Title: The wheat microbiome under four management strategies, and potential for endophytes in disease protection.

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Abstract:

Manipulating plant-associated microbes to reduce disease or improve crop yields requires a thorough understanding of interactions within the phytobiome. Plants were sampled from a wheat/maize/soybean crop rotation site that implements four different crop management strategies. We analyzed the fungal and bacterial communities of leaves, stems, and roots of wheat throughout the growing season using 16S and ITS2 rRNA gene amplicon sequencing. The most prevalent operational taxonomic units (OTUs) were shared across all samples, although levels of the low-abundance OTUs varied. Endophytes were isolated from plants, and tested for antagonistic activity toward the wheat pathogen *Fusarium graminearum*. Antagonistic strains were assessed for plant protective activity in seedling assays. Our results suggest that microbial communities were strongly affected by plant organ and plant age, and may be influenced by management strategy.

Introduction:

The basis for interactions among microorganisms as communities are beginning to be elucidated, including bacteria, fungi, protists, and viruses growing on and within host organisms. Microbes interact with plants in a variety of ways: pathogens utilize the plants as a food source, symbiotic mycorrhizae and rhizobia exchange nutrients with their hosts, endophytes live inside plant cells asymptomatically and some species can provide protection to the plants against harsh environmental conditions (Rodriguez et al. 2009). Yet, basic knowledge about the structure of microbial communities across plant organs is still lacking.

Wheat is a staple food globally, and is one of the most commonly grown crops, with approximately two billion bushels produced in the United States annually (USDA 2016). The fungal pathogen *Fusarium graminearum* has resulted in devastating yield losses, estimated at \$2.491-7.67 billion between 1993 and 2001 (McMullen et al. 2012). There are few control options as fungicides have low efficiency against *F. graminearum* and there are no strongly resistant varieties (reviewed by Wegulo et al. 2015). One potential method that can contribute to an integrated approach to control is manipulation of plant microbial communities to suppress pathogen populations. The most practical method to achieve this goal involves colonization with parasitic or competitive endophytes that will kill or displace pathogens of interest.

The term endophyte is used to describe microbial organisms that spend the majority or entirety of their life cycle living within a host plant (Rodriguez et al. 2009). Endophytic fungi have been documented to benefit their plant hosts in diverse conditions. They can improve salt and heat tolerance in wild grasses (Rodriguez et al. 2008). In wheat, improved germination rates have been attributed to endophytes (Hubbard et al. 2012), and protective effects of endophytes against *Stagonospora* infection have been documented (Sieber et al. 1988). Recently, bacterial endophytes have been shown to reduce disease and mycotoxin production by pathogens in millet (Mousa et al. 2016). Identification of wheat endophytes may provide novel strains to improve crop health and reduce disease.

Previous studies of wheat microbiomes have largely focused on identifying microbes in the roots or rhizosphere (for example, Hartmann et al. 2014; Mahoney et al. 2017; Ofek et al. 2013; Yin et al. 2017), while noticeably fewer studies have focused on aboveground organs (Granzow et al. 2017; Huang et al. 2016; Karlsson et al. 2017). To our knowledge, there are no published studies which have surveyed the entire wheat microbiome, including both above- and below-ground plant organs, with high throughput sequencing techniques. Here we classify the bacterial and fungal microbiomes of three wheat organs (stems, leaves, and roots) grown under four land management strategies (conventional tillage, no-till, low input, and organic). Microbial communities were dependent on type of plant organ, and community composition changed as plants matured. We then used the wheat microbiome analysis as the context for identifying and testing potential biocontrol strains isolated from the experimental plots for protective abilities against *F. graminearum* seedling damping-off.

Materials and Methods:

Microbiome Sample Collection

Wheat plants were collected from the Michigan State University (MSU) W.K. Kellogg Biological Station (KBS) Long-Term Ecological Research (LTER) main crop rotation site located in Hickory Corners, Michigan, USA (42.411085 N 85.377078 W; https://lter.kbs.msu.edu). The soils of the KBS-LTER site are Typic Hapludalfs of the Kalamazoo (fine-loamy, mixed, mesic) and Oshtemo (coarse-loamy, mixed, mesic) series, developed on glacial outwash (Crum and Collins 1995). Soil series were mapped onto the sample site using the USDA Official Soil Series Descriptions: https://soilseries.sc.egov.usda.gov/osdname.aspx (Fig. S1). All wheat/maize/soybean rotation plots were of the soil series Kalamazoo, except plots T1-R3, T3-R3, and T4-R4 which were Oshtemo. The site has been under continuous wheat/maize/soybean rotations since 1993 (Robertson 2015) and is organized in randomized, replicated one-hectare plots under four land management strategies with six replicates of each conventional till, no-till, reduced chemical inputs with an alfalfa cover crop, and organic with an alfalfa cover crop (Fig. S1). In the fall of 2012, seeds of soft red winter wheat, variety 25R39 (Pioneer Hi-Bred International, Inc., Johnston, IA), treated with Gaucho fungicide (Bayer Corp., Pittsburgh, PA), were planted in plots of all management strategies, except the organic plots, which were sown with untreated seeds of the same variety. Weeds were controlled by tilling in organic plots and chemically controlled in the other plots.

Plants were collected at the following Zadocks stages (Zadocks et al. 1974) and dates: stage 30 (vegetative) on May 1, 2013; stage 45 (late boot, early flowering) on May 30, 2013; stage 83 (early seed development) on July 5, 2013. Six random plants, with intact roots, were removed from each of the 24 plots for microbiome analysis. Plants were bagged in pairs; henceforth each pair of plants was treated as one biological replicate. Roots and aboveground tissues were placed in separate sterile sample collections bags (Nasco Whirl-Pak®, Fort Atkinson, WI) and maintained on ice during transport. Plants were stored at -80°C then lyophilized. Lyophilized tissue was stored at room temperature under a desiccant until processed for DNA isolation.

rRNA Gene Amplification and Sequencing

Approximately 50 mg of 0.2 mm^2 pieces of leaf, stem, or root tissues (fine and thick) were transferred into ClavePak 1.1 mL tubes (Denville Scientific, Holliston, MA) containing 5/32” (3.97 mm) stainless steel ball bearings (Grainger, Lansing, MI). DNA extractions were performed in triplicate for each biological replicate using the Mag-Bind® Plant DNA Plus Kit (Omega Bio-tek, Norcross, GA) following the manufacturer’s protocols with a Retsch Oscillating Mill M400 (Verder Scientific, Newtown, PA) and a KingFisher™ Flex (ThermoFisher Scientific, Waltham, MA). Phusion High Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA) was used to amplify the 16S V4 and ITS2 rRNA gene regions of bacteria and fungi, respectively (see Table S1 for primers). PCR amplification of each sample was performed in triplicate, PCR products were pooled and purified with Wizard Gel and PCR Clean-up Kit (Promega, Madison, WI). Amplicons were sequenced at the MSU Research Technology Support Facility (East Lansing, MI) using a dual-index barcode strategy (Kozich et al. 2013) and Illumina MiSeq 2x250 bp chemistry. Barcodes were used to distinguish between samples from replicate plots of each management strategy, growth stage, and plant organ. Reads are available in NCBI Small Read Archive under BioProject PRJNA356450 and accession number SRP102192.

Bioinformatics

The USEARCH pipeline (version v8.1.1861) was used for quality filtering, trimming, Operational Taxonomic Unit (OTU)-clustering, and chimera detection. The cluster threshold was set to 97% similarity (Edgar 2010; Edgar et al. 2011; Edgar and Flyvbjerg 2015). The Ribosomal Database Project Naive Bayesian Classifier was used for taxonomic assignment with the 16S and UNITE fungal training sets (Wang et al. 2007; Cole et al. 2013; Kõljalg et al. 2013). OTUs belonging to Archaea, Plantae, and Protista were discarded. Samples were normalized with variance-stabilizing normalization and significant OTUs were identified using the 'DESeq2' package (McMurdie and Holmes 2014; Love et al. 2014). To determine within-sample diversity, alpha diversity statistics were calculated with the 'phyloseq' package (McMurdie and Holmes 2013; Rideout et al. 2014), significance was tested with ANOVA and Tukey's HSD in the R statistical computing environment. To determine between-sample diversity, ordination analyses on Bray-Curtis and Jaccard distances, PERMANOVA to test community centroids and homogeneity of variance to test community variance, were calculated using the 'adonis' and 'betadisp' functions in the 'vegan' package (Oksanen et al. 2016). Graphs were generated with 'ggplot2' (Wickham 2009). Analyses were completed using R version 3.3.2 (R Core Team 2016).

Microbial Isolation and Identification

Two additional plants for microbe isolations were collected at each growth stage, following the methods as described above. Plants were stored at 4 °C, and processed as described below within 48 hours of sample collection. Endophytic fungi were isolated as previously described (Arnold et al. 2000) with slight modifications: ten 2 mm^2 pieces of tissue were removed from roots, stems, or leaves and surface sterilized by soaking in 10% sodium hypochlorite with 0.1% Tween 80 for 2 minutes, followed by a rinse in 70% ethanol for 2 minutes, and a quick rinse in sterile distilled water. Surface sterilized tissue was incubated at room temperature in Nutrient Broth Yeast Extract agar medium (Suay et al. 2000) or 2% Malt Extract Agar (MEA; Amresco, Solon, OH) appended with 50 µg/ml ampicillin to reduce contaminating bacteria. Emerging fungi were transferred singly to MEA and sub-cultured twice to obtain a homogeneous culture.

Endophytic bacteria were isolated as follows: roots or intact aboveground tissues of vegetative stage plants were surface sterilized as described above. For plants from the two later growth stages, leaves, roots, and stems were individually surface sterilized and ground in 0.85% aqueous NaCl with glass beads in a mortar and pestle (Compant et al. 2011). Three 10X serial dilutions of the extraction wash were generated and duplicates of each dilution were streaked onto R2A medium (Reasoner and Geldreich, 1985) supplemented with 40 µg/ml cycloheximide to reduce contamination by eukaryotic microbes.

Non-endophytic microbes were isolated by cutting tissue into small fragments, approximately 5 mm^2, and transferring them to selective media. MEA or Rose Bengal agar medium supplemented with 50 µg/ml ampicillin was used to capture a diverse population of fungi. Fungal colonies were sub-cultured as described above. To select for bacteria, R2A medium supplemented with 40 µg/ml cycloheximide. Bacterial colonies were re-streaked at least three times, and single colonies were isolated to ensure cultures were genetically homogeneous.

Fungal isolates used in plant protection assays, and morphotypes of isolates used in *in vitro* competition assays were identified by sequencing of the full ITS rRNA gene region. DNA extraction was performed on lyophilized mycelium of isolates used in plant protection assays and isolates of representative morphotypes with the Extract-N-Amp kit (Sigma-Aldrich, St. Louis, MO) as modified by Bonito et al. (2011). The ITS region was amplified with the ITS1F and ITS4 primer pair (Table S1) and Phusion High Fidelity DNA Polymerase, following manufacturer’s recommendations (New England Biolabs, Ipswich, MA). PCR products were purified with EXOSAP-IT (Affymetrix, Santa Clara, CA) and sequenced at the MSU Research and Technology Support Facility. Sequences were identified via BLAST matches to the NCBI database (Altschul et al. 1990).

In vitro *Competition Assays*

A Michigan isolate of *F. graminearum* (PH-1, NRRL #31084, FGSC #9075, Trail and Common, 2000) was used in all *in vitro* competition and plant protection assays. Petri dishes (35 mm diameter) containing MEA were simultaneously inoculated with *F. graminearum* and the antagonistic fungus at opposite poles. Bacterial isolates were streaked across the midline of one-half of the dishes 24 hours after inoculation of *F. graminearum*. Fungal interactions were recorded starting at 48 hours after inoculation. Interactions were classified based on observed phenotypes for up to 10 days post-inoculation (Fig. S2).

Plant Protection Assays

Endophytic isolates that exhibited inhibitory activity *in vitro* were used *in planta* to determine if endophytes could protect against seedling blight. Wheat seeds (cultivar Wheaton) were surface sterilized in 95% ethanol for 10 seconds, rinsed in sterile distilled water for 10 seconds followed by a 3-minute wash in 5% sodium hypochlorite, and three rinses in sterilized distilled water. Seeds were germinated on 6 cm MEA plates colonized with 2-3 day-old cultures of isolates of the endophytic fungi, as described in Hubbard et al. (2012). After three days, endophyte-inoculated seeds were transferred to 50 ml cone-tainers (Steuwe and Sons, Inc., Tangent, OR.) with potting mix (Suremix Perlite, Michigan Grower Products, Inc., Galesburg, MI). Plants were challenged by inoculation with *F. graminearum* with the addition of colonized agar; ⅙ of a 10 cm diameter Petri dish containing Synthetic Nutrient-poor Agar (SNA) medium (Baldwin et al. 2010). Control plants were potted with sterile SNA (Fig. S3). The assay was replicated three times, independently, each isolate was tested with 10 plants per replicate. Isolates were scored for plant protective abilities, based on disease incidence, as calculated by lesion presence at the base of the stem or deceased plants.

Results:

Microbiome Composition across Plant Organs and Land Management Strategies

The ITS2 rRNA gene sequencing of all samples, from wheat organs at three growth stages and under four management strategies, generated 31,507,778 reads. Of 216 samples, sequences from 214 resulted in usable sequences that passed quality filtering. Sequence processing was performed with the USEARCH pipeline and identified 3,164 ITS2 OTUs at 97% similarity for clustering (Table S2).

Taxonomic composition changed across growth stage, and root communities contained more unique members than phyllosphere communities. Recently, several usages for "phyllosphere" have appeared in the literature. For clarity, in this publication we will use "phyllosphere" to refer to aerial parts of the plant, as previously defined (Ruinen 1956; Vorholt 2012). During the vegetative growth stage under all management strategies, approximately 40% of fungal OTUs were classified as Dothideomycetes, followed by a decrease in abundance at flowering, and then an increase to 50-90% of all observed sequences at seed development (Fig. 1, Table S3). Dothideomycetes were classified as members of the Pleosporaceae, Phaeosphaeriaceae, or Leptosphaeriaceae, but many Dothideomycete OTUs remained unclassified at the family level (Fig. S4A). Across the growing season, we observed a decrease from vegetative to flowering stages in the relative abundance of low-abundance OTUs, such as

Tremellomycetes, Microbotryomycetes, Leotiomycetes, and Agaricomycetes. Agaricomycetes populations were largely members of the Ceratobasidiaceae and Marasmiaceae (Fig. S4B). Microbotryomycetes populations were dominated by the Leucosporidiaeae family (Fig. S5A). At flowering we observed a higher proportion of Eurotiomycetes in leaf samples, specifically the Herpotrichiellaceae and Massariaceae (Fig. S5B), compared with vegetative and seed development stages. Also at flowering, leaf and stem samples were dominated by the Leotiomycetes, specifically the Erysiphaceae (Fig. S6A).

The majority of OTUs, across all samples, belonged to a few groups (Dothideomycetes, Sordariomycetes, Agaricomycetes), however, abundances of some OTUs differed across management strategy. Notably, in conventional and no-till plots, we observed a greater abundance of Microbotryomycetes in leaves and stems (Fig. 1). Roots, compared with leaves or stems at all growth stages and under all management strategies, had higher numbers of unidentified OTUs, with nearly 50% of the relative abundance of OTUs from conventional and low input plots (Fig. 1). The no-till samples had more unique OTUs, and approximately 25% of the observed OTUs belonged to the Sordariomycetes. Sordariomycete populations were largely composed of members of the Nectriaceae and Lasiosphaeriaceae across all management strategies, except the Glomerellaceae dominated the leaves and stems of low input and organic management types during seed development (Fig. S6B). Pairwise comparison of unique OTUs in the roots revealed that *Periconia* sp. was enriched in no-till and organic plots when compared to all other management strategies. *Tetracladium* sp. was enriched in all low-input plots, and *Fusarium* sp. was enriched in all organic plots (Fig. S7). The same analysis of the phyllosphere samples showed enrichment of *Fusarium* sp. in organic and no-till plots, enrichment of *Ceratobasidium* sp. in low-input plots when compared to all other management strategies. *Puccinia* sp. was enriched in conventional, low input, and organic plots when these management strategies were compared to no-till (Fig. S8).

The 16S rRNA gene sequencing generated 28,082,995 sequences that clustered into 7,906 OTUs. All 216 samples generated high quality sequences. Proteobacteria was the most abundant bacterial Phylum, followed by Bacteroidetes and Firmicutes (Table S4). At the Class level, Alphaproteobacteria, Actinobacteria, Gammaproteobacteria, Deltaproteobacteria, Planctomycetia, and Sphingobacteria were the most abundant OTUs (Fig. 2; Table S5). Fungal alpha diversity was generally consistent across organs and management strategies, then slightly decreased during seed development (Fig. 3A). The fungal diversity of plant organs, as calculated by the Shannon Index (*H'*), were not significantly different during vegetative or flowering growth stages. But at the seed development stage, *H'* of stems was significantly lower than roots from the organic plots ($p \leq 0.05$; Fig. 3A; Table S6). This correlates with the large increase in the dominance of Dothideomycetes in all plant organs as the most abundant OTUs during seed development. In contrast to the fungal samples, the bacterial alpha diversity in roots was significantly higher than in stems and leaves when compared ($p \leq 0.05$) across growth stages and management strategies, but stems and leaves were not significantly different from each other (Fig. 3B; Table S6).

To determine if beta diversity of microbial communities differed, ordination analysis was conducted within management types and organs. As stated by Kelly et al. (2015), 90% power for PERMANOVA tests can be achieved with five independent samples in microbiome studies; here we tested groups of six independent samples. PERMANOVA tests showed that within each management strategy, fungal communities from different growth stages ($p<0.001$) and organs ($p<0.05$) had significantly different centroids (Fig. 4A; Table S7). Tests of homoscedasticity for growth stage were not significant, but were significant for organ in no-till and low input plots ($p<0.05$). Fungal communities differ at each growth stage, and communities may differ across wheat organ. Centroids of bacterial communities were significantly different for organ and growth stage ($p \leq 0.001$; Fig. 4B; Table S7). Dispersions tests were significant for organs within all management strategies except no-till ($p \leq 0.01$), and were significant for growth stages within all management strategies except conventional ($p \leq 0.05$). The differences in group dispersions may cause observed clustering of bacterial communities by organ.

In contrast to the analysis of communities under different management strategies, PERMANOVA tests within each organ, showed fungal communities from different growth stages and management strategies had significantly different centroids ($p \leq 0.01$; Fig. S9A; Table S7). Homoscedasticity tests on management strategy were not significant in any organ, but were significant for growth stage in stems ($p \leq 0.001$). The fungal communities of all organs except stems, differ across management strategies and at each growth stage. Bacterial communities had significantly different centroids for management strategy and growth stage ($p \leq 0.05$; Fig. S9B; Table S7). Homoscedasticity tests were significant for growth stage ($p \leq 0.01$) and management strategy in leaves ($p \leq 0.05$). The observed differences in bacterial communities across growth stage may be due to differences in group dispersions, but it appears that management strategy affects the bacterial communities within different plant organs.

Microbe Isolations and In vitro *Assays*

1,634 fungal and 1,112 bacterial isolates were cultured from all wheat organs and management strategies across the three growth stages (Table 1). A subset of these isolates, 711 fungi and 715 bacteria were screened with an *in vitro* confrontation assay paired with *F. graminearum*. Based on observed interaction phenotypes, five categories were identified and all isolates used for the *in vitro* confrontation assay were placed in one of the five categories (Table 2): Type 1: Zone of inhibition between colonies, excessive pigment production by *F. graminearum*. Type 2: Zone of inhibition between colonies, no *F. graminearum* pigment observed in inhibition zone. Type 3: No zone of inhibition, mycelium deadlock between fungal colonies, or hyphal proliferation at fungal-bacterial colony interface. Type 4: *F. graminearum* hyphae grew over top of competing microbe. Type 5: Hyphae of competing fungus grew over top *F. graminearum* colony, or competing bacterial colony covered more than 50% of the petri dish. The fungal isolates within each of these categories were then grouped into morphotypes, isolates of similar morphological appearance (Table 2). Morphotypes were identified as *Alternaria* sp., *Bipolaris* sp., *Cochliobolus* sp., *Fusarium* sp., *Colletotrichum* sp., *Talaromyces* sp., *Trichoderma* sp., *Parastagnospora* sp., *Penicillium* sp., among others (Table S8).

Greenhouse Assay and Isolate Identification

Endophytic fungi (78 strains) that generated Type 1 interactions during *in vitro* confrontation assays were tested in a seedling assay. Fifteen of these strains showed biocontrol activity during an initial test, and these strains were pursued further. Many of the strains were identified as species known to be saprotrophs, weak grass pathogens, or pathogens of other plant species (strains and ID numbers can be found in Table 3). Positive controls (plants inoculated with *F. graminearum* in the absence of an endophyte) had an average 82% disease in the seedling assay (Fig. 5). Negative controls (plants not inoculated with an endophyte and without *F. graminearum* in the soil) had an average of 3% disease. The positive and negative controls were significantly different from each other ($\alpha = 0.05$; Fig. 5). Endophyte inoculated seedlings challenged with *F. graminearum* in soil had a 10 to 41% reduction in disease compared with the positive control (Fig. 5). Strain 40 had significantly reduced disease compared to the positive control ($\alpha = 0.05$). All other endophyte inoculated plants, besides strain 40, had reduced disease, but due to high variance of the means the disease reduction was not significant. Two endophyte strains, 11 and 34, had higher or nearly equal disease rates in control plants when compared with *F. graminearum* challenged-plants (Fig. 5), indicating that these endophyte strains may be pathogens of wheat.

Fungal microbiome data was mined for OTUs closely related to the genera of the isolates used in the greenhouse assay. *Alternaria* sp. was found in nearly all samples, with the highest abundances in leaf samples (Fig. S10). *Microdochium* sp. and *Fusarium* sp. were the next most abundant OTUs, with highest abundances specifically in root samples at the flowering and seed development growth stages (Fig. S11). *Phoma* sp. were most abundant during seed development in conventional and no-till plots, and *Talaromyces* sp. were most abundant during flowering, but both *Phoma* sp. and *Talaromyces* sp. were found in relatively low abundances across multiple samples (Fig. S12). *Aspergillus* sp. and *Penicillium* sp. were found in very few samples, namely the roots of no-till plants during flowering and vegetative stages, respectively (Fig. S13).

Discussion:

One of the proposed applications of microbiome research is the manipulation of microbial communities to reduce pathogen pressure and increase yield. Before this can successfully occur, the composition of a plant-associated community that can induce these effects must be determined. Toward this goal, the present study provides the first in-depth analysis of the wheat microbiome -- phyllosphere, roots, and rhizosphere.

Contrary to our expectations, management strategy did not have a strong influence on plant microbial communities. Previous studies compared soil and root microbial communities across management strategies, such as conventional versus organic including wheat in the rotation. These studies have reached varied conclusions regarding the impact of management strategies on soil or plant associated microbial communities (Hartmann et al. 2014; Lenc et al. 2014; Li et al. 2012). Studies which found that management strategy influenced the microbial community compared sites in which the rotated crops differed across management strategy and/or compared sites which were separated by significant geographic distances (Hartmann et al. 2014; Lenc et al. 2014; Li et al. 2012; Rascovan et al. 2016). Similar to the present study, a previous study on the wheat phyllosphere used sites with a maximum distance of 10 km between fields of differing management strategies (Karlsson et al. 2017). The majority of the of the high-abundance OTUs from leaf microbiomes were detected across all fields and management strategies, but the authors found significant differences in low-abundance OTUs. Previous surveys of plant microbiomes comparing the same genotypes across multiple locations found that geography has a stronger influence on microbial community than management strategy or plant genotype (Chen et al. 2016; Copeland et al. 2015; Finkel et al. 2011; Peiffer et al. 2013). As discussed by Peay et al. (2016), decay-by-distance patterns for fungal communities are commonly observed, indicating geography and associated environmental factors impose strong effects on fungal community composition. These same factors would likely also impose these effects on the bacterial community.

The structure of the KBS-LTER site is nearly ideal for testing management effects -- all of the plots are located within 2 km^2 and have been planted with the same 3-crop rotation for almost three decades. Studies of soil microbial communities conducted at the KBS-LTER site found similar results to the present study. For example, Lauber et al. (2013) sampled the 16S soil community of the conventional and low-input plots at the KBS-LTER. They observed similar community composition among management strategies, and also observed increased soil diversity throughout the growing season (Lauber et al. 2013). Xue et al. (2013) investigated the functional differences in soil communities of the KBS-LTER plots using the Geochip, an array based on genes involved in biogeochemical processes of soil microbes. They surveyed conventional, no-till, and low input plots, but found no significant differences in community function (Xue et al. 2013). Seed treatments could influence these outcomes. In our study, plots from three of the management strategies were planted with fungicide-treated seed, and the seed for the organic plots was not treated. This did not appear to affect the outcome, in that there were no major differences between the microbiomes of the plants in these plots. However, previous studies have shown that fungicide treatments affect rhizosphere and phyllosphere microbiomes of maize and soybean tested during the vegetative growth phase (Nettles et al. 2016). Further research is warranted to detect the impact of seed fungicides on the extant microbiome, and to determine the degree the fungicide, inherited microbes, or environment impact the microbiome of a mature plant. In the present study, the absence of strong management effects in any of the three growth stages, together with the survey of published data discussed above, suggest a strong influence of geographical factors on wheat microbiomes. However, it is likely that a combination of climate, cultivar, land use history, and management strategies all impact plant microbial communities in ways we may not be able to detect at this time.

Our study found that bacterial alpha diversity in roots was higher than in stems and leaves, whereas fungi show consistent diversity across plant organs. These results are consistent with previous studies showing soil and rhizosphere bacterial diversity were greater than diversity of the phyllosphere (Coleman-Derr et al. 2015; de Souza et al. 2016; Knief et al. 2011), and others showing rhizosphere and phyllosphere fungal diversity were similar (Coleman-Derr et al. 2015). The observed consistencies in fungal diversity across plant organs may be due to systemic colonization of crops by endophytic fungi, or due to aerial dispersal and subsequent phyllosphere colonization by fungal spores. Microclimate effects on plant organs that inhibit colonization by microbes may be one explanation for the low diversity of phyllosphere bacterial communities on wheat.

Our experimental design did not distinguish between epiphytic and endophytic microbes. Other studies that examined microbial communities of epiphytic and endophytic plant compartments found that the plant organs had a stronger effect than compartment on fungal community assembly (Coleman-Derr et al. 2015; de Souza et al. 2016). In contrast, the rhizosphere and the leaf episphere, had higher bacterial diversity than corresponding endophytic compartments (Coleman-Derr et al. 2015; de Souza et al. 2016). Thus, it is not surprising that microbial communities of stems and leaves in the present study were very similar. It is possible that in the rhizosphere and in the root endophytic compartment, the plant genotype imparts a greater selective force on community assembly (Coleman-Derr et al. 2015; Mendes et al. 2014; Ofek et al. 2013; Peiffer et al. 2013).

We observed increased microbial community diversity over the growing season. This is a consistent pattern found across other plant microbiome studies of cereals and fruits (Bakker et al. 2017; Donn et al. 2014; Shade et al. 2013). However, the observed increase in diversity is in contrast with the results of Copeland et al. (2015) who found a decrease in phyllosphere bacterial diversity throughout the growing season in beans and canola. The increase in diversity could be explained by ecological succession within the plant microbiome through the growing season, as emerging surfaces on crops bringing new habitats and an expansion of niche breadth. As plants grow and age, the community complexity and diversity would then increase. In addition, the observed increase in diversity could be a direct response to signals between the plants and microbes; that is, colonization by saprotrophic organisms that are able to colonize the plant host at earlier growth stages. The increased diversity could also reflect microbial responses to complex metabolites produced by mature plants. These interactions would not be limited to pathogens or saprotrophs. A more comprehensive study of the colonization of older plants is needed.

The bacterial communities of wheat at the KBS-LTER site were composed of common wheat- and plant-associated taxa. Previous wheat-microbiome studies, which were limited to studies of soils of wheat fields, roots, or rhizosphere, found similar fungal and bacterial community composition as the study presented here. Proteobacteria, Firmicutes, and Actinobacteria were the dominant bacterial taxa in the KBS-LTER plots, similar to other wheat rhizosphere studies (Donn et al. 2014; Hartmann et al. 2014; Lenc et al. 2014; Ofek et al. 2013). Dothideomycetes, Leotiomycetes, Sordariomycetes were the most common fungal taxa observed in the present study as well as previous studies (Karlsson et al. 2017; Kwaśna et al. 2010; Lenc et al 2014). Future studies are needed to investigate genotype-, inflorescence-, and developing seed-specific microbial communities.

The limited approaches available for control of *F. graminearum* suggest implementation of an integrated approach combining crop rotation and use of partially resistant varieties as the optimum control strategy. Biologicals used as soil or seed amendments would provide another tool to control a difficult disease. As reviewed recently, the phyllosphere microbiome contains organisms that influence plant defense and carbon cycling, among other functions (Vorholt 2012; Bulgarelli et al. 2013; Rastogi et al. 2013). We used damping-off of seedlings to test our strains for protective effects. In wheat seedlings, damping-off is currently controlled with fungicide-coated seeds (Wegulo et al. 2015), but fungicide resistance in *F. graminearum* is an increasing problem. We tested single protective strains, however multi-strain protection from disease is likely to make a more robust application in the field, as has been reported (Slininger et al. 2010). We speculate that these tested strains, isolated in Michigan, are likely to be more successful protectors than products derived from strains native to other geographic regions, based on previous work with *Trichoderma* sp. biological controls (Chaverri et al. 2015; Grondona et al. 1997; Sharma et al. 2009) and aflatoxin control strains (Adhikari et al. 2016).

For more than 150 years, plant pathologists have shown us that individual microbes have adapted to specific niches on their hosts (Stakman and Harrar, 1957). Our ability to perform high-throughput sequencing of these niches has revealed large numbers of microbes forming communities that can affect disease outcomes. More research is required to better understand the composition of organisms in these niches, the interactions among members of these communities, and how the communities impact plant health. A fundamental understanding of the plant microbiome is necessary for successful manipulation for agricultural benefit.

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Table 1. Microbe isolates recovered from wheat plants.

	Number isolated[a]	
Growth stage	**Fungi**	**Bacteria**
Early Vegetative	456	510
Boot	527	211
Kernel Development	651	391

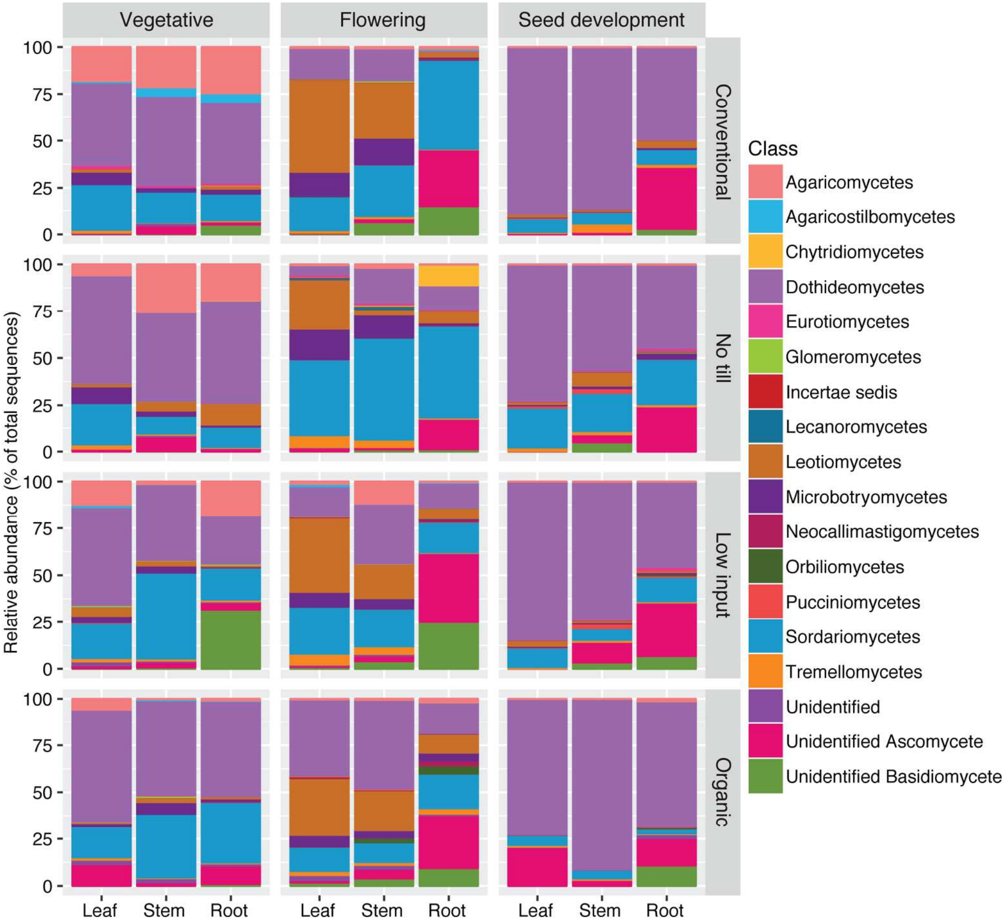
[a] Numbers presented here are totals and do not account for duplicates of morphotypes.

Table 2. Results of in vitro co-culture competition assay with *Fusarium graminearum*.

	Number observed					
		Bacteria		**Fungi**		
Category	Total	Total	Percent	Total	Percent	**Observed morphotypes**
Type 1	175	74	10.3%	101	14.2%	15
Type 2	308	113	15.8%	195	27.4%	16
Type 3	442	175	24.5%	267	37.6%	11
Type 4	463	341	47.7%	122	17.2%	15
Type 5	38	12	1.7%	26	3.7%	10

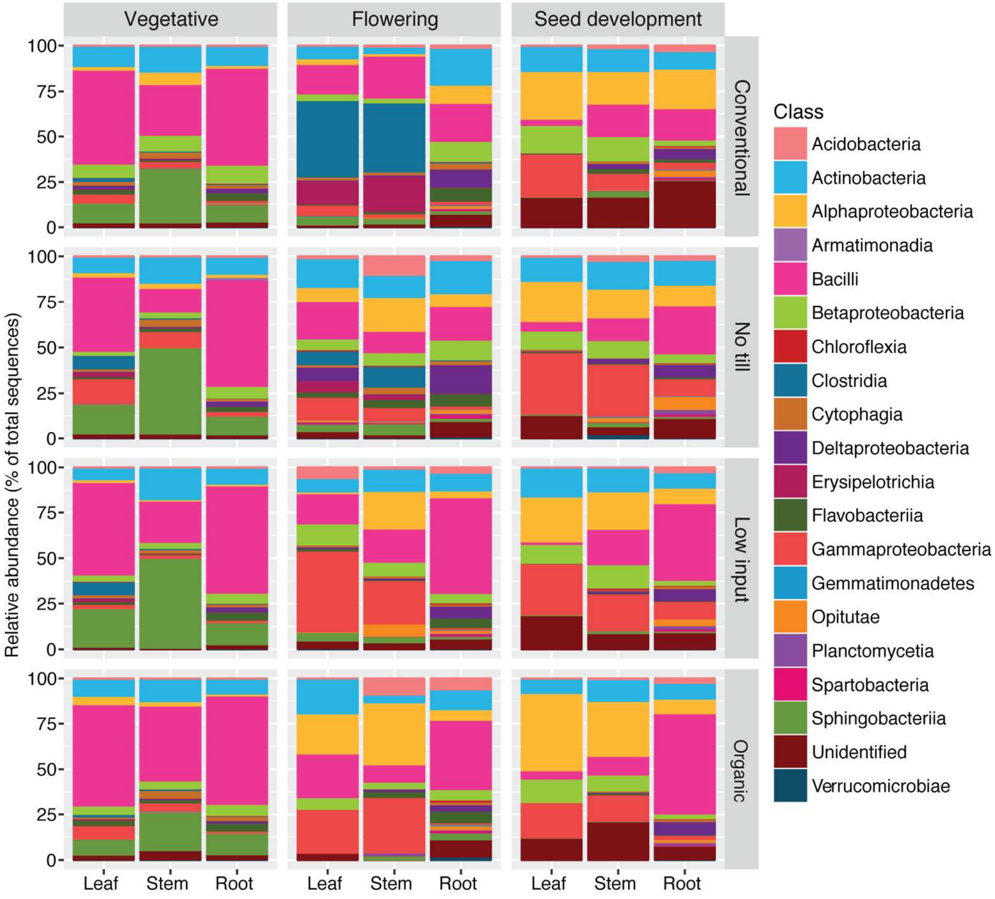
Table 3. Identification of most protective fungal strains used in greenhouse plant assays.

Strain ID	ID by full length ITS locus
11	*Microdochium bolleyi*
30	*Alternaria tenuissima*
34	*Alternaria* sp.
35	*Talaromyces trachyspermus*
36	*Aspergillus niger*
37	*Alternaria tenuissima*
38	*Fusarium solani*
40	*Fusarium* sp.
44	*Fusarium* sp.
45	*Penicillium reticulisporum*
51	*Phoma* sp.
57	*Phoma* sp.
59	*Fusarium* sp.
70	*Fusarium oxysporum*
88	*Penicillium commune*



Class-level relative abundance of fungal communities across growth stage, plant organ, and crop management strategies.

176x163mm (300 x 300 DPI)



Class-level relative abundance of bacterial communities across growth stage, plant organ, and crop management strategies.

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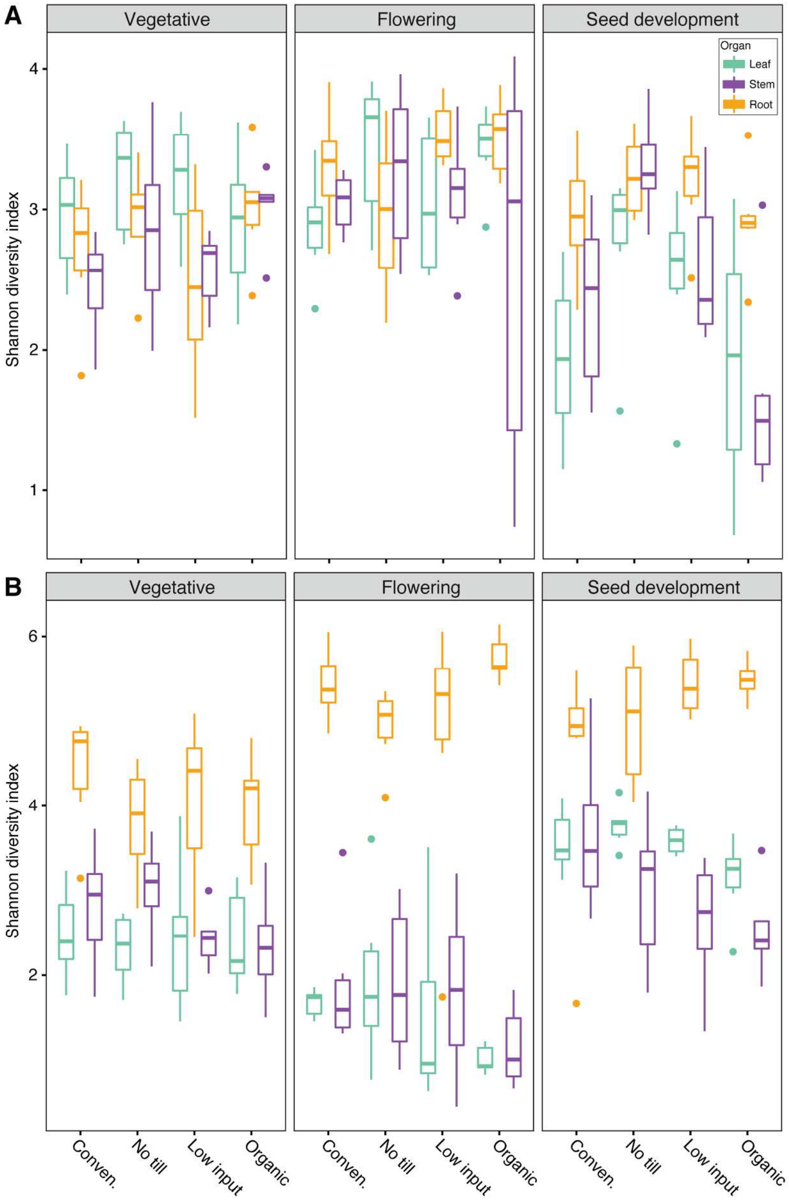
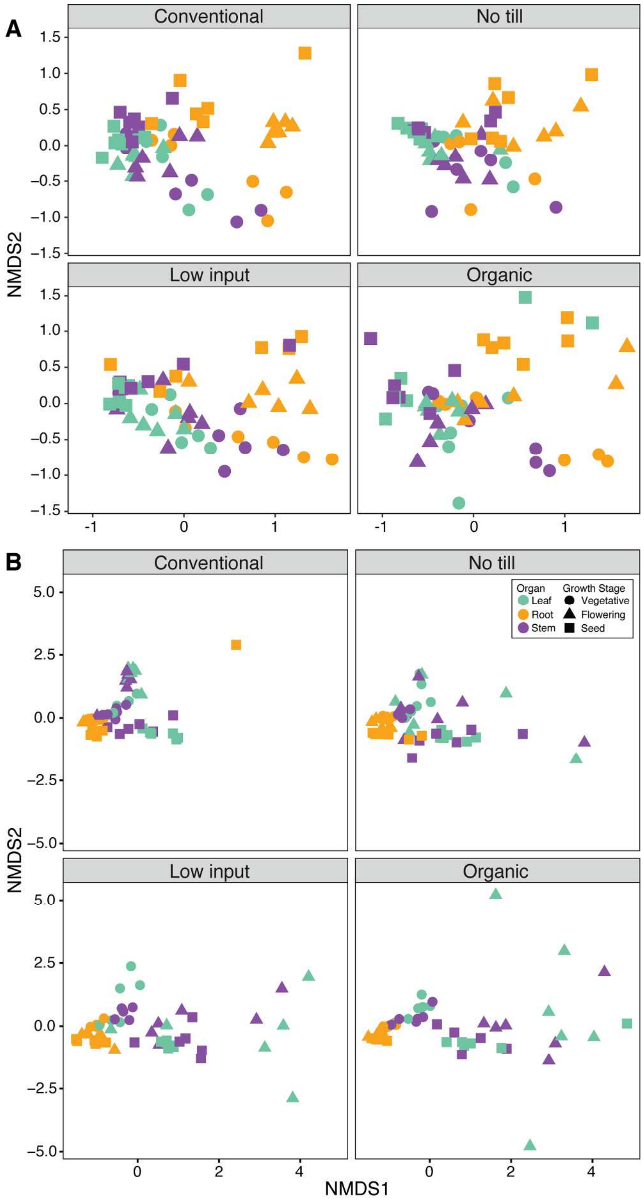


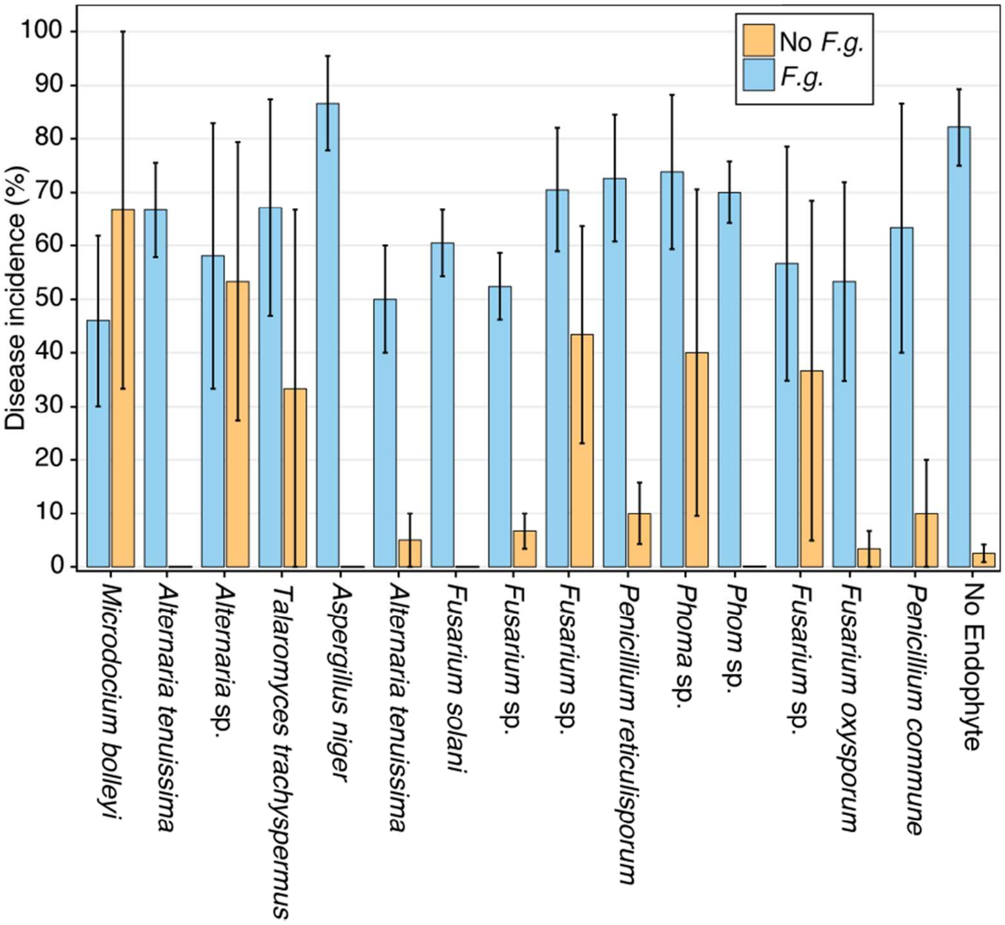
Alpha diversity of fungi (A) and bacteria (B) found across all samples estimated by Shannon diversity index. Data are represented by six replicates from each stage-management-organ combination. Center line of boxes represents median of samples. The upper and lower sides of the boxes represent the third and first quartiles, respectively. Whiskers represent ±1.5 times the interquartile range. Data points beyond whiskers represent outliers. ANOVA and Tukey's HSD were used to test significance ($p < 0.05$). Statistical support is detailed in Table S6. Conven = conventional.

159x243mm (300 x 300 DPI)



Effect of management strategies on beta diversity of fungal (A) and bacterial (B) communities originating from plant organs. Non-metric multidimensional scaling (NMDS) calculated by Bray-Curtis distance. Difference between centroids were tested using PERMANOVA and homoscedasticity to test variance (Oksanen et al. 2016). Stress values of fungal data (A), which reveals goodness of fit, were 0.115, 0.117, 0.117, and 0.121 for conventional, no till, low input, and organic NMDS plots, respectively. Stress values of bacterial data (B) were 0.086, 0.092, 0.086, and 0.113 for conventional, no till, low input, and organic NMDS plots, respectively. Statistical support is detailed in Table S7.

131x247mm (300 x 300 DPI)



Mean disease incidence (percentage of replicate) of endophyte inoculated seedlings ± standard error of the mean. Ten plants per replicate, three independent replicates. Labels on x-axis indicate strain identification numbers. F.g. = Fusarium graminearum. ANOVA and Tukey's HSD were used to test significance ($p < 0.05$).

80x73mm (300 x 300 DPI)

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