myxococcales | Proteobacteria; Deltaproteobacteria; Myxococcales | -5.93** | 0.06 |

| OTU | log2 Fold Change [a] | Silva Annotation | Phylum; Class; Order | LFC with RGR[b] | LFC with N uptake[b] |
|---|---|---|---|---|---|
| OTU.2367 | 1.65 (3.25) | uncultured Sorangiineae | Proteobacteria; Deltaproteobacteria; Myxococcales | -3.29 | 0.3** |
| OTU.133 | 1.19 (3.72) | uncultured Sinobacteraceae | Proteobacteria; Gammaproteobacteria; Xanthomonadales | -4.93* | 0.22** |
| OTU.143 | 1.89 (4.24) | Opitutus | Verrucomicrobia; Opitutae; Opitutales | 2.6 | 0.04 |
| OTU.79 | 2.06 (4.23) | Luteolibacter | Verrucomicrobia; Verrucomicrobiae; Verrucomicrobiales | -3.24 | 0.04 |
| OUT.1476 | 1.61 (3.29) | uncultured OPB35 | Verrucomicrobia; OPB35_soil_group | -2.52 | 0.04 |

[a] $\log_2$-fold (LFC) change in abundance with unit change in sample score along CAP axis 1

[b]LFC change in abundance per unit change in RGR or rate of N uptake in rhizosphere samples from all sample dates (* $p < 0.05$, ** $p < 0.01$; BH adjusted).

# APPENDIX C

# SUPPLEMENTARY MATERIAL FROM CHAPTER 3

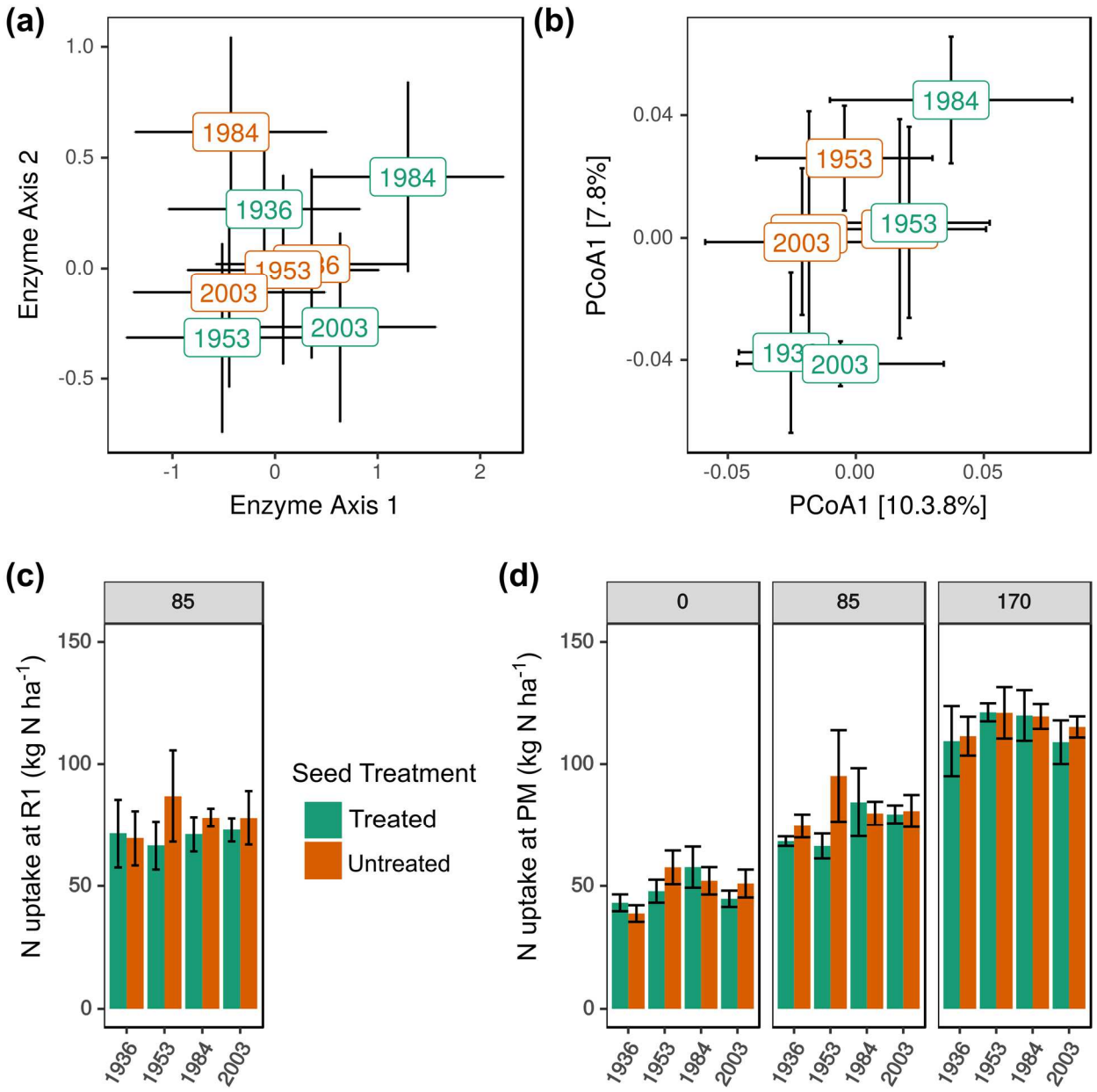


Figure C1: Seed treatment did not influence rhizosphere or growth metrics of four maize hybrids: Extracellular enzyme activity in the rhizosphere at anthesis (a), rhizosphere beta-diversity as measured by Bray-Curtis dissimilarity of square root transformed relative abundance (b), plant N uptake at flowering (c) and plant N uptake at physiological maturity (d). Samples are from plots receiving 85 kg N $ha^{-1}$ (a,b,c) and 0, 85, and 170 kg N $ha^{-1}$ plots (d). Points and bars indicate means ± 1 s.e.m. (n=4). See Table C2 and C3 for effect tests.

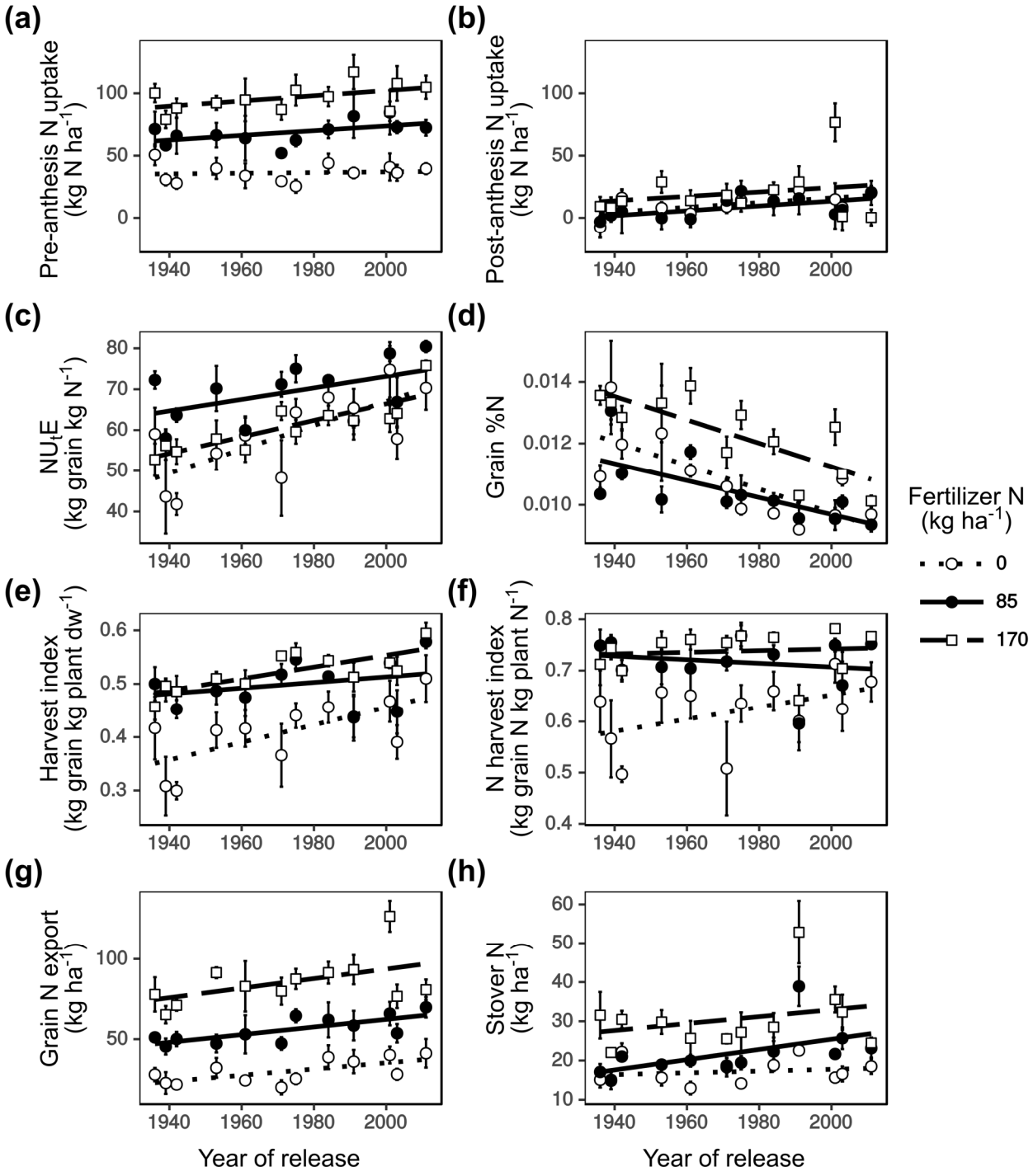


Figure C2: Changes in pre- and post-anthesis nitrogen (N) uptake, nitrogen utilization efficiency ($NU_tE$), grain percent N, harvest index, total N export in grain and retention in stover with year of hybrid release under 0, 85 and 170 kg N $ha^{-1}$. Points are hybrid means ± 1 s.e.m. for each treatment (n = 4).

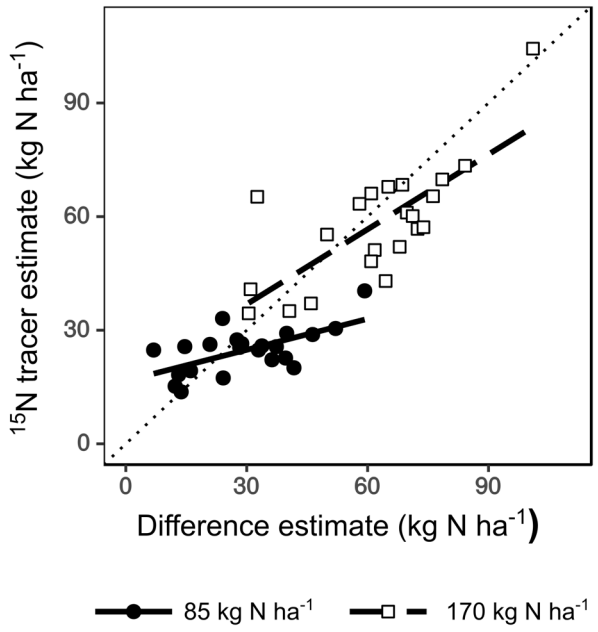


Figure C3: Comparison of plant fertilizer uptake estimates using a $^{15}$N tracer vs the difference between plant nitrogen uptake in fertilized and unfertilized plots.

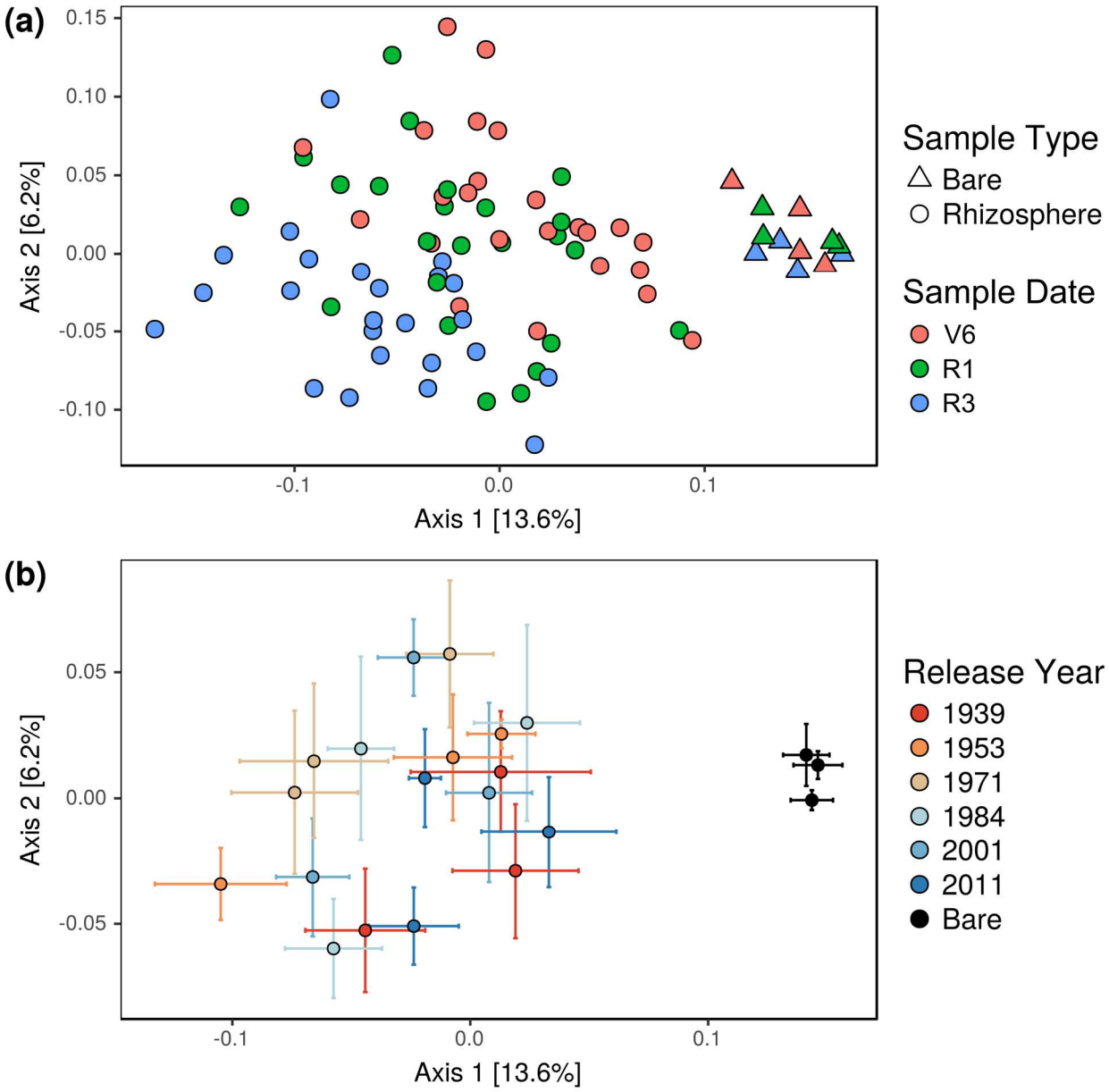


Figure C4: Principal coordinate analysis of beta-diversity in bare soil and rhizosphere samples colored by sampling date (V6 – red, R1 – green, and R3 – blue) (a) and by hybrid release date (b). In panel (b) points represent centroid ± 1 s.e.m. of each hybrid's samples on each sample date. Beta-diversity measured by Bray-Curtis dissimilarity calculated on square root transformed relative abundance. Only data from plots sampled on all three sampling dates displayed.

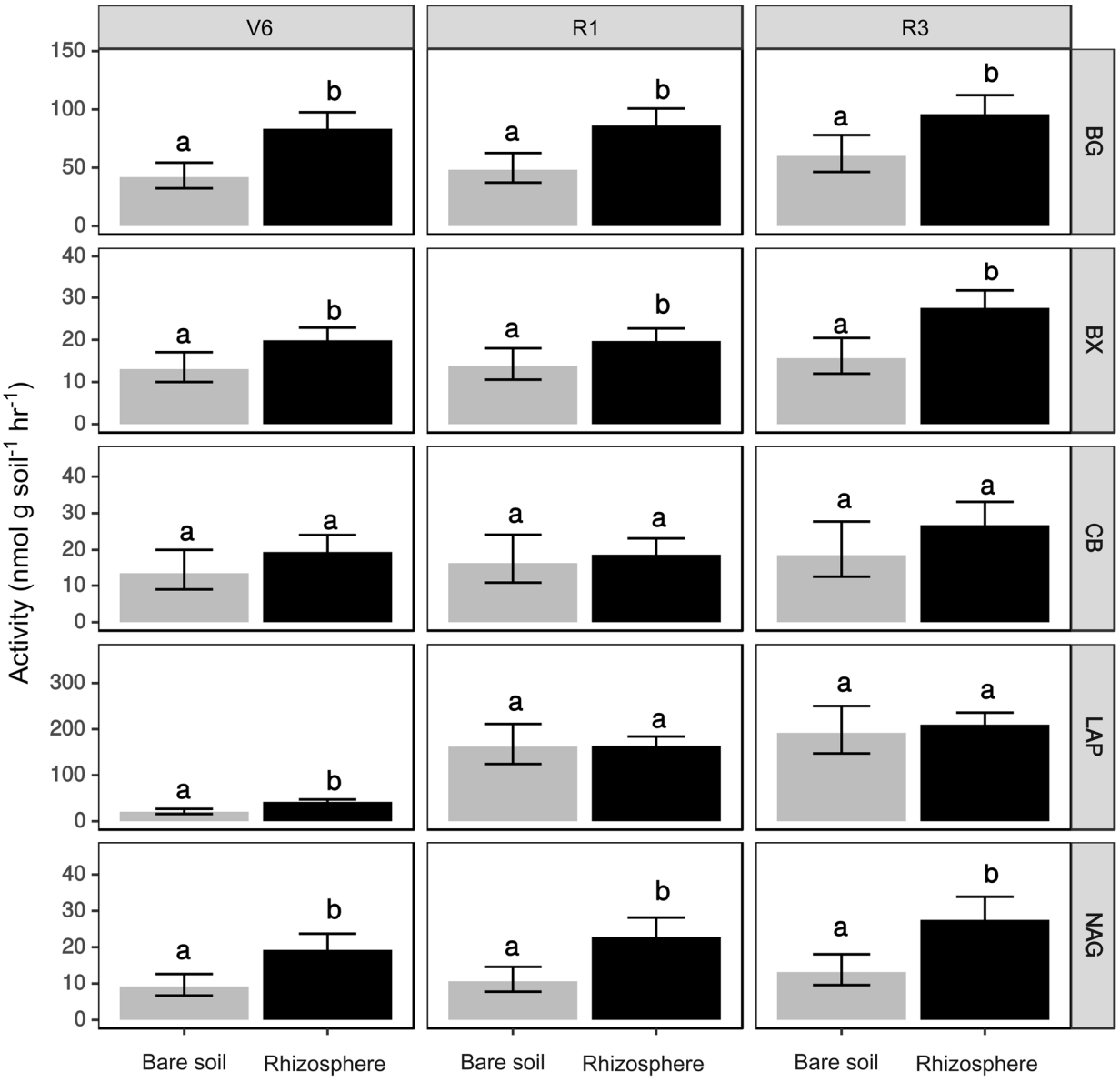


Figure C5: Rhizosphere effect on potential activity of extracellular enzymes on three sample dates. Enzyme abbreviations: beta-1,4-glucosidase (BG), beta-xylosidase (BX), cellobiohydrolase (CB), leucine-aminopeptidase (LAP), *N*-acetylglucosaminidase (NAG). Bars represent means of core plots sampled on each timepoint ± 95% CI. Bars with different letters indicate a significant difference in potential activity between bulk and rhizosphere soil ($p < 0.05$).

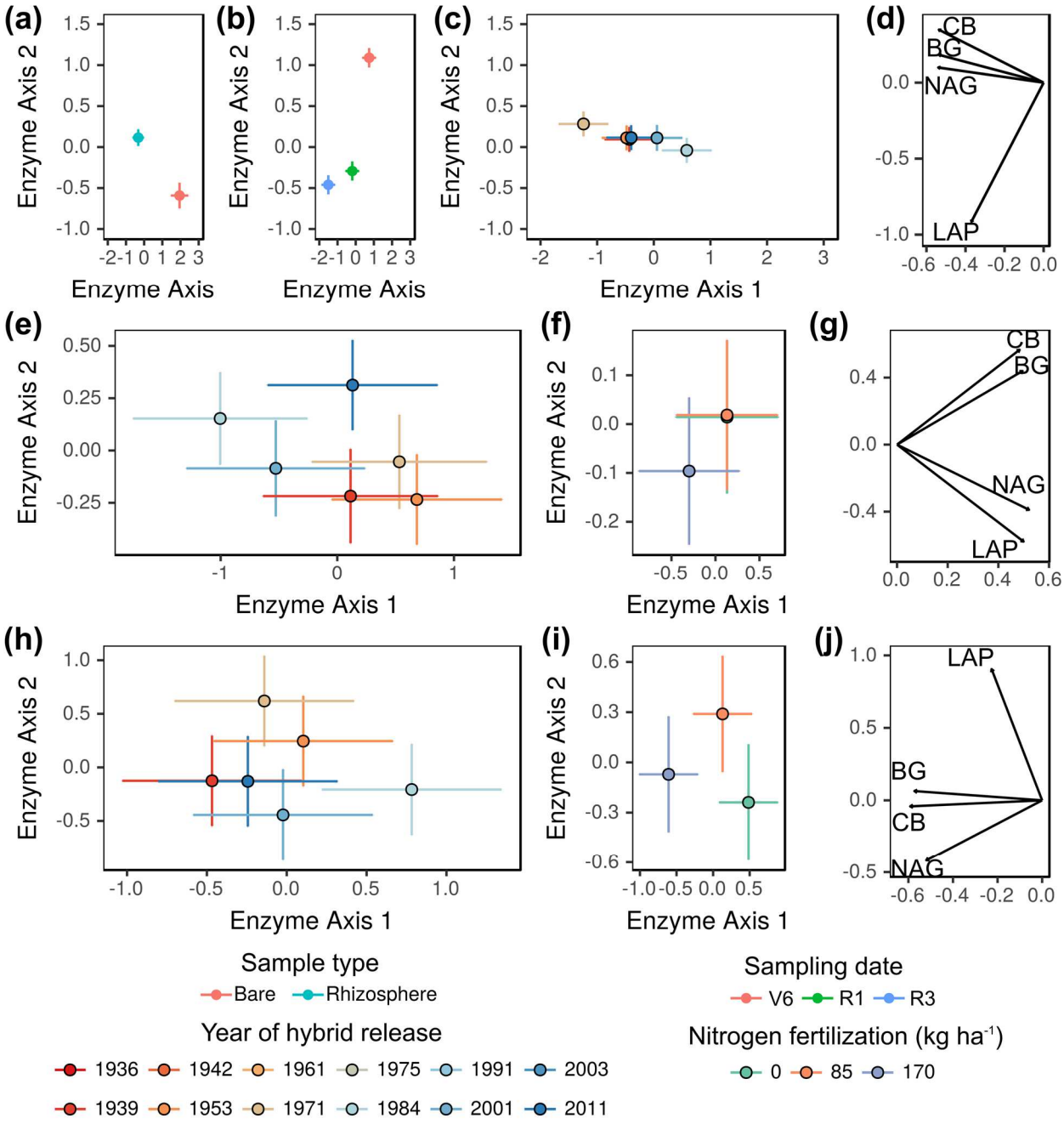


Figure C6: Potential extracellular enzyme profile of core rhizosphere and bare soil samples influenced by sample type (rhizosphere vs bare soil) (a), sampling date (b) and maize hybrid (c). Enzyme profile of samples at V6 influenced by maize hybrid (e) and fertilization (f), and samples at R3 influenced by maize hybrid (h) and fertilization (i). Enzyme loadings on principal components for core samples (d) samples from V6 (g)and R3 (j). Points are lsmeans of sample scores on PCA axes ±1 s.em. (n = 4). Enzyme abbreviations: beta-1,4-glucosidase (BG), beta-xylosidase (BX), cellobiohydrolase (CB), leucine-aminopeptidase (LAP), *N*-acetylglucosaminidase (NAG).

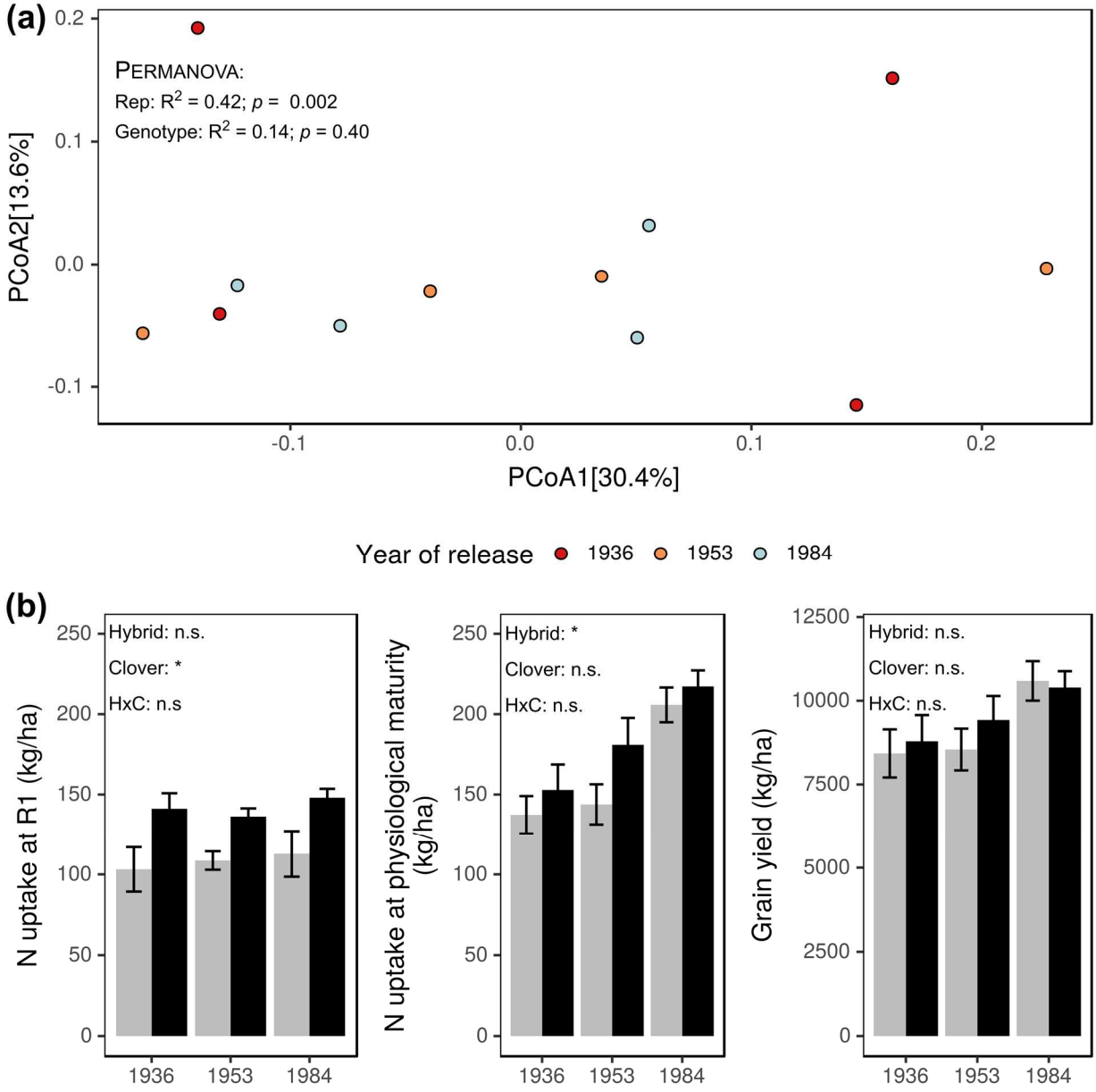


Figure C7: Principal coordinate analysis of rhizosphere samples from three ERA maize hybrids planted in a field with 20-year history of complex rotations and leguminous nitrogen sources (a) and growth metrics of same three hybrids in plots with + and – above ground clover transfer (black and grey bars respectively) of approximately 55kg N $ha^{-1}$ (b). Bars represent sample mean) ± 1 s.e.m. (n = 4). Effect test of model terms showed above (* $p < 0.05$; ns $p \geq 0.05$).

Table C1**:** Summary of treatments and sample collection in field experiment.

| | | | V6 | | | VT/R1 | | | R3 | | | PM |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Year** | **Fertility Treatments (kg N ha$^{-1}$)** | **$^{15}$N** | **Enzymes** | **DNA** | **Biomass** | **Enzymes** | **DNA** | **Biomass** | **Enzymes** | **DNA** | **Biomass** | **Final Yield** |
| 1936 | 0/85/170 | Natural | | | ■ | □ | □ | ■ | | | | ■ |
| 1939 | 0/85/170 | 15N | ■ | ■ | ■ | □ | □ | ■ | ■ | ■ | ■ | ■ |
| 1942 | 0/85/170 | Natural | | | ■ | □ | □ | ■ | | | | ■ |
| 1953 | 0/85/170 | 15N | ■ | ■ | ■ | □ | □ | ■ | ■ | ■ | ■ | ■ |
| 1961 | 0/85/170 | Natural | | | ■ | □ | □ | ■ | | | | ■ |
| 1971 | 0/85/170 | 15N | ■ | ■ | ■ | □ | □ | ■ | ■ | ■ | ■ | ■ |
| 1975 | 0/85/170 | Natural | | | ■ | □ | □ | ■ | | | | ■ |
| 1984 | 0/85/170 | 15N | ■ | ■ | ■ | □ | □ | ■ | ■ | ■ | ■ | ■ |
| 1991 | 0/85/170 | Natural | | | ■ | □ | □ | ■ | | | | ■ |
| 2001 | 0/85/170 | 15N | ■ | ■ | ■ | □ | □ | ■ | ■ | ■ | ■ | ■ |
| 2003 | 0/85/170 | Natural | | | ■ | □ | □ | ■ | | | | ■ |
| 2011 | 0/85/170 | 15N | ■ | ■ | ■ | □ | □ | ■ | ■ | ■ | ■ | ■ |
| 1936[u] | 0/85/170 | Natural | | | | □ | □ | □ | | | | ■ |
| 1953 [u] | 0/85/170 | Natural | | | | □ | □ | □ | | | | ■ |
| 1984 [u] | 0/85/170 | Natural | | | | □ | □ | □ | | | | ■ |
| 2003 [u] | 0/85/170 | Natural | | | ■ | □ | □ | □ | | | | ■ |
| Bare | 0/85/170 | Natural | ■ | ■ | | □ | □ | | □ | □ | | |

[u]Planted with untreated seeds; □ only 85kg N ha$^{-1}$ plots sampled; ■ all plots sampled

Table C2: Analysis of variance testing seed treatment and interactions with maize hybrid and fertilizer addition on biomass accumulation, N uptake and extracellular enzyme profile in rhizosphere samples.

| | Hybrid | Fertilizer | Seed Treatment | F x S | FxH | S x H |
|---|---|---|---|---|---|---|
| Grain yield | $F_{(3, 20)} = 5.09$* | $F_{(2, 4)} = 169.1$* | $F_{(1,17)} = 0.47$ | $F_{(2,45)} = 0.06$ | $F_{(6,52)} = 1.35$ | $F_{(3,20)} = 0.90$ |
| Total N uptake | $F_{(3, 20)} = 1.65$ | $F_{(3, 5)} = 97.51$* | $F_{(1, 20)} = 0.39$ | $F_{(2,53)} = 0.32$ | $F_{(6,50)} = 0.16$ | $F_{(3,20)} = 0.70$ |
| R1 biomass | $F_{(3, 21)} = 0.31$ | -- | $F_{(1, 21)} = 2.29$ | -- | -- | $F_{(3, 21)} = 0.16$ |
| R1 N uptake | $F_{(3, 20)} = 0.17$ | -- | $F_{(1, 20)} = 1.01$ | -- | -- | $F_{(3, 20)} = 0.46$ |
| Enzyme Axis 1 R1 | $F_{(3, 21)} = 0.17$ | -- | $F_{(1,21)} = 0.46$ | -- | -- | $F_{(3, 21)} = 0.78$ |
| Enzyme Axis 2 R1 | $F_{(3, 21)} = 1.17$ | -- | $F_{(1,21)} = 0.12$ | -- | -- | $F_{(3, 21)} = 0.16$ |

* $p < 0.05$, ** $p < 0.01$

[a]Only 85 kg $ha^{-1}$ plots sampled at R1

Table C3: Permutational multiple analysis of variance testing main effects of replicate block, maize hybrid and seed treatment on Bray-Curtis dissimilarity of rhizosphere bacterial community at anthesis.

| | SS | DF | *F* | *R2* | p |
|---|---|---|---|---|---|
| Rep | 0.20 | 3 | 1.80 | 0.16 | **<0.01** |
| Hybrid | 0.12 | 3 | 1.07 | 0.10 | 0.21 |
| Seed treatment | 0.04 | 1 | 1.02 | 0.03 | 0.35 |
| H x St | 0.10 | 3 | 0.95 | 0.08 | 0.67 |
| Residuals | 0.76 | 21 | | | |

Table C4: F-tests from analysis of variance testing fixed effects of year of hybrid release, nitrogen fertilizer (0,85 and 170 kg N $ha^{-1}$) and interaction on nitrogen use efficiency of maize.

| | Year | N | YxN |
|---|---|---|---|
| NUtE | $F_{(1,10)} = 19.8$ ** | $F_{(2,31)} = 15.28$ *** | $F_{(2,91)} = 2.75$ $^{+}$ |
| Grain N export | $F_{(1,10)} = 9.11$ * | $F_{(2,31)} = 69.73$ *** | $F_{(2,91)} = 0.58$ |
| Grain %N | $F_{(1,10)} = 27.62$ *** | $F_{(2,28)} = 15.02$ *** | $F_{(2,91)} = 0.90$ |
| Stover N kg ha | $F_{(1,10)} = 2.09$ | $F_{(2,32)} = 12.89$ *** | $F_{(2,92)} = 2.98$ $^{+}$ |
| Stover %N | $F_{(1,10)} = 0.08$ | $F_{(2,32)} = 8.30$ ** | $F_{(2,92)} = 1.80$ |
| Harvest index (dw) | $F_{(1,10)} = 9.30$ * | $F_{(2,32)} = 36.3$ *** | $F_{(2,92)} = 3.98$ * |
| Harvest index (N) | $F_{(1,10)} = 0.42$ | $F_{(2,31)} = 30.93$ *** | $F_{(2,91)} = 4.86$ ** |
| V6 biomass | $F_{(1,10)} = 1.18$ | $F_{(2,11)} = 1.43$ | $F_{(2,87)} = 0.11$ |
| V6 N uptake | $F_{(1,10)} = 2.46$ | $F_{(2,9)} = 3.15$ $^{+}$ | $F_{(2,86)} = 0.21$ |
| R3 biomass | $F_{(1,4)} = 9.03$ * | $F_{(2,18)} = 16.63$ *** | $F_{(2,40)} = 1.84$ |
| R3 N uptake | $F_{(1,4)} = 1.85$ | $F_{(2,22)} = 2.36$ | $F_{(2,40)} = 0.15$ |

$^{+}$ $p < 0.1$, * $p < 0.05$, ** $p < 0.01$, ***$p < 0.001$

NUtE: nitrogen utilization efficiency (kg grain kg $N^{-1}$), harvest index (kg grain kg $plant^{-1}$ and kg grain N kg plant $N^{-1}$)

Table C5: Permutational multiple analysis of variance testing main effects of replicate block, sample type (rhizosphere vs bare soil), date of sampling and interactions on bacterial community Bray-Curtis dissimilarity matrix for core plots sampled at three time points.

| | SS | DF | *F* | *R2* | p |
|---|---|---|---|---|---|
| Core plots | | | | | |
| Rep | 0.23 | 3 | 1.96 | 0.07 | **<0.01** |
| Sample type | 0.33 | 1 | 8.37 | 0.09 | **<0.01** |
| Time point | 0.21 | 2 | 2.69 | 0.06 | **<0.01** |
| St x T | 0.07 | 2 | 0.87 | 0.02 | 0.83 |
| Residuals | 2.93 | 75 | | | |

Table C6: Relative abundance of dominant families in rhizosphere samples from three sampling dates with number of OTUs significantly enriched in the rhizosphere / number of OTUs within family.

| Rhizosphere composition at V6: | | |
|---|---|---|
| Family | % | n Rhiz / n OTU |
| Unclassified DA023 | 6.3 | 0/103 |
| Unclassified Gaiellales | 4.2 | 0/85 |
| Planctomycetaceae | 3.9 | 0/567 |
| Comamonadaceae | 3.1 | 9/29 |
| Unclassified DA023 | 2.6 | 0/38 |
| Gaiellaceae | 2.4 | 0/13 |
| Unclassified Chloroflexi | 2.1 | 0/25 |
| Streptomycetaceae | 2.1 | 4/31 |
| Nocardioidaceae | 2 | 1/36 |
| Gemmatimonadaceae | 1.9 | 1/105 |
| Sinobacteraceae | 1.6 | 1/53 |
| Nitrosomonadaceae | 1.6 | 0/43 |
| Sphingomonadaceae | 1.3 | 9/39 |
| Bacillaceae | 1.3 | 3/21 |
| Chitinophagaceae | 1.3 | 4/95 |
| Unclassified Acidobacteria | 1.3 | 0/36 |
| Xanthobacteraceae | 1.3 | 0/21 |
| Unclassified GR-WP33-30 | 1.3 | 0/29 |
| Verrucomicrobiaceae | 1.3 | 5/34 |
| 480-2 | 1.3 | 0/34 |

| Rhizosphere composition at R1: | | |
|---|---|---|
| Family | % | nRhiz / n OTU |
| Unclassified DA023 | 5.7 | 0/103 |
| Streptomycetaceae | 5.5 | 6/31 |
| Unclassified Gaiellales | 4 | 0/85 |
| Planctomycetaceae | 3.7 | 0/567 |
| Comamonadaceae | 3.4 | 7/29 |
| Unclassified DA023 | 2.5 | 0/38 |
| Nocardioidaceae | 2.4 | 1/36 |
| Gaiellaceae | 2.3 | 0/13 |
| Unclassified KD4-96 | 1.9 | 0/25 |
| Gemmatimonadaceae | 1.8 | 1/105 |
| Oxalobacteraceae | 1.8 | 4/14 |
| Sphingomonadaceae | 1.7 | 7/39 |
| Verrucomicrobiaceae | 1.7 | 3/34 |
| Sinobacteraceae | 1.6 | 1/53 |
| Nitrosomonadaceae | 1.5 | 0/43 |
| Unclassified OPB35 | 1.3 | 1/74 |
| Pseudonocardiaceae | 1.3 | 2/22 |
| Cytophagaceae | 1.3 | 6/76 |
| Solirubrobacteraceae | 1.2 | 0/24 |
| Unclassified DA023 | 1.2 | 0/36 |

| Rhizosphere composition at R3 | | |
|---|---|---|
| Family | % | nRhiz / n OTU |
| Streptomycetaceae | 7.3 | 12/31 |
| Unclassified DA023 | 5.1 | 0/103 |
| Comamonadaceae | 4.4 | 14/29 |
| Unclassified Gaiellales | 3.5 | 0/85 |
| Planctomycetaceae | 3.4 | 0/567 |
| Nocardioidaceae | 2.5 | 10/36 |
| Pseudonocardiaceae | 2.5 | 7/22 |
| Unclassified DA023 | 2.3 | 0/38 |
| Gaiellaceae | 2.1 | 0/13 |
| Unclassified KD4-96 | 2 | 0/25 |
| Sphingomonadaceae | 1.9 | 11/39 |
| Gemmatimonadaceae | 1.7 | 3/105 |
| Sinobacteraceae | 1.6 | 6/53 |
| Unclassified OPB35 | 1.5 | 0/74 |
| Cytophagaceae | 1.5 | 16/76 |
| Oxalobacteraceae | 1.5 | 7/14 |
| Nitrosomonadaceae | 1.5 | 1/43 |
| Verrucomicrobiaceae | 1.4 | 10/34 |
| Chitinophagaceae | 1.2 | 12/95 |
| Microbacteriaceae | 1.2 | 3/13 |

| Rhizosphere composition at V6: | | |
|---|---|---|
| Family | % | n Rhiz / n OTU |
| Unclassified MB-A2-108 | 1.2 | 0/29 |
| Solirubrobacteraceae | 1.2 | 0/24 |
| Micrococcaceae | 1.2 | 0/10 |
| Cytophagaceae | 1.2 | 9/76 |
| Unclassified OPB35 | 1.1 | 2/74 |
| Bradyrhizobiaceae | 1.1 | 0/13 |
| Nitrospirales 0319-6A21 | 1.1 | 0/22 |
| Intrasporangiaceae | 1 | 0/6 |
| Candidatus_Alysiosphaera | 1 | 0/14 |
| Micromonosporaceae | 1 | 2/43 |

| Rhizosphere composition at R1: | | |
|---|---|---|
| Family | % | nRhiz / n OTU |
| Micrococcaceae | 1.2 | 0/10 |
| Chitinophagaceae | 1.2 | 4/95 |
| Thermoleophilia 480-2 | 1.2 | 0/34 |
| Xanthobacteraceae | 1.2 | 0/21 |
| Intrasporangiaceae | 1.1 | 0/6 |
| Microbacteriaceae | 1.1 | 0/13 |
| Unclassified MB-A2-108 | 1.1 | 0/29 |
| Unclassified GR-WP33-30 | 1.1 | 0/29 |
| Bradyrhizobiaceae | 1.1 | 0/13 |
| Xanthomonadaceae | 1 | 5/46 |

| Rhizosphere composition at R3 | | |
|---|---|---|
| Family | % | nRhiz / n OTU |
| Micromonosporaceae | 1.2 | 3/43 |
| Solirubrobacteraceae | 1.1 | 1/24 |
| 480-2 | 1.1 | 0/34 |
| Unclassified DA023 | 1.1 | 0/36 |
| Bradyrhizobiaceae | 1.1 | 2/13 |
| Xanthobacteraceae | 1 | 0/21 |
| Intrasporangiaceae | 1 | 0/6 |
| Xanthomonadaceae | 1 | 10/46 |
| Micrococcaceae | 1 | 1/10 |
| Rhizobiaceae | 1 | 6/10 |

Table C7: Mean relative abundance of dominant OTUs in rhizosphere samples from three sampling dates with OTUs significantly enriched compared to bulk soil highlighted in grey.

| Rhizosphere taxa at T1: | % | Rhizosphere taxa at T2: | % | Rhizosphere taxa at T3 | % |
|---|---|---|---|---|---|
| Unclassified KD4-96: | 1.79 | Streptomycetaceae: Streptomyces | 1.91 | Streptomycetaceae: Streptomyces | 2.88 |
| Gaiellaceae: Gaiella | 1.2 | Streptomycetaceae: Streptomyces | 1.72 | Comamonadaceae: Pelomos | 1.97 |
| Unclassified MB-A2-108: | 1.01 | Unclassified KD4-96: | 1.62 | Unclassified KD4-96: | 1.67 |
| Intrasporangiaceae: Janibacter | 1 | Comamonadaceae: Pelomos | 1.26 | Streptomycetaceae: Streptomyces | 1.64 |
| Unclassified Gaiellales: | 0.91 | Verrucomicrobiaceae: Luteolibacter | 1.12 | Pseudonocardiaceae: Lentzea | 1.56 |
| Comamonadaceae: | 0.91 | Oxalobacteraceae: | 1.11 | Streptomycetaceae: Streptomyces | 1.55 |
| Unclassified GR-WP33-30: | 0.86 | Gaiellaceae: Gaiella | 1.07 | Gaiellaceae: Gaiella | 1.01 |
| Micrococcaceae: Arthrobacter | 0.85 | Intrasporangiaceae: Janibacter | 1.06 | Intrasporangiaceae: Janibacter | 1 |
| Comamonadaceae: Pelomos | 0.85 | Comamonadaceae: | 0.94 | Comamonadaceae: | 0.94 |
| Unclassified DA023: | 0.8 | Unclassified Gaiellales: | 0.9 | Oxalobacteraceae: | 0.91 |
| Unclassified DA023: | 0.74 | Micrococcaceae: Arthrobacter | 0.87 | Sphingomonadaceae: Sphingobium | 0.88 |
| 288-2: | 0.7 | Unclassified MB-A2-108: | 0.84 | Microbacteriaceae: Agromyces | 0.87 |
| Nocardioidaceae: Marmoricola | 0.69 | Unclassified DA023: | 0.81 | Unclassified Gaiellales: | 0.73 |
| Solirubrobacteraceae: Solirubrobacter | 0.69 | Sphingomonadaceae: Sphingobium | 0.78 | Micrococcaceae: Arthrobacter | 0.71 |
| MSB-1E8: | 0.67 | Microbacteriaceae: Agromyces | 0.75 | Verrucomicrobiaceae: Luteolibacter | 0.68 |
| Unclassified Elev-16S-573: | 0.65 | Streptomycetaceae: Streptomyces | 0.72 | Unclassified DA023: | 0.68 |
| Xiphinematobacteraceae: Candidatus_Xiphinematobacter | 0.64 | Unclassified GR-WP33-30: | 0.71 | Unclassified MB-A2-108: | 0.67 |
| Bacillaceae: Bacillus | 0.62 | Nocardioidaceae: Marmoricola | 0.69 | Unclassified DA023: | 0.63 |

| Rhizosphere taxa at T1: | % |
|---|---|
| Unclassified JG30-KF-CM45: | 0.61 |
| Streptomycetaceae: Streptomyces | 0.6 |
| Xanthobacteraceae: | 0.6 |
| Candidatus_Alysiosphaera: | 0.56 |
| Blastococcus: | 0.56 |
| Gaiellaceae: Gaiella | 0.54 |
| Bradyrhizobiaceae: Bradyrhizobium | 0.54 |
| Microbacteriaceae: Agromyces | 0.54 |
| Streptomycetaceae: Streptomyces | 0.52 |
| Verrucomicrobiaceae: Luteolibacter | 0.52 |
| Unclassfied Rhizobiales: | 0.5 |
| Gaiellaceae: Gaiella | 0.45 |

| Rhizosphere taxa at T2: | % |
|---|---|
| Solirubrobacteraceae: Solirubrobacter | 0.67 |
| Unclassified DA023: | 0.67 |
| Pseudonocardiaceae: Lentzea | 0.64 |
| 288-2: | 0.63 |
| Unclassified Elev-16S-573: | 0.6 |
| MSB-1E8: | 0.56 |
| Gaiellaceae: Gaiella | 0.55 |
| Xiphinematobacteraceae: Candidatus_Xiphinematobacter | 0.54 |
| Blastococcus: | 0.53 |
| Xanthobacteraceae: | 0.52 |
| Bradyrhizobiaceae: Bradyrhizobium | 0.52 |
| Unclassified JG30-KF-CM45: | 0.5 |

| Rhizosphere taxa at T3 | % |
|---|---|
| Nocardioidaceae: Marmoricola | 0.61 |
| Unclassified GR-WP33-30: | 0.58 |
| Solirubrobacteraceae: Solirubrobacter | 0.58 |
| Bradyrhizobiaceae: Bradyrhizobium | 0.55 |
| 288-2: | 0.53 |
| Gaiellaceae: Gaiella | 0.51 |
| Unclassified Elev-16S-573: | 0.51 |
| Rhizobiaceae: Shinella | 0.5 |
| Blastococcus: | 0.47 |
| Xanthobacteraceae: | 0.47 |
| Xiphinematobacteraceae: Candidatus_Xiphinematobacter | 0.45 |
| Unclassified JG30-KF-CM45: | 0.45 |

Table C8: Differentially abundant OTUs in ERA hybrids compared to early release reference.

| Taxa: | Mean abundance | $Log_2$-fold change | hybrid | Intersection* |
|---|---|---|---|---|
| V6 – Reference 1939 | | | | |
| Actinobacteria: Propionibacteriaceae Jiangella | 2.3E-04 | 1.49 (0.35) | 1971 | |
| Proteobacteria: Oxalobacteraceae Massilia | 2.5E-04 | -1.7 (0.4) | 1971 | F |
| Proteobacteria: Pseudomodaceae Pseudomonas | 3.0E-04 | -1.64 (0.41) | 1971 | F |
| Proteobacteria: Pseudomodaceae Pseudomonas | 4.4E-04 | -1.64 (0.39) | 1971 | |
| Proteobacteria: Rhodocyclaceae uncultured | 4.0E-04 | -1.56 (0.38) | 2001 | |
| Proteobacteria: Rhodospirillaceae Defluviicoccus | 1.1E-04 | -1.66 (0.35) | 2001 | |
| R1 – Reference 1936 | | | | |
| Acidobacteria: uncultured BPC102 | 5.1E-04 | -1.52 (0.4) | 1971 | |
| Actinobacteria: 480-2 uncultured Solirubrobacterales | 8.8E-04 | 1.38 (0.35) | 1975 | |
| Actinobacteria: Iamiaceae Iamia | 4.1E-04 | -1.64 (0.44) | 1971 | |
| Actinobacteria: Iamiaceae Iamia | 3.3E-04 | 1.85 (0.5) | 1971 | E |
| Actinobacteria: Nocardioidaceae Marmoricola | 6.9E-03 | 0.74 (0.2) | 1975 | |
| Actinobacteria: Nocardioidaceae Nocardioides | 1.5E-03 | 1.33 (0.37) | 1975 | |
| Actinobacteria: Nocardioidaceae Nocardioides | 1.8E-03 | 1.65 (0.38) | 1975 | F |
| Actinobacteria: Streptomycetaceae Streptomyces | 4.3E-03 | 2.77 (0.55) | 1961 | |
| Bacteroidetes: Cytophagaceae Adhaeribacter | 1.8E-03 | 1.51 (0.32) | 1975 | |
| Bacteroidetes: Cytophagaceae Flexibacter | 7.4E-04 | 1.68 (0.41) | 1971 | |
| Bacteroidetes: Cytophagaceae Flexibacter | 1.7E-04 | 2.65 (0.61) | 1971 | |
| Chloroflexi: Aerolineaceae | 9.2E-05 | 2.24 (0.62) | 1971 | |
| Chloroflexi: Herpetosiphoceae Herpetosiphon | 1.4E-04 | 2.2 (0.59) | 1975 | |
| Chloroflexi: Herpetosiphoceae Herpetosiphon | 1.4E-04 | 2.78 (0.58) | 1971 | |
| Firmicutes: Thermoactinomycetaceae Shimazuella | 4.8E-04 | 2.65 (0.63) | 1961 | F |
| Gemmatimodetes: uncultured AT425-EubC11_terrestrial_group | 1.0E-04 | 2.43 (0.63) | 1971 | |
| Gemmatimodetes: Gemmatimodaceae uncultured | 2.7E-04 | 1.62 (0.45) | 1971 | |
| Gemmatimodetes: Gemmatimodaceae uncultured | 3.0E-04 | 1.97 (0.48) | 1971 | |
| Planctomycetes: uncultured OM190 | 3.4E-04 | -2.2 (0.46) | 1975 | |
| Planctomycetes: uncultured Pla4_lineage | 2.7E-04 | -1.94 (0.53) | 1975 | |
| Proteobacteria: uncultured Xanthomodales | 1.3E-04 | 2.37 (0.63) | 1971 | |
| Proteobacteria: Caulobacteraceae Asticcacaulis | 2.4E-04 | 2.62 (0.64) | 1971 | FE |
| Proteobacteria: Comamodaceae uncultured | 4.2E-04 | 2.22 (0.64) | 1971 | |
| Proteobacteria: Enterobacteriaceae | 4.4E-04 | 2.67 (0.64) | 1961 | F |
| Proteobacteria: Erythrobacteraceae Altererythrobacter | 4.0E-04 | 1.85 (0.42) | 1971 | E |
| Proteobacteria: nnocystineae Haliangiaceae | 3.0E-04 | -1.44 (0.39) | 1975 | |

| Taxa: | Mean abundance | $Log_2$-fold change | hybrid | Intersection* |
|---|---|---|---|---|
| Proteobacteria: Oxalobacteraceae Massilia | 3.5E-03 | 2.7 (0.62) | 1961 | F |
| Proteobacteria: Rhizobiaceae Rhizobium | 4.8E-04 | 2.73 (0.64) | 1961 | F |
| Proteobacteria: Rhodobacteraceae | 2.4E-04 | 2.67 (0.64) | 1971 | |
| Proteobacteria: Rhodobacteraceae Rhodobacter | 6.0E-04 | 1.57 (0.43) | 1975 | |
| Proteobacteria: Rhodobacteraceae Rhodobacter | 6.0E-04 | 1.84 (0.43) | 1971 | |
| Proteobacteria: Rhodocyclaceae Dechloromos | 6.5E-04 | 2.19 (0.52) | 1971 | |
| Proteobacteria: Sorangiineae uncultured | 5.2E-04 | 1.93 (0.45) | 1971 | |
| Proteobacteria: uncultured_bacterium uncultured B1-7BS | 1.4E-03 | -1.11 (0.32) | 1971 | |
| Proteobacteria: uncultured_bacterium uncultured Sh765B-TzT-29 | 2.1E-04 | -1.75 (0.48) | 1975 | |
| Proteobacteria: Xanthomodaceae Stenotrophomos | 1.6E-04 | 2.66 (0.61) | 1971 | F |
| Verrucomicrobia: uncultured OPB35_soil_group | 3.6E-04 | -1.66 (0.45) | 1975 | |
| Verrucomicrobia: uncultured OPB35_soil_group | 7.9E-04 | 1.5 (0.4) | 1971 | F |
| Verrucomicrobia: Verrucomicrobiaceae uncultured | 8.1E-05 | 2.17 (0.6) | 1975 | |
| Verrucomicrobia: Xiphinematobacteraceae Candidatus_Xiphinematobacter | 5.5E-03 | -0.93 (0.26) | 1971 | |
| R3 – Reference 1939 | | | | |
| Actinobacteria: Streptomycetaceae Streptomyces | 2.2E-04 | 1.89 (0.42) | 2001 | |
| Actinobacteria: uncultured_bacterium uncultured MB-A2-108 | 2.7E-04 | -1.03 (0.23) | 1971 | |
| Actinobacteria: uncultured_bacterium uncultured MB-A2-108 | 2.7E-04 | -0.99 (0.23) | 3475 | |
| Proteobacteria: 0319-6G20 uncultured_bacterium | 2.8E-04 | 1.71 (0.41) | 3475 | |
| Proteobacteria: Xanthomodaceae Lysobacter | 2.6E-03 | 1.02 (0.21) | 3475 | F |

*OTUs also differentially abundant between fertilizer treatments at R3 (F) or correlated with enzyme activity (E)

Table C9: OTUs differentially abundant in rhizosphere of fertilized plots compared to unfertilized controls at R3.

| Taxa: | Mean abundance | $Log_2$-fold change | Fertilizer - Contrast | Intersection * |
|---|---|---|---|---|
| Actinobacteria: uncultured Frankiales | 1.2E-04 | -1.15 (0.29) | 170 vs 0 | |
| Actinobacteria: uncultured Gaiellales | 2.3E-04 | 0.91 (0.2) | 170 vs 0 | |
| Actinobacteria: Glycomycetaceae | 2.4E-03 | 2.51 (0.32) | 170 vs 0 | |
| Actinobacteria: Intrasporangiaceae | 1.5E-04 | 1.31 (0.3) | 170 vs 0 | |
| Actinobacteria: Micrococcaceae Arthrobacter | 3.2E-04 | 1.4 (0.31) | 170 vs 0 | |
| Actinobacteria: Micrococcaceae Arthrobacter | 7.2E-03 | 0.64 (0.16) | 170 vs 0 | |
| Actinobacteria: Micromonosporaceae Dactylosporangium | 1.4E-03 | -0.54 (0.15) | 170 vs 0 | |
| Actinobacteria: Nocardiaceae Nocardia | 2.3E-04 | 1.16 (0.36) | 170 vs 0 | |
| Actinobacteria: Nocardioidaceae Kribbella | 2.5E-03 | 0.64 (0.16) | 170 vs 0 | |
| Actinobacteria: Nocardioidaceae Nocardioides | 1.8E-04 | 1.19 (0.37) | 170 vs 0 | |
| Actinobacteria: Nocardioidaceae Nocardioides | 4.3E-04 | 1.14 (0.26) | 170 vs 0 | |
| Actinobacteria: Nocardioidaceae Nocardioides | 1.2E-04 | 1.02 (0.32) | 170 vs 0 | |
| Actinobacteria: Nocardioidaceae Nocardioides | 2.7E-04 | 0.89 (0.24) | 170 vs 0 | |
| Actinobacteria: Nocardioidaceae Nocardioides | 1.5E-03 | 1.75 (0.21) | 170 vs 0 | |
| Actinobacteria: Nocardioidaceae Nocardioides | 8.6E-04 | 0.58 (0.18) | 170 vs 0 | |
| Actinobacteria: Nocardioidaceae Nocardioides | 2.0E-03 | 0.62 (0.19) | 170 vs 0 | H |
| Actinobacteria: Pseudonocardiaceae | 3.9E-05 | 1.6 (0.38) | 170 vs 0 | |
| Actinobacteria: Pseudonocardiaceae | 4.6E-04 | 0.88 (0.27) | 170 vs 0 | |
| Actinobacteria: Pseudonocardiaceae Actinosynnema | 7.3E-04 | 1.15 (0.31) | 170 vs 0 | |
| Actinobacteria: Pseudonocardiaceae Lentzea | 1.6E-02 | 0.72 (0.22) | 170 vs 0 | |
| Actinobacteria: Pseudonocardiaceae Pseudonocardia | 6.2E-04 | -0.49 (0.15) | 170 vs 0 | |
| Actinobacteria: Streptomycetaceae Streptomyces | 2.2E-04 | 1.11 (0.28) | 170 vs 0 | |
| Actinobacteria: Streptomycetaceae Streptomyces | 6.0E-05 | 1.01 (0.32) | 170 vs 0 | |
| Actinobacteria: Streptomycetaceae Streptomyces | 1.3E-04 | 0.99 (0.27) | 170 vs 0 | |
| Actinobacteria: Streptomycetaceae Streptomyces | 1.0E-03 | 1.69 (0.29) | 170 vs 0 | |
| Actinobacteria: Streptomycetaceae Streptomyces | 3.0E-02 | 1.14 (0.18) | 170 vs 0 | |
| Actinobacteria: Streptomycetaceae Streptomyces | 1.6E-02 | 1.71 (0.25) | 170 vs 0 | |
| Actinobacteria: Streptomycetaceae Streptomyces | 1.7E-02 | 0.54 (0.14) | 170 vs 0 | |
| Actinobacteria: Streptomycetaceae Streptomyces | 5.9E-04 | 1.58 (0.24) | 170 vs 0 | |
| Actinobacteria: Thermomonosporaceae Actinomadura | 1.3E-03 | 1.52 (0.27) | 170 vs 0 | |
| Bacteroidetes: uncultured Cytophagales | 1.3E-04 | 1.12 (0.3) | 170 vs 0 | |
| Bacteroidetes: Sphingobacteriaceae | 2.5E-04 | 1.92 (0.37) | 170 vs 0 | |
| Bacteroidetes: Sphingobacteriaceae Sphingobacterium | 6.3E-04 | 1.97 (0.34) | 170 vs 0 | |
| Candidate_division_TM7: uncultured Candidate_division_TM7 | 2.7E-05 | 1.23 (0.38) | 170 vs 0 | |

| Taxa: | Mean abundance | $Log_2$-fold change | Fertilizer - Contrast | Intersection * |
|---|---|---|---|---|
| Candidate_division_TM7: uncultured Candidate_division_TM7 | 2.2E-05 | 1.29 (0.38) | 170 vs 0 | |
| Chloroflexi: Chloroflexaceae Roseiflexus | 5.7E-04 | -0.65 (0.21) | 170 vs 0 | |
| Chloroflexi: Oscillochloridaceae Oscillochloris | 3.5E-04 | -1.11 (0.23) | 170 vs 0 | |
| Firmicutes: Thermoactinomycetaceae Shimazuella | 2.7E-04 | 0.94 (0.29) | 170 vs 0 | H |
| Planctomycetes: Planctomycetaceae Pirellula | 1.1E-03 | -0.47 (0.13) | 170 vs 0 | |
| Proteobacteria: uncultured Betaproteobacteria | 4.0E-05 | 1.3 (0.37) | 170 vs 0 | |
| Proteobacteria: uncultured Burkholderiales | 3.1E-05 | 1.32 (0.39) | 170 vs 0 | |
| Proteobacteria: uncultured DB1-14 | 1.0E-04 | 1.61 (0.38) | 170 vs 0 | |
| Proteobacteria: uncultured GR-WP33-30 | 1.7E-03 | 0.31 (0.09) | 85 vs 0 | |
| Proteobacteria: Bacteriovoracaceae Bacteriovorax | 8.9E-05 | 1.4 (0.31) | 170 vs 0 | |
| Proteobacteria: Bdellovibrioceae Bdellovibrio | 1.2E-04 | 2.03 (0.38) | 170 vs 0 | |
| Proteobacteria: Bdellovibrioceae Bdellovibrio | 5.0E-05 | 1.47 (0.38) | 170 vs 0 | |
| Proteobacteria: Caulobacteraceae Asticcacaulis | 7.2E-04 | 1.33 (0.3) | 170 vs 0 | HE |
| Proteobacteria: Caulobacteraceae Brevundimos | 8.1E-05 | 1.2 (0.36) | 170 vs 0 | |
| Proteobacteria: Caulobacteraceae Caulobacter | 2.5E-03 | 1.41 (0.29) | 170 vs 0 | |
| Proteobacteria: Comamodaceae Hydrogenophaga | 1.0E-04 | 1.7 (0.34) | 170 vs 0 | |
| Proteobacteria: Comamodaceae Pelomos | 2.1E-02 | 1.09 (0.2) | 170 vs 0 | |
| Proteobacteria: Comamodaceae uncultured | 3.1E-03 | 1.24 (0.29) | 170 vs 0 | |
| Proteobacteria: Comamodaceae uncultured | 3.6E-03 | 0.59 (0.16) | 170 vs 0 | |
| Proteobacteria: Comamodaceae uncultured | 9.0E-05 | 1.2 (0.34) | 170 vs 0 | |
| Proteobacteria: Comamodaceae uncultured | 4.5E-04 | 1.52 (0.3) | 170 vs 0 | |
| Proteobacteria: Comamodaceae Variovorax | 3.8E-05 | 1.19 (0.36) | 170 vs 0 | |
| Proteobacteria: Coxiellaceae Aquicella | 5.5E-05 | -1.1 (0.33) | 170 vs 0 | |
| Proteobacteria: Cystobacterineae uncultured | 3.2E-04 | -0.79 (0.21) | 170 vs 0 | |
| Proteobacteria: Enterobacteriaceae | 5.3E-04 | 1.14 (0.36) | 170 vs 0 | H |
| Proteobacteria: Enterobacteriaceae Escherichia-Shigella | 5.1E-04 | 1.42 (0.39) | 85 vs 0 | |
| Proteobacteria: Hyphomicrobiaceae Devosia | 2.1E-04 | 1.25 (0.31) | 170 vs 0 | |
| Proteobacteria: Hyphomicrobiaceae Devosia | 2.0E-04 | 1.82 (0.34) | 170 vs 0 | |
| Proteobacteria: Hyphomicrobiaceae Filomicrobium | 6.0E-04 | 1.33 (0.29) | 170 vs 0 | |
| Proteobacteria: Hyphomicrobiaceae uncultured | 6.2E-04 | 0.89 (0.24) | 170 vs 0 | |
| Proteobacteria: Methylophilaceae | 2.4E-04 | 1.11 (0.34) | 170 vs 0 | |
| Proteobacteria: nnocystineae Haliangiaceae | 3.1E-05 | -1.34 (0.38) | 170 vs 0 | |
| Proteobacteria: nnocystineae nnocystaceae | 4.1E-05 | -1.29 (0.37) | 170 vs 0 | |
| Proteobacteria: Oxalobacteraceae Massilia | 3.0E-04 | 1.56 (0.29) | 170 vs 0 | |
| Proteobacteria: Oxalobacteraceae Massilia | 2.2E-03 | 1.89 (0.3) | 170 vs 0 | H |
| Proteobacteria: Oxalobacteraceae Massilia | 1.7E-04 | 1.86 (0.34) | 170 vs 0 | H |
| Proteobacteria: Oxalobacteraceae Massilia | 2.7E-04 | 1.32 (0.36) | 170 vs 0 | |

| Taxa: | Mean abundance | $Log_2$-fold change | Fertilizer - Contrast | Intersection * |
|---|---|---|---|---|
| Proteobacteria: Oxalobacteraceae Massilia | 1.5E-03 | 1.75 (0.33) | 170 vs 0 | |
| Proteobacteria: Oxalobacteraceae uncultured | 9.6E-03 | 1.92 (0.2) | 170 vs 0 | |
| Proteobacteria: Phyllobacteriaceae Phyllobacterium | 1.5E-04 | 1.07 (0.31) | 170 vs 0 | |
| Proteobacteria: Pseudomodaceae Pseudomonas | 1.5E-04 | 1.43 (0.39) | 170 vs 0 | |
| Proteobacteria: Pseudomodaceae Pseudomonas | 1.4E-03 | 1.24 (0.31) | 170 vs 0 | |
| Proteobacteria: Pseudomodaceae Pseudomonas | 1.4E-04 | 1.38 (0.36) | 85 vs 0 | H |
| Proteobacteria: Rhizobiaceae Rhizobium | 9.4E-04 | 1.71 (0.34) | 170 vs 0 | H |
| Proteobacteria: Rhizobiaceae Rhizobium | 1.0E-03 | 2.24 (0.32) | 170 vs 0 | |
| Proteobacteria: Rhizobiaceae Rhizobium | 1.0E-04 | 1.3 (0.36) | 170 vs 0 | |
| Proteobacteria: Rhizobiaceae Rhizobium | 4.6E-05 | 1.36 (0.37) | 170 vs 0 | |
| Proteobacteria: Rhizobiaceae Rhizobium | 2.8E-03 | 1.25 (0.22) | 170 vs 0 | |
| Proteobacteria: Rhizobiaceae Shinella | 5.2E-03 | 1.09 (0.19) | 170 vs 0 | |
| Proteobacteria: Rhodospirillaceae Dongia | 1.3E-04 | -1.39 (0.33) | 85 vs 0 | |
| Proteobacteria: SM2D12 uncultured SM2D12 | 6.1E-05 | -1.15 (0.33) | 170 vs 0 | |
| Proteobacteria: SM2D12 uncultured SM2D12 | 9.2E-04 | 0.84 (0.26) | 170 vs 0 | |
| Proteobacteria: Sorangiineae Phaselicystidaceae | 2.2E-04 | -1.3 (0.29) | 170 vs 0 | |
| Proteobacteria: Sorangiineae Polyangiaceae | 2.7E-04 | -0.9 (0.28) | 170 vs 0 | |
| Proteobacteria: Sphingomodaceae Sphingobium | 9.5E-03 | 1 (0.27) | 170 vs 0 | |
| Proteobacteria: Sphingomodaceae Sphingomos | 6.7E-04 | 0.59 (0.18) | 170 vs 0 | |
| Proteobacteria: Sphingomodaceae Sphingomos | 8.8E-04 | 1.17 (0.29) | 170 vs 0 | |
| Proteobacteria: uncultured_delta_proteobacterium uncultured GR-WP33-30 | 9.9E-05 | -0.96 (0.28) | 170 vs 0 | |
| Proteobacteria: Xanthobacteraceae uncultured | 5.1E-04 | -0.46 (0.14) | 170 vs 0 | |
| Proteobacteria: Xanthobacteraceae uncultured | 2.3E-03 | -0.32 (0.09) | 170 vs 0 | |
| Proteobacteria: Xanthomodaceae Arenimos | 7.3E-04 | 1.21 (0.25) | 170 vs 0 | |
| Proteobacteria: Xanthomodaceae Lysobacter | 2.6E-03 | 0.67 (0.15) | 170 vs 0 | H |
| Proteobacteria: Xanthomodaceae Stenotrophomos | 5.9E-04 | 1.58 (0.38) | 170 vs 0 | |
| Proteobacteria: Xanthomodaceae Stenotrophomos | 2.0E-04 | 1.21 (0.36) | 170 vs 0 | H |
| Proteobacteria: Xanthomodaceae Thermomos | 5.8E-04 | 0.79 (0.21) | 170 vs 0 | |
| Proteobacteria: Xanthomodaceae Thermomos | 6.1E-04 | 1.12 (0.25) | 170 vs 0 | |
| Proteobacteria: Xanthomodaceae Xanthomos | 1.3E-04 | 1.36 (0.37) | 170 vs 0 | |
| Verrucomicrobia: uncultured OPB35_soil_group | 7.9E-04 | 0.68 (0.17) | 85 vs 0 | H |
| Verrucomicrobia: uncultured OPB35_soil_group | 4.9E-04 | -0.68 (0.16) | 170 vs 0 | |
| Verrucomicrobia: uncultured OPB35_soil_group | 1.6E-03 | 0.49 (0.13) | 85 vs 0 | |
| Verrucomicrobia: Chthoniobacteraceae Chthoniobacter | 1.0E-03 | -0.47 (0.15) | 170 vs 0 | |
| Verrucomicrobia: Opitutaceae Opitutus | 5.1E-04 | -1.13 (0.27) | 170 vs 0 | |
| Verrucomicrobia: Verrucomicrobiaceae Haloferula | 1.4E-03 | 0.68 (0.19) | 170 vs 0 | |

| Taxa: | Mean abundance | $Log_2$-fold change | Fertilizer - Contrast | Intersection * |
|---|---|---|---|---|
| Verrucomicrobia: Verrucomicrobiaceae Luteolibacter | 7.3E-03 | 2.33 (0.25) | 170 vs 0 | |
| Verrucomicrobia: Verrucomicrobiaceae Prosthecobacter | 6.3E-05 | -1.36 (0.34) | 85 vs 0 | |
| Verrucomicrobia: Verrucomicrobiaceae uncultured | 1.1E-04 | -1.15 (0.29) | 170 vs 0 | |
| WCHB1-60: uncultured WCHB1-60 | 3.7E-05 | -1.47 (0.38) | 170 vs 0 | |

*OTUs also differentially abundant between maize hybrids (H) or correlated with enzyme activity (E)

Table C10: OTUs correlated with primary or secondary principle components of variation in enzyme profiles of rhizosphere samples at one of three time points.

| Taxa: | Mean abundance | $Log_2$-fold change | Test | Intersection* |
|---|---|---|---|---|
| Acidobacteria: uncultured DA023 | 3.1E-04 | 0.26 (0.05) | T1: PCA1 | |
| Acidobacteria: uncultured DA023 | 1.5E-04 | 0.19 (0.05) | T1: PCA1 | |
| Acidobacteria: uncultured DA023 | 2.2E-03 | -0.14 (0.04) | T3: PCA1 | |
| Acidobacteria: uncultured DA023 | 1.2E-03 | -0.13 (0.03) | T3: PCA1 | |
| Acidobacteria: uncultured DA023 | 6.0E-04 | -0.13 (0.04) | T3: PCA1 | |
| Acidobacteria: uncultured Holophagae | 4.8E-04 | 0.14 (0.04) | T1: PCA1 | |
| Actinobacteria: uncultured Acidimicrobiia | 1.5E-03 | 0.1 (0.03) | T3: PCA1 | |
| Actinobacteria: uncultured Gaiellales | 2.3E-04 | 0.18 (0.05) | T1: PCA1 | |
| Actinobacteria: uncultured Gaiellales | 1.0E-03 | 0.12 (0.04) | T3: PCA1 | |
| Actinobacteria: 0319-6M6 uncultured Solirubrobacterales | 2.0E-03 | -0.08 (0.02) | T1: PCA1 | |
| Actinobacteria: Candidatus_Microthrix | 1.3E-04 | 0.18 (0.05) | T1: PCA1 | |
| Actinobacteria: Cellulomodaceae Cellulomos | 8.2E-04 | -0.14 (0.04) | T3: PCA1 | |
| Actinobacteria: Iamiaceae Iamia | 3.0E-04 | -0.16 (0.05) | T3: PCA1 | H |
| Actinobacteria: Microbacteriaceae Microbacterium | 1.4E-03 | -0.14 (0.04) | T3: PCA1 | |
| Actinobacteria: Propionibacteriaceae Jiangella | 1.4E-03 | 0.17 (0.05) | T3: PCA1 | |
| Actinobacteria: Streptomycetaceae Streptomyces | 3.9E-04 | 0.17 (0.05) | T3: PCA1 | |
| Actinobacteria: uncultured_bacterium uncultured PeM15 | 3.0E-04 | -0.27 (0.05) | T3: PCA1 | |
| Bacteroidetes: Chitinophagaceae Ferruginibacter | 7.6E-05 | 0.63 (0.12) | T2: PCA2 | |
| Chloroflexi: uncultured KD4-96 | 3.3E-04 | 0.16 (0.04) | T1: PCA1 | |
| Chloroflexi: uncultured S085 | 4.0E-04 | 0.13 (0.04) | T1: PCA1 | |
| Chloroflexi: uncultured TK10 | 3.9E-04 | 0.14 (0.04) | T1: PCA1 | |
| Chloroflexi: Chloroflexaceae Roseiflexus | 1.0E-03 | 0.19 (0.05) | T3: PCA1 | |
| Chloroflexi: uncultured_bacterium uncultured C0119 | 4.0E-04 | 0.16 (0.04) | T3: PCA1 | |
| Chloroflexi: uncultured_bacterium uncultured JG30-KF-CM45 | 1.1E-03 | -0.18 (0.05) | T3: PCA1 | |
| Gemmatimodetes: uncultured S0134_terrestrial_group | 1.6E-04 | -0.21 (0.05) | T1: PCA1 | |
| Nitrospirae: 0319-6A21 uncultured 0319-6A21 | 3.0E-04 | 0.18 (0.05) | T3: PCA1 | |
| Planctomycetes: Planctomycetaceae uncultured | 5.8E-04 | 0.13 (0.04) | T3: PCA1 | |
| Proteobacteria: uncultured SC-I-84 | 1.0E-04 | 0.25 (0.05) | T1: PCA1 | |
| Proteobacteria: Caulobacteraceae Asticcacaulis | 3.3E-04 | -0.37 (0.07) | T2: PCA1 | HF |
| Proteobacteria: Erythrobacteraceae Altererythrobacter | 4.3E-04 | -0.19 (0.04) | T3: PCA1 | H |
| Proteobacteria: Nitrosomodaceae uncultured | 1.3E-03 | 0.11 (0.03) | T3: PCA1 | |
| Proteobacteria: Rhodobacteraceae uncultured | 2.8E-04 | 0.14 (0.04) | T1: PCA1 | |
| Verrucomicrobia: uncultured OPB35_soil_group | 4.0E-04 | 0.17 (0.05) | T1: PCA1 | |
| Verrucomicrobia: Verrucomicrobiaceae uncultured | 6.4E-04 | 0.16 (0.04) | T1: PCA1 | |

*OTUs also differentially abundant between maize hybrids (H) or fertilizer treatments at R3 (F)

Notes C1: Breeding influence on maize nitrogen use efficiency and implications for rhizosphere traits

Increased N uptake of modern hybrids was a result of combined increase in both pre- and post-anthesis N uptake, though neither term was statistically significant on its own ($p = 0.10$ and $p = 0.06$, respectively; Figure S2; see Table S4 for effect tests). In addition to the observed increase in nitrogen (N) uptake, there was also an increase in the efficiency with which plant N is converted to grain yield (Nitrogen utilization efficiency (NUtE); $p < 0.01$), which reflects several changes in biomass and N partitioning in modern hybrids. Modern hybrids produced more grain per unit plant N than older hybrids at low and high N availability, but NUtE was relatively constant at the intermediate N fertilization level (F x Y: $p = 0.06$). The interaction points to different mechanisms supporting NUtE at high and low fertility. The increase in NUtE at 0 kg N $ha^{-1}$ was associated with an increased fraction of plant biomass and nitrogen partitioned to grain production in modern hybrids (Figure S2). This is consistent with the widely observed improvement in kernel set and decrease in barren plants of modern hybrids under N stress (Haegele *et al.*, 2013; DeBruin *et al.*, 2017). Under higher N conditions the fraction of biomass partitioned to grain increased with year of release, while N partitioning remained constant. This contrast reflects the greater total N retained in the stover of modern hybrids under fertilized conditions (Y x N: $p = 0.01$). Under adequate fertility, delayed senescence of leaves in modern hybrids decreases remobilization of leaf N (DeBruin *et al.*, 2017), which results in a lower N harvest index and higher amount of N retained in stover at physiological maturity (Figure S2). This continued leaf activity provides for greater C transfer to the ear and for continued root activity indicated by increased post-anthesis N uptake ($p = 0.06$; Figure S2). Meanwhile, percent

N content of grain has decreased over year of hybrid release ($p < 0.01$) indicating that more grain can be produced per unit N translocated to the ear. However, total N exported in grain was still greater for modern hybrids as a result of the higher yields. These changes in maize physiology have been important to yield improvements in maize (Duvick, 2005) and can also inform our understanding of how breeding may interact with rhizosphere processes. Changes in signaling and a decrease in the anthesis-silking interval improve kernel set and provide a sink for plant C and N (Duvick *et al.*, 2004; Haegele *et al.*, 2013; DeBruin *et al.*, 2017), but does necessarily impact root traits. Thus, yield improvements under stressful conditions imposed by high plant density, low nitrogen availability or water limitation are possible without altering plant root traits. On the other hand, delayed senescence of leaves prolongs photosynthetic and root activity in the growing season during periods where soil N supply is more limited, which may provide a positive selection pressure on root and rhizosphere traits that support plant acquisition from soil N pools during this period.

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