CROP MICROBIOMES AND THE SEARCH FOR EFFECTIVE BIOCONTROL OF *FUSARIUM GRAMINEARUM* ON WHEAT

By

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ABSTRACT

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Manipulation of naturally occurring microbial communities to reduce plant diseases or increase crop yields requires a thorough understanding of interactions within the phytobiome, in particular, how microbial communities change as plants age, across plant species and organs, and under different land management regimes. Plants were sampled from a wheat-maize-soybean crop rotation site that implements four different land management strategies (conventional, no-till, reduced inputs, and organic). The fungal and bacterial communities of leaves, stems, and roots of wheat, maize, and soybean throughout the growing season were analyzed using fungal internal transcribed spacer and bacterial 16S rRNA gene amplicon sequencing. Analysis of sequence-based fungal communities has some limitations due to the unreliable phylogenetic resolution of DNA sequence alignments. To improve this deficiency, a tool that improved phylogenetic resolution was developed. This tool increases the number of operational taxonomic units which are identified at genus and species levels. Endophytes were isolated from the wheat plants used for microbial community analysis and tested for antagonistic activity toward the wheat pathogen *Fusarium graminearum* during wheat seedling and head infection. Endophytes on crops can be developed to manage disease, and endophyte-based biocontrols could solve current limitations in *F. graminearum* disease control. Additionally, functional analysis of *F. graminearum* secondary metabolite genes provides insight into the function of their gene products for this fungal pathogen. Microbial community structure is affected by various genetic factors of the host plant, environmental factors, and interactions with other organisms. Understanding community

responses to these factors is necessary for targeted manipulation of communities to reduce plant disease.

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KEY TO ABBREVIATIONS

ANOVA	Analysis of variance
ARISA	Automated ribosome intergenic spacer analysis
BLAST	Basic Local Alignment Search Tool
CONSTAX	Consensus Taxonomy Tool
DGGE	Denaturing gradient gel electrophoresis
FGDB	*Fusarium graminearum* Genome Database
ITS	Internal transcribed spacer
KBS	Kellogg Biological Station
LTER	Long-term ecological research
MIPS	Munich Information Center for Protein Sequences
NCBI	National Center for Biotechnology Information
NMDS	Non-metric multi-dimensional scaling
OTU	Operational taxonomic unit
PERMANOVA	Permutational analysis of variance
TPS	Terpene synthase
T-RFLP	Terminal restriction fragment polymorphism
Tukey's HSD	Tukey's honest significant difference test

CHAPTER 1

LITERATURE REVIEW

Introduction

Growing interest in the manipulation of naturally occurring microbial communities to reduce plant diseases or increase crop yields has caused the plant microbiome and phytobiome fields to blossom in recent years. Before manipulation of plant or soil microbial communities can be successfully implemented, we must have a thorough understanding of the composition of a healthy microbial community, in particular, how microbial communities change as plants age, how communities differ across plant species, genotype and organ, under different land management regimes, and across various climates. This review will focus on the origins of the plant microbiome and phytobiome fields, how phytobiome research will be useful for changing preferences in disease management, how an increasing understanding of distributions of plant microbiomes and endophytes on crops will be useful to manage disease, and how endophyte based biocontrols could solve current weaknesses in *Fusarium graminearum* Schwabe (teleomorph *Gibberella zeae* [Schwein.] Petch) disease control.

Emergence of Phytobiome Field

Historically, before the onset of low-cost high-throughput DNA sequencing, microbes and plants were studied almost exclusively in paired isolations (for example, a pathogen plus the plant host). Studies of these limited plant-pathogen systems are also restricted by what we can

reasonably culture and grow in a laboratory setting. In the 1990s and early 2000s, techniques such as automated ribosome intergenic spacer analysis (ARISA), terminal restriction fragment polymorphism (T-RFLP), or denaturing gradient gel electrophoresis (DGGE) were used in microbial ecology to glean information about community composition, structure, and function. ARISA was used to distinguish between microbial communities, with PCR-amplified intergenic spacers that were separated on polyacrylamide gels. Analysis of these gels assumed each member of the community had a different length intergenic spacer (Fisher and Triplett 1999). T-RFLP is a technique based on DNA extraction and fluorescently-labelled PCR amplification followed by an enzyme digestion that can provide information about community membership (Liesack et al. 2004). When applied to complex microbial communities, researchers gained insight into diversity, but T-RFLP often oversimplified the picture of a microbial community, thus was often complemented with analysis of clone libraries; which is very is laborious. DGGE also required DNA extraction and PCR amplification of 16S or 18S rRNA genes. PCR products were separated on a denaturing gel based on DNA fragment composition instead of size (Strathdee and Free 2013). All of these techniques are laborious and time intensive. They also can provide oversimplified pictures of the communities analyzed because they largely rely on distinguishing between sized-based band patterns.

The invention of "next-generation" sequencing (such as 454-Roche pyrosequencing or Illumina sequencing, now more appropriately termed sequence-by-synthesis), quickly replaced previous microbial community analysis methods such as ARISA, T-RFLP, or DGGE because DNA marker gene sequencing generated larger volumes of data generated for equal or reduced sample preparation costs. The first paper using pyrosequencing of the 16S rRNA gene to analyze the composition of a microbial community was published in 2002 (Jonasson et al. 2002). This

technique was popularized by the publication of tools such as Dotur, Mothur, and the RDP Classifier (Schloss and Handelsman 2005, Schloss et al. 2009, Cole et al. 2013). Entire research initiatives have arisen from these new technologies – Human Microbiome Project (Turnbaugh et al. 2007), Microbiome of the Built Environment (Lax et al. 2014), Earth Microbiome (Gilbert et al. 2014), and most recently the Phytobiome Initiative (APS 2016).

Reduced sequencing costs and consistently increasing dataset sizes generated in a single sequencing run, coupled with the increased ease-of-access to high-performance computer clusters, has enabled a culture-independent era of microbial ecology research. These technologies have given scientists the ability to identify thousands of "species" or operational taxonomic units (OTUs) from a small sample, such as one gram of soil. Sequencing of barcode or marker regions, such as the fungal internal transcribed spacer (ITS) region and bacterial 16S rRNA gene coupled with high-throughput sequencing technologies allows researchers to obtain an in-depth picture of the community structure. The numbers of studies using these techniques have exploded in recent years with 14,570 results for a "microbiome" topic search on Web of Science, with the oldest publication dated in 2002.

The term microbiome was first defined as a "characteristic microbial community" (Whipps et al. 1988). In the early days of genomics and metagenomics, the use of "microbiome" was co-opted to refer to the microbial meta-genome (Lederberg 2004, Huss 2014). Metagenomics – the sequencing of total DNA from a sample, instead of one gene or a single species' genome, allows bioinformaticians to reconstruct many genomes simultaneously. Using this technique, we can interpret or hypothesize functions and lifestyles of organisms that we have not yet isolated in the lab. This information can be used to culture some previously difficult-to-culture or unculturable species and use these organisms for experimental manipulation in the laboratory (Browne et al.

2016). There has been recent formal acknowledgement of the original definition of the term microbiome as usage of this term is becoming more common (Shade et al. 2012, Marchesi et al. 2015, Leach et al. 2017, Prescott et al. 2017, Schlatter et al. 2017).

As with the terms microbiome and metagenome, there has been some confusion regarding "phytobiome." Phytobiome does not equate to "plant microbiome," it actually has a more holistic definition; incorporating environmental factors, pathogens, herbivores, plant genetics, and the interactions among all aforementioned items on plant health. Due to methodological development from the previous microbiome research initiatives, plant microbiome researchers are at an advantage and can skip some of the methodological development that comes with an emerging field. But plant microbiome researchers cannot skip the descriptive community steps. There has been considerable work conducted toward describing the microbial communities of rhizosphere and root-associated taxa of major crops and model plants. There has been less focus on identifying the communities associated with phyllosphere (aboveground plant-associated) communities. Work on model plant systems has shown that the microbes inhabiting the aboveground organs of plants influence plant health and nutrient cycling (Delmotte et al. 2009, Ryffel et al. 2016, Vogel et al. 2016)

From the limited number of studies that have investigated microbes across multiple organs of the same plant – it appears that there are organ-specific plant microbiomes (reviewed by Vandenkoornhuyse et al. 2015). From the diversity captured by previous studies, a core plant microbiome (microbes shared across all plants) does not appear to be present, but a meta-analysis of all the plant microbiome studies conducted to date may reveal otherwise. Although there is not yet a clearly established metadata guidelines for plant microbiome work – it would be advantageous for the plant microbiome field to establish them. Such guidelines have been

established for the Human (Turnbaugh et al. 2007), and Earth (Gilbert et al. 2014) Microbiome Projects. Plant microbiome researchers should strongly consider incorporating Earth Microbiome Project metadata guidelines into their studies, including measurements such as altitude, biome, host taxonomy, and growth conditions which will aid in future meta-analyses of sequence data (Earth Microbiome guidelines are available at http://www.earthmicrobiome.org/protocols-and-standards/metadata-guide/).

It has also been demonstrated that transport and storage methods affect quality of samples (U'Ren et al. 2014). Immediate freezing of samples best preserves plant and microbial DNA for extraction and future re-sampling. Incubation and transport in extraction buffer from field site to lab is the next best ideal method, but one method commonly used (drying and room temperature storage) leads to a significant drop in DNA quantity and subsequently OTU recovery (U'Ren et al. 2014). Studies on plants in urban or agricultural habitats lend themselves to easy adaptation of best-practice methods (it is practical to drive in a cooler of dry ice) but studies of plant microbiomes in remote habitats such as mountains, deserts, rainforests, or tundra, using such best-practices can be inhibitory. Without modifying current lab protocols – researchers can streamline communication across the phytobiome research field. For example, consistent growth stage sampling for descriptive analyses, such as during flowering; consensus on plant organ names, specifically, root-associated, rhizosphere, and rhizoplane, would make cross-study comparisons and microbial meta-analyses more straightforward. Although, these examples are straightforward when considering crop plants, they quickly become complicated when considering non-crop plants because of the sheer diversity in plant natural histories. Also, due to the diversity of plant tissue structure, no single extraction method can universally be used across all plants, for example, woody or fibrous tissues require more complex extraction methods. Furthermore, chemicals in

some leaves, including phenolic compounds, can complicate DNA, RNA, and metabolite extraction methods (Couch and Fritz 1990). But standardization of methods for major cash crop systems such as wheat (*Triticum aestivum*), maize (*Zea mays*), or soybean (*Glycine max*) would be more practical to establish.

Several fungal barcode regions, 18S, SSU, LSU, were tested and the results published before a consensus was established to use the ITS region (which comprises the ITS1, 5.8S, and ITS2 segments) as the formal barcode marker for fungi (Kõljalg et al. 2013, Hibbett et al. 2016). Due to the short nature of Illumina reads, only one of the ITS segments is often used. Neither the ITS1 or ITS2 regions are superior and use of either of these markers yields similar pictures of fungal communities (Dentinger at al. 2011, Bazzicalupo et al. 2013, Blaalid et al. 2013). Other researchers use primers for the full length ITS region and analyze the reads without merging the forward and reverse reads. This strategy essentially generates two complementary datasets of the fungal community that are analyzed in parallel, one for ITS1 and one for ITS2 (Benucci et al 2016, Johansen et al. 2016).

Across the diverse plant habitats where microbiome research occurs – tropical, boreal, deciduous, agricultural – adopting one best-practice method for sample collection and processing across such diverse habitats is not feasible, or even recommended. A better solution would be the adoption of standard methods within each sub-field. For example, researchers interested in crop microbiomes should adopt the following methods: (1) Immediate freezing of samples in the field, or immediate suspension of samples into DNA extraction buffer when freezing is inhibitory due to practicality (U'ren et al. 2014); (2) Freeze-drying tissue samples and subsequent room temperature storage under desiccant; (3) Use of peptide nucleic acid clamps (which bind to specific DNA and prevent PCR amplification) to reduce chloroplast contamination of sequence libraries

(Sakai and Ikenaga 2013). Use of ITS2 fungal primers to reduce bias against early-diverging fungal lineages that may be created with ITS1 primer sets (Toju et al. 2012, Bazzicalupo et al. 2013, Blaalid et al. 2013), or use of full length ITS primers that provide amplicons for both ITS regions (Benucci et al. 2016, Johansen et al. 2016). (4) Report of environmental conditions, such as weather, for the growing season and at the time of sampling; (5) Report of soil type, pH, and series of location sampled. The soil is often assumed to be the primary source of microbial inoculum, and Evans et al. (2016) have demonstrated that the diversity and composition of microbial inoculum is often linked to the diversity and composition of the final community. (6) Report disease history or symptoms on plant, where applicable; (7) Report land use history, e.g., the number of years farmed and types of crops farmed; (8) Current and previous crop management strategies used, including details regarding: fertilizers, pesticides, tillage, cover crops, and planting or harvesting equipment; (9) Clean tools between sample collections to prevent cross-contamination; (10) Collection of an appropriate number of plants from a field to obtain statistical power (Kelly et al. 2015); (11) Pool replicate PCRs for each DNA extraction and set-up libraries to achieve deep sequence coverage of each sample (Manter et al. 2010, Smith and Peay 2014); (12) Standardization of methods to isolate endophytic (within or between plant cells) or epiphytic (surface) microbes, preferably after a systematic analysis of various methods. If all plant microbiome researchers would adapt these points when designing and conducting their studies, it would greatly increase the frequency with which researchers could re-analyze and generate hypotheses about larger ecological plant microbiome patterns.

Shifting Attitudes in Disease Management

Plant defense responses can be specific to one pathogen species, but plants also have generic defense systems that are triggered by conserved microbial cues such as flagellin and chitin (reviewed by Durrant and Dong, 2004). The increasing use of "-omics" technologies is changing our understanding from pairwise plant-pathogen or plant-symbiont interactions to more intricate and complex systems that show evidence of a shared evolutionary arms race. There is some evidence that plants can recruit other organisms to assist with defense against pathogen attacks. For example, plants emit volatile compounds under herbivore predation that can attract parasitoid wasps (De Moraes et al. 1998) or predatory nematodes of the herbivores (Rasmann et al. 2005). There are a few examples of specific inter-Kingdom interactions that aid the plant with defense, scientists also have numerous examples of non-specific interactions that cause reduced pathogen pressure in crop fields; the phenomenon of disease-suppressive soils.

Disease suppressive soils are observed to inhibit pathogens across numerous years of monoculture (reviewed by Schlatter et al. 2017). There are two categories of suppressive soils: general and specific. General suppression occurs when an apparently diverse soil community causes a natural decline in the previously dominant pathogen population, and this is believed to be due to microbial competition. Specific suppression is believed to be due to antagonistic interactions between the pathogen and one or few other microbial species. The phenomenon of disease-suppressive soils has been observed in diverse crop systems such as take-all of wheat or barley, Phytophthora root rot of avocado, and crown gall of almonds; but the mechanisms behind suppressive soils are not yet understood (New and Kerr 1972, Baker and Cook 1974, Weller et al. 2002). There are limited examples where the suppressive-soil phenomenon is transferable. Transference is possible by inoculating the population of suppressive-soil to a conducive soil

(reviewed by Schlatter et al. 2017). It has been demonstrated that diverse microbial populations have suppressive qualities (Kinkel et al. 2011, Kinkel et. al 2012, Bakker et al. 2014). Additionally, complex plant communities, regardless of plant species, selected for soil microbes that were more effective at inhibiting *Fusarium* spp. (Essarioui et al. 2017). Microbial community analysis techniques are beginning to be applied in suppressive-soil systems to help elucidate the mechanisms – with the goal of transferring or inducing general suppression (Poudel et al. 2016). Researchers hope to analyze microbial community and big-data techniques to understand suppressive soils and begin to manipulate them for agricultural benefit. These techniques could also be applied to different populations of microbes, such as endophytes to protect against disease.

Endophytes Offer Plant Protection

The term endophyte is used to describe microorganisms that spend the majority or entirety of their life cycle living within a host plant. This classification is not taxon-specific, it is determined by organism lifestyle (Rodriguez et al. 2009). There is some debate about whether this classification is a valid lifestyle descriptor (e.g., saprotroph, necrotroph) or if it is better suited as a descriptor for a growth stage (van Overbeek and Saikkonen 2016). It has been proposed that endophytes may be latent pathogens or saprotrophs (van Kan et al. 2014), but it appears they instead are close relatives of pathogens (reviewed by Porras-Alfaro and Bayman 2010). Endophytic fungi have been documented to benefit their plant hosts in diverse conditions. There are several examples of endophytic fungi that do benefit the plant; such as providing heat, drought, or salt tolerance (Macia-Vicente et al. 2008, Rodriguez et al. 2008, reviewed by Rodriguez et al. 2009, Hubbard et al. 2012, Murphy et al. 2014, Mousa et al. 2016). They can improve salt and heat tolerance in wild grasses (Rodriguez et al. 2008). Epichloë endophytes prevent colonization of

smuts in grasses (Vignale et al. 2013). Endophytes can increase polyketide production of their plant hosts (Chagas et al. 2013). Seed-borne diseases decreased in barley when inoculated with endophytes (Murphy et al. 2014). In wheat, improved germination rates have been attributed to endophytes (Hubbard et al. 2012), as have protective effects against *Stagonospora* infection (Sieber et al. 1988). Recently, bacterial endophytes have shown disease and mycotoxin protective abilities in millet (Mousa et al. 2016).

There are numerous biocontrol products on the market that prevent pathogen colonization, for example, BASF manufactures a *Bacillus* sp. seed coating for cotton that has anti-fungal properties, Serifel® (BASF, Florham Park, NJ, USA) and a *Bacillus amyloliquefaciences* strain from Valent to control nematodes (Valent, Liberytville, IL, USA). There are also biologically-based products that protect plants against abiotic stress, including the Epivio™ line from Syngenta (Syngenta, Basel, Switzerland). All products from Indigo Agriculture (Boston, MA, USA), and the mycorrhizal inoculant MycoGold (MycoGold, Cincinnati, OH, USA) provide plant growth-promotion.

Biocontrol and plant-growth promoting products can have limited efficacy and may be effective for a limited range of pests or growing regions. When the mechanism of a biocontrol strain is known, it is usually only active for a narrow range of pathogens (reviewed by Fravel 2005). Evolutionary pressures or environmental factors can overcome the parasitic ability of a biocontrol strain. Implementing combinations of multiple biocontrol strains is sometimes more effective than inoculation with single strains (Xu et al. 2011). The first biocontrol strain, *Agrobacterium radiobacter* strain K84, was registered in the United States in 1979 for control of crown gall (Fravel 2005). Historically, identification and testing of biocontrol strains was time consuming. The current direction of the plant-microbiome field – use of high-throughput

technologies to analyze microbial communities and the interactions between community members, and the subsequent use of those data to identify important taxa in microbial networks – will likely provide more robust and sustainable control products (Poudel et al. 2016, Schlatter et al. 2017).

Superior biocontrol products can be developed if the deployment of the strains is conducted in a manner which reduces competition or environmental stress. Implementation of endophytic biocontrol strains which can be vertically transmitted to the next generation of seed would reduce stress on these microbes from environmental factors or competition from soil and rhizosphere microbes and may make for more robust products on the market. Vertical transmission has been demonstrated previously in wheat (Huang et al. 2016), and should improve product-viability, but was not investigated in the strains used in this work. Vertical transmission of endophytic bacteria has been demonstrated in maize, soybean, pepper, and wheat (Mitter et al. 2017). Also, investigations of endophyte pathogenicity on non-host crops to prevent interference with rotations or neighboring fields is required to ensure efficacy of such biologically-based treatments.

Previous Crop Microbiome Studies

Of the three crop species (wheat, maize, soybean) studied here, wheat may have the most well-studied microbial community in terms of diversity of plant compartments and microbial groups. Across these crops there has been a general bias toward studying prokaryotes and conducting community analysis with 16S-based methods (Table 1-1). Investigations of fungal and eukaryotic microbial communities were less common. There are examples of culture-based and non-sequence-based methods (such as DGGE) that investigated the eukaryotic communities of maize (Saravanakumar et al. 2017) and soybean (Hamid et al. 2017), but only a few high-throughput sequence-based studies analyzed the fungal communities associated with these crop

plants, and these studies only investigated the rhizosphere fungal communities (Table 1-1). In addition to the bias against fungal communities, there is also a bias against analysis of phyllosphere microbial communities. Poor recovery of relatively low-abundance microorganisms, such as on a leaf surface, may be partly the cause, especially when community analysis methods available before next-generation sequencing were used. To our knowledge, only one previous study investigated maize leaf communities using high-throughput amplicon sequencing (Johnston-Monje et al. 2016), others used T-RFLP or ARISA (Balint-Kurti et al. 2010, Nettles et al. 2016). Similarly in soybean, there were fewer studies of leaf-associated microbial communities of field plants using amplicon sequencing (Copeland et al. 2015) or other techniques (Nettles et al. 2016), when compared with the number of root or root-associated studies. Thanks to the deep sequence coverage available with current methods, it is possible to recover and identify previously low-abundance and unknown organisms. Culture-based methods and other historic techniques, have yielded a preliminary picture of microbial community composition on crop plants, with the increased amounts of data generated with next-generation sequence methods, we know that culture-based and other techniques have only captured a small glimpse of microbial communities. There is a large gap in our knowledge regarding the detailed composition of microbial communities associated with various organs of crop plants. Filling in these gaps will provide a valuable resource for microbiome engineering to improve agricultural outputs.

Despite the limitations of current and previous methods, and gaps in our understanding of community composition, we have a general understanding of community composition in specific scenarios. Previous studies have indicated that soil microbial communities differ when compared across management strategy (Berthrong et al. 2013, Xue et al. 2013, Hartmann et al. 2014) but the plant-associated or phyllosphere communities show less difference (Coleman-Derr et al. 2015,

Karlsson et al. 2017). The plant species in a rotation series appear to have an effect (Hartmann et al. 2014). Land-use history also influences the community, but with time, communities of restored landscapes become more similar to native communities (Jangid et al. 2011).

Table 1-1. Highlights of plant-microbiome studies of wheat, maize, or soybean conducted to date.

Crop	Organ/ Compartment	Management[a]	Rotation	Gene[b]	Technique[c]	Reference
wheat	soil	conventional	rice, wheat	16S	454	Zhao et al. 2016
wheat	soil	organic	rice, wheat	16S	454	Zhao et al. 2016
wheat	soil	organic (NOFERT)	yes	16S, ITS	454	Hartmann et al. 2014
wheat	soil	organic (BIODYN)	yes	16S, ITS	454	Hartmann et al. 2014
wheat	soil	organic (BIOORG)	yes	16S, ITS	454	Hartmann et al. 2014
wheat	soil	conventional (CONMIN)	yes	16S, ITS	454	Hartmann et al. 2014
wheat	soil	conventional (CONFYM)	yes	16S, ITS	454	Hartmann et al. 2014
wheat	soil	conventional	no	16S	454	Chávez-Romero et al. 2016
wheat	soil	reduced inputs	no	16S	454	Chávez-Romero et al. 2016
wheat	soil	rotation	wheat, maize	16S	454	Jiménez-Bueno et al. 2016
wheat	soil, rhizosphere	no-till	winter, spring wheat	16S	454	Yin et al. 2017
wheat	soil, rhizosphere	conventional	wheat, fallow	16S	454	Yin et al. 2017
wheat	soil, rhizosphere	greenhouse	no	16S	microarray	Bouffaud et al. 2014
wheat	soil, rhizosphere	no-till	no	16S	TRFLP, 454	Donn et al. 2014
wheat	soil, rhizosphere		rice, wheat		GeoChip	Li et al. 2012

Table 1-1 (cont'd)

wheat	soil, rhizosphere	greenhouse	no	16S	454	Ofek et al. 2013
wheat	soil, rhizosphere, root	organic	wheat, clover + forage	bacteria, fungi, oomycetes	culture	Lenc et al. 2014
wheat	soil, rhizosphere, root	integrated	potato, wheat, bean, wheat	bacteria, fungi, oomycetes	culture	Lenc et al. 2014
wheat	soil, rhizosphere, root	conventional	wheat, oil seed rape, wheat	bacteria, fungi, oomycetes	culture	Lenc et al. 2014
wheat	rhizosphere	growth chamber	no	18S	cloning	Smit et al. 1999
wheat	rhizosphere	conventional	no	16S	Illumina	Mahoney et al. 2017
wheat	rhizosphere, root	no-till	no	16S	454	Rascovan et al. 2016
wheat	rhizosphere, root	conventional	chickpea, pea, lentil, wheat	ITS, 18S	454	Borrell et al. 2017
wheat	root	conventional	no	ITS	cloning	Kwaśna et al. 2010
wheat	soil, rhizosphere, leaf, endospheres	greenhouse	wheat, bean	16S, ITS	Illumina	Granzow et al. 2017
wheat	soil, rhizosphere, leaf, endospheres	greenhouse	no	16S, ITS	Illumina	Granzow et al. 2017
wheat	soil, rhizosphere, root, leaf, endosphere	growth chamber	no	16S	454	Liu et al. 2017
wheat	leaf	organic, conventional	no	ITS	454	Karlsson et al. 2017
wheat	leaf	reduced inputs	no	ITS	454	Sapkota et al. 2017
wheat	seed	greenhouse	no	16S, ITS	Illumina	Huang et al. 2016

Table 1-1 (cont'd)

maize	soil	conventional	no		PFLA	Lazcano et al. 2013
maize	soil	integrated	no		PFLA	Lazcano et al. 2013
maize	soil	organic	no		PFLA	Lazcano et al. 2013
maize	soil	conventional	no	16S	Ion Torrent	Lupatini et al. 2017
maize	soil	organic	no	16S	Ion Torrent	Lupatini et al. 2017
maize	soil	no-till	no	metagenomic	454	Souza et al. 2013
maize	soil	conventional	no	metagenomic	454	Souza et al. 2013
maize	soil	rotation	yes	metagenomic	454	Souza et al. 2013
maize	soil	no-till	no	metagenomic	454	Souza et al. 2015
maize	soil	conventional	no	metagenomic	454	Souza et al. 2015
maize	soil	rotation	yes	metagenomic	454	Souza et al. 2015
maize	soil			16S	454	Duncan et al. 2016
maize	soil	rotation	wheat, maize	16S	454	Jiménez-Bueno et al. 2016
maize	soil, rhizosphere	greenhouse	no	16S	454	Ofek et al. 2013
maize	soil, rhizosphere	conventional	no	16S	454	Peiffer et al. 2013
maize	soil, rhizosphere	organic	no	16S	454	Peiffer et al. 2013

Table 1-1 (cont'd)

maize	soil, rhizosphere	greenhouse	no	16S	microarray	Bouffaud et al. 2014
maize	rhizosphere	conventional	no	16S, ITS	ARISA	Nettles et al. 2016
maize	rhizosphere	greenhouse	no	16S, ITS	454	Saravanakumar et al. 2017
maize	rhizosphere	greenhouse	no	16S	454	Zhu et al. 2016
maize	rhizosphere	greenhouse	no	16S	454	Zhu et al. 2016
maize	soil, rhizosphere, root	growth chamber	no	16S	Illumina	Niu et al. 2017
maize	root	conventional	no	18S	TRFLP	Gosling et al. 2013
maize	root, rhizosphere, leaf episphere	greenhouse	no	16S	Illumina	Johnston-Monje et al. 2016
maize	leaf endosphere	conventional	no	ITS	ARISA	Nettles et al. 2016
maize	leaf episphere		no	16S	TRFLP	Balint-Kurti et al. 2010
maize	leaf		no	16S	Illumina	Mashiane et al. 2017
maize	seed		no	16S	cloning	Liu et al. 2012
soybean	soil	no-till	no	16S	454, DGGE	Babujia et al. 2016
soybean	soil		no	ITS	Illumina	Han et al. 2017
soybean	soil		no	16S	Illumina	Hernandez et al. 2017
soybean	soil	reduced inputs	maize, soybean	fungi, nematodes	DGGE	Liu et al. 2015
soybean	soil, rhizosphere		no	16S	Illumina	Lu et al. 2017
soybean	soil, rhizosphere	greenhouse	no	metagenomic	454	Mendes et al. 2014

Table 1-1 (cont'd)

soybean	rhizosphere	conventional	no	16S, ITS	ARISA	Nettles et al. 2016
soybean	rhizosphere	greenhouse	no	16S, ITS	Illumina	Hamid et al. 2017
soybean	soil, rhizosphere, root	greenhouse	no	16S	Illumina	Xiao et al. 2017
soybean	soil, root	greenhouse	no	bacteria	TRFLP	Sun et al. 2017
soybean	rhizosphere, root	no-till	no	16S	454	Rascovan et al. 2016
soybean	root	conventional	no	18S	TRFLP	Gosling et al. 2013
soybean	root	various	yes	oomycetes (ITS)	Illumina	Rojas et al. 2017
soybean	soil, leaf		no	16S	Illumina	Copeland et al. 2015
soybean	leaf endosphere	conventional	no	ITS	ARISA	Nettles et al. 2016

[a]blank cells due to missing information in publications.
[b]16S: prokaryotic marker gene, ITS: fungal marker gene, 18S: eukaryotic marker gene, blank cells did not use DNA or sequence-based technique.
[c]454: 454-Roche pyrosequencing, Illumina: Illumina MiSeq.

Microbes in Agriculture

Microorganisms, including archaea, bacteria, fungi, protists, and others, are ubiquitous in natural, agricultural, and built environments. Microbes are responsible for nutrient cycling, carbon and nitrogen cycling, organic matter degradation, and other ecological processes. Agriculturally relevant microbes are often pathogens such as *F. graminearum*, but microbes can also play a beneficial role in plant health and crop productivity. Microorganisms can interact with their plant hosts in numerous ways, ranging from beneficial or commensal to pathogenic or parasitic. For example, rhizobia and mycorrhizal fungi exchange nutrients such as nitrogen, carbon, and phosphorus, in addition to micronutrients during their symbioses (Bhuvaneswari et al. 1980, van der Heijden et al. 1998, Smith and Read 2008, Lira et al. 2015). Commercial strains of rhizobia and mycorrhizal fungi are available for purchase as growth-promoting supplements on crops, for example rhizobia-coated seeds sold by Smith Seed Services (Halsey, OR, USA), and Mykos spores from mycorrhizal fungi sold by Xtreme Gardening (Gilroy, CA, USA). There are also a range of neutral plant-microbe interactions, endophytic fungi grow asymptomatically between cells of a plant, and do not always provide a clear benefit to the plant hosts. There are beneficial or protective endophytes, as discussed above. Growing concerns about sustainability of modern agricultural systems has driven biocontrol research for both growth-promoting and pathogen control applications. Biocontrol products could fill a void in current control methods for certain pathogens, such as *F. graminearum*, for which no effective chemically based control is available.

Fusarium graminearum Head Blight of Wheat

F. graminearum is a filamentous fungal pathogen of cereal crops. Head Blight of wheat, also known as Head Scab, was first reported in the United States in 1891 (Arthur 1891). Fungi

have historically been given unique Latin binomials for each phase of growth, the anamorphic (asexual) and the telomorphic (sexual) growth stages. Use of the telomorphic name, *Gibberella zeae*, was recently dropped during the 'one fungus, one name' initiative (Taylor 2011, Wingfield et al. 2012). Head Blight of wheat is actually a disease complex and be caused by simultaneous or independent infections of *F. graminearum* and several other *Fusarium* spp. (*Fusarium culmorum, Fusarium avenacum*) or *Microdochium* species, but in the United States, this disease is most commonly caused by *F. graminearum* (Ioos et al. 2005, Xu et al. 2005, Siou et al. 2015). Previously, distinct populations of *F. graminearum* were believed to cause root and head infections. But due to genetic evidence, the *F. graminearum* clade was split into two species, *F. graminearum* and *Fusarium pseudograminearum* (Aoki and O'Donnell 1999). *F. pseudograminearum* is soil-borne, causing root and crown infections primarily in Australia (Aoki and O'Donnell 1999, Burgess et al. 2001). *F. graminearum* can cause seedling damping-off in fields with high inoculum levels and is the primary pathogen responsible for crown infections in the United States (Sutton 1982, Wiese 1987).

Fusarium Head Blight is an economically devastating disease, causing an estimated $2-3 billion yield loss in the last several decades (McMullen et al. 1997, 2012). Although Head Blight can have such a severe impact, there is not one completely efficient control strategy. Head Blight is currently controlled with an integrated approach, there are no strongly resistant plants available, and fungicides are only moderately effective (Wegulo et al. 2015). Resistance mechanisms include prevention of initial infection, or prevention of spread throughout florets (Schroeder and Christensen 1963, Cook 1981, Mesterhazy 1995). Fungicides are usually sprayed at flowering to coincide with the time of *F. graminearum* ascospore release. Plants infected with *F. graminearum*

may still able to complete their life cycle, but the seed can be shriveled and depleted and may not be fit for food or feed due to mycotoxin contamination.

F. graminearum produces an arsenal of mycotoxins, which are small molecules detrimental to health. Deoxynivalenol (DON), the primary mycotoxin produced by *F. graminearum* causes gastrointestinal distress (reviewed by Sobrova et al. 2010). Zearalenone, another mycotoxin, binds to the mammalian estrogen receptor and can interfere with animal development (Miksicek 1994, reviewed by Desjardins and Proctor 2007). Aurofusarin is a red-pigmented secondary metabolite produced by *F. graminearum* that has been linked to problems with egg development in the poultry industry and has weak antimicrobial activity (Mendentsev et al. 1993, Dvorska et al. 2001). Contamination of malting barley with *F. graminearum* interferes with malting process used during beer production. Hydrophobins from the fungus cause a chemical reaction colloquially termed "gushing" due to the explosive effect it can generate when beer is released from a container (Schwarz 2017).

Conclusions

Traditional microbiology and ecology have provided scientists with insight into the structure of microbial communities across various natural habitats. With the development of culture-independent microbial ecology techniques, scientists are able to understand the dynamics of microbial communities with ever-increasing detail. Although our understanding of microbial communities of natural habitats is continually improving, there are some gaps in our knowledge that the work presented in this dissertation aims to fill: the structure of fungal and bacterial communities across multiple plant organs and how it varies across growth stages, and land management strategies (Chapter 2-3). One of the intended applications of crop microbial

community analysis is the manipulation of these communities to reduce plant disease or increase crop productivity. I describe use of my findings from the wheat microbiome to initiate control of Fusarium Head Blight with beneficial endophyte strains (Chapter 2, Appendix A). I have initiated a functional analysis of *F. graminearum* terpene synthase genes to provide insight into the function of terpenes as signals for community manipulation and self-defense molecules for this fungal pathogen (Appendix B). Community structure is affected by various genetic and environmental factors, and understanding these factors is necessary for targeted manipulation of communities to reduce plant pathogen presence.

CHAPTER 2

THE WHEAT MICROBIOME UNDER FOUR MANAGEMENT STRATEGIES, AND POTENTIAL FOR ENDOPHYTES IN DISEASE PROTECTION

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 fungal internal transcribed spacer and bacterial 16S rRNA gene amplicon sequencing. The most prevalent operational taxonomic units were shared across all samples, although levels of the low-abundance operational taxonomic units 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.

Source

For a full text of this work go to: Gdanetz K, Trail F. 2017. The wheat microbiome under four management strategies, and potential for endophytes in disease protection. Phytobiomes. 1:158-168. https://apsjournals.apsnet.org/doi/full/10.1094/PBIOMES-05-17-0023-R

CHAPTER 3

HUB TAXA AND CORE MICROBIAL COMMUNITIES OF ROTATION CROPS UNDER COMMON LAND MANAGEMENT STRATEGIES

Abstract

Manipulation of naturally occurring microbial communities to reduce plant diseases or increase crop yields requires a thorough understanding of interactions within the phytobiome. This paper presents the first in-depth comparative analysis of fungal and bacterial microbial communities in these economically important row crops. Plants were sampled from a long-term wheat-maize-soybean crop rotation research site under four different land management strategies. The fungal and bacterial communities of leaves, stems, and roots of all crops throughout the growing season were analyzed using fungal internal transcribed spacer and bacterial 16S rRNA gene amplicon sequencing. Our analysis showed that microbial communities differed across growth stages and plant organs of each host; this effect was most pronounced in the bacterial communities of wheat and maize aboveground organs. Roots consistently had the most unique bacterial OTUs. Fungal OTU composition across leaves and stems of wheat and soybean were similar, but the most notable changes in fungal OTU composition of the microbial communities were between roots of maize and soybean. Network analysis identified important members of the microbial communities of the three crops. Understanding the microbial community structure across various organs of crop plants and identifying important taxa will provide a critical resource for researchers for future experimental manipulations with plant-associated communities.

Introduction

Microorganisms are ubiquitous in natural environments, including nearly all plant organs and tissues. These microbes have diverse functions, for example, rhizobia and mycorrhizae form symbioses with roots and provide nutritional advantages to their plant hosts (Bhuvaneswari et al., 1980, ven der Heijden et al. 1998). Phyllosphere microbes can fix carbon (Abanda-Nkpatt et al. 2006, Vorholt 2012) and stimulate plant defenses (Vogel et al. 2016). Plant pathogens are another guild of microorganisms that infect plant organs; root pathogens cause rots, foliar pathogens infect and destroy healthy leaf tissues, and many pathogens can infect the developing seed. The emergence of the plant-microbiome and phytobiome fields of study are causing plant pathologists to take a more integrated approach toward researching plant diseases; for example, biotic and abiotic factors are considered together, and microbial pathogens are viewed as part of a community instead of in isolation (Vorholt 2012, Mueller and Sachs 2015, Berg et al. 2016, Ellis 2017, Leach et al. 2017). Part of this research initiative requires identification of plant-associated microbial communities, or microbiomes. There is growing interest in the plant pathology field to treat plant diseases via manipulation of whole microbial communities. Before experimental manipulation of communities can occur, the core communities, microbes shared across habitats or niches, on various crop species or various plant organs must be identified, and better understood in terms of functional interactions between the host plant and the microbiome. The three most commonly grown crops in the United States are maize, soybean, and wheat with approximately 88, 83, and 47 million respective acres planted (USDA 2016). This paper presents the first in-depth comparative analysis of fungal and bacterial microbial communities in these economically important row crops.

The Michigan State University W. K. Kellogg Biological Station (KBS) Long-Term Ecological Research (LTER) site has been managed under the current strategies and crop rotations for more than 25 years (Robertson 2015). It is an ideal research site to test effects of management strategies on microbial communities since the different strategies are laid out in a replicated and randomized field. The wheat community data used here (Gdanetz and Trail 2017) have been previously investigated. In this study, we analyzed the fungal and bacterial microbial communities, with amplicon sequencing of maize and soybean, grown under four common management strategies. We also analyzed the changes in microbial communities at three growth stages for each crop, over the course of one three-year rotation cycle.

The KBS-LTER site hosts a multi-year three-crop rotation series of wheat, maize, and soybean. Wheat is planted prior to maize, to avoid high disease pressure on the wheat crop since maize is also a host for wheat head blight pathogen, *Fusarium graminearum*, and the maize crop residue remains in the soil for two years, hosting pathogen propagules. Soybean follows maize, providing an opportunity for the rhizobia to replenish nitrogen in the soil. Prior to use as a research field site, the area was farmed privately. The crop rotation site is divided into plots containing the rotation series under four common crop management strategies; conventional, no-till, low inputs, and organic (Robertson, 2015). The KBS-LTER site provides a nearly ideal site to study the effects of agriculture on crop-associated microbial communities because of the long-term treatments and random replicate plot design. The goal of this multi-year study was to establish a detailed profile of a healthy microbial community on economically important crops.

Materials and Methods

Site Description and Sample Collection

The study site is located at the Kellogg Biological Station Long-Term Ecological Research (KBS LTER) site in Hickory Corners, Michigan, USA (42.411085, -85.377078). Annual precipitation for this site is 1,005 mm, with about half falling as snow (NCDC 1980-2010 climate normals for the Gull Lake Biological Station, https://lter.kbs.msu.edu/research/site-description-and-maps/general-description/climate-normals/). The annual precipitation during the study was 1177, 932, and 1153 mm, during wheat, maize, and soybean growing years, respectively. The KBS-LTER crop rotation site has been planted in the wheat-maize-soybean rotation series since 1993, and is organized in randomized, replicated plots under four land management strategies with six replicate plots for each strategy: T1 = conventional till, T2 = no-till, T3 = reduced chemical inputs with alfalfa cover crop, T4 = organic with alfalfa cover crop (Figure 3-1). Detailed management information can be found at https://lter.kbs.msu.edu/research/. Wheat seeds of the same cultivar, Pioneer 25R39 Soft Red Winter Wheat (Pioneer High-Bred International, Inc., Johnston, IA, USA), were planted in all plots, but wheat seeds for plots T1-T3 were coated with Gaucho fungicides (Bayer Crop Science, Research Triangle Park, NC, USA). A commercial maize hybrid cultivar, Dekalb DKC52-59 Corn Hybrid (Monsanto Company, St. Louis, MO, USA), was planted in plots T1-T3, and an organic-approved cultivar, Blue River Hybrids 25M75 Organic Corn (Blue River Organic Seed, Ames, IA, USA), was planted in T4 plots. A commercial soybean cultivar, Pioneer P22T69R (Roundup Ready®) Soybean Seed (Pioneer High-Bred International, Inc., Johnston, IA, USA), was planted in T1-T3 plots, and Viking Organic Soybean Seed, Variety 0.2265 (Albert Lea Seed, Albert Lea, MN, USA), was planted in T4 plots. Plants were collected at three analogous developmental stages for each crop: late vegetative growth, flowering, and early

seed/ear/pod development (Table 3-1, Figure 3-2). At each developmental stage, three intact plants were removed from each of the 24 plots. Fine and thick roots, and above-ground tissues from each plant were placed in separate sterile sample collections bags (Nasco Whirl-Pak®, Fort Atkinson, WI, USA) and maintained on ice during transport. Roots were rinsed to remove loosely attached soil, and plants were stored at -80°C, then lyophilized. Lyophilized plant tissue was stored at room temperature under a desiccant.

Sample Processing

Lyophilized plant tissue was ground with a Retsch Oscillating Mill M400 (Verder Scientific, Newtown, PA, USA) and DNA was extracted using the Mag-Bind® Plant DNA Plus Kit (Omega Bio-tek, Norcross, GA, USA) following the manufacturer's protocols with the KingFisher™ Flex (ThermoFisher Scientific, Waltham, MA, USA). Fungal ITS2 and bacterial 16S rRNA gene libraries were generated as described previously and sequenced using Illumina MiSeq 2x250 bp chemistry (Table 3-2; Gdanetz and Trail 2017). Maize microbiome sequences are available with the National Center for Biotechnology Information (NCBI) Small Reach Archive project accession numbers for wheat, maize and soybean are SRP102192, SRP102245, and SRP120500, respectively.

Sequence Processing and Community Analysis

Forward and reverse read pairs from each library were merged with USEARCH (version v8.1.1861; Edgar 2010, Edgar et al. 2011, Edgar and Flyvbjerg 2015). Low quality reads, (fastq expected error set to 1.0), and read pairs without mates were discarded (read length of 250 bp for 16S and 380 bp for ITS sequences). The 16S and ITS2 libraries from all three crops were concatenated into one large dataset for each barcode region. Sequences were processed as described previously (Gdanetz and Trail 2017). Briefly, the USEARCH pipeline was implemented

for chimera filtering and OTU assignment, with the cluster threshold set to 97% similarity. The Ribosomal Database Project Classifier was used for taxonomic assignment, with the 16S and the UNITE ITS training sets for bacteria and fungi, respectively, customized to include the plant hosts (Wang et al. 2007, Cole et al. 2013, Deshpande et al. 2016). OTUs matching plants, mitochondria, chloroplasts, or unidentified at Kingdom level were discarded. Samples were filtered to include OTUs that occurred in at least five samples and this trimmed dataset was used for all downstream analyses.

Venn diagrams and tables for core taxa analysis were generated using the *gplots* package (Warnes et al. 2016). Before generating barplots, taxa were merged at Class level. Taxa that were present in less than 3% of the samples were removed. Relative abundances of taxa were calculated as a percentage of total sequences in each sample. Alpha diversity statistics (abundance transformation, observed OTUs, and Shannon's Index) were calculated with the *Phyloseq* package (McMurdie and Holmes 2013) in the R statistical computing environment (version 3.3.3; R Core Team 2016). Non-rarefied data were used to calculate the Shannon Diversity Index (*H'*), an alpha diversity metric that measures species diversity within a sample (McMurdie and Holmes 2014), and analysis of variance (ANOVA) and Tukey's honest significant difference test (HSD) were used to determine significance. Non-metric multi-dimensional scaling (NMDS) ordination analysis was conducted using Bray-Curtis distance values, a measure of species diversity between communities. Permutational analysis of variance (PERMANOVA) tests were completed using the *adonis* function in the *vegan* R package (Oksanen et al. 2016). Heterogeneity of the variances was calculated using the *betadispersion* function of the *vegan* package. Figures were generated with the *ggplot2* package (Wickham 2009). Microbial hubs, species that influence the presence of others in an environment, were identified as highly connected nodes, with ten or more connections.

Fungal and bacterial OTU tables were concatenated before calculating the co-occurrence network using the *SpiecEasi* package in R (Kurtz et al. 2015). Network statistics and network plots were generated using Cytoscape (version 3.5.1, http://www.cytoscape.org).

Table 3-1. Collection dates of plant growth stages.

Crop	Stage Name[a]	Age	Management[b]	Collection Date
Wheat	30	Vegetative (C1)	T1-T4	1-May-2013
Wheat	45	Boot/flowering (C2)	T1-T4	30-May-2013
Wheat	83	Early seed development (C3)	T1-T4	5-July-2013
Maize	V7	Vegetative (C1)	T1, T2	24-Jun-2014
			T3	01-Jul-2014
			T4	09-Jul-2014
Maize	VT	Flowering (C2)	T1, T2	16-Jul-2014
			T3	23-Jul-2014
			T4	06-Aug-2014
Maize	R1	Early ear development (C3)	T1, T2	20-Jul-2014
			T3	06-Aug-2014
			T4	19-Aug-2014
Soybean	V2	Vegetative (C1)	T1-T3	19-Jun-2015
			T4	29-Jun-2015
Soybean	R1	Flowering (C2)	T1-T3	15-Jul-2015
			T4	24-Jul-2015
Soybean	R6	Early pod development (C3)	T1-T4	01-Sep-2015

[a]Wheat growth stages were rated on Zadoks scale (Zadoks et al. 1947).
[b]T1: conventional, T2: no-till, T3: reduced inputs, T4: organic.

Table 3-2. Primer sequences of microbiome target loci.

Primer	Sequence	References
515f	5' GTG CCA GCM GCC GCG GTAA 3'	16S v4 PCR primers from (Kozich et al. 2013).
806r	5' TAA TCT WTG GGV HCA TCA GG 3'	
ITS3_KYO4	5' GAT GAA GAA CGY AGY RAA 3'	ITS2 PCR primers from (Toju et al. 2012).
ITS4_KYO3	5' CTB TTV CCK CTT CAC TCG 3'	
ITS1F	5' CTT GGT CAT TTA GAG GAA GTA A 3'	Full length ITS primers (White et al. 1990, Bruns & Gardes 1993).
ITS4	5' TCC TCC GCT TAT TGA TAT GC 3'	

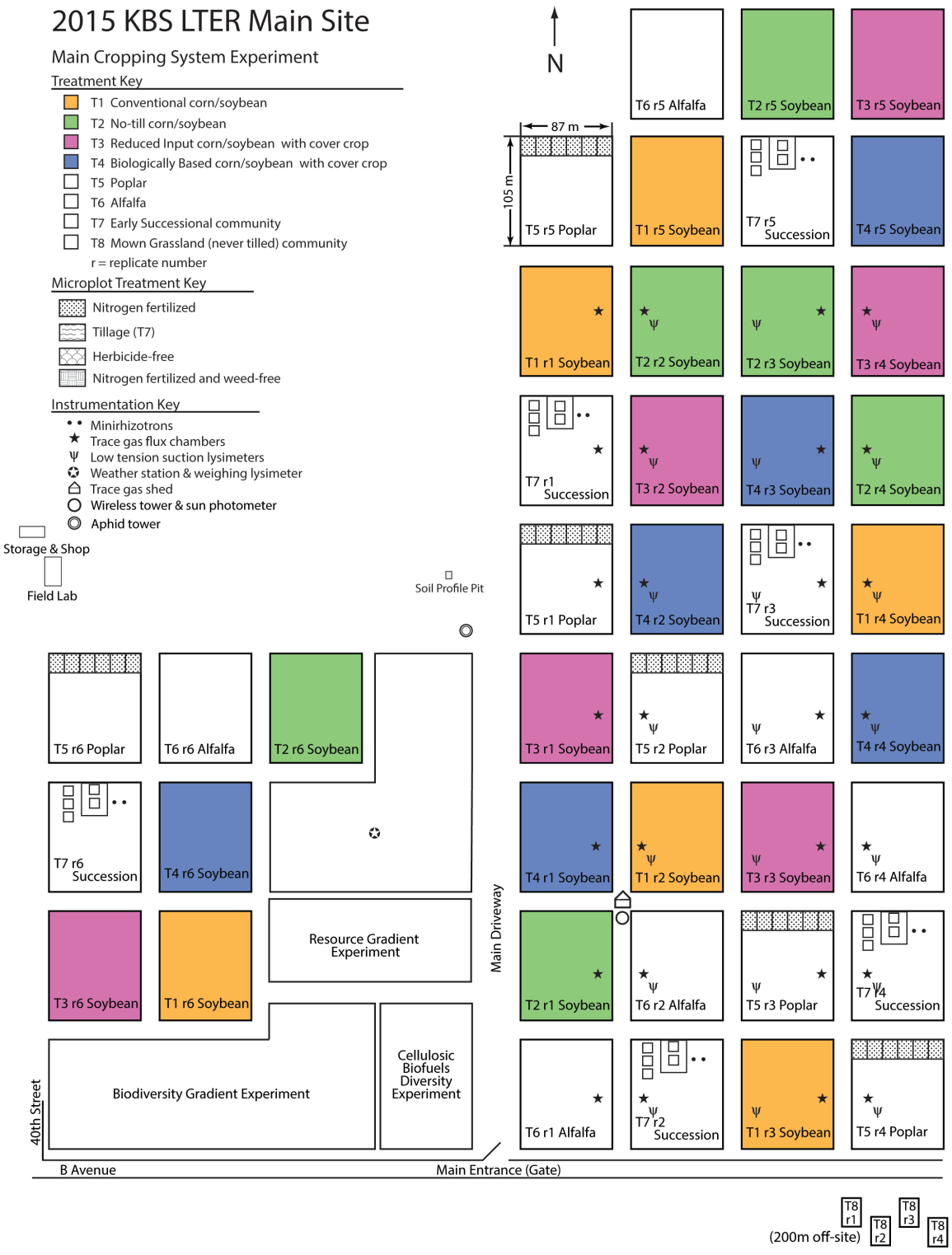

Figure 3-1. Layout of management strategy replicates at Michigan State University KBS LTER field site. Colored plots are planted in a three-year wheat-maize-soybean rotation cycle. Modified from http://lter.kbs.msu.edu/research/annual-plot-maps/.

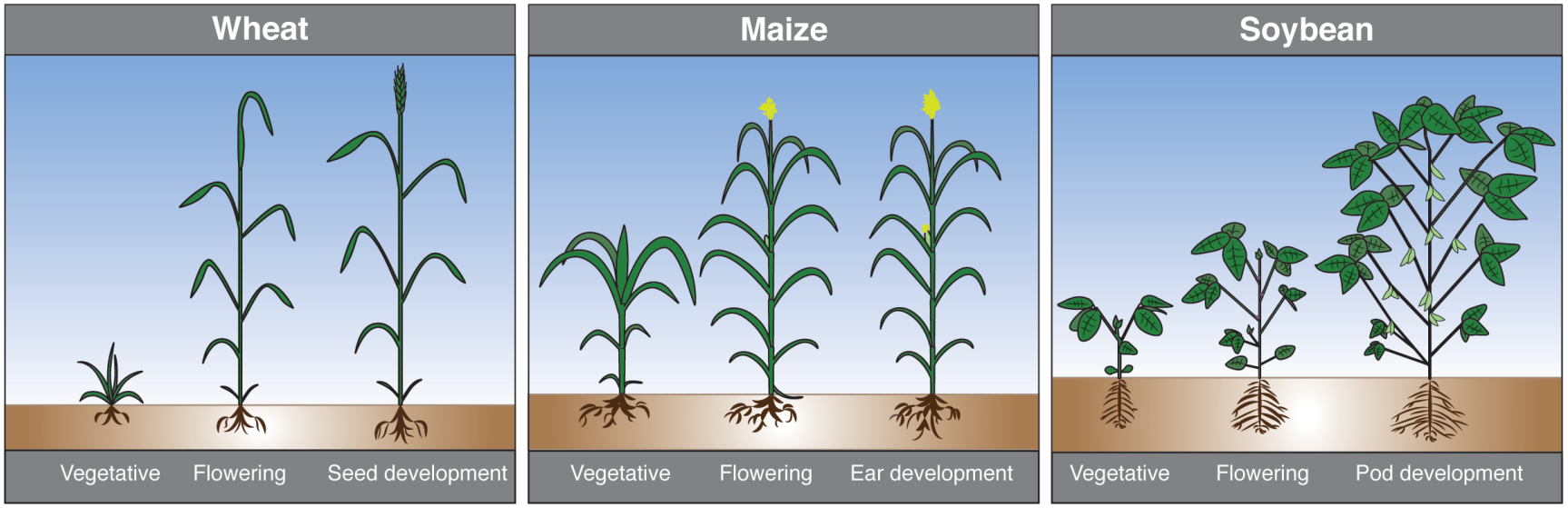

Figure 3-2. Overview of plants and growth stages. For plant collection dates and formal growth stage names see Table 3-1.

Results

Observed and Core Taxa

We analyzed bacterial and fungal communities of maize and soybean grown under four different management strategies over a two-year period. After filtering and quality control, there were 14,395,302 and 27,341,137 high quality reads for fungi and bacteria, respectively, with means of 21,245 fungal and 42,166 bacterial reads per sample. No fungal sequences from soybean T4 stems and roots passed quality control. 7,728 fungal and 13,770 bacterial OTUs were identified from the combined data of all crops (Table 3-3). A total of 4,739 fungal OTUs and 8,942 bacterial OTUs were identified after trimming and filtering. Of the predicted bacterial and fungal OTUs, fewer OTUs were unique to soybean plants (Figure 3-3). Roots consistently had the most unique bacterial OTUs, with higher numbers of bacterial OTUs unique to roots than shared among organs in maize and soybean (Figure 3-4).

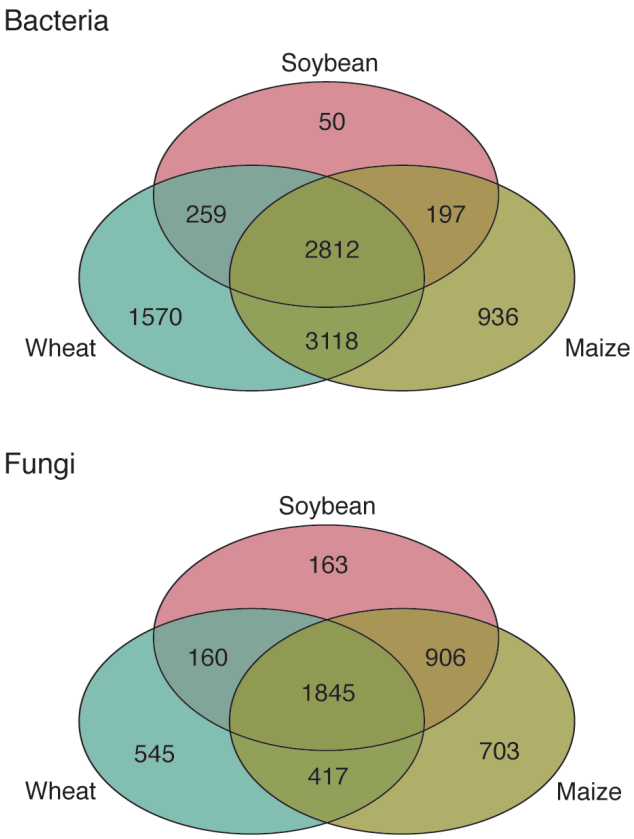

Figure 3-3. Shared and unique bacterial or fungal operational taxonomic units among all crops.

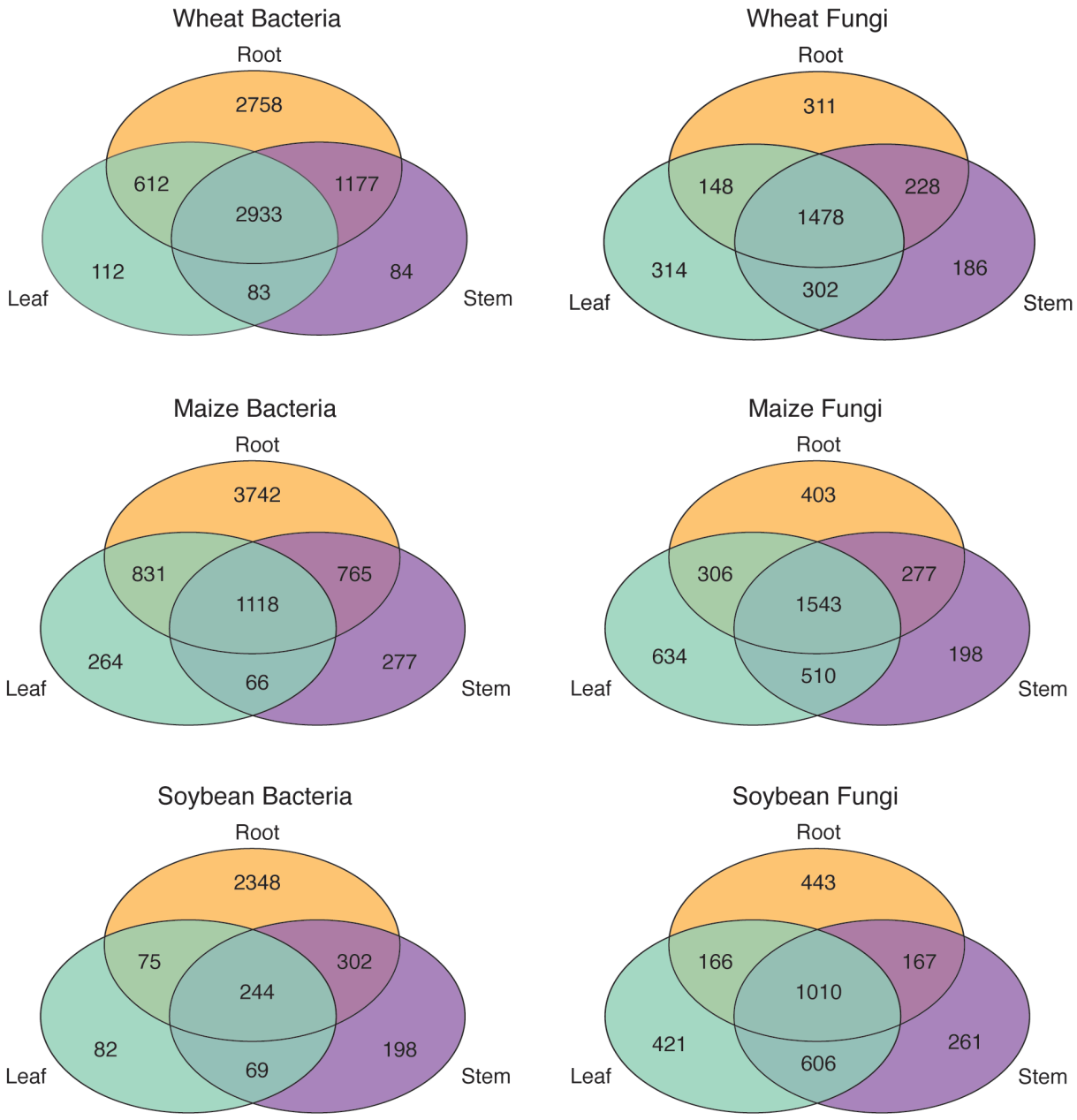

Figure 3-4. Shared and unique operational taxonomic units across plant organs.

Table 3-3. Sequence processing statistics and OTU assignment statistics.

Step in Pipeline	ITS library	Wheat	Maize	Soybean	16S library	Wheat	Maize	Soybean
Merged read pairs	14,395,302	4,538,082	6,998,733	2,760,569	27,341,137	14,047,252	8,746,925	4,546,960
Reads after dereplication	3,794,174	1,451,825	1,268,377	1,055,034	1,801,827	1,067,099	462,196	272,532
Total OTUs	7,728				13,770			
OTUs after chimera removal	7,574				13,387			

Table 3-4. Samples without high-quality reads after sequence processing.

Sequence Library	Wheat ITS	Maize ITS	Soybean ITS	Wheat 16S
Samples with zero high-quality reads[a]	C1,T2,R6,L	C1,T4,R3,S	C3,T3,R6,R	C3,T4,R6,R
	C1,T2,R6,R	C2,T3,R2,S	C3,T4,R1,R	C3,T4,R6,S
		C2,T3,R4,S	C3,T4,R1,S	
			C3,T4,R2,R	
			C3,T4,R2,S	
			C3,T4,R3,R	
			C3,T4,R3,S	
			C3,T4,R4,R	
			C3,T4,R4,S	
			C3,T4,R5,R	
			C3,T4,R5,S	
			C3,T4,R6,R	
			C3,T4,R6,S	

[a]C-number indicates growth stage, T-number indicates treatment, R-number indicates replicate plot; L, S, R indicate leaf, stem, or root samples. Growth stage descriptions can be found in Table 3-1.

Within a crop species, OTUs were generally conserved across growth stages and organs. Highly-abundant bacterial OTUs, belonging to Bacilli, Alphaproteobacteria, and Gammaproteobacteria, were generally shared across all crops (Figure 3-5). The phyllosphere organs, leaves and stems, of each crop had similar bacterial taxa at the Class level when compared with that crop's roots (Figure 3-5, 3-6). Maize roots contained the highest number of unique bacterial taxa compared to other maize organs and other crops (Figure 3-4). OTUs found in maize leaf and stem samples were consistent across all management types at the oldest growth stage (Figure 3-7). Soybean root samples were dominated by Alphaproteobacteria. Bacterial communities also changed with growth stage; this effect was most pronounced in wheat and maize aboveground organs (Figure 3-5, 3-7). Both soybean leaf and stem samples had many unique taxa at the youngest and oldest growth stages (Figure 3-8). Although bacterial communities were similar across all management strategies within a crop at the Class level (Figure 3-6), we observed differences among crops and organs (Figure 3-5). OTUs found in maize stems were more consistent between no-till and low input samples. Sphingobacteria were not abundant under any managements, except maize organic (Figure 3-7). Unique taxa (Actinobacteria, Flavobacteriia, Opitutae) were observed in no-till and low input soybean samples at the vegetative stage (Figure 3-8).

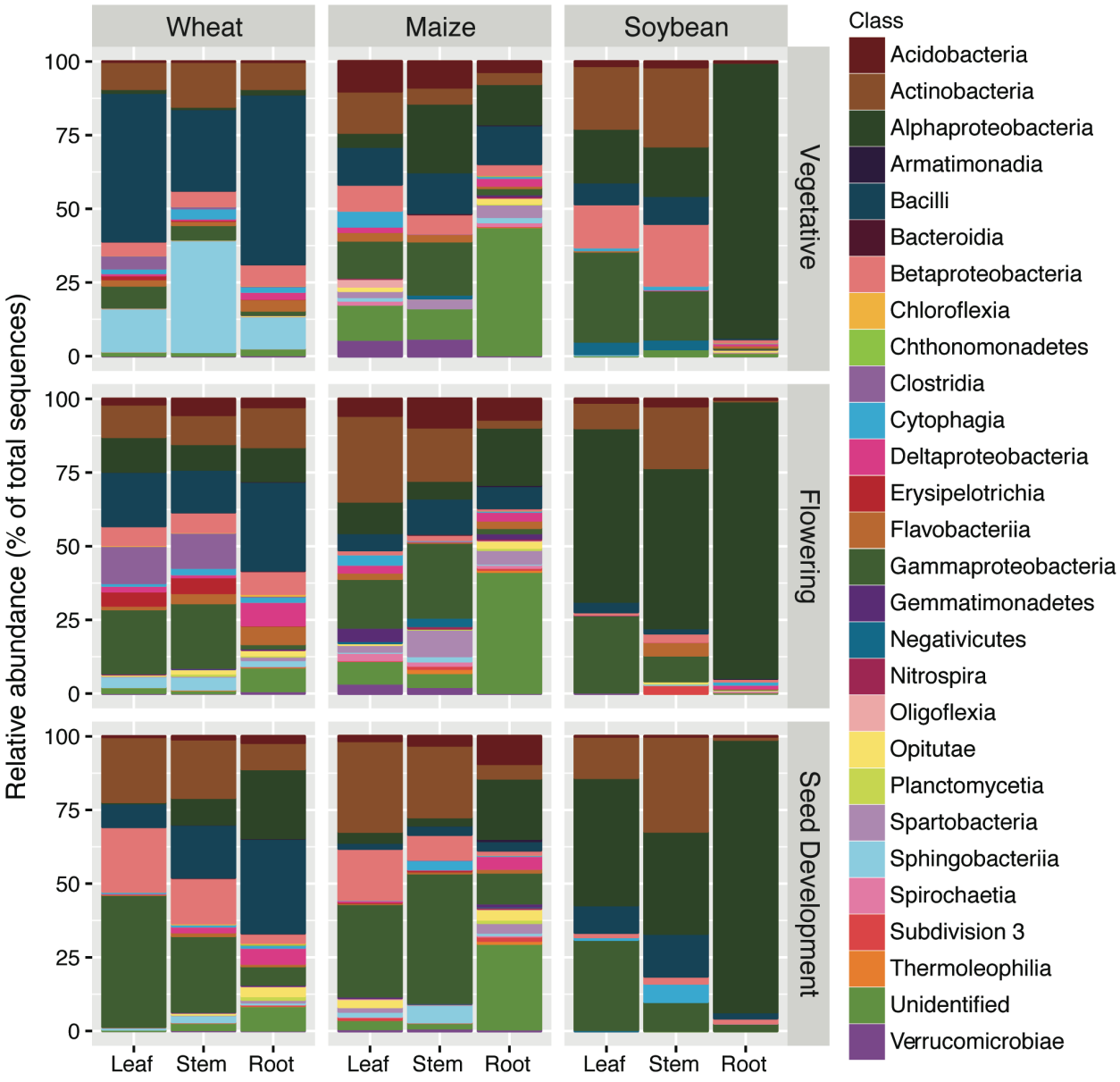

Figure 3-5. Class-level relative abundance of operational taxonomic units in bacterial communities across crop, organ, and growth stage.

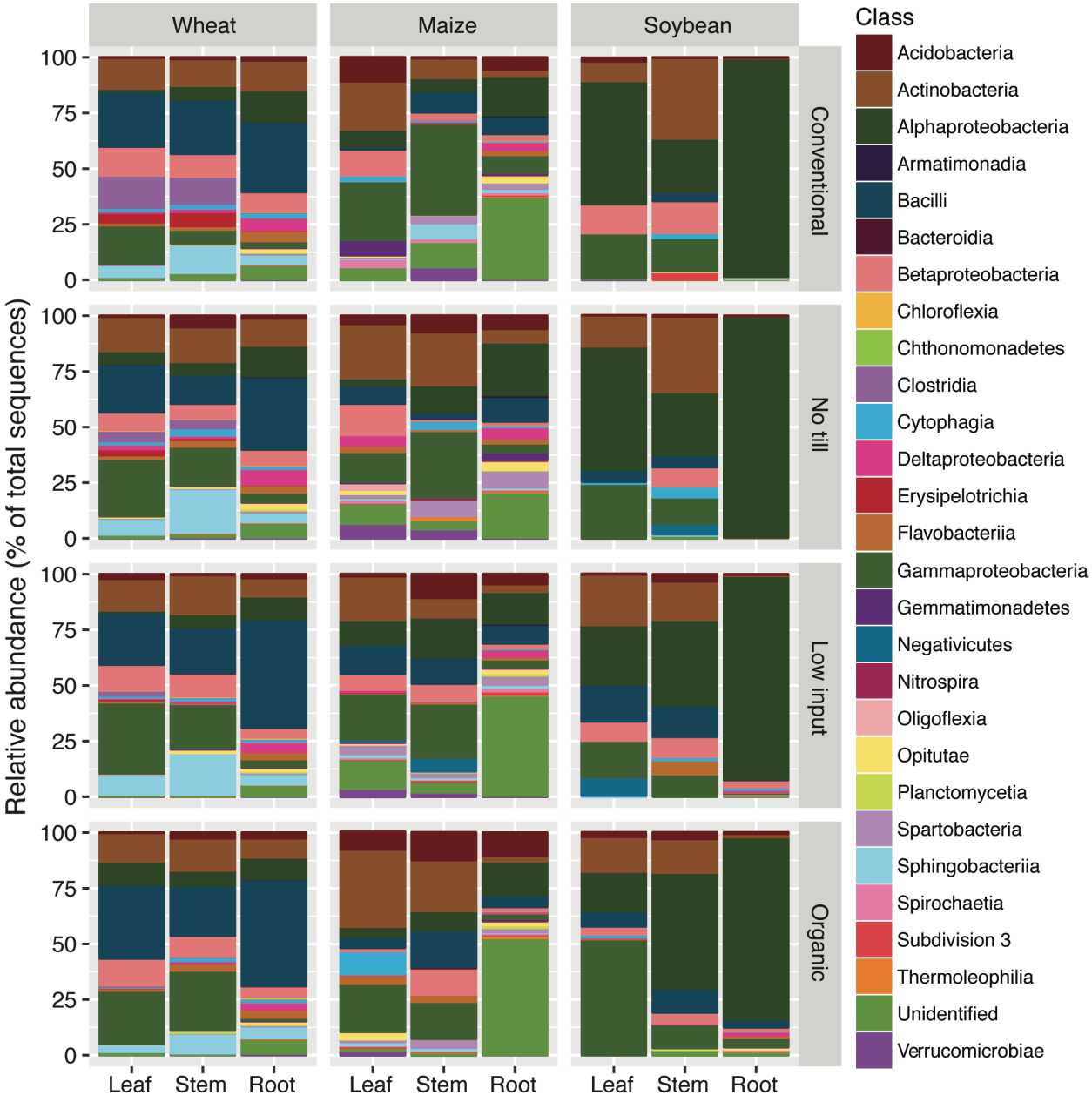

Figure 3-6. Class-level relative abundance of operational taxonomic units in bacterial communities across crop, organ, and management strategies.

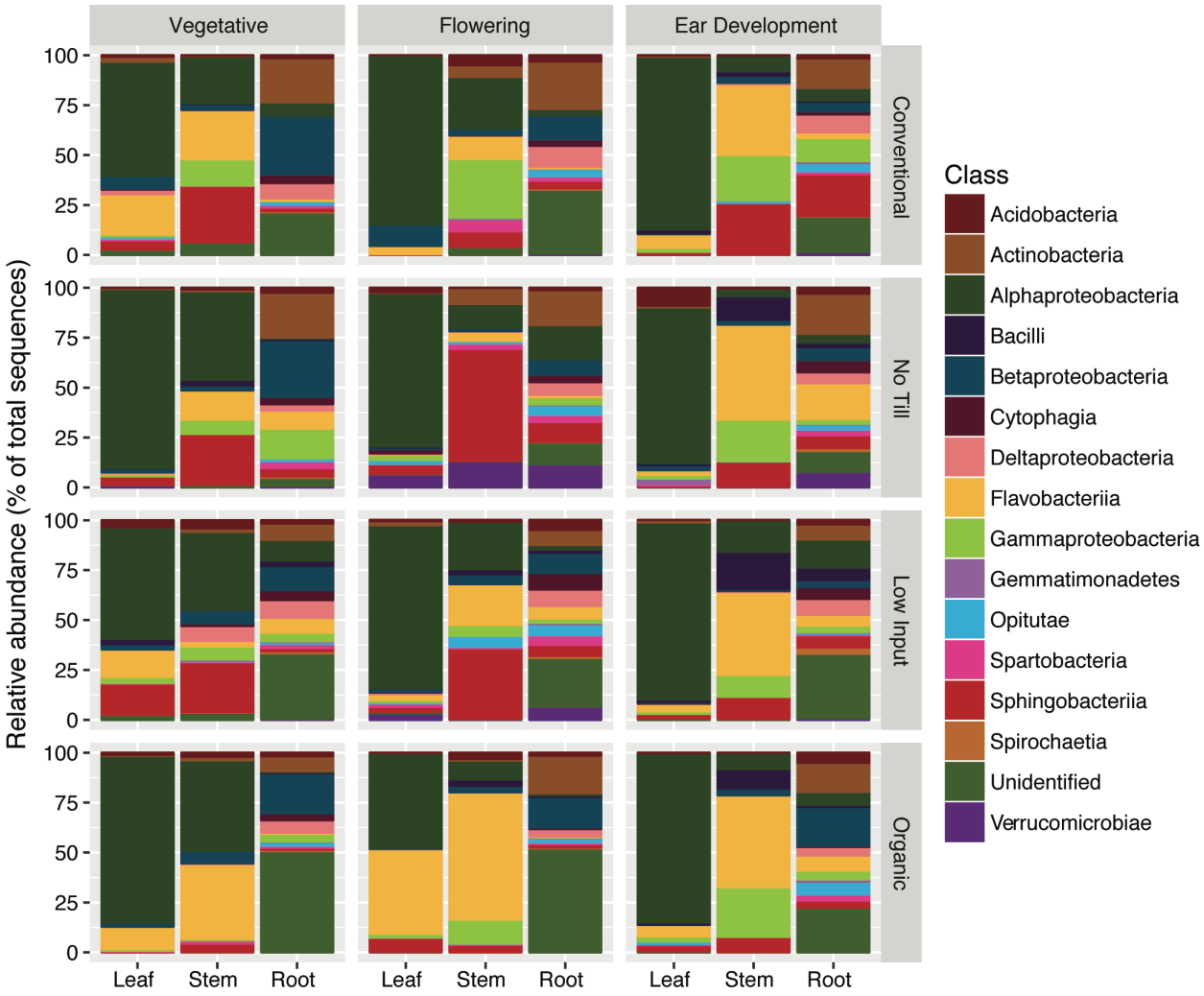

Figure 3-7. Class-level relative abundance of operational taxonomic units in bacterial communities across maize growth stage, organ, and management strategies.

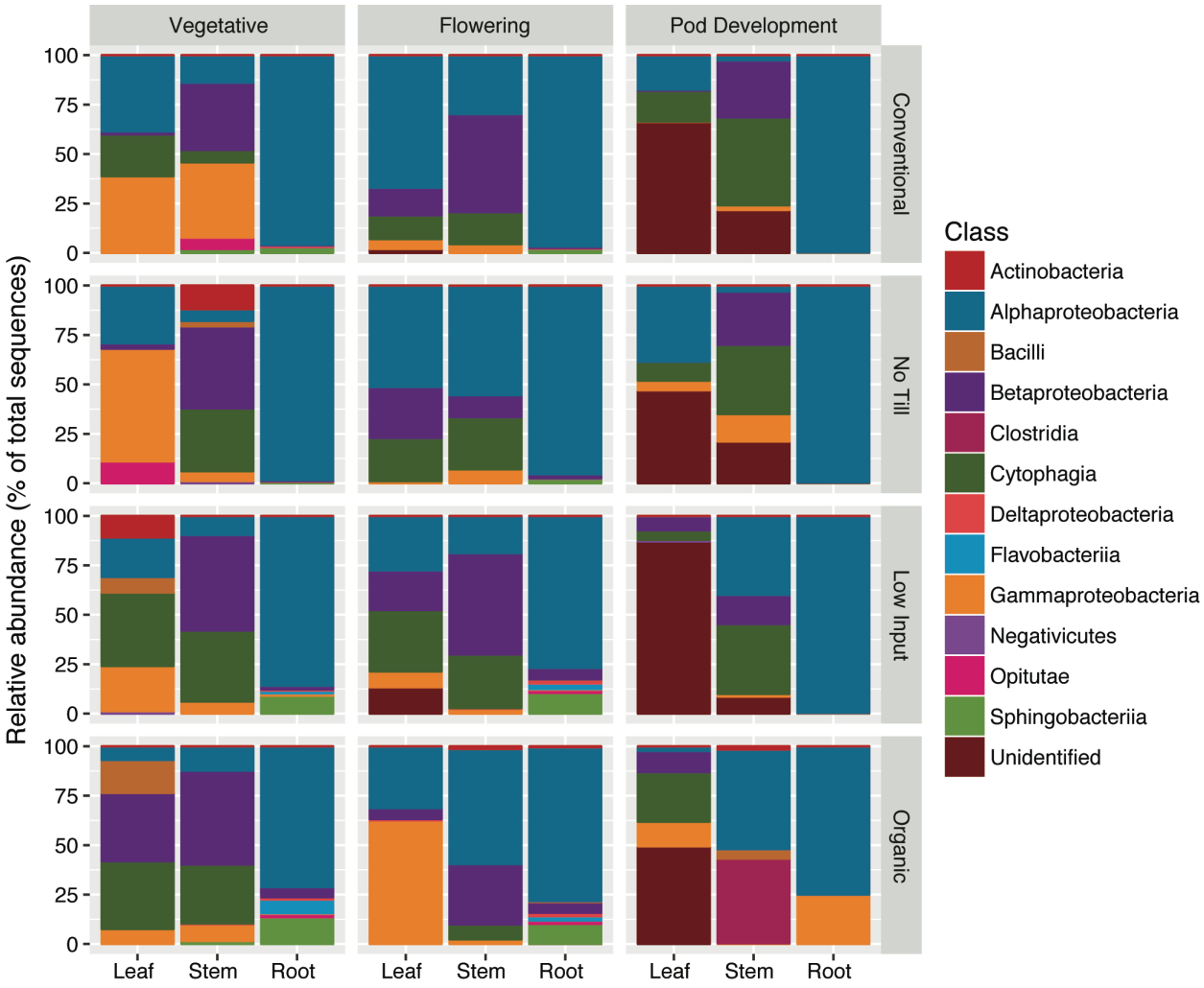

Figure 3-8. Class-level relative abundance of operational taxonomic units in bacterial communities across soybean growth stage, organ, and management strategies.

Fungal OTUs were more uniformly represented across crops than bacteria. The Dothideomycetes and Sordariomycetes were the most abundant fungal classes observed (Figure 3-9). Fungal OTU composition of leaves and stems of wheat and soybean were similar, but the most notable changes in fungal OTU composition of the microbial communities were between roots of maize and soybean (Figure 3-9). Root samples of all crops had a higher abundance of Agaricomycetes than phyllosphere organs (Figure 3-9, 3-10). Tremellomycetes were observed in phyllosphere organs on maize, but in no other samples (Figure 3-9, 3-11). Differences in some of the low-abundance taxa were observed across all growth stages (Figure 3-9). Fungal community members changed across growth stages on maize (Figure 3-11). Taxa more common and abundant in soybean, specifically Glomeromycetes and Orbiliomycetes, were observed only at vegetative and flowering growth stages in soybean roots (Figure 3-9, 3-12). As observed for bacteria, fungal communities were similar across all management strategies within each crop (Figure 3-10, 3-11, 3-12).

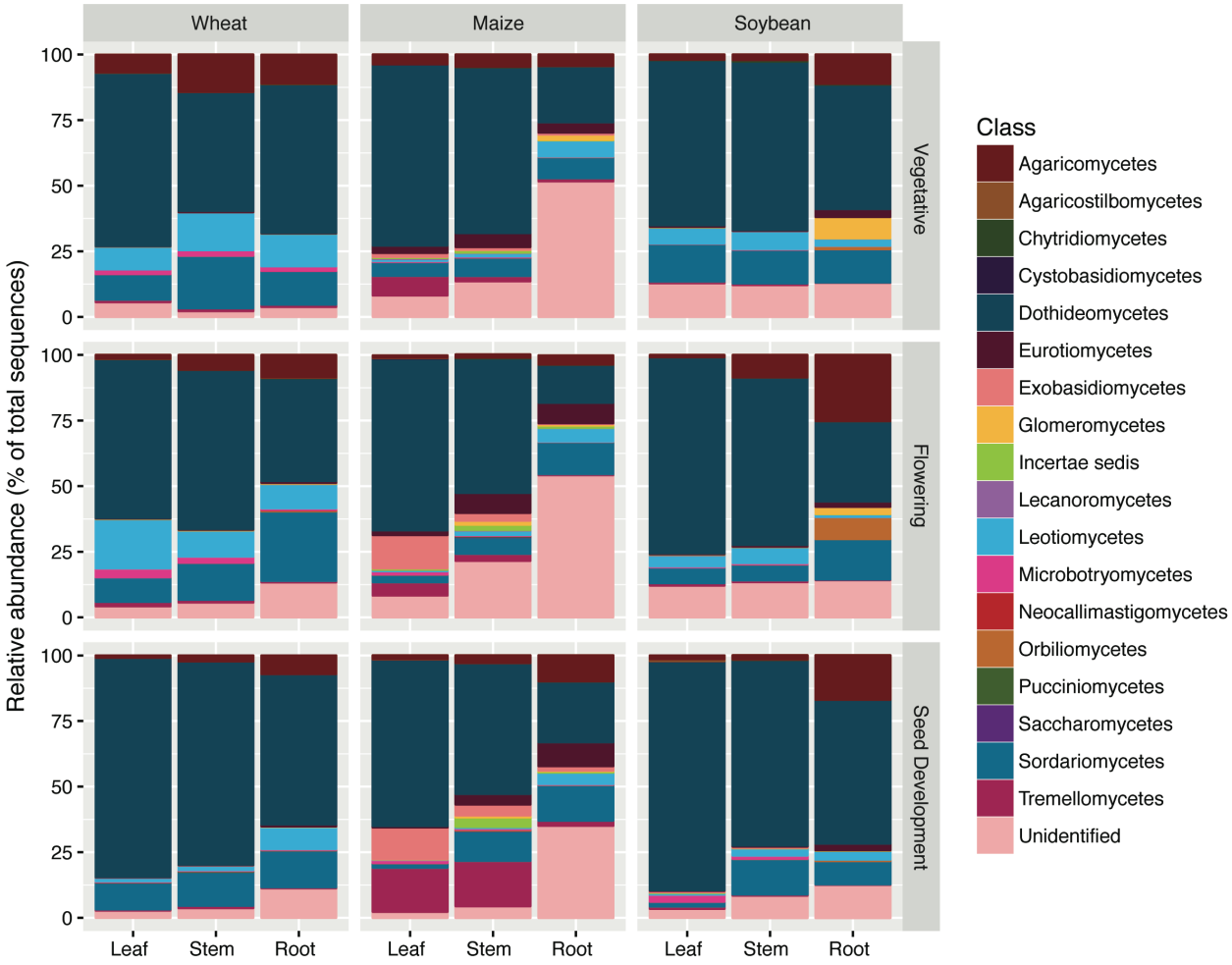

Figure 3-9. Class-level relative abundance of operational taxonomic units in fungal communities across crop, organ, and growth stage.

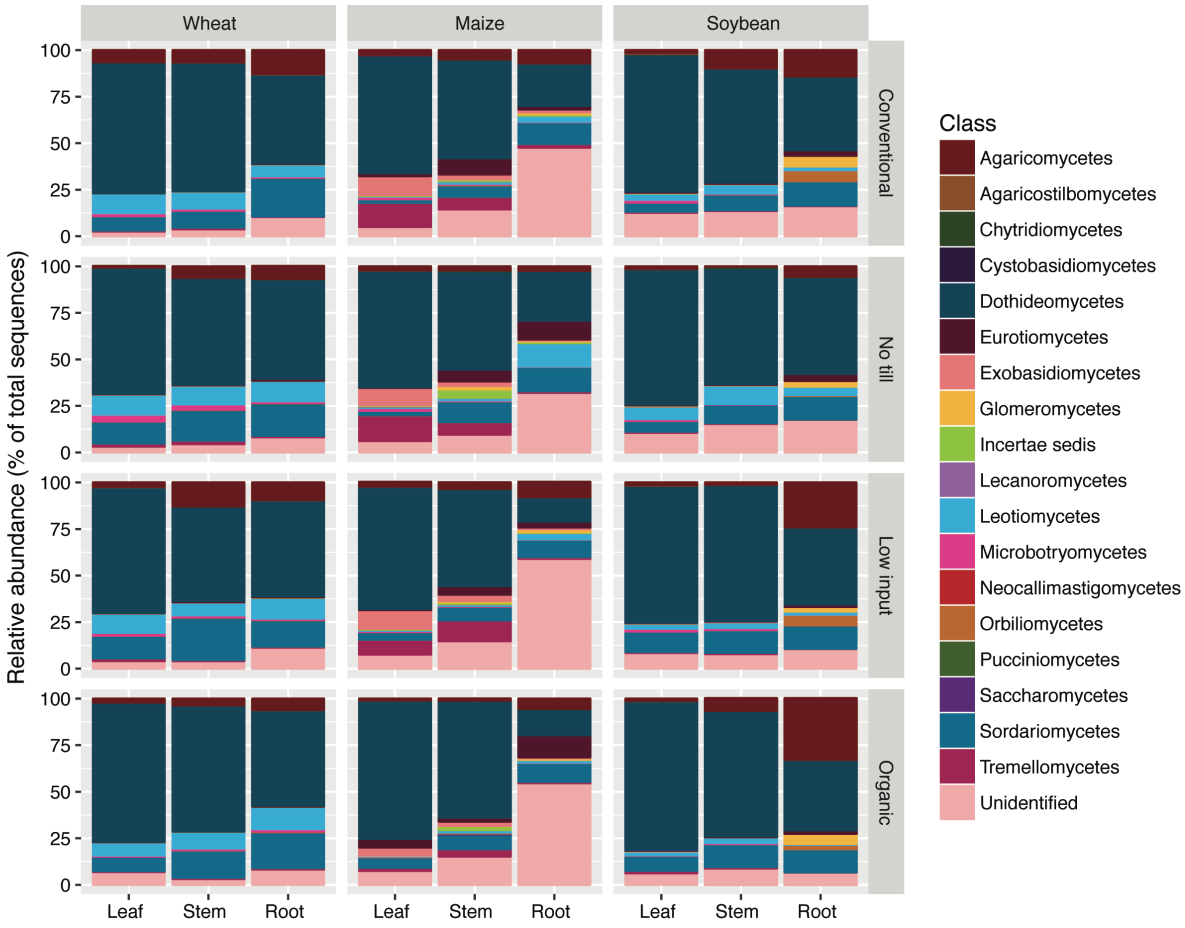

Figure 3-10. Class-level relative abundance of operational taxonomic units in fungal communities across crop, organ, and management strategies.

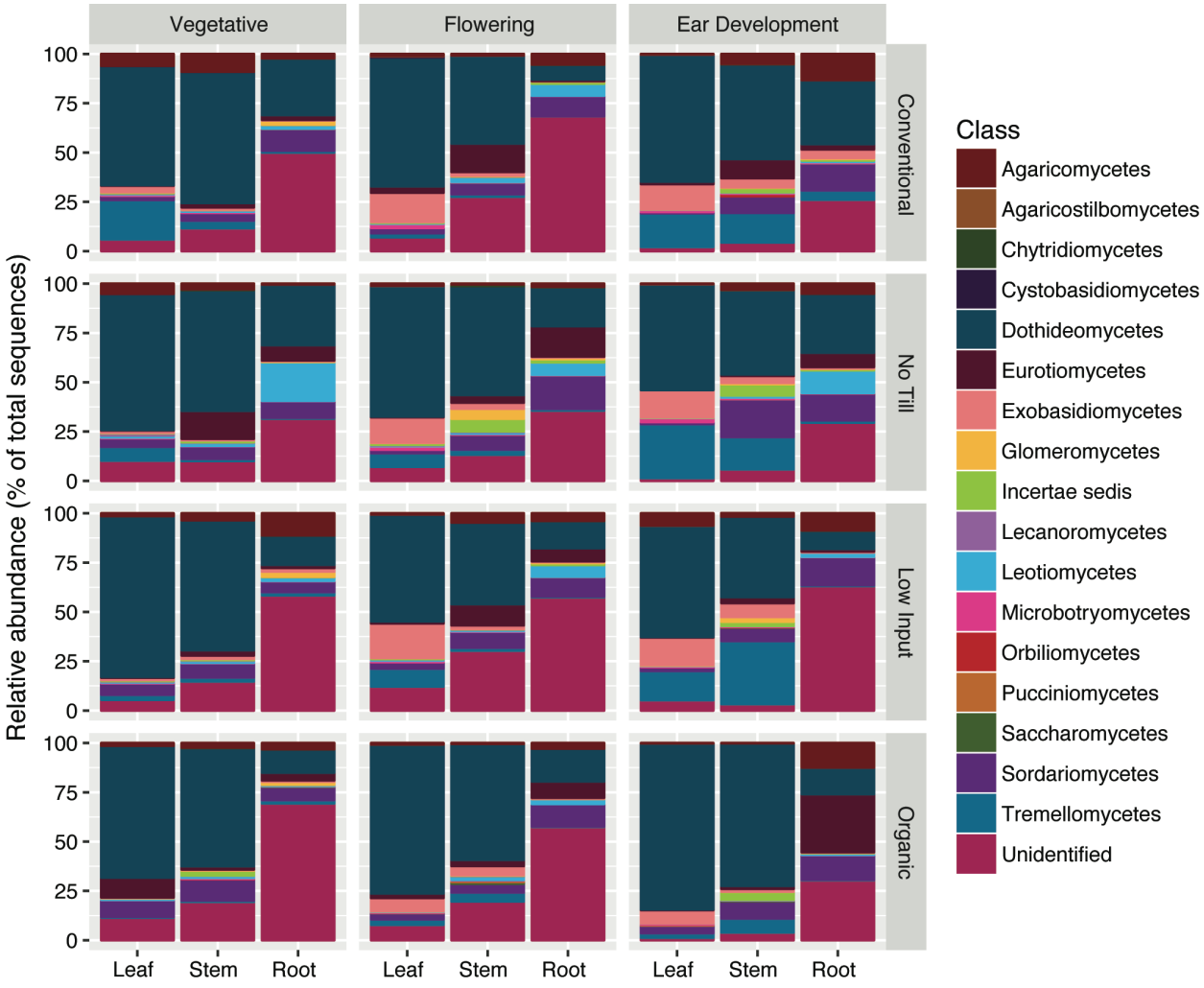

Figure 3-11. Class-level relative abundance of operational taxonomic units in fungal communities across maize growth stage, organ, and management strategies.

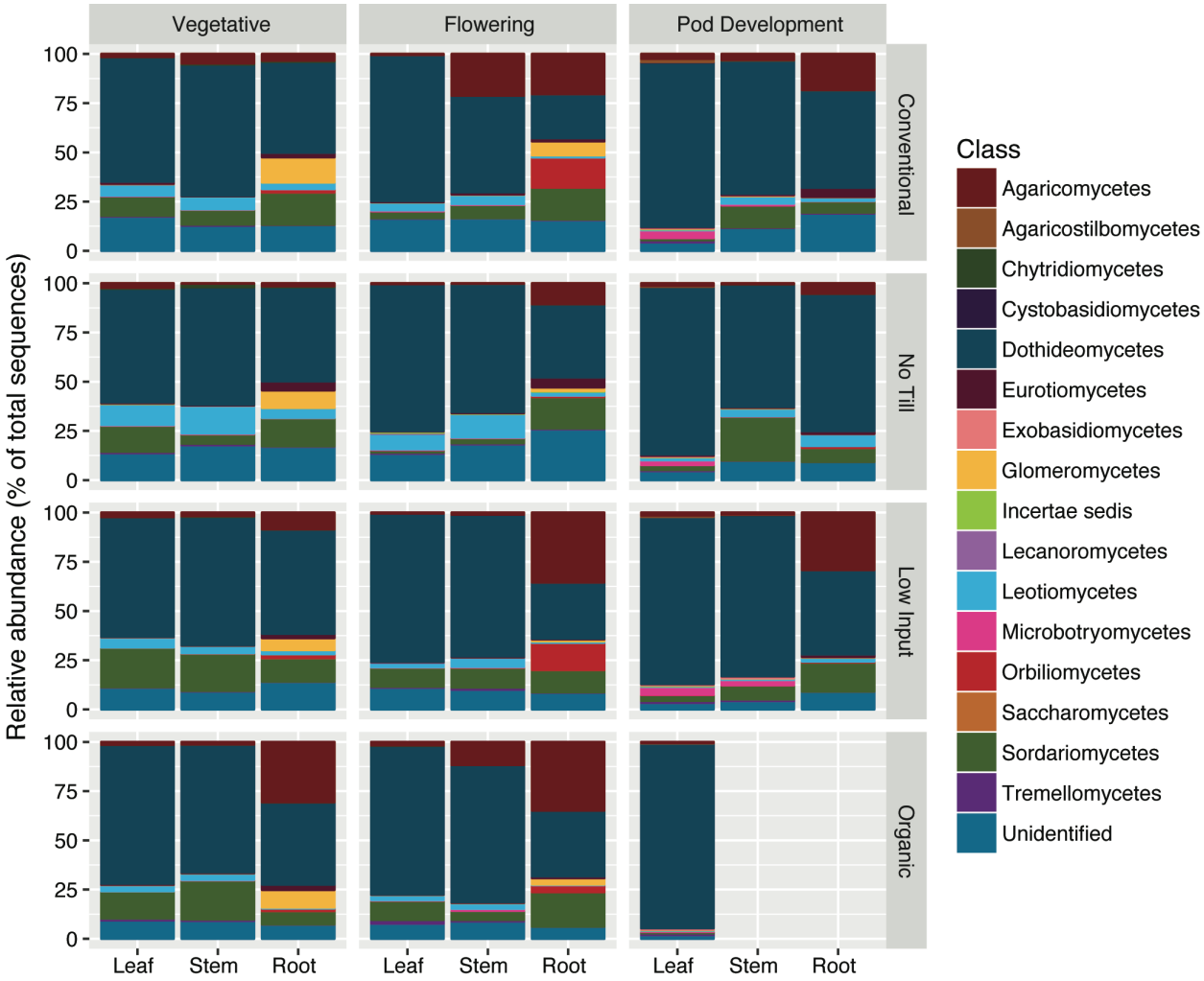

Figure 3-12. Class-level relative abundance of operational taxonomic units in fungal communities across soybean growth stage, organ, and management strategies.

Community Diversity

Bacterial *H*' of roots was generally significantly higher than *H'* of leaves and stems, but there was not a significant difference in *H'* across organs of fungal communities (Figure 3-13, Table 3-5). The mean fungal diversity of maize roots was lower than aboveground organs at the vegetative stage but was significantly lower only when compared with leaves from conventional plots. (Figure 3-14A, Table 3-5). Whereas fungal diversity in soybean roots decreased as plants aged, this trend was only significant when compared to leaves from low input plots during the flowering stage (Figure 3-14B, Table 3-5). Bacterial *H'* of maize roots was higher than aboveground organs at all growth stages and under all management strategies, this was a significant increase except in T1 leaves and T1-T3 stems at the vegetative stage (Figure 3-14C, Table 3-5). The bacterial diversity of soybean roots was more variable than maize, but also decreased across the growing season (Figure 3-14D). Soybean leaves and stems during pod development from all management strategies had significantly higher *H'* compared to roots, except stems from T4 (Table 3-5).

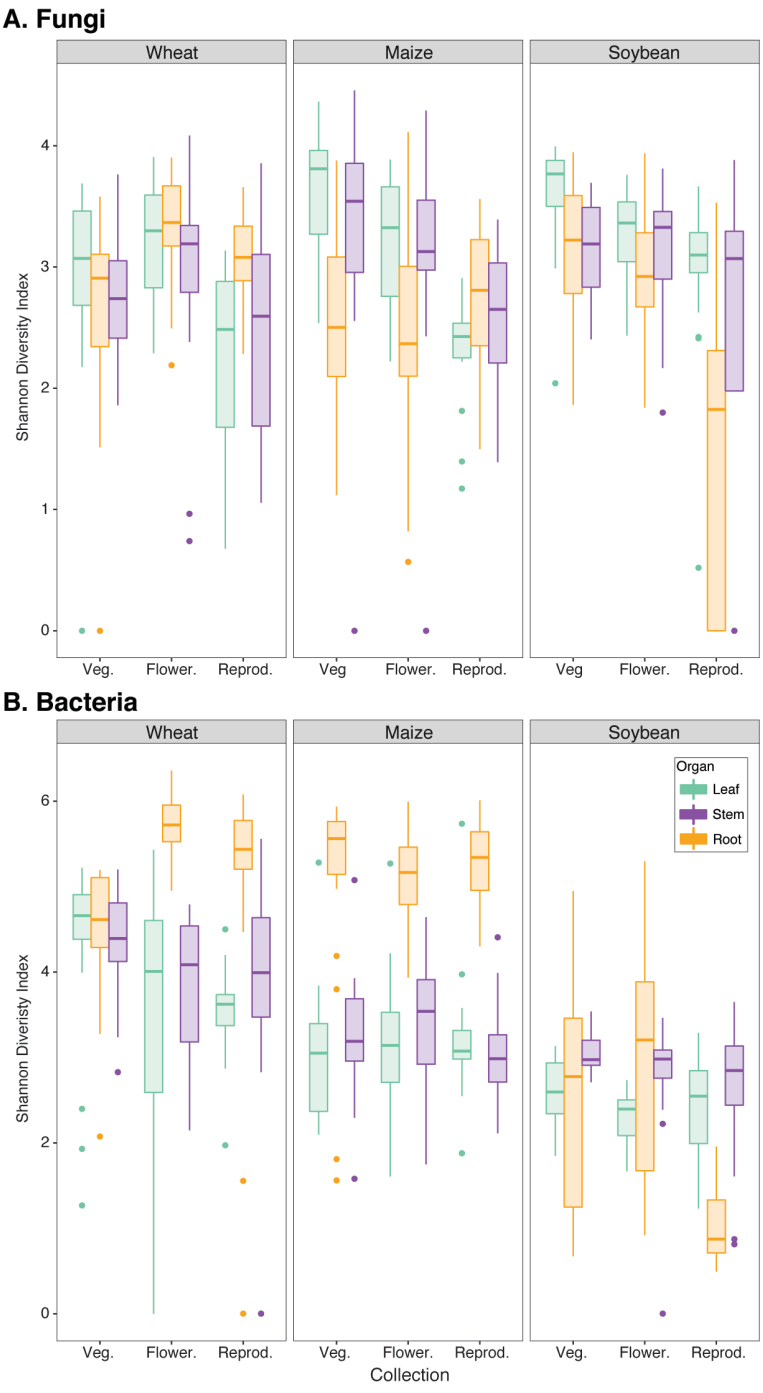

Figure 3-13. Global alpha diversity patterns calculated by Shannon Diversity Index (H'). All treatments and replicates were pooled within growth stages for (A) fungi and (B) bacteria. Data are represented by 24 replicates from each crop-growth stage-organ combination. Center line of boxes represents 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. Analysis of variance and Tukey's honest significant difference were used to test significance ($P < 0.05$). Statistical support is detailed in Table 3-5.

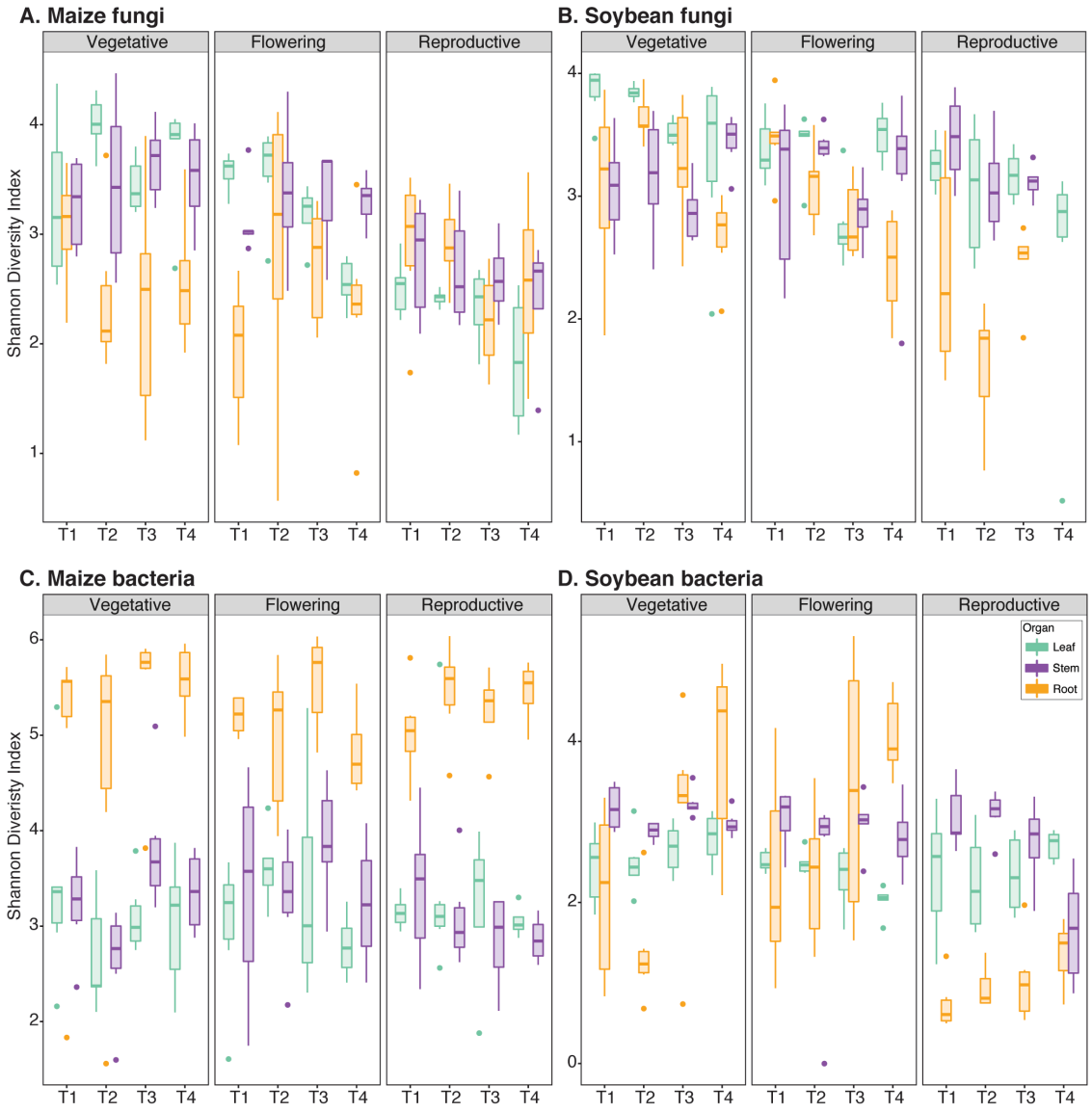

Figure 3-14. Alpha diversity maize fungi (A), soybean fungi (B), maize bacteria (C), soybean bacteria (D) found on different plant organs across growth stages, and under four different management strategies, estimated by Shannon Diversity Index. T1=conventional, T2=no till, T3=low input, T4=organic. Reproductive growth stage indicates early ear development, and early pod fill for maize and soybean, respectively. 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. Analysis of variance and Tukey's honest significant difference were used to test significance ($P < 0.05$). Statistical support is detailed in Table 3-5.

Table 3-5. Mean estimated Shannon diversity ± standard deviation. Superscripts indicate significant differences within a growth stage and management style, after Tukey's HSD.

		Maize							
		Fungi				Bacteria			
Sample[c]		T1	T2	T3	T4	T1	T2	T3	T4
	L	1.86±6.17[a]	2.45±0.35	2.06±0.22	2.42±0.34	3.42±1.03	2.66±0.58[a]	3.09±0.37[a]	3.01±0.66[a]
V.	S	1.99±0.32	2.20±0.66	2.44±0.16	2.62±0.49	3.21±0.52	2.58±0.57	3.80±0.69	3.34±0.42[a]
	R	3.10±0.30	2.30±1.06	2.96±0.48	3.18±0.27	4.88±1.52	4.64±1.61	5.46±0.82	5.55±0.37
	L	2.38±0.30	2.27±0.26	2.22±0.31	1.47±0.22	2.97±0.73[a]	3.59±0.38[a]	3.41±1.19[a]	2.79±0.31[a]
F.	S	2.15±0.33	2.34±0.52	2.24±0.50	2.12±0.20	3.38±1.13[a]	3.84±1.03[a]	3.87±0.65[a]	4.33±0.59[a]
	R	2.49±0.64	2.44±0.73	2.68±0.24	2.33±0.54	5.20±0.21	4.95±0.79	5.54±0.50	4.80±0.42
	L	1.66±0.14	1.59±0.04	1.57±0.28	0.95±0.36	3.14±0.17[a]	3.44±1.15[a]	3.20±0.92[a]	3.05±0.18[a]
S.	S	1.89±0.47	1.76±0.34	1.69±0.36	1.59±0.57	3.36±0.75[a]	3.08±0.50[a]	2.83±0.54[a]	2.86±0.26[a]
	R	2.37±0.28	2.44±0.45	2.28±0.38	1.79±1.16	5.02±0.49	5.44±0.50	5.24±0.48	5.44±0.35
		Soybean							
		Fungi				Bacteria			
		T1	T2	T3	T4	T1	T2	T3	T4
	L	2.57±0.24	2.72±0.07	2.18±0.12	2.17±0.64	2.44±0.46	2.48±0.36	2.66±0.31	2.77±0.34
V.	S	1.89±0.51	2.15±0.61	1.71±0.24	2.32±0.14	3.17±0.29	2.88±0.11[a]	3.22±0.17	2.97±0.17
	R	2.72±0.86	2.75±0.20	3.09±0.32	2.08±0.90	2.10±1.09	1.37±0.66	3.13±1.27	3.85±1.20
	L	2.18±0.22	2.30±0.32	1.55±0.19[a]	2.30±0.19	2.74±0.13	2.48±0.15	2.32±0.39	2.00±0.17[a]
F.	S	2.47±0.21	2.30±0.33	1.68±0.20	2.30±0.18	3.04±0.35	2.97±0.11	2.99±0.34	2.80±0.44
	R	2.51±0.41	2.54±0.39	2.21±0.32	2.15±0.42	2.30±1.24	2.34±0.84	3.39±1.62	4.06±0.50
	L	1.96±0.15	1.81±0.59	1.99±0.13	1.61±0.73	2.37±0.79[a]	2.24±0.61[a]	2.34±0.49[a]	2.70±0.19[a]
S.	S	2.43±0.34	2.54±0.37	2.00±0.16		3.05±0.41[a]	3.10±0.27[a]	2.74±0.50[a]	1.64±0.70[b]
	R	2.21±0.76	1.75±0.96	2.32±0.47		0.73±0.31	0.93±0.26	1.02±0.52	1.37±0.40

[a]significantly different from roots ($P<0.05$).

[b]significantly different from leaves ($P<0.001$).

[c]V.=vegetative, F.=flowering, S.=seed development, L=leaf, S=stem, R=root

NMDS plots generated from Bray-Curtis distances were used to visualize beta diversity of microbial communities. PERMANOVA was used to determine if centroids of clusters were significantly different. Analysis of homogeneity of variances was used to determine if differences among centroids may be due to high variances across clusters. Global analysis revealed clusters for each crop species and segregation was clear for aboveground and belowground samples in fungi (Figure 3-15A). At the vegetative growth stage, wheat bacterial communities formed a clear cluster, while maize and soybean communities shared a similar pattern; however, this pattern was lost in the later growth stages (Figure 3-15B). Within maize (Figure 3-16A-B) and soybean (Figure 3-16C-B), there was clustering of fungal and bacterial communities by plant organs. Centroids were significant, but variances were also significantly different ($P < 0.001$, Table 3-6). Within each growth stage, there was not clear clustering of fungal and bacterial communities by management strategy; although, root samples formed clusters separate from aboveground organs (Figure 3-17). Management had significant centroids ($P < 0.01$); significantly different variances were observed for fungal communities ($P < 0.05$) but were not significant for bacterial communities (Table 3-6).

Table 3-6. PERMANOVA of the communities presented in Figures 3-15, 3-16, and 3-17, considering all factors and their interactions.

		All Crops									
		Fungi					Bacteria				
	Factor[a]	DoF, Res	F	R^2	*P*		DoF, Res	F	R^2	*P*	
Global	**Crop**	2, 509	114.249	0.176	0.001	***	2, 499	86.883	0.146	0.001	***
	Organ	2, 509	63.804	0.098	0.001	***	2, 499	55.909	0.094	0.001	***
	Management	3, 509	8.561	0.020	0.001	***	3, 499	2.814	0.007	0.001	***
	Growth Stage	2, 509	19.809	0.030	0.001	***	2, 499	14.659	0.025	0.001	***
	Crop:Organ	4, 509	22.692	0.070	0.001	***	4, 499	29.333	0.098	0.001	***
	Crop:Management	6, 509	5.596	0.026	0.001	***	6, 499	2.524	0.013	0.001	***
	Organ:Management	6, 509	2.599	0.012	0.001	***	6, 499	2.105	0.011	0.001	***
	Crop:Growth Stage	4, 509	13.611	0.042	0.001	***	4, 499	13.943	0.047	0.001	***
	Organ:Growth Stage	4, 509	5.008	0.015	0.001	***	4, 499	4.707	0.016	0.001	***
	Management:Growth Stage	6, 509	2.502	0.012	0.001	***	6, 499	2.103	0.011	0.001	***
	Crop:Organ: Management	12, 509	2.356	0.022	0.001	***	12, 499	1.970	0.020	0.001	***
	Crop:Organ: Growth Stage	8, 509	4.707	0.029	0.001	***	8, 499	4.911	0.033	0.001	***
	Crop:Management: Growth Stage	12, 509	2.174	0.020	0.001	***	12, 499	1.797	0.018	0.001	***
	Organ:Management: Growth Stage	12, 509	1.356	0.013	0.006	**	12, 499	1.530	0.015	0.001	***
	Crop:Organ: Management: Growth Stage	22, 509	1.463	0.025	0.001	***	24, 499	1.456	0.029	0.001	***

Table 3-6 (cont'd)

		Maize									
		Fungi					Bacteria				
	Factor[a]	DoF, Res	F	R^2	*P*		DoF, Res	F	R^2	*P*	
Global	**Growth Stage**	2, 162	10.940	0.082	0.001	**	2, 161	3.429	0.027	0.001	
	Organ	2, 162	21.514	0.161	0.001	***	2, 161	25.316	0.199	0.001	***
	Mgt[b]	3, 162	2.613	0.035	0.001		3, 161	1.766	0.026	0.004	
	Organ:Mgt	6, 162	2.312	0.039	0.001		6, 161	1.769	0.039	0.001	
	Growth Stage:Organ	4, 162	3.425	0.051	0.001		4, 161	2.270	0.036	0.001	
	Growth Stage:Mgt	6, 162	1.775	0.048	0.002						
	Growth Stage:Organ:Mgt	12, 162	1.574	0.052	0.002						
Leaf	**Growth Stage**	2, 55	20.524	0.307	0.001	***	2, 53	3.457	0.091	0.001	**
	Mgt	3, 55	5.969	0.134	0.001		3, 53	2.224	0.088	0.001	
	Growth Stage:Mgt	6, 55	3.308	0.148	0.001		6, 53	1.579	0.125	0.001	
Stem	**Growth Stage**	2, 52	7.978	0.197	0.001	***	2, 54	2.832	0.081	0.001	***
	Mgt						3, 54	1.558	0.067	0.003	
	Growth Stage:Mgt	6, 52	1.467	0.109	0.011						
Roots	**Growth Stage**	2, 55	1.923	0.050	0.019		2, 54	2.017	0.056	0.001	*
	Mgt	3, 55	2.967	0.116	0.001	*	3, 54	2.225	0.093	0.001	
	Growth Stage:Mgt	6, 55	1.453	0.114	0.016		6, 54	1.183	0.099	0.021	
T1	**Growth Stage**	2, 45	5.505	0.121	0.001	*	2, 44	1.929	0.057	0.008	
	Organ	2, 45	12.775	0.282	0.001	**	2, 44	7.786	0.228	0.001	
	Growth Stage:Organ	4, 45	2.290	0.101	0.002						
T2	**Growth Stage**	2, 45	3.575	0.084	0.002		2, 44	1.942	0.055	0.005	
	Organ	2, 45	12.786	0.300	0.001	***	2, 44	8.056	0.229	0.001	***
	Growth Stage:Organ	4, 45	1.877	0.088	0.003		4, 44	1.557	0.089	0.005	

Table 3-6 (cont'd)

T3	**Growth Stage**	2, 34	6.717	0.151	0.001		2, 35	1.714	0.060	0.021	*
	Organ	2, 34	15.603	0.351	0.001		2, 35	6.895	0.241	0.001	
	Growth Stage:Organ	4, 34	2.557	0.115	0.003						
T4	**Growth Stage**	2, 38	5.269	0.126	0.001		2, 38	2.295	0.064	0.005	
	Organ	2, 38	12.330	0.295	0.001		2, 28	10.632	0.298	0.001	
	Growth Stage:Organ	4, 38	2.566	0.123	0.001		4, 38	1.856	0.104	0.004	
		Soybean									
		Fungi					**Bacteria**				
	Factor[a]	**DoF, Res**	**F**	**R^2**	***P***		**DoF, Res**	**F**	**R^2**	***P***	
Global	**Growth Stage**	2, 169	8.889	0.061	0.001		2, 173	3.429	0.027	0.001	***
	Organ	2, 169	32.577	0.222	0.001	***	2, 173	25.316	0.199	0.001	***
	Mgt	3, 169	8.115	0.101	0.001	**	3, 173	1.766	0.026	0.005	
	Organ:Mgt	6, 169	5.059	0.060	0.001		6, 173	2.835	0.039	0.002	
	Growth Stage:Organ	4, 169	4.070	0.056	0.001		4, 173	2.270	0.036	0.001	
	Growth Stage:Mgt	6, 169	1.805	0.045	0.001						
	Growth Stage:Organ:Mgt	10, 169	2.088	0.041	0.001		12, 173	1.992	0.055	0.001	
Leaf	**Growth Stage**	2, 60	28.047	0.319	0.001	*	2, 56	7.866	0.188	0.001	*
	Mgt	3, 60	12.998	0.222	0.001		3, 56	1.757	0.063	0.006	
	Growth Stage:Mgt	6, 60	3.451	0.118	0.001						
Stem	**Growth Stage**	2, 55	9.723	0.154	0.001		2, 58	8.439	0.180	0.001	***
	Mgt	3, 55	14.542	0.346	0.001		3, 58	1.644	0.053	0.004	
	Growth Stage:Mgt	5, 55	1.585	0.063	0.022		6, 58	2.288	0.147	0.001	
Roots	**Growth Stage**	2, 54	6.436	0.145	0.001	***	2, 58	6.896	0.124	0.001	
	Mgt	3, 54	3.513	0.119	0.001		3, 58	7.536	0.203	0.001	***
	Growth Stage:Mgt	5, 54	2.261	0.127	0.001		6, 58	2.837	0.153	0.001	

Table 3-6 (cont'd)

T1	**Growth Stage**	2, 45	6.207	0.111	0.001		2, 44	4.428	0.074	0.004	
	Organ	2, 45	20.694	0.369	0.001	***	2, 44	28.616	0.481	0.001	***
	Growth Stage:Organ	4, 45	3.349	0.119	0.001		4, 44	2.221	0.075	0.013	
T2	**Growth Stage**	2, 45	8.095	0.128	0.001	*	2, 43	3.969	0.064	0.003	
	Organ	2, 45	23.571	0.373	0.001		2, 43	31.774	0.514	0.001	***
	Growth Stage:Organ	4, 45	4.529	0.143	0.001		4, 43	2.287	0.074	0.012	
T3	**Growth Stage**	2, 44	8.908	0.127	0.001		2, 45	3.654	0.076	0.001	
	Organ	2, 44	28.901	0.412	0.001	***	2, 45	17.464	0.365	0.001	**
	Growth Stage:Organ	4, 44	5.176	0.148	0.001		4, 45	2.112	0.088	0.009	
T4	**Growth Stage**	2, 35	5.295	0.124	0.001		2, 41	6.343	0.130	0.001	
	Organ	2, 35	18.135	0.425	0.001		2, 41	12.638	0.260	0.001	
	Growth Stage:Organ						4, 41	4.589	0.189	0.001	

[a]Only values significant at * ($P \leq 0.10$), ** ($P \leq 0.05$), *** ($P \leq 0.01$) shown.
[b]Mgt = management

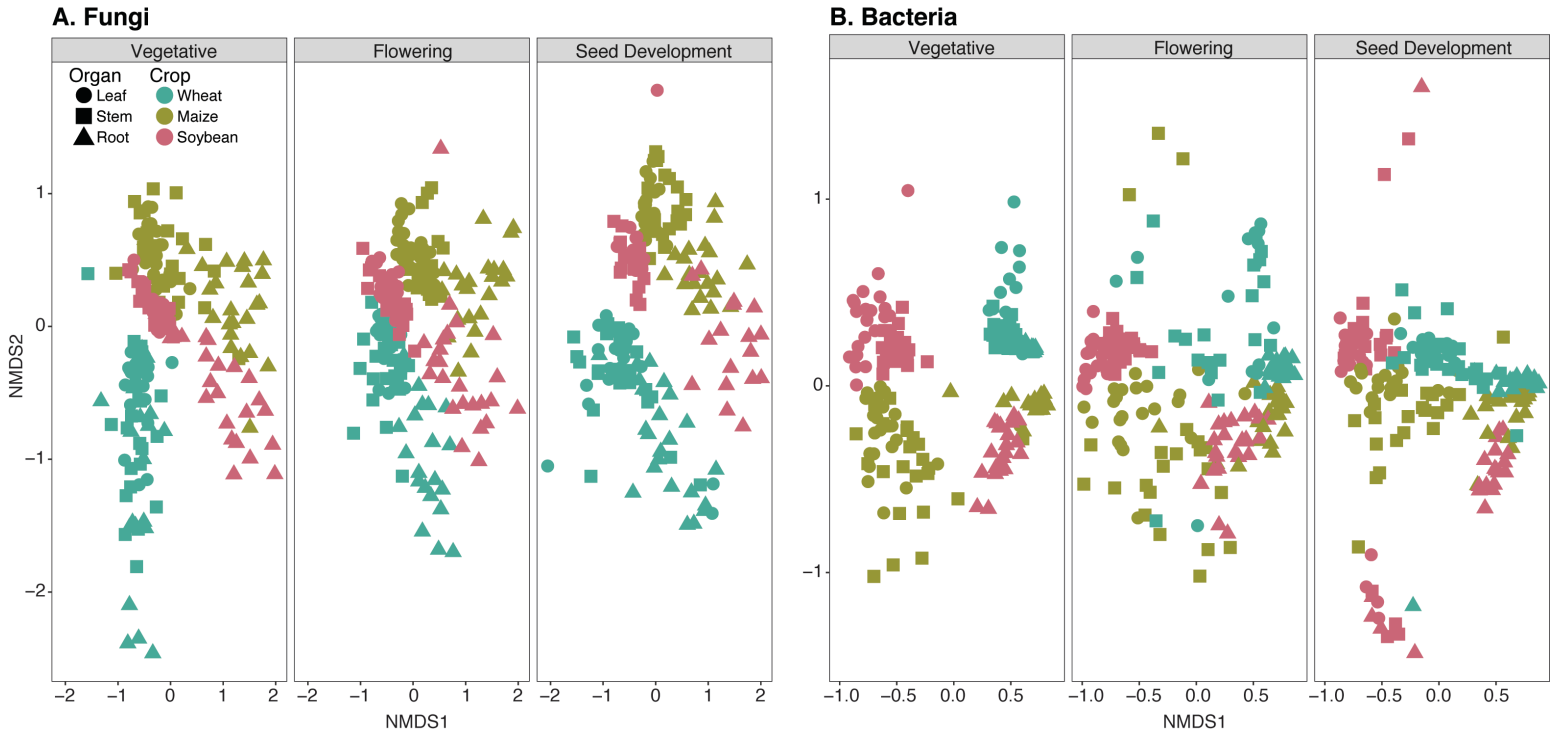

Figure 3-15. Effect of growth stage on beta diversity of fungal (A) and bacterial (B) communities on all crops. Points colored by crop species, shapes indicate plant organ. Non-metric multidimensional scaling (NMDS) calculated by Bray-Curtis distance. Statistical support is detailed in Table 3-6.

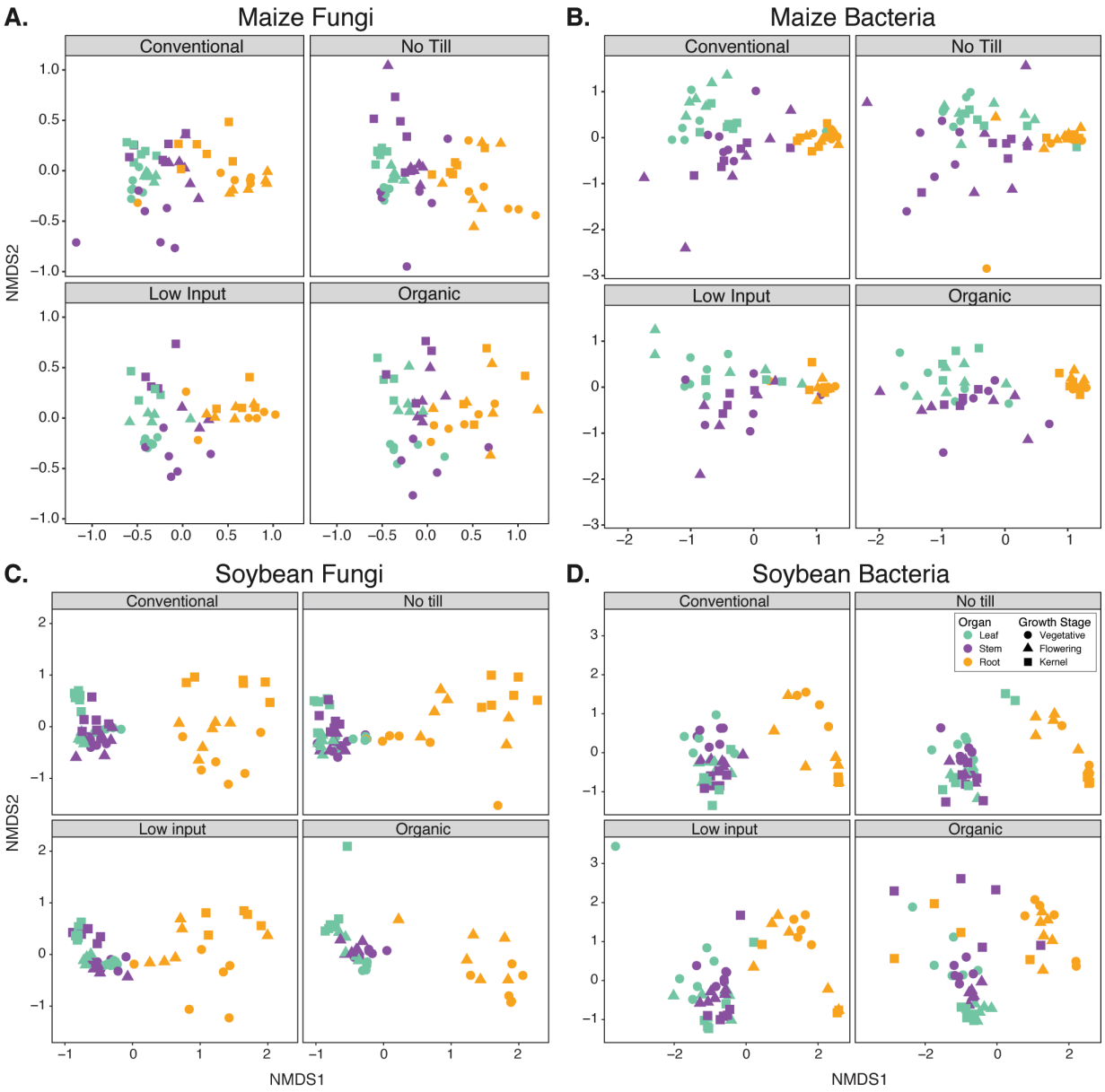

Figure 3-16. Influence of management strategies on beta diversity of maize fungal (A), maize bacterial (B), soybean fungal (C), soybean bacterial (D) communities originating from each plant organ. NMDS calculated by Bray-Curtis distance. Statistical support is detailed in Table 3-6.

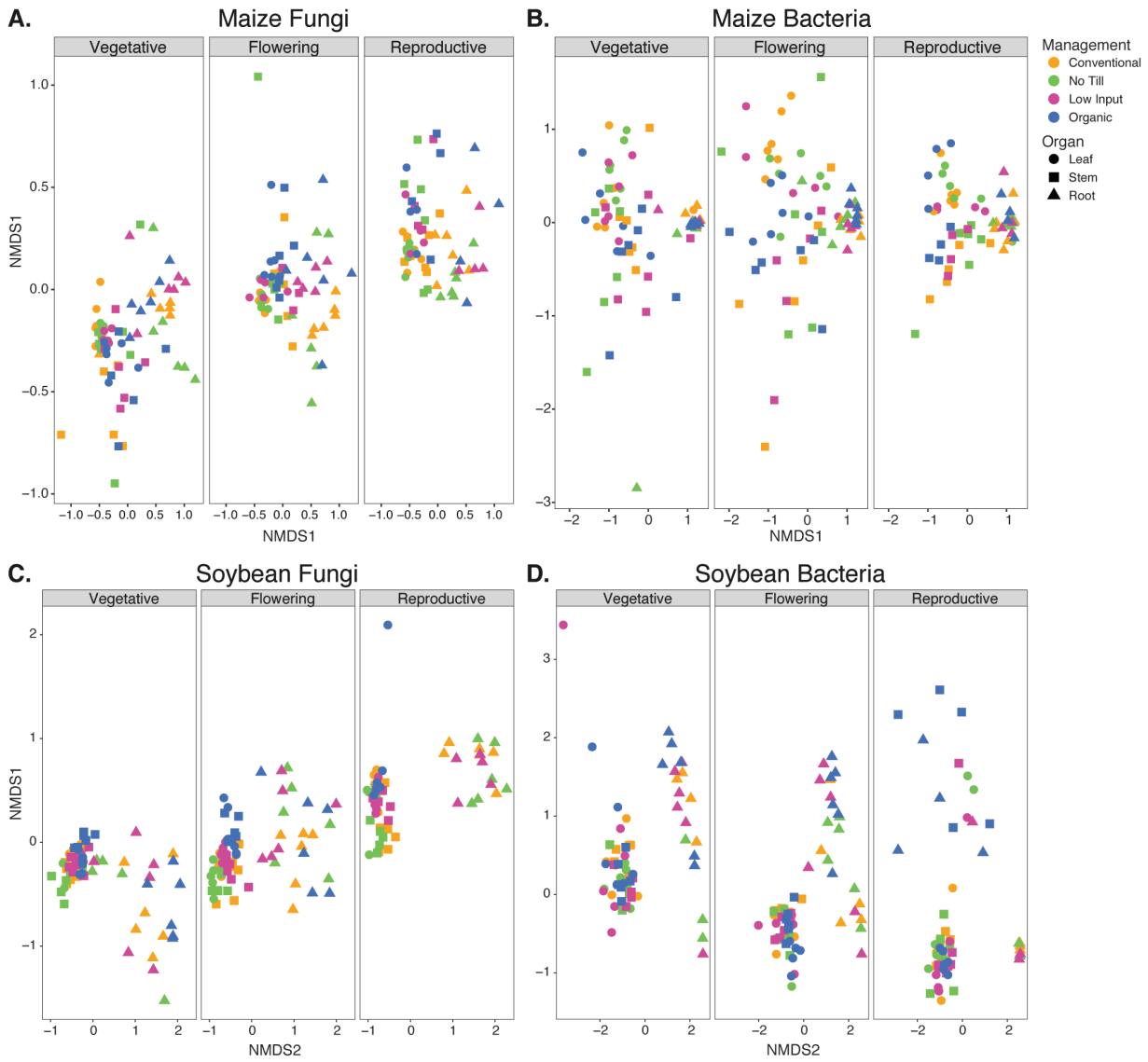

Figure 3-17. Effect of growth stage on beta diversity of maize fungal (A), maize bacterial (B), soybean fungal (C), and soybean bacterial (D) communities originating from each management style. NMDS calculated by Bray-Curtis distance. Statistical support is detailed in Table 3-6.

Microbial Networks

Microbial hubs, species that influence the presence of others in an environment, were defined as highly connected nodes with ten or more connections and 35 hubs were discerned across all crops (Table 3-7). Of the hub taxa, 12 bacterial hubs and no fungal hubs were shared between wheat roots and phyllosphere (Figure 3-18, 3-19). All hub taxa in wheat roots had at least one negative correlation with a non-hub taxon. The two hubs *Dongia* sp. and *Cytophaga* sp. were negatively correlated with each other in wheat roots (Figure 3-18). *Chalatospora* sp. was the only hub taxon whose presence was not correlated with the other hub taxa in wheat roots (Figure 3-18). Nine of the hub taxa in the wheat phyllosphere were correlated with other hub taxa, and *Pedobacter* sp. co-occurred with seven of them (Figure 3-19). Of the wheat phyllosphere hub species, *Sedimentibacter* sp., *Mucilaginibacter* sp., and *Ferruginibacter* sp. did not have any negative correlations (Figure 3-19).

The hub taxon in maize roots, *Pseudoduganella* sp., was predominantly correlated with other bacterial species, but negatively influenced the presence of two fungal taxa; *Brachyphoris* sp. and *Rhexocercosporidium* sp. (Figure 3-20A). Glomeromycota, a group of fungi that form mycorrhizal symbioses with plants, formed a small isolated network not connected with the main maize root network (Figure 3-20A). Hub taxa in the maize phyllosphere were all identified as fungi except one, and no negative correlations containing hubs were identified (Figure 3-21). *Devosia* sp. was identified as a hub taxon in both wheat and maize phyllospheres. We did not identify hub taxa that were shared across maize or soybean organs. Only one hub taxon was observed in each soybean root (*Stagonospora pseudovitensis*; Figure 3-20B) and phyllosphere (*Bensingtonia subrosea;* Figure 3-22) networks. Interestingly, the hub taxa in soybean roots was positively correlated with fungi and was negatively correlated with bacteria (Figure 3-20B). Closely related

taxa, such as several nodule-forming bacterial OTUs (*Bradyrhizobium*, *Mesorhizobium*, *Neorhizobium*) frequently occurred together in the networks. Although not identified as a hub, *Fusarium* sp. was present in the co-occurrence networks. The ITS barcode marker cannot be used to definitively identify *Fusarium* sp. down to genus, we do not know if the *Fusarium* OTUs recovered by microbiome sequencing are plant pathogens. *Fusarium* and/or *Gibberella* OTUs were found in all networks except maize roots.

All bacterial hub taxa, except OTU 148, were shared across all crops. OTU 148 was not found in soybean. Twenty-one OTUs were identified as hub taxa on wheat; seven of these shared hub taxa status across the root and phyllosphere networks. All 21 OTUs can be found in leaves, stems, and roots of wheat. This phenomenon was observed for maize and soybean networks as well. Although hub taxa may not share hub status across crops or plant organs, these taxa are common OTUs found across plant organs.

Table 3-7. Hub taxa of KBS LTER crops.

Crop	Root	Phyllosphere
Wheat	*Chalastospora ellipsoidea*[a]	*Devosia* sp.
	Bradyrhizobium sp.	*Discula destructiva*[a]
	Cytophaga sp.	*Dyadobacter* sp.
	Dongia sp.	*Ferruginibacter* sp.
	Dyadobacter sp.	*Flavobacterium* sp.
	Ferruginibacter sp.	*Methylophilus* sp.
	Massilia sp.	*Mucilaginibacter* sp.
	Pedobacter sp.	*Pedobacter* sp.
	Polaromonas sp.	*Polaromonas* sp.
	Ralstonia sp.	*Rhizobium* sp.
	Salinibacterium sp.	*Rhodopseudomonas* sp.
	Sedimentibacter sp.	*Salinibacterium* sp.
	Sphingomonas sp.	*Sedimentibacter* sp.
	Variovorax sp.	
Maize	*Pseudoduganella* sp.	*Schizophyllum commune*[a]
		Discula destructiva[a]
		Neofavolus alveolaris[a]
		Ophiognomonia sogonovii[a]
		Devosia sp.
Soybean	*Stagonospora pseudovitensis*[a]	*Bensingtonia subrosea*[a]

[a]fungal OTU

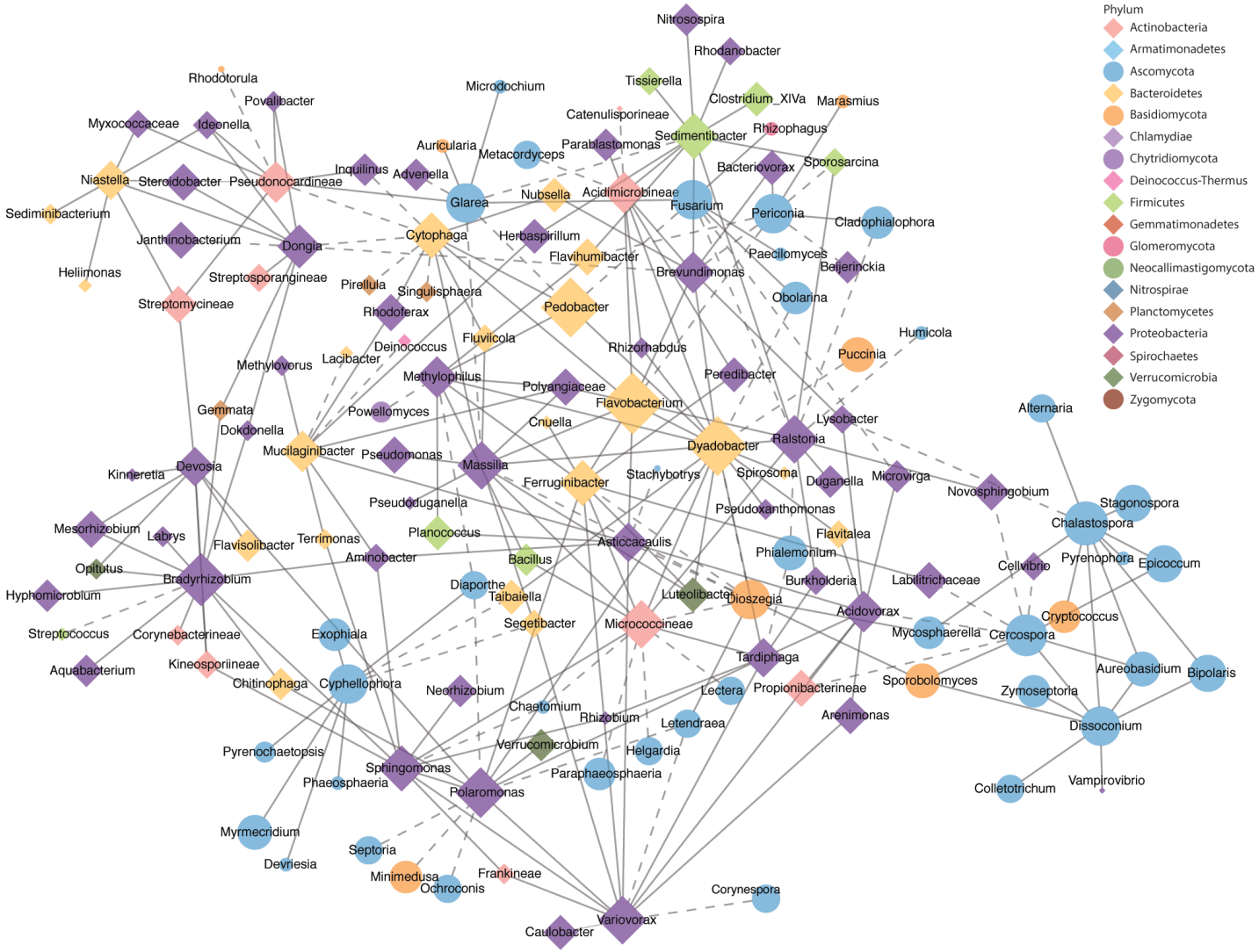

Figure 3-18. Co-occurrence network of taxa in wheat roots. Bacterial OTUs are represented by diamond shapes, fungal OTUs are represented by circles, and nodes are colored by class. Solid lines indicate positive correlation, and dashed lines indicate a negative correlation between OTUs. Node size indicates degree of connectivity.

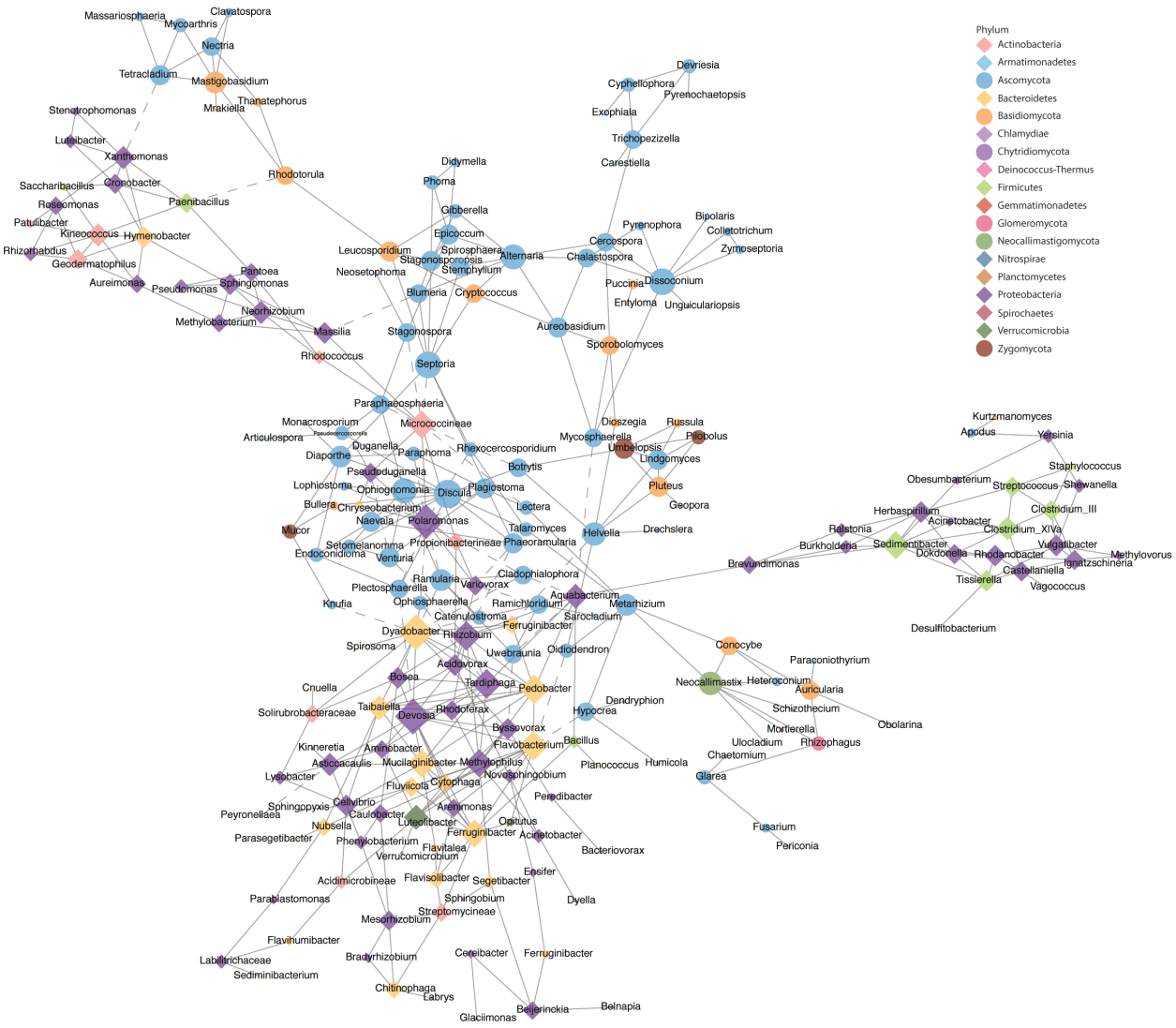

Figure 3-19. Co-occurrence network of taxa in wheat phyllosphere. Bacterial OTUs are represented by diamond shapes, fungal OTUs are represented by circles, and nodes are colored by class. Solid lines indicate positive correlation, and dashed lines indicate a negative correlation between OTUs. Node size indicates degree of connectivity.

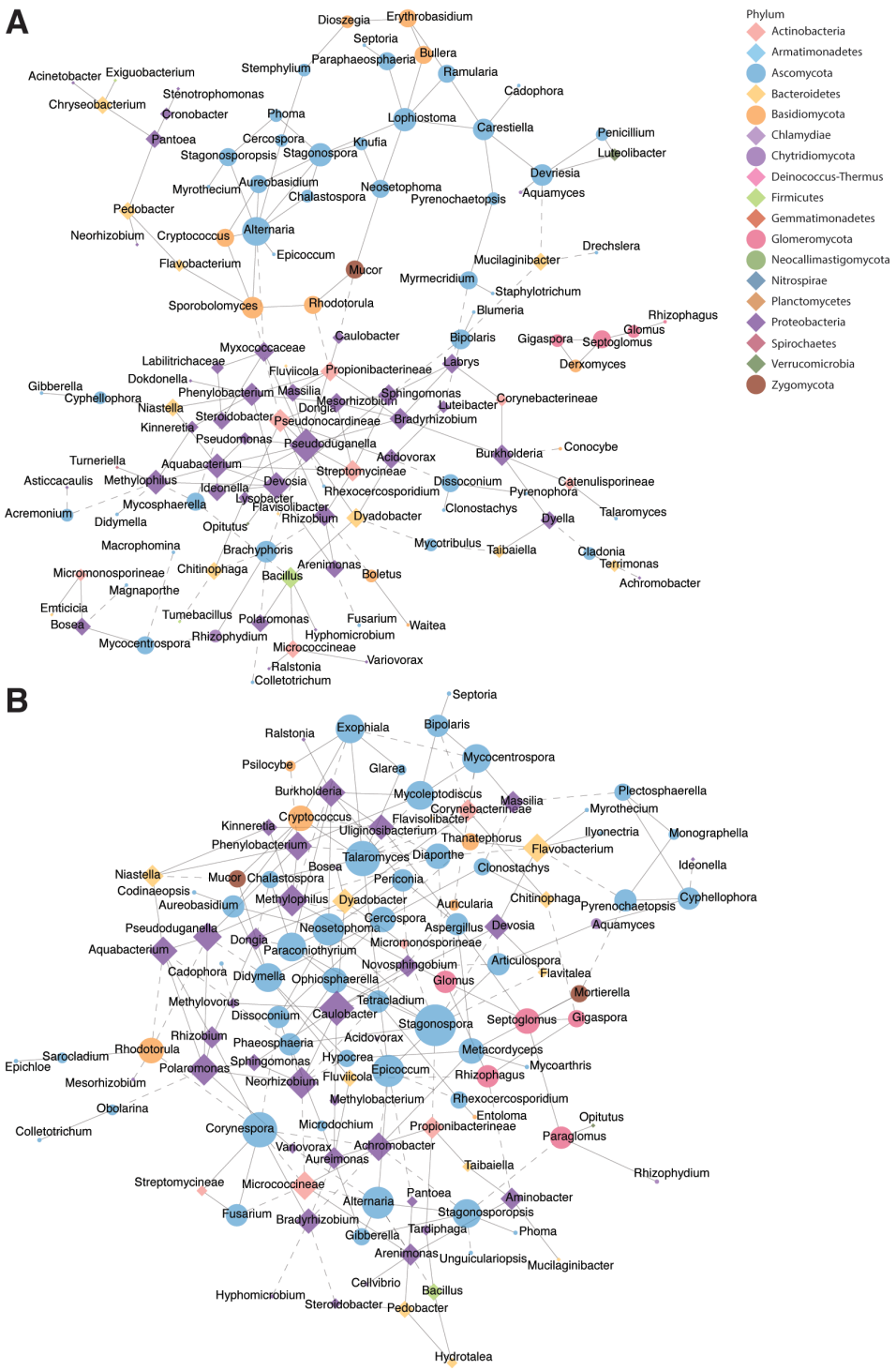

Figure 3-20. Co-occurrence network of taxa in maize (A) and soybean (B) roots. Bacterial operational taxonomic units (OTUs) are represented by diamond shapes, fungal OTUs are represented by circles, and nodes are colored by class. Solid lines indicate positive correlation, and dashed lines indicate a negative correlation between OTUs. Node size indicates degree of connectivity.

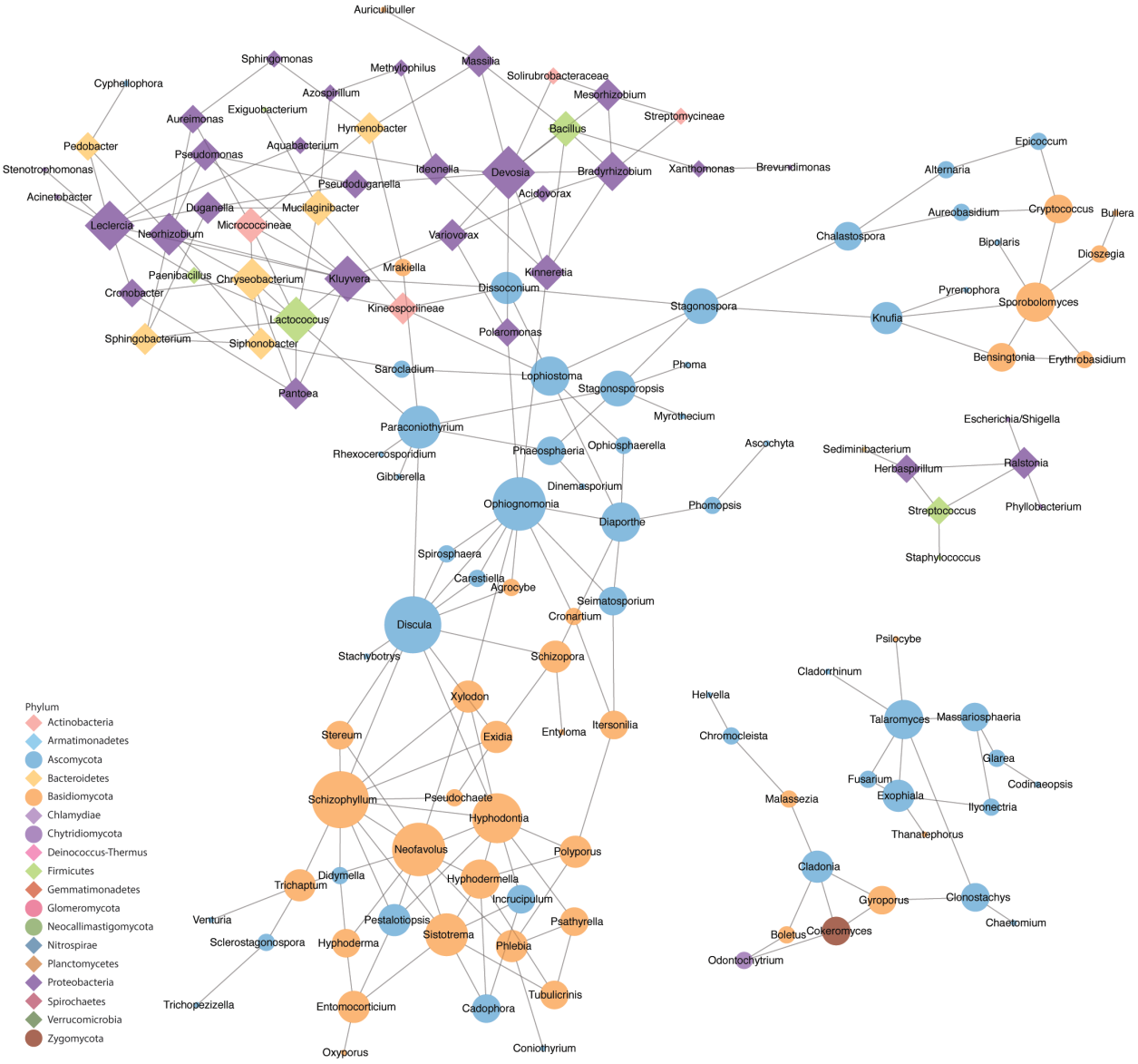

Figure 3-21. Co-occurrence network of taxa in maize phyllosphere. Bacterial OTUs are represented by diamond shapes, fungal OTUs are represented by circles, and nodes are colored by class. Solid lines indicate positive correlation, and dashed lines indicate a negative correlation between OTUs. Node size indicates degree of connectivity.

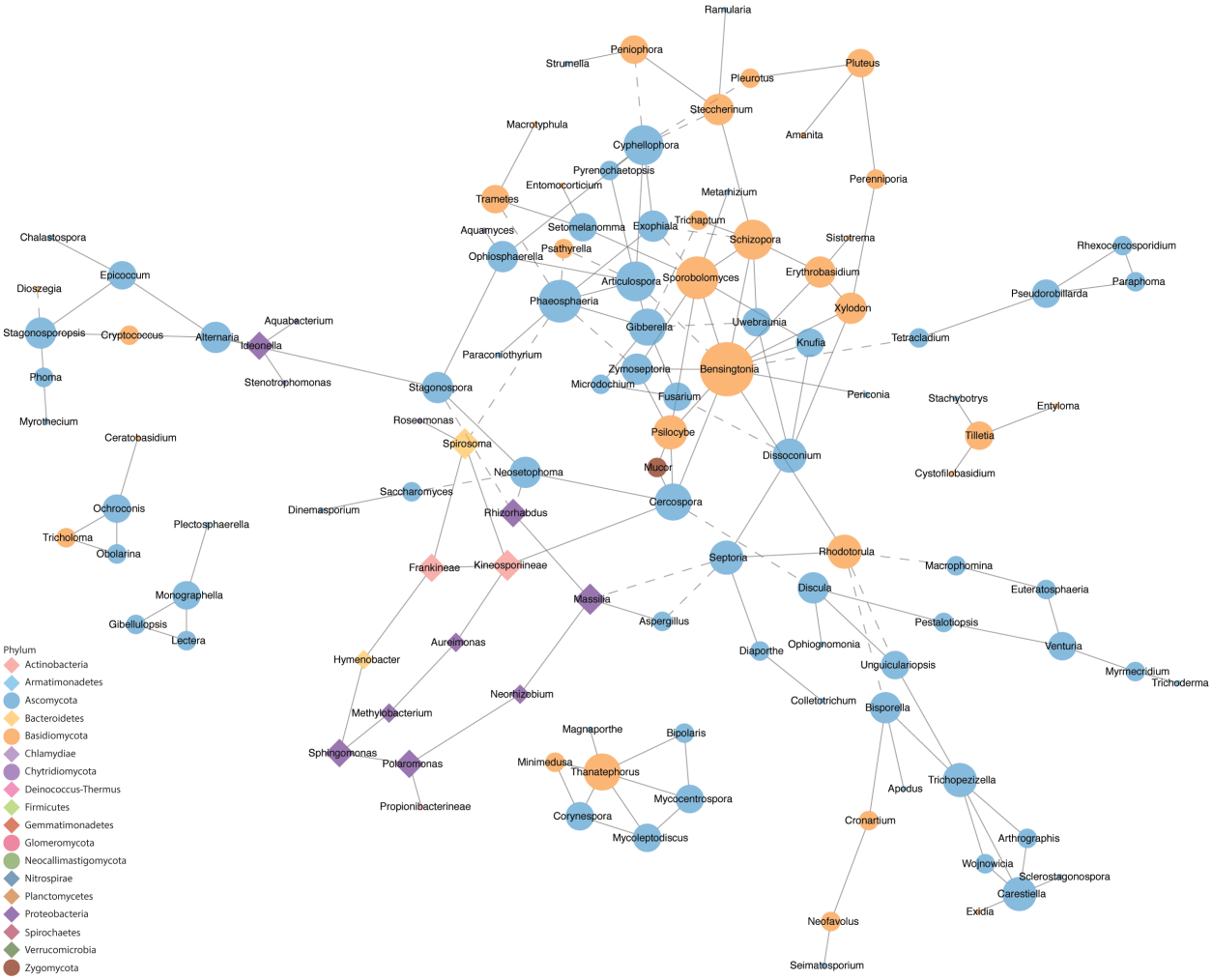

Figure 3-22. Co-occurrence network of taxa in soybean phyllosphere. Bacterial OTUs are represented by diamond shapes, fungal OTUs are represented by circles, and nodes are colored by class. Solid lines indicate positive correlation, and dashed lines indicate a negative correlation between OTUs. Node size indicates degree of connectivity.

Discussion

Here we present the first in-depth comparative analysis of fungal and bacterial microbial communities in the economically important row crops, wheat, maize, and soybean. The study demonstrates that microbial communities differ across three growth stages and organs of one plant host, and between plant hosts. Although the plant hosts studied here share some microbial taxa, the communities are distinct between each crop species, as determined by core taxa analysis and diversity metrics.

Contrary to expectations, no large differences in plant microbial diversity among land management strategies were observed. Previous studies that explored differences in microbial communities among management strategies (such as conventional vs. organic management) analyzed rhizospheric soil communities or conducted comparisons of fields that were large distances apart (for example Bernard et al. 2012, Peiffer et al. 2013, Hartmann et al. 2014, Souza et al. 2015). Studies which surveyed plant microbiomes of the same genotypes across multiple locations found that geography and environment have a stronger influence on microbial community than management and plant genotype (Finkel et al. 2011, Peiffer et al. 2013, Copeland et al. 2015, Chen et al. 2016). The microbial reservoir of the soil may be the biggest influencing factor on the final microbial community composition. Analyses of diverse plant taxa showed that plant species and genotype (cultivar) influenced root microbial communities. Based on previous studies and the present study, land management affects soil and rhizosphere microbial community composition but appears to have a weaker impact on plant or phyllosphere microbial community composition (Bálint et al. 2013, Bonito et al. 2014, Coleman-Derr et al. 2015, Sapkota et al. 2015, Naylor et al. 2017).

The present study did not use fungicides directly on the crops, and T1-T3 managements used fungicide-treated seed. We did not have controls for these processes to assess the direct effect of fungicides on the microbiome. Previous studies have demonstrated that fungicide seed treatments affect phyllosphere microbiomes of crops tested during the vegetative growth phase (Nettles et al. 2016). It is not known if this observation would persist to reproductive growth stages. Fungicides have also been known to affect mycorrhizal associations and reproduction (Marx and Rowan 1981). However, Plante (2017) argues that crop management strategies, such as applications of fertilizers and pesticides, are not perturbation events in the traditional ecological sense that should alter the microbiota found on plants. Plante implies the application of pesticides may be a chronic stress for the native microbiota instead of a perturbation event (2017). However, the data presented in the current study indicates that fungicides may be secondary influencers of microbial community composition, with geography and genotype as the primary influencing factors.

The microbial community composition of the wheat, maize, and soybean growth at our study site is distinct for each host crop and plant organ. Some of the most abundant OTUs found on wheat, maize, and soybean (Dothideomycetes, Sordariomycetes, Alphaproteobacteria, Gammaproteobacteria) are also found as some of the most abundant taxa in previous studies (Rascovan et al. 2016, Gdanetz and Trail 2017, Karlsson et al. 2017). Studies that analyze microbial communities across multiple organs or tissues on the same plant often show that there are distinct communities across these niches (for example, Ottesen et al. 2013, Coleman-Derr et al. 2015). Plant-microbe interactions can influence a plant's susceptibility or resistance to pathogens by stimulating plant defense responses (reviewed by Durrant and Dong 2004). There is a bias in the literature toward analyzing soil or rhizosphere microbial communities (Table 1-1).

Work on model systems shows that phyllosphere microbes can influence disease progression and nutrient cycling (Delmotte et al. 2009, Vorholt 2012, Agler et al. 2016, Ryffel et al. 2016, Vogel et al. 2016). We demonstrated that root-associated microbial communities are often distinct from those of the phyllosphere or aboveground organs. Although the rhizosphere has a higher abundance and diversity of plant-associated microbes compared to the phyllosphere (Ottesen et al. 2013, Coleman-Derr et al. 2015, Zarraonaindia et al. 2015), microbes inhabiting other plant surfaces may also be important to plant health. Neglecting aboveground plant surfaces, in favor of the soil and rhizosphere, during microbial community analyses will exclude microbes that have a life cycle which is not captured by a soil or root-associated phase, for example, important plant pathogens such as rust fungi.

Our findings indicate that maize and soybean fungal diversity decreases across the growing season while bacterial diversity remains constant. Previous studies examining changes in plant-associated microbial communities over the course of a growing season have been inconclusive. Some studies showed increasing community diversity over time (Shade et al. 2013, Gdanetz and Trail, 2017) whereas others showed a decrease (Copeland et al. 2015, de Souza et al. 2016), or no significant change (Sapkota et al. 2017). The diversity patterns of the soybean fungal communities we examined reinforced the temporal patterns observed on maize from the KBS LTER study site, and in previous studies of soybean from other field sites (Copeland et al. 2015). Further studies are needed to examine the plant microbial community dynamics over time to determine if the observed differences throughout the growing season are driven by host plant or environmental factors. Some aspects of soil diversity, specifically the alpha-diversity in this study, appear to vary temporally across the seasons, but this may also be an artifact of seasonal changes such as moisture (Lauber et al. 2013). Further investigation should be conducted into the degree to which random

colonization by microbes affects the end community composition. Computer modelling of the plant microbiome suggests the initial colonizers influence final community structure (Evans et al. 2016). An understanding of the influence of the initial colonizers on final community composition will have implications for community manipulation in field crops and synthetic environments such as greenhouses or hydroponic systems.

Community structure is dictated by environmental microbial diversity. However, there are certain members of microbial communities, hub taxa, which can strongly influence community composition. In the current study, hub taxa identified in the plant organ(s) of one crop did not share hub taxa status across all crops and plant organ(s). However, these OTUs are commonly occurring and are found across all crops and conditions. These organisms are targets for future studies with synthetic microbial communities. Most of the wheat microbial hubs identified in this study are bacterial OTUs. This may be due to low sequencing coverage from maize and soybean 16S rRNA gene libraries.

Fungal hub taxa were also identified for all crops, with many fungal hubs in the maize phyllosphere, indicating that fungi are important members of plant microbial communities. Some of the fungal hub species identified in the current study are pathogens of other plant species, for example, *Discula destructiva* a hub species of both the wheat and maize phyllospheres, causes dogwood anthracnose. The presence of a non-host pathogen hub may prevent the infection of another pathogen, as demonstrated by Agler et al. (2016). *Fusarium graminearum* disease incidence was low during the study years, this may be due to the presence of hub taxa, specifically *D. destructiva*. However, some of the OTUs identified as hub species in the microbial co-occurrence networks may be assigned incorrect taxonomies due to incomplete reference databases, instead they may be closely related species. As observed in this study and as described by Berry

and Widder (2014), closely related OTUs such as Rhizobia, wood rot fungi, and mycorrhizal fungi frequently co-occur in network analysis. Taxonomic assignment of OTUs to species-level resolution is possible with the ITS region of the rRNA gene in some fungal lineages, but it is not possible for OTUs identified by the 16S rRNA gene. Further work on development and testing of network analysis methods (as discussed by Poudel et al. 2016, Schlatter et al. 2017), followed by experimental validation of microbial communities is needed.

Microbial hub taxa are important members of the plant-associated microbiota that can influence final community composition and plant health (Agler et al. 2016). Identification of hub taxa and understanding their influence on final community composition will be important for growing plants in synthetic environments such as hydroponic systems or on the International Space Station (Foster et al. 2014). For the interest of microbial manipulation in agriculture, future work should determine if there is a degree of phylogenetic or functional conservation in microbial hub species. If function proved to be a more important characteristic, this may make selection and development of microbial cocktails to improve crop yields or reduce plant diseases, easier to develop. Analyses such as the hub taxa identified here will be a valuable resource for researchers wishing to experimentally manipulate these microbial communities. Future work in our lab will test the hub taxa identified here in synthetic community experimental manipulations with crop plants for protective outcomes.

This study shows that multiple organs (root, leaf, and stem) on the same plant often have distinct microbial communities. The microbial reservoir of the soil and land management strategy are factors which can influence the plant-associated microbial communities; however, plants are still able to select for specific microbes. This selection may be in the form of root exudates that recruit microbes (Wu et al. 2015, Biere and Goverse 2016), or leaf chemistry that is hostile toward

germination of spores which land on a leaf surface. Detailed understanding of the composition of plant-associated microbial communities will allow growers to add in the influence of the microbiota when calculating pesticide or fertilizer applications in precision agriculture approaches (Tackenberg et al. 2016, Pineda et al. 2017).

As we demonstrated here with inferred microbial networks, and as others have shown previously (Agler et al. 2016, Mark Welch et al. 2016), microorganisms interact with each other across Kingdoms. To better emulate natural microbial community composition, controlled experiments with synthetic plant microbial communities should include microbes from across the tree of life. Although not investigated in the current study Archaea, Protists, Oomycetes, and viruses can interact with other members of microbial communities – exciting research opportunities lie ahead of those attempting to understand these complex microbial communities composed of organisms from multiple Kingdoms across the tree of life (Anonymous 2017). We also want to state the value of culture-based work in this era of computational and "-omics" biology; researchers generating culture collections from the samples or field sites at which they conduct microbiome studies are building an important resource useful for experimental testing of the hypotheses or questions generated by microbial ecology work.

CHAPTER 4

FUNGAL MICROBIOMES: A STRATEGY FOR IMPROVED TAXONOMIC RESOLUTION OF ENVIRONMENTAL ITS SEQUENCES

Abstract

One of the most crucial steps in high-throughput sequence-based microbiome studies is the taxonomic assignment of sequences belonging to operational taxonomic units (OTUs). Without taxonomic classification, functional and biological information of microbial communities cannot be inferred or interpreted. The internal transcribed spacer (ITS) region of the ribosomal DNA is the conventional marker region for fungal community studies. While bioinformatics pipelines that cluster reads into OTUs have received much attention in the literature, less attention has been given to the taxonomic classification of these sequences, upon which biological inference is dependent. Here we compare how three common fungal OTU taxonomic assignment tools (RDP Classifier, UTAX, and SINTAX) handle ITS fungal sequence data. The classification power, defined as the proportion of assigned OTUs at a given taxonomic rank, varied among the classifiers. Classifiers were generally consistent (assignment of the same taxonomy to a given OTU) across datasets and ranks; a small number of OTUs were assigned unique classifications across programs. We developed CONSTAX (CONSensus TAXonomy), a Python tool that compares taxonomic classifications of the three programs and merges them into an improved consensus taxonomy. This tool also produces summary classification outputs that are useful for downstream analyses. Our results demonstrate that independent taxonomy assignment tools classify unique members of the

fungal community, and greater classification power is realized by generating consensus taxonomy of available classifiers with CONSTAX.

Source

For the full text of this work see: Gdanetz K, Benucci GMN, Vande Pol N, Bonito G. CONSTAX: a tool for improved taxonomic resolution of environmental fungal ITS sequences. BMC Bioinformatics. 18:538.

https://bmcbioinformatics.biomedcentral.com/articles/10.1186/s12859-017-1952-x

APPENDICES

APPENDIX A

EVALUATION OF PROTECTIVE ENDOPHYTES AGAINST *FUSARIUM GRAMINEARUM* HEAD BLIGHT

Introduction

Fusarium Head Blight of cereals is a complex of diseases caused by *F. graminearum* (reviewed by Trail 2009, Xu and Nicholson 2009, Wegulo et al. 2015), and a consortium of species (*Fusarium culmorum, Fusarium avenaceum, Fusarium poae, Microdochium majus,* and *Microdochium nivale*). Multiple fungal species can be isolated from a single head (Ioos et al. 2005, Xu et al. 2005, Xu and Nicholson 2009). While wheat, barley, oats, and rye are all susceptible crops, Head Blight is best characterized in wheat and barley. Not only does this disease cause yield losses, but it also degrades the quality of harvested and stored grains resulting in the accumulation of mycotoxins (Brown and Proctor 2013).

Currently, there are no completely resistant varieties of wheat or barley available. Partially resistant varieties in combination with foliar fungicides, is the most effective plant protection strategy against *F. graminearum* infection (Wegulo et al. 2015). Due to changing social pressures and the growing risk of fungicide resistance, there is an increasing interest in the manipulation of microbial communities to increase agricultural output and reduce disease (Chaparro et al. 2012, APS 2016, Busby et al. 2017, Schlatter et al. 2017). Manipulation of microbes associated with crops to improve agricultural outcomes is not a new concept. Scientists have known that microbes, such as rhizobia and mycorrhizae, are beneficial to plant growth for over a century (Young and Haukka 1996, Berch et al. 2004), and research on beneficial microorganisms has resulted in species and strains of microbes that have shown efficacy in productivity improvement or disease protection. For example, Alfa-Guard® to control *Aspergillus* infection of peanuts (Fravel 2005, Dorner and Lamb 2006), and *Trichoderma harzianum* (RootShield®, Trichodex®, Binab T®) to control various infections in crops (Fravel 2005). Additionally, there are biocontrol bacterial strains such as *Bacillus subtilis* to control various soil borne fungal diseases, and *Pseudomonas*

spp. to control diseases on various plants ranging from ornamentals, turf grass, to tree fruits (Fravel 2005). More recently, scientists have been attempting to identify microbial consortia that provide general suppression of plant and soil pathogens. However, there are limited stories of success with this strategy (reviewed by Schlatter et al. 2017). Difficulties may be due to competition with native microbes in the soil, as in the case of a biocontrol applied via microbial seed coatings; or the harsh environmental conditions such as UV light on leaf surfaces and rapidly changing moisture conditions (Lindow and Brandl 2003), for a foliar or spray-based biocontrol. Use of endophytic microbes with protective abilities may make for more effective control products on the market as there would be less exposure to harsh environmental conditions such as light and water stress (Lindow and Brandl 2003, Gdanetz and Trail 2017). *Fusarium graminearum* causes both flower and crown infections (Trail 2009, Wang et al. 2015). Fungal endophytes with protective abilities against seedling infection were tested in Chapter 2; here the protective ability of these strains during Head Blight initiation was tested.

Materials and Methods

Strains and Growth Conditions

All fungal cultures were maintained on agar medium under 35% glycerol at -80ºC. Endophyte strains were isolated from field-grown wheat plants as described previously (Gdanetz and Trail 2017). A Michigan wild-type strain of *F. graminearum,* PH-1 (NRRL #31084, FGSC #9075; Trail and Common 2000), was used as pathogen inoculum. Endophytic fungal strains tested for protective ability by Gdanetz and Trail (2017) were used in the current study. Identity of endophytes was determined via sequencing of the ITS rRNA gene after DNA amplification with the primer pair ITS1F and ITS4, as described previously (White et al. 1990, Bruns and Gardes

1993, Gdanetz and Trail 2017). Fungal strains were grown on malt extract agar medium (2% malt extract (Difco, Detroit, MI, USA), 1.5% agar) for four days prior to plant inoculation. A 4-mm diameter borer was used to generate agar plugs of each fungal culture. The uncolonized portion of the plug was removed and the colonized part of each plug was cut into quarters.

Plant Inoculation and Disease Rating

Wheat plants of the *F. graminearum*-susceptible cultivar Wheaton were grown in a greenhouse (21-23ºC with 16 hours of supplemental lighting). Plants were inoculated with protective endophytes and/or the pathogen during flowering (Table A-1). Forceps were used to place the colonized agar blocks into the middle floret of each plant (Guenther and Trail 2005, Hallen-Adams et al. 2011b). Agar plugs of endophyte cultures and fresh malt extract agar plugs (control plants) were inserted into florets, then plants were placed into a misting chamber (misting every 15 minutes) for 24 hours to stimulate fungal growth. Then *F. graminearum* colonized agar plugs were added to the same florets containing the endophyte-colonized plugs and to control plant florets (Figure A-1). Plants were returned to the misting chamber for an additional 48 hours. Each plant was rated for Head Blight symptoms (necrosis, premature bleaching, bent awns) for 15 days post inoculation.

Disease incidence, the number of infected florets per plant, was compared between endophyte-inoculated plants and the endophyte-free controls. Statistics on mean disease incidence were calculated in R Studio (R Core Team 2016) and plots were created with the ‘ggplot2’ package (Wickham, 2009).

Table A-1. Protective endophyte strains used for head inoculations (Gdanetz and Trail 2017).

Strain ID	ID by full length ITS locus
11	*Microdochium bolleyi*
30	*Alternaria tenuissima*
34	*Alternaria* sp.
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*

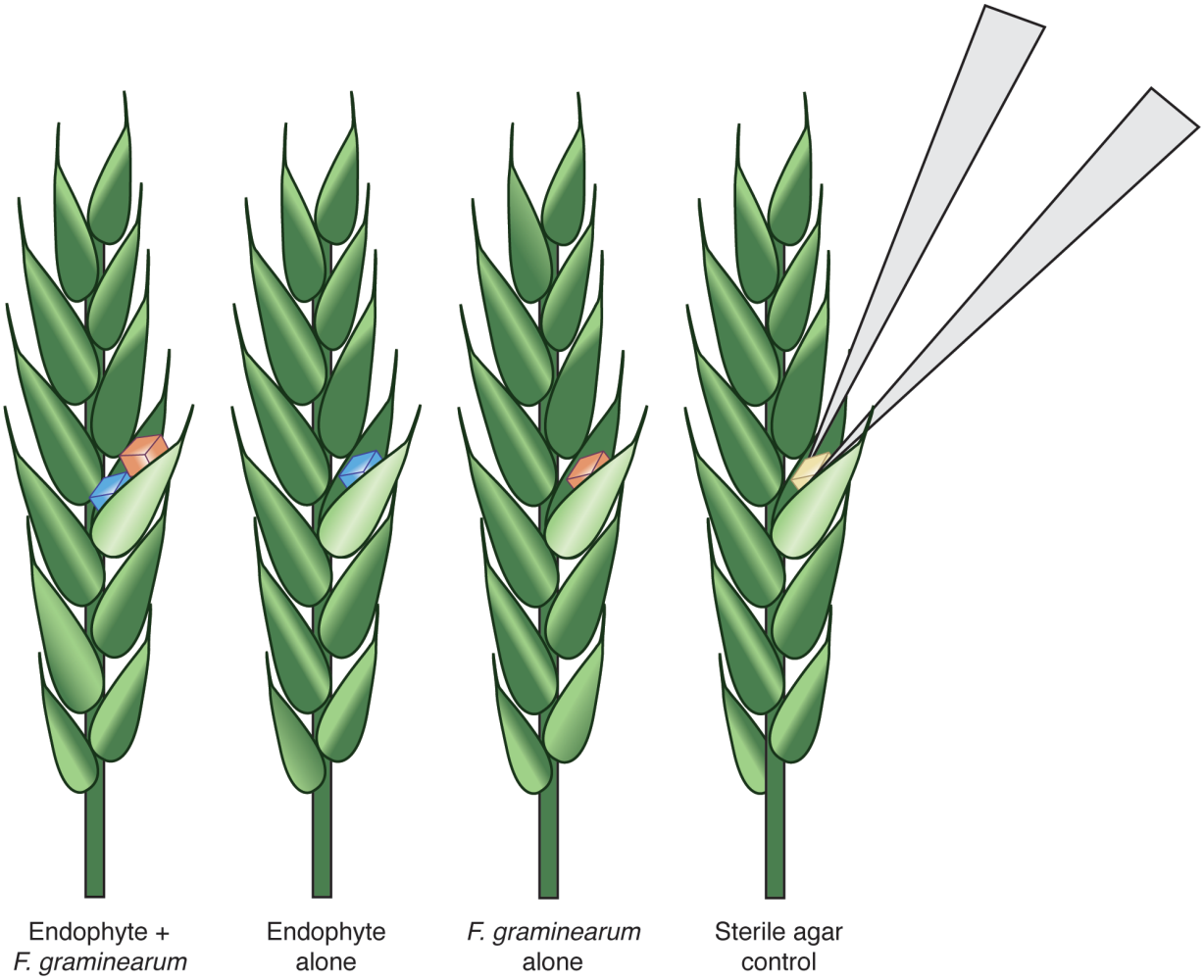

Figure A-1. Inoculation method and experimental design of protective endophytes.

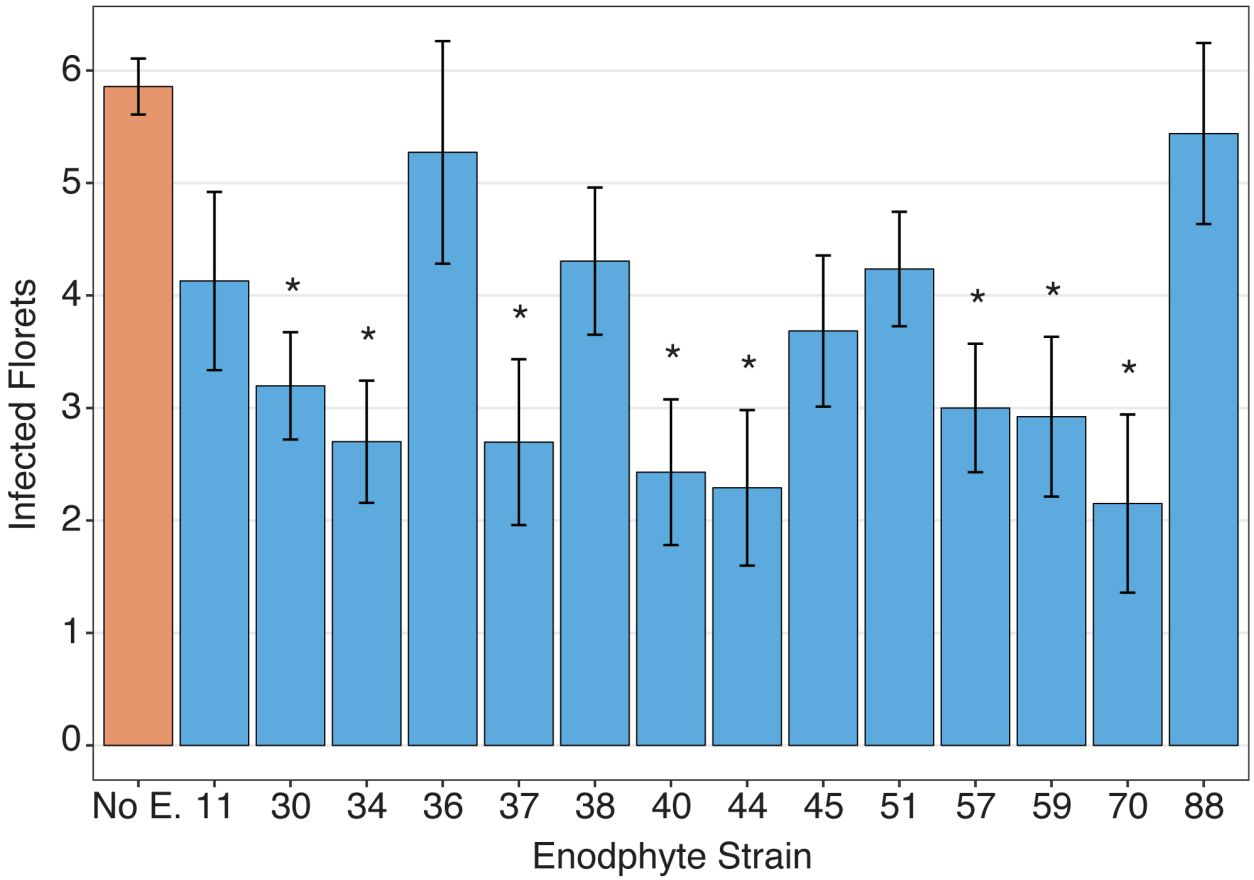

Figure A-2. Fusarium Head Blight disease incidence. Wheat inoculated with endophytes (No E.: endophyte-free control) and challenged with *Fusarium graminearum*. Error bars represent standard error of the mean (3 independent replicates of 8 plants). Strains marked with asterisk had significantly reduced disease compared to endophyte free control ($p < 0.05$).

Results and Discussion

Endophyte strains that demonstrated protective ability against *F. graminearum* damping-off in a seedling assay (Gdanetz and Trail, 2017) were tested for protective ability against Head Blight infection. Endophyte strains that demonstrated protective ability in seedlings also demonstrated protective ability in wheat heads. Plants inoculated with strains 37 (*Alternaria tenuissima*), 40 (*Fusarium* sp.), and 70 (*Fusarium oxysporum*) had reduced disease when compared with endophyte-free plants (Figure A-2) in both mature plants and seedlings. Five additional strains showed protective ability in mature plants but not seedlings: 30 (*Alternaria tenuissima*), 34 (*Alternaria* sp.), 57 (*Phoma* sp.), 59 (*Fusarium* sp.), and 44 (*Fusarium* sp.). Strain 38 (*Fusarium solani*) was the only endophyte that caused reduced disease in seedlings, but not in mature plants.

Changing consumer demands are creating a renewed interest in biocontrol organisms for agriculture. Several candidate strains that may be used as protective endophytes in wheat were identified (Gdanetz and Trail 2017), although, further work is required to identify the mechanism of action of these strains. It is not yet known if the fungi tested here protect by creating a physical barrier (colonizing and taking up space between plant cells which blocks *F. graminearum* growth); stimulating systemic or induced plant defenses which then indirectly prevents *F. graminearum* from colonizing the plant; or by directly reducing growth or pathogenicity of *F. graminearum* via hyphal parasitism or antibiotic production.

Field implementation of a protective spray just prior to or during flowering (as when protective fungicides are applied to wheat) is possible, however the use of spray-based biocontrols is not ideal. Weather conditions could alter spray efficacy, and spray implementation generates

additional costs for growers (preparing solutions, and additional passes of equipment over the field).

Most of the strains tested here were protective when inoculated directly into the floret. However, we do not yet know which protective mechanism, as described above, is used by these strains. Future work on the identification of the mechanism, in addition to mapping the spread of colonization through the plant (one or more plant organs) is required. Due to sensitivity of microbial strains to abiotic factors, and competition from other microbes, inoculations with protective microbial cocktails would increase chances of success. Siou et al. (2015) have demonstrated that competition between fungal species commonly found on infected heads can affect the disease outcomes and have reported that co-inoculation of heads with multiple strains does not increase mycotoxin disease risk (Siou et al. 2015). Future work with these strains should include seed inoculations with combinations of microbes (microbes that utilize different methods of protection) to increase likelihood of successful protection. Once successful combinations of protective strains have been established, field trials should be pursued.

APPENDIX B

FUNCTIONAL ANALYSIS OF KNOCKOUTS OF TERPENE SYNTHASE GENES IN *FUSARIUM GRAMINEARUM*

Introduction

The ascomycete fungus *F. graminearum* Schwabe causes Head Blight, also known as Head Scab, of cereals. Head Blight has caused devastating yield losses in the last several decades (McMullen et al. 1997, 2012). Each spring this soil-borne fungus fires ascospores from perithecia, which land on the plant and colonize from the head down through the stem. *F. graminearum* hyphae and perithecia-initials overwinter on crop debris remaining on the soil surface (Trail 2009). Due to soil conservation efforts, there has been an increase in the prevalence of no-till farming, however, this comes with a tradeoff – higher disease pressure from *F. graminearum* (McMullen et al. 2012). *F. graminearum* is well-known not only for causing yield losses in crops, but also because it can produce an array of secondary metabolites, including mycotoxins, that impact both the agriculture industry and human health.

The genome of *F. graminearum* was sequenced in 2003 [www.broad.mit.edu/annotation/genome/fusarium_graminearum/Home.html]. The first draft of the annotation encoded 51 secondary metabolite genes (Cuomo et al. 2007). Secondary metabolites are chemicals produced during cell growth that are not involved in essential cell functions such as glycolysis, respiration, or cell division. There are four classes of enzymes producing secondary metabolites commonly produced by the ascomycete fungi and synthesized by *F. graminearum* – non-ribosomal peptide synthases, polyketide synthases terpene synthases (TPS), and cytochrome P450s (reviewed by Tudzynski 2005). Some of the most notable secondary metabolites in *F. graminearum* are deoxynivalenol, nivalenol, and their derivatives. Deoxynivalenol and nivalenol are trichothecene mycotoxins, made by terpene synthases, that have been extensively studied and affect the health of humans and livestock (Bennett and Klich 2003, Rocha et al. 2005). Zearalenone, which is synthesized by a polyketide synthase, is an estrogen-mimic which also causes health issues for

livestock and humans (Miksicek 1994, reviewed by Desjardins and Proctor 2007), and has been shown to be toxic to some fungal species (Utermark and Karlovsky 2007). Aurofusarin is a red pigment produced by polyketide synthase enzymes that gives *F. graminearum* mycelium their characteristic pink color (Kroken et al. 2003). Deoxynivalenol and zearalenone have been shown to stimulate *Alternaria* mycotoxin production and fungal growth (Müller et al. 2014). *Aspergillus fumigatus* epipolythiodioxopiperazine secondary metabolites (disulfide-containing cyclic peptides) also have been shown to have anti-fungal activity (Patron et al. 2007, Coleman et al. 2011).

We hypothesize that *F. graminearum* uses secondary metabolites to deter competitor microbes while overwintering on crop debris, as it has been demonstrated in other fungi that secondary metabolites can serve as antibiotic or self-defense molecules. Studies of *F. graminearum* growth while overwintering on crop debris would provide insight into how this fungus survives on dead plant tissue, a nutrient source which would be very attractive to, and under attack from saprotrophic microbes. An understanding of the cues that trigger production of *F. graminearum* secondary metabolites and their genetic regulation could provide insight into another possible control point in the life cycle of Head Blight.

Materials and Methods

Identification of Terpene Synthase Genes

A recent re-annotation of the *F. graminearum* genome classified 17 previously unannotated genes as TPS (Sieber et al. 2014). Previously published Affychip (Affymetrix, Santa Clara, CA, USA) microarray data from *F. graminearum* colonized wheat plants and straw (Hallen et al. 2007, Hallen and Trail 2008, Guenther et al. 2009, Hallen-Adams et al. 2011b) was re-normalized and

analyzed using the 'limma' package in R (Ritchie et al. 2015, R Core Team 2016). Gene expression patterns across the 17 TPS genes were analyzed. Corresponding GeneChip (Affymetrix, Santa Clara, CA) probe IDs and gene IDs from the current and previous versions of the *F. graminearum* genome are available in Table A-2 (King et al. 2015).

Table A-2. TPS gene IDs with greatest log-fold change in expression.

FGDB Gene ID	Current Gene ID	Affy Chip ID
FGSG_03066	FGRAMPH1_01G11973	fgd147-240_at
FGSG_06784	FGRAMPH1_01G23179	fgd275-400_at
FGSG_09381	FGRAMPH1_01G27225	fgd383-420_at
FGSG_10933	FGRAMPH1_01G20835	fgd455-20_at
FGSG_12186	FGRAMPH1_01G06541	fg02725_s_at, fgd136-30_at
FGSG_17725	FGRAMPH1_01G07277	fgd418-70_at

Homologous Gene Analysis

Gene sequences were downloaded from the *F. graminearum* genome browser: Munich Information Center for Protein Sequences (MIPS) *Fusarium graminearum* Genome Database (FGDB) version 3.2 [http://mips.helmholtz-muenchen.de/genre/proj/FGDB/]. NCBI Basic Local Alignment Search Tool (BLAST) (Altschul et al. 1990) was used to identify homologous genes in other *Fusarium* spp. Protein alignments were created using MAFFT version 7.351b (Katoh et al. 2017) and statistics on the alignment were calculated using Geneious version 6 (Biomatters Inc., Newark, NJ). Phyre2 was used to identify structural and functional domains of the predicated proteins (Kelley et al. 2015). NCBI BLASTp was used to search for paralogs within the genome of *F. graminearum*.

Generation and Genetic Analysis of Transformants

The wild-type *F. graminearum* strain PH-1 (NRRL #31084, FGSC #9075, Trail and Common 2000) was used for all genetic experiments. After confirmation of transformant strains, cultures were maintained as freezer stocks under 35% glycerol at -80°C. TPS Candidate genes were chosen based on log-fold changes in gene expression between the two infection stages analyzed. Primers used to generate and confirm fragments for putative transformants are listed in Table A-3. A split-marker strategy, which combined the flanking regions upstream and downstream of the target gene with one half of the hygromycin phosphatase (*hph*) gene (Carroll et al. 1994), was used to generate DNA constructs for deletion mutants of one of the six candidate genes (*FGSC_12186*). Homologous recombination, with *hph* as the selectable marker, was used to introduce the split-marker DNA fragments into *F. graminearum* protoplasts following the polyethylene glycol mediated transformation protocols of (Cavinder and Trail 2012). PCR screening by size shift was used for confirmation of putative transformants

Phenotypes of two confirmed knockout strains of *FGSC_12186* gene were determined as described below. To determine whether or not TPSs are involved in fungal self-defense, mutant cultures were challenged *in vitro* as described by Gdanetz and Trail (2017). Briefly, cultures were co-inoculated on malt extract agar medium plates and were scored into one of five interaction categories (Gdanetz and Trail 2017). Pathogenicity of mutants was assessed with wheat head infections; the middle floret was inoculated with 1×10^5 conidia ml^{-1}, as described previously (Hallen-Adams et al. 2011a, 2011b). Pathogenicity was also tested in seedling infections as described by Baldwin et al. (2010) and modified by Gdanetz and Trail (2017). Briefly, wheat seedlings were grown in vermiculite containing *F. graminearum* inoculum. A subset of seedlings was inoculated with protective fungal endophytes; 37 (*Alternaria tenuissima*), 38 (*Fusarium*

solani), 40 (*Fusarium* sp.), and 70 (*Fusarium oxysporum*); as described in Gdanetz and Trail (2017).

Perithecial development on wheat straw was induced by placing inoculated straw on the surface of moist sterile vermiculite, as described previously (Guenther et al. 2009). Perithecium formation on wheat straw was quantified as the percent nodes along the length of the stem with visible perithecia. *In vitro* perithecium production was induced on Carrot Agar medium (Klittich and Leslie 1988) by the addition of 900 ul of tween-60 as described previously (Trail and Common 2000). Quantity of perithecia generated on Carrot Agar medium were measured by counting the number of fruiting bodies on the surface of an agar plug taken with a 7-mm borer. Fruiting-body and ascospore development at 72, 96, and 120 hours post-tweening were measured as described previously (Cavinder et al. 2012). Conidia cultures were initiated with 1-ml of 1×10^5 conidia ml^{-1} into 100-ml of carboxymethyl cellulose medium (Cappellini and Peterson 1965). Conidia cultures were shaken at 225 rpm in a 25°C incubator, and conidia collected via filtering through three layers of sterile Miracloth (Millipore-Sigma, Burlington, MA, USA) before estimating concentration with a hemocytometer. Conidial production curves were calculated after measuring conidia concentrations at 48, 72, 96, and 120 hours.

Table A-3. Primers used to generate terpene synthase gene knockouts.

Primer	Sequence 5' to 3'	Description	PCR Product Length
12186-L5	TCT TTT GGA TGT TTG GAT G	[a]FP for flanking region upstream of *12186*	909
12186-L3	CGT CAG ATC GAT GGT AGT TGT CGT CGA CTC AAA GGC TGT GAA TTA TGT GTA G	[b]RP flanking region upstream of *12186* with 5' overhang for *hph*	
12186-R5	ACA CTG GTG ACG GCT AAC CAG AAC TGT CAA ACA TAT TCG AGA TAT AAC CCC	RP flanking region downstream of *12186* with 3' overhang for *hph*	588
12186-R3	GGG TGT GCT TCA TTT CAT	FP flanking region downstream of *12186*	
HYG-F	AGTCGACGACAACTACCATCG ATCTGACGACGCCTGGTTGCTA CGCCTGAATAAGTG	FP to amplify *hph* from pCB1004	1,867
HYG-R	TGACAGTTCTGGTTAGCCGTCA CCAGTGTAACGCTGGTGAAAG TAAAAGATGCTGAAGAT	RP to amplify *hph* from pCB1004	
YG-F	GTATTGACCGATTCCTTGCGGT CCGAA	FP for *hph* overlapping region, pair with HYG-R	1,217
HY-R	CGATGTAGGAGGGCGTGGATA TGTCC	RP for *hph* overlapping region, pair with HYG-F	966

[a]FP = forward primer
[b]RP = reverse primer

Results and Discussion

Identification of Terpene Synthase Genes and Homologous Gene Analysis

Six of the 17 putative TPS *F. graminearum* genes had increased levels of gene expression on straw, when compared to gene expression during plant infection (Figure A-3). Orthologs of *F. graminearum* TPS genes were identified throughout the *Fusarium* genus, and functional information was available for four of the six TPS orthologues in *Fusarium fujikuroi* (Homann et al. 1996, Mende et al. 1997, Linnemannstöns et al. 2002, Zhao et al. 2010). Nucleotide alignments of the TPS genes and their orthologs indicated they are conserved across *Fusarium* spp. (Table A-4). Phyre2 analysis of *F. graminearum* TPS protein sequences and the corresponding *F. fujikuroi* homologs indicated 73% of the residues modelled at >90% confidence, indicating secondary and tertiary structures of *F. graminearum* and *F. fujikuroi* TPS are similar. Based on Phyre2 analysis, we hypothesize the TPS homologs in *F. graminearum* and *F. fujikuroi* could have similar functions. Functional and chemical analysis of *F. graminearum* gene products is needed to confirm this hypothesis.

Table A-4. Summary of *Fusarium* spp. sequence alignments.

Gene ID	Num. in MSA	Length (bp)	Identical sites (bp)	Pairwise % Identity
FGSG_03066	9	619	331	83.6
FGSG_06784	6	907	280	83.3
FGSG_09381	9	463	366	91.6
FGSG_10933	10	457	396	95.3
FGSG_12186	9	526	243	76.4
FGSG_17725	9	497	325	91.1

Confirmation of Mutant Phenotypes

Pathogenicity of one mutant strain, Δ*12186.3B*, during head infection was significantly reduced when compared with wild-type (Figure A-4A). Perithecium development on straw was not significantly different between wild-type and knockout strains. Perithecia were formed through stomata down to the second node in all strains (Figure A-4B). Numbers of *in vitro* perithecium produced were not significantly different across the strains (Figure A-4C). Disease incidence, during seedling infection or after challenging against protective endophytes, did not differ across wildtype and mutant strains (Figure A-5). Conidia production of the wild-type strain was higher than the production of both mutant strains (Figure A-6). The rates of production of conidia by both mutant strains were significantly reduced from the wild-type when measured at 72 hours (Figure A-6).

The function of the *F. graminearum* TPS gene, *FGSC_12186,* does not appear to be involved in pathogenicity or perithecium development. However, it may be involved in conidial germination or growth, as the mutant strains Δ*12186.2B* and Δ*12186.3B* had reduced conidial production compared to wild-type *F. graminearum*. The rates of conidial production differed between the two mutant strains, therefore generation and phenotypic analysis of additional Δ*12186* strains will confirm the reduced conidia production phenotype. Complementation of the FGSC_12186 gene in the mutant strains is also required to verify the conidial production phenotype. Future work should focus on chemical or metabolite analysis of the wild-type and the Δ*12186* strains. Additionally, comparison of *F. graminearum* TPS gene products to the known products of *F. fujikuroi* TPS genes would confirm if the sequence homology and subsequent similar structural predictions from Phyre2 analysis are accurate.

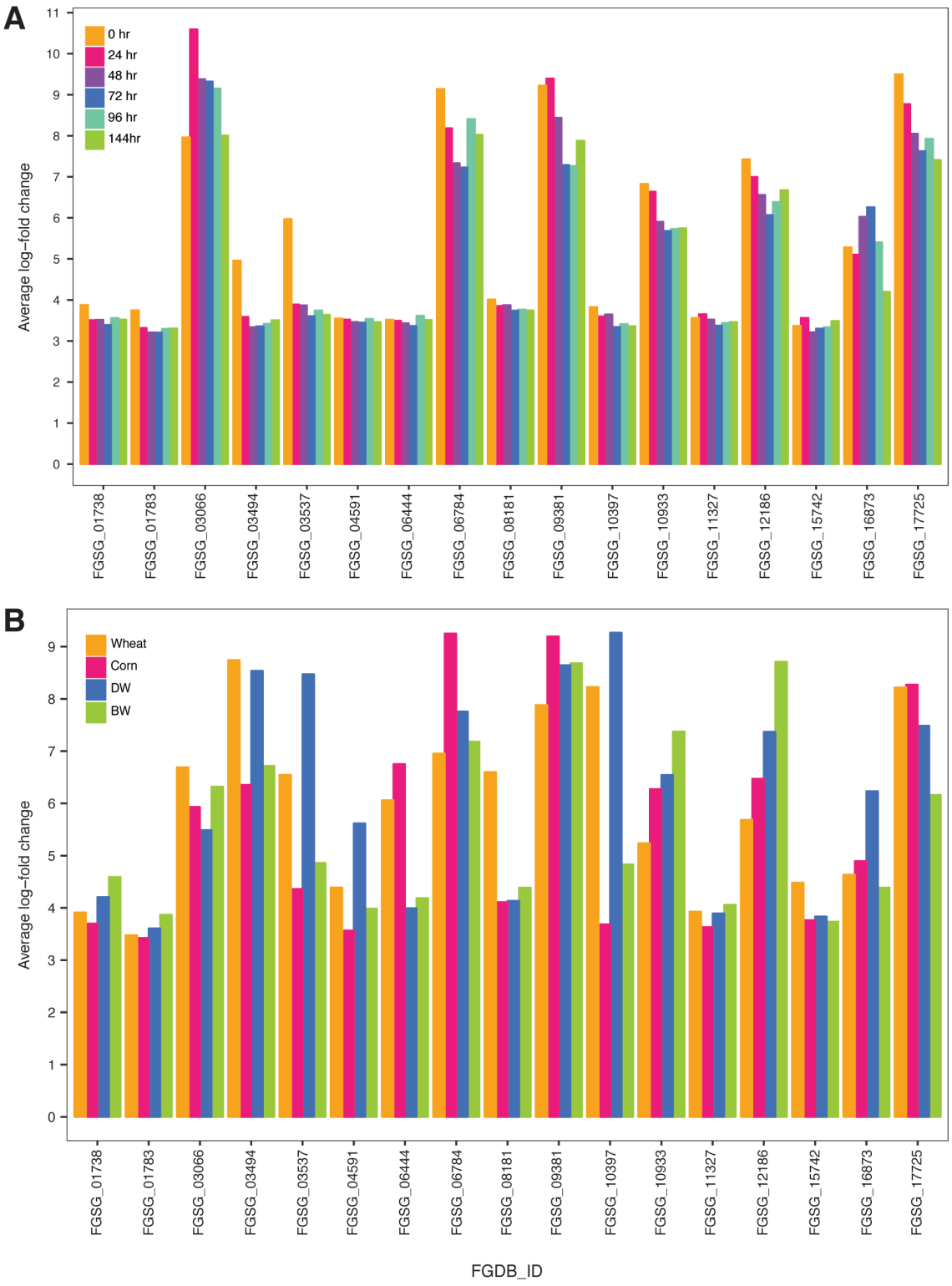

Figure A-3. Relative gene expression of *F. graminearum* terpene synthase genes. Expression levels during perithecia development on straw (A), and during plant infection (B), DW and BW indicate barley cultivars. Expression data from Hallen et al. 2007, Hallen and Trail 2008, Guenther et al. 2009, Hallen-Adams et al. 2011b.

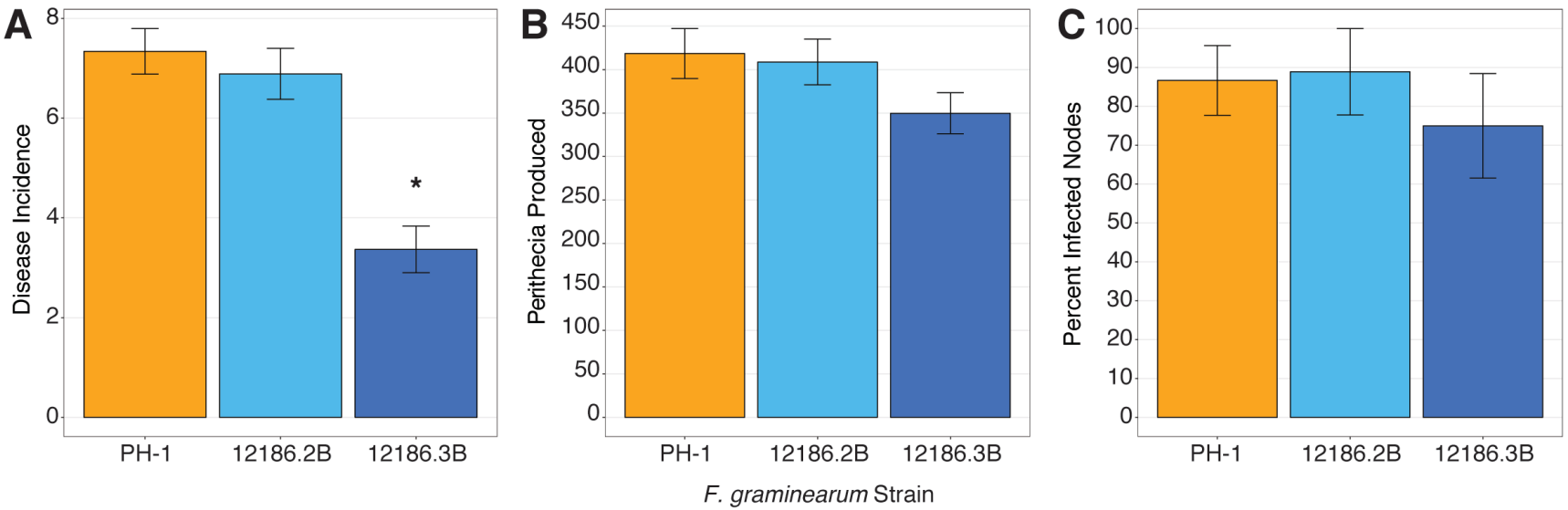

Figure A-4. Disease incidence of wild-type (PH-1) and independent knockout strains of FGSC_12186 during wheat head infection (A), distribution of perithecia production on wheat straw (B), and *in vitro* perithecia production (C). Bars in panel A represents the average of ten or more inoculated plants, repeated three independent times. Bars in panel B represent three sets of inoculate stems, repeated three independent times. Bars in panel C represent the number of perithecia on two cores per plate, six independent plates. Errors bars indicate standard deviation of the mean. Analysis of variance and Tukey's honest significant difference were used to test significance; asterisk indicates significance ($p < 0.001$).

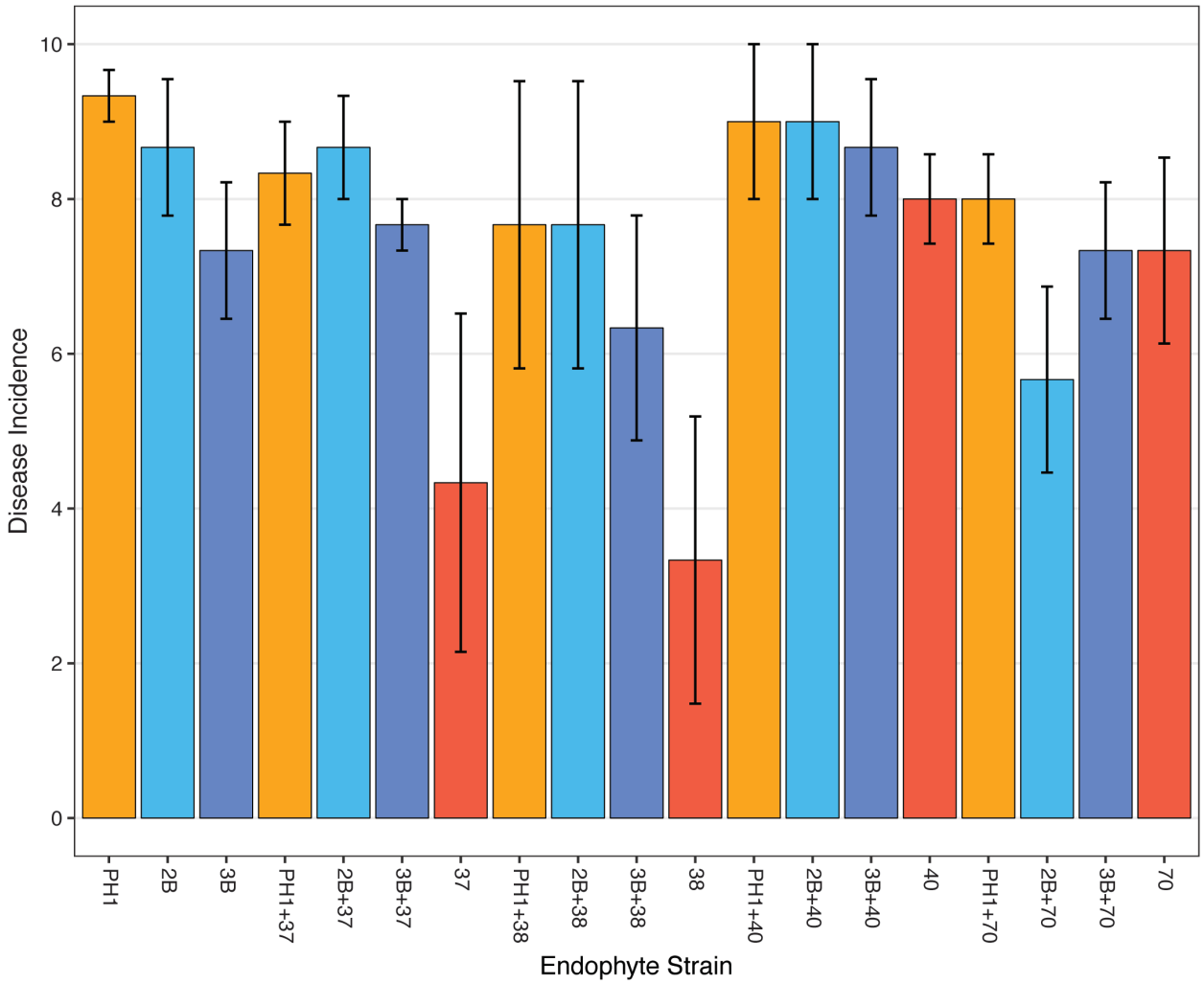

Figure A-5. Disease incidence of wild-type (PH1) and knockout strains during seedling infection. Subset of seedlings were also inoculated with best protective endophytes. Each bar represents ten plants repeated two independent times. 2B = Δ*12186.2B*; 3B = Δ*12186.3B*; 37, 38, 40, 70 are fungal endophyte strains from Gdanetz and Trail, 2017.

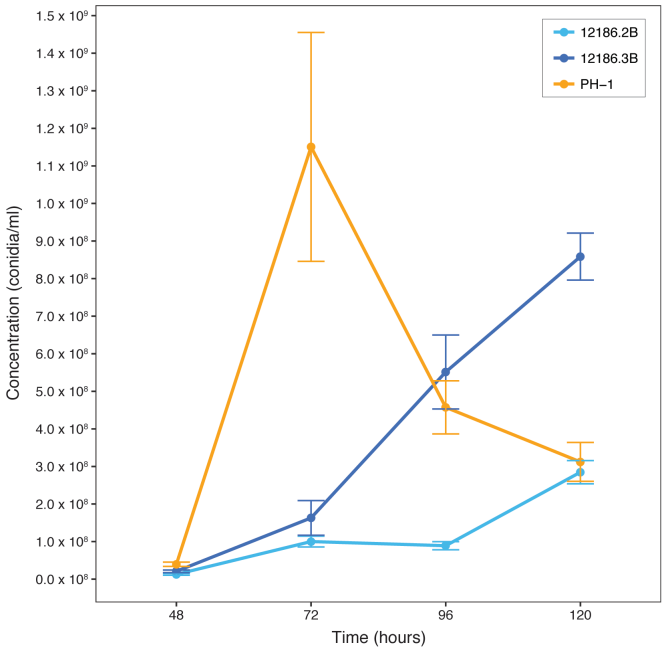

Figure A-6. Conidia growth curves of wild-type (PH-1) and knockout strains. Each point represents the average of three independent replicates, ± standard error of the mean. Analysis of variance and Tukey's honest significant difference were used to test significance. PH-1 displayed significantly higher rate of conidia production than both knockout strains at 72 hours, and higher conidia production than Δ*12186.2B* at 96 hours ($p < 0.05$). Strain Δ*12186.3B* displayed significantly higher conidia production than PH-1 and Δ*12186.2B* at 120 hours ($p < 0.05$).

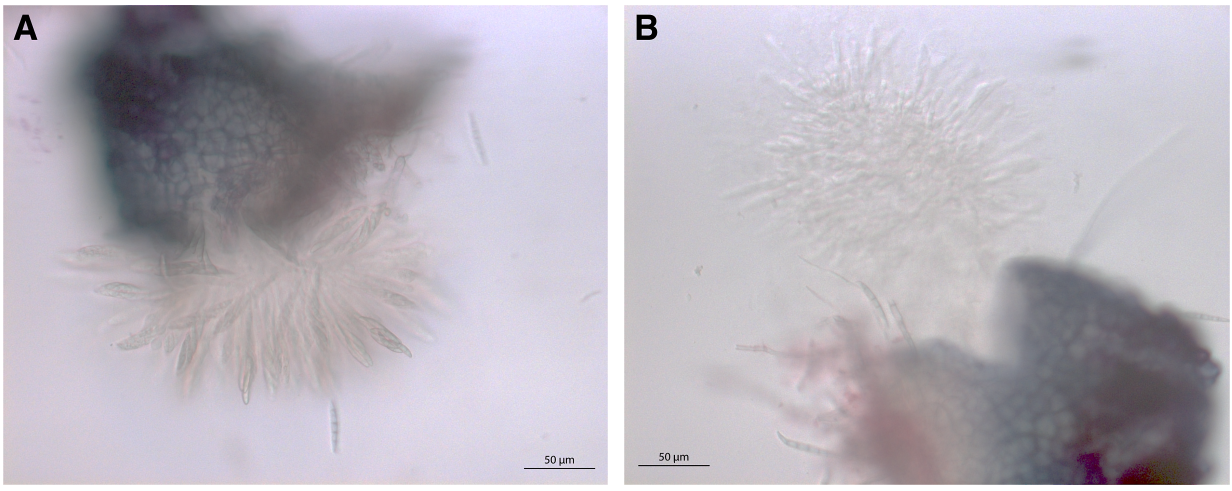

Figure A-7. *In vitro* perithecium development. Squash mounts of *F. graminearum* perithecia at 120 hours post-induction of sexual development, showing mature asci of wild-type (A) and delayed development of knockout strain *12186.3B* (B).

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