

1 Title: Crop diversity increases disease suppressive capacity of soil microbiomes

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10 Running Head: disease suppression in soil microbiomes

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13

14 Abstract

15 Microbiomes can aid in the protection of hosts from infection and disease, but the mechanisms

16 underpinning these functions in complex environmental systems remain unresolved. Soils

17 contain microbiomes that influence plant performance, including their susceptibility to disease.

18 For example, some soil microorganisms produce antimicrobial compounds that suppress the

19 growth of plant pathogens, which can provide benefits for sustainable agricultural management.

20 Evidence shows that crop rotations increase soil fertility and tend to promote microbial diversity,

21 and it has been hypothesized that crop rotations can enhance disease suppressive capacity, either

22 through the influence of plant diversity impacting soil bacterial composition or through the

23 increased abundance of disease suppressive microorganisms. In this study, we used a long-term

24 field experiment to test the effects of crop diversity through time (i.e., rotations) on soil  
25 microbial diversity and disease suppressive capacity. We sampled soil from seven treatments  
26 along a crop diversity gradient (from monoculture to five crop species rotation) and a spring  
27 fallow (non-crop) treatment to examine crop diversity influence on soil microbiomes including  
28 bacteria that are capable of producing antifungal compounds. Crop diversity significantly  
29 influenced bacterial community composition, where the most diverse cropping systems with  
30 cover crops and fallow differed from bacterial communities in the 1-3 crop species diversity  
31 treatments. While soil bacterial diversity was about 4% lower in the most diverse crop rotation  
32 (corn-soy-wheat + 2 cover crops) compared to monoculture corn, crop diversity increased  
33 disease suppressive functional group *prnD* gene abundance in the more diverse rotation by about  
34 9% compared to monocultures. Identifying patterns in microbial diversity and ecosystem  
35 function relationships can provide insight into microbiome management, which will require  
36 manipulating soil nutrients and resources mediated through plant diversity.

37

38 Key words: Crop rotation; disease suppression; microbial diversity; structure-function  
39 relationships

40

41 Abbreviations

42 2,4-diacetylphloroglucinol (DAPG); plant growth promoting rhizobacteria (PGPR); plant  
43 pathogen suppression (PPS); pyrrolnitrin (PRN)

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47 Introduction

48 Microbiomes are collections of microorganisms that live in close association with plants  
49 and animals. Certain microorganisms can confer benefits because they contain genes that aid in  
50 nutrient acquisition (Chaparro et al. 2012, Berendsen et al. 2012) while other microorganisms  
51 can protect hosts by preventing colonization by pathogens (Latz et al. 2012, Schlatter et al.  
52 2017). For example, soils harbor a diverse collection of microorganisms that affect the evolution  
53 and ecology of plant populations (Lau and Lennon 2012, van der Putten et al. 2013, 2016). Many  
54 soil microorganisms establish intimate associations with plant roots, which can result in  
55 enhanced plant growth through many mechanisms (Mendes et al. 2013, 2015). One important  
56 mechanism through which soil microorganisms increase plant performance and fitness is via  
57 disease suppression. In this case, a healthy and robust soil microbiome can serve as a first line of  
58 defense for plants against soil-borne pathogens within the resident soil microbial community  
59 (Mendes et al. 2013, van der Putten et al. 2016) either directly through antibiosis or parasitism,  
60 or indirectly through enhancing plant immune responses (Mendes et al. 2013).

61 Plant-soil feedback theory provides a framework for assessing the mechanisms and  
62 outcomes microbiome dynamics. More specifically, there are many ways soil microbiomes can  
63 be managed to influence soil pathogens. One way is through crop selection. Specifically,  
64 individual crops can affect pathogen populations by altering chemical, physical, or biological  
65 properties in their rhizosphere (Raaijmakers et al. 2009, Berendsen et al. 2012). Further, recent  
66 attention is being paid to ecological intensification of farms (Tilman et al. 2011), and one of the  
67 promising specific management practices under this strategy is to diversify farms by rotating  
68 crops (Smukler et al. 2010, Lin 2011). The colloquial use of the term “rotation effect” has a long  
69 history of agronomic research (Karlen et al. 1994), and its origins are from the overwhelming

70 evidence that rotating crops increase crop yield (Liebman and Dyck 1993, Karlen et al. 1994).  
71 From a management or conservation perspective, crop rotations are not the traditional form of  
72 increasing biodiversity. At any given time, the species richness on a farm using crop rotations is  
73 often one (i.e., monoculture), but there is a diverse suite of biochemical inputs from crops  
74 planted at different times. There is mounting evidence that this form of ‘temporal biodiversity’  
75 may provide some of the same beneficial ecosystem functions as traditional spatial biodiversity  
76 (Zak et al. 2003), such as carbon sequestration, pest control, and nutrient cycling (Ball et al.  
77 2005, McDaniel et al. 2014b, Tiemann et al. 2015, Venter et al. 2016). Despite this frequently  
78 found “rotation effect”, the underlying mechanism(s) in support of crop rotations are largely  
79 unknown, but might be related to crop diversity promoting plant-pathogen-suppressing  
80 microorganisms.

81       Often, plant pathogen suppression (PPS) is associated with soil microbial communities  
82 that have the capacity to produce antimicrobial compounds. Specifically, antibiosis has been  
83 linked to disease suppressive capacity, whereby the abundance of antagonistic bacteria has been  
84 associated reductions in fungal pathogens through competitive inhibition (Weller et al. 2002,  
85 Haas and Défago 2005). For example, bacterial production of secondary metabolites 2,4-  
86 diacetylphloroglucinol (DAPG) and pyrrolnitrin (PRN) are two potent toxins known to suppress  
87 fungal pathogens in soils (Garbeva et al. 2004a, 2004b, Haas and Défago 2005). However, the  
88 extent to which abiotic and biotic factors influence the abundance of such microbes remains  
89 unclear. Abiotic factors (e.g., salt, moisture, nutrients) can limit the strength and alter the  
90 direction of plant-soil feedbacks (Bever et al. 1997, Mills and Bever 1998, Packer and Clay  
91 2000, Kulmatiski et al. 2008). It has been argued that edaphic features may be important or even  
92 required for PPS and might influence species interactions. In addition, aboveground features

93 such as plant diversity could influence PPS. Specifically, plant diversity could increase the total  
94 soil bacterial diversity giving way to the “sampling effect” where species-rich ecosystems  
95 contain species that function at high levels (e.g., Tilman et al. 2002, Naeem and Wright 2003).  
96 Specifically, plant diversity could increase the probability of harboring PPS in the soil microbial  
97 community. Alternatively, plant diversity could modify soil microbial communities without  
98 influencing total diversity but rather through selecting for microorganisms that perform certain  
99 functions such as disease suppression. Some evidence suggests that PPS microorganisms are  
100 influenced by competition for iron, antibiosis, lytic enzymes, and induction of systemic  
101 resistance with host plant (Doornbos et al. 2012). For example, antibiosis has been linked to  
102 disease suppressive capacity, whereby the abundance of antagonistic bacteria has been associated  
103 with reductions in fungal pathogens through competitive inhibition (Weller et al. 2002, Haas and  
104 Défago 2005). Therefore, the abundance of PPS microbes may be a reflection of the total  
105 diversity of the soil microbial community, but this hypothesis has not been rigorously evaluated.

106         Given the unknown effect of crop diversity on PPS, we used a long-term (12 y) crop  
107 rotation study at the Kellogg Biological Station LTER to examine the effect of crop diversity on  
108 soil bacterial biodiversity and PPS potential. Specifically, our study addresses the following  
109 questions: (1) what is the relationship between crop diversity and soil microbial community  
110 composition and PPS? and (2) what is the role of changes in soil physicochemical properties on  
111 the crop diversity effect on soil microbial community composition, and PPS? We hypothesized  
112 that increased crop diversity would increase the diversity of the soil microbial community, and  
113 also increase the PPS in the soil through supporting a higher proportion of disease suppressive  
114 microbial taxa.

115

116 Methods

117

118 *Site description and experimental design*

119 We collected soils from the Biodiversity Gradient Experiment  
120 (<http://lter.kbs.msu.edu/research/long-term-experiments/biodiversity-gradient/>) at W.K. Kellogg  
121 Biological Station Long-Term Ecological Research (KBS LTER) site in southwest, Michigan,  
122 USA. Mean annual temperature is about 10 °C and mean annual precipitation is about 1000 mm  
123 yr<sup>-1</sup> (Robertson and Hamilton 2015). The soils are Kalamazoo (fine-loamy) and Oshtemo  
124 (coarse-loamy) mixed, mesic, Typic Hapludalfs formed under glacial outwash (Crum and  
125 Collins 1995). The crop rotation treatments at the Biodiversity Gradient Experiment included:  
126 monoculture corn (*Zea mays*, mC), corn with 1 red clover (*Trifolium pretense* L.), cover crop  
127 (C<sub>1cov</sub>), corn-soy (*Glycine max*, CS), corn-soy-wheat (*Triticum aestivum*, CSW), CSW with red  
128 clover (CSW<sub>1cov</sub>), CSW with red clover and cereal rye (*Secale cereal* L., CSW<sub>2cov</sub>), and a spring  
129 fallow treatment that was just plowed every spring but contains 7-10 naturally-occurring plant  
130 species in the region (Table 1). This spring fallow treatment is considered the benchmark for  
131 plant diversity in the region, and under same tillage. Plantings of cover crop were dependent on  
132 the main crop in rotation (Smith and Gross 2006, 2007). The experiment was in a randomized  
133 complete block design, which included four blocks or replicates of each treatment. All plots  
134 received the same tillage at 15 cm depth, and no fertilizer or pesticides were applied to these  
135 plots.

136

137 *Soil sampling*

138 We sampled soil from six crop diversity treatments, but to eliminate any immediate crop  
139 effect all the treatments were sampled in the corn phase and a spring fallow treatment (Table 1)  
140 on November 1, 2012. In each plot, we collected five soil cores (5 cm diameter, 10 cm depth)  
141 and then homogenized the cores in the field. A subsample from each composite sample was  
142 sieved through 4 mm in the field, flash frozen in the field in liquid nitrogen, and stored at -80 °C  
143 prior to molecular-based microbial analyses.

144

#### 145 *Soil physicochemical analyses*

146 From the same soil samples that were flash frozen for DNA extraction, soil chemical  
147 properties (total carbon, total nitrogen, ammonium, nitrate, pH, texture). These soils were  
148 previously analyzed and soil physicochemical characteristics reported (McDaniel et al. 2014a,  
149 McDaniel and Grandy 2016). Labile C was measured as permanganate oxidizable C (POXC)  
150 according to (Culman et al. 2012). Overall biological activity and amount of potentially  
151 mineralizable carbon (PMC) and nitrogen (PMN) were analyzed using a 120 d aerobic  
152 incubation (McDaniel and Grandy 2016).

153

#### 154 *Bacterial community sequencing*

155 To examine the relationship between crop diversity and soil microbial diversity, we used  
156 16S rRNA targeted amplicon sequencing of the soil bacterial community. We extracted DNA  
157 using the MoBio Power Soil DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA).  
158 DNA concentration was adjusted to a standard concentration of 20 ng  $\mu\text{l}^{-1}$  and used as template.  
159 To characterize bacterial taxonomic diversity, we used barcoded primers (515f/806r primer set)  
160 developed by the Earth Microbiome Project to target the V4-V5 region of the bacterial 16S

161 subunit of the ribosomal RNA gene (16S rRNA) (Caporaso et al. 2012). For each sample, PCR  
162 product combined from three 50 µl reactions, concentration quantified, and PCR product from  
163 each soil sample was combined in equimolar concentrations for paired-end 250×250 sequencing  
164 using the Illumina MiSeq platform according to details in (Muscarella et al. 2014). Briefly, we  
165 assembled the paired-end 16S rRNA sequence reads using the Needleman algorithm (Needleman  
166 and Wunsch 1970). All sequences were subjected to systematic checks to reduce sequencing and  
167 PCR errors. High quality sequences (i.e., >200 bp in length, quality score of >25, exact match to  
168 barcode and primer, and contained no ambiguous characters) were retained. In addition, we  
169 identified and removed chimeric sequence using the UCHIME algorithm (Edgar et al. 2011). We  
170 aligned our sequence data set with the bacterial SILVA-based bacterial reference database  
171 (Yilmaz et al. 2014). During data analysis, operational taxonomic units (OTUs) were binned at  
172 97% sequence identity and phylogenetic classifications of bacterial sequences performed.  
173 Sequences were processed using the software package *mothur* v.1.35.1 (Schloss et al. 2009,  
174 Kozich et al. 2013). A total of 12,539,359 sequence reads were generated, and we analyzed  
175 47,261 OTUs for bacterial community analyses.

176

### 177 *Composition and abundance of disease suppression genes*

178 To characterize the subset of the microbiome associated with disease suppressive  
179 potential, we targeted disease suppressive taxa as the subset of soil microorganisms possessing  
180 genes that are required for the production of antifungal compounds 2,4-diacetylphloroglucinol  
181 (DAPG) (von Felten et al. 2011) (see supplemental material) and pyrrolnitrin (PRN) (Garbeva et  
182 al. 2004b, Haas and Défago 2005).

183 We assessed the relative abundance of disease suppressive functional genes by targeting



184 *prnD* using quantitative PCR (qPCR) (Garbeva et al. 2004b). The partial *prnD* gene abundance  
185 was quantified using a SYBR green assay with primers *prnD*-F (5'-  
186 TGCACTTCGCGTTCGAGAC-3') and *prnD*-R (5'-GTTGCGCGTCGTAGAAGTTCT-3')  
187 (Garbeva et al. 2004b). The 25  $\mu$ L PCR reaction contained 1 $\times$  GoTaq Colorless Master Mix  
188 (Promega, Madison, WI), 0.4  $\mu$ M of each primer, and 5  $\mu$ L of template DNA. Cycling conditions  
189 were as following: initial cycle 95  $^{\circ}$ C for 10 min, and 30 cycles of 95  $^{\circ}$ C for 15 s and 60  $^{\circ}$ C for 1  
190 min. For the qPCR standard curve, *prnD* gene was amplified from soil genomic DNA. PCR  
191 fragments were cloned to pGEM-T Easy Vector System according to the manufacturer's manual  
192 (Promega, Madison, WI). Plasmids were extracted using the QIAprep Spin Miniprep kit (Qiagen,  
193 Valencia, CA), and cloned fragments were verified by PCR and agarose gel electrophoresis.  
194 Dilutions of plasmid DNA containing *prnD* gene were used to generate standard curves in  
195 quantities ranging from  $5.0 \times 10^2$  to  $5.0 \times 10^7$  copies. We quantified the *prnD* gene in 25  $\mu$ L  
196 reaction volumes containing about 20 ng DNA template, 1 $\times$ TaqMan Environmental Master Mix  
197 2.0 (Applied Biosystems, Valencia, CA), 1 $\times$  SYBR green I, and 0.4  $\mu$ M of each primer.  
198 Fragments were amplified with an initial denaturation step at 95  $^{\circ}$ C for 10 min, followed by 40  
199 cycles of 95 $^{\circ}$ C for 15 s, 60  $^{\circ}$ C for 1 min. For each sample, PCR reactions were run in triplicate.  
200 We obtained standard curves based on serial dilutions of mixed PCR product amplified from soil  
201 samples. Reactions were analyzed on a BIO-RAD CFX-96Real-Time System (Bio-Rad,  
202 Hercules, California, USA).

203

#### 204 *Statistical analyses*

205 We examined microbiome differences among crop diversity treatments by comparing  
206 total community diversity and composition as well as disease suppression markers. We tested for

207 differences in total bacterial diversity (based on Shannon Diversity Index  $H'$ , bacterial species  
208 richness, and Pielou's Evenness Index  $J'$ ) and *prnD* gene abundance in response to crop diversity  
209 treatment using analysis of variance (ANOVA). We checked that data met assumptions of  
210 analyses, and we treated crop diversity treatment as a fixed factor and block as a random effect.  
211 We used Tukey's Honestly Significant Difference (HSD) tests to identify between-group  
212 differences in bacterial diversity and *prnD* gene abundance.

213 To visualize patterns of microbial community composition, we used Principal  
214 Coordinates Analysis (PCoA) of the microbial community composition based on the Bray-Curtis  
215 dissimilarity coefficient for each possible pair of samples using the R statistical package (R Core  
216 Development Team 2015). To test for differences in total bacterial communities and a subset of  
217 previously identified biocontrol bacterial taxa (i.e., *Pseudomonas* spp. and *Streptomyces* spp.)  
218 among crop diversity treatments, we used non-parametric permutational multivariate analysis of  
219 variance (PERMANOVA) implemented with the *adonis* function in the R Statistics Package R  
220 version 3.2.3 (R Development Core Team 2015). PERMANOVA was also used to assess the  
221 contribution of soil factors to the variation in bacterial community composition. The  $R^2$  value  
222 reported refers to the treatment sums of squares divided by the total sums of squares for each soil  
223 factor in the model. Because the *adonis* function carries out sequential tests (similar to Type I  
224 sums of squares) (Oksanen et al. 2010), the effect of the last soil factor or soil biological activity  
225 factor of the model was included in the final PERMANOVA model summary (Peralta et al.  
226 2012). We also performed a similarity percentage analysis (SIMPER) using the *simper* function  
227 (R Statistics Package R version 3.2.3) (Clarke 1993, Warton et al. 2012) to identify the bacterial  
228 OTUs responsible for community differences between monoculture corn and other crop diversity  
229 treatments and is based on the contribution of individual taxa to the average Bray-Curtis

230 dissimilarity. We also performed multiple linear regression (gene abundance ~ crop number +  
231 total soil carbon + soil moisture + soil ammonium + soil nitrate) to test the influence of soil  
232 factors and crop diversity number on abundance of disease suppression/biocontrol gene *prnD*  
233 using the *lm* function in the R Statistics Package R version 3.0.2 (R Core Development Team  
234 2015).

235

## 236 Results

237

### 238 *Bacterial community composition and soil function relationships*

239 The crop diversity treatment significantly influenced soil microbiomes represented by the  
240 bulk soil bacterial community composition ( $R^2 = 0.37$ ,  $p < 0.001$ ; Appendix S1: Table S2, Fig.  
241 1). Bacterial communities from the fallow plots and the most diverse crop rotations (CSW,  
242 CSW<sub>1cov</sub>, CSW<sub>2cov</sub>) were more similar to each other than the lower crop diversity treatments  
243 (C<sub>1cov</sub>, CS) (Fig. 1). The monoculture corn (mC) treatment was more distinct in bacterial  
244 community composition than all other crop diversity treatments (Fig. 1).

245 Bacterial diversity, as measured using Shannon Diversity Index ( $H'$ ), was surprisingly  
246 greater under lower crop diversity systems than higher crop diversity systems, but highest in  
247 fallow treatments the most diverse non-cropping system (crop rotation:  $F_{6,20}=10.16$ ,  $p<0.0001$ ;  
248 block:  $F_{1,20}=0.20$ ,  $p=0.6600$ ; Fig. 2). Among, the corn cropping systems, mC had the highest  
249 Shannon Diversity Index in the most diverse rotation of corn-soybean-wheat with two cover  
250 crops (CSW<sub>2cov</sub>). In addition, bacterial species richness and Pielou's Evenness Index ( $J'$ ) revealed  
251 similar patterns across crop diversity treatments (evenness:  $F_{6,18}=2.36$ ,  $p=0.073$ ; richness:  
252  $F_{6,18}=2.61$ ,  $p=0.053$ ; Fig. 2). Across all diversity metrics, the longest crop rotation (CSW<sub>2cov</sub>)

253 showed the lowest richness and evenness values, and fallow soils generally had the highest  
254 values (Fig. 2).

255 Soil physicochemical properties and soil function were related bacterial community  
256 composition to varying degrees. A summary of soil attributes is presented in Appendix S1: Table  
257 S1 and elsewhere (McDaniel and Grandy 2016). Bacterial community composition was best  
258 explained by soil texture, which varied across the experiment site from 9 to 38 % clay  
259 ( $R^2=0.066$ ,  $p<0.05$ , Table 3a). However, bacterial community composition was also marginally  
260 affected by soil moisture ( $R^2=0.048$ ,  $p<0.10$ , Table 2). Labile C as measured with permanganate  
261 oxidization was related to bacterial community composition ( $R^2=0.074$ ,  $p<0.05$ ), but potentially  
262 mineralizable C did not. Potentially mineralizable nitrogen (PMN), however, which is produced  
263 in the same aerobic incubation as PMC and an indicator of nutrient-supplying power of a soil (a  
264 biologically available N pool), was significantly correlated with bacterial community  
265 composition ( $R^2=0.063$ ,  $p<0.05$ , Table 3).

266

#### 267 *Disease suppression functional potential*

268 Crop diversity affected PPS potential in soils. The *prnD* gene abundances in cropping  
269 systems were higher than under fallow conditions (crop rotation:  $F_{6,20}=7.51$ ,  $p=0.0003$ ; Fig. 3).  
270 In cropping systems, the *prnD* gene in CSW<sub>2cov</sub> treatment was the most abundant, and the gene  
271 abundance was significantly higher than in CSW and fallow treatments (Fig. 3). Our diversity  
272 benchmark, the fallow treatment (i.e., lowest crop diversity), showed the lowest *prnD* gene  
273 abundances (Fig. 3). Based on multiple linear regression analysis, plant and soil factors  
274 significantly related to *prnD* abundance (Adjusted  $R^2=0.40$ ,  $F=4.571$ ,  $p=0.005$ ). Crop species  
275 number ( $p=0.003$ ), soil carbon ( $p=0.002$ ), and soil moisture ( $p=0.0005$ ) appeared to be

276 significant predictors of *prnD* gene abundance (Table 3). We also observed a shift in the  
277 composition of disease-suppression microorganisms (represented by *phlD* gene fingerprint  
278 analysis using terminal restriction length polymorphism, T-RFLP) along the crop diversity  
279 gradient. The *phlD* community composition in the fallow treatment was different from other  
280 cropping systems (Appendix S1: Fig. S1).

281

### 282 *Soil bacterial-disease suppressive function relationship*

283 The bacterial taxa primarily responsible for treatment differences between mC and the  
284 other crop diversity treatments were *Sphingomonadales* spp. and *Acidobacteria* subgroup Gp6  
285 (Appendix S1: Table S3). When we compared a subset of taxa representing broad biocontrol  
286 bacterial community (composed of *Streptomyces* spp. and *Pseudomonas* spp.), there was no  
287 significant pattern in community composition across the crop diversity treatment  
288 (PERMANOVA; crop rotation:  $R^2=0.321$ ,  $p=0.132$ ; Appendix S1: Table S4).

289

## 290 Discussion

291

292 Soil microbiomes represent microbial communities living in close association with host  
293 plants and can protect host organisms from infection and disease. In this study, we found that  
294 crop rotation history impacted soil microbiomes and altered disease suppression potential in  
295 agricultural soils. However, we found some unexpected results that contrasted with our  
296 hypothesis. Contrary to our hypothesis, bacterial diversity decreased with increasing cropping  
297 diversity (Fig. 2). However, the PPS capability of the soil microbial community increased with  
298 crop diversity, but surprisingly the lowest PPS was in the diverse fallow treatments (Fig. 3). We

299 observed that without crop plants (as reflected in the no crop fallow treatment), disease  
300 suppressive potential was significantly diminished compared to crop treatments, possibly due to  
301 reduced selection for soil microorganisms with disease suppression traits. The composition of the  
302 soil microbial community may be more important than diversity to soil suppressive function.  
303 Thus, crop rotation has the potential to impact diseases suppressive function, providing evidence  
304 for facilitation of fungal pathogen protection of plants in diverse crop rotation systems.

305

### 306 *Crop diversity effects on soil bacterial diversity*

307       Crop rotation history decreased soil bacterial diversity over this 12-year crop diversity  
308 study. The pattern of reduced bacterial diversity (based on 16S rRNA gene sequencing) was  
309 lower in soils with higher cropping diversity. There are two most parsimonious explanations for  
310 this unexpected finding. First, this pattern in belowground biodiversity might be due to increased  
311 abundance of weedy plant species in low diversity treatments, but especially the monoculture  
312 corn. In other words, while we were considering the corn treatment as a single species; there  
313 could ostensibly have been up to 13 weed species per m<sup>2</sup>, as measured in an earlier study from  
314 this experiment (Smith and Gross 2007). On the other hand, this same study showed the most  
315 diverse cropping systems (CSW<sub>2cov</sub>) had only 5 or 6 weeds per m<sup>2</sup>. Second, perhaps there was  
316 not an artifact from the weeds and that soil bacterial diversity does decrease with increasing crop  
317 diversity, but other members of the soil microbial community (e.g., fungi, archaea) may be  
318 increasing in diversity with longer crop rotations. Despite decreased bacterial taxonomic  
319 diversity, a previous study based on the same soils that we used in this study, found that  
320 catabolic evenness (a measure of the diversity of catabolic function) also decreased with  
321 increasing crop diversity (McDaniel and Grandy 2016). This indicates that the trend in lower

322 bacterial diversity with increasing crop diversity is not just structural, but also functional, and  
323 may indicate carbon resource specialization among bacteria since they are probably the major  
324 contributor to C catabolism in these substrate-induced respiration methods (Goldfarb et al. 2011,  
325 Allison et al. 2014). Based on phospholipid fatty acid analysis, a previous study showed that  
326 bacterial biomass in the micro-aggregate soil organic matter fraction was greatest in high  
327 compared low crop diversity treatments at this long-term experiment during a different sampling  
328 date (Tiemann et al. 2015). In addition, a previous meta-analysis revealed that the crop rotation  
329 effect increased soil bacterial diversity (i.e., Shannon Diversity Index H') most notably in the  
330 first five years of treatment, but crop rotations longer than five years were more variable in  
331 diversity and not significantly different (Venter et al. 2016). Other studies do find significant  
332 negative effects of crop rotations on soil microbial diversity (Berg and Smalla 2009, Yin et al.  
333 2010, Kulmatiski and Beard 2011, Reardon et al. 2014). The reason for these findings remain  
334 unknown but may be a combination of diversity impacts on other soil organisms not evaluated in  
335 this study or due to length of time associated with crop diversity treatment.

336

### 337 *Crop diversity and plant pathogen suppression relationship*

338 We found that the increased crop diversity, via rotation, increased the abundance and  
339 altered the composition of a specific plant pathogen suppression gene (Figs. 3, Appendix S1: Fig.  
340 S1). Our results suggest that crop diversity may increase the disease suppression of agricultural  
341 soils, and are consistent with previous studies suggesting that plant diversity can enhance  
342 protection against soil-borne pathogens by fostering antagonistic soil bacterial communities  
343 (Latz et al. 2012, van der Putten et al. 2016). One potential explanation for the negative plant  
344 diversity and disease suppressive function relationship is due to facilitation, where changes in

345 plant root exudation may lead to enrichment of plant growth promoting rhizobacteria (PGPRs)  
346 (Lugtenberg and Kamilova 2009, Badri et al. 2009, Chaparro et al. 2012). In previous studies,  
347 microbial interactions among the total microbial community and soil-borne pathogens in the  
348 plant rhizosphere have influenced both plant growth and productivity (Bakker et al. 2010, Penton  
349 et al. 2014).

350         The addition of cover crops to rotations strongly increased disease suppressive potential.  
351 This along with evidence from previous studies shows that crop rotations may prevent many  
352 forms of crop disease caused by *Fusarium* spp., *Phytophthora*, and *Rhizoctonia* spp.  
353 (Raaijmakers et al. 2009, van der Putten et al. 2016). Soil microbial diversity has been implicated  
354 as important for soil disease suppression; sterilized soils lose suppressive capacity, and adding  
355 soil microorganisms to sterilized soil facilitates disease suppression functional capacity (Garbeva  
356 et al. 2006, Brussaard et al. 2007, Postma et al. 2008). Biocontrol bacteria can also provide  
357 disease suppression against plant pathogens by way of the following mechanisms: competition  
358 for iron, antibiosis, lytic enzymes, and induction of system resistance of host plants (Doornbos et  
359 al. 2012, Schlatter et al. 2017). Plants can also facilitate recruitment of specific biocontrol  
360 microorganisms in some cases. A previous study suggests that beneficial pseudomonads are  
361 recruited depending on the most dominant soil-borne pathogen infecting crop species (Mavrodi  
362 et al. 2012, Berendsen et al. 2012). In the present study, we analyzed a subset of previously  
363 reported biocontrol bacterial taxa (e.g., *Pseudomonas* spp. and *Streptomyces* spp.) across the  
364 crop diversity gradient; however, we did not detect distinct changes in putative biocontrol  
365 community composition (Appendix S1: Table S4).

366         Our study revealed that cover crops in combination with corn-soy-wheat rotations  
367 increased abundance of the *prnD* gene, which is responsible for producing antifungal compound



368 pyrrolnitrin (PRN) (Garbeva et al. 2004b, Haas and Défago 2005), by about 9 % compared to the  
369 other cropping system. Cover crop species may have important effects on the *prnD* gene  
370 abundance and disease suppressive functional potential in soils, but only in combination with  
371 corn-soy-wheat because the cover crop with corn only did not show high *prnD* abundance (Fig.  
372 3). The *prnD* gene abundance in all cropping systems was higher than in fallow treatment (or  
373 most diverse treatment). The abundance of DAPG and PRN producers increasing with plant  
374 diversity has been previously observed (Latz et al. 2012). Compared to agricultural soils, the  
375 PRN producers were more frequently detected in grassland or grassland-derived plots (Garbeva  
376 et al. 2004a, 2004b). In a previous study, the *prnD* gene abundance increased in the presence of  
377 grasses, but the legume species tended to decrease the DAPG and PRN producer abundance  
378 (Latz et al. 2012). Without crops (as reflected in the fallow treatment), we observed that disease  
379 suppressive potential significantly declined. This observation may be indicative of species-  
380 specific facilitation of PPS soil microorganisms. This disease suppressive phenomenon is known  
381 to have important implications for sustainable biocontrol of soil-borne pathogens. In addition, it  
382 is possible that when plant diversity is high, there is less soil-borne pathogen pressure on plant  
383 hosts due to decreased competition for resources among pathogenic and non-pathogenic soil  
384 microorganisms.

385

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

387 Disease suppression may have a major role in what is colloquially referred to as “the  
388 rotation effect.” Our study provided evidence that crop diversity alters soil bacterial community  
389 composition and population of PPS microbes, but the mechanisms through which this occurs can  
390 include physical, chemical, and biological changes to the soil environment. Crops can influence

391 soil properties and soil microbiomes in a variety of ways, including physically and chemically.  
392 Cover crops are the most salient feature of these crop rotations affecting the soil bacterial  
393 community in general. This is not surprising, since cover crops have been shown to influence  
394 several soil properties, which likely have indirect effects on the soil bacterial community  
395 composition. In addition, previous studies showed cover crops can have immediate impacts on  
396 soil microbial communities (Wiggins and Kinkel 2005, Finney et al. 2017). Soil properties like  
397 total C, total N, pH, and bulk density and porosity have all been shown to increase with cover  
398 crops (Bullock 1992, Liebman and Dyck 1993, Tilman et al. 2002, McDaniel et al. 2014b,  
399 Tiemann et al. 2015). Physically, crop diversity (especially rotations) can enhance soil properties  
400 like improving plant water availability by lowering bulk density, increasing soil pore space, and  
401 increasing soil aggregate formation (Tilman et al. 2002, McDaniel et al. 2014b, Tiemann et al.  
402 2015), which could have indirect influence over the soil bacterial community as well.  
403 Chemically, cover crops are providing more carbon to the soil through residues, but also root  
404 exudation of recently assimilated photosynthate, composed of soluble, low molecular weight  
405 organic compounds (Neumann and Romheld 2007). As a consequence, the increased C flow  
406 from cover crop root exudates can stimulate soil microbial activity. Changes in root exudates  
407 have been observed to shift microbial community composition and stimulate a diverse microbial  
408 community (Hooper et al. 2000, Stephan et al. 2000, Paterson et al. 2009, Dijkstra et al. 2010).  
409 Biologically, some soil microorganisms can provide PPS through competition for nutrients,  
410 antibiosis, and induction of system resistance of host plants (Doornbos et al. 2012). Our study  
411 focused on soil bacterial community composition. It has been identified that crop rotation also  
412 influences soil fungal and faunal communities, which are also important members of the soil  
413 food web (McLaughlin and Mineau 1995). For example, increased protist predation on soil

414 bacteria has resulted in indirect effect on disease suppression function (Jousset et al. 2008, 2010).  
415 These studies revealed that increased predator pressure by soil protists have been linked to  
416 increased biocontrol function through enhanced bacterial DAPG production (Jousset et al. 2008,  
417 2010).

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

437 suggest that the land-use regime, plant diversity, and plant species influence disease suppressive  
438 microbial communities.

439

#### 440 Conclusions

441 We and others demonstrate links between crop diversity soil ecosystem functions;  
442 however, the mechanisms underpinning this relationship require further study for more  
443 predictive soil microbiome management (Lauber et al. 2008, Jangid et al. 2008, McDaniel et al.  
444 2014b, Orr et al. 2015, Tiemann et al. 2015, Venter et al. 2016). Crop diversity may facilitate the  
445 abundance of PPS organisms even though both our study and previous study show decreases in  
446 structural diversity and functional evenness (McDaniel and Grandy 2016). We observed that the  
447 soil microbial community composition may be more important than soil microbial diversity to  
448 soil disease suppression. Crop rotations may also provide other important benefits like enhanced  
449 nutrient provisioning to plants, improvement of soil physical properties, increases in soil C, and  
450 increases in soil microbial and faunal activity that also could be responsible for the increased  
451 yields responsible for the rotation effect (Ball et al. 2005, van der Putten et al. 2016). Additional  
452 research focused on identifying patterns in soil microbial diversity and ecosystem function  
453 relationships can inform microbiome management, which will involve defined management of  
454 soil nutrients and plant diversity.

455

#### 456 Acknowledgements

457 This work was supported by the U.S. Department of Agriculture National Institute of Food and  
458 Agriculture Postdoctoral Fellowship (2012-67012-19845 to A.L.P.) and the National Science  
459 Foundation (DEB 1442246 to J.T.L.). The funders had no role in study design, data collection

460 and interpretation, preparation of the manuscript, or decision to submit the work for publication.  
461 Support was also provided by the NSF Long-term Ecological Research Program (DEB 1027253)  
462 at the Kellogg Biological Station and by Michigan State University AgBioResearch. We would  
463 like to thank the Kellogg Biological Station LTER for logistical support and use of sampling  
464 sites. We also acknowledge the logistical support of K.L. Gross and G.P. Robertson, who  
465 originally established these sites. We also thank M. Muscarella, J. Ford, S. Krahnke, and M.  
466 Brewer for microbial analyses support and B. O'Neill, A.S. Grandy and T.M. Schmidt Labs for  
467 field and soil analyses support. All code and data used in this study can be found in a public  
468 GitHub repository (<https://github.com/PeraltaLab/CropDiversity>) and the NCBI SRA  
469 (BioProject).

470

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707 Tables

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709 Table 1. Cropping diversity treatments at the Kellogg Biological Station Long-term Ecological

710 Research (KBS LTER) Biodiversity Gradient Experiment Plots. Plant treatments were

711 established in 2000. Treatments were composed of monoculture, two-crop rotation, three-crop

712 rotation +/- cover crops, and fallow plots (early successional) and soil collected during the corn

713 phase of the rotation. Treatment abbreviations are in parentheses.

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<b>Crop diversity treatment description</b>	<b>Number of crop species</b>
(1) Continuous monoculture (mC)	1
(2) Continuous monoculture, one cover crop ( $C_{1cov}$ )	2
(3) Two-crop rotation (CS)	2
(4) Three-crop rotation (CSW)	3
(5) Three-crop rotation, one cover crop ( $CSW_{1cov}$ )	4
(6) Three-crop rotation, two cover crops ( $CSW_{2cov}$ )	5
(7) Spring Fallow/early successional field (fallow)	10

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721 Table 2. Summary of the contribution of (A) soil factors (original data from McDaniel et al.  
722 2014) and (B) soil biological activity (original data from McDaniel and Grandy 2016) on  
723 bacterial community variation at the KBS Biodiversity Gradient Experimental Plots based on  
724 permutational MANOVA (PERMANOVA). Soil factor effects were considered to significantly  
725 contribute to community variation at  $P < 0.05$ .

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727 (a) Soil Factors

Effect	df	SS	MS	<i>F</i>	<i>R</i> <sup>2</sup>	<i>p</i> -value
Sand	1	0.088	0.088	2.243	0.066	0.014
Silt	1	0.088	0.088	2.239	0.066	0.020
Clay	1	0.087	0.087	2.207	0.065	0.024
pH	1	0.057	0.057	1.444	0.043	0.143
Nitrate	1	0.023	0.023	0.593	0.018	0.893
Ammonium	1	0.019	0.019	0.496	0.015	0.966
Nitrogen	1	0.043	0.043	1.086	0.032	0.326
Carbon	1	0.036	0.036	0.921	0.027	0.491
Moisture	1	0.064	0.064	1.622	0.048	0.078
Residuals	18	0.707	0.039		0.534	
Total	27	1.325			1	

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733 (b) Soil Biological Activity

Effect	df	SS	MS	<i>F</i>	<i>R</i> <sup>2</sup>	<i>p</i> -value
PMN	1	0.083	0.083	1.821	0.063	0.049
PMC	1	0.062	0.062	1.358	0.047	0.146
POXC	1	0.097	0.097	2.125	0.074	0.028
Residuals	24	1.100	0.046		0.830	
Total	27	1.325			1	

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

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Factor	Estimate	Std error	t-value	<i>p</i> -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

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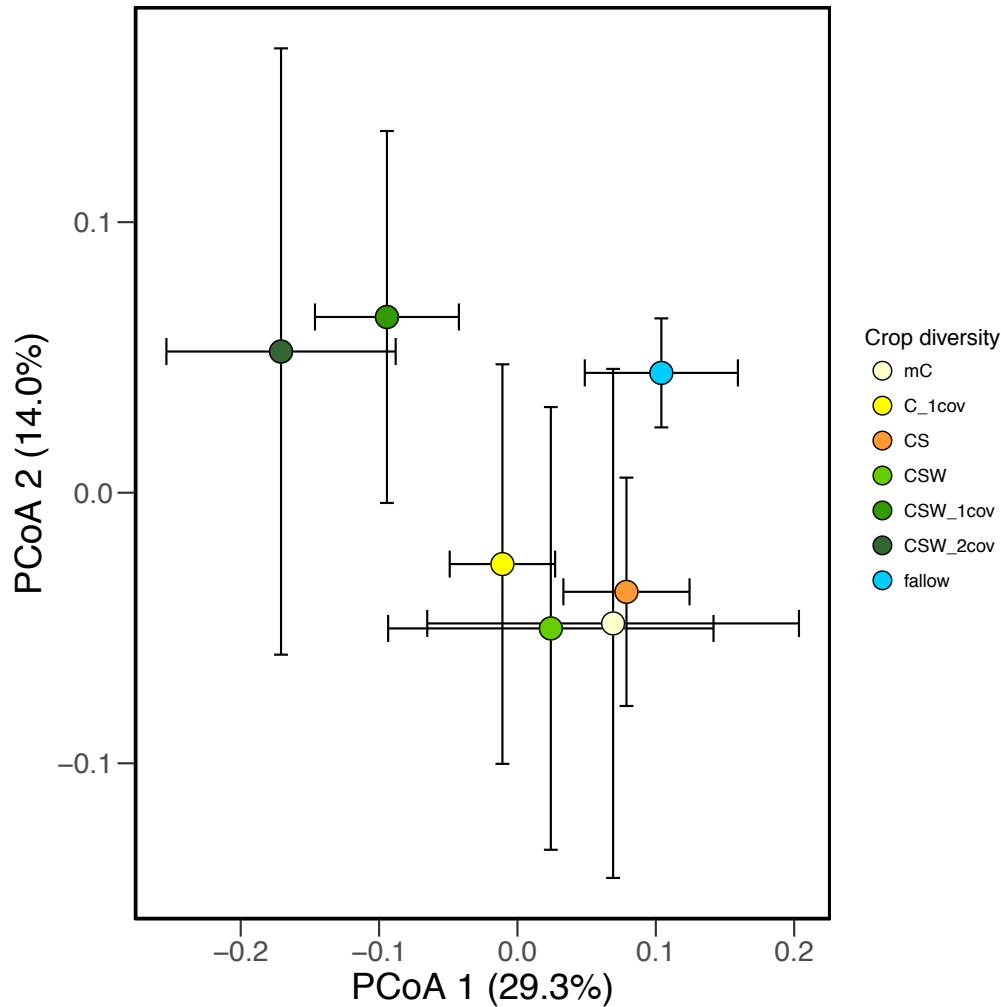
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765 Figures

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767 Figure 1



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769 Figure 1. Ordination from Principal Coordinates Analysis depicting soil bacterial communities

770 along a cropping diversity gradient. Symbols are colored according to cropping diversity

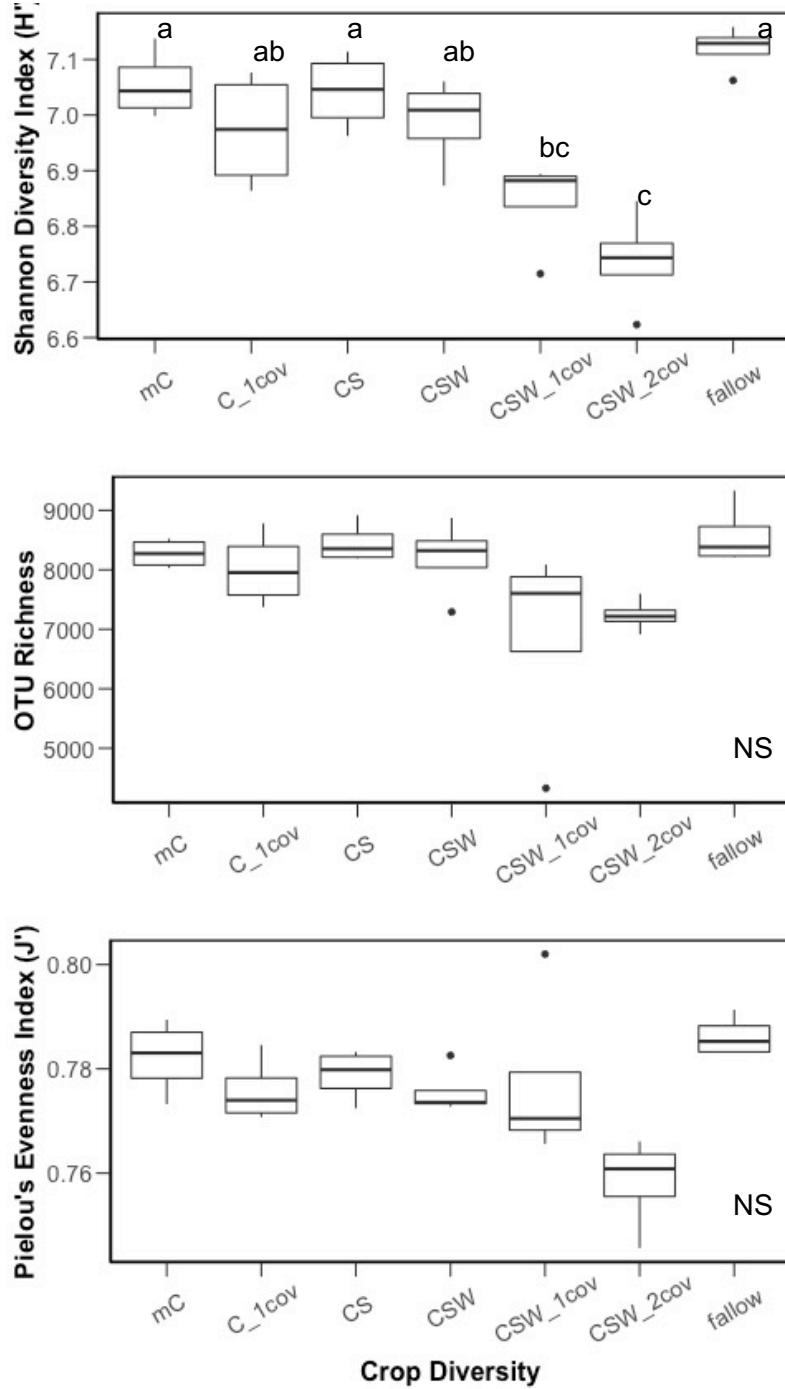
771 treatment (mC=monoculture corn; C<sub>1cov</sub>=corn/1 cover crop; CS=corn/soy;

772 CSW=corn/soy/wheat; CSW<sub>1cov</sub>=corn/soy/wheat/1 cover crop; CSW<sub>2cov</sub>=corn/soy/wheat/2 cover

773 crops; fallow=spring fallow, tilled annually).

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775 Figure 2



795 Figure 2. Total bacterial diversity (mean  $\pm$  SEM based on Shannon Diversity Index  $H'$ ), richness,  
796 and evenness (Pielou's Evenness Index  $J'$ ) in response to long-term crop diversity treatment.

797 Different letters above points reflect significant differences in gene abundance along crop  
798 diversity gradient at  $p < 0.05$  (Tukey's HSD *post-hoc* analysis).

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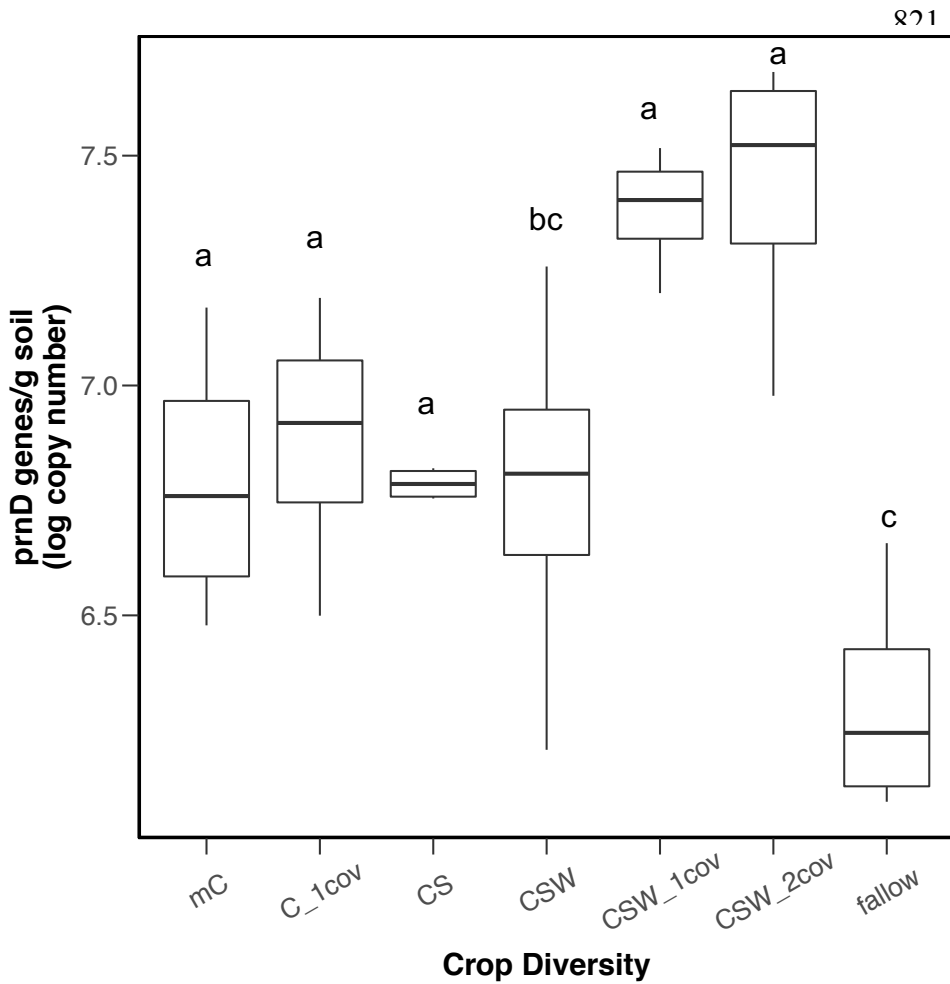
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820 Figure 3



835 Figure 3. Abundance of *prnD* gene (PRN producers) in response to crop diversity treatment  
836 analyzed using quantitative PCR and expressed as log copy number of *prnD* gene. Different  
837 letters above points reflect significant differences in Different letters above boxplots considered  
838 significantly different in gene abundance at  $p < 0.05$  (Tukey's HSD *post-hoc* analysis).

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