Title: Rhizosphere microbial communities explain positive effects of diverse crop rotations on corn and soybean performance

Running title: microbiome explain benefits of diverse rotations

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Abstract

In agricultural systems, crop rotation diversity influences soil microbial communities and often increases crop productivity. Yet the specific contributions of microorganisms to crop rotation benefits are unknown. We studied corn (*Zea mays* L.) and soybean (*Glycine max* L.) within a two-year corn-soybean rotation and four, four-year, four-crop rotations with varying crop sequences. We hypothesized that rhizosphere microbial communities would predict crop productivity contingent on rotation diversity and previous crop legacy. Sampling at seedling and flowering stages, we assessed rhizosphere bacterial and fungal communities, plant tissue nutrients, aboveground biomass, and yield. Rhizosphere communities varied with rotation diversity and previous crop legacy. Concurrently, corn and soybean yields and biomasses were larger in more diverse rotations and with different crop legacies, but not tissue nutrients. Fungal communities predicted the suppression of corn seedlings when following soybean, and soybean seedlings when following corn, independently of rotation effects. This fungal effect ultimately predicted suppressed corn yield in the corn-soybean rotation, while in more diverse rotations, bacterial communities predicted corn would fully recover from a soybean legacy by flowering. These results suggest that corn-soybean rotations select for yield-suppressive microbial communities and highlight a microbial mechanism behind the benefits of diverse rotations.

Introduction

Microorganisms mediate essential ecological processes in agricultural ecosystems. Agroecosystems depend on the activity of microorganisms to mineralize organic inputs and sustain nutrient cycling, promote soil structure and stabilization of organic matter pools, and support plants through symbioses (1). Microorganisms directly interact with plants, in particular in the area physically closest to and under greater influence of the plant root, the rhizosphere (2). Beyond acting as plant pathogens, rhizosphere microorganisms can stimulate plant growth through different mechanisms, including nutrient cycling and increased nutrient availability to the plant, synthesis plant growth promoting organic compounds, priming of plant resistance, and antagonism of pathogen and pest activity (3–6). Hence, the diversity and function of rhizosphere-associated microorganisms, which are intimately related to plant performance, are important assets in the sustainable intensification of agriculture, with the goal of achieving increased crop yields without compromising the environment (7–9).

In annual cropping systems, which typically contain monocultures of a single crop grown per year, one way to alter the diversity and function of rhizosphere-associated microorganisms is through the selection and sequencing of plants grown in a multi-year rotation. For instance, incorporating additional crops into a continuous monoculture or a two-year rotation can help manage both soil fertility and pathogen and pest pressure (9). This rotation effect happens at multiple timescales. First, a plant species releases a variety of root exudates, which modulate rhizosphere microorganisms in a plant species-specific manner (10,11). These changes may directly impact the subsequent crop as a legacy effect. Legacy effects of crop history have been described for crop health and soil processes (12,13), soil and rhizosphere microbial communities (14–16) and individual microorganisms (17,18). This is followed by an effect observed on a

longer timescale wherein the overall diversity and plant species composition of a rotation impact microbial communities. Generally, diversified rotations – those with more than two crops grown in sequence across at least two years – have higher microbial diversity and altered microbial activities than simple two-year rotations (19).

In parallel with microbial effects, diversified rotations can increase productivity and stability over time under less favorable conditions and without additional inputs (20–22). Additionally, shifting from low-diversity rotations such as corn-soybean to incorporate additional crops can substantially reduce fertilizer use and greenhouse gas emissions, and improve air quality, without compromising economic or agronomic performance (23). Therefore, the careful design of cropping practices that enable crop diversification, such as crop rotations, plays an important role in the development of sustainable agriculture (24). However, while a number of microbial community changes and processes have been implicated in driving greater crop productivity, the importance of microorganisms in mediating the effect of rotation diversity on crop productivity is poorly understood (25). This knowledge gap hinders the monitoring and development of crop rotations that promote key interactions leading to greater crop establishment, vigor and yield (26).

Previously, we demonstrated that both the previous crop legacy and rotation sequence composition impact rhizosphere-associated microbial communities in corn using soils from this field study (12). In the current study we evaluate the in-field predictive power of microorganisms on crop performance in response to the effects of crop rotation diversity and previous crop legacy. We studied a 16-year old experiment comparing a two-year, corn and soybean rotation to multiple four-year rotations. We have documented greater yield of both corn and soybeans in at least one of the four-year rotations compared to the two year rotation (20,27). Hence, we

hypothesized that both the diversity of rotation and the legacy of the preceding crop independently affected corn and soybean yield. Further, we hypothesized that rotational diversity and legacy effects could be better predicted by concurrent changes in rhizosphere microbial communities, in comparison to plant tissue nutrient contents. In testing these hypotheses, we a) derive indicators of yield-supporting or yield-suppressing rhizosphere bacterial and fungal communities and b) identify rotation characteristics that would discourage yield-suppressive rhizosphere communities. These indicators and rotation characteristics are critical to facilitating the design and monitoring of cropping systems that alter soil and rhizosphere microbial communities to support sustainable intensification in agriculture.

Materials and Methods

Site description

A long-term research experiment was established in 2000 at the Eastern South Dakota Soil and Water Research Farm in Brookings, South Dakota, USA (44°21' N; 96°48' W) to evaluate the benefits of four-year rotation sequences. Thirty-year mean annual precipitation is 580mm and mean annual temperature is 6.2°C. The Mollisol soils are a moderately drained, Barnes sandy clay loam with organic carbon content of 18 g C kg^{-1} soil (0–15 cm). Rotation treatments were established in a randomized complete block design with four replications; each crop in a rotation sequence was present each year. The plots were no-till, with 85% locally recommended nitrogen fertilization based on fall soil tests and crop yield goals ((corn:7.84 Mg ha^{-1}; winter wheat: 4.03 Mg ha^{-1}; spring wheat: 3.36 Mg ha^{-1}; oat: 3.94 Mg ha^{-1}), phosphorus inputs of 17.6 kg P ha^{-1} at planting, and herbicide-based weed management as needed (28). Corn (*Zea mays* L.) and soybean (*Glycine max* L. Merr.) in the following rotation sequences were examined: CS: corn –

soybean; CPWwS: corn - pea *(Pisum sativum L.)* - winter wheat (*Triticum aestivum* L.) – soybean; COWwS: corn - oat (*Avena sativa* L.) - winter wheat – soybean; CSSwSf: corn - soybean– spring wheat – sunflower *(Helianthus annus* L.); and CSSwP: corn – soybean – spring wheat – pea. CS is a two-year rotation; the others are four-year rotations. Corn follows soybean in CS, CPWwS, and COWwS; and soybean follows corn in CS, CSSwSf, and CSSwP. See Supplementary Methods.

Sample collection and plant measurements

Plant and soil samples were collected from corn and soybean plots within each rotation in 2016 and 2017, coinciding with the completion of four cycles of four-year rotations. At seedling (vegetative stage 1-3), six corn and soybean plants were destructively sampled from each plot, and roots with surrounding soils were collected to a depth of 15 cm with a hand trowel. Plants were sampled again at flowering (corn – VT/R1, soybean – R2). Four plants per plot were cut at the base and associated root and soil were sampled using golf cup cutters (10.8 cm diameter) to 15 cm depth. Sampling tools were disinfected with 70% ethanol between plots. Samples were maintained on ice packs in the field and refrigerated (4°C) until processing. In the laboratory, the root and associated rhizosphere soils (i.e. roots plus adhering soils after shaking), herein collectively referred to as the rhizosphere, were collected for microbial community analyses and stored at -80 °C.

Aboveground plant tissue was collected and dried at 60° C for biomass measurements and tissue nutrient analysis. Dried tissue was ground to ☒ 2 mm sieve and analyzed for plant nutrient concentrations by inductively-coupled plasma emission spectrometry (AgLab Express, Sioux

Falls, SD). Yields were collected with a combine and corrected to standard moisture (corn – 155 g kg^{-1}; soybean – 130 g kg^{-1}).

Amplicon sequencing of bacterial and fungal ribosomal gene markers

Prior to DNA extraction, approximately 0.2 g of rhizosphere sample was freeze-dried and ground with a bead beater for 30 s. Total rhizosphere DNA was extracted using Qiagen's DNeasy® Plant-96 kit. Library preparation for amplicon sequencing in the Illumina MiSeq (San Diego, CA, USA) platform was performed using a two-step PCR protocol (29), (30). The 16S rRNA gene V4 region (16S) and the internal transcribed spacer 2 (ITS2) were used for bacterial and fungal amplicon sequencing of rhizosphere samples using primers 515fm and 806rm (31,32); and ITS3mix1-5,10 and ITS4 (33) that included an overhang tag for Nextera-kit indexing (Table S1) . Gene-specific amplification was performed following Benitez et al. (12) for each individual plant per plot, and individual PCR reactions were pooled by field plot prior to indexing. Indexed amplicons were cleaned, quantified and pooled in equimolar concentrations for sequencing in a 2x 300 MiSeq sequencing run. In 2016, amplicon libraries were prepared in house and sequenced at the University of Minnesota Genomics Center, Microbiome Sequencing Services (UMGC, Saint Paul, MN, USA). In 2017, both library preparation and sequencing were performed at UMGC.

Read processing to amplicon sequence variants

Primers and adapters were trimmed from pair-ended, de-multiplexed fastq reads using cutadapt v1.8 (34). Truncated reads were processed to amplicon sequence variants (ASVs) in R ,3.6 (35) following the DADA2 pipeline (36). Taxonomic assignment was based on the SILVA database

nonredundant training set version 132 (37) for bacteria and UNITE all eukaryotes v8.2 (38) for fungi. Non-bacterial and fungal sequences were removed. Dataset statistics, including diversity metrics, were calculated using *phyloseq* (38) (Table S2).-

Statistical Analyses

We planned a multi-step analysis to compare two explanations of differences in corn and soybean yield across rotations: nutrient content or microbial community differences (Figure S1). All analyses were performed in R (35) v3.6. Unless otherwise noted, analyses used base R functions, figures were made with *ggplot2 (39)*, and structural equation models (SEM) used *lavaan* (40). Permutations and bootstrapped SEM errors used 999 iterations.

Yield responses to rotation and rotation contrasts

First, we tested whether corn and soybean yields for these two years (2016-17) varied with rotation, using the *Anova*() command from *car* (41). Then, pre-defined contrasts were used to identify aspects of the five rotations that seemed to affect yield (Table S3). The three contrasts included aspects of rotation diversity and crop legacy between rotations, and were partially orthogonal: First, rotation diversity (two versus four species) compared the CS rotation with the more diverse COWwS, CPWwS, CSSwSf, and CSSwP. Within the four-year rotations, we further contrasted two types of attributes of the previous crop. The first was whether corn followed soybean versus either pea or sunflower, or whether soybean followed corn versus winter wheat. This contrast separated the legacy effect of the previous crop from the effect of rotation diversity. The second previous crop attribute tested the effects of sunflower prior to corn,

based on the results from Benitez et al (12) and the effect of pea in a rotation in soybean. We tested contrasts with *glht*() in *multcomp* after accounting for year effects (42).

Plant vigor as yield predictors

Next, we tested whether plant vigor at both seedling and flowering stages were predictive of each crop yield using SEM. Plant vigor at seedling and flowering stages were indicated by biomass and tissue nitrogen, phosphorus, and potassium concentrations. First, a definition of plant vigor was tested using confirmatory factor analysis (43). This tested whether more vigorous plants had greater tissue nutrient concentrations as well as greater biomass, which would indicate nutrient limitation. This definition of plant vigor simplified to biomass as the sole indicator based on a negative correlation between biomass and nutrient concentrations, as well as Akaike information criteria (AIC) and $\chi 2$ deviance (Figure S2). We tested this reduced model as an SEM.

Microbial community responses to rotation and rotation contrasts

In analyzing microbial communities, we employed a compositional analysis approach, which mitigates many of the challenges of analyzing high-throughput sequencing data (44–46). We first removed rare taxa, defined as ASVs that were present in fewer than 5% of total samples, and sparse samples, defined as samples with fewer than three unique 16S or ITS2 ASVs. We also excluded *Bradyrhizobium* from all 16S communities. Next, to enable logarithmic transformations with minimal compositional data distortion, we replaced zeros in our community tables by multiplicative replacement using the *cmultRepl*() command in *zCompositions* (47). Next, we converted counts to relative abundances and transformed to centered-log-ratios with *CLR*() from *easyCODA* (48). We then performed a weighted log-ratio analysis (LRA; (49)), analogous to

redundancy analysis, to test the effect of rotation on community structure after partialing year effects, using the *pcwOrd*() function of *pcwOrd* (50). *pcwOrd* has been validated against both *easyCODA* and *vegan* (48,51). Both samples and taxa were weighted to reflect certainty in relative abundances of taxa across samples – taxa by mean relative abundance across samples, and samples by $\log_{10}$(total reads). Without weighting, uncertainty of rare taxa or poorly-sequenced samples dominates solutions of correspondence analysis and log-ratio analysis (44–46,49). Finally, we tested whether communities responded to rotation using a permutational multivariate analysis of variance (PERMANOVA; (52)).

This analysis was performed for each microbial amplicon (16S and ITS2) under each crop (corn and soybean) at each sampling time (seedling and flowering). For those communities found responsive to rotation, we then substituted rotation with the above-defined, significant contrasts into the LRAs to test whether the community responded to rotation diversity or rotation sequence (Table S3). These arranged samples along a single axis constrained by the contrast, which we tested using with PERMANOVA.

2.4.3 Microbial community predictors of biomass and yield

If a community responded to the rotation contrast at 90% confidence, we extracted the constrained axis score for each sample. We then tested whether the 16S or ITS2 community axes that responded to the rotation contrast also mediated rotation effects on crop biomass by comparing three classes of structural equation models (Figure S3). The first, a “no mediation” model, hypothesized that the rotation contrast predicted the microbial community as defined by the axis score, and also predicted crop biomass. The “full mediation” model hypothesized that

the rotation contrast predicted only the microbial community, and only the microbial community predicted crop biomass. The "partial mediation" model hypothesized that the rotation contrast predicted both the microbial community and crop biomass, and the community also predicted biomass. In all models, year was included as a covariate predictor of biomass; seedling biomass was also included as a predictor of flowering biomass. These models were compared using total deviance (χ^2) and Akaike information criterion (AIC).

If either the full or partial mediation model were most parsimonious, we visualized the relationship between the microbial community and crop biomass by plotting the marginal biomass, i.e. after removing year, rotation, and seedling biomass effects, against the community's ordination axis score. We tested all ASVs' responses to the rotation contrast using multiple permutational ANOVAs, with adjusted *p* values to report the false discovery rate (53) and identified the ASVs that contributed at least 1% of the community shift with rotation. For fungi, we interpreted the potential function of contrast-responsive taxa using FunGUILD (54).

Finally, we constructed a full SEM that combined the vigor-predicting-yield SEM (Figure S2) with rotation- and microbes-predicting-crop biomass SEM (Figure S3), which tested whether microbial communities that predicted early- and mid-season crop biomass ultimately predicted crop yield differences.

Data Availability

Data analyzed during this study are included in this article and its Supplementary Information. Raw sequences generated in this work have been deposited in NCBI's Sequence Read Archive

under BioProject numbers PRJNA655937, PRJNA655936, PRJNA656563 for soybean, corn and sequencing controls, respectively. Analysis code is available at https://github.com/pmewing/brookings_rotation_rhizobiomes.

Results

3.1 Corn

Corn's rhizosphere bacterial and fungal communities varied with rotation. At the seedling stage, rotation explained a credible amount of variance in both bacterial ($F_{5,34}$ = 1.14; R^2 = 0.11; p = 0.06) and fungal ($F_{5,34}$ = 2.08, R^2 = 0.16, p = < 0.001) communities (Figure 1; Table S4). Both communities were different depending on whether they originated in long, diverse rotations (bacteria: $F_{2,37}$ = 1.33, R^2 = 0.03, p = .04; fungi: $F_{2,37}$ = 3.31, R^2 = 0.07, p < 0.001). For fungi, the soybean legacy was also important ($F_{2,37}$ = 1.83, R^2 = 0.04, p = 0.03). Corn rhizosphere communities also diverged among rotations at the flowering stage (bacteria: $F_{5,32}$ = 1.27; R^2 = 0.13, p = 0.004; fungi: $F_{5,34}$ = 1.60, R^2 = 0.14, p = 0.002). However, only the diversity of rotation emerged as a driver of these community responses (bacteria: $F_{2,35}$ = 1.33, R^2 = 0.03, p = 0.05; fungi: $F_{2,37}$ = 1.67, R^2 =0.04, p= 0.03).

Concurrently with rhizosphere responses, corn yields varied significantly with rotation ($F_{4,34}$ = 276; p < 0.001; Figure 2; Table S5). Specifically, corn yielded significantly less in the two-year (CS) rotation, by an average of 1400 +/- 300 kg/ha (t = -4.9; p < 0.001) compared to the longer, more diverse rotation (Table S6). The lower corn yield in the CS rotation was independent of a soybean legacy, as within the four-year rotations corn following soybean did not result in lower yields (t = 0.2; p= 1.0). A sunflower legacy reduced corn yields marginally compared to legacies

of other crops (t = -2.4, p = 0.07). Corn vigor at seedling and flowering stages predicted corn yield. However, corn tissue nutrient concentrations were not a driver of this, as they were negatively correlated with biomass and produced a less parsimonious model than biomass alone (final model χ^2 = 0.69, p = 0.41; Figure S2).

At both stages, biomass varied with rotation (seedling: $F_{4,34}$ = 4.36, p < 0.001; flowering: $F_{4,34}$ = 3.63, p = 0.01; Figure 2; Table S5). Rotation diversity again emerged as an important driver of corn biomass, with corn growing in the two-year rotation being smaller than in four-year rotations (seedling: -0.06 +/- 0.02 g/plant, t = -2.8, p = 0.02; flowering: -18.5 +/- 5.7 g/plant, t = -3.24, p = 0.007; Table S5). Corn at the seedling stage also had a marginally lower biomass with a soybean legacy in four-year rotations (-0.05 +/- 0.02 g/plant, t = -2.95, p = 0.07). The sunflower legacy did not affect corn biomass at either stage (seedling: t = 1.56, p = 0.29; flowering: t = -1.18, p= 0.52).

Shifts in bacterial and fungal communities with rotation predicted corn biomass at both stages better than either rotation diversity or the previous crop's legacy (Figure 3). At the seedling stage, when corn was grown in a diverse rotation, the resulting shifts in both rhizosphere communities better predicted corn seedling biomass than rotation diversity alone (partial mediation: AIC = 221, χ^2 = 0.0008; Table S7). However, ignoring a microbial effect was most parsimonious (No mediation: AIC = 219; χ^2 = 1.62, p = 0.8). On the other hand, the fungal community fully and parsimoniously explained the effect of a soybean legacy on corn seedling biomass (Full mediation: AIC = 149, χ^2 = 0.4, p = 0.8; No mediation: AIC = 164, χ^2 = 15.2). The taxa that were more prevalent with a soybean legacy include the likely pathogens, *Giberella acuminata* (Booth),

292 a member of *Diaporthe*, and a member of *Drechslera* (Table S8). Additionally, three variants of
293 *Olpidium brassicae* (Woronin) were associated with corn following either sunflower or wheat.
294 The relative proportions of groups of taxa together predicted a lower corn seedling biomass when
295 soybean preceded (standard coefficient = -0.38, z = -3.9, $p < 0.001$; Figure 3).
296
297 At the flowering stage, whether corn was grown in a two-year or four-year rotations shaped
298 bacterial communities that fully and parsimoniously mediated the effect of rotation diversity on
299 corn biomass (Full Mediation: AIC = 216, χ^2 = 2.32, p = 0.9; No Mediation: AIC = 220, χ^2 =
300 7.23; Figure 3; Table S7). Concurrent differences in fungal communities generally reduced
301 model quality versus the No Mediation model. Among the bacterial taxa that changed with
302 rotation diversity, various strains of the ubiquitous *Candidatus Udeobacter* were more prevalent
303 in the long, diverse rotations, while various *Proteobacteria* as well as a number of
304 *Actinobacteria* were more prevalent in the corn-soybean rotation (Table S8). This bacterial
305 community shift predicted lower corn biomass in the short rotation (standard coefficient = -0.28,
306 z = -3.2, p = 0.001) independently of corn biomass at the seedling stage (Figure 3).
307
308 Overall, the suppression of corn yield in the two-year, corn-soybean rotation relative to the
309 diverse, four-year rotations was best understood as resulting from the combined effects of a
310 soybean legacy and rotation diversity on rhizosphere microbial communities (fungi with soybean
311 legacy at seedling: standard coefficient = -0.05, z = -1.9, p = 0.054; bacteria with the simple
312 rotation at flowering: standard coefficient = -0.04, z = -1.5, p = 0.13; overall model χ^2 = 25.4, p =
313 0.044; Figure 3).
314

315 **3.2 Soybean**

316 In contrast with corn, the structure of soybean's rhizosphere bacterial community was not
317 significantly different among rotations (seedling: $F_{5,33} = 1.14$, $R^2 = 0.11$; $p = 0.16$; flowering:
318 $F_{5,34} = 1.12$, $R^2 = 0.10$; $p = 0.23$; Figure 4; Table S4). Fungal communities, however, were
319 different among rotations at both time points (seedling: $F_{5,33} = 2.32$, $R^2 = 0.20$, $p < 0.001$;
320 flowering: $F_{5,33} = 1.62$, $R^2 = 0.16$, $p = 0.01$). At the seedling stage, the fungal community varied
321 with crop legacy ($F_{2,36} = 3.14$, $R^2 = 0.07$, $p < 0.001$). At the flowering stage, fungal communities
322 again differed with the legacy of corn ($F_{2,36} = 2.89$, $R^2 = 0.07$, $p = 0.004$) and with rotation
323 diversity ($F_{2,36} = 1.22$, $R^2 = 0.03$, $p = 0.004$).
324
325 Concurrently with rhizosphere fungal communities, soybean yields varied significantly among
326 rotations ($F_{4,28} = 8.72$, $p < 0.001$; Figure 5; Table S5). Soybean yields were 830 +/- 160 kg/ha
327 lower in the two-year rotation than in the diverse, four-year rotations ($t = -5.2$, $p < 0.001$; Table
328 S6). This result was independent of a legacy of corn, as when following corn in four-year
329 rotations, soybean yields were 780 +/- 174 kg/ha smaller ($t = -4.5$, $p < 0.001$) than when
330 following wheat. In contrast to our hypothesis, a rotation with pea did not affect soybean yields (t
331 $= -0.65$, $p = 0.88$). Soybean vigor at seedling and flowering stages predicted soybean yield.
332 However, as with corn, tissue nutrient concentrations were not a driver of this: they were
333 negatively correlated with biomass and produced a less parsimonious model than biomass alone
334 ($\chi^2 = 4.0$; $p = 0.05$; Figure S2).
335
336 At both stages, soybean biomass varied with rotation (seedling: $F_{4,33} = 6.0$, $p < 0.001$; flowering:
337 $F_{4,33} = 16.2$, $p < 0.001$; Figure 5; Table S5). At the seedling stage, a corn legacy reduced soybean

biomass by 0.08 +/- 0.02 g/plant (t = -3.53; p = 0.004; Table S6). This response was maintained through flowering, when the corn legacy reduced soybean biomass by 4.5 +/- 0.7 g/plant (t = -6.2, $p < 0.001$). Rotation diversity reduced this biomass suppression soybean growing in the 4-year rotations was 3.2 +/- 0.8 g/plant larger than in the two-year rotation (t = -3.7, p = 0.002). The rotations with pea only affected soybean biomass at the seedling stage, increasing biomass by 0.07 +/- 0.02 g/plant (seedling: t = 3.1, p = 0.01; flowering: t = 0.78, p = 0.8).

Shifts in the fungal community predicted soybean biomass (Figure 6). At the seedling stage, the fungal community fully and parsimoniously explained the corn legacy effect on soybean biomass (Full mediation: AIC = 165; χ^2 = 0.60, p = 0.74; No mediation: AIC = 168; χ^2 = 3.68; Table S7). A number of fungal taxa varied credibly with the previous crop under soybean seedlings but did not follow clear functional patterns (Table S8). Nonetheless, these taxa together predicted lower biomass of soybean seedlings when corn preceded (standard coefficient = -0.43, z = -3.63, p < 0.001; Figure 6).

At the flowering stage, differences in fungal communities resulting from rotation diversity partially mediated the effect of rotation diversity on soybean biomass, moderately improving fit (AIC = 73.6, χ^2 = 7.11; Table S7). However, the no mediation model was more parsimonious (AIC = 71.6 χ^2 = 7.16, p = 0.07). Therefore, we did not attempt to identify taxa behind a fungal effect on soybean flowering biomass. Although fungal communities did not credibly predict yield (seedling fungi: standard coefficient = -0.04, z = -1.4, p = 0.15; model χ^2 = 6.8, p = 0.56; Figure 6), the lower soybean seedling biomass in the two-year rotation relative to the four-year, diverse rotation was predicted by crop legacy effects on rhizosphere fungal communities.

Discussion

Soil and rhizosphere microbial communities are known to respond to crop rotation both compositionally and functionally (12,55–57). Whether changes in microbial communities explain concurrent increases in crop productivity in diverse rotations is less documented. Consistent with other studies, we observed changes in bacterial and fungal community structure, crop biomass, and crop yield between different rotations (e.g. (17,19,56,57). However, these rotations differed in both the diversity of the rotation and the sequence of crop species, which impact microbial communities and soil processes on different timescales. In general, we found that crop legacy effects (i.e. previous crop effects) on rhizosphere communities were strongest at the seedling stage, and rotation diversity effects were most consistent at flowering. The choice of the previous crop in a rotation can shape the rhizosphere microbial communities of subsequent crops – especially fungi – but this effect is transient as the following crop also influences rhizosphere microorganisms. These crop influences happen within the context of more permanent shifts in the rhizosphere microbial community with increased rotation diversity.

To facilitate monitoring soil biological processes that support vigorous and high yielding crops we employed SEM to test whether microbial community responses to rotation diversity and crop legacy predicted differential plant performance across rotations. Structural equation modeling has gained popularity in recent years for its ability to both communicate and test complex hypotheses of system dynamics as readily-understood graphs (58,59). Inferences from SEM are most robust when comparing competing, positive hypotheses (60) as we did by a) testing whether tissue nutrient concentrations explained crop performance; and b) whether knowledge of the microbial communities improved prediction of crop performance. Our results provide strong

evidence that alterations of the rhizosphere-associated bacterial and fungal communities are partially responsible for greater crop performance in more diverse rotations: we could better predict crop performance when incorporating microbial communities to the analysis, compared to crop rotation only. Overall, a) fungal communities shaped by the previous crop predicted the biomass of corn and soybean seedlings, b) bacterial communities in long rotations predicted corn biomass at tasseling, and c) neither bacterial nor fungal communities predicted soybean biomass at flowering.

Just as crop health may affect the structure of rhizosphere communities, microbial communities also impact crop health. Additionally, other factors might also contribute to crop vigor across rotations. For example, differences in soil water usage by the previous crop can reduce water availability the following season, inducing drought losses (61). Our site is rarely water limited, and the Palmer Drought Severity Index was greater than 2.0 for the duration of the study, suggesting moisture depletion was unlikely to be a major factor in crop performance (62). Alternatively, while nutrient availability might affect crop performance (14,63,64), we found no evidence for nutrient limitation as biomass was consistently negatively correlated with nitrogen and phosphorus concentrations. Overall, our analysis suggests that crop resource use efficiency was suppressed in the two-year rotation by elements of rhizosphere communities across contexts of annual weather variation. Still, these microbial communities are conservatively understood as indicators of overall changes in soil properties with diversified rotations that improve crop performance.

We report putative identities and/or functions for the fungal (including plant pathogens) and bacterial taxa (largely ubiquitous soil/plant-associated) that predicted crop responses to rotation, while emphasizing caution when interpreting these assignments. This is due to the ambiguity of precisely identifying taxa from ITS and 16S rRNA fragments, the relatively unknown roles of those taxa that were identified at genus or species equivalents, and the difficulty of reproducing exact matches to taxa across locations and years (54,65,66). Instead, we note that continuous monocultures of various plant species often are characterized by the accumulation of plant parasitic and pathogenic bacteria and fungi which suppress the growth of the monoculture plant host (67–69). Moreover, a single year of rotation with a non-host crop is not sufficient to meaningfully reduce some pathogen populations, such as *Fusarium verguliforme* (O'Donell & T. Aoki), the causative agent of soybean sudden death syndrome (70). These corn-soybean plots had grown only two species for 16 years, and in general, the US Corn Belt has been increasingly dominated by two species across its 650,000 km^2 since World War II (71,72). This creates a strong, persistent, and extensive selective pressure on crop-associated microbial communities that are more likely detrimental than beneficial (55,73). Our results, especially for the identified fungal taxa, are consistent with this hypothesis.

Whether this selection pressure on microbial communities in two-year rotations has practical implications remains a pressing question. In natural systems, more diverse plant communities support more diverse and higher-functioning microbial communities, which further support plant growth (e.g. (74)) For corn grown in North America, the continuous cropping yield penalty is 4.3% versus a corn-soybean rotation (75). However, the corn-soybean yield penalty versus

longer rotations may be 6-fold larger (20). Understanding the mechanisms that underly these benefits of diverse rotations on crop performance is an urgent need.

Our results identify communities of microbes that predict suppressed corn and soybean yield in short rotations. To our knowledge, these are the first field results that directly implicate microbial communities found in corn-soybean rotations with a corn-soybean yield penalty. This study lays a foundation for monitoring microbial communities with the goal of manipulating crop rotation. Further study is necessary to test whether the communities of potentially crop-suppressive taxa we identified are recoverable across years or fields, and also reproduce predictions of crop performance. Still, the microbial taxa identified in this work represent preliminary indicators of crop-supportive and crop-suppressive rhizosphere communities which are sensitive to management. Identification of these types of biological indicators is a major bottleneck in the monitoring of soil health, and in the design and implementation of sustainable cropping systems (25,76).

Conclusion

Continued innovation in agricultural management is necessary to reduce environmental impacts of agriculture while continuing to meet global food demands (77). Microbial activity may support these goals, but significant challenges remain, notably, quantifying the agronomic significance of changes in microbial communities, and developing approaches to monitor these communities such that outcomes can be predicted. We address these challenges by identifying suites of bacterial and fungal taxa that are sensitive to management and predict meaningful increases in the growth and performance of corn and soybean, independent of plant tissue

nutrient concentrations. These bacteria and fungi were altered by both crop sequencing and the overall diversity of a rotation, which highlights that these rotation characteristics are complimentary in designing sustainable and productive agricultural systems. These results will facilitate the incorporation of microorganisms into cropping systems design and monitoring, and ultimately support the sustainable intensification of agriculture.

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Competing Interests

The author(s) declare no competing financial interests.

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Figures

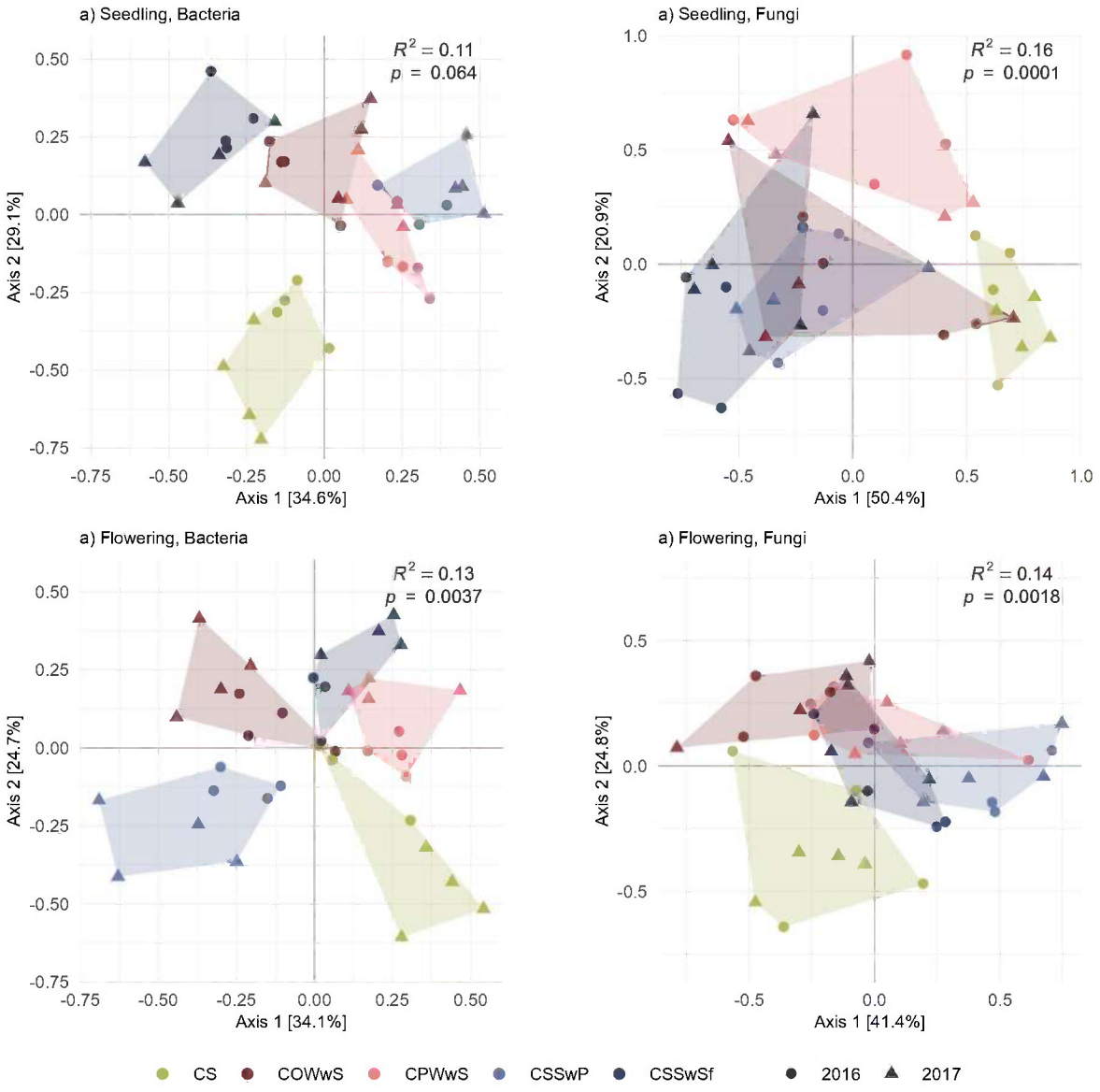

Figure 1: Constrained log-ratio analysis of bacterial (16S) and fungal (ITS) communities at the seedling and flowering stages of corn. Data points are shaped by sampling year and colored by rotation. Additionally, the two-year rotation is yellow, and a soybean legacy in 4-year rotations are shades of red. Rotations: CS, Corn-Soy; COWwS, Corn-Oats-Winter wheat-Soy; CPWwS, Corn-Pea-Winter wheat-Soy; CSSwP, Corn-Soy-Spring wheat-Pea; CSSwSf, Corn-Soy-Spring wheat-Sunflower. Axis variances refer to the proportion of constrained variance shown along each axis. R^2 refers to the Pearson correlation coefficient. *p*-values are based on a pseudo-*F* test across 9999 permutations.

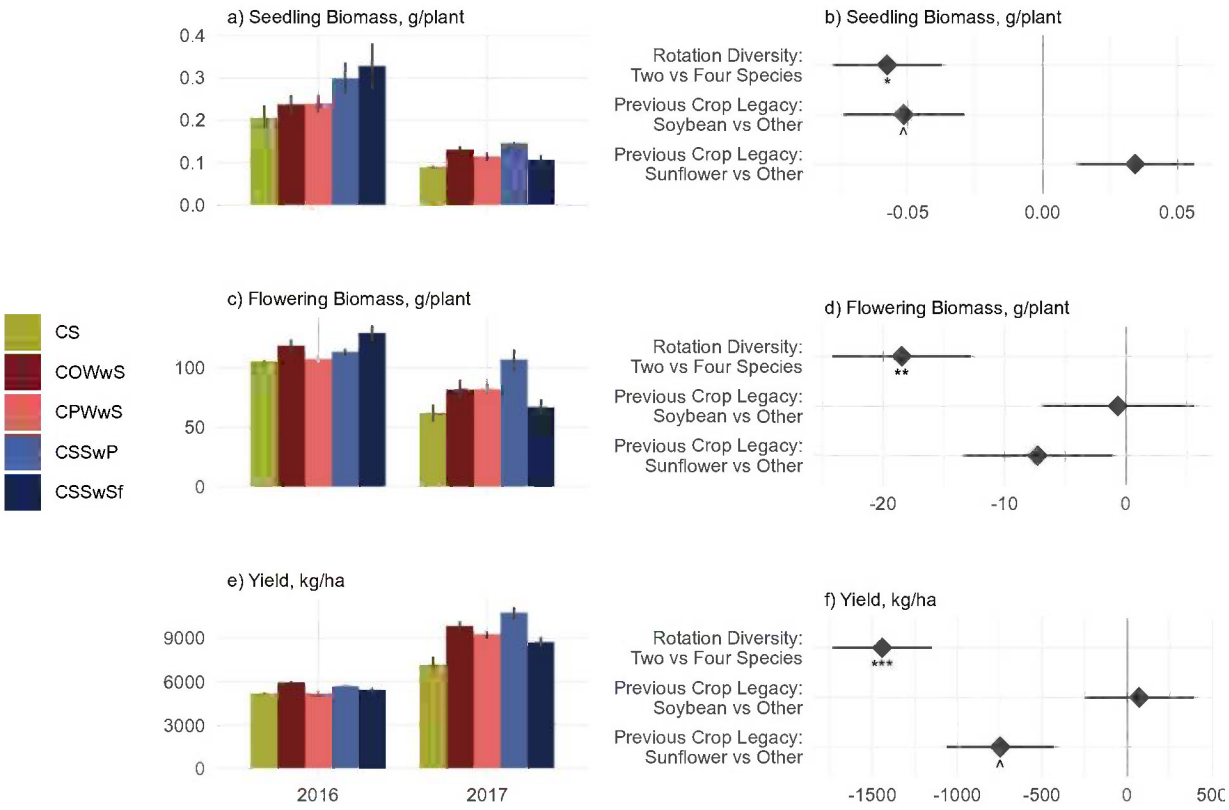

Figure 2: Corn vigor in response to rotation and rotation characteristics. a, b) biomass at seedling; c, d) biomass at flowering; e, f) yield in each year of study. In a), c), and e), colors represent rotation as in Figure 1. Panels b), d), and f) show mean effects of rotation characteristics on the measured parameters across years. Rotation characteristics correspond to the following contrasts: Rotation diversity, gold (2-yr) versus red and blue bars (4-yr); Previous Crop Soybean, red versus blue bars (other previous crop except soybean); Previous Crop Sunflower, dark blue vs light blue and red bars (other previous crop except sunflower). Significance of contrasts are: ^ - $p < 0.1$; * - $p < 0.05$; ** - $p < 0.01$; *** - $p < 0.001$. Errors are standard errors.

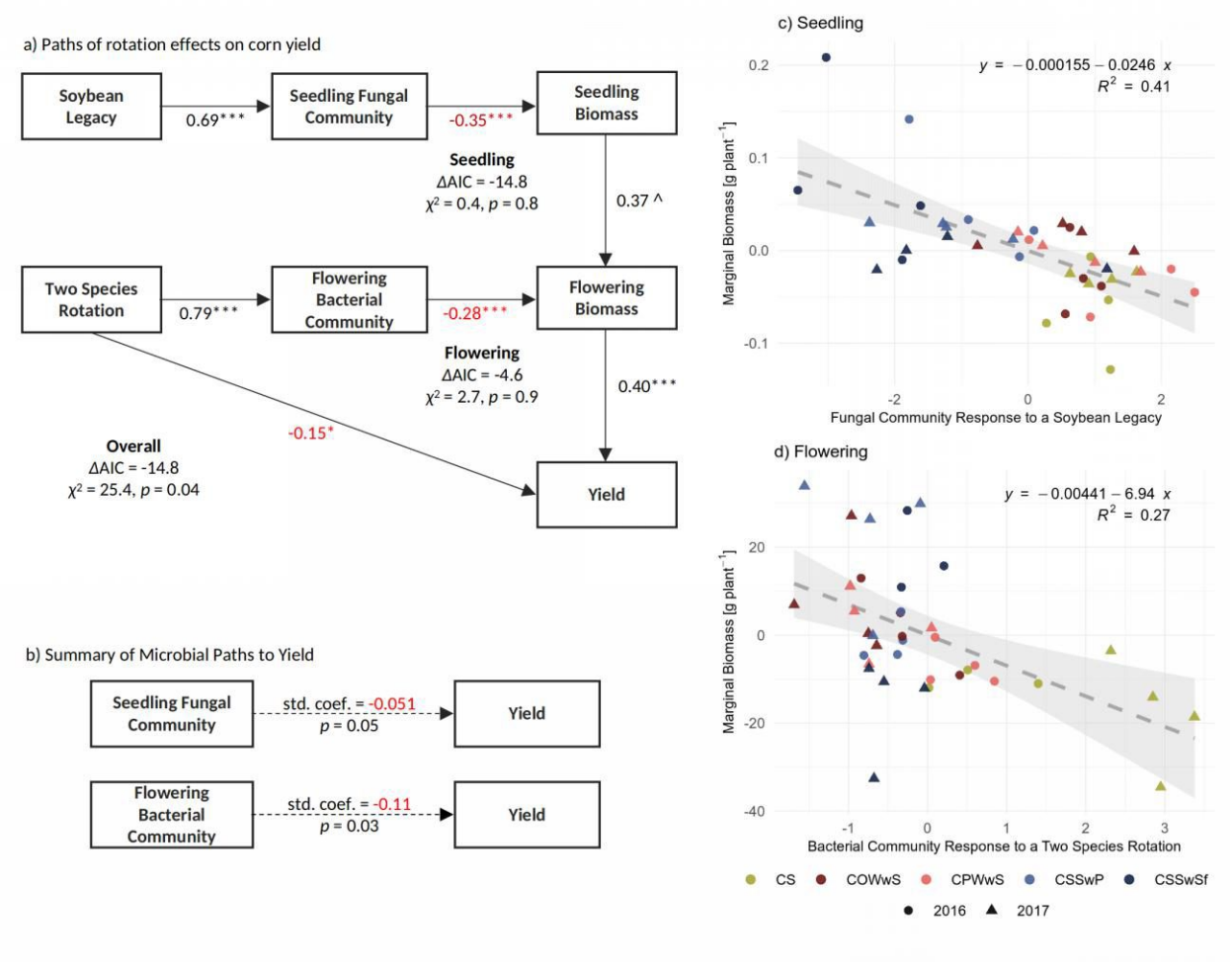

Figure 3: Role of microbial communities in mediating effects of rotation on corn vigor and yield. a) Structural equation model describing these relationships. *Δ*AIC refers to the change in Akaike information criterion by including the microbial community as a predictor for corn biomass at either the seedling or flowering stage versus just using rotation information. Contrasts are as in Figure 2. The χ^2 statistics and the corresponding p values for each biomass sub-model and the full model are also indicated. Values adjacent to arrows are standardized regression coefficients; red are negative relationships, black are positive. Bootstrapped significance of paths are as in Figure 2; gray arrows were not significant at $p < 0.1$. Year was included as a covariate for biomass and yield measurements (not shown). b) Summary of paths between the indicated microbial community and corn yield, as mediated by biomass. c) Partial plot of the response of corn seedling biomass to the seedling fungal community as shaped by following soybean. The horizontal axis are principal sample scores along the first axis of an ordination constrained by the indicated rotation contrast after removing year effects. The vertical axis is marginal biomass after removing year effects. Shapes and colors are as in Figure 1. The gray shaded area indicates the standard error. R^2 is the Spearman correlation coefficient. d) Effect of the bacterial community shaped by rotation diversity on corn biomass at tasseling. Marginal biomass is after removing effects of year and seedling biomass. All symbology is as in c).

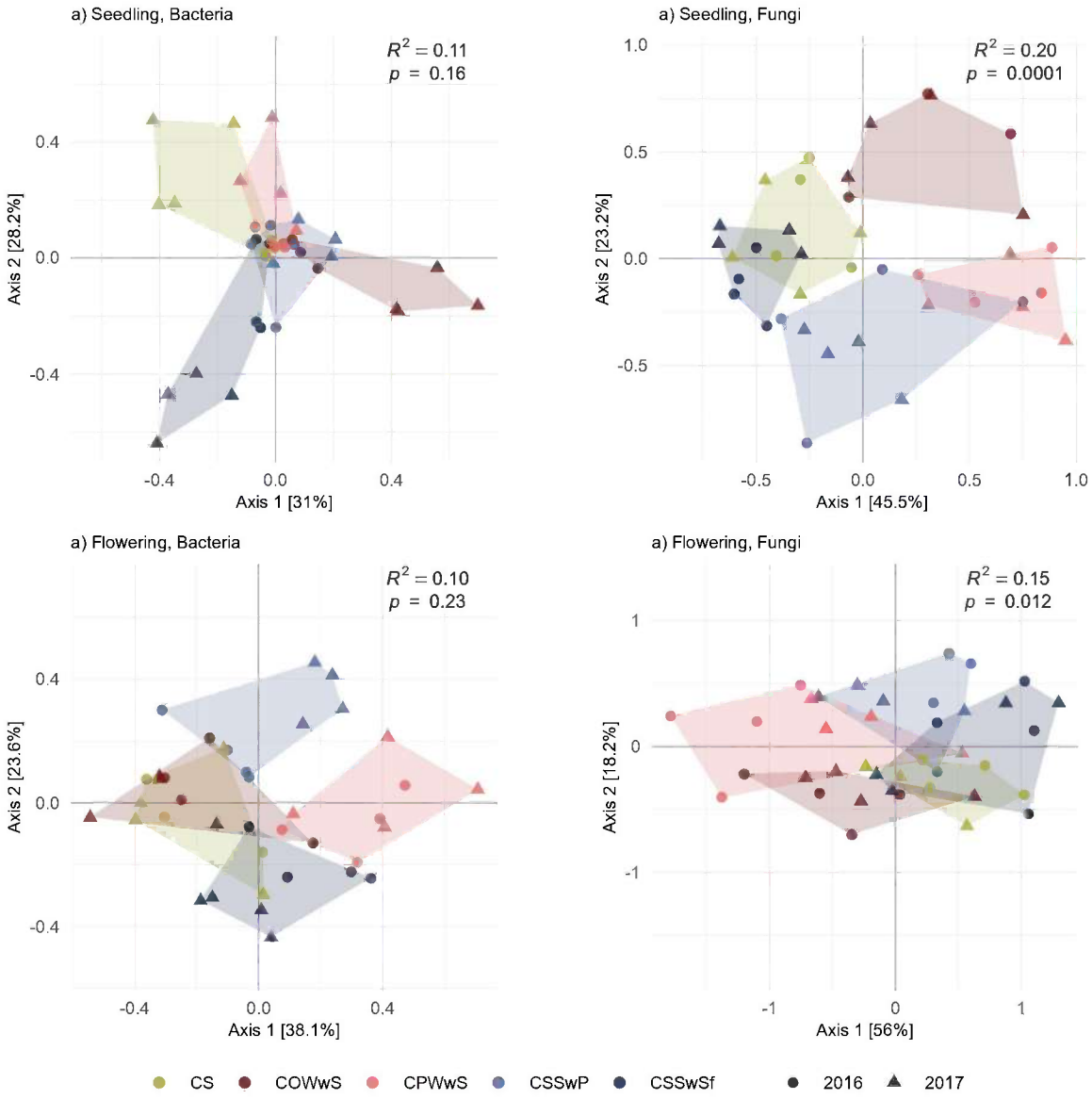

Figure 4: Constrained log-ratio analysis of bacterial (16S) and fungal (ITS) communities at the seedling and flowering stages of soybean. Symbology is as in Figure 1. Additionally, a legacy of corn in 4-year rotations is indicated by shades of blue.

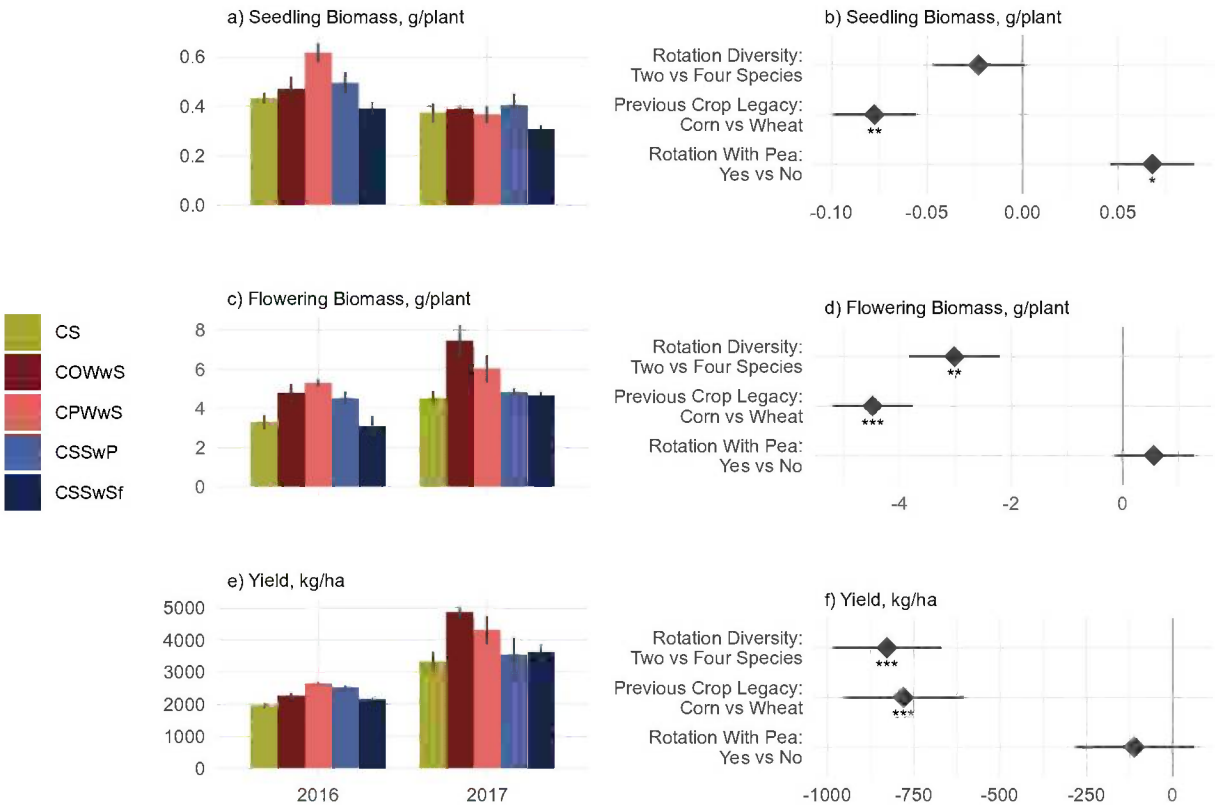

Figure 5: Soybean vigor in response to rotation and rotation characteristics. a, b) biomass at seedling; c, d) biomass at flowering; e, f) yield in each year of study. In a), c), and e), colors represent rotation as in Figure 1. Panels b), d), and f) show mean effects of rotation characteristics on these parameters across years. Rotation contrasts are as follows: Rotation diversity, gold (two-yr) versus red and blue bars (four-yr); Previous Crop Corn, blue versus red bars (other previous corn, except corn); Rotation with pea: light blue versus dark blue and red bars (rotation does not include pea). Significance of contrasts are: ^ - $p < 0.1$; * - $p < 0.05$; ** - $p < 0.01$; *** - $p < 0.001$. Errors are standard errors.

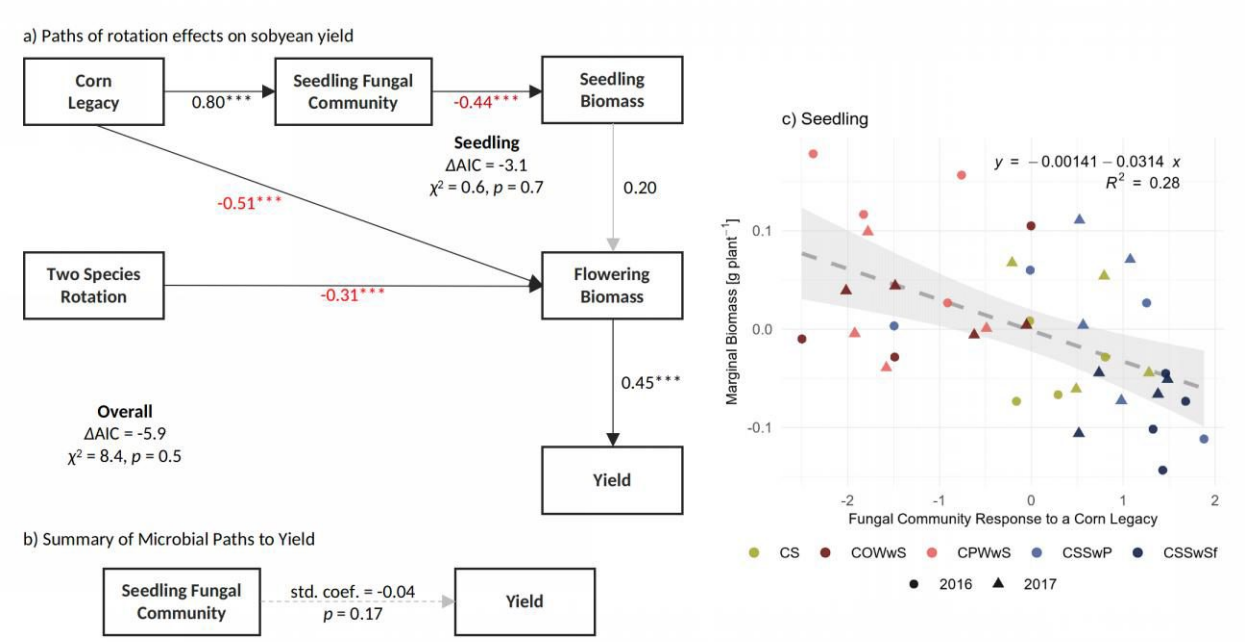

Figure 6: Role of microbial communities in mediating effects of rotation on soybean vigor and yield. a) Structural equation model describing these relationships. Year was included as a covariate for biomass and yield measurements (not shown). b) Summary of paths between the indicated microbial community and corn yield, as mediated by biomass. c) Partial plot of the response of corn seedling biomass to the seedling fungal community as shaped by following soybean. Symbology for each panel is as in Figure 3 and contrasts are as in Figure 5.