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Influence of pesticide seed treatments on rhizosphere fungal and bacterial communities and leaf fungal endophyte communities in maize and soybean

Rachel Nettles¹, John Watkins¹, Kevin Ricks¹, Monica Boyer¹, Megan Licht¹, Lesley W. Atwood², Matthew Peoples³, Richard G. Smith², David A. Mortensen³, Roger T. Koide^{1,3}

¹Brigham Young University, Department of Biology

²University of New Hampshire, Department of Natural Resources and the Environment

³The Pennsylvania State University, Department of Plant Science

Corresponding author:

Roger T. Koide

rogerkoide@byu.edu

Tel: 1 (801) 358-3582

Fax: 1 (801) 422-0048

Abstract

Insecticide and fungicide seed treatments are commonly used to control pests and pathogens in conventional maize and soybean culture, but little is known about their effects on the communities of non-target microbes that inhabit the rhizospheres or leaves of these crops. Because rhizosphere bacterial and fungal communities influence carbon and nutrient turnover, nutrient transformation, nutrient uptake and disease suppression, and because leaf endophyte fungal communities influence many aspects of stress tolerance in plants, any effect of a pesticide seed treatment on these microbial communities could have unintended and possibly adverse effects on seedling performance. We conducted a three-year field experiment in which maize (2013, 2015) and soybean (2014) were grown in rotation from seeds that were either coated or not coated with common pesticide treatments, which included contact and systemic fungicides and systemic insecticides. We sampled seedling rhizosphere soil (maize in 2013, soybean in 2014) and seedling leaves (soybean in 2014, maize in 2015) and characterized their microbial communities. For maize, the rhizosphere fungal and bacterial communities were significantly affected by the seed treatment, but leaf endophytic fungal communities were not. For soybean, the rhizosphere fungal community was significantly affected, as was the leaf endophytic fungal community, but not the rhizosphere bacterial community. These results show that pesticide seed treatments may affect rhizosphere soil microbial communities and endophytic leaf fungal communities more than one month after planting and, therefore, may have significant, unintended effects on non-target organisms. Additional research must determine the consequences of these effects and the nature of their context dependency.

Keywords: rhizosphere bacterial community; rhizosphere fungal community; leaf endophytic fungal community; non-target effects; pesticide seed treatment; sustainable agriculture

1. Introduction

The seeds of agriculturally important plant species have been coated with various materials at least since the 1920s (Lambert et al., 1926). Seed treatments are now commonly used for maize (*Zea mays* (L.), soybean (*Glycine max* (L.) Merr., wheat (*Triticum aestivum* (L.) and cotton (*Gossypium spp.* (L.) (Jeschke et al., 2011; Douglas and Tooker, 2015) in order to deliver chemicals such as fungicides and insecticides that target pathogens and other pests of seeds or seedlings (Taylor and Harman, 1990).

While pesticides applied to seeds may enter the air during planting and have potentially negative consequences on above-ground insects (Hallmann et al., 2014; Krupke et al., 2012; Marzaro et al., 2011), most of the chemicals in seed coatings are delivered to the region of the soil in which the roots of the seedling develop (Thompson, 2010), the rhizosphere. While these pesticides are frequently effective against targeted, soil-borne pathogens and herbivores (Baird et al., 1994), they are not species-specific and, unfortunately, we currently know little about their effects on communities of non-target microorganisms.

Roots interact with complex communities of non-pathogenic fungi and bacteria, each member of which may benefit the plant in direct or indirect ways. Microorganisms that may be negatively impacted by pesticide seed treatments include those that are directly beneficial to plants such as those involved in nutrient mineralization, nutrient transformation, N-fixation, P-solubilization, plant nutrient uptake, and plant hormone production (Lugtenberg and Kamilova, 2009). Other potentially impacted microorganisms include those that indirectly improve crop vigor, such as competitors of pathogens or those

that produce antibiotics (Weller, 1988; Mendes et al., 2011). Still others include those that promote the formation of beneficial symbioses (Hameeda et al., 2007).

Because most fungicides in seed treatments are systemic, it is possible for them to influence endophytic fungi. Complex communities of endophytic fungi occur in the living tissues of every plant species investigated thus far (Arnold et al., 2003; Arnold and Lutzoni, 2007; Rodriguez et al., 2009). Endophytic fungi have been shown to confer on their hosts increased resistance to herbivory (Carroll, 1988; Cheplick and Clay, 1988; Clay, 1987, 1988), disease (Carroll, 1988; Arnold et al., 2003), heat stress (Rodriguez et al., 2004; Marquez et al., 2007) and water stress (Clay and Shardl, 2002; Rodriguez et al., 2004; Penuelas et al., 2012), as well as increased rates of photosynthesis, growth, reproduction and seed germination (Clay, 1987; 1988). In stressful environments endophytic fungi may even be essential for plant survival (Rodriguez et al., 2004).

Thus, while the net effect of pesticide seed treatments on crop vigor is frequently positive due to their impact on targeted pathogens, parasites and herbivores, unplanned adverse effects on beneficial microorganisms may attenuate their net benefit. We, therefore, documented the effects of commonly used pesticide seed treatments (mixtures of insecticides and fungicides applied prophylactically) on rhizosphere fungal and bacterial communities and leaf endophytic fungal communities of maize and soybean. Because beneficial microorganisms may exert impacts on plants via their effects on soil enzymes, which influence nutrient transformation, litter mineralization and nutrient uptake by roots, we also determined the influence of the seed treatments on rhizosphere enzyme activities, including an enzyme that influences P mineralization (acid phosphatase), one that influences N mineralization (β -N-acetylglucosaminidase), and one that influences C

mineralization (1,4- β -cellobiohydrolase). We further documented the effects of the pesticide seed treatments on shoot N and P concentrations and shoot weight. Both maize and soybean form associations with arbuscular mycorrhizal fungi that may strongly influence the P economy of their hosts (Koide, 1991). A number of fungicides can significantly retard the development of mycorrhizal fungi (Menge, 1982) but it is unclear whether any fungicide delivered as a seed treatment significantly affects the colonization of roots by arbuscular mycorrhizal fungi. Therefore, we also documented the effect of seed treatment on colonization by mycorrhizal fungi.

2. Materials and methods

2.1 Site Description

This study was conducted at the Pennsylvania State University R.A. Larson Agricultural Research Center at Rock Springs, PA, USA (40°43' N, 77°55' W, 350 m elevation). Soils at the site are shallow, well-drained lithic Hapludalfs formed from limestone residuum, and the dominant soil type is a Hagerstown silt loam (fine, mixed, semiactive, mesic Typic Hapludalf) (Braker, 1981). The soil is characterized by a silt loam surface texture and subsurface textures of silty clay loam and silty clay. In the five years preceding this study the field was planted with the following crops: no-till maize for grain in 2008 and 2009, no-till soybean in 2010, no-till spring oats in 2011, and barley and wheat crops in 2012. The field was plowed prior to planting in fall of 2011. For the purposes of this study, the field was divided into ten plots, each 3 meters wide (encompassing four experimental crop rows) by 6 meters long and each randomly assigned to one of the two

treatments (treated or untreated seeds, see below). Plot treatment assignments were maintained throughout the 3-year duration of this study.

2.2 2013 (maize) methods

On 26 April 2013 1520 g ha⁻¹ glyphosate (in the form of the potassium salt) and 1400 g ha⁻¹ dichlorophenoxyacetic acid (2,4-D) was applied for weed control. On 14 May 2013 the field was S-tined. On 15 May 2013 the field was disked and cultimulched. On 16 May 2013 the field was planted with maize hybrid TA510-18 (TA Seeds, Jersey Shore, PA, USA) in 76 cm-spaced rows at a seed density of 78,300 seeds ha⁻¹. Untreated seeds were planted in five randomly selected plots, and treated seeds were planted in the other five plots. The seed treatment was CruiserMaxx Corn 250 (Syngenta, Greensboro, NC, USA), which is a mixture of the systemic insecticide thiamethoxam (class neonicotinoid), the contact fungicide fludioxonil, and the systemic fungicides mefenoxam, azoxystrobin, and thiabendazole. The same maize genotype was used for both treatments. On 31 May 2013 the field was fertilized using urea with Agrotain® at the rate of 358 kg N ha⁻¹. On 20 June 2013 a post-emergence application of 1390 g ha⁻¹ of glyphosate was made to control emerged weeds.

Two pooled rhizosphere soil samples were taken from each plot between 30 May and 3 June 2013, resulting in a total of ten pooled rhizosphere soil samples per treatment. For each pooled sample from each plot, 5 seedlings were randomly chosen and removed from the soil while attempting to recover as much of the root system as possible. The root system was then manually shaken to remove excess soil. The soil that remained adhering to the root system was considered rhizosphere soil. Rhizosphere soil was removed

manually from the root system. Rhizosphere soil samples from each of the five seedlings were pooled and sealed in a plastic bag and stored overnight at 5°C until they were used for enzyme analyses and for DNA extraction on the next day.

Following soil collection, root systems were separated from shoots, pooled by plot, and stored in 50% ethanol. Later, roots were cleared in 10% KOH (w:v), acidified in dilute HCl and stained in a solution of acetic acid and trypan blue (Sharda and Koide, 2008). Mycorrhizal colonization was quantified by line-intercept method as in Koide and Mooney (1987). Shoots were rinsed under distilled water and dried at 70°C until they attained a constant weight.

The day after collection, three soil subsamples per plot were used for the analyses of acid phosphatase (Pase, EC 3.1.3.2), 1,4- β -cellobiohydrolase (CBase, EC 3.2.1.91) and β -N-acetylglucosaminidase (NAGase, EC 2.4.1.255) according to Peoples and Koide (2012). On the same day, genomic DNA was extracted from a fourth subsample of each soil sample. To extract genomic DNA, approximately 0.25g of soil from each sample were placed in extraction tubes from the PowerSoil DNA Isolation Kit (Mo Bio Laboratories Inc., Carlsbad, CA, USA). Extraction tubes were vortexed for a total of 15 min. All other steps were completed as outlined in the PowerSoil protocol. Extracted genomic DNA was then stored at -20 C until amplification by PCR.

To amplify the bacterial and fungal genomic DNA, Sigma Jumpstart Taq (Sigma–Aldrich, St. Louis, MO, USA) was used. Fungal DNA was amplified using ITS1F[Hex] and ITS4 primers (Gardes and Bruns, 1993), while bacterial DNA was amplified using 1406F[FAM] and 23SR primers (Fisher and Triplett, 1999). The thermal cycling program used to amplify fungal DNA was: 30 cycles of 95°C for 40 s, 49°C for 30 s, and 72°C for 48 s

with final elongation at 72°C for 7 min. The thermal cycling program used to amplify bacterial DNA was: 30 cycles of 95°C for 40 s, 55°C for 30 s, and 72°C for 2 min with final elongation at 72°C for 7 min.

Hex-labeled and FAM-labeled PCR products were separated on an ABI 3730xl genetic analyzer using MapMarker ROX-1000xl ladder (BioVentures, Inc. Murfreesboro, TN). The ROX-1000xl ladder was used because the vast majority of PCR products were less than 1000 bases long. GeneMapper Software (Applied Biosystems, Grand Island, NY, USA) was used to analyze fungal and bacterial ribosomal intergenic spacers (ARISA). The raw ARISA peaks were binned into operational taxonomic units (OTUs) with a bin width of 5 bp. Bins created by spurious peaks (usually ladder bleed-through) were manually eliminated prior to analysis. Peak areas were used in subsequent multivariate analyses. Binary (presence/absence) data were used in the calculations of C-scores and species richness.

2.3 2014 (soybean) methods

Soybean (TS2849R2S, TA Seeds) was planted directly into the previous year's maize residue on 30 May 2014 without tillage, following a pre-plant application of 1520 g ha⁻¹ glyphosate (in the form of the potassium salt) and 1400 g ha⁻¹ 2,4-D on 27 May. Treated soybean seeds were planted into the plots previously containing treated maize and untreated soybean seeds were planted in the plots previously containing untreated maize. The same soybean genotype was used for both treatments. The seed treatment consisted of CruiserMaxx Beans with Vibrance (Syngenta) + Gaucho (Bayer Crop Science LP, Research Triangle Park, NC, USA), which is a mixture of the systemic insecticides

thiamethoxam and imidacloprid (both class neonicotinoid), and the systemic fungicides mefenoxam and sedaxane. Soybean was planted in 76 cm-spaced rows at 432,000 seeds ha⁻¹. On 16 June 2014 a post-emergence application of 1390 g ha⁻¹ of glyphosate was made to control emerged weeds.

Rhizosphere soil was collected on 16 June 2014 from 10 randomly selected seedlings per plot following the same method used in the maize phase of the rotation, for a total of 50 treated seedlings and 50 control seedlings. Soil samples from individual seedlings were not pooled prior to DNA extraction, PCR and subsequent analysis, which were carried as for maize in 2013. The processing of root systems and shoots was the same as in 2013.

On 17 June 2014, two leaf disks were sampled using paper hole punchers from the largest leaf of each seedling. The two disks from each plant were placed into a single MoBio Powerplant Pro DNA extraction tube (Mo Bio Laboratories) and then placed in a -20°C freezer until they were extracted within the week following the standard protocol for the Powerplant Pro DNA extraction kits (Mo Bio Laboratories). Extracted genomic DNA was then stored at -20 °C until amplification by PCR and subsequent analysis as for maize in 2013.

2.4 2015 (maize) methods

Following pre-plant herbicide (a postemergence application of 1320 g ha⁻¹ of glyphosate and 700 g ha⁻¹ of 2,4-D), treated maize seed was no-till planted into the former treated soybean plots and untreated maize was planted into the former untreated soybean plots on 13 May 2015. The seed treatment was CruiserMaxx Corn 250 (Syngenta) as in

2013. The same genotype of maize (FC 397 3122, First Choice Seeds, IN, USA) was used for both treatments. The maize was planted in 76 cm-spaced rows at 78,600 seeds ha⁻¹.

We sampled leaves on 10 June 2015 for fungal DNA by taking two small disks each from the second and third true leaves from seven randomly selected seedlings per plot using paper hole punchers. The four leaf disks from each plant were placed into a single MoBio Powerplant Pro DNA extraction tube (Mo Bio Laboratories). Each sampled shoot was then cut at soil level. The shoots were dried for 3 d at 60°C, pooled by plot and weighed. The leaf disks were kept refrigerated overnight and the next day fungal DNA from the leaf disks was extracted following the standard protocol for the Powerplant Pro DNA extraction kits (Mo Bio Laboratories). Extracted genomic DNA was then stored at -20°C until amplification by PCR and subsequent analysis as for maize in 2013.

2.5 Univariate statistical analyses

Shoot weights and nutrient concentrations, mycorrhizal colonization, enzyme activities, and yields were subjected to analysis of variance in Minitab 16 Statistical Software (Minitab Inc., 2010).

2.6 Multivariate community analyses

Bacterial and fungal communities were analyzed separately. Peak areas from the automated ribosomal intergenic spacer analysis (ARISA) were Hellinger-transformed prior to these analyses in order to relativize the peak areas for each sample (Yannarell et al., 2014). Significant differences in community structure were determined using permutational multivariate analysis of variance (PerMANOVA) in the R statistical

environment (R Core Team, 2013) with the Vegan package (Oksanen et al., 2015). Bray-Curtis distances were used. Variation in community structure associated with seed treatment was visualized using ordination (nonmetric multidimensional scaling, NMDS, with a principal component analysis, PCA, final rotation) in Canoco 5 (Ter Braak and Šmilauer, 2012). NMDS was performed using Bray-Curtis distances, 150 perturbations and three axes.

2.7 Richness estimates, Co-occurrence (C-Scores), Associations among species

The Chao2 estimate of richness is an incidence-based estimator of richness and was computed in Estimate S (Colwell, 2013).

Little is known about the organization of rhizosphere microbial communities. Therefore we performed the following tests to determine the extent to which the bacterial and fungal OTUs were structured in terms of non-random co-occurrence of OTUs within samples (C-scores), and in terms of positive or negative correlations among OTUs within samples (Spearman rank correlations). The checkerboard-score or C-score (Stone and Roberts, 1990) is a measure of co-occurrence of species within a sample among all possible species pairs within the community. The C-scores were calculated using the co-occurrence module of the Ecosim program (Gotelli and Entsminger, 2004). Ecosim calculates the C-score for the actual community and compares it to a distribution of C-scores calculated from a user-defined number of simulated matrices (5,000 in this study) assuming a constrained, randomized distribution of species. Simulated matrices were generated maintaining the same row sums as in the actual data matrix and equiprobable column sums, in which the occurrences for each species are distributed randomly among the

different samples. An observed C-score that is significantly larger than the C-score calculated for a community with randomly distributed species, for example, indicates that within samples there is significantly less co-occurrence (more avoidance) among the species than expected.

The degree to which individual OTUs were correlated (positively or negatively) with each other within soil samples was determined by calculating Spearman rank correlations using rank-transformed peak area data in Statistica 5 (Statsoft, Tulsa, OK, USA) for the 30 most frequently occurring OTUs. Correlations were considered significant when $P \leq 0.05$.

3. Results

3.1 Effects of seed treatment on seedling weights, nutrient concentrations, etc.

In 2013, treated maize seedling shoot weights were significantly greater than controls (Table 1). Maize shoot N and P concentration, arbuscular mycorrhizal colonization, grain yield, and enzyme activities did not differ significantly between the two treatments (Table 1). In 2014, soybean seedling shoot weight and arbuscular mycorrhizal colonization were not significantly affected by treatment (Table 2). In 2015, maize seedling shoot weights were not significantly affected by treatment: control 89.8 mg, treated 80.5 mg, $n=5$, ANOVA $p=0.44$.

3.2 Effects of seed treatment on the rhizosphere microbial communities

When rhizosphere fungal communities for maize (2013) and soybean (2014) were analyzed together, year and seed treatment were both significant factors, as was their interaction (Table 3). The fungal community was significantly differentiated by the seed

treatment during both the maize (2013) and soybean phases of the rotation (Table 3, Figures 1, 2). The biplot (Figure 1) of significant taxa on the fungal community ordination from the maize (2013) phase indicated that the fungal community associated with the treated seeds was enriched in the OTUs 613, 716 and 692, while the fungal community from the control seeds was enriched in the OTU 482 (values are the fragment lengths in base pairs). The biplot (Figure 2) from the soybean (2014) phase was less capable of illustrating the taxa responsible for distinguishing between treated and control treatments, but these included OTUs 411, 731, 599, 579 and 692.

For rhizosphere bacterial communities (maize 2013 and soybean 2014 together), year and seed treatment were both significant, but not their interaction (Table 3). The rhizosphere bacterial communities were significantly differentiated due to seed treatment in maize (2013) but not in soybean (2014) (Table 3). For maize (2013), the rhizosphere bacterial community associated with the treated seeds was enriched in the OTUs 543, 455, 423, 617 and 565 in (Figure 2).

There was a significant effect of the seed treatment on soybean leaf endophyte fungal community in 2014 (Table 3, Figure 4). The OTUs most responsible for this were 627, 822, 585, 613 and 634, but it is not clear from the biplot how their abundances differed between the treatments. There was no significant effect of the seed treatment on the maize leaf endophyte fungal community in 2015 (PerMANOVA, $p=0.56$).

3.3 Richness estimates

In maize (2013), the ARISA detected a total of 53 fungal OTUs. The mean Chao 2 richness was 57.4 for all samples, including treated and control samples (Table 4).

According to the 95% confidence intervals, the mean Chao 2 richness for the treated samples alone (44.5) and for the control samples alone (52.3) were not significantly different, suggesting that fungal richness was not significantly affected by the seed treatment. ARISA detected a total of 95 bacterial OTUs. The mean Chao 2 richness was 95.1 for all samples, including treated and control samples (Table 4). According to the 95% confidence intervals, the mean Chao 2 richness for the treated samples alone (89.9) and for the control samples alone (96.5) were not significantly different, suggesting that bacterial richness was not significantly affected by the seed treatment.

In soybean (2014), the ARISA detected a total of 100 fungal OTUs. The mean Chao 2 richness was 100.0 for all samples, including treated and control samples (Table 4). According to the 95% confidence intervals, the mean Chao 2 richness for the treated samples (100.4) alone and for the control samples alone (98.0) were significantly different, suggesting that fungal richness was significantly (but only slightly) increased by the pesticide seed treatment. In contrast to fungal richness, the ARISA detected a total of 132 bacterial OTUs. The mean Chao 2 richness for the bacterial community was 132.0 for all samples, including treated and control samples (Table 4). According to the 95% confidence intervals, the mean Chao 2 richness for the treated samples alone (132.2) and for the control samples alone (130.0) were not significantly different, suggesting that fungal richness was not significantly affected by the seed treatment.

3.4 Co-occurrence (*C-Scores*) and associations among species

The observed C-scores of the fungal and bacterial communities in maize (2013) were 6.52 and 11.8, respectively, while the means of the corresponding simulated C-scores

were 9.01 and 33.7, respectively. Thus, co-occurrence among both fungal and bacterial OTUs was significantly greater than expected (Table 5). The significant co-occurrence was manifest in the Spearman rank correlations. Of the 450 possible unique pairings of fungal OTUs in the Spearman rank correlation matrix, 44 OTU pairs exhibited a significantly positive correlation, while only 4 OTU pairs exhibited a significantly negative correlation (Fig. S1). Similarly, of the 450 possible unique pairings of bacterial OTUs in the Spearman rank correlation matrix, 105 OTU pairs exhibited a significantly positive correlation, while only 7 OTU pairs exhibited a significantly negative correlation (Fig. S2).

The observed C-scores for the fungal and bacterial communities in soybean (2014) were 146.6 and 128.9, respectively, while the mean of the simulated C-scores was 175.4 and 310.6, respectively. Again, co-occurrence among both fungal and bacterial OTUs was significantly greater than expected (Table 5). Of the 450 possible unique pairings of fungal OTUs in the Spearman rank correlation matrix, 82 OTU pairs exhibited a significantly positive correlation, while only 48 OTU pairs exhibited a significantly negative correlation (Fig. S3). Of the 450 possible unique pairings of bacterial OTUs in the Spearman rank correlation matrix, 139 species pairs exhibited a significantly positive correlation, while only 4 species pairs exhibited a significantly negative correlation (Fig. S4).

4. Discussion

The pesticide seed treatments significantly influenced the rhizosphere microbial communities in 2013 (maize) and 2014 (soybean). Because significant concentrations of the fungicides find their way from the seed coat to the rhizosphere (Hassan and Cox, 1947) it is perhaps not too surprising to find that fungal communities of the rhizosphere were

significantly affected. Seed treatment fungicides could have direct effects on susceptible fungi as well as indirect effects on less susceptible fungi due to their release from competition when competitor fungal species are suppressed. Moreover, it was clear from our results that the effect of seed pesticide treatments could last longer than a month. While long half-lives of the fungicides are important for protracted protection of the crop, long half-lives also increase the likelihood that the pesticides exert unintended effects on non-target organisms.

The effect of the seed treatments on rhizosphere bacterial communities was unexpected. The treatments consist of insecticides and fungicides; they do not contain pesticides that specifically target bacteria. The mechanism by which these pesticides influenced the bacteria is not clear. It is possible that by having a negative effect on some fungi, certain bacteria are released from competition, or that commensal interactions between bacteria and fungi (Roesti et al., 2005) are interrupted by the loss of the fungi. Other mechanisms whereby fungicides impact bacteria have been offered including direct effects of fungicides on bacteria and indirect effects of fungicides on bacteria-grazing animals (Siddiqi and Alexander, 1991). Soil bacterial community structure has also been altered by foliar applications of fungicides, insecticides, and herbicides (Lupwayi et al., 2004; Lupwayi et al., 2009). In any case, because some rhizosphere bacteria promote plant growth (Lugtenberg and Kamilova, 2009), effects of seed treatments on bacteria could have unintended consequences for plant vigor.

In this study, the significant effects of the seed treatments on the microbial communities were largely unrelated to changes in OTU richness. Therefore, differences in community structure that we observed are likely indicative of seed treatments causing

shifts in the relative abundances of taxa or shifts in community species composition rather than changes in species richness *per se*. Other studies of foliar applications of fungicides, insecticides, and herbicides also showed no reductions in soil bacterial richness (Lupwayi et al., 2004; Lupwayi et al., 2009).

Little is known about the structure of rhizosphere microbial communities. Some microbial communities exhibit structure in the form of greater than expected co-occurrence among taxa within samples, possibly the result of multiple OTUs possessing similar environmental requirements or mutualistic interactions among OTUs, or less than expected co-occurrence, possibly the result of competitive exclusion (Koide et al., 2005). In this study, considerable structure existed among both bacterial and fungal communities of the rhizospheres of both maize and soybean. This structure was manifest in the form of non-random associations among OTUs as evidenced by significantly more than expected co-occurrence and significant Spearman rank correlations among OTUs, most of which were positive. Because the soil samples for DNA analysis were very small (0.25 g), these non-random associations must have occurred on a relatively small spatial scale.

Because of the systemic nature of some of the fungicides in the seed treatments, we expected that leaf endophyte fungal communities would also be altered by the seed treatment fungicides. In fact, the seed treatment did significantly affect the soybean (2014) leaf endophyte fungal community but not the maize (2015) leaf endophyte fungal community. Leaf endophyte fungal communities may promote drought tolerance (Morse et al., 2002; Ren et al., 2014), produce defense compounds to guard against pathogens (Hartley et al., 2015), and increase adaptability to environmental stresses (Zhang et al.,

2015). Therefore, the significant effect on leaf endophyte fungal communities deserves further investigation.

Pesticide seed treatments are used to increase germination, seedling survival and vigor and, ultimately, agronomic yield (Taylor and Harmon, 1990). In 2013, maize seedling dry weight benefited significantly from the seed treatment. In 2015, however, maize seedling dry weight was not significantly affected by seed treatment. This suggests, not surprisingly, that benefit from seed treatments is context dependent. The mechanism by which maize benefited from the seed treatment in 2013 is not clear. One possibility is that it reduced populations of disease organisms or detrimental insects in a year in which those pressures were significant, as intended. The beneficial effect of seed treatment on maize (2013) was not mediated by significant increases in seedling N or P concentrations, or by increased rhizosphere activities of acid phosphatase, acetyl glucoaminidase or cellobiohydrolase, or by stimulation of mycorrhizal colonization. We do not know whether the treatment altered the structure of the community of mycorrhizal fungi colonizing the root system, which could itself impact plant vigor (van der Heijden et al., 1998), but the treatment at least did not reduce the amount of colonization, as had previously been observed with other fungicides (Spokes et al., 1989).

For soybean (2014) seedlings, the seed treatment was not significantly beneficial in terms of growth or yield. Possibly soybean pest pressure was lower in 2014 than maize pest pressure was in 2013 such that in 2013 but not in 2014, the benefits of the pesticides outweighed their costs (in terms of their effects on non-target organisms and their associated agroecosystem functions). Another possibility is that the effect of the seed treatments on maize and soybeans were different because the pesticides were different.

The pesticides for soybean (CruiserMaxx Beans with Vibrance + Gaucho) includes the systemic insecticides thiamethoxam and imidacloprid, and the systemic fungicides mefenoxam and sedaxane, while the seed treatment pesticides for corn (CruiserMaxx Corn 250) includes the systemic insecticide thiamethoxam, the contact fungicide fludioxonil, and the systemic fungicides mefenoxam, azoxystrobin, and thiabendazole. In any case, this result suggests, again, that the benefits from seed treatments are context dependent.

These data represent the first evidence of which we are aware that pesticide seed treatments influence rhizosphere fungal and bacterial communities and leaf endophyte fungal communities. The consequences of these findings are not entirely clear. In this study we chose a simple and cost-effective method (ARISA) to characterize the effects of seed pesticides on rhizosphere microbes. High-throughput sequencing could also be used to help determine the bacterial and fungal taxa involved in this effect and thus to possibly identify potentially important taxa that increase or decrease in abundance due to seed pesticide use. Because rhizosphere microbes are an integral part of soil food webs and are intimately involved in important ecosystem processes such as nutrient mineralization, nutrient transformation, nutrient uptake, carbon sequestration (Li et al., 2014) and disease suppression (Weller, 1988; Mendes et al., 2011), the consequences should be investigated further. In particular it will be important to know the magnitude of the impact of seed treatment pesticides on these ecosystem properties, as well as how their impact is affected by crop species and other environmental factors.

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Table 1. Effects of pesticide seed treatments during the maize phase of the rotation (2013). Seedling data were collected 30 May 2013. Mean (standard error), n = 5.

Rhizosphere enzyme activities								
	Acid phosphatase	Acetyl glucos- aminidase	Cellobio- hydrolase	AM colonization (%)	Shoot P (mg g ⁻¹)	Shoot N (mg g ⁻¹)	Seedling shoot weight (mg)	Yield (Bu ac ⁻¹)
Control	2.58 (0.18)	0.727 (0.054)	1.06 (0.06)	10.4 (2.9)	3.17 (0.23)	37.8 (1.42)	74.8 (3.4)	244.0 (3.8)
Treated	2.41 (0.18)	0.840 (0.054)	1.02 (0.06)	14.7 (2.9)	2.80 (0.23)	35.7 (1.42)	105.0 (3.4)	254.9 (3.8)
<i>P</i>	<i>0.522</i>	<i>0.174</i>	<i>0.649</i>	<i>0.322</i>	<i>0.279</i>	<i>0.320</i>	<i>0.0001</i>	<i>0.078</i>

Table 2. Effects of pesticide seed treatment during the soybean phase of the rotation (2014). Mean (standard error), n = 5.

	AM colonization (%)	Seedling shoot weight (mg)	Yield (Bu ac ⁻¹)
Control	50.2 (3.9)	264 (9.3)	72.5 (2.8)
Treated	53.5 (3.9)	283 (9.3)	74.8 (2.8)
<i>P</i>	<i>0.563</i>	<i>0.205</i>	<i>0.582</i>

Table 3. Summary of multivariate analyses.

Year	Crop	Community	Contrast	PerMANOVA P value (Seed treatment)	PerMANOVA P value (Year)	PerMANOVA P value (Interaction)	NMDS
2013/2014	Maize/Soybean	Rhizosphere fungi	Seed treatment x year	0.001	0.005	0.001	
2013	Maize	Rhizosphere fungi	Seed treatment	0.001			Fig. 1
2014	Soybean	Rhizosphere fungi	Seed treatment	0.015			Fig. 2
2013/2014	Maize/Soybean	Rhizosphere bact.	Seed treatment x year	0.047	0.001	0.142	
2013	Maize	Rhizosphere bact.	Seed treatment	0.003			Fig. 3
2014	Soybean	Rhizosphere bact.	Seed treatment	0.170			
2014	Soybean	Leaf fungi	Seed treatment	0.048			Fig. 4
2015	Maize	Leaf fungi	Seed treatment	0.560			

Table 4. Calculated Chao2 estimates of species richness of the fungal and bacterial components of the soil microbial community during the maize and soybean phases of the rotation.

Year - Crop	Community	Treatment	Mean Chao2	sd	95% CI lower limit	95% CI upper limit
2013 maize	Fungal	Both treatments	57.4	3.7	54.0	71.3
2013 maize	Fungal	Treated only	44.5	5.4	39.1	63.6
2013 maize	Fungal	Control only	52.3	4.5	47.8	68.1

2013 maize	Bacterial	Both treatments	95.1	0.4	95.0	97.7
2013 maize	Bacterial	Treated only	89.9	2.2	88.3	99.3
2013 maize	Bacterial	Control only	96.5	4.5	92.3	113.6

2014 soybean	Fungal	Both treatments	100.0	0.4	100.0	100.9
2014 soybean	Fungal	Treated only	100.4	0.9	100.0	108.5
2014 soybean	Fungal	Control only	98.0	0.2	98.5	99.4

2014 soybean	Bacterial	Both treatments	132.0	0.3	132.0	132.8
2014 soybean	Bacterial	Treated only	132.2	0.6	132.0	136.0
2014 soybean	Bacterial	Control only	130.0	0.8	130.0	132.3

Table 5. Results from C-score analyses. An observed C-score that is significantly smaller than the C-score calculated for a community with randomly distributed species, for example, indicates that within samples there is significantly more co-occurrence (less avoidance) among the species than expected.

Year - Crop	Community	Co-occurrence	p value
2013 maize	Fungal	greater than expected	$p < 0.0001$
2013 maize	Bacterial	greater than expected	$p < 0.0001$
2014 soybean	Fungal	greater than expected	$p < 0.0001$
2014 soybean	Bacterial	greater than expected	$p < 0.0001$

Figure 1. Ordination of 2013 maize rhizosphere fungal communities from treated and control plants. Vectors represent the 5 most influential OTUs contributing to the distinction between treatments. Stress reached 0.095 after 43 iterations.

Figure 2. Ordination of 2014 soybean rhizosphere fungal communities from treated and control plants. Vectors represent the 5 most influential OTUs contributing to the distinction between treatments. Stress reached 0.193 after 41 iterations.

Figure 3. Ordination of 2013 maize rhizosphere bacterial communities from treated and control plants. Vectors represent the 5 most influential OTUs contributing to the distinction between treatments. Stress reached 0.086 after 39 iterations.

Figure 4. Ordination of 2014 soybean leaf endophyte fungal communities from treated and control plants. Vectors represent the 5 most influential OTUs contributing to the distinction between treatments. Stress reached 0.113 after 37 iterations.