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Rooting for Microbes: Impacts of Plant Breeding on the Kernza Rhizosphere

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ABSTRACT

Aims Kernza (*Thinopyrum intermedium)* is among the first perennial grain crops ever bred and has the potential to address challenges associated with annual agriculture, in part due to its robust microbial partnerships and soil health benefits. However, breeding programs generally select plants based on aboveground traits such as seed size, resulting in unintended consequences on belowground traits such as root exudation and microbial symbioses. This study aimed to investigate the impact of plant breeding on the rhizosphere environment and microbial community of the novel grain crop Kernza.

Methods This study is the first to investigate a crop's rhizosphere environment across breeding cycles, starting with a wild-type population. We collected rhizosphere soil from Kernza plants across nine cycles of selection for increased yield and harvestability, and analyzed labile organic matter pools, potential enzyme activities, phospholipid fatty acids, and DNA from bacteria and arbuscular mycorrhizal fungi.

Results We found that plant breeding altered bacterial and arbuscular mycorrhizal fungal communities in the rhizosphere. Selection also reduced rhizosphere labile organic matter and microbial biomass, but not microbial activity, which instead increased across later breeding cycles.

Conclusions Our results suggest that the Kernza breeding program created a less abundant but more active rhizosphere microbial community, potentially due to impacts on plants' stress tolerance, resource allocation, or resource-use strategies.

INTRODUCTION

Perennial grasslands build some of the world's most fertile soils, in part by supporting robust microbial communities (Crews 2021; Sprunger et al. 2024). However, over 40% of global prairie ecosystems have been converted to croplands (70% of which grow annual grains), making these ecosystems highly vulnerable to erosion and loss of soil organic matter (White et al. 2000; Crews 2021). Unlike perennial prairie, annual crops generally rely on external inputs such as fertilizers and pesticides and lose topsoil over time (Rodgers et al. 2021; Sprunger et al. 2024). Annual crops also cannot provide the same ecosystem services as perennials; they tend to be more vulnerable to climatic changes, use water and nutrients less efficiently, and support less biodiversity (de Vries et al. 2012; Culman et al. 2013; DuPont et al. 2014). In comparing a harvested perennial grassland to a winter wheat field in Kansas, Glover et al. (2010) found that the perennial grassland yielded more nitrogen (N), supported higher insect

and microbial biodiversity, and stored more soil organic carbon (SOC), while only requiring 8% of the in-field energy costs. In the past 30 years, efforts were launched to breed perennial grain crops that can mimic native ecosystems and their associated ecosystem services, an approach known as Natural Systems Agriculture.

Kernza, the first developed perennial grain crop, is bred from intermediate wheatgrass (*Thinopyrum intermedium*) and has been shown to reverse land degradation attributed to annual grain cropping (Bell et al. 2010; Duchene et al. 2019). Kernza is generally grown for three to five years and can substitute annual grains in products such as breads, cereals, and beer. Its extensive root system supports soil health, efficient nutrient cycling, and a soil microbiome more similar to undisturbed perennial prairie than to annual wheat (Sprunger et al. 2019; McKenna et al. 2020; Peixoto et al. 2020). The soil microbiome is key to the success of any crop; microorganisms associated with plant roots transform nutrients into plant-available forms, protect plants from pathogens, and stabilize carbon (C) (Sharma et al. 2013; Trivedi et al. 2020). These plant-microbial interactions occur mainly in the rhizosphere, the soil zone surrounding roots, and are as integral to plant health as the gut microbiome is to human health (Ramírez-Puebla et al. 2013). A robust microbiome helps plants adapt to variable conditions such as drought, creating food systems that are more resilient in the face of climate change (Vries and Wallenstein 2017; IPCC 2019).

However, plant domestication has profoundly altered plant-microbial interactions and in some cases created crops less resilient to environmental stresses than their wild relatives (Wissuwa et al. 2009; Philippot et al. 2013). Plants exude up to 20% of fixed C and 15% of N through their roots (Sasse et al. 2018), and exudation increases when plants experience stressors such as drought and nutrient deficiency (Preece and Peñuelas 2020). Plant breeding for increased yield alters root exudate composition and root morphology, which in turn shapes the rhizosphere microbial community (Walker et al. 2003; Preece and Peñuelas 2020; Nerva et al. 2022). If crops are bred in low-stress, high-nutrient environments, breeding programs can inadvertently weaken stress tolerance and reduce reliance on microbial partners such as arbuscular mycorrhizal fungi (AMF) (Martín-Robles et al. 2018; Preece and Peñuelas 2020). Additionally, breeding for higher seed yield has been shown to shift resource allocation away from roots and reduce nutrient-use efficiency in favor of fast growth (Vilela and González-Paleo 2015; Pastor-Pastor et al. 2019; Gonzalez-Paleo et al. 2023). However, interest has been growing in developing plant breeding programs to fortify plant-microbial associations, in order to reduce reliance on agricultural inputs and bolster resilience to climate change (Sessitsch and Mitter 2015; Vries and Wallenstein 2017; Trivedi et al. 2020). Before these breeding efforts

can be successful, we must first understand what constitutes a "healthy" rhizosphere and how it has been impacted by plant breeding.

Our study is the first to compare the rhizosphere environment of a novel crop across breeding cycles, starting from a wild-type population. We analyzed labile organic matter, microbial biomass, enzyme activity, and bacterial and AMF community composition in rhizosphere soil from plants representing nine populations of the Kernza breeding program, ranging from wild-type intermediate wheatgrass (cycle 0) to a population that had undergone nine cycles of selection for traits related to yield and harvestability (cycle 9). Our results indicate that the Kernza breeding program decreased rhizosphere labile organic matter and microbial biomass and altered microbial community composition and activity, creating a plant with a more active, though less abundant, rhizosphere microbiome.

MATERIALS AND METHODS

2.1 Site Description & Experimental Design

Soil samples were collected in July 2022 in research plots managed by The Land Institute in Salina, Kansas (38°46'14.2"N, 97°34'06.4"W, 380m elevation). The soil is classified as a mixed, active, mesic Udorthentic Haplustoll (Soil Survey Staff, NRCS 2023) with a silt loam texture, containing 22.5% clay, 2.2% SOC, 1.8% calcium carbonate, and pH of 7.96. The region experiences a humid continental climate, with mean annual precipitation of 786 mm and mean monthly temperatures ranging from -0.6 ºC in January to 27.0 ºC in July (Kansas State University 2023). Seed for the experiment came from The Land Institute's seed vault (stored at 9.4 °C and 19% humidity) and included plants from each of the first nine breeding cycles. The Land Institute's breeding program began with a population of wild-type intermediate wheatgrass (*Thinopyrum intermedium)* (cycle 0) planted in 2002. This population was bred for traits including yield, seed size, shatter resistance, and threshability. Selections for each cycle took 1-3 years, with cycle 9 planted in 2019. Grain yield increased by 140% across the first five cycles, with continued increases thereafter. Details on the breeding program are available in DeHaan et al. (2018). To initiate this study, 4-6 plants per cycle were planted into greenhouse pots containing PRO-MIX BX potting soil (Premier Tech, Rivière-du-Loup, Quebec) in spring 2019. In October 2019, plants were transplanted into an outdoor field in a randomized order, fertilized at 70 kg ha^{-1} N, 50 kg ha^{-1} P, and 15.7 kg ha^{-1} S, which represents a

97 standard rate for similar crops, and left to grow for an additional 2.5 years before sampling. Subsurface irrigation
98 was applied as needed to prevent wilting.
99 *2.2 Rhizosphere Soil Sampling*
100 In July 2022, roots were excavated from beneath 3-5 plants from each breeding cycle (total n = 45) to a
101 depth of 30 cm. Roots were shaken to remove soil that did not adhere tightly, and the remaining soil adhering to the
102 roots (referred to as rhizosphere soil) was brushed and shaken into sterilized buckets by hand, then transferred into
103 sterile Whirl-Pak bags. Soil was stored on ice for transport to the laboratory and then sieved to 2 mm. Field-moist
104 analyses were initiated on subsamples within 36 hrs (gravimetric water content, dissolved organic C (DOC),
105 dissolved organic N (DON), nitrate (NO_3^-), and potentially mineralizable N (PMN)). Additional subsamples were
106 frozen at -20 °C for microbial analyses (phospholipid fatty acids (PLFA), DNA, and enzyme activities) and the
107 remainder was air-dried for analysis of pH, electrical conductivity (EC), soil protein, permanganate oxidizable C
108 (POXC), total C and N, inorganic C, and texture.
109 *2.3 Soil Analyses*
110 Gravimetric moisture was analyzed by oven-drying at 105 °C for 24 hrs. DOC, DON, and NO_3^- were
111 extracted with 0.5 M potassium sulfate (K_2SO_4) in a 1:5 (w:v) soil:solution mixture, horizontally shaken for 30
112 minutes at 200 rpm, filtered through Q5 filter paper (Fisher Scientific, Waltham, MA). Extracts were stored at -20
113 ^oC until analysis. DOC and DON were quantified using combustion catalytic oxidation/non-dispersive infrared
114 (NDIR) detection on a Shimadzu TOC-VCSH (Shimadzu Corporation, Kyoto, Japan) (Newcomb and Carrilo 2011).
115 NO_3^- was analyzed by reaction with vanadium chloride (VCl_3) and quantification on a Synergy HTX multimode
116 reader with absorbance measurement at 540 nm (BioTek, Inc., Winooski, VT) (Doane and Horwáth 2003). PMN
117 was analyzed by 14-day anaerobic incubation and quantification on a Lachat QuickChem 8500 Series 2 Flow
118 Injection Analysis System (Lachat Instruments, Milwaukee, WI) (Hart et al. 1994). Soil pH and EC were analyzed
119 by electrode in a 1:2 (w:v) soil:deionized water mixture (Thermo Scientific Orion Start A215). Soil protein was
120 analyzed using the autoclaved citrate-extractable (ACE) protein method and quantified on a Synergy HTX
121 multimode reader with absorbance measurement at 562 nm (BioTek, Inc., Winooski, VT) (Hurisso et al. 2018).
122 POXC was analyzed by reaction with 0.2 M potassium permanganate ($KMnO_4$) and quantification on a Synergy
123 HTX multimode reader at 550 nm (BioTek, Inc., Winooski, VT) (Weil et al. 2003). Total C & N were analyzed by
124 dry combustion analysis on a LECO C/N Analyzer 928 Series (LECO Corporation, St. Joseph, MI). Inorganic C was

analyzed by reaction with 6 M HCl with 3% $FeCl_2$ and CO_2 quantification on a LI-COR LI-820 CO_2 Gas Analyzer (LI-COR Biosciences, Lincoln, NE) (Sherrod et al. 2002). SOC was calculated as the difference between total C and inorganic C. Texture was analyzed by hydrometer on three composite rhizosphere samples, each consisting of two samples from the north end, south end, or middle of the field (Gee and Bauder 1986).

PLFAs were extracted from 1 g of freeze-dried soils using a modified Bligh-Dyer method following the NEON protocol (Jackoway 2020) and analyzed on an Agilent 6890 gas chromatograph with autosampler, split-splitless injector, and flame ionization detector (Agilent, Santa Clara, CA). Peak identification was done using the MIDI Sherlock software, with quantification using the internal standard 19:0 and peak matching to microbial functional groups according to Joergensen (2022). Extracellular enzyme activities were analyzed by reaction with the appropriate 4-methylumbelliferone (MUB) or 7-amido-4-methylcoumarin (AMC) labeled substrate and fluorescence measurement on a Synergy HTX multimode reader (BioTek Instruments, Inc., Winooski, VT) at excitation wavelength of 360 nm and an emission wavelength of 450 nm, as in Custer et al. (2020). Fluorescence conversions were based on measurements of standards (10 µM) of MUB and AMC. The seven enzymes measured are involved in the degradation of a range of organic compounds: cellobiohydrolase (CBH) and β-glucosidase (BG) target cellulose, β-xylosidase (BX) targets hemicellulose, α-glucosidase (AG) targets starch, leucine aminopeptidase (LAP) targets polypeptides, phosphatase (PHOS) targets phosphate esters, and arylsulfatase (SUL) targets ester sulfates.

2.4 DNA Extraction, sequencing, and processing

DNA was extracted from 0.25 g of soil using a Qiagen DNeasy PowerSoil Pro Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions, and stored at -20 °C until library prep. Concentration and quality of extracted DNA was checked on a Synergy HTX multi-mode reader (BioTek Instruments, Inc., Winooski, VT). Library preparation and PCR were conducted with a 1-step library protocol, with 9 µl of PCR master mix (3 µl 5X KAPA HiFi Buffer, 0.45 µl 10M dNTPs, 0.3 µl Kapa HiFi HotStart DNA Polymerase, and 5.25 µl HPLC H_2O) combined with 2 µl of 0.75 µM paired primers and 2 µl of normalized (10 ng μl^{-1}) DNA. Unique barcodes for each sample were contained within the primers. The amplification step was performed using the following PCR conditions for bacteria: 95 °C for 3 min (1 cycle), 98 °C for 30 sec (35 cycles), 62 °C for 30 sec (35 cycles), 72 °C for 30 sec (35 cycles), 72 °C for 5 min (1 cycle), and hold at 4 °C. For AMF, the PCR conditions were: 95 °C for 10 min (1 cycle), 95 °C for 30 sec (35 cycles), 55 °C for 30 sec (35 cycles), 72 °C for 60 sec (35 cycles), 72 °C for 9

153 min (1 cycle), and hold at 4 °C. Following PCR, samples were purified using modified manual AxyPrep MagBead
154 PCR Clean-Up, and molar concentrations were checked on a Synergy HTX multimode reader using a Take3 Micro-
155 Volume Plate (BioTek Instruments, Inc., Winooski, VT). Samples were sequenced at the University of Wyoming
156 Genome Technologies Lab on a NextSeq 2000 using paired-end 2 x 300 chemistry. Bacteria were assessed by
157 amplifying the V4 region of the 16S rRNA gene using the primer set 515F (5'-GTGYCAGCMGCCGC GGTAA-3')
158 (Parada et al. 2016) and 806R (5'-GGACTACNVGGGTWTCTAAT-3') (Apprill et al. 2015). AMF were assessed
159 by amplifying a region of the 18S rRNA gene using the primer set AMV4.5NF (5'-
160 AAGCTCGTAGTTGAATTTCG-3') and AMDGR (3'-CCCAACTATCCCTATTAATCA-5') (Sato et al. 2005).
161 Initial sequence data processing was performed using Vsearch (Rognes et al. 2016). Raw reads were
162 trimmed of primers, forward and reverse reads were merged, and paired sequences were filtered, discarding reads
163 with more than one expected error. Reads were clustered into exact sequence variants (ESVs) using a 100%
164 similarity threshold. Contaminants were identified as taxa present at a higher mean abundance in blanks than
165 samples, and excluded from further analysis. Taxonomy was assigned for bacteria with the RDP 16s database
166 version 18 (Maidak et al. 1996) and for AMF using the MaarjAM 2019 database (Öpik et al. 2010).
167 *2.5 Statistical Analyses*
168 Data were analyzed using program R (version 4.3.1). All data and scripts are available at
169 github.com/hrodgers5/Kernza_Rhizosphere_2024, and genomic sequences are available on the NCBI sequence read
170 archive under BioProject ID PRJNA1173191. Soil chemistry, organic matter pools, enzyme activities, and PLFAs
171 were analyzed using generalized additive models (GAMs) in the *mgcv* package (Wood 2023), with model validation
172 using the *DHARMa* package (Hartig and Lohse 2022) and data visualization using *ggplot2* (Wickham et al. 2019).
173 GAMs included breeding cycle as a smoothed predictor term, five knots, and a Gamma or Gaussian distribution.
174 Enzymes that degrade C compounds (AG, BG, BX, CBH) were highly correlated (mean r = 0.88), and a scaled
175 mean (centered at 5) was calculated to represent C-cycling enzyme activity. P-cycling enzyme activity refers to
176 PHOS, N-cycling enzyme activity to LAP, and S-cycling enzyme activity to SUL. The sum of all PLFA biomarkers
177 was used to indicate total microbial biomass. Biomarkers were assigned to AMF, firmicutes, actinobacteria,
178 saprotrophic fungi (Zygomycota, Ascomycota, and Basidiomycota), total fungi, and total bacteria according to
179 Joergensen (2022), with the sum of assigned biomarkers indicating the biomass of each group. In addition, the ratio
180 of fungi: bacteria was calculated as fungal biomass / bacterial biomass and the ratio of AMF: saprotrophic fungi as

AMF biomass / saprotrophic fungal biomass. PLFA biomarker composition was analyzed using permutational multivariate analysis of variance (PERMANOVA) in the *vegan* package (Oksanen et al. 2022). Enzyme activity was also normalized per unit microbial biomass by dividing enzyme activities by the sum of all PLFAs.

For sequence data, alpha diversity was analyzed after rarefaction using the *phyloseq* package (McMurdie and Holmes 2013), including ESV richness, Shannon, Simpson, Fisher, Chao1, and ACE indices. Beta diversity was analyzed by conducting PERMANOVA on Bray-Curtis distance matrices and visualized by performing non-metric multidimensional scaling (NMDS) and correlating environmental variables with NMDS1 and 2 using the *vegan* package. Only environmental variables that were significantly impacted by plant breeding were correlated (DOC, DON, PMN, total microbial biomass, AMF biomass, and C, phosphorus (P) and S-cycling enzyme activities). Alpha and beta phylogenetic diversity were analyzed using Faith's phylogenetic diversity in the *picante* package and UniFrac distance in the *phyloseq* package, respectively.

Differential relative abundance of taxa across breeding cycles were analyzed using the *LinDA* package, which fits linear regression models on centered log-transformed data and corrects bias due to compositional effects (Zhou et al. 2022). Differential relative abundance was analyzed at the phylum and family level for bacteria and at the class and family level for AMF (AMF families perfectly matched the genus level). Ecological functions of the bacterial community were assigned according to FAPROTAX (Louca et al. 2016) using the *microeco* package (Liu et al. 2021), only considering functions involved in C- or N-cycling with mean relative abundance > 0.2% and excluding two redundant functions (methylotrophy and aerobic nitrite oxidation). The impact of breeding cycle on alpha diversity and ecological functions were analyzed using GAMs, as described above. Finally, distance decay analysis was performed in the *microeco* package to assess the correlation between samples' differences in microbial community (Bray-Curtis dissimilarity) and breeding cycle, and between differences in microbial community and environment (Euclidean distance of enzyme activities, organic matter pools, and total microbial biomass).

RESULTS

3.1 Effects of breeding cycle on rhizosphere organic matter and microbial biomass

Plant breeding (selection for yield and harvestability) decreased highly labile organic matter pools: PMN and DON decreased linearly across breeding cycles ($p = 0.035$ and $p = 0.047$, respectively) whereas DOC responded quadratically, with a minimum concentration at cycle 6 ($p = 0.012$) (Fig. 1, Table S1) . However, breeding cycle did

not affect semi-labile organic matter pools (soil protein or POXC), stable organic matter pools (SOC or total N), or soil chemistry (pH, EC, or NO_3^-) (Table S1). Based on PLFA measurements, total microbial biomass decreased linearly across breeding cycles by around 50%, with the steepest decline from cycle 0 to 5 ($p < 0.001$) (Fig. 2a, Table S1). The biomass of microbial functional groups (firmicutes, actinobacteria, AMF, saprotrophic fungi, total fungi, and total bacteria) were highly correlated with each other (mean r = 0.95) and each experienced a similar decline (Fig. 2b, Table S1). Breeding cycle did not impact the ratio of fungi:bacteria or of AMF:saprotrophic fungi (Table S1), but did affect the composition (PERMANOVA $p < 0.001$) and dispersion ($p = 0.020$) of PLFA biomarkers, with more homogeneous PLFA profiles in later breeding cycles (Fig. S1).

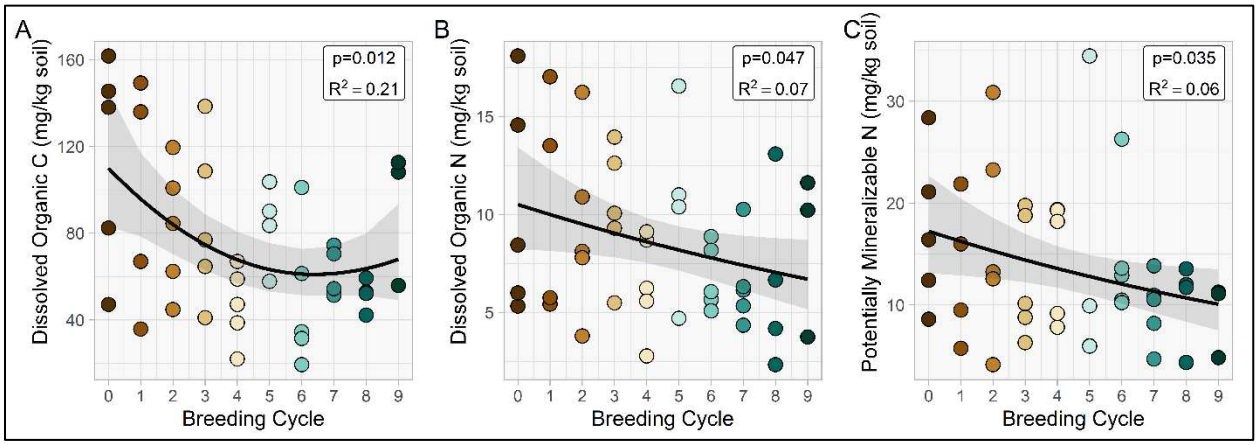

Figure 1. Effect of breeding cycle on dissolved organic C (A), dissolved organic N (B), and potentially mineralizable N (C). Predicted lines, confidence intervals, R^2 and p-values are from generalized additive models (GAMs) with breeding cycle as a smoothed term. C is carbon and N is nitrogen.

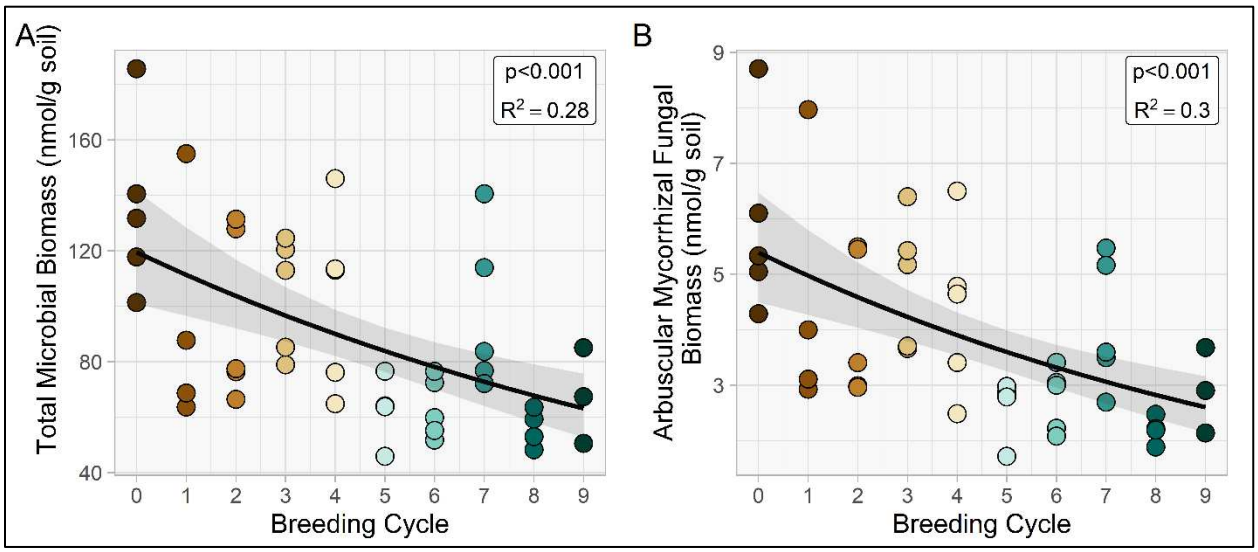

Figure 2. Effect of breeding cycle on total microbial biomass (A) and arbuscular mycorrhizal fungal (AMF) biomass (B) by phospholipid fatty acids (PLFAs). Predicted lines, confidence intervals, R^2 and p-values are from generalized additive models (GAMs) with breeding cycle as a smoothed term.

3.2 Effects of breeding cycle on rhizosphere microbial diversity and community composition

For bacteria, samples had between 24,000 and 109,000 total reads from 20 phyla. ESVs unassigned at the phylum level were removed (3,791 ESVs representing 11.0% of total reads), resulting in 18,262 total bacterial ESVs. For AMF, two samples with fewer than 1,670 reads were removed. Remaining AMF samples had between 6,800 and 106,000 total reads, all in the phylum *Mucoromycota*. ESVs unassigned at the class level were removed (963 ESVs representing 20.5% of total reads), resulting in 4,568 total AMF ESVs. For bacterial alpha diversity, breeding cycle decreased Chao1 and ACE diversity ($p = 0.034$ and $p = 0.037$, respectively) and marginally decreased ESV richness, Fisher diversity ($p = 0.073$ and $p = 0.075$, respectively), and Faith's phylogenetic diversity ($p = 0.060$) (Table S2). For AMF alpha diversity, breeding marginally affected Simpson diversity only ($p = 0.056$). For beta diversity, breeding cycle altered both bacterial and AMF community composition (PERMANOVA $p = 0.027$ and $p = 0.049$, respectively) (Fig. S2). Breeding cycle did not affect the dispersion of bacterial ESVs, and marginally decreased the dispersion of AMF ESVs ($p = 0.088$).

Breeding cycle marginally impacted AMF phylogenetic beta diversity (UniFrac distance) ($p = 0.068$). Mantel's distance decay analysis revealed that Bray-Curtis dissimilarity of community composition correlated to difference in breeding cycle for AMF (Mantel $p = 0.007$, $r = 0.14$) but not bacteria ($p > 0.05$). Conversely, Bray-Curtis dissimilarity of community composition correlated to environmental distance for bacteria ($p < 0.001$, $r = 0.39$) but not AMF ($p > 0.05$).

Breeding cycle did not impact the relative abundances of any taxa at the phylum level for bacteria or class level for AMF (Fig. 3). However, breeding decreased the relative abundance of two out of 192 total bacteria families (*Kribbellaceae* and *Pseudonocardiaceca*, $p_{adj} = 0.047$ and 0.028, respectively) and one out of seven total AMF families (*Glomeraceae*, $p_{adj} = 0.011$) (Fig. 4).

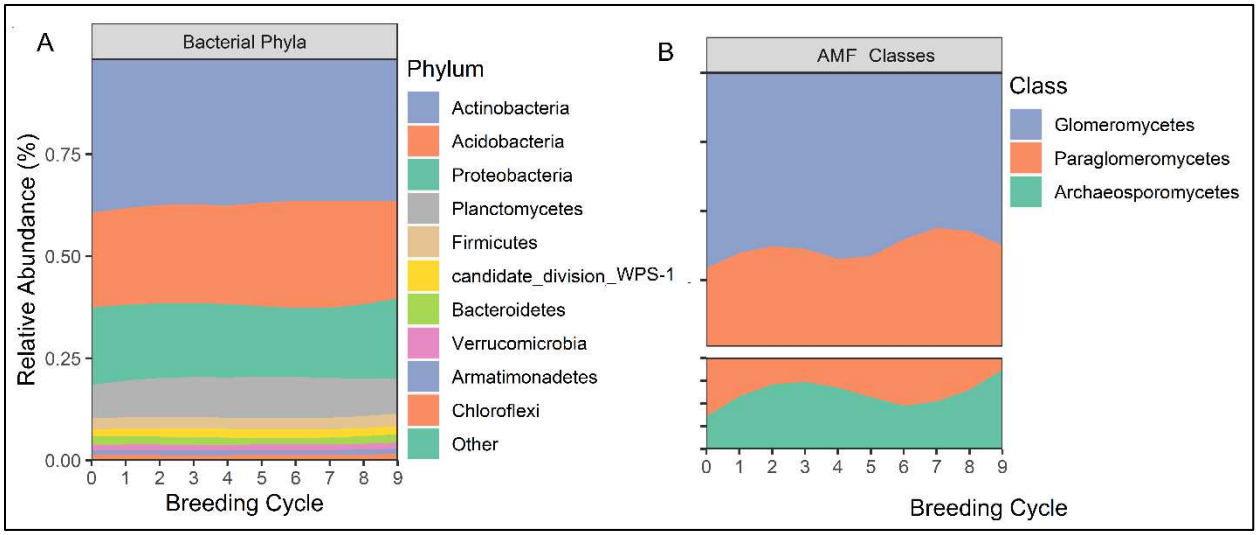

Figure 3. Relative abundance of bacterial phyla and arbuscular mycorrhizal fungi (AMF) classes across breeding cycles. "Other" represents the ten bacterial phyla of lowest abundance combined. Breeding cycle did not significantly impact the relative abundance of any bacterial phyla or AMF classes. Boundary lines between phyla represent smoothed means.

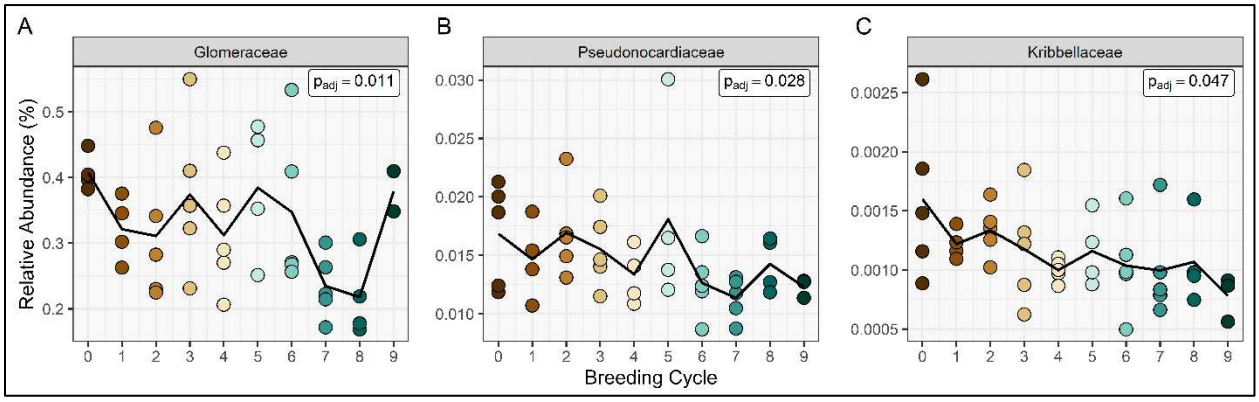

Figure 4. One arbuscular mycorrhizal fungi (AMF) family (*Glomeraceae,* A) and two bacterial families (*Pseudonocardiaceae and Kribbellaceae,* B & C*)* significantly impacted by breeding cycle. P-values are from linear regression models on centered log-transformed data with bias corrected due to compositional effects according to the *LinDA* package. Black line represents the smoothed mean.

3.3 Effects of breeding cycle on potential rhizosphere microbial activity and functional capacity

Regarding enzyme activities, PHOS decreased linearly across breeding cycles ($p < 0.001$), while LAP, SUL, and mean C-cycling activity responded nonlinearly, increasing from cycles 7-9 ($p = 0.067$, $p < 0.001$, and $p = 0.008$, respectively) (Fig. 5, Table S1). In contrast, for enzyme activity divided by microbial biomass, LAP and mean C-cycling activity per unit biomass increased linearly across breeding cycles ($p = 0.015$ and $p = 0.008$, respectively), while SUL per unit biomass responded nonlinearly, again increasing from cycles 7-9 ($p = 0.007$) (Fig. 6, Table S1). Microbial functional capacity predicted using genomic data indicated that two of the six functions

analyzed were marginally impacted by breeding cycle: hydrocarbon degradation and methanotrophy both responded nonlinearly ($p = 0.059$ and $p = 0.060$), increasing from cycles 7-9 (Fig. 7).

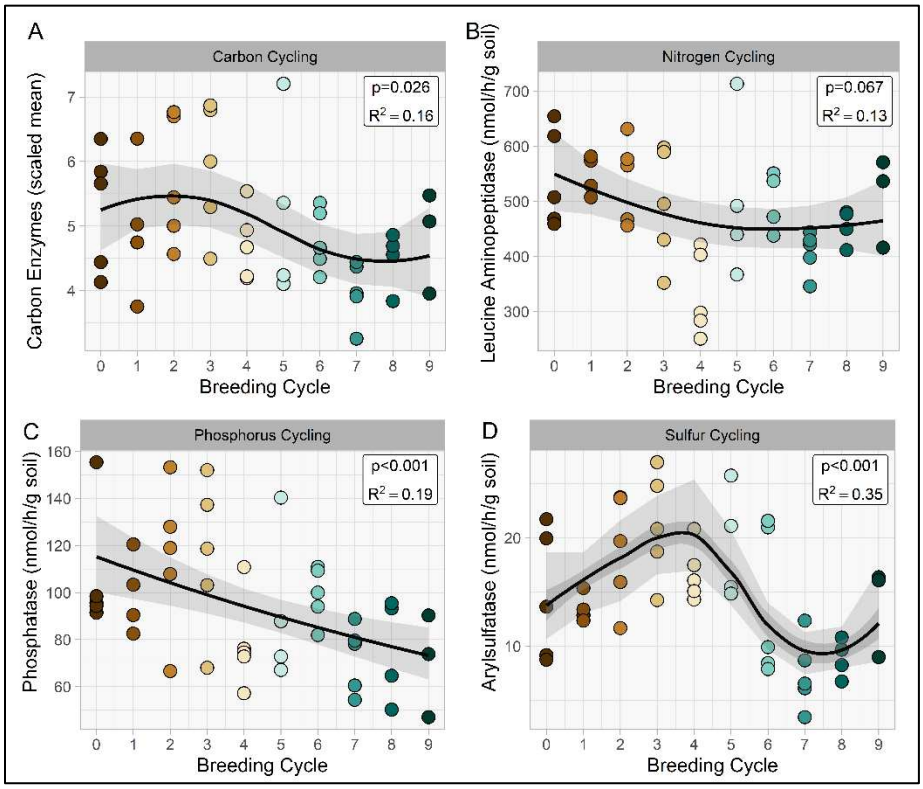

Figure 5. Effect of breeding cycle on enzyme activities. Carbon enzymes represents the scaled mean of α- and β-glucosidase, β-xylosidase, and cellobiohydrolase, which all relate to the degradation of carbon compounds and responded similarly to plant breeding. Predicted lines, confidence intervals, and p-values are from generalized additive models (GAMs) with breeding cycle as a smoothed term.

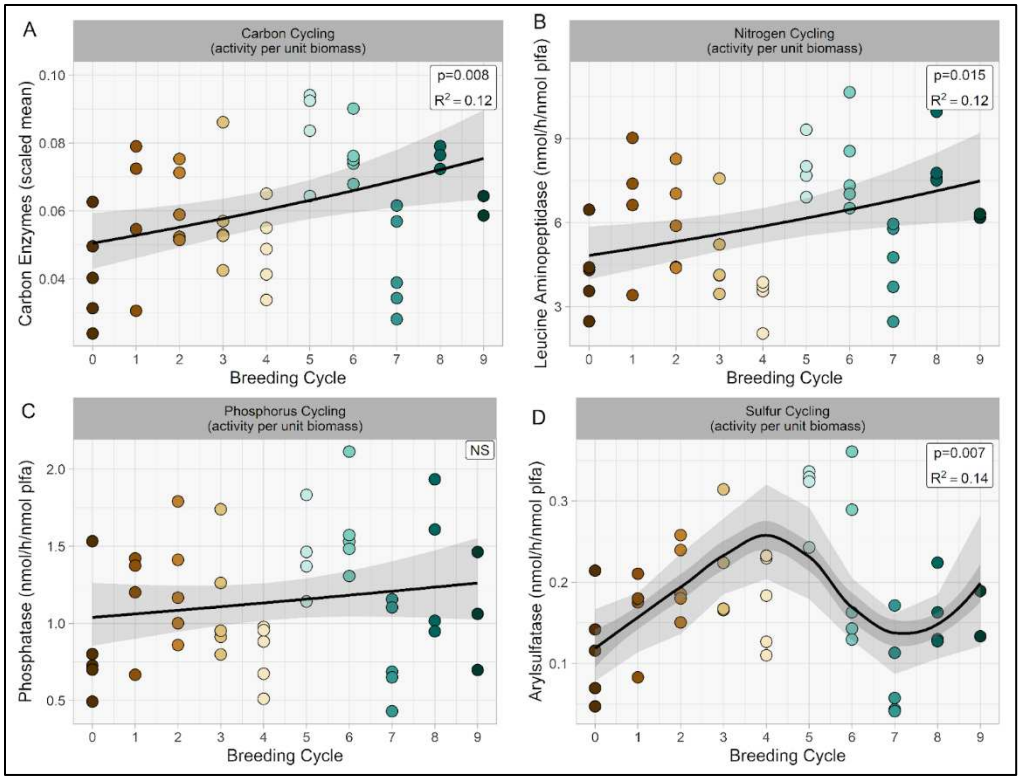

Figure 6. Effect of breeding cycle on enzyme activities per unit microbial biomass by total phospholipid fatty acids (PLFAs). Carbon enzymes represents the scaled mean of α- and β-glucosidase, β-xylosidase, and cellobiohydrolase, which all relate to the degradation of carbon compounds and responded similarly to plant breeding. Predicted lines, confidence intervals, and p-values are from generalized additive models (GAMs) with breeding cycle as a smoothed term.

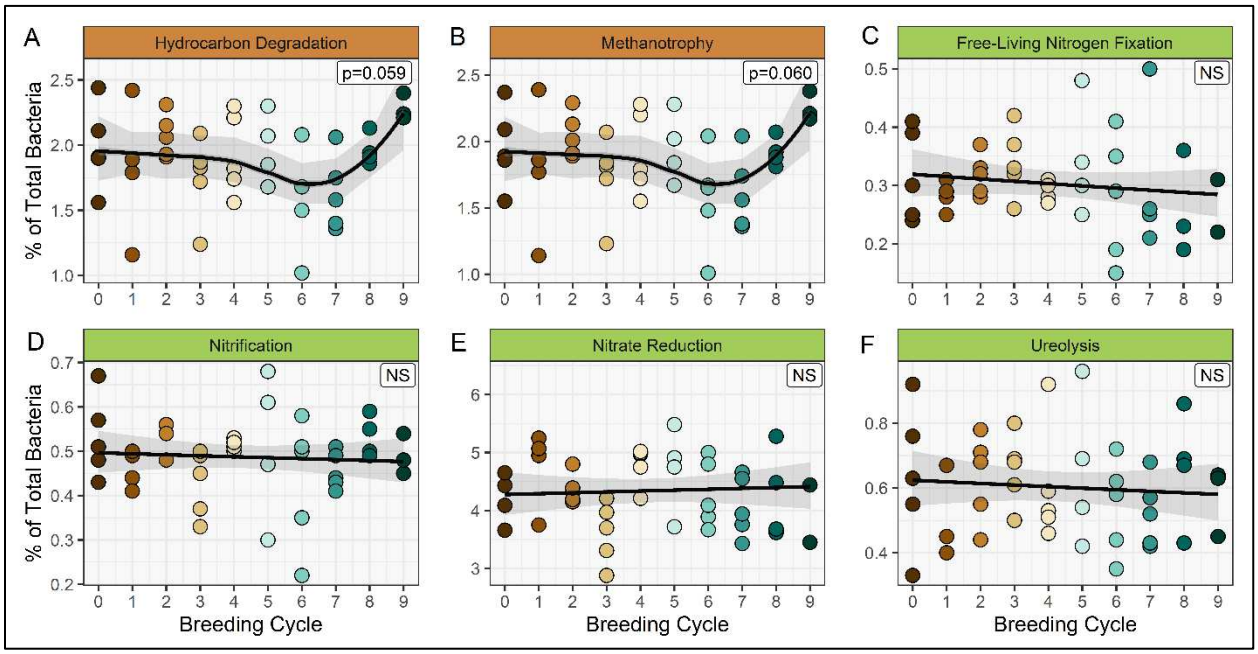

Figure 7. Effect of breeding cycle on predicted functional capacity. Y-axis shows the percentage of bacteria in the community able to carry out each function. Only functions involved in nutrient cycling with a mean relative abundance > 0.2% are shown. Functions with brown labels relate to carbon cycling and those with green labels relate to nitrogen cycling. Predicted lines, confidence intervals, and p-values are from generalized additive models (GAMs) with breeding cycle as a smoothed term.

DISCUSSION

4.1 Rhizosphere organic matter pools and microbial community

Plant breeding (selection for yield and harvestability) decreased highly labile organic matter pools in the rhizosphere (DOC, DON, and PMN) but not semi-labile or stable pools (protein, POXC, and total C and N) (Fig. 1, Table S1). Labile organic matter pools serve as a microbial food source, and their decline likely explains the similar decline found in rhizosphere microbial biomass (Fig. 2). Root exudates contribute to these labile organic matter pools, especially to DOC and DON (Liu et al. 2022). Plant breeding has been found to impact root exudation, with different cultivars of the same crop varying in the quantity and composition of root exudates (Pastor-Pastor et al. 2019; Seitz et al. 2022). Furthermore, these differences in root exudation have been shown to alter the structure and functioning of the rhizosphere microbiome (Seitz et al. 2022; Nerva et al. 2022; Pantigoso et al. 2022), as was observed in the current study.

In our study, plant breeding altered the community composition of both AMF and bacteria and decreased the relative abundance of one AMF family (*Glomeraceae*, the largest AMF family) and two bacterial families (*Kribbellaceae* and *Pseudonocardiaceae,* both gram-positive chemoorganotrophic actinobacteria) (Fig S2, Fig. 5). Though little is known about the functional importance of these families in relation to other actinobacteria or AMF, future research could investigate whether they may be key drivers of microbiome changes during breeding. Plant breeding also decreased bacterial alpha diversity, possibly due to increased competition for the lower amount of available labile organic matter (Bauer et al. 2018).

4.2 Plant growth strategy and plant-microbial interactions

Though no previous studies have measured the rhizosphere microbiome of one crop population over several cycles of selection, studies comparing different cultivars of the same crop, or comparing a crop to its wild ancestor, can provide some insights. Selection for yield tends to shift plants from a conservative to an acquisitive resource-use strategy, benefiting nutrient uptake, reproductive capacity, and fast growth at the expense of nutrient-use efficiency and resource storage (Vilela and González-Paleo 2015; Gonzalez-Paleo et al. 2023). For example, cultivars of the perennial oilseed silflower (*Silphium integrifolium)* bred for yield allocate 50% more N to seeds and less N to crowns and roots than wild-type silflower (Gonzalez-Paleo et al. 2023). Though studies are mixed as to

how this resource-use shift impacts root exudation, Iannucci et al. (2021) proposed higher root exudation and plant-microbial cooperation as a key marker of an acquisitive resource-use strategy.

Our study found that plant breeding impacted potential microbial activity in the Kernza rhizosphere, both in the form of enzyme activities and predicted functional capacity. Though P-cycling enzyme activity decreased across breeding cycles, many other measures of microbial activity increased: C and N-cycling enzyme activity per unit microbial biomass increased across all breeding cycles (Fig. 6), while C and sulfur-cycling enzyme activity, methanotrophy, and hydrocarbon degradation increased across the last three cycles (Fig. 5, Fig. 7). This data suggests that plants from later breeding cycles might support a smaller but more active microbial community, possibly due to an "acquisitive" resource-use strategy that promotes fast nutrient-uptake.

Research on crop-microbial partnerships has mainly focused on AMF. Though crops bred in highly fertile conditions tend to become less dependent on AMF, many modern breeding efforts instead breed crops adapted to resource-poor environments. Kernza breeding was mainly done in unfertilized dryland fields, with plants subjected to frequent water limitation (DeHaan et al. 2018). In wheat, Iannucci et al. (2021) and Kavamura et al. (2020) found more rhizosphere bacteria and AMF colonization in higher-exuding cultivars. Martin-Robles et al. (2018) compared 27 crop species to their wild progenitors and found that while both benefited from AMF symbioses under P-limited conditions, domestication reduced crop response to AMF in soils with adequate P.

A meta-analysis on 320 crop genotypes found that newer crop cultivars tended to be less intensively colonized but more responsive to AMF compared to ancestral genotypes (Lehmann et al. 2012), which seems to be the case for Kernza. Koziol et al. (2019) used AMF isolated from a nearby native prairie to inoculate Kernza plants from cycle 1 and 5. The study found a larger growth response to inoculation in cycle 5, despite the fact that our study found 42% *lower* AMF biomass in cycle 5 than in cycle 1. Together, these findings could suggest that later cycles of Kernza maintain a more efficient community of AMF, though future research is needed.

4.3 Possible reasons for impacts of plant breeding on the rhizosphere

Several hypotheses might explain the observed impacts of plant breeding on the Kernza rhizosphere. **Resource Allocation:** Selection for yield may have encouraged plants to allocate more energy aboveground and less to root growth and exudation. However, a recent study found no evidence for trade-offs between root abundance and aboveground biomass or seed yield in Kernza, though this relationship could have been masked by overall variation between plants (Griffin 2023). **Genomic Selection:** The Kernza breeding program changed from phenotypic

selection in cycles 0-6 to genomic selection in cycles 7-9. In phenotypic selection, plant breeders select outstanding individuals in the field and cross them to produce the next generation, allowing biotic factors to be passed directly to offspring through seed. In genomic selection, plant breeders instead select individuals from the greenhouse based on predicted breeding values using genetic markers and a field-based training population (Crain et al. 2020, 2021). Genomic selection is a relatively new process, and could potentially shape plant-microbial associations differently than traditional phenotypic selection. **Disease & Stress Resistance:** During the Kernza breeding program, cycle 6 experienced flooding and root disease followed by drought in cycles 6 and 7 (DeHaan et al. 2018; Crain et al. 2021). Artificial selection during these years likely favored plants better able to withstand these environmental stressors, possibly due to plant-microbial symbioses or enhanced discrimination between pathogens and mutualists. This hypothesis might explain why microbial activity, but not biomass, increased across cycles 7-9.

Additional research is needed to confirm these findings and evaluate implications for soil and plant health. Extracellular enzyme activity and predicted functional capacity are not perfect measures of microbial activity, and the rhizosphere environment likely undergoes seasonal changes that depend on plant growth stage and strategy. Furthermore, our study did not measure total root biomass or exudation per plant, making it difficult to comment on broader implications for soil health. Finally, selection criteria vary widely between plant breeding programs, each of which could impact the crop rhizosphere in unique ways.

4.4 Conclusions

Selection for yield and harvestability impacted the Kernza rhizosphere environment, decreasing labile organic matter and microbial biomass but not microbial activity. Some measures of microbial activity instead increased, especially across later breeding cycles, suggesting that these higher-yielding plants might associate with a less abundant but more active microbial community. These findings could be due to a variety of physiological changes in the host plants, potentially involving resource-use strategy or tolerance to drought and disease. Ultimately, the impact of plant breeding on the rhizosphere is not linear, and depends on the specific environmental conditions and selection criteria used. In order to develop breeding programs that consider plant-microbial symbionts, future research could evaluate how selection under specific stressors, such as drought or nutrient limitation, influences these plant-microbial symbioses.

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STATEMENTS & DECLARATIONS

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

Hannah Rodgers, Jay Norton, and Linda van Diepen contributed to the study conception and design. Material preparation, data collection and analysis, and preparation of the first draft of the manuscript were performed by Hannah Rodgers. All authors revised and edited the manuscript and read and approved the final version.

Data Availability

The datasets and programming scripts generated during and analyzed during the current study are available at github.com/hrodgers5/Kernza_Rhizosphere_2024, and genomic sequences are available on the NCBI sequence read archive under BioProject ID PRJNA1173191

Supplementary Files

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