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Crop rotational diversity increases disease suppressive capacity of soil microbiomes

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Abstract. Microbiomes can aid in the protection of hosts from infection and disease, but the mechanisms underpinning these functions in complex environmental systems remain unresolved. Soils contain microbiomes that influence plant performance, including their susceptibility to disease. For example, some soil microorganisms produce antimicrobial compounds that suppress the growth of plant pathogens, which can provide benefits for sustainable agricultural management. Evidence shows that crop rotations increase soil fertility and tend to promote microbial diversity, and it has been hypothesized that crop rotations can enhance disease suppressive capacity, either through the influence of plant diversity impacting soil bacterial composition or through the increased abundance of disease suppressive microorganisms. In this study, we used a long-term field experiment to test the effects of crop diversity through time (i.e., rotations) on soil microbial diversity and disease suppressive capacity. We sampled soil from seven treatments along a crop diversity gradient (from monoculture to five crop species rotation) and a spring fallow (non-crop) treatment to examine crop diversity influence on soil microbiomes including bacteria that are capable of producing antifungal compounds. Crop diversity significantly influenced bacterial community composition, where the most diverse cropping systems with cover crops and fallow differed from bacterial communities in the 1–3 crop species diversity treatments. While soil bacterial diversity was about 4% lower in the most diverse crop rotation (corn–soybean–wheat + 2 cover crops) compared to monoculture corn, crop diversity increased disease suppressive functional group *prnD* gene abundance in the more diverse rotation by about 9% compared to monocultures. In addition, disease suppressive potential was significantly diminished in the (non-crop) fallow treatment compared to the most diverse crop rotation treatments. The composition of the microbial community could be more important than diversity to disease suppressive function in our study. Identifying patterns in microbial diversity and ecosystem function relationships can provide insight into microbiome management, which will require manipulating soil nutrients and resources mediated through plant diversity.

Key words: crop rotation; disease suppression; microbial diversity; structure–function relationships.

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INTRODUCTION

Microbiomes are collections of microorganisms that live in close association with plants and animals. Certain microorganisms can confer benefits because they contain genes that aid in nutrient acquisition (Berendsen et al. 2012, Chaparro et al. 2012), while other microorganisms can

protect hosts by preventing colonization by pathogens (Latz et al. 2012, Schlatter et al. 2017). For example, soils harbor a diverse collection of microorganisms that affect the evolution and ecology of plant populations (Lau and Lennon 2012, van der Putten et al. 2013, 2016). Many soil microorganisms establish intimate associations with plant roots, which can result in enhanced plant growth through many mechanisms (Mendes et al. 2013, 2015). One important mechanism through which soil microorganisms increase plant performance and fitness is via disease suppression. In this case, a healthy and robust soil microbiome can serve as a first line of defense for plants against soil-borne pathogens within the resident soil microbial community either directly through antibiosis or parasitism (Mendes et al. 2013, van der Putten et al. 2016), or indirectly through enhancing plant immune responses (Mendes et al. 2013).

Plant–soil feedback theory provides a framework for assessing the mechanisms and outcomes of microbiome dynamics. More specifically, there are many ways soil microbiomes can be managed to influence soil pathogens. One way is through crop selection. Specifically, individual crops can affect pathogen populations by altering chemical, physical, or biological properties in their rhizosphere (Raaijmakers et al. 2009, Berendsen et al. 2012). Further, recent attention is being paid to ecological intensification of farms (Tilman et al. 2011, Bommarco et al. 2013, Tittonell 2014, Garratt et al. 2018), and one of the promising specific management practices under this strategy is to diversify farms by rotating crops (Smukler et al. 2010, Lin 2011).

The colloquial use of the term "rotation effect" has a long history of agronomic research (Karlen et al. 1994), and its origins are from the overwhelming evidence that rotating crops increases crop yield (Liebman and Dyck 1993, Karlen et al. 1994). At any given time, the species richness on a farm using crop rotations is often one (i.e., monoculture), but there is a diverse suite of biochemical inputs from crops planted at different times. There is mounting evidence that this form of temporal biodiversity may provide some of the same beneficial ecosystem functions as traditional spatial biodiversity (Zak et al. 2003), such as carbon sequestration, pest control, and nutrient cycling (Ball et al. 2005, McDaniel et al. 2014*b*, Tiemann et al. 2015, Venter et al. 2016). Despite being frequently observed, the underlying mechanism(s) driving the increased yield in more diverse crop rotations (i.e., rotation effect) are largely unknown. One possible mechanism is that increased crop diversity decreases soil pathogens—however, the evidence is mixed. While declines in pathogen abundances occur when non-host crops are in rotation (Bennett et al. 2012), alternative hypotheses associated with increased abundance of biocontrol producing *Pseudomonas* spp. conferring disease suppression of soil-borne pathogens of wheat (i.e., take-all disease) are also observed in monoculture wheat fields (Kwak and Weller 2013). Thus, the benefits of crop rotation are, therefore, mixed; and the variation may be related to crop diversity promoting plant-pathogen-suppressing microorganisms.

Often, plant pathogen suppression (PPS) is associated with soil microbial communities that have the capacity to produce antimicrobial compounds. Specifically, antibiosis has been linked to disease suppressive capacity, whereby the abundance of antagonistic bacteria was associated with reductions in fungal pathogens through competitive inhibition (Weller et al. 2002, Haas and Défago 2005). For example, bacterial production of secondary metabolites 2,4-diacetylphloroglucinol (DAPG) and pyrrolnitrin (PRN) are two potent toxins known to suppress fungal pathogens in soils (Garbeva et al. 2004*a*, *b*, Haas and Défago 2005). However, the extent to which abiotic and biotic factors influence the abundance of such microbes remains unclear. Abiotic factors (e.g., salt, moisture, nutrients) can limit the strength and alter the direction of plant–soil feedbacks (Bever et al. 1997, Mills and Bever 1998, Packer and Clay 2000, Kulmatiski et al. 2008, Mavrodi et al. 2012). It has been argued that changes in soil physicochemical properties may be important or even required for PPS and might influence species interactions. In addition, aboveground features such as plant diversity could influence PPS. Specifically, plant diversity could increase the total soil bacterial diversity giving way to the sampling effect where species-rich ecosystems contain species that function at high levels (Tilman et al. 2002, Naeem and Wright 2003). Plant diversity could increase the

probability of harboring PPS in the soil microbial community. Alternatively, plant diversity could modify soil microbial communities without influencing total diversity but rather through selecting for microorganisms that perform certain functions such as disease suppression. Some evidence suggests that PPS microorganisms are influenced by competition for iron, antibiosis, lytic enzymes, and induction of systemic resistance in the host plant (Doornbos et al. 2012). Therefore, the abundance of PPS microorganisms could be a reflection of the total diversity of the soil microbial community, but this hypothesis has not been rigorously evaluated.

Given the unknown effect of crop diversity on PPS, we used a long-term (12 yr) crop rotational diversity study at the W.K. Kellogg Biological Station Long-Term Ecological Research (KBS LTER) site to examine the effect of crop diversity on soil bacterial biodiversity and PPS potential. Specifically, our study addresses the following questions: (1) What is the relationship between crop diversity and soil microbial community composition and PPS potential? (2) What is the role of changes in soil physicochemical properties on the crop diversity effect on soil microbial community composition and PPS populations? We hypothesized that increased crop diversity would increase the diversity of the soil microbial community, and also increase the PPS potential in the soil through supporting a higher proportion of disease suppressive microbial taxa.

Methods

Site description and experimental design

We collected soils from the Biodiversity Gradient Experiment (http://lter.kbs.msu.edu/research/long-term-experiments/biodiversity-gradient/) at the KBS LTER site in southwest, Michigan, USA. Mean annual temperature is about 10°C, and mean annual precipitation is about 1000 mm/yr (Robertson and Hamilton 2015). The soils are Kalamazoo (fine-loamy) and Oshtemo (coarse-loamy) mixed, mesic, Typic Hapludalfs formed under glacial outwash (Crum and Collins 1995). The crop rotation treatments at the Biodiversity Gradient Experiment included the following: monoculture corn (*Zea mays*, mC), corn with red clover (*Trifolium pretense* L.), cover crop (C_{1cov}), corn–soybean (*Glycine max*, CS), corn–soybean–wheat (*Triticum aestivum*, CSW), CSW with red clover (CSW_{1cov}), CSW with red clover and cereal rye (*Secale cereal* L., CSW_{2cov}), and a spring fallow treatment that was just plowed every spring but contains 7–10 naturally occurring plant species in the region (Table 1). This spring fallow treatment is considered the benchmark for plant diversity in the region, and under same tillage. Plantings of cover crop were dependent on the main crop in rotation (Smith and Gross 2006, 2007). The experiment was conducted in a randomized complete block design, which included four blocks or replicates of each treatment. All plots received the same tillage at 15 cm depth, and no fertilizer or pesticides (i.e., herbicides, insecticides) were applied to these plots. Because of the lack of nutrient or weed management, there was no significant bare ground during crop growth; and the presence of weeds was highest in the monoculture (mC) compared to the other crop rotation treatments.

Soil sampling

We sampled soil from six crop diversity treatments, but to eliminate any immediate crop effect, all the treatments were sampled in the

Table 1. Crop rotation treatments at the Kellogg Biological Station Long-term Ecological Research Biodiversity Gradient Experiment.

Crop rotation treatment description	Number of crop (or plant) species
(1) Continuous monoculture corn (mC)	1
(2) Continuous monoculture corn + one cover crop (C_{1cov})	2
(3) Two-crop rotation, corn–soybean (CS)	2
(4) Three-crop rotation, corn–soybean–wheat (CSW)	3
(5) Three-crop rotation + one cover crop, corn–soybean–wheat + red clover cover crop (CSW_{1cov})	4
(6) Three-crop rotation + two cover crops, corn–soybean–wheat + red clover and cereal rye cover crops (CSW_{2cov})	5
(7) Spring Fallow/early successional field (fallow, no cash crops)	7–10

Notes: Plant treatments were established in 2000. Treatments were composed of monoculture, two-crop rotation, three-crop rotation ± cover crops, and fallow plots (early successional), and soil was collected during the corn phase of the rotation. Treatment abbreviations are in parentheses.

corn phase and a spring fallow treatment (Table 1) on 1 November 2012. During the time of sampling, cover crops had been planted but showed no growth yet. By sampling after the crop harvest, we were able to get the integrated representation of the soil bacterial community rather than plant-specific influences during the growing season. In each plot, we collected five soil cores (5 cm diameter, 10 cm depth) and then homogenized the cores in the field. A subsample from each composite sample was sieved through 4 mm in the field, flash frozen in the field in liquid nitrogen, and stored at −80°C prior to molecular-based microbial analyses.

Soil physicochemical analyses

From the same soil samples that were flash frozen for DNA extraction, soil chemical properties (total carbon, total nitrogen, ammonium, nitrate, pH, texture) were previously analyzed and reported (McDaniel et al. 2014*a*, McDaniel and Grandy 2016). Labile C was measured as permanganate oxidizable C according to (Culman et al. 2012), and overall biological activity and amount of potentially mineralizable carbon (PMC) and nitrogen (PMN) were analyzed using a 120-d aerobic incubation (McDaniel and Grandy 2016).

Bacterial community sequencing

To examine the relationship between crop diversity and soil microbial diversity, we used 16S rRNA targeted amplicon sequencing of the soil bacterial community. We extracted DNA using the MoBio Power Soil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, California, USA). DNA concentration was adjusted to a standard concentration of 20 ng/μL and used as template. To characterize bacterial taxonomic diversity, we used barcoded primers (515f/806r primer set) developed by the Earth Microbiome Project to target the V4–V5 region of the bacterial 16S subunit of the ribosomal RNA gene (16S rRNA; Caporaso et al. 2012). For each sample, PCR product combined from three 50-μL reactions, concentration quantified, and PCR product from each soil sample was combined in equimolar concentrations for paired-end 250 × 250 sequencing using the Illumina MiSeq platform according to details in Muscarella et al. (2014). Briefly, we assembled the paired-end 16S rRNA sequence reads using the Needleman algorithm (Needleman and Wunsch 1970). All sequences were subjected to systematic checks to reduce sequencing and PCR errors. High-quality sequences (i.e., >200 bp in length, quality score of >25, exact match to barcode and primer, and contained no ambiguous characters) were retained. In addition, we identified and removed chimeric sequences using the UCHIME algorithm (Edgar et al. 2011). We aligned our sequence data set with the bacterial SILVA-based bacterial reference database (Yilmaz et al. 2014). During data analysis, operational taxonomic units (OTUs) were binned at 97% sequence identity and phylogenetic classifications of bacterial sequences performed. Sequences were processed using the software package mothur v.1.35.1 (Schloss et al. 2009, Kozich et al. 2013). We rarefied to 200,000 sequences prior to calculating bacterial richness, evenness, and diversity metrics. To normalize sample-to-sample variation in sequence depth, we took the relative abundance of each OTU and divided by the total number of OTUs for each soil bacterial community prior to statistical analyses.

Composition and abundance of disease suppression genes

To characterize the subset of the microbiome associated with disease suppressive potential, we targeted disease suppressive taxa as the subset of soil microorganisms possessing genes that are required for the production of antifungal compounds DAPG (von Felten et al. 2011; see Appendix S1: Fig. S1) and PRN (Garbeva et al. 2004*b*, Haas and Défago 2005). Microbial defense against fungal pathogens is known to affect both corn and wheat. For example, *Fusarium* spp. produce mycotoxins and can cause yield losses in wheat and maize (Luongo et al. 2005). Therefore, we assessed the relative abundance of disease suppressive functional genes by targeting the *prnD* gene using quantitative PCR (qPCR; Garbeva et al. 2004*b*). The partial *prnD* gene abundance was quantified using a SYBR green assay with primers prnD-F (5′-TGCACTTCGCGTTC GAGAC-3′) and prnD-R (5′-GTTGCGCGTCGT AGAAGTTCT-3′; Garbeva et al. 2004*b*). For the qPCR standard curve, the *prnD* gene was amplified from soil genomic DNA. The 25-μL PCR reaction contained 1× GoTaq Colorless Master

Mix (Promega, Madison, Wisconsin, USA), 0.4 μmol/L of each primer, and 5 μL of template DNA. Cycling conditions included an initial cycle 95°C for 10 min, and 30 cycles of 95°C for 15 s and 60°C for 1 min. PCR fragments were cloned using the pGEM-T Easy Vector System according to the manufacturer's protocol (Promega). Plasmids were extracted using the QIAprep Spin Miniprep kit (Qiagen, Valencia, California, USA), and cloned fragments were verified by PCR and agarose gel electrophoresis. Dilutions of plasmid DNA containing *prnD* gene were used to generate standard curves in quantities ranging from 5.0×10^2 to 5.0×10^7 copies. We quantified the *prnD* gene in 25 μL reaction volumes containing about 20 ng DNA template, 1× TaqMan Environmental Master Mix 2.0 (Applied Biosystems, Valencia, California, USA), 1× SYBR green I, and 0.4 μmol/L of each primer. Fragments were amplified with an initial denaturation step at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 1 min. For each sample, PCR products were run in triplicate. We obtained standard curves based on serial dilutions of mixed PCR product amplified from soil samples. Reactions were analyzed on a BIO-RAD CFX-96 Real-Time System (Bio-Rad, Hercules, California, USA).

Statistical analyses

We examined microbiome differences among crop rotation treatments by comparing total bacterial community diversity and composition as well as disease suppression markers. We tested for differences in total bacterial diversity (based on Shannon Diversity Index H', bacterial species richness, and Pielou's Evenness Index J') and *prnD* gene abundance in response to crop diversity treatment using ANOVA. We checked that data met assumptions of analyses, and we treated crop rotation treatment as a fixed factor and block as a random effect. We used Tukey's honestly significant difference (HSD) tests to identify between-group differences in bacterial diversity and *prnD* gene abundance.

We ran all statistical analyses in the R environment (R v.3.4.3, R Core Development Team 2017). To visualize patterns of microbial community composition, we used principal coordinates analysis of the bacterial community composition based on the Bray–Curtis dissimilarity coefficient for each possible pair of samples. To test for differences in total bacterial communities and a subset of previously identified biocontrol bacterial taxa (i.e., *Pseudomonas* spp. and *Streptomyces* spp.) among crop rotation treatments, we used non-parametric permutational multivariate analysis of variance (PERMANOVA) implemented with the adonis function in the vegan package (Oksanen et al. 2018). Permutational multivariate analysis of variance was also used to assess the contribution of soil factors to the variation in bacterial community composition. The R^2 value reported refers to the treatment sums of squares divided by the total sums of squares for each soil factor in the model. Because the adonis function carries out sequential tests (similar to Type I sums of squares; Oksanen et al. 2018), the effect of the last soil factor or soil biological activity factor of the model was included in the final PERMANOVA model summary (Peralta et al. 2012). We related bacterial community dissimilarity (based on Bray–Curtis dissimilarity) to disease suppression gene abundance using a distance-based redundancy analysis (dbrda function in the vegan package; Oksanen et al. 2018). We used indicator species analysis to identify bacterial taxa that were most representative of each crop rotation treatment. We included bacterial taxa with a relative abundance >0.05 when summed across all plots. We performed the indicator species analysis (Dufrêne and Legendre 1997) using the indval function in the labdsv package (Roberts 2016). We also performed multiple linear regression (gene abundance ~ crop number + total soil carbon + soil moisture + soil ammonium + soil nitrate) to test the influence of soil factors and crop diversity number on abundance of disease suppression/biocontrol gene *prnD* using the lm function.

Results

Bacterial community composition and soil function relationships

The crop diversity treatment significantly influenced soil microbiomes represented by the soil bacterial community composition ($R^2 = 0.37$, $P < 0.001$; Fig. 1; Appendix S1: Table S2). Bacterial communities from the fallow plots and the most diverse crop rotations (CSW, CSW_{1cov}

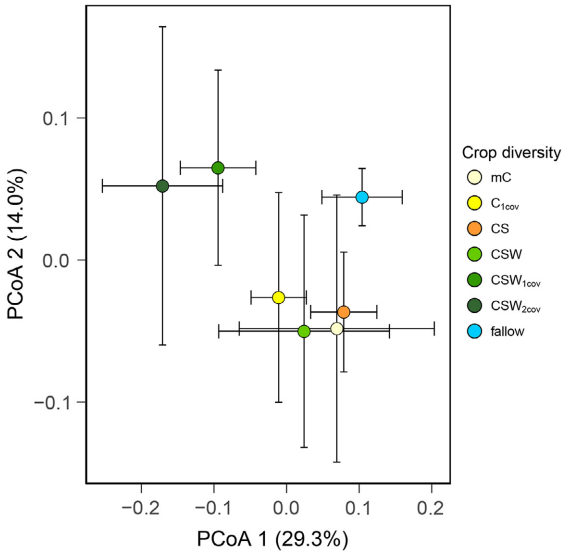

Fig. 1. Ordination from principal coordinates analysis depicting soil bacterial communities along a crop diversity gradient. Symbols are colored according to crop diversity treatment (mC, monoculture corn; C_{1cov}, corn/1 cover crop; CS, corn/soybean; CSW, corn/soybean/wheat; CSW_{1cov}, corn/soybean/wheat/1 cover crop; CSW_{2cov}, corn/soybean/wheat/2 cover crops; fallow, spring fallow, tilled annually).

CSW_{2cov}) were more similar to each other than the lower crop diversity treatments (mC, C_{1cov}, CS; Fig. 1).

Bacterial diversity, as measured using Shannon Diversity Index (H'), was surprisingly greater under lower crop diversity systems than higher crop diversity systems, but highest in fallow treatments the most diverse non-cropping system (crop rotation: $F_{6,20} = 10.16$, $P < 0.0001$; block: $F_{1,20} = 0.20$, $P = 0.660$; Fig. 2). Among, the corn cropping systems, the shorter crop rotations (mC, C_{1cov}, CS, CSW) had the highest Shannon Diversity Index compared to the most diverse rotation of corn–soybean–wheat with two cover crops (CSW_{2cov}). In addition, bacterial species richness and Pielou's Evenness Index (J') revealed similar patterns across crop diversity treatments (evenness: $F_{6,18} = 2.36$, $P = 0.073$; richness: $F_{6,18} = 2.61$, $P = 0.053$; Fig. 2). Across all diversity metrics, the longest crop rotation (CSW_{2cov}) showed the lowest richness and evenness values, and fallow soils generally had the highest values (Fig. 2).

Soil physicochemical properties and soil function were related to bacterial community composition to varying degrees. A summary of soil attributes is presented in Appendix S1: Table S1 and elsewhere (McDaniel and Grandy 2016). Bacterial community composition was best

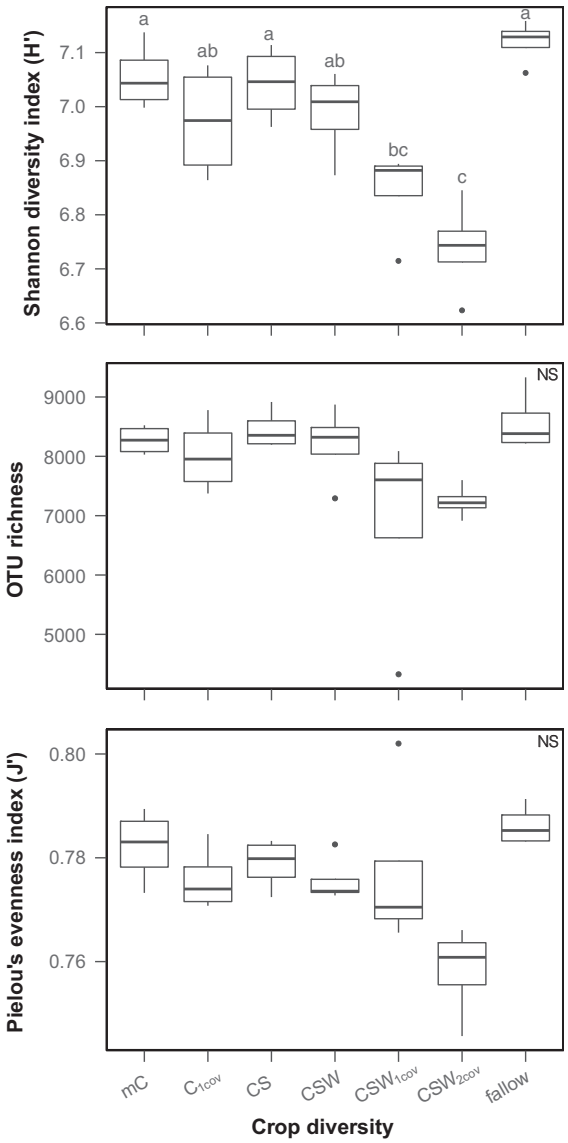

Fig. 2. Boxplots representing total bacterial diversity (Shannon Diversity Index H'), richness, and evenness (Pielou's Evenness Index J') along a crop diversity gradient. Different letters above boxplots reflect significant differences between crop diversity treatments at $P < 0.05$ (Tukey's honestly significant difference post-hoc analysis).

Table 2. Summary of the contribution of (a) soil factors (original data from McDaniel et al. 2014*a*) and (b) soil biological activity (original data from McDaniel and Grandy 2016) on bacterial community variation at the Kellogg Biological Station Biodiversity Gradient Experimental Plots based on permutational multivariate analysis of variance.

Effect	df	SS	MS	*F*	R^2	*P*-value
(a) Soil factors						
Sand	1	0.089	0.089	2.381	0.073	0.014
Silt	1	0.089	0.089	2.363	0.073	0.008
Clay	1	0.086	0.086	2.301	0.071	0.016
pH	1	0.059	0.059	1.568	0.048	0.091
Nitrate	1	0.023	0.023	0.620	0.019	0.884
Ammonium	1	0.019	0.019	0.505	0.016	0.968
Nitrogen	1	0.042	0.042	1.128	0.035	0.281
Carbon	1	0.037	0.037	0.975	0.030	0.438
Moisture	1	0.063	0.063	1.686	0.052	0.072
Residuals	17	0.636	0.037		0.524	
Total	26	1.215			1	
(b) Soil biological activity						
PMN	1	0.083	0.083	1.821	0.063	0.055
PMC	1	0.062	0.062	1.359	0.047	0.153
POXC	1	0.097	0.097	2.129	0.074	0.039
Residuals	24	1.094	0.046		0.830	
Total	27	1.318			1	

Notes: PMC, potentially mineralizable carbon; PMN, potentially mineralizable nitrogen; POXC, permanganate oxidizable C. Soil factor effects were considered to significantly contribute to community variation at $P < 0.05$.

explained by soil texture, which varied across the experiment site from 9% to 38% clay ($R^2 = 0.071$, $P < 0.05$; Table 2a). However, bacterial community composition was also marginally affected by soil moisture ($R^2 = 0.052$, $P < 0.10$; Table 2a). Labile C as measured with permanganate oxidization was related to bacterial community composition ($R^2 = 0.074$, $P < 0.05$), but potentially mineralizable C did not. However, PMN, which is produced in the same aerobic incubation as PMC and an indicator of nutrient-supplying power of a soil (i.e., a biologically available N pool), was significantly correlated with bacterial community composition ($R^2 = 0.063$, $P = 0.055$; Table 2b).

Disease suppression functional potential

Crop rotation affected PPS potential in soils. The *prnD* gene abundances in cropping systems were higher than under fallow conditions (crop rotation: $F_{6,20} = 7.51$, $P = 0.0003$; Fig. 3). In cropping systems, the *prnD* gene in CSW_{2cov} treatment was the most abundant, and the gene abundance was significantly higher than in CSW and fallow treatments (Fig. 3). Our diversity benchmark, the

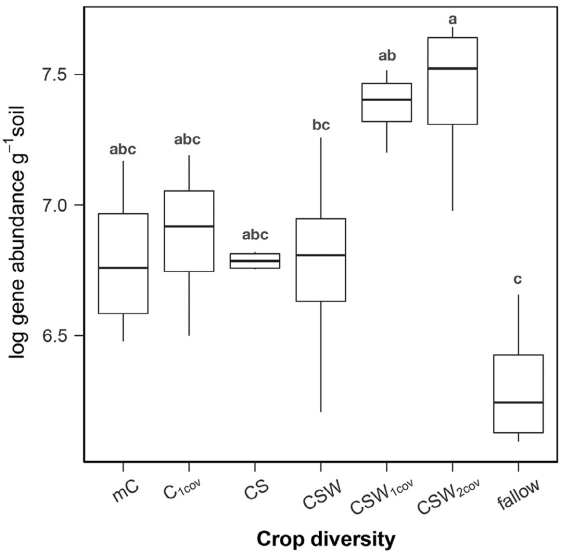

Fig. 3. Abundance of *prn*D gene (pyrrolnitrin producers) along a crop diversity gradient analyzed using quantitative PCR of the *prn*D gene. Different letters above boxplots reflect significant differences between crop diversity treatments at $P < 0.05$ (Tukey's honestly significant difference post-hoc analysis).

Table 3. Summary of multiple linear regression to test the influence of soil factors and crop rotation treatment on disease suppressive functional potential (*prnD* gene abundance).

Factor	Estimate	Std error	*t*-value	*P*-value
Intercept	7.444	0.420	17.728	<0.001
Crop_number	−0.085	0.025	−3.355	0.003
Carbon	0.180	0.050	3.618	0.002
Moisture	−11.564	2.817	−4.105	<0.001
Ammonium	−0.701	0.948	−0.739	0.468
Nitrate	0.093	0.136	0.684	0.501

fallow treatment (i.e., lowest crop diversity), showed the lowest *prnD* gene abundances (Fig. 3). Based on multiple linear regression analysis, plant and soil factors significantly related to *prnD* abundance (Adjusted $R^2 = 0.40$, $F = 4.571$, $P = 0.005$). Crop species number ($P = 0.003$), soil carbon ($P = 0.002$), and soil moisture ($P = 0.0005$) appeared to be significant predictors of *prnD* gene abundance (Table 3). We also observed a shift in the composition of disease suppression microorganisms (represented by *phlD* gene fingerprint analysis using terminal restriction length polymorphism, T-RFLP) along the crop diversity gradient. The *phlD* community composition in the fallow treatment was different from other cropping systems ($R^2 = 0.519$, $P = 0.062$; Appendix S1: Fig. S1).

Soil bacterial disease suppressive function relationship

There was no relationship between bacterial community composition and *prnD* gene abundance (dbRDA: $R^2 = 0.003$, $F_{1,26} = 1.08$, $P = 0.329$). When we compared a subset of taxa representing broad biocontrol bacterial community (composed of *Streptomyces* spp. and *Pseudomonas* spp.), there was no significant pattern in community composition across the crop diversity treatments (PERMANOVA; crop rotation: $R^2 = 0.321$, $P = 0.132$; Appendix S1: Table S3). Indicator species analysis suggested that there were bacterial taxa (OTUs) unique to C_{1cov}, CS, CSW_{1cov}, and CSW_{2cov} treatments and a total of 15 indicator taxa were identified (Table 4). Most indicator taxa were associated with the most diverse cropping treatment (CSW_{2cov}). The indicators are taxonomically diverse and represented by Proteobacteria phyla (including orders Burkholderiales and Myxococcales for C_{1cov}; Acidobacteria (Gp 4,6) for CS; Acidobacteria (Gp 4) for CSW_{1cov}; and

Table 4. Bacterial taxa (OTUs) unique to crop rotation treatment according to indicator species analysis.

OTU	Cluster	IndVal	Prob	Phylum/Class/Order/Family/Genus
Otu000013	C1cov	0.179	0.015	Proteobacteria/Betaproteobacteria/Burkholderiales/unclassified/unclassified
Otu000015	C1cov	0.204	0.003	Proteobacteria/Betaproteobacteria/unclassified/unclassified/unclassified
Otu000022	C1cov	0.186	0.018	Proteobacteria/unclassified/unclassified/unclassified/unclassified
Otu000076	C1cov	0.203	0.009	Proteobacteria/Deltaproteobacteria/Myxococcales/unclassified/unclassified
Otu000002	CS	0.189	0.016	Acidobacteria/Acidobacteria_Gp6/Acidobacteria_Gp6_order_incertae_sedis/Acidobacteria_Gp6_family_incertae_sedis/Gp6
Otu000060	CS	0.235	0.044	Acidobacteria/Acidobacteria_Gp4/Acidobacteria_Gp4_order_incertae_sedis/Acidobacteria_Gp4_family_incertae_sedis/Gp4
Otu000046	CSW1cov	0.240	0.007	Acidobacteria/Acidobacteria_Gp4/Acidobacteria_Gp4_order_incertae_sedis/Acidobacteria_Gp4_family_incertae_sedis/Gp4
Otu000001	CSW2cov	0.227	0.001	Proteobacteria/Alphaproteobacteria/Sphingomonadales/Sphingomonadaceae/unclassified
Otu000021	CSW2cov	0.245	0.026	Proteobacteria/Alphaproteobacteria/Rhizobiales/unclassified/unclassified
Otu000027	CSW2cov	0.234	0.041	Proteobacteria/Alphaproteobacteria/unclassified/unclassified/unclassified
Otu000034	CSW2cov	0.219	0.008	Proteobacteria/Betaproteobacteria/unclassified/unclassified/unclassified
Otu000038	CSW2cov	0.197	0.047	Proteobacteria/Betaproteobacteria/Burkholderiales/Oxalobacteraceae/unclassified
Otu000051	CSW2cov	0.194	0.041	Proteobacteria/Betaproteobacteria/unclassified/unclassified/unclassified
Otu000061	CSW2cov	0.265	0.023	Proteobacteria/Alphaproteobacteria/Sphingomonadales/Sphingomonadaceae/unclassified
Otu000072	CSW2cov	0.248	0.009	Proteobacteria/Alphaproteobacteria/unclassified/unclassified/unclassified

Note: C1cov, corn/1 cover crop; CS, corn/soybean; CSW1cov, corn/soybean/wheat/1 cover crop; CSW2cov, corn/soybean/wheat/2 cover crops; OTU, operational taxonomic unit.

Proteobacteria phyla which included groups of unclassified Alpha- and Betaproteobacteria, Sphingomonadales, Rhizobiales, and Burkholderiales for CSW_{2cov} (Table 4).

Discussion

Soil microbiomes represent microbial communities living in close association with host plants and can protect host organisms from infection and disease. Crop rotations can contribute to disease suppression function by reducing pathogen abundances when non-host crops are in rotation (Bennett et al. 2012); however, disease suppression can also arise in continuous wheat fields due to increased abundance of DAPG producing *Pseudomonas* spp. which is particularly antagonistic on the take-all fungal wheat pathogen (Kwak and Weller 2013). In this study, we found that crop rotation history impacted soil microbiomes and altered disease suppression potential in agricultural soils. However, we found some unexpected results that contrasted with our hypothesis. Contrary to our hypothesis, bacterial diversity decreased with increasing crop diversity (Fig. 2). However, the PPS capability of the soil microbial community increased with crop diversity, but surprisingly the lowest PPS was in the diverse fallow treatments (Fig. 3). We observed that without crop plants (as reflected in the no crop fallow treatment), disease suppressive potential was significantly diminished compared to the most diverse crop rotation treatments, possibly due to reduced selection for soil microorganisms with disease suppression traits. The composition of the soil microbial community may be more important than diversity to soil suppressive function. Thus, crop rotation has the potential to impact diseases suppressive function, providing evidence for facilitation of fungal pathogen protection of plants in diverse crop rotation systems as supported in past studies (Reynolds et al. 2003, Raaijmakers et al. 2009, van der Putten et al. 2013).

Crop diversity effects on soil bacterial diversity

Crop rotation history decreased soil bacterial diversity over this 12-yr crop diversity study. The pattern of reduced bacterial diversity (based on 16S rRNA gene sequencing) was lower in soils with higher crop diversity. There are two most parsimonious explanations for this unexpected finding. First, this pattern in belowground biodiversity might be due to increased abundance of weedy plant species in low diversity treatments, but especially the monoculture corn. In other words, while we were considering the corn treatment as a single species, there could ostensibly have been up to 13 weed species per m^2, as measured in an earlier study from this experiment (Smith and Gross 2007). On the other hand, this same study showed the most diverse cropping systems (CSW_{2cov}) had only 5–6 weeds per m^2 (Smith and Gross 2007). These annual ruderal weedy species are generally less invested in defense traits, in contrast to current crop varieties (Coley et al. 1985, Reynolds et al. 2003, Gaba et al. 2017). Second, perhaps there was not an artifact from the weeds and that soil bacterial diversity does decrease with increasing crop diversity, but other members of the soil microbial community (e.g., fungi, archaea) may be increasing in diversity with longer crop rotations. Despite decreased bacterial taxonomic diversity, a previous study based on the same soils that we used in this study found that catabolic evenness (a measure of the diversity of catabolic function) also decreased with increasing crop diversity (McDaniel and Grandy 2016). This indicates that the trend in lower bacterial diversity with increasing crop diversity is not just structural, but also function. This may indicate carbon resource specialization among bacteria since they are probably the major contributor to C catabolism in these substrate-induced respiration methods (Goldfarb et al. 2011, Allison et al. 2014). Variation in production of biocontrol compounds between *Streptomyces* isolates against fungal pathogen (*Fusarium* spp.) may depend on competition for carbon (Bressan and Figueiredo 2008). Based on phospholipid fatty acid analysis, a previous study showed that bacterial biomass in the micro-aggregate soil organic matter fraction was greatest in high compared low crop diversity treatments at this long-term experiment during a different sampling date (Tiemann et al. 2015). In addition, a previous meta-analysis revealed that the crop rotation effect increased soil bacterial diversity (i.e., Shannon Diversity Index H') most notably in the first five years of treatment, but crop rotations longer than five years were more variable in diversity and not

significantly different (Venter et al. 2016). This study is unique due to the long-term examination of the crop rotation treatment on the soil bacterial community. Other studies do find significant negative effects of crop rotations on soil microbial diversity (Berg and Smalla 2009, Yin et al. 2010, Kulmatiski and Beard 2011, Reardon et al. 2014). The reason for these findings remains unknown but may be a combination of diversity impacts on other soil organisms not evaluated in this study or due to length of time associated with crop diversity treatment.

Crop diversity and PPS relationship

We found that the increased crop diversity, via rotation, increased the abundance and altered the composition of a specific PPS gene (Figs. 3; Appendix S1: Fig. S1). Our results suggested that crop diversity may increase the disease suppression of agricultural soils, and are consistent with previous studies suggesting that plant diversity can enhance protection against soil-borne pathogens by fostering antagonistic soil bacterial communities (Latz et al. 2012, van der Putten et al. 2016). One potential explanation for the negative plant diversity and disease suppressive function relationship is due to facilitation, where changes in plant root exudation may lead to enrichment of plant growth promoting rhizobacteria (Lugtenberg and Kamilova 2009, Badri et al. 2009, Chaparro et al. 2012). In previous studies, microbial interactions among the total microbial community and soil-borne pathogens in the plant rhizosphere have influenced both plant growth and productivity (Bakker et al. 2010, Penton et al. 2014).

The addition of cover crops to rotations strongly increased disease suppressive potential. This along with evidence from previous studies shows that crop rotations may prevent many forms of crop disease caused by *Fusarium* spp., *Phytophthora,* and *Rhizoctonia* spp. (Raaijmakers et al. 2009, van der Putten et al. 2016). Soil microbial diversity has been implicated as important for soil disease suppression; sterilized soils lose suppressive capacity, and adding soil microorganisms to sterilized soil facilitates disease suppression functional capacity (Garbeva et al. 2006, Brussaard et al. 2007, Postma et al. 2008). Biocontrol bacteria can also provide disease suppression against plant pathogens by way of the following mechanisms: competition for iron, antibiosis, lytic enzymes, and induction of system resistance of host plants (Doornbos et al. 2012, Schlatter et al. 2017). Plants can also facilitate recruitment of specific biocontrol microorganisms in some cases. A previous study suggested that beneficial pseudomonads are recruited depending on the most dominant soil-borne pathogen infecting crop species (Berendsen et al. 2012, Mavrodi et al. 2012). In the present study, we analyzed a subset of previously reported biocontrol bacterial taxa (e.g., *Pseudomonas* spp. and *Streptomyces* spp.) across the crop diversity gradient; however, we did not detect distinct changes in putative biocontrol community composition (Appendix S1: Table S3) nor was there a correlation between bacterial composition and *prnD* gene abundance. While this was observed, there is great diversity in biosynthetic genes of these PPS bacterial groups which are likely overlooked based on our amplicon sequencing analysis. Strain-specific antifungal metabolite production depends on plant cultivar–bacterial strain relationship (Okubara and Bonsall 2008). We did identify a subset of taxa that were representative of particular crop rotation treatments. For example, *Burkholderia* spp. were relatively abundant in crop rotation treatments planted with cover crops (C_{1cov}, CSW_{2cov}; Table 4). These taxa have been associated with antagonistic microbial properties (Salles et al. 2004, Elliott et al. 2006, Postma et al. 2008) and associated with maize cropping systems (Salles et al. 2004, Li et al. 2014). In addition, the order Myxococcales represented the C_{1cov} treatment. This group is known to specialize in degradation of biomacromolecules and can efficiently produce exoenzymes and secondary metabolites and provide these bacteria a competitive advantage in nutrient limited environments (Reichenbach 1999).

Our study revealed that cover crops in combination with corn–soybean–wheat rotations increased abundance of the *prnD* gene, which is responsible for producing antifungal compound PRN (Garbeva et al. 2004*b*, Haas and Défago 2005), by about 9% compared to the other cropping systems. Cover crop species may have important effects on the *prnD* gene abundance and disease suppressive functional potential in soils, but only in combination with corn–soybean–wheat because the cover crop with corn only did not show high *prnD* abundance (Fig. 3).

In past studies, cover crops such as cereal rye have been implicated at increasing corn pathogen densities leading to reduced disease suppressive soil function (Acharya et al. 2016, Bakker et al. 2016).

Without crops (as reflected in the fallow treatment), we observed that disease suppressive potential significantly declined. The *prnD* gene abundance in all cropping systems was generally higher than in fallow treatment. The abundance of DAPG and PRN producers increasing with plant diversity has been previously observed (Latz et al. 2012), but with greater spatial diversity in grassland species. Compared to agricultural soils, the PRN producers were more frequently detected in grassland or grassland-derived plots (Garbeva et al. 2004*a*, *b*). In a previous study, the *prnD* gene abundance increased in the presence of grasses, but the legume species tended to decrease the DAPG and PRN producer abundance (Latz et al. 2012). In our study, we did not examine plant species community composition in the fallow treatment that year; however, previous research (Smith 2006) indicated that fallow plots were dominated by later-emerging forbs and C3 grasses (i.e., forbs *Arabidopsis thaliana* (L.) Heynh, *Ambrosia artemisiifolia* L., and grass *Elytrigia repens* (L.) Nevski). Also, a large amount of biomass from perennial legumes like *Trifolium pratense* L. was represented in fallow plots. The abundance of perennial legumes could also be contributing to declines in DAPG in our study (Smith 2006) as observed in previous study by Latz et al. 2012.

This observation of reduced disease suppressive potential may be indicative of species-specific facilitation of PPS soil microorganisms. This disease suppressive phenomenon is known to have important implications for sustainable biocontrol of soil-borne pathogens. In addition, it is possible that when plant diversity is high, there is less soil-borne pathogen pressure on plant hosts due to decreased competition for resources among pathogenic and non-pathogenic soil microorganisms (Reynolds et al. 2003, van der Putten et al. 2013).

Proposed mechanisms for crop diversity effects on soil bacterial diversity and PPS abundance

Disease suppression may have a major role in what is colloquially referred to as "the rotation effect." Our study provided evidence that crop diversity alters soil bacterial community composition and populations of putative PPS bacterial taxa, but the mechanisms through which this occurs can include physical, chemical, and biological changes to the soil environment. Crops can influence soil properties and soil microbiomes in a variety of ways, including physically and chemically. Cover crops are the most salient feature of these crop rotations affecting the soil bacterial community in general. This is not surprising since cover crops have been shown to influence several soil properties, which likely have indirect effects on the soil bacterial community composition. In addition, previous studies showed cover crops can have immediate impacts on soil microbial communities (Wiggins and Kinkel 2005, Finney et al. 2017). Soil properties like total C, total N, pH, bulk density, and porosity have all been shown to increase with cover crops (Bullock 1992, Liebman and Dyck 1993, Tilman et al. 2002, McDaniel et al. 2014*b*, Tiemann et al. 2015). Physically, crop diversity (especially rotations) can enhance soil properties like improving plant water availability by lowering bulk density, increasing soil pore space, and increasing soil aggregate formation (Tilman et al. 2002, McDaniel et al. 2014*b*, Tiemann et al. 2015), which could have indirect influence over the soil bacterial community as well. A correlation between soil factors (silt, clay and soil nutrients) and expression of the antimicrobial genes has been observed in past studies (Postma et al. 2008, Raaijmakers et al. 2009, Imperiali et al. 2017). Chemically, cover crops are providing more carbon to the soil through residues, but also root exudation of recently assimilated photosynthate, composed of soluble, low molecular weight organic compounds (Neumann and Romheld 2007). As a consequence, the increased C flow from cover crop root exudates can stimulate soil microbial activity. Changes in root exudates have been observed to shift microbial community composition and stimulate a diverse microbial community (Hooper et al. 2000, Stephan et al. 2000, Paterson et al. 2009, Dijkstra et al. 2010). Biologically, some soil microorganisms can provide PPS through competition for nutrients, antibiosis, and induction of system resistance of host plants (Doornbos et al. 2012). Our study focused on soil bacterial community

composition, but past studies have identified that crop rotation also influences soil fungal and faunal communities, which are also important members of the soil food web (McLaughlin and Mineau 1995). For example, increased protist predation on soil bacteria has resulted in indirect effect on disease suppression function (Jousset et al. 2008, 2010). These studies revealed that increased predator pressure by soil protists has been linked to increased biocontrol function through enhanced bacterial DAPG production (Jousset et al. 2008, 2010).

However, disease suppression traits such as antifungal production may not be needed and are not maintained in the community when crops are no longer planted. Several explanations could underpin our observations. When agricultural management is absent, there is reduced selection for soil microorganisms with disease suppression traits. Higher plant diversity reflected in longer crop rotations was expected to support overall diversity, resulting in an increased probability of getting more disease suppressive microorganisms. However, we observed that overall taxonomic diversity decreased with increasing crop diversity, indicating alternative mechanisms may be involved in this diversity–function relationship. One argument is that in monocultures, the selection for fungal pathogen defense is weakened and microorganisms that are (constitutively or facultatively) making defense compounds are paying a cost and are replaced by microorganisms that do not invest in the defense strategy. In addition, fluctuating environments can influence selection of traits (Heath et al. 2010, Akçay and Simms 2011). For example, high variation in carbon compounds such as under diverse crop rotations could alter selection of defense traits, whereby crop plants facilitate PPS or other defense traits that are adaptive only when crop plants are present. Increasing plant diversity such as in fallow, non-cropping systems, provides opportunity for microbial community members to partition according to diverse (and more even) carbon resources rather than crop inputs driving selection of microbial communities and defense traits (Reynolds et al. 2003, Hartmann et al. 2009). In other words, when you are in a resource-rich soil under fallow, there is no need for PPS gene production and maintenance. Our findings combined with previous studies suggest that the land-use regime, plant diversity, and plant species influence disease suppressive microbial communities.

Conclusions

We and others demonstrate links between crop diversity and soil ecosystem functions; however, the mechanisms underpinning this relationship require further study for more predictive soil microbiome management (Jangid et al. 2008, Lauber et al. 2008, McDaniel et al. 2014*b*, Orr et al. 2015, Tiemann et al. 2015, Venter et al. 2016). Crop rotations may facilitate the abundance of PPS organisms even though both our study and a previous study show decreases in structural diversity and functional evenness (McDaniel and Grandy 2016). We observed that the soil microbial community composition may be more important than soil microbial diversity to soil disease suppression. Crop rotations may also provide other important benefits like enhanced nutrient provisioning to plants, improvement of soil physical properties, increases in soil C, and increases in soil microbial and faunal activity that also could be responsible for the increased yields responsible for the rotation effect (Ball et al. 2005, van der Putten et al. 2016). Additional research focused on identifying patterns in soil microbial diversity and ecosystem function relationships can inform microbiome management, which will involve defined management of soil nutrients and plant diversity.

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Supporting Information

Additional Supporting Information may be found online at: http://onlinelibrary.wiley.com/doi/10.1002/ecs2.2235/full

Ecosphere

Crop rotational diversity increases disease suppressive capacity of soil microbiomes

Appendix S1

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Table S1. Soil properties measured along the KBS crop diversity gradient averaged over four replicate blocks. We acknowledge M.D. McDaniel and A.S. Grandy for these results (†McDaniel et al. 2014).

Treatment	Total C (g C kg^{-1} soil)	Total N (g N kg^{-1} soil)	NH_4^+ (mg N kg^{-1} soil)	NO_3^- (mg N kg^{-1} soil)	pH	Clay (%)	Silt (%)	Sand (%)
Fallow	8.74 ± 2.41	0.77 ± 0.16	0.07 ± 0.04	1.50 ± 0.31	6.70 ± 0.28	25 ± 9	39 ± 19	36 ± 11
CSW-2cov	8.98 ± 1.81	0.91 ± 0.12	0.09 ± 0.04	1.24 ± 0.50	6.25 ± 0.11	21 ± 10	51 ± 22	29 ± 12
CSW-1cov	9.63 ± 1.29	0.91 ± 0.04	0.06 ± 0.02	1.09 ± 0.51	6.37 ± 0.25	24 ± 10	46 ± 17	31 ± 7
CSW	7.43 ± 1.58	0.70 ± 0.10	0.07 ± 0.03	1.26 ± 0.62	6.54 ± 0.21	21 ± 10	45 ± 21	34 ± 12
CS	7.70 ± 2.11	0.73 ± 0.26	0.10 ± 0.09	1.16 ± 0.74	6.66 ± 0.12	23 ± 11	41 ± 24	36 ± 14
C-1cov	9.09 ± 1.86	0.93 ± 0.16	0.06 ± 0.02	1.50 ± 0.65	6.46 ± 0.25	27 ± 8	36 ± 19	38 ± 12
C	8.09 ± 1.24	0.71 ± 0.15	0.16 ± 0.16	1.31 ± 0.36	6.58 ± 0.38	25 ± 9	36 ± 19	38 ± 11

†McDaniel MD, Grandy AS, Tiemann LK, Weintraub MN. 2014. Crop rotation complexity regulates the decomposition of high and low quality residues. Soil Biol Biochem 78:243-254.

Table S2. Effects of crop rotation on total bacterial community composition (16S rRNA gene amplicon sequencing) based on permutational MANOVA (PERMANOVA) results. Rotation effect was considered to significantly contribute to community variation at $p<0.05$.

	df	SS	MS	*F*	R^2	*p*-value
Rotation	6	0.493	0.082	2.08	0.372	<0.001
Residuals	21	0.832	0.040		0.628	
Total	27	1.32			1	

Methods

We targeted *phlD* and *prnD*, which are known to code for a subset of DAPG producers and PRN producers, respectively in environmental samples (according to methods in Latz et al. 2012). We assessed the composition of disease suppressive microorganisms by targeting the *phlD* gene using terminal restriction fragment length polymorphism (T-RFLP) (von Felten et al. 2011). For *phlD* gene amplification, the forward primer B2BF (5′-ACCCACCGCAGCATCGTTTATGAGC-3′) and reverse primer FAM-BPR4 (5′-CCGCCGGTATGGAAGATGAAAAAGTC-3′) yielded a 629 bp product. In each 25 μL PCR reaction, we combined 5% dimethylsulfoxide, 0.8 mg ml^{-1} bovine serum albumin, 1× GoTaq Colorless Master Mix (Promega, Madison, WI), 0.2 μM of each primer and 5 μL of template DNA. Reactions were cycled with an initial denaturation at 94 °C for 2 min, followed by 35 cycles of 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 2 min, with a final extension carried out at 72 °C for 10 min (von Felten et al. 2011). The amplified PCR products were purified using the QIAquick PCR purification kit (Qiagen, Valencia, CA). After purification, amplicons generated from each sample were digested in multiple restriction enzymes overnight in 12 μL reaction mixtures containing 4 μL of PCR product, 1×enzyme buffer (von Felten et al. 2011). After digestion, the enzymes were inactivated for 5 min at 80 °C, and the digested products were purified according to the purification kit protocol (Qiagen, Valencia, CA). For T-RFLP analysis, we combined 1.5 μL of the digested product with 9 μL of HiDi formamide (Applied Biosystems, Foster City, CA) and 0.4 μL of internal size standard ABI GeneScan LIZ 600 (Applied Biosystems, Foster City, CA). The

samples were incubated for 3 min at 96 °C and then stored on ice prior to fragment analysis. We determined the length and relative abundance of terminal restriction fragments (T-RFs) using an ABI 3130×1 Genetic Analyzer (Applied Biosystems, Foster City, CA). Electrophoresis conditions were 60 °C and 15 kV with a run time of 41 min using POP-7 polymer. The resulting data were analyzed using the GeneMapper Software 4.0 (Applied Biosystems, Foster City, CA). The peak detection limit was set to 50 fluorescence intensity units.

Figure S1.

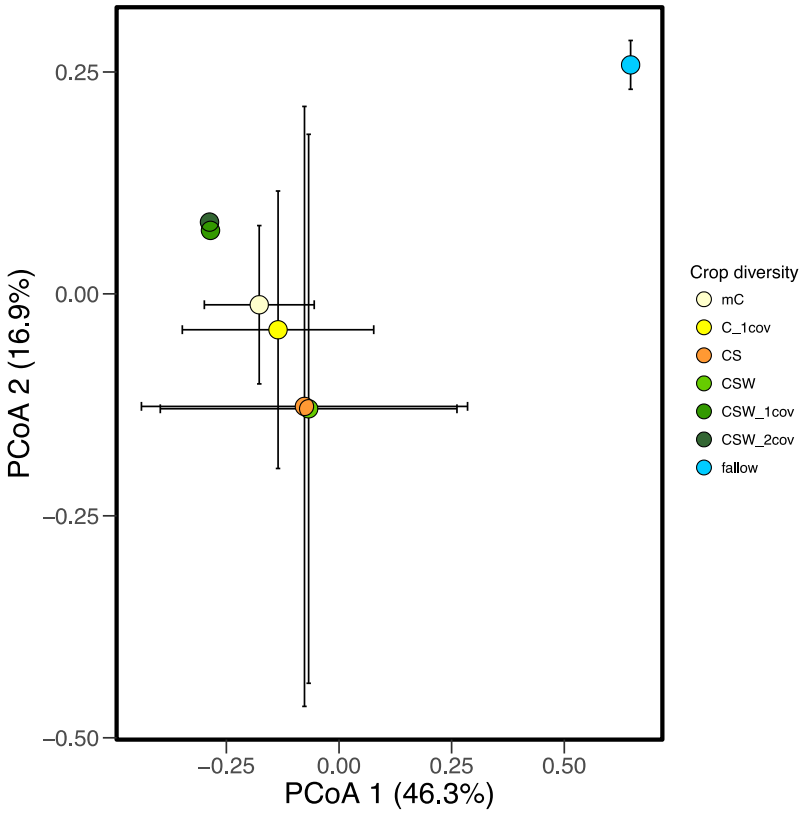

Figure S1. Ordination from Principal Coordinates Analysis of disease suppressive community based on T-RFLP of *phlD* gene (DAPG producers) T-RF relative abundance along crop diversity gradient.

Figure S2.

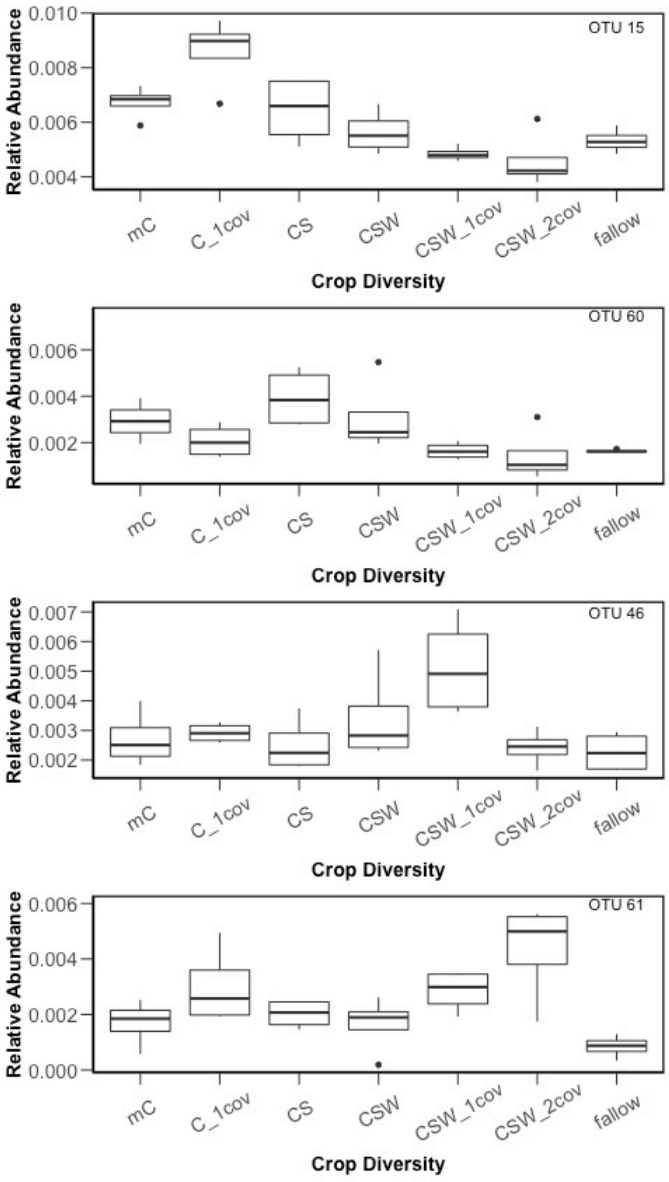

Figure S2. Boxplots of indicator bacteria relative abundance along crop diversity gradient based on indicator species analysis for a subset of taxa displayed in Table 4. [OTU15 = unclassified Betaproteobacteria represented C_{1cov} treatment; OTU60 = Acidobacteria Gp4 represented CS treatment; OTU46 = Acidobacteria Gp4 represented CSW_{1cov} treatment; OTU61 = unclassified Sphingomonadaceae represented CSW_{2cov}]