ON GERMS AND GERMINATION: UNCOVERING THE HIDDEN ECOLOGY OF SEEDBORNE BACTERIA AND FUNGI IN OPEN-POLLINATED MAIZE

by

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DISSERTATION ABSTRACT

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Plants are inhabited by diverse species of bacteria and fungi, which affect plant health and fitness. Endophytes are bacteria or fungi that live within plant host tissues without causing symptoms of disease, and mediate important plant traits in agriculture, such as nutrient acquisition, disease resistance, and abiotic stress tolerance. However, we know little about the general ecology of endophytes, including which factors determine their compositions within plants. Seedborne transmission may represent an important source of bacterial and fungal endophytes, which can significantly impact the plant microbiome and plant traits. However, seeds are also a vehicle for transmission of plant pathogens. Seeds are commonly treated to control against seedborne pathogens, and increasingly bacteria and fungi are inoculated onto seeds to serve as biological control against pathogens. My dissertation explores the theoretical and applied ecology of seedborne endophytes of maize, including their interactions with pathogenic *Fusarium* fungi, and with seed treatments designed to control *Fusarium*.

In Chapter II, I examine factors that affect the transmission of seedborne fungal endophytes and *Fusarium* into maize seedlings, including the influence of soil microbiota, and the impact of disinfection and biological control seed treatments. In Chapter III, I determine the long-term effects of seed disinfection and biological control

inoculants on maize bacterial and fungal endophytes and *Fusarium* pathogenicity across three different farms. In Chapter IV, I recruit maize seed growers across the Pacific Northwest and U.S. to participate in a broad scale study of seedborne endophytes. Across submitted seed samples, I find commonly occurring seedborne endophyte taxa, and delineate how maize varieties interact with environmental factors to affect the composition and diversity of seed-associated endophytes in seeds. Throughout these chapters, I explore the potential applications of seedborne endophytes in agriculture, particularly as a source for biological control against *Fusarium* in maize, and speculate how seed treatments can have significant, lasting impacts on the plant microbiome.

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This work is dedicated to my wife Alese Colehour,
and our yet unborn baby Cassidy, whom she carried
while I finalized the dissertation chapters herein.
To turning the page, and planting seeds for
this exciting new chapter in our lives.

TABLE OF CONTENTS

LIST OF FIGURES

Figure Page

LIST OF TABLES

CHAPTER I

INTRODUCTION TO SEEDBORNE ENDOPHYTES

Plant associated microbes are integral to plant functioning and survival, influencing important plant traits such as nutrient acquisition, secondary metabolite production (Mousa & Raizada, 2013; Schmidt et al., 2014), immune functioning (Doornbos, Loon, & Bakker, 2011), pathogen resistance (Cavaglieri et al.,2005), and abiotic stress tolerance (Rodriguez et al., 2008). The plant microbiome represents all microbes that live in and on the plant. Scientists have delineated the plant microbiome into three interacting spheres, (i) the rhizosphere, (ii) the phyllosphere, and (iii) the endosphere, each denoting distinct microbial habitats in a plant (Lundberg et al., 2012; Partida-Martínez & Heil, 2011). The rhizosphere and phyllosphere represent the microbial habitats on the plant's surfaces in contact with the soil and the air, respectively. In contrast, the endosphere is considered any microbial habitat that is embedded within plant tissues, whether between plant cells, in the apoplast (inter-cell compartment), or within the plant vascular system. Microbes inhabiting the endosphere without causing disease are collectively known as ***endophytes*** (Stone, Bacon, & White, 2000)**.** Thus, known plant pathogens are generally not regarded to be endophytes. However, it is difficult to categorize whether a particular endophyte is generally beneficial or harmful to the plant; endophyte effects on plant fitness exist on a continuum, which can be highly context-dependent. For example, the fungus *Fusarium verticillioides* has been observed to protect maize from maize smut fungus *Ustilago maydis* in some cases (Lee, Pan, & May, 2009), but it is also classified a potent pathogen of maize (Bacon, Glenn, & Yates, 2008).

In practice, scientists identify endophytes as those microbes that remain in plant tissues after sterilizing the surface of the plant with bleach, ethanol, detergent, and/or sonication (Bodenhausen, Horton, & Bergelson, 2013; Schlaeppi et al.,2014). Once the surface-associated microbes have been removed, the remaining, 'endophytic' fraction of microbes may be identified either via (i) culture-dependent isolation or culture-independent DNA-based methods. Culturing methods typically entail placing surface-sterilized plant tissues on microbial growth media, where the microbes will grow out of the tissue onto the media. However, culture-based methods only recover the subset of bacterial and fungal endophytes that are able to grow on a given culture medium (Pereira et al.,2011). By performing DNA extraction, we are able to retain all microbial DNA that is present in the plant, and should theoretically be able to classify all microbes present in a given plant tissue. However, primer sets used to amplify microbial DNA are biased in which species they will amplify, possibly resulting in inaccurate microbial community representations due to missing taxa (Kennedy et al.,2014). Furthermore, the presence of a microbial DNA sequence does not indicate whether the microbe is alive or active (Singer, Wagner, & Woyke, 2017), and we cannot definitely confirm microbial functional traits using this method. Culture-independent methods ultimately rely on databases produced by culture-dependent methods for taxonomic identification and putative microbial functional characterization (Langille et al., 2013; Louca, Parfrey, & Doebeli, 2016). Both methods are complementary and ideally are best applied together, to optimally characterize the endophyte microbiome.

Scientists use the term ***competent endophyte*** to distinguish microbes adapted to persist in the plant environment from those more transient, incidental colonizers of the

plant. By definition, competent endophytes those that are equipped with the underlying genes necessary for the maintenance of endophytic symbiosis with the plant (Hardoim, van Overbeek, & Elsas, 2008). Common traits that may distinguish competent endophytes from incidental ones include motility mechanisms (e.g., flagella in bacteria) for moving through the plant, and biochemical capacity to modulate the plant's immune response (e.g., ethylene), or degrade phytotoxins (Hardoim et al., 2008; Saunders & Kohn, 2009). Comparative genomics offers some insight into the genomic strategies that microbes use to achieve an endophytic lifestyle (Taghavi et al., 2010). For example, one study compared an endophytic *Azoarcus* sp. strain to a similar strain that naturally occurs in soils, and suggested that endophytes may have a streamlined genome adapted to evading the plant's innate immune system and growing in an otherwise energy-rich, benign plant environment (Krause et al., 2006).

TRANSMISSION OF ENDOPHYTES IN SEEDS

For plant-endophyte symbioses to persist through evolutionary time, endophytic microbes must effectively transmit from plant parents to their offspring. Many endophytes are capable of horizontal transmission into a plant host via dispersal in air or soil, from other hosts or other habitats in the environment (Carroll, 1988; Thomas et al., 2016). Alternatively to horizontal transmission, endophytes may undergo vertical transmission across plant generations via the seed. Seedborne vertical transmission has been well-documented in both fungal and bacterial plant pathogens, e.g., *Fusarium* spp. in maize (Munkvold, McGee, & Carlton, 1997), in addition to *Epichloë* endophytes in fescue (Schardl, Leuchtmann, & Spiering, 2004). A broad diversity of viable

microorganisms have been isolated from the seeds of many plant species, including grasses, legumes, conifers, and cacti, many of which appear to exhibit plant-beneficial traits, such as nutrient solubilization, production of growth hormones, nitrogen fixation, and suppression of pathogens (Ganley & Newcombe, 2006; Johnston-Monje & Raizada, 2011; Liu et al., 2012a; López-López et al., 2010; Okunishi et al., 2005; Puente, Li, & Bashan, 2009). However, very little is known about the general ecology of seedborne endophytes, in part because it is difficult to ascertain the efficiency of vertical transmission of seedborne endophytes to seedlings (Tintjer, Leuchtmann, & Clay, 2012), and the short- and long-term impacts they have on plants once inherited (Shade, Jacques, & Barret, 2017).

Although vertical transmission may, in theory, be an effective means for endophytes to persist in plant populations, seedborne endophytes face several challenges. First of all, endophytes need to navigate the plant's defenses to enter the seed. Then, microbes must be able to survive desiccation and high osmotic stress of the drying seed, which coincides with a marked decline in the diversity of seed-inhabiting bacteria (Liu et al., 2012a). Finally, microbes must be able to proliferate during seed germination, which requires rapid growth and competition with other microorganisms from the seed and soil for a limited niche (Barret et al., 2014; Nelson, 2004). Vertical transmission is thus considered to be an inherently risky ecological strategy for endophytes (Afkhami & Rudgers, 2008), so the representation of seedborne endophytes that can survive across generations may be relatively small, compared to horizontally transmitted soilborne and airborne microbes.

If seedborne transmission is a viable life history strategy for endophytes, it opens

up the theoretical possibility for co-evolution between plants and their symbionts, in a process described as **hereditary symbiosis** (Clay, 1994). The seedborne transmission strategy is predicated on the plant host producing viable offspring, i.e., it is intimately tied to host fitness, suggesting that a host-symbiont mutualism is possible through partner fidelity feedback. Indeed, there are field observations to support this association, e.g., between the seedborne fungal endophtye *Neotyphodium* and fescue grasses *Lolium arundinaceum* and *Festuca* spp. (Clay & Schardl, 2002). However, hereditary symbiosis does not necessarily imply a mutualistic relationship, with seedborne pathogens being a clear example. If a seedborne endophyte has efficient dispersal mechanisms, i.e., a high efficiency of horizontal transmission between hosts, then it can persist in host meta-populations through evolutionary time without necessitating a positive contribution to plant fitness (Saikkonen, Ion, & Gyllenberg, 2002). Moreover, whether a seedborne endosymbiont is a mutualist or a parasite is strongly context-dependent. Previous studies have observed that seedborne transmission of the fungal endophyte increases when environmental factors cause it to be beneficial to plants in the system, and decreases when it no longer serves the plant. For example, *Neotyphodium* can protect fescue plants against herbivores due to production of mycotoxins and high herbivore pressure can result in a 30% increase in seedborne transmission rates (Clay, Holah, & Rudgers, 2005). Additionally, this fungal association enables plants to flower sooner in water limited conditions, allowing plants to escape from drought, thus perpetuating a mutualistic relationship between plant and seedborne endophytes during drought (Davitt, Chen, & Rudgers, 2011). However, under more ideal plant conditions, such as low herbivore pressure or higher water availability, the endophyte functions more as a plant

parasite, and a lower transmission of seedborne endophytes is observed.

Although little is yet known about the significance of seedborne bacterial endophytes in agriculture, there has been important research in maize. For example, Johnston-Monje and Raizada (2011) collected diverse maize seeds, including modern hybrids, ancestral teosintes, and indigenous landraces. Using DNA-based community profiling methods, the group observed that seedborne bacterial communities differ in accordance with the genetic distance of maize lines, even after planting all maize lines in a common field to account for environmental effects. Moreover, bacteria isolated from these seeds exhibited a range of plant-beneficial traits, including phosphate solubilization, nitrogen fixation, and plant growth promotion, and were able to proliferate in roots and the rhizosphere after being injected in the stem of plants. In subsequent experiments, it was found that the majority of bacterial 16S rDNA sequences recovered from the endosphere and rhizosphere of maize seedlings exactly matched bacterial 16S rDNA sequences that were present in seeds at planting (Johnston-Monje et al., 2016; Johnston-Monje et al., 2014), suggesting that the maize microbiome has a high degree of vertical transmission of seedborne bacteria. There is also evidence for significant vertical transmission of bacteria in wheat (Robinson et al., 2016), rice (Hardoim, van Overbeek, & van Elsas, 2012) and *Arabidopsis* (Truyens et al., 2014). However, DNA-sequence matching methods cannot definitely account for other, unmeasured sources of endophytes such as the soil or air, so scientists caution that this circumstantial evidence must be confirmed by tagging seedborne endophytes using molecular methods to directly observe transmission across plant generations (Shade et al., 2017).

FUSARIUM AND MAIZE

Fungi from the genus *Fusarium* are highly associated with maize, nearly ubiquitous as an endophyte or pathogen. A common seedborne microbe, *Fusarium* species notoriously produce fumonisins, T2-toxin and zealerones, among other deadly mycotoxins. *Fusarium* and maize appear to have a long evolutionary history, as supported by the observation that maize's teosinte ancestor shares the same *F. verticillioides* mating type (i.e., mating population A) as modern maize (Kvas et al., 2009). This evolutionary association may be attributed to the fungus' vertical transmission in seeds, its ability to survive in soils or in maize residues, and its biochemical capacity to degrade key defensive toxins of maize, known as benoxazinoids (BX). Over a dozen species of *Fusarium* endophytes isolated from maize share the ability to tolerate BOA (2-benzoxazolinone, a toxic byproduct of BX) by degrading it into benign products (Saunders & Kohn, 2009). BOA-tolerant Fusaria include *F. verticillioides, F. subglutinans, F. proliferatum,* and *F. graminearum*. Interestingly, the abundance of *Fusarium* species was observed to be 35 times higher in the leaves of BX-producing maize than non-BX producing maize (Saunders, Glenn, & Kohn, 2010). So positive is the association between *Fusarium* species and BX, that Glenn et al., were able utilize a toxigenic BX derivative, BOA, in Nash-Schneider medium as a selective medium that is highly specific to *Fusarium* endophytes, particularly *F. verticilliodes*, from maize (Glenn et al., 2001). Practically speaking, the near-ubiquity of *Fusarium* as a maize endophyte suggests that eradication is not a tractable solution, and efforts should be made to understand its ecology so that it may be managed to effectively curb its over-dominance as an endophyte, and mitigate its tendencies to become pathogenic and

produce mycotoxins.

OVERVIEW OF DISSERTATION CHAPTERS

This dissertation is a summary of my research efforts to explore the basic and applied ecology of seedborne endophytes, presented in three chapters that are intended to become stand-alone publications. The chapters are arranged along a continuum of spatial and temporal scales that intersect with the ecology of seedborne endophytes. I begin in Chapter II with a maize seedling experiment that addresses short-term interactions between seedborne endophytes, biological and antimicrobial seed treatments, and the soil microbiome. In Chapter III, I explore the impacts of seed treatments on the microbiome of adult plants and the seeds that they produce, across three different farms in the Willamette Valley, OR. Lastly, Chapter IV describes a broad census of seed-associated microbes sourced from multiple seed growers, maize varieties and years. Across all of these spatial and temporal scales I assess the potential importance of the seedborne microbiome for the plant, their interactions with the highly ubiquitous fungal endophyte *Fusarium*, and generalizable patterns across multiple plants and farms. My study system is situated within a local network of plant breeders, farmers and seed savers. I focused primarily on a flint corn variety, Cascade Ruby Gold, that is bred to produce well in the Willamette Valley, and to have high culinary value for gardeners, chefs and local distributors, and I studied this maize variety among organic farmers who are actively growing it. Furthermore, as the Pacific Northwest is internationally recognized for organic seed production, I was able to recruit seed savers to participate through extensive networks established and maintained by the organic seed movement. Thus, my

dissertation contributes to fundamental theory regarding the ecology and evolution of endophytes, and simultaneously has the potential to directly benefit the plant breeders, farmers, and seed savers involved in this study, and in the broader community of which they are a part.

CHAPTER II

SEED AND SOIL TREATMENTS EFFECT VERTICAL TRANSMISSION RATES OF FUNGAL SEEDBORNE ENDOPHYTES IN MAIZE SEEDLINGS

INTRODUCTION

The plant microbiome is an important determinant of plant traits, and thus has been referred to as the plant's second genome (Berg, 2009). The plant microbiome includes surface-associated bacteria and fungi present at the root-soil interface (i.e., the rhizosphere), aboveground plant surfaces (i.e., the phyllosphere), and the inside of the plant, (i.e., the endosphere). Microbes inhabiting the endosphere without causing disease are collectively called endophytes (Stone et al., 2000). Endophytes are known to mediate agronomically-important plant traits, including plant nutrient use efficiency, abiotic stress tolerance, and pest and pathogen resistance (Hardoim et al., 2008; R. J. Rodriguez, White, Arnold, & Redman, 2009). Endophyte microbiome assembly appears to be influenced by a combination of factors including plant host age, genotype, environment, and genotype by environment (GxE) interactions (David, Seabloom, & May, 2017; Lundberg et al., 2012; Porras-Alfaro & Bayman, 2011; Wagner et al., 2016). Much of our conceptual understanding of the endophyte microbiome is based on the observation that endophytes colonize plants via dispersal in the soil or the air, (i.e., via horizontal transmission). However, fungal and bacterial endophytes can also enter the plant via seeds in a process called vertical transmission, effectively transmitting across plant host generations (Gundel, Rudgers, & Ghersa, 2011; Truyens et al., 2014). The process of vertical transmission of endophytes introduces an additional layer of complexity to our

understanding of the forces that shape the endophyte microbiome. For example, seeds may carry endophytes sourced from different environments in which plants were grown, confounding our interpretation of genotype, environment, and GxE interactions. Furthermore, seedborne endophytes may have a disproportionate influence on plant microbiome assembly and functioning, due to their presence during the initial formation of the plant microbiome (Shade et al., 2017).

Seedborne endophytes have been described in a number of plant types, including maize (Johnston-Monje & Raizada, 2011; Liu et al., 2012b), rice (Hardoim et al., 2012), wheat (Coombs & Franco, 2003; Robinson et al., 2016), beans (López-López et al., 2010; Parsa et al., 2016), forbes (Hodgson et al., 2014) and cacti (Puente et al., 2009). These studies and others have found that many seedborne endophyte isolates exhibit plant-beneficial traits. Based on culture-independent DNA sequencing methods, it has been claimed that the majority of both rhizosphere and endosphere bacteria in maize seedlings originate from the seed (Johnston-Monje et al., 2016, 2014). However, it is difficult to definitively ascertain the general rate of vertical transmission of seedborne bacteria and fungi, and their impacts on the plant microbiome and plant fitness. Much of what we know about the effects of seedborne fungal endophytes comes from research regarding the vertically transmitted fungal endophyte *Neotyphodium/Epichloë* which infects grass species. The *Neotyphodium* endophyte significantly increases the fitness of fescue grasses in cases of high herbivore pressure (Clay et al., 2005) or drought stress (Davitt et al., 2011), but exhibits a neutral or negative effect on plant fitness in other environmental contexts. Furthermore, the rate of vertical transmission of fungal endophytes may be highly variable and context-dependent (Afkhami & Rudgers, 2008; Tintjer et al., 2012).

Seedborne pathogens remain a perennial problem in agriculture, prompting seed companies and farmers to treat seeds against pathogens (Maude, 1996; Nega et al., 2003). For example, fungi from the genus *Fusarium* are nearly ubiquitous in maize (Saunders & Kohn, 2008), and commonly vertically transmitted in the seed, in addition to horizontal transmission via soil or aerial spores (Munkvold et al., 1997). Furthermore, seedborne *Fusarium* can produce mycotoxins, including fumonisins, trichothecenes and zealerones, which negatively impact the health of people and livestock when ingested (Munkvold, 2003). In some cases, seedborne *Fusarium* in maize can be controlled using hot water seed treatment methods (Rahman et al., 2008), fungicides (Pscheidt & Ocamb, 2017) and biological control inoculants (Bacon et al., 2001; Cavaglieri et al., 2005). However, antimicrobial seed treatments may disrupt the vertical transmission of other endophytes, theoretically leaving open niche space for the colonization of soilborne pathogens, including soilborne *Fusarium*. Biological control inoculants hold promise for controlling pathogens such as *Fusarium* through direct antagonism. However, inoculants are often inconsistent, likely due to the complex ecology associated with establishment and maintenance of inoculants in the plant microbiome (Hawkes & Connor, 2017). In theory, seedborne endophytes may be a good source for biocontrol against seedborne pathogens, as the symbionts are likely to be competing for access to limited niche space within the seed and the germinating seedling.

We conducted a seedling experiment to determine the importance of seedborne fungal endophytes in the development and assembly of the maize microbiome, and additionally to ascertain if seed treatments would affect their vertical transmission rates. To determine the relative contribution of seedborne vs. soilborne fungi to the maize

seedling microbiome, we planted seeds into either raw soil or gamma-irradiated soil, and subjected the seeds to various seed treatments, including disinfection, biocontrol inoculation, and fungicides. During preliminary research, we had isolated and identified bacterial endophytes from maize seeds, and then screened them to find those that were antagonistic to a seed-associated Fusarium pathogen. Once we found a suitable biocontrol bacterium, we enlisted it in the seedling experiment. We also inoculated a subset of soils with a pathogenic Fusarium oxysporum strain to determine the interactions between seedborne endophytes, seed treatments and pathogenesis. We hypothesized that (i) seedlings grown in irradiated soil will contain a higher proportion of seed-associated endophytes than those grown in untreated, raw soil, and that (ii) seed disinfection would reduce seedborne transmission of endophytes, making seedlings more susceptible to horizontal transmission of soil microbes (including pathogens) into the endosphere. Furthermore, we hypothesized that (iii) antimicrobial and biocontrol seed treatments would significantly alter the assembly of the maize fungal endophyte microbiome, and finally that (iv) seed disinfection would increase the effectiveness of the biocontrol inoculant.

MATERIALS AND METHODS

Seed Source

We chose the locally-grown northern flint type maize variety, Cascade Ruby-Gold, for this study, due to its local popularity among growers. This open-pollinated maize variety was bred locally by Carol Deppe (Corvallis, OR, personal comm.) through recurrent selection, i.e., the traditional method used to prevent inbreeding depression.

From a field population of hundreds of openly interbreeding plants, tens to hundreds of plants are selected for ideal traits (yield, color, ear quality, flavor) and the process is repeated with each successive generation. Although we did not measure plant genetics, this open-pollinated variety is presumed to be significantly more genetically-diverse than hybrids and inbreds. We assumed the higher plant genetic diversity would also provide us with a greater diversity of seedborne endophytes (Peiffer et al., 2013).

Bacterial Endophyte Isolation and Screening

During previous field studies we isolated bacterial endophytes from maize crown, seed and stem tissue. We surface-sterilized seeds using a standard protocol (Thomas et al., 2016), by soaking them in 3% hypochlorite solution for 3 minutes, followed by a rinse in 95% ethanol, and then with sterile water, and placed seeds for 1 min on Potato Dextrose Agar (Difco/Beckton Dickonson, Franklin Lakes, NJ, USA) plates before transferring them to test for the absence of surface-associated microbes. In some cases, we first soaked seeds overnight, and split seeds in halves and quarters using a sterile scalpel. To isolate endophytes from 4-week old seedling stems and 8-week old crowns, we made a transverse cut down the plant stem, splitting it in two halves. Crowns were cut in the field while imaging for disease (see below), and young stems were cut aseptically in a biosafety cabinet. As we assumed crown surfaces were contaminated, we rinsed them with 95% ethanol and them flame-sterilized the open surface. Then, we scraped off the surface with a sterile scalpel and extracted tissue underneath. Excised crown and stem tissue and surface sterilized seeds were placed on half strength Potato Dextrose Agar for up to two weeks.

We selected bacterial isolates as they became visible on the growth media,

streaking them on a new PDA plate to obtain a single strain. To identify isolates, we extracted DNA, PCR-amplified the 799F-1792R 16S rDNA region, and sent samples for Sanger sequencing at Functional Biosciences (Madison, WI), as outlined in the honors thesis by Wesley Horton (Horton, 2015). Based upon sequences matched in the BLAST nucleotide database (blast.ncbi.nlm.nih.gov), we were able to identify most isolates to the species level (Table S1). We also determined if any of the recovered isolate sequences matched bacterial endophyte sequences recovered by culture-independent Illumina MiSeq sequencing of 16Sr DNA amplicons from crown and seed samples obtained during the 2013 and 2014 field season (See Chapter III).

We next screened for bacteria that would be competent in the maize endosphere by determining the effect of maize phytotoxin BOA (Alfa Aesar, Harverhill, MA; 0.5 mg/mL BOA in Potato Dextrose Broth) on their growth in liquid media. Selecting the four most BOA-tolerant bacterial isolates, we screened their capacity to slow the growth of *Fusarium* spp. in vitro, both in PDA plates, and PDA amended with BOA. Finally, the top three bacterial endophytes that inhibited a pathogenic *Fusarium subglutinans* isolate F96 were screened directly *in planta* via a 7-day, soil-free maize seedling trial. Seeds were soaked in bacterial endophyte inoculant before they were germinated on water agar, and a *Fusarium* spore suspension was added directly to their emerging radicals. Based on this trial, bacterial isolate *Arthrobacter ilicis* strain M97 significantly reduced the severity of disease symptoms in 7-day-old maize seedlings (Horton, 2015).

Seedling Experimental Setup

The seedling experiment setup involved seedlings planted in Ray Leach "cone-tainers" (RLC4; Stuewe and Sons, Corvallis, OR), arranged in a tray (Product RL98)

within a refrigerator incubator maintained at 25°C, equipped with overhead fluorescent lighting (four T5 bulbs). There were five seed treatments: Control, Disinfection, Inoculation, Disinfection + Inoculation, and Fungicide. These treatments were planted into four soil treatments, Raw, Irradiated, Raw + Pathogen, Irradiated + Pathogen. Each seed treatment-soil treatment combination contained 8 replicates, for a total of 160 seedlings. We randomized treatments into replicate blocks across the tray to account for spatial heterogeneity (Figure 1). We sterilized seedling containers before the experiment, by soaking for 1 hour in 0.3% sodium hypochlorite solution.

Soil was sourced from the upper 20 cm of topsoil of Moondogs organic farm near Marcola, OR, and is classified as a Cloquato silty clay loam. The soil was first passed through a 2 mm sieve, homogenized, and kept at 4°C for two weeks before the experiment. We sterilized a subsample of soil using gamma-irradiation, at a dose of 2.5 Mrad (Oregon State TRIGA Reactor).

At planting, we first added soil to half the volume of the containers, at which point we added our liquid inoculum of pathogenic *Fusarium oxysporum* isolate 37. The *F. oxysporum* cultures had been grown in Schneider and Nash Agar medium (SNA) amended with sterilized filter paper cuttings to induce sporulation. We acquired a spore suspension from the culture by pipetting sterile water over the culture and aspirating the suspension, and diluting it to $1x10^7$ spores per mL. We pipetted 20 uL of the spore suspension, ~$5x10^7$ spores, into each soil receiving the Pathogen treatment, and non-pathogen controls received 20 uL of sterile water. After pathogen inoculation, we filled containers with soil to 75% capacity, added our treated seeds, and enough water to reach soil water holding capacity. Finally, we added the remaining soil to bury the seeds.

We maintained soil moisture level gravimetrically with distilled water on days 5, 7, 10, 13, and 15 after planting. On days 5 and 6 we measured the height of the emergent petiole, and on Days 7, 10, and 14, we measured maize seedling height from the soil surface to the tallest exposed leaf node. On Day 15, we destructively harvested half the seedlings, processed a subset of them for culture-independent microbial analysis, and measured root and shoot wet and dry biomass. We maintained the remaining seedlings in containers for one more week before transplanting into 2-gallon pots of potting soil. All replicate plants receiving the same soil and seed treatments were planted together in the same pot. Pots were maintained in the greenhouse at OSU for one month, until destructive sampling at 7 weeks post-planting.

Seed Treatments

All seeds were soaked for 24 hours in a rotary shaker (100 rpm) prior to planting. The Control treatment entailed soaking seeds in sterile water (60 g seeds in 100 mL) the entire 24 hours without any further treatment. We developed a disinfection treatment for maize seeds as an organic alternative to pesticide seed treatments, using Organic Materials Review Institute (OMRI) approved peracetic acid (PAA) combined with a modified hot water protocol (Rahman et al., 2008). Disinfected seeds were first soaked for 4 hours in a solution of 240 ppm PAA in the rotary shaker, before rinsing them off three times in distilled water. These surface-sterilized seeds were then placed into a water bath heated to 60°C for 5 minutes, stirring every minute, and then immediately plunged into an ice bath for two minutes to bring their internal temperature back down. After the 4 hour Disinfection treatment, the seeds were either soaked in distilled water or in a suspension of bacterial inoculant (Disinfection + Inoculation treatment) for the

remaining 20 hours. Seed receiving the Inoculation treatment were soaked in sterile water for 4 hours before we added the bacterial inoculant. The Fungicide treatment was applied on planting day to seeds that had soaked for 24 hours in distilled water. The fungicidal cocktail included MaximXL (Syngenta, Basil, Switzerland) at a rate of 0.024 mL per lb of seed and Dynasty (Syngenta) at a rate of 0.045 mL per lb of seed. To create the inoculant of *Arthrobacter ilicis*, we grew up a liquid culture in Potato Dextrose Broth, centrifuged the cells at 1000x g, and resuspended in sterile water. We added the inoculant to the soaking seeds at a rate of $1.5x10^7$ bacterial cells per 100 mL.

Extra seeds receiving the five seed treatments were air-dried and then the following day were planted at Oregon State University Botany and Plant Pathology Field Lab, which is maintained to have abundant pathogenic, soilborne *Fusarium* through introduction of pathogenic strains and conventionally cropping with susceptible crop varieties, primarily maize, since 2001 (Cindy Ocamb, personal comm.). We planted seeds in a randomized block design, and watered regularly with overhead irrigation.

Scoring Crown Rot

We measured *Fusarium* pathogenesis by scoring the severity of crown rot during the pollination stage, at 7 weeks post-planting in the case of greenhouse plants, and 9 weeks for plants grown at OSU-BPP Field Lab. Crown rot in maize appears as darkened crown tissue, due to necrosis of plant cells caused by *Fusarium* spp. Our scoring method was based upon the methods of Miller and Ocamb (Miller & Ocamb, 2009). Plants were dug up and cleaned of dirt around their roots, and then cut longitudinally through the middle of the stem, slicing through the crown at the base of the stem. We photographed the transverse sections of crowns directly upon harvesting via a smartphone camera (HTC

One M8), equipped with a custom-built imaging apparatus, outlined in detail in Chapter III. We scored each crown photo using ImageJ software (Schneider, Rasband, & Eliceiri, 2012) by converting each image to grayscale and measuring the mean value within the area of the photo representing the crown tissue, comparing its value to the black and white standards in each photo. The crown rot index represents a darkness value between these standards, where "white" is a value of 0, and "black" is a value of 1.

DNA Extraction from Plants and Soil

Each seed DNA sample represents the aggregate of 20 seeds, randomly sampled from the pool of all seeds. We took three replicate seed samples each of the original sourced seed, in addition to seeds harvested at the OSU Field Lab, representing seeds from plants that had been treated with each of the five seed treatments. To remove superficially-associated bacteria and fungi, we surface-sterilized seeds based on a protocol developed by Johnson-Monje and Raizada (2011). We submerged seeds in the following solutions for 10-minute intervals: 0.1% Tween-20 detergent, 3% sodium hypochlorite (two washes for 20 minutes total), and 95% ethanol, followed by rinsing in autoclaved nanopure water. Seeds were allowed to air-dry in a biosafety cabinet before grinding them. We ground seeds using a pre-sterilized hand-crank ceramic-burr coffee grinder (Porlex, Osaka, Japan). The grinder was first disassembled and disinfected before each use by scrubbing in detergent (Labtone, VWR, Randor, PA, USA), submerging and rinsing in MoBio Ultraclean Lab Cleaner which removes contaminating DNA and RNA (MoBio Laboratories, Carlsbad, CA, USA), and then flame-sterilizing ceramic burrs and stainless steel parts with 95% ethanol. Plastic parts were air-dried after the ethanol rinse. Once disinfected, we reassembled grinders with sterile, gloved hands and flame-sterilized

tools.

From a subset of seedlings on day 15 of the study, we excised a 1-cm long portion of each stem directly above the seed and stored at -80°C until DNA extraction. We surface-sterilized the stem pieces by submerging and agitating them in sterilized 5-ml glass culture tube using the same sterilization method of seeds, described above. Stem samples were then pulverized in each glass tube using a flame-sterilized stainless steel handle as a pestle. Samples were pulverized directly into lysis buffer of the MoBio PowerPlant Pro DNA extraction kit (MoBio Laboratories, Carlsbad, CA).

After sectioned crown tissues were imaged for crown rot, a smaller subset of them were placed on ice, stored at 4°C overnight, and processed the next day in a biosafety cabinet. Exposed crown tissue was rinsed with distilled water, and then flame-sterilized in 95% ethanol. We used a flame-sterilized scalpel to scrape off the exposed top layer, and then carved out a wedge of previously unexposed crown tissue, which was stored in -80C until DNA extraction.

We extracted DNA from stem samples, crown tissue pieces and ground-up seeds using a DNeasy PowerPlant Pro kit (MoBio Laboratories, Carlsbad, CA) modifying the protocol to improve cell lysis from fungi and bacteria as follows. To the standard 1mm steel beads, we added 0.3ml of 0.1mm glass beads (BioSpec, Bartlesville, OK, USA). After adding the (~50 mg) plant tissue sample and the kit's cell lysis solution (i.e., solutions PB1, PB2, and RNaseA) to each tube, we put samples through two freeze-thaw cycles, alternating between liquid nitrogen until frozen and a 65°C water bath for 2 minutes. Samples were then homogenized in a FastPrep 24 homogenizer (MP Biomedicals, Santa Ana, CA, USA) for two 25 sec cycles at 5.5 power setting. Lastly,

samples were left in the 65°C water bath for 10 minutes before continuing with the standard protocol. We extracted soil DNA from both raw and irradiated soil using the MoBio PowerSoil DNA isolation kit (MoBio Laboratories, Carlsbad, CA, USA), and utilized the same freeze-thaw protocol described above. We quantified the purified plant and soil DNA using qubit fluorometric quantitation (Thermo Fisher Scientific) and used DNA sample aliquots diluted to 5 ng/ul for downstream DNA analyses.

Quantitative PCR for *Fusarium* Abundance

To determine the abundance of *Fusarium* in each crown sample in the greenhouse at 7 weeks, we utilized a quantitative PCR (qPCR) approach, using the KAPA SYBR Fast kit (KAPA Biosystems, Wilmington, MA, USA), and primers that targeted a sequence of the fungal intergenic spacer (IGS) specific to the genus *Fusarium* (Jurado et al., 2006). To each 10 ul qPCR reaction, we added 10 ng of template DNA and 0.2 nM of forward and reverse primers, the KAPA SYBR Fast master mix, and diluted in PCR grade water. For qPCR analysis we used a BioRad (Hercules, CA, USA) CFX96 Touch instrument with the following protocol: 10 minutes at 98°C, followed by 40 cycles at 98°C (denaturation) and 60°C (annealing plus extension) for 15 and 30 seconds, respectively, and finishing with a 2-minute extension step at 72°C. To ensure qPCR product purity, amplicons were also subjected to a melting curve analysis, and a subset was independently confirmed on a gel to ensure the correct size. We calculated baseline fluorescence and qPCR doubling efficiencies on a per-sample basis by fitting raw fluorescence reads to a log-linear equation, using the LinRegPCR software (Ramakers et al., 2003). We standardized copy number estimates of samples by comparing the PCR amplification rate to that of genomic DNA from a *Fusarium verticillioides* isolate,

assuming a genome size of 41.74 Mbp (Ma et al., 2010).

Preparation and Processing of ITS Amplicon Sequences

We used a DNA metabarcoding approach to characterize fungi present in soil and plant tissue, through amplification of a region of the fungal Internal Transcribed Spacer (ITS1), using custom PCR primers for Illumina MiSeq (Illumina, San Diego, CA, USA), followed by sequencing (Table S2). The Illumina sequencing primers contained plant-DNA-excluding ITS1F forward primer matched with ITS2 reverse primer to amplify the ITS1 region of interest. Also on each end were 8-bp indexing barcodes, allowing us to match different combinations of forward and reverse reads. For PCR amplification of the Illumina library, we used KAPA HiFi High Fidelity HotStart ReadyMixPCR (KAPA Biosystems), pooling two replicate PCR runs per sample. Each 10 ul PCR run contained 10 ng of DNA template and 300 pM of each primer. We used an Eppendorf Mastercyler ProS (Hamburg, Germany) for PCR amplification with the following protocol: 10 minutes at 98°C, and 35 cycles of 98°C for 15 seconds, 55°C for 15 seconds and 72°C 30 seconds, and ending on a 5-minute extension step at 72°C. After pooling, we performed a magnetic bead PCR cleanup and size selection (Agencourt Ampure XL; Beckman Coulter Inc., Brea, CA, USA) to remove non-target primer dimers. Our size selection protocol exploited the fact that larger DNA fragments have a greater affinity for the magnetic beads than smaller DNA fragments, at limiting concentrations (Quail, Swerdlow, & Turner, 2009). Following bead purification, pooled equimolar amounts of ITS amplicons from each sample into the same Illumina library. We used the Illumina MiSeq v3 reagent kit, allowing for paired sequencing of 300 bp in both directions, and sequenced samples over three replicate sequencing runs, at University of Oregon

Genomics and Cell Characterization Core Facility (C3F; Eugene, OR, USA).

Illumina Sequencing Analysis Pipeline

For unknown reasons, we obtained sub-optimal DNA sequences from two repeated MiSeq sequencing runs. The forward read was excellent quality, but there was no information from its barcode sequence; the reverse reads were of exceptionally poor quality, but contained the reverse index sequence. We could only obtain the barcode sequence from reverse reads that sequenced up to the forward index sequence. Both forward and reverse index sequences are necessary for correct assignment of the sample from where it originated. A technician at the University of Oregon C3F wrote a script that salvaged about 20% of the sequencing dataset. However, as an artifact of this near-failed sequencing run, we were necessarily limited to ITS1 amplicons less than 285 bp in length. Based on previous sequencing with ITS (See Chapter III), this implies a possible 24% reduction in diversity of fungal species present in this dataset. However, ITS1 amplicon length does not directly correlate with phylogenetic distance, and we are still able to observe community-scale changes and important taxa of interest, including *Fusarium.*

Illumina sequencing processing was performed in R (R Core Team, 2017). Separately for each amplicon and sequencing run, we utilized the workflow of the DADA2 package (Benjamin J. Callahan et al., 2016), which explicitly models sequencing errors, and used it to infer exact amplicon sequence variants (ASVs) based on a probabilistic model. Throughout this paper, we will refer to estimated amplicon sequence variants broadly as ASVs. ASVs have advantages over OTUs (Operational Taxonomic Units), which are based upon clustering methods (e.g., 97% similarity clustering), do not

explicitly calculate sequencing error, and often overestimate true taxonomic richness due to sequencing artifacts (Callahan, McMurdie, & Holmes, 2017). Using only the forward-read sequences, we filtered out any paired-end assemblies containing an "expected error" rate greater than 2 nucleotides (i.e., maxEE=2), and truncated sequences where the quality score dropped to 2. We estimated the sequencing error rates by pooling all samples present in the sequencing run, and used this broadscale error rate in the dada() function of the DADA2 package. Non-default arguments to the dada() function included pooling all samples per run, and increasing the OMEGA_A parameter to 1E-4, which allows detection of more rare variants, though with higher probability of false positives. We assigned fungal taxonomy via DADA2 package's naive Bayesian classifier method using the UNITE database (Community, 2017) . The ITS sequence tables, taxonomy tables, and DNA sample metadata were analyzed together mainly using the phyloseq package in R (McMurdie & Holmes, 2013).

Microbiome Data Normalization

High-throughput sequencing datasets typically exhibit high variance in sequencing depth across samples. As a result, these datasets will have a confounding mean-variance relationship, i.e., taxa occurring in samples with a greater mean value also contain a higher variance across samples. To overcome this heteroscedasticity, we used two transformation methods. In the first method, we normalized raw ASV counts using a variance-stabilizing transformation (VST), which adjusts count data by fitting each taxon to a mean-variance curve (Love, Huber, & Anders, 2014). In the alternative method, we normalized taxa counts to relative abundances of the total sequence count in each sample (total sum scaling, TSS), and then adjusted relative abundances to their centered log-ratio

(CLR), which effectively transforms the relative abundance data for use in euclidean space (Lê Cao et al., 2016). With the exception of NMDS ordination comprising all sample types, we normalized crown and seed endophyte ASVs separately from each other.

Calculation of Alpha and Beta Diversity

Using raw ASV counts, we calculated a normalized metric of ASV richness in each sample, controlling for read abundance using the *phyloseq rarefy_even_depth* function. For each soil or plant sample, we iteratively resampled 100 random sequences from the total pool of sequences in that sample, repeating the process 100 times to acquire a mean value of ASV richness per 100 reads for each sample. We assessed compositional, i.e., beta-diversity, differences in fungal and bacterial ASVs between samples using Bray-Curtis dissimilarity of VST-transformed counts, visualizing paired sample dissimilarity measures using NMDS ordination biplots. Furthermore, we used principal components analysis (PCoA) to collapse pairwise dissimilarity matrices onto principal components axes, using sample scores projected on each of the first two principal component enet axes as a one-dimensional quantification of community dissimilarity. In addition to using ecological community dissimilarity metrics based on PCoA of VST-transformed count data, we also inferred community dissimilarity between samples by performing PCoA on CLR-transformed taxa abundances, as this scaled and centered transformation is valid to use directly in PCoA, without using conventional distance dissimilarity metrics.

Modeling the Effects of Seed Treatments

To test for the effects of soil and seed treatments on plant growth, disease, yield

measures, and qPCR of *Fusarium* we used restricted maximum likelihood (REML) linear mixed models, with Soil, Treatment, Pathogen, Disinfection, Inoculation, and Fungicide as fixed factors, and replicate blocks on the seedling tray or the field as random effects, using the lme4 package in R (Bates 2015). When assessing factor effects, we found that the *F. oxysporum* pathogen treatment had no appreciable effect on plant measures, including *Fusarium* abundance, and thus eliminated the term from the models. The first model included Soil (i.e., raw vs. irradiated) and the Disinfection and Inoculation seed treatments, and all interactions between the three factors, including a Disinfection x Inoculation interaction and Soil x Disinfection x Inoculation three-way interaction. The second model was set up to determine main effects and interactions between Soil treatment and Fungicide treatment, excluding other seed treatments except for the control. We also used REML linear mixed models to assess the effects of seed treatments on fungal (ITS) alpha- and beta- diversity measures. The models were identical to those used for plant measures, except we included llumina sampling depth of each sample as a covariate in the model. Statistical significance of fixed and random effects were determined using the lmerTest package in R (Kuznetsova, Brockhoff, & Christensen, 2017). Statistical significance of fixed effects was calculated using Type III ANOVA with Satterthwaite's approximation of denominator degrees of freedom, based on Type 3 sums of squares, and significance of each random error term was tested with the Chi-squared statistic of a likelihood ratio LRT.

Endophyte Community Composition Analysis

To generate a metric of community dissimilarity between the seedling stem endophyte community and the initial seed endophyte community, we used the mean

Bray-Curtis distance between each stem sample and the three different seed samples, and subtracted the mean Bray Curtis index from 1 to get a similarity score. We also investigated the dynamics of a smaller subset of endophytic taxa found in the stem that we determined to be more seed-associated. This subset included ASVs that were uniquely found in seeds and not found in soil. In addition, we selected the top taxa that discriminated seed samples from soil samples, using partial least squares discriminant analysis from the package mixOmics (Le Cao et al., 2017). The taxonomic abundances were first normalized and centered using CLR transformation, then ordinated using principal components analysis constrained to discriminate the greatest variation between seed and soil. We defined seed-associated taxa as those having the top 10% of loading scores along the discriminating axis between seed samples than to soil samples, and then filtered out all non-seed-associated taxa from stem endophyte communities. Using this method, we identified 14 seed-associated ASVs.

RESULTS

Seedling Growth Measures

On average, only 50% of seeds receiving the disinfection treatment germinated in the growth chamber (Figure 1A). In the field experiment, disinfected seeds germinated at a rate of 75% (Figure 1B) which was statistically indistinguishable from the germination rates of the other seedlings. Disinfection was associated with reduced seedling height on days 5, 6, 7 and 10 (Figure 2A). The Inoculation and Fungicide seed treatments were also associated with lower seedling height, an effect which disappeared after 6 days (Figure 2A). When a subset of seedlings were harvested on Day 15, there was no there was no

significant effect of disinfection on seedling height, as disinfected seedlings receiving the bacterial inoculant were similar height to the non-disinfected seedlings. On average, seedlings receiving the Inoculation treatment were 11% taller than plants not receiving the inoculant (Figure 2A, B). Across all seed treatments, seedlings grown in irradiated soil had a 20% higher biomass on average than seedlings grown on raw soil ($P=0.026$), although this trend differed significantly with respect to seed treatment ($P=0.04$), and was most pronounced in seedlings receiving the fungicide treatment (Figure 2C).

Figure 1. Germination rates in growth chamber and the field. Seedlings receiving the disinfection treatment had a reduced germination rate in growth chambers (A), but not in the field (B).

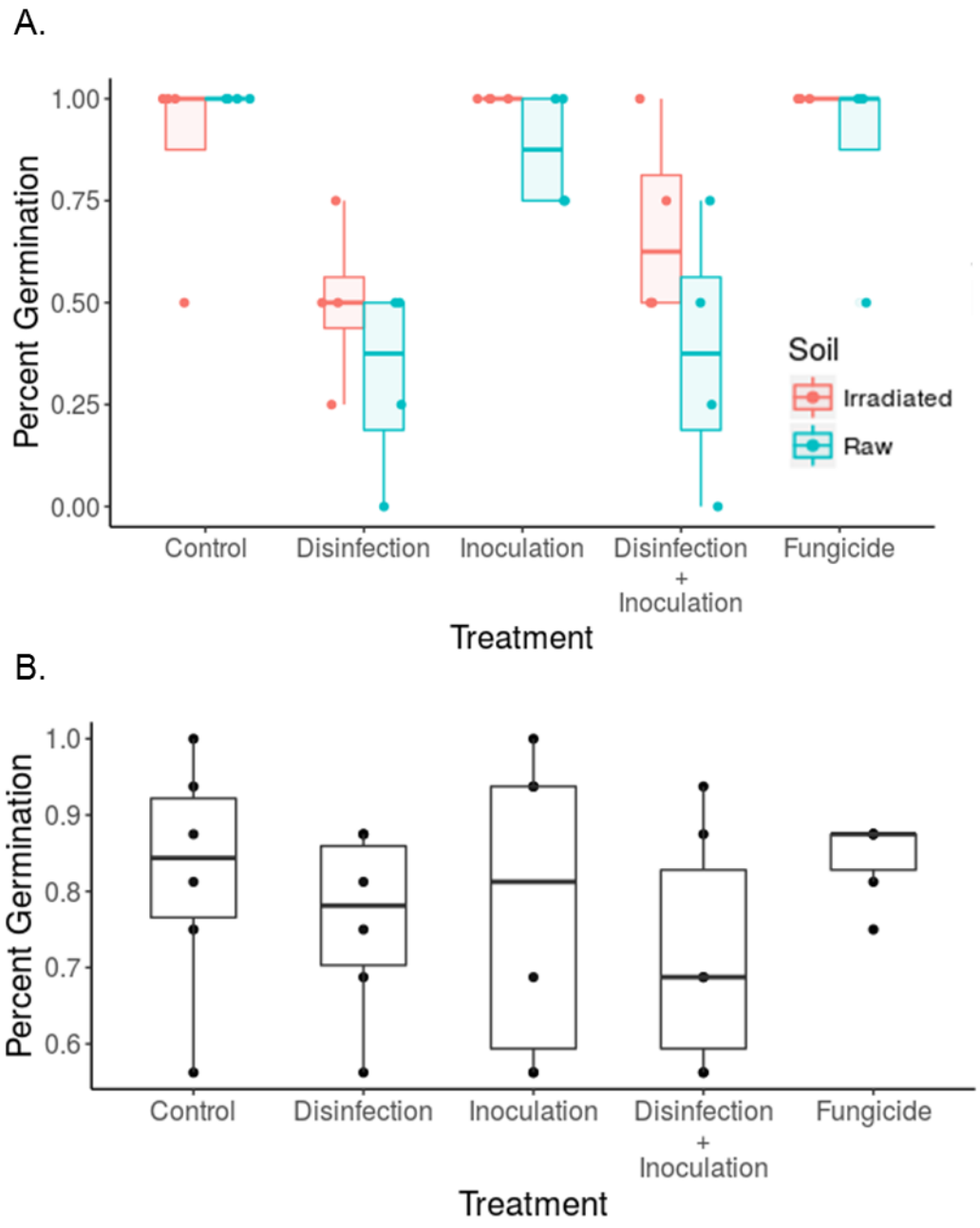
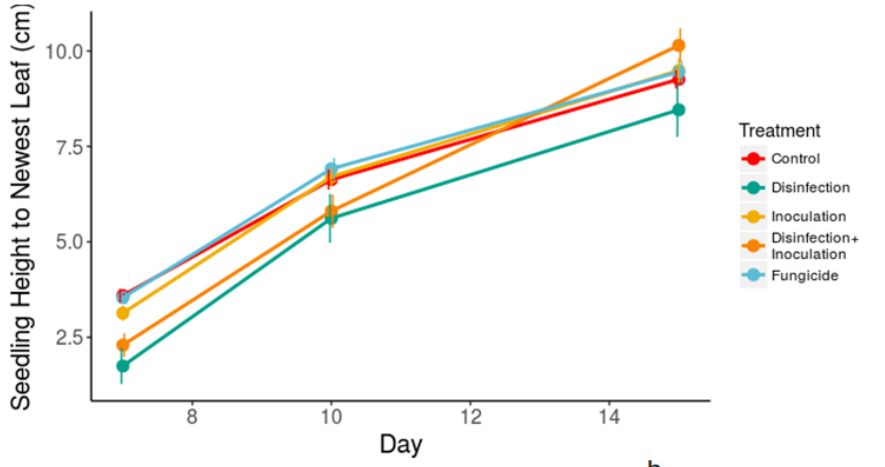


Figure 2. Seedling measures. Soil and Seed treatment effects on seedling measures, listed in *p*-values of REML linear mixed model terms, with direction of effect in parentheses, where applicable. (A). Disinfected seedlings were shorter in height, but those receiving the inoculant recovered in height (B). On Day 15, seedlings planted in sterile soil generally had higher biomass (C).

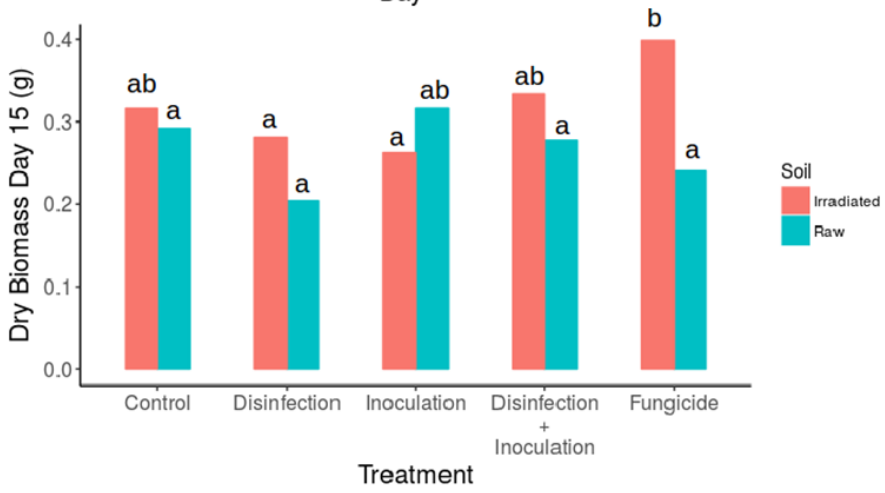
A.

	Height of Petiole		Height to Newest Leaf Node		
Disinfection & Inoculation	**Day 5**	**Day 6**	**Day 7**	**Day 10**	**Day 15**
Sterile Soil	0.392	0.841	0.749	0.458	0.580
Disinfection	**0.000 (–)**	**0.000 (–)**	**0.000 (–)**	**0.014 (–)**	0.633
Inoculation	**0.005 (–)**	0.070	0.968	0.601	**0.012 (+)**
Soil x Disifection	0.772	0.399	0.169	0.772	0.552
Soil x Inoculation	0.863	0.878	0.935	0.385	0.551
Disinfection x Inoculation	0.060	**0.033**	0.067	0.772	**0.050**
Soil x Disinfection x Inoculation	0.920	0.761	0.592	0.899	0.829
Block	1.000	0.558	1.000	1.000	0.654
Row	0.669	1.000	1.000	1.000	1.000
Fungicide					
Sterile Soil	0.388	0.122	**0.037 (+)**	0.122	0.312
Fungicide	**0.001 (–)**	**0.001 (–)**	0.702	0.503	0.576
Soil x Fungicide	0.959	0.476	0.900	0.208	0.600
Block	0.371	0.796	1.000	1.000	1.000
Row	0.756	0.250	0.898	1.000	1.000

B.



C.



No Apparent Pathogen Effects

Plants growing in the *F. oxysporum* pathogen treated soils exhibited no symptoms associated with pathogenesis, nor distinguishable changes in growth rate, seedling biomass, crown rot, or *Fusarium* abundance in crowns. We removed this as a factor from models to increase statistical power to detect other important relationships.

Crown Rot Index and *Fusarium* abundance

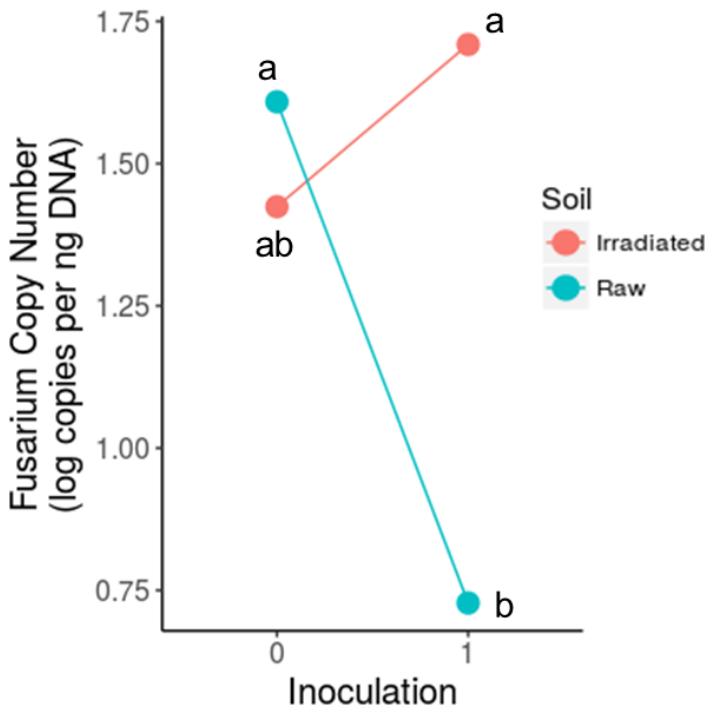
There were no significant main seed treatment effects on Crown Rot Index in 7-week-old plants transplanted into the greenhouse. However, the abundance of *Fusarium* in greenhouse crown tissue samples could be explained by a Soil x Inoculation effect and a Soil x Disinfection x Inoculation effect (Figure 3A, B). Inoculation significantly reduced the abundance of *Fusarium* in the crown of plants whose seeds had both been disinfected and planted in raw control soil (contrast t_{22}=-2.01: P = 0.057), while no significant effects of Inoculation were detected in raw soil and non-disinfection seed treatments. Neither crown rot measures nor yield measures differed with respect to seed treatment for plants growing at the OSU Field Lab.

Figure 3. Abundance of Fusarium in crown tissue of 7 week old seedlings that were transplanted into a greenhouse, based on qPCR copy number abundance of the IGS_Fus gene. Linear mixed model results (A) indicated that the effect of the inoculant depended on soil treatment and seed disinfection. The inoculant significantly reduced Fusarium abundance in crown tissue in seedlings planted in raw soil, but not when planted in irradiated soil (B).

A.

Fusarium Copy Number	Model Term	F or X2 statistic	P-value
	Soil	F1,5=2.43	0.179
	Disinfection	F1,26=0.25	0.621
	Inoculation	F1,25=0.98	0.333
	Soil x Disinfection	F1,26=0.32	0.579
	Soil x Inoculation	F1,25=4.39	**0.047**
	Disinfection x Inoculation	F1,25=0.44	0.512
	Soil x Disinfection x Inoculation	F1,25=6.23	**0.020**
	block	X21	0.898
	row	X21	1.000

B.

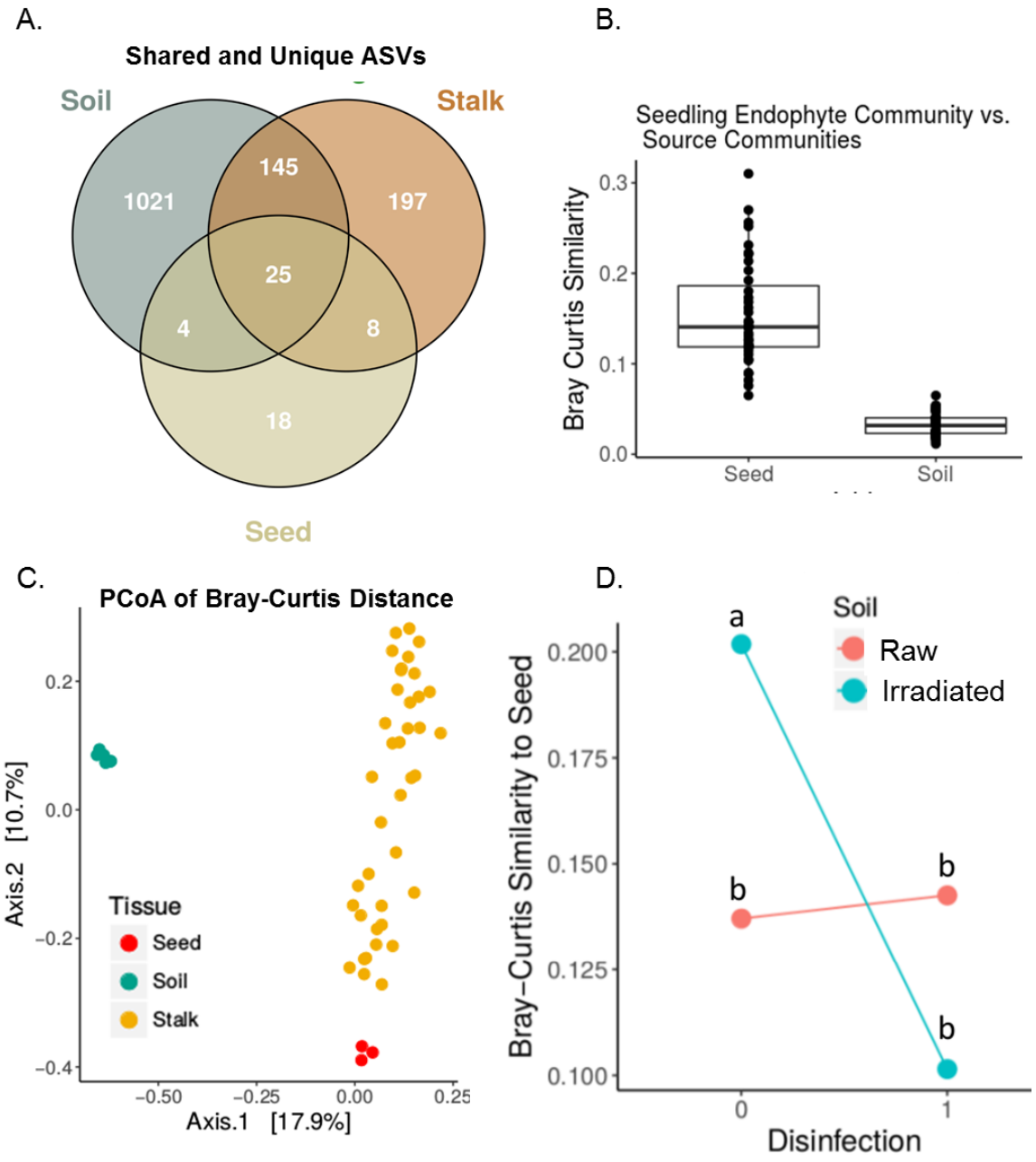


Seedling Fungal Endophyte Community Measures

The gamma-irradiated soil contained over 40% fewer fungal amplicon sequence variants compared to raw soil, although the ASVs could either be viable organisms or simply fungal DNA. Seedling endophytes shared most fungal ASVs with the soil (Figure 4A). However, seedling fungal endophyte communities were generally more similar to the seedborne endophyte communities than the soil fungal communities, based on Bray Curtis community dissimilarity measures (Figure 4B,C). Fungal endophyte communities in maize seedlings growing in irradiated soil more closely resembled the fungal endophyte communities measured in the seed, compared to endophytes of seedlings grown in raw soil. This trend did was not observed in seedlings whose seeds were first disinfected (Figure 4D).

Seedlings contained the same fungal endophyte species richness, regardless of whether seeds were treated or planted into raw or irradiated soil. However, the Inoculation treatment was associated with a greater species evenness of seedling endophytes, as measured by the Inverse Simpson Index (P=0.029). The first principle component of Bray-Curtis distance indicated the fungal endophyte community structure in seedlings was marginally affected by whether they were planted into raw or sterilized soil (P =0.054), and the fungicide treatment significantly affected the community structure of seedling endophyte communities, indicated on the second principal component of Bray Curtis distances (P =0.049).

Figure 4. Comparison of seedling stalk fungal endophyte communities to fungi in the seeds and the soil. Although seedling stalks overall contained more ASVs found in the soil (A), the endophyte communities of seedlings were more similar to the seedborne endophytes than the soil based on the Bray-Curtis (dis)similarity metric (B,C). Seedling endophyte communities become significantly more similar to the seed when planted in in sterile soil, except when disinfected (D).



Seed-Associated and Soil-Associated ASVs in Seedlings Respond Differently to Seed and Soil Treatments

We divided our analysis of seedling endophytes into those categorized as either seed-associated or soil-associated by sPLS-DA. The disinfection seed treatment resulted in a 28% reduction in seed-associated endophytes (P=0.05; Table II.1). Compared to raw soil, irradiated soil was associated with a lower evenness of seed-associated seedling endophytes in the stem (P=0.031, Table II.1). This shift in evenness with respect to soil sterilization is accompanied with an alteration in the community structure of seed-associated endophytes in stems, based upon Bray Curtis distances between samples (Table II.1). While Disinfection seed treatment was not by itself associated with any significant community-wide changes in stem endophytes, seed disinfection appeared to increase the effect of the inoculant, with a significant Disinfection x Interaction term on both principal component axes of Bray Curtis Distance metric. Soil-associated seedling endophytes were not significantly affected by soil irradiation treatment. However, both the Inoculation and Fungicide treatments significantly affected the composition of soil-associated fungal endophytes in the seedlings (Table II.1). Moreover, the Fungicide treatment was associated with a 32% reduction in soil-associated fungal endophytes in the seedling, a trend that was marginally significant (P=0.056, Table II.1).

Table 1. Linear mixed model P-table for seed and soil treatment effects on alpha and beta diversity measures. Numbers are p-values of each term corresponding to each diversity metric. The sign in the parentheses indicates the direction of the effect, where applicable. Bolded values indicate significant p-values. Block and row effect

	Seed-Associated Endophytes in Stems			
Model Terms	ASV Richness	Inverse Simpson	Bray Curtis PC1	Bray Curtis PC2
Model 1				
Irradiated Soil	0.133	**0.031 (–)**	**0.007**	0.078
Disinfection	**0.050 (–)**	0.804	0.173	0.346
Inoculation	0.587	0.949	0.054	0.202
Soil x Disifection	0.625	0.849	0.456	0.939
Soil x Inoculation	0.394	0.691	0.848	0.870
Disinfection x Inoculation	0.378	0.178	**0.008**	**0.046**
Soil x Disinfection x Inoculation	0.764	0.444	0.108	0.363
Sequence Depth	**0.006**	0.668	0.064	0.485
Block Effect	1.000	1.000	1.000	1.000
Row Effect	0.519	1.000	1.000	1.000
Model 2				
Irradiated Soil	0.877	0.214	0.989	0.856
Fungicide	0.558	0.506	0.108	0.544
Soil x Fungicide	0.946	0.986	0.992	0.086
Sequence Depth	0.121	0.831	0.323	0.580
Block Effect	1.000	1.000	0.962	1.000
Row Effect	0.872	1.000	1.000	1.000
	Soil-Associated Endophytes in Stems			
Model Terms	ASV Richness	Inverse Simpson	Bray Curtis PC1	Bray Curtis PC2
Model 1				
Irradiated Soil	0.970	0.601	0.063	0.390
Disinfection	0.206	0.283	0.873	0.194
Inoculation	0.739	0.663	**0.013**	0.177
Soil x Disifection	0.101	0.092	0.082	0.236
Soil x Inoculation	0.918	0.586	0.253	0.759
Disinfection x Inoculation	0.162	0.297	0.648	0.213
Soil x Disinfection x Inoculation	0.967	0.422	0.092	0.058
Sequence Depth	0.144	0.447	0.104	0.782
Block Effect	1.000	0.518	1.000	1.000
Row Effect	1.000	0.838	1.000	1.000
Model 2				
Irradiated Soil	0.365	0.494	0.283	0.519
Fungicide	0.056 (–)	0.663	0.871	**0.040**
Soil x Fungicide	0.784	**0.038**	0.175	**0.018**
Sequence Depth	0.712	0.360	0.813	0.433
Block Effect	0.581	0.156	1.000	0.700
Row Effect	1.000	0.882	0.855	1.000

In a given maize seedling, seed-associated endophytes comprised an average of 11% of all fungal endophyte ASVs, and 20% of all sequences recovered (Figure 5A). However, the vertical transmission rate substantially differed among seed treatments, soil treatments, and taxa. The most abundant and common seed-associated endophyte sequence variant found in seedling stems was a *Fusarium* sp., which was classified as *Fusarium_denticulatum_1* using the SILVA taxonomy database, though it shares 100% ITS sequence similarity with other Fusaria including *F. verticillioides* and *F. oxysporum*, and it may in fact represent several species of *Fusarium*. This *Fusarium_denticulatum_1* ASV was also highly abundant (approximately 40% of all sequences) in stock seeds, but rare in the soil at a frequency of less than 0.01% (Figure 5). The *Fusarium* strain was significantly more abundant in seedlings grown in irradiated soil (P=0.04), except in seedlings receiving the Disinfection treatment. Overall, Disinfection reduced *Fusarium_denticulatum_1* abundance in seedlings (P=0.01; Figure 5).

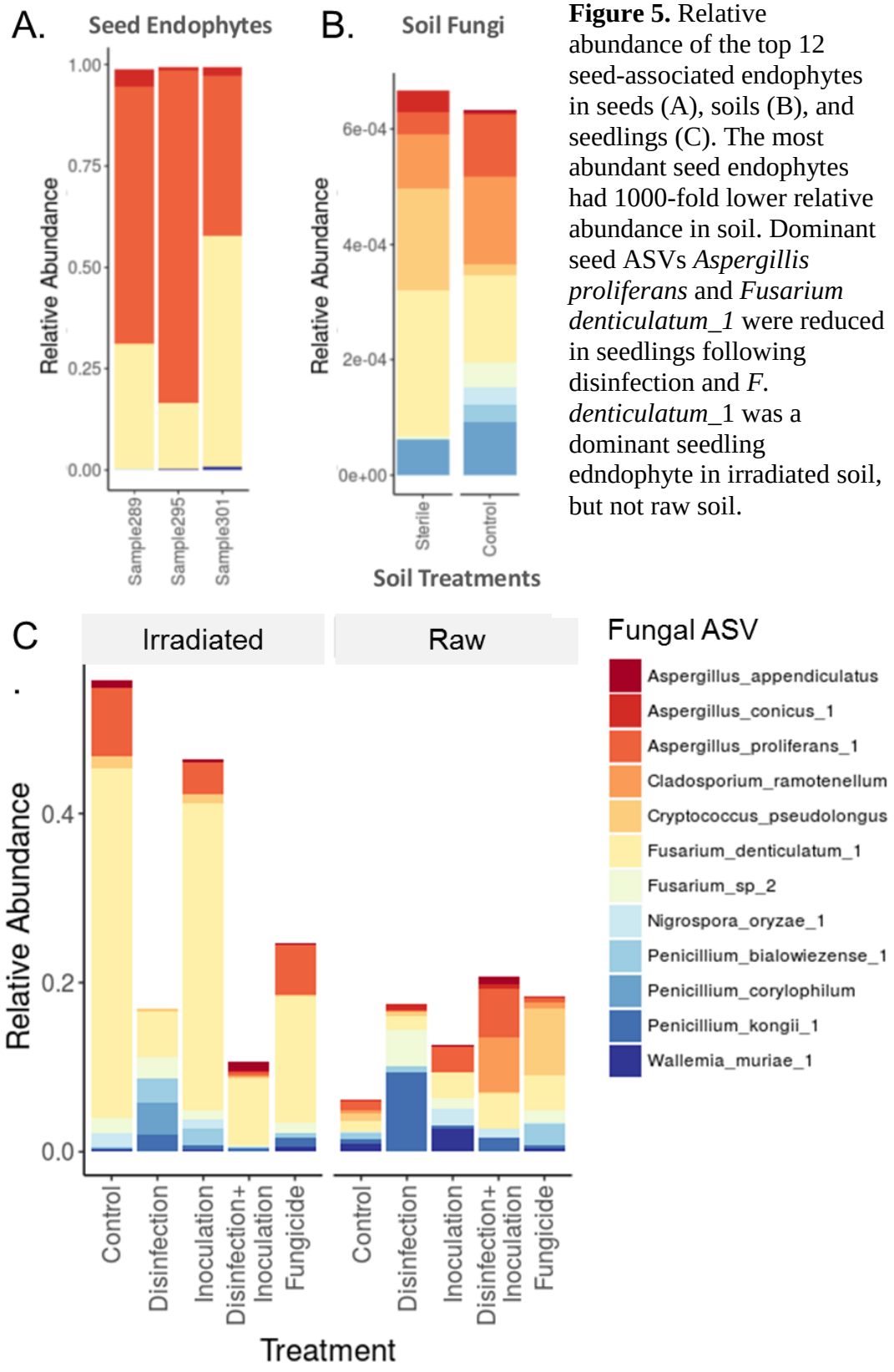


Figure 5. Relative abundance of the top 12 seed-associated endophytes in seeds (A), soils (B), and seedlings (C). The most abundant seed endophytes had 1000-fold lower relative abundance in soil. Dominant seed ASVs *Aspergillis proliferans* and *Fusarium denticulatum_1* were reduced in seedlings following disinfection and *F. denticulatum*_1 was a dominant seedling edndophyte in irradiated soil, but not raw soil.

DISCUSSION

The aim of this study was to determine the relative contribution of seedborne versus soilborne fungal endophytes of maize seedlings, and to modify the relative contributions of each potential endophyte source through sterilization of seed and soil. Furthermore, we tried to assess possible effects these alterations to seedborne microbes may have on plant growth, plant susceptibility to *Fusarium* disease, and yield.

In this study, we found that the majority of ASVs found in seed also existed in the soil environment, so we could not base our analysis on the presence or absence of soil or seed ASVs. Instead, we used the Bray-Curtis dissimilarity metric to determine if plant endophytes more closely represented soil or seed endophyte communities, taking into account the relative abundance of each taxon rather than its presence or absence. Furthermore, we delineated those taxa that were more strictly seed-associated, i.e., had a many-fold higher relative abundance in the seed than in the soil. In alpha diversity measures, we assume that any of these seed-associated taxa present in seedlings originated in the seed. However, we should note that as some of these fungal taxa are also present in the soil, it is possible that not all seed-associated endophytes in fact originated from the seed.

Seedborne Fungal Endophytes Compete with Soil Microbes

In agreement with our hypothesis, seedlings grown in irradiated soils contained fungal endophyte communities that more closely resembled seed-associated fungal endophytes, compared to seedlings grown in raw soils. However, sterilizing the soil did not significantly increase the richness of seed-associated endophytes, and was instead associated with a loss of species evenness, wherein some seed-associated endophytes

became disproportionately enriched. In theory, seedborne endophytes experience heavy competition in the microbe-rich soil environment, for a limited niche in the germinating seedling, and would likely face less competition in irradiated soil. Contrary to our expectations, competition with soil microbes did not significantly reduce vertical transmission efficiency of seed-associated taxa, measured by species richness, but instead reduced the ability of any one seedborne fungal endophyte to become a dominant member of the seedling. This is evident in the case of the *Fusarium_denticulatum_1* ASV. This taxon was particularly dominant in seeds, and became disproportionately abundant in seedlings planted in irradiated soil, but remained in relatively low proportional abundance in seedlings planted in irradiated soil (Figure 5).

Our observation that soil biology had no effect on the vertical transmission efficiency is consistent with the study of Davitt et al. (2011), who found no effect of irradiated vs. raw soil on the vertical transmission efficiency of *Epochloe* in *Agrostis hyemalis*. Interestingly, Davitt and colleagues found a lower biomass of plants grown on raw soil than irradiated soil, which is consistent with our own observations (Figure 2C). They propose that seedborne symbiosis may be costly to plants grown in raw soil. Alternatively, soilborne microbes may have a growth-suppressive effect on maize seedlings, or seedborne endophytes may have a growth promoting effect. We found no significant correlation between the *Fusarium_denticulatum_1* ASV abundance and seedling dry biomass, indicating this taxon was not the cause for increased dry biomass in seedlings grown in irradiated soil.

Seed Disinfection Reduces Vertical Transmission and Increases Inoculation Treatment Effects

Also consistent with our hypothesis, seedlings whose seeds were disinfected contained endophyte populations that less closely resembled those of the seed (Figure 4D). Moreover, seed disinfection reduced the number of seed-associated endophyte taxa found in stems, but did not significantly affect the composition of seed-associated endophytes in stems. Furthermore, in agreement with our hypotheses, Disinfection of seeds increased the effects of the Inoculation treatment, in terms of the biocontrol inoculant effects on plant growth rate (Figure 2A,B), capacity to mediate *Fusarium* abundance in crowns (Figure 3A,B), and additionally its effects on the community structure of seedling fungal endophytes (Table II.1).

The disinfection treatment was associated with lower seedling germination and reduced seedling vigor in the growth chamber. The hot water treatment had been optimized previously to have no significant effects on germination rate. However, optimization had been conducted using larger seedlots, and it may be that in the smaller batch of seeds used in this study each seed absorbed more thermal radiation. Additionally, seeds may have responded negatively to soaking for 20 hours following the treatment and then planting into a warm, saturated soil. Combined with moisture and warmth, the seeds may have succumbed to an aggressive seedborne or soilborne microbe. Surviving seedlings grew up to have a comparable biomass. The disinfection treatment did not significantly reduce germination rate in the field, or disease or yield measures, suggesting the growth chamber environment may have been a factor. Therefore, we caution that seedlings receiving the disinfection treatment underwent more stress than

those that did not, which may be a confounding factor in this study.

Biocontrol Capacity of *Arthrobacter ilicis*

We developed the *Arthrobacter ilicis* biological control inoculant to be antagonistic to *Fusarium* spp. in maize plants. We found that the inoculant was only effective at reducing *Fusarium* in plants grown in raw soil, when seeds were first Disinfected (Figure 3). First of all, this observation suggests that the inoculant may have been unable to compete with seedborne *Fusarium*, and only after reducing their abundance via seed disinfection did the inoculant out-compete *Fusarium*. During our screening method for the endophyte biocontrol ability, we provided it with a head start, inoculating seeds with the bacterium before inoculating emergent radicles with the pathogenic *Fusarium* two days later. That the biocontrol inoculant only reduced *Fusarium* abundance in raw soil suggests that its biological control effect relied on the presence of other soil microorganisms.

Despite its weak biocontrol capacity in seedlings, the inoculant significantly enhanced plant growth measures and altered soil-associated endophyte community structure, regardless of the soil in which it was planted. A consistent Disinfection x Inoculation effect on growth measures indicated that seedlings inoculated with *Arthrobacter ilicis* recovered better from the Disinfection treatment than those that did not. *A.ilicis* was associated with a greater species evenness in seedlings, suggesting it may have the capacity to prevent any single fungal taxon from becoming a dominant member of the endophyte community.

Fungicide Treatment

We hypothesized the fungicide treatment would reduce the diversity of seedborne

fungi. Seedlings that receiving the fungicidal treatment had a significant but transient reduction in seedling vigor. Also, the Fungicide had no measurable effect on seed-associated endophyte communities, but significantly affected soil-associated endophyte diversity and composition (Table II.1). The fungicide appears to have had a minimal effect on vertical transmission of fungi, but may have inhibited the ability of certain soil fungi to colonize the seedling. It is possible the fungicidal treatment does not work as well in seeds that are already imbibed with water, as the fungicide is less likely to enter the seed and may wash off into the soil. Furthermore, the potency of fungicides is transient, only lasting for 10-14 days (Cindy Ocamb, personal comm.).

CONCLUSION

While there are a number of case studies of the vertical transmission of specific seedborne endophytes, such as *Fusarium* and *Neotyphodium* in grasses, little is known about the general rate of seedborne transmission of fungi. Our work suggests that seedborne fungal endophytes generally represent 25% of all fungi found in aboveground tissues of maize, and comprise 20% of the seedling endophyte species diversity. However, the membership of seedborne fungal taxa in each seedling differs due to imperfect vertical transmission rates across taxa. We found that taxa significantly differ with respect to their capacity for vertical transmission across host generations. *Fusarium*, *Aspergillis* and *Penicillium* spp., commonly occurring seedborne fungi, appear to have the highest vertical transmission efficiency in this system. These fungi are also well known for having a high dispersal rate and horizontal transmission ability, which implies they may not be the best candidates for seedborne mutualists. Nevertheless, seedlings

planted in irradiated soil, with greater representation of seedborne endophytes gained more biomass on average, suggesting either a growth-promoting effect of seedborne fungi of a suppressive effect of soilborne fungi. Future research is needed to determine if vertically transmitted fungal symbionts have the capacity for a heritable mutualistic relationship.

In this chapter, we observed that seed treatments influenced alpha and beta measures of seed-associated fungal endophytes in seedlings, in addition to *Fusarium* abundance and disease susceptibility in the greenhouse setting. In the next chapter, we will explore the general long-term effects of seed treatments on the composition of fungal and bacterial endophytes, across different agricultural fields.

CHAPTER III

SEED TREATMENTS HAVE LASTING EFFECTS ON THE COMPOSITION OF BACTERIAL AND FUNGAL ENDOPHYTES OF MAIZE

INTRODUCTION

The plant microbiome is integral to the determination of plant phenotype and fitness. Bacteria and fungi comprising the plant microbiome can inhabit one or more described plant-associated habitat types, including the rhizosphere (the root-soil interface), the phyllosphere (the aerial surfaces of the plant), and the endosphere, (tissues within the plant host, including inner portions of roots, shoots, leaves, seeds, flowers and fruits). Endophytes are microbes that inhabit regions of the plant endosphere, without causing any apparent disease symptoms (Hardoim et al., 2008; R. J. Rodriguez et al., 2009). Bacterial and fungal endophytes are known to mediate agronomically-important plant traits, such as disease resistance (Busby, Ridout, & Newcombe, 2016; Cavaglieri et al., 2005; Mousa, et al., 2015), abiotic stress tolerance (Rodriguez et al., 2008; Worchel, Giauque, & Kivlin, 2013), nutrient acquisition (Montañezet al., 2008; Puente et al., 2009; Roesch et al., 2007), and plant growth promotion (Forchetti et al., 2007; Hardoim et al., 2008). However, the effect of a given endophyte on its plant host is context-dependent, mediated by plant host genotype, environmental factors, and microbe-microbe interactions (Busby et al., 2017).

Until recently, plant breeding and cropping practices have functioned without knowledge of their effects on the plant microbiome. There is evidence that intensive crop domestication may have altered the abilities of crops to interact with microbes. For

instance, crop domestication has been associated with a breakdown in plant symbiosis with mycorrhizae (Hetrick, Wilson, & Cox, 1993; Xing et al., 2012) and rhizobia (Pérez-Jaramillo, Mendes, & Raaijmakers, 2016), presumably due to selection of plants that respond to fertilizer inputs. Moreover, recent efforts have observed differences in microbiome assembly and functioning between wild and domesticated cultivars. For example, sunflower domestication is associated with a shift in the composition of fungal root endophytes (Leff et al., 2017), and rhizosphere bacteria of modern sugar beets exhibit less disease-suppressive traits than those found in wild plant relatives (Zachow et al., 2014). In response to these observations, scientists are looking to wilder versions of domesticated crops, such as teosinte (a wild relative of maize), as sources of beneficial endophytes (Estrada et al., 2002).

Seed treatments represent one modern management tool that may affect the plant microbiome in unforeseen ways. Seeds in U.S. commercial agriculture are commonly treated using fungicides or other antiseptic methods to remove seedborne pathogens or to prevent soilborne pathogens from entering the germinating seedling. Similar to the use of antibiotics in humans and livestock, antimicrobial methods to treat for seedborne pathogens or prevent infection by soil pathogens may strongly affect the composition and functioning of the host microbiome. Increasingly, microbes are also applied to seeds as a means of enhancing nutrient acquisition or biological control against pathogen attack (Pratap & Bahadur, 2016). Although antimicrobial or biological seed treatments are effective and often necessary practices for controlling pathogen outbreaks, little is known about how these treatments affect non-target microbes in the plant microbiome.

Importantly, seeds contain multiple species of endophytic bacteria and fungi that

may affect seedling success and plant microbiome assembly. Indeed, many seedborne endophytes exhibit plant-beneficial traits. For example, bacterial seedborne endophytes of cardon cactus increase seedling success by solubilizing rock minerals (Puente et al., 2009), and seed-transmitted *Epichloë festucae* enhances drought tolerance in *Festuca rubra* (red fescue) (Davitt et al., 2011). Seedborne endophytes are known to colonize the rhizosphere in addition to roots and aboveground plant tissues (Barret et al., 2014; Johnston-Monje et al., 2014). The literature on maize seed-associated endophytes suggests that bacterial endophytes of maize have multiple plant-beneficial traits, including nitrogen fixation, phosphate solubilization, production of growth hormones, and biocontrol against fungal disease (Johnston-Monje & Raizada, 2011; Kohl et al., 2015; Links et al., 2014; Rijavec et al., 2007). Although little is known about the general vertical transmission efficiency of seedborne endophytes, recent circumstantial evidence suggests that the majority of bacterial endophytes in maize seedlings may actually come from seed-associated endophytes (Johnston-Monje et al., 2016, 2014).

Fungi of the genus *Fusarium* are ubiquitous in maize, and can occur as asymptomatic endophytes, but the genus also contains pathogenic strains known to cause seedling blight, crown rot, and ear rot (Miller & Ocamb, 2009). *Fusarium* also occurs commonly in maize seeds, and vertical transmission via seeds represents a key dispersal strategy for the genus (Munkvold et al., 1997). The fungus can also survive in soil, and infect plants via horizontal transmission (Fernandez et al., 2008). *Fusarum* can build up in maize and other crops over several generations without exhibiting disease symptoms, and then become pathogenic as levels increase or when plant defenses are weakened by unfavorable environmental conditions (Munkvold & White, 2016). Furthermore, some

species of *Fusarium* produce mycotoxins within seeds, including trichothecene (T-2) and zearalenone, a carcinogen and estrogen agonist, respectively, which are toxic to humans and livestock when infected grain kernels are ingested (Munkvold, 2003). Due to the pernicious nature of *Fusarium* in maize, seed companies often treat seeds as a precaution. Although synthetic fungicides are not an option for organic systems, organic seed companies may utilize approved disinfectants and biological control inoculants to mitigate the negative effects of seedborne *Fusarium* (Hopkins et al., 2003; Nega et al., 2003; Tinivella et al., 2009).

Organic maize represents a small, but rapidly growing sector of U.S. agriculture. There is a lack of hybrid maize varieties available on the market that perform consistently well under more complex and diversely managed organic production systems, so organic growers and plant breeders have been utilizing open-pollinated (OP) maize varieties (Shelton & Tracy, 2015). Open-pollinated maize varieties are bred such that populations consisting of multiple genotypes are allowed to cross-fertilize openly in the field. While this strategy offers less uniformity and typically lower yield than F1 inbred hybrids, it provides several advantages to farmers. OP systems allow for on-farm selection of complex traits such as disease resistance and drought tolerance, which are known to involve many interacting genes, and exhibit strong genotype x environment interactions. OP varieties also represent genetically diverse populations, allowing farmers and plant breeders to select locally-adapted plants while maintaining a diverse gene pool that allows for subsequent adaptation to unpredictable climate patterns and pest outbreaks, or movement of germplasm to other regions (Shelton & Tracy, 2015; Tester, 2011). In the context of seedborne endophytes, studying OP varieties of seed saving

farmers provides an opportunity to follow the composition of seedborne microbes over several successive generations of on-farm selection.

With this study, we sought to determine the effects of antimicrobial and biological seed treatments on bacterial and fungal endophytes of organic, OP maize, and potential feedback effects on plant traits. We tested seed treatments across three different farms in Oregon, USA, two of which are certified organic, and the third farm is an experimental farm, Oregon State University Botany and Plant Pathology Field Lab (OSU), which is managed to have a high incidence of soilborne *Fusarium* pathogens (Miller, 2007). Although seed disinfection methods are limited in certified organic systems, we formulated a new method based on peracetic acid and hot water treatment. We also sourced a locally developed bacterial inoculant that is antagonistic to *Fusarium*. Both the disinfection and inoculant treatments were approved by Oregon Tilth organic certifiers prior to planting.

We wanted to investigate the effects of seed treatments on plant measures related to crown rot and yield, hypothesizing that (i) disinfection of seeds would decrease symptoms of crown rot by reducing incidence of seedborne *Fusarium*, but disinfection of seeds would conversely increase crown rot symptoms at the OSU farm, as disinfected seedlings would be more susceptible to pathogenic soil borne *Fusarium*; (ii) biocontrol inoculation would generally reduce symptoms of crown rot and increase yield; and (iii) disinfection of seeds prior to inoculation would increase the effectiveness of inoculation treatments. In addition to plant measures, we sought to determine the effects of seed treatments on the composition of endophyte communities of adult plants and the seeds that they produced. We predicted that (iv) disinfection and inoculation seed treatments

would reduce alpha diversity of bacterial and fungal endophytes through reduction in seedborne microbial abundance, and (v) significantly alter endophyte microbiome community structure, by disproportionately affecting some taxa over others.

MATERIALS AND METHODS

Experimental Design

We treated maize seeds in a full factorial design including a disinfection treatment and a biocontrol inoculant, i.e., *Control, Disinfection, Inoculation, and Disinfection + Inoculation*. Treated seeds were planted on the respective farms from where they originated, and additionally planted together at the OSU Field Lab, to serve as a common garden and to challenge plants with high pressure from pathogenic strains of soilborne *Fusarium*. We planted seeds a day after seed treatments, in mid-to-late-May of 2014 on the organic farms, and a month later at OSU, to increase seedling susceptibility to pathogenic *Fusarium* induced by heat stress (Munkvold & White, 2016). At each farm site, we planted seeds directly into the soil at 1 ft spacing, into replication plots using a randomized block design to account for spatial variation across fields. Depending on the farm, there were 15-20 replicate plots for each treatment, and each replication plot was approximately 400-600 sq ft, containing 16-24 plants each. When plants reached pollination stage, approximately 8-9 weeks after planting, we destructively harvested a subset of plants for measurements of crown rot and DNA sample collection of crown tissue. Once mature, plants were allowed to dry in the field before harvesting of ears. We removed husks in the field, and dried ears indoors at 22°C. We pooled all ears in a given replication plot, and reported all yields based upon per-plot measures, normalized to the

number of plants planted in each plot. We also took DNA samples from subsets of seeds, as explained below.

Maize seed source

The maize used in this study is a short-season (90-day), open-pollinated flint variety called Cascade Ruby-Gold, bred near Corvallis, Oregon, USA, by plant breeder Carol Deppe. Cascade Ruby-Gold was derived from New England flint maize varieties Abenaki (a.k.a. Roy's Calais) and Byron. It was selected for this study due to its popularity among organic maize growers in the Pacific Northwest. Seed was sourced from the two organic farms involved in this study, Open Oak Farm (OF1), and Pitchfork and Crow (OF2). Both farms had sourced seed from the same stock (Open Oak Farm stock seed, 2012), before growing it on their respective farms in 2013, one year prior to this study. Thus, any differences between seed sources would have occurred over a single growing season on separate farms, or possibly due to sampling effects. Although we did not test the genetics, we assume the maize seed sources to be both genetically diverse and genetically indistinguishable, as they are open-pollinated populations, and did not undergo heavy selection during the one year they were grown on either farm. Seeds did not significantly differ in average weight.

Farm Sites

All farms sites in this study are situated in Linn County, Oregon and share similar soil types. Located near Brownsville, OR, Open Oak's soil is classified as Abiqua silty clay loam, and was amended with 100 lbs per acre of Perfect Blend 4:4:2 pelletized chicken and fish fertilizer prior to the study. Pitchfork and Crow is located near Lebanon, OR. Its soil type is classified as Malabon silty clay loam, and was amended with

Stutzman's 4:3:2 fertilizer at a rate of 1 ton per acre. Both OF1 and OF2 were certified organic by Oregon Tilth at the time of the study - OF1 for one year, and OF2 for three years - although both farms were managed under organic practices for at least 4 years prior. The OSU field site was managed to have high abundance of pathogenic *Fusarium* spp. in the soil, through incorporating *Fusarium* infested crop residues from susceptible plants since 2002 (Ocamb, personal comm.). The OSU field soil type is classified as Chehalis silty clay loam; it is managed conventionally, and was amended with 12:29:10 chemical fertilizer at a rate of 450 lbs. per acre prior to planting.

Seed Treatments

Our custom disinfection treatment began with soaking seeds for 4 hours in a solution of 240 ppm peracetic acid (PAA) in distilled water on a rotary shaker, then rinsing them off in distilled water. We used PAA as an alternative to hydrogen peroxide because it is approved by the Organic Material Review Institute for use on seeds (OMRI Products List; www.omri.org). These surface-sterilized seeds were then placed into a water bath heated to 60°C for 5 minutes, stirring every minute, and then immediately plunged into an ice bath for two minutes to bring their internal temperature back down. This hot water protocol was modified from a protocol developed by Rahman et al., (Rahman et al., 2008) to reduce the abundance of seedborne *Fusarium*. Non-disinfected controls were simply shaken in distilled water for 4 hours, and kept wet for the same period of time as the disinfected seeds. A small subset of seeds was placed on water agar plates (1.5%) and allowed to germinate for 7 days to determine treatment effects on germination rate and seedling vigor.

Directly after the disinfection treatment, we inoculated the still-wet seeds with

1ml per 100 g of seeds of liquid bacterial biocontrol inoculant, by shaking seeds until fully-coated.Seeds were subsequently air-dried in a biosafety cabinet. The inoculant was developed by Ocamb for protection of plants from *Fusarium* pathogens, including resistance to crown rot in sweet corn. The biocontrol formulation is produced by TerraMax (Bloomington, MN) under the label MicroAF. MicroAF contains a cocktail of 8 bacterial strains isolated from plant rhizospheres. The eight bacterial strains were identified as *Methylobacterium mesophiliccum*, *Rhodococcus erythropolis*, *Kocuria varians*, *Pseudomonas diminuta*, *Streptomyces violacceusniger* subsp. violaceusniger, *Streptomyces roches* subsp. rochei, S*treptomyces lavendulae* and *Bacillius megaterium*,, based on fatty-acid analysis conducted in 1994 (Ocamb, personal comm.)

Measuring Crown Rot in Maize

Crown rot in maize appears as darkened crown tissue, due to necrosis of plant cells caused by pathogenesis (Munkvold & White, 2016). During the pollination stage on each farm, we photographed crowns in the field and determined the relative degree of tissue darkening through image analysis, based on the methods of Miller and Ocamb (Miller & Ocamb, 2009). Plants harvested for crown rot were first cleaned of dirt around their roots, and then cut downwards along the middle of the stem, slicing through the crown at the base of the stem (Figure 6). We photographed the transverse sections of crowns in the field via a smartphone camera (HTC One M8), equipped with a custom-built imaging apparatus to standardize crown photos. The viewing apparatus was essentially a 10cm-long cardboard cylinder with a viewing window at the end. The internal viewing window was bordered with white and black swatches to serve as standards during image analysis (Figure 6). All photos were taken using the camera flash

setting as the sole light source. To limit interference of indirect light, we painted the inside of the cylinder with black acrylic, and shielded outside light using a black cloth. We scored each crown photo using ImageJ software (Schneider et al., 2012) by converting each image to grayscale and measuring the mean value within the area of the photo representing the crown tissue, comparing its value to the black and white swatches in each photo. The crown rot index represents a darkness value between these standards, where "white" is a value of 0, and "black" is a value of 1.

Figure 6. Scoring Crown Rot. The Crown Rot Index was scored by photographing the base of transverse stalk sections in the field and analyzing them with image software. The dashed triangle on the right represents an area of the crown selected for image analysis for the degree of darkness by comparing it to the neighboring black and white swatches. Left photo credit Cindy Ocamb.

DNA Extraction from Soil and Plant Samples

At the time of planting on each farm, we collected soil cores from the top 20 cm of topsoil, and pooled them together into a composite sample for each site. Soil samples were air-dried, and then passed through a 2-mm sieve, after which we selected two

subsamples from each site for DNA extraction. We extracted soil DNA using the MoBio PowerSoil DNA isolation kit (MoBio Laboratories, Carlsbad, CA, USA), according to standard protocol.

After sectioned crown tissues were imaged for crown rot, a subset of samples were placed on ice, stored at 4°C overnight, and processed the next day in a biosafety cabinet. Exposed crown tissue was rinsed with distilled water, and then flame-sterilized in 95% ethanol. We used a flame-sterilized scalpel to scrape off the exposed top layer, and then carved out a wedge of previously unexposed crown tissue, which was stored at -80°C until DNA extraction.

Each seed DNA sample represented the aggregate of 20 seeds, randomly sampled from the pool of all seeds harvested from each plot-level treatment replicate. Thus, each seed DNA sample represents a subsample of multiple harvested plants (15-20 plants, depending on the replication plot size) belonging to a single replicate plot. To reduce the influence of surface-associated bacteria and fungi, we surface-sterilized seeds using a protocol adapted from Johnston-Monje and Raizada (2011) by submerging and shaking them in the following solutions for 10-minute intervals: 0.1% Tween-20 detergent, 3% sodium hypochlorite (twice for 20 minutes total), and 95% ethanol, followed by rinsing in autoclaved nanopure water. Seeds were air-dried in a biosafety cabinet before grinding. We ground seeds using a pre-sterilized hand-crank ceramic-burr coffee grinder (Porlex, Osaka, Japan). The grinder was first disassembled and disinfected before each use, first cleaned by scrubbing in detergent (Labtone, VWR, Randor, PA, USA), and then soaking it in MoBio Ultraclean Lab Cleaner (MoBio Laboratories, Carlsbad, CA, USA) which neutralizes contaminating DNA and RNA. The Lab Cleaner was rinsed off in 95%

ethanol, and ceramic burrs and stainless steel parts we flame-sterilized. Plastic parts were air-dried after the ethanol rinse. Once disinfected, the grinders were reassembled with sterile, gloved hands and flame-sterilized tools.

We extracted DNA from crown tissue pieces and ground-up seeds using a DNeasy PowerPlant Pro kit (MoBio Laboratories) modifying the protocol to improve cell lysis from fungi and bacteria, as follows. To the standard 1mm steel beads, we added 0.3ml of 0.1mm glass beads (BioSpec, Bartlesville, OK, USA). After adding the (~50 mg) plant tissue sample and the kit's cell lysis solution (i.e., solutions PB1, PB2, and RNaseA) to each tube, we put samples through two freeze-thaw cycles, alternating between liquid nitrogen until frozen and a 65°C water bath for 2 minutes. Samples were then homogenized in a FastPrep 24 homogenizer (MP Biomedicals, Santa Ana, CA, USA) for two 25 sec cycles at 5.5 power setting. Lastly, samples were left in the 65°C water bath for 10 minutes before continuing with the standard protocol.

Quantitative PCR

We quantified the purified plant and soil DNA using qubit fluorometric quantitation (Thermo Fisher Scientific) and used sample aliquots diluted to 5 ng/ul for downstream DNA analyses. To determine the abundance of *Fusarium* in each DNA sample, we utilized quantitative PCR (qPCR), using the KAPA SYBR Fast kit (KAPA Biosystems, Wilmington, MA, USA), and primers that targeted a sequence of the fungal intergenic spacer IGS gene specific to the genus *Fusarium* (Jurado et al., 2006). To each 10 ul qPCR reaction, we added 10 ng of template DNA and 0.2 nM of forward and reverse primers, to the KAPA SYBR Fast master mix, and diluted in PCR grade water. For qPCR analysis we used a BioRad (Hercules, CA, USA) CFX96 Touch instrument

with the following protocol: 10 minutes at 98°C, followed by 40 cycles at 98°C (denaturation) and 60°C (annealing plus extension) for 15 and 30 seconds, respectively, and finishing with a 2-minute extension step at 72°C. To ensure qPCR product purity, amplicons were also subjected to a melting curve analysis, and a subset was independently confirmed on a gel to ensure the correct size. We calculated baseline fluorescence and qPCR doubling efficiencies on a per-sample basis by fitting raw fluorescence reads to a log-linear equation, using the LinRegPCR software (Ramakers et al., 2003). We standardized copy number estimates of samples by comparing PCR amplification rate to genomic DNA from a *Fusarium verticillioides* isolate, assuming a genome size of 41.74 Mbp (Ma et al., 2010).

Preparation and Processing of 16S rDNA and ITS Amplicon Sequences

We used a DNA metabarcoding approach to characterize microbes present in soil and plant tissue, through amplification of the bacterial 16S ribosomal DNA (rDNA) and fungal Internal Transcribed Spacer (ITS) genes. We designed custom PCR primers for Illumina MiSeq (Illumina, San Diego, CA, USA) sequencing of bacteria and fungi in plant and soil samples. Both primer sets relied on a 2-step PCR process, using Nextera sequencing primer design. The first step of the PCR reaction involved amplification of bacterial or fungal DNA, using variable-length primers to reduce the likelihood of poor clustering efficiency of the sequencer due to low read diversity, as an alternative to spiking amplicon libraries with *PhiX*. Each set of forward and reverse primers contained a Nextera Illumina tag on the 5’ end, followed by a random spacer of 1 to 5 nucleotides, and then the amplicon-specific primer. The second-step PCR primers contained homologous sequence to the first-step primers, in addition to barcode sequences and

standard sequence to bind to the Illumina flowcell. Each forward and reverse 2nd-step primer contained a custom 8-bp barcode, allowing for multiple combinations of 16 bp barcodes. All primer sequences are included in the supplementary material (Table S2).

For amplification of fungi, step-1 forward and reverse primers contained the plant-excluding ITS1F and ITS2 primer sequences, respectively, which target the fungal ITS1 region. For amplification of bacterial 16S rDNA from plant and soil DNA extracts, step-1 primers contained the 799F/1193R universal primer pair, which amplifies the V5,V6 and V7 regions of 16S rDNA gene. The 799F primer excludes plant host plasmid DNA (and consequentially cyanobacterial DNA). Furthermore the 799F/1193R primar pair allows us to distinguish bacterial amplicons from host mitochondrial amplicons due to size differences: maize mitochondrial amplicons are approximately 350 bp longer in this region due to an insertion which is not present in bacteria. To further limit the interference of maize mitochondria, prior to amplification of 16S rDNA we performed a restriction digest of the template DNA, using *NdeI* enzyme (New England Biolabs, Ipswich, MN, USA). Per 10uL reaction, we added 5ul of genomic DNA (50 ng total) to 20 Units of NdeI, and digested overnight. *NdeI* targets a DNA sequence (CATATG), which is present in the maize 16S mitochondrial amplicon, occurs in less than 0.001% of bacteria in the V5-V7 region, and is non-specific across bacterial taxa.

For PCR amplification of the Illumina library, we used KAPA HiFi High Fidelity HotStart ReadyMixPCR (KAPA Biosystems), pooling two replicate PCR runs per sample. Each 10 ul PCR run contained 10 ng of DNA template and 300 pM of each primer. We used an Eppendorf Mastercyler ProS (Hamburg, Germany) for PCR amplification with the following protocols. The 16S protocol for 16S and ITS was as

follows: 98°C for 10 minutes, then 35 cycles of 98°C for 20 seconds, 50°C for 15 seconds, and 72°C for 30 seconds, followed by a final extension step of 72°C for 5 minutes. Duplicate PCR amplicons from step 1 PCR were pooled, and we added 1 uL of pooled 16S and ITS amplicons to each 20 uL second step reaction, which contained Step-2 Nextera primers (Table S2). We amplified using the same protocol as step 1, except the annealing temperature was 63°C, and we only used 10 cycles. Following step 2 PCR, we performed a magnetic bead PCR cleanup and size selection (Agencourt Ampure XL; Beckman Coulter Inc., Brea, CA, USA) to remove non-target primer dimers and host mitochondrial DNA (in the case of 16S amplicons). Our size selection protocol exploited the fact that larger DNA fragments have a greater affinity for the magnetic beads than smaller DNA fragments, at limiting concentrations (Quail et al., 2009). Following bead purification, we pooled equimolar amounts of 16S and ITS amplicons into the same Nextera Illumina Library. We used the Illumina MiSeq v3 reagent kit, allowing for paired sequencing of 300 bp, and sequenced samples over three sequencing runs, at Oregon Health & Science University Molecular and Cell Biology Core (Portland, OR, USA), Oregon State University Center for Genome Research and Computing (Corvallis, OR, USA) and University of Oregon Genomics and Cell Characterization Core Facility (Eugene, OR, USA). There were no significant effects of sequencing run on 16S amplicon composition across samples, so we merged runs together in analysis. However, the ITS amplicon sequences significantly differed based on sequencing run. Fortunately, ITS sample representation almost completely overlapped between runs. Therefore, we used the proportion of sequences from each run as a factor in our statistical analysis.

Illumina Sequencing Analysis Pipeline

Raw MiSeq sequences were first aligned with host *Zea mays* B73 inbred genome DNA using *bowtie2* (Langmead & Salzberg, 2012), to remove maize DNA from the dataset. Then, paired-end reads of fungal and bacterial sequences were merged using PEAR (Paired End reAd mergeR), which assembles reads based on a maximum probability score across a range of overlap lengths (Zhang et al., 2014). On assembled sequences, we used a custom python script to separate bacterial (16S) sequences from fungal (ITS) sequences based on their match to original primer sequences, and trimmed off variable length nucleotide spacers from each end. The remainder of Illumina sequencing processing was performed in R (R Core Team, 2017). Separately for each amplicon and sequencing run, we utilized the workflow of the *DADA2* package (Benjamin J. Callahan et al., 2016), which explicitly models sequencing errors and infers exact amplicon sequence variants (ASVs) based on a probabilistic model. Throughout this paper, we will refer to estimated amplicon sequence variants broadly as ASVs. ASVs have advantages over OTUs (Operational Taxonomic Units) based upon clustering methods (e.g., 97% similarity clustering); for example, OTU clustering methods do not explicitly calculate sequencing error and often overestimate true taxonomic richness due to sequencing artifacts (Benjamin J. Callahan et al., 2017). We filtered out any paired-end assemblies containing an "expected error" rate greater than 2 nucleotides (i.e., maxEE=2). Then, we estimated the sequencing error rates by pooling all samples present in each of the separate sequencing runs. We inferred sequence variants based upon the estimated error rate of each run, by pooling all samples per run, and increasing the OMEGA_A parameter to 1E-4, which allows detection of more rare variants, although

with higher probability of false positives. After determining ASVs, we used the DADA2 *removeBimeraDenovo* function to remove chimeras. Most (88%) of inferred sequence variant sequences were shared across runs, and all ASV's not shared between runs were rare (< 1% of sequences). Therefore, we merged ASV's from both sequencing runs to generate our final sequence table. We assigned taxonomy via DADA2 package's naive Bayesian classifier method. Bacterial ASV's were assigned using the SILVA reference database (SILVA, 2017), and fungal ASV's were assigned using the UNITE database (Community, 2017) . We aligned 16S ASVs using the DECIPHER package (Wright, 2016) and constructed a phylogenetic tree using the "phangorn" package (Schliep et al., 2017), as outlined in the Bioconductor Workflow (Callahan et al., 2016). The 16S and ITS ASV tables, taxonomy tables, taxonomic trees (only 16S), and DNA sample metadata were analyzed together using the *phyloseq* package in R (McMurdie & Holmes, 2013).

Microbiome Data Normalization

High-throughput sequencing datasets typically exhibit high variance in sequencing depth across samples. As a result, these datasets will have a confounding mean-variance relationship, i.e., taxa occurring in samples with a greater mean value also contain a higher variance across samples. To overcome this heteroscedasticity, we used two transformation methods. In the first method, we normalized raw ASV counts using a variance-stabilizing transformation (VST) in the *DESeq* package, which adjusts count data by fitting each taxon to a mean-variance curve (Love et al., 2014). In the alternative method, we normalized taxa counts to relative abundances of the total sequence count in each sample (total sum scaling, TSS), and then adjusted relative abundances to their

centered log-ratio (CLR), which effectively transforms the relative abundance data for use in euclidean space (Lê Cao et al., 2016). With the exception of NMDS ordination comprising all sample types, we normalized crown and seed endophyte ASVs separately from each other.

Calculation of Alpha and Beta Diversity

Using raw ASV counts, we calculated a normalized metric of ASV richness in each sample, controlling for read abundance using *phyloseq rarefy_even_depth* function. For each soil or plant sample, we iteratively resampled 100 random sequences from the total pool of sequences in that sample, repeating the process 100 times to acquire a mean value of ASV richness per 100 reads for each sample. We assessed compositional, i.e., beta-diversity, differences in fungal and bacterial ASVs between samples using Bray-Curtis dissimilarity of VST-transformed counts. Additionally, for the 16S amplicons, we approximated phylogenetic similarity between taxa by determining weighted and unweighted unifrac distances between samples, which either weight scores by the relative abundance of each ASV, or simply uses presence/absence of ASV, respectively. These unifrac phylogenetic dissimilarity metrics were not performed with ITS1 because they are variable length amplicons containing tandem repeats, and therefore difficult to align, and furthermore one cannot accurately ascertain taxonomic similarity between ITS1 amplicons based on sequence similarity alone. We visualized paired dissimilarity measures between samples, using 2-dimensional NMDS ordination plots. Furthermore, we used PCoA to collapse pairwise dissimilarity matrices principal components axes, using sample scores on each of the top 2 principal axes as a one-dimensional quantification of community dissimilarity. In addition to using ecological community

dissimilarity metrics based on PCoA of VST-transformed count data, we also inferred community dissimilarity between samples by performing PCoA of CLR-transformed taxa abundances.

Linear Mixed Models

To test for the effects of farm site and seed treatments on plant disease and yield measures, we used restricted maximum likelihood (REML) linear mixed models, with farm, disinfection, inoculation, and seed source as fixed factors and within-farm replication plot as a random factor, using the *lme4* package in R (Bates et al., 2015). When assessing all farms together, we retained in the model all possible interactions between the fixed factors of Farm, Disinfection and Inoculation including the three-way interaction term. As seed source was only varied at OSU, we retained Seed Source factor as a non-interacting, main effect in the all-farm model. We independently assessed interactions between Seed Source, Disinfection and Inoculation treatments on plant measures by subsetting the data to only the OSU site, modeling all possible interactions between the three terms. Statistical significance of fixed and random effects was determined using the *lmerTest* package in R (Kuznetsova et al., 2017). Statistical significance of fixed effects was calculated using Type III ANOVA with Satterthwaite's approximation of denominator degrees of freedom, based on Type 3 sums of squares. Statistical significance of each random error term was tested with the Chi-squared statistic of a likelihood ratio LRT. We saved residuals from these models to confirm assumptions of normality, and for use in downstream analysis to determine if endophytes can improve model goodness of fit (see next section).

Similar to plant measures, we assessed the effects of seed treatments on bacterial

(16S) and fungal (ITS) alpha- and beta- diversity measures in addition to *Fusarium* abundance obtained by qPCR, using REML linear mixed models. The models were identical to those used for plant measures, except we included llumina sampling depth of each sample as a covariate in the model, and used a different random error term. As DNA samples represented a smaller subset of all plant samples in the field, to maintain a full-rank model in our analysis, each error term represents fewer, broader field blocks of each farm that still contained all combinations of factors in the model. For models involving the ITS1 amplicon, we additionally used SeqRun as a covariate, which represents the relative proportion of reads obtained from each Illumina MiSeq sequencing run of the ITS amplicon.

Identifying Taxonomic Features

To identify bacterial and fungal taxa that are associated with experimental factors or plant measures, we compared several different techniques, and different taxonomic levels, including ASV, genus, family, class, order, and phylum. We limited the search to more common taxa, defined as taxa occurring in at least 10% of the samples of interest, and with read abundances greater than 0.01% of the total read abundances. In the first method, we modeled raw count data using negative binomial generalized linear models, implemented in *DESeq2* package, via the strategy of Wagner et. al 2016 (Wagner et al., 2016). With this approach, we were able to isolate effects of different experimental factors on the abundance of each taxon by performing a likelihood ratio test (LRT) to compare deviation between full and reduced models. For example, to isolate the effect of disinfection treatments, we compared a full model of Farm + Disinfection + Inoculation + Seed Source to a reduced model of Farm + Inoculation + Seed Source, and determined

which taxa were significantly impacted by the change in the model. To investigate interaction terms, we performed LRT between the model containing only main terms to a model that included the interaction term of interest. We used this method to generate contrasts in taxon read abundance with respect to the variables of interest.

We used CLR-transformed relative abundance counts for all other taxa-discrimination methods, which employed R packages *randomForest* (Liaw & Wiener, 2002), *ALDEx2* (Fernandes et al., 2013), and *mixOmics* (Le Cao et al., 2017). The random forest algorithm uses decision trees on randomly subsampled predictor variables to determine the taxonomic features that best predict the response variables of interest. To maximize certainty in this method, we utilized three different random forest feature selection techniques, the standard *out-of-bag* method (*randomForest* package; selecting the top 5 features), *recursive feature elimination* implemented through the *caret* package (Wing et al., 2017), and an *all-relevant-features* feature selection method implemented in package *Boruta* (Kursa & Rudnicki, 2010). The ALDEx2 method creates a probability distribution of compositional data using Monte Carlo simulations to enable ANOVA-like differential expression analysis. Using this package, we determined discriminating taxa based upon the Benjamini-Hocherg corrected Welch's t-test ($p < 0.05$). Lastly, we utilized sparse partial least squares discriminant analysis sPLS-DA through the *mixOmics* package. This method produces PCoA ordinations of samples based upon community composition, constrained by the variable of interest. We limited PCoAs in this analysis to a single axis spanning most of the variation in the predictor variable of interest, and ranked important taxa based upon their loading scores on this axis.

To identify endophyte taxa that help predict plant crown rot and harvest measures,

we used residuals produced by respective the linear mixed models as the response variable. We either set these residuals as continuous response variables, or converted them to categorical response variables, i.e., whether the residual from that DNA sample positively or negatively deviated from the linear mixed model prediction. Taxa that consistently differed with respect to residuals were added to linear mixed models to determine whether they could be used to more accurately predict plant disease status or yield.

RESULTS

Plant Measures

The Crown Rot Index at the pollination stage was significantly higher in plants growing at Oregon State University (OSU) than at the organic farms Open Oak (OF1) and Pitchfork and Crow (OF2), which did not significantly differ between each other with respect to crown rot ($P<0.001$). Maize ears produced at OF2 and OSU had a high degree of lepidopteran maize earworm damage, which often resulted in moldy ears. Entire ears, or portions of ears, containing mold or damage were discarded at harvest, largely contributing to differences in harvest measures between farms (Figure 7). On average, plants grown at OF1 yielded three times more seeds per plant, and 25% heavier ears.

Linear mixed models across the three farms indicated that both disinfection and inoculation of seeds affected plant measures in a context-specific manner. Although there was no consistent fixed effect across all farms, the severity of crown rot was mediated by an interaction between seed disinfection and seed inoculation (Figure 7 A,B; $P<0.05$). Specifically, the biocontrol inoculant only reduced crown rot when seeds were first

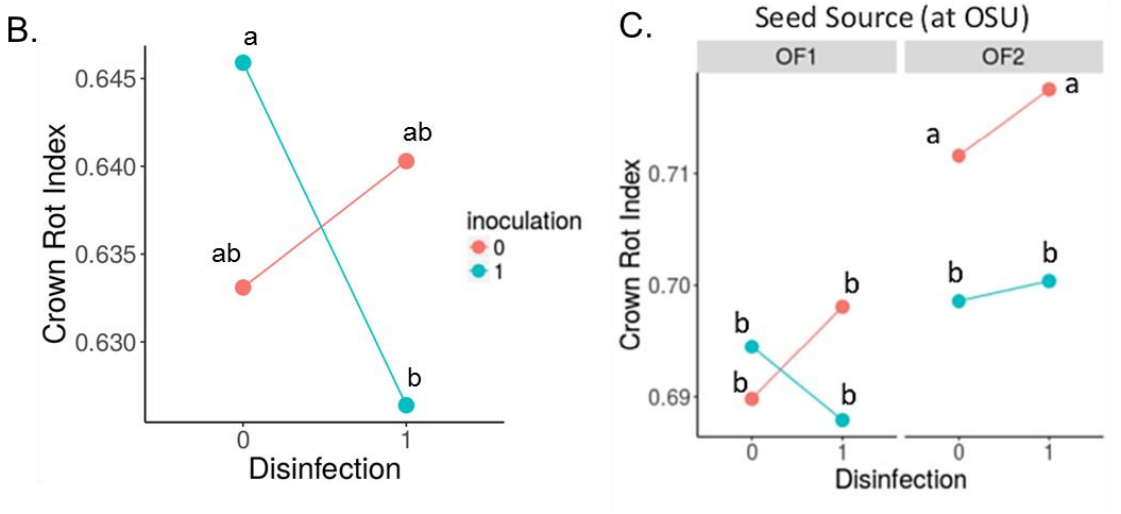
disinfected. Plants grown from seeds sourced from OF2 were associated with higher crown rot than seeds from OF1 across all farms (P=0.054), a trend that was more significant in a linear mixed model that only included the common garden at OSU (Figure 7A,C P=0.020).

The bacterial inoculant differentially affected crop yield measures with respect to both farm and seed source (Figure 7A). Inoculated seeds planted at OF1 generally tended to yield more seeds and heavier ears, while seeds planted at OF2 and OSU tended to yield marginally less than, or equal to, non-inoculated controls. At OSU, yields of OF1-sourced seeds responded positively to both Disinfection and Inoculation, while seeds sourced from OF2 tended to produce lower yields in response to the seed treatments.

Figure 7. Summary of linear mixed model results of plant disease and harvest measures, including ear weight and yield per plant. The results table (A) indicates strong site-specific and seed source specific responses to seed treatments. Significant p-values (P<0.05) are in bold. Across all farms, the inoculation treatment reduced Crown Rot Index only after disinfection. The effect of Inoculation on crown rot severity also depended significantly on Seed source (C).

A.

	Crown Rot Index		Cob Weight		Yield Per Plant	
Across all farms	**Statistic**	**P-value**	**Statistic**	**P-value**	**Statistic**	**P-value**
R2	0.350		0.107		0.699	
Farm	F2,51=62.65	**1.64E-14**	F2,56=44.44	**2.88E-12**	F2,60=138.61	**2.2E-16**
Disinfection	F1,427=0.96	0.327	F1,4201=0.40	0.528	F1,226=0.26	0.608
Inoculaiton	F1,427=0.01	0.928	F1,4191=0.42	0.517	F1,226=1.83	0.178
Seed Source	F1,427=3.73	**0.054**	F1,4170=0.48	0.488	F1,225=0.04	0.838
Farm x Disinfection	F2,427=0.65	**5.23E-01**	F2,4197=0.85	0.426	F2,226=2.94	0.055
Farm x Inoculation	F2,427=0.66	0.520	F2,4188=4.61	**0.010**	F2,226=4.20	**0.016**
Disinfection x Inoculation	F1,427=4.43	**0.036**	F1,4191=0.64	0.422	F1,225=1.96	0.162
Farm x Disinfection x Inoculation	F2,427=1.70	0.184	F2,4189=0.59	0.556	F2,225=3.30	**0.039**
Replicate Plot	X21=11	**1.04E-03**	X21=54.01	**1.99E-13**	X12=22.72	**1.88E-06**
OSU Only						
R2	0.004		0.013		0.030	
Disinfection	F1,4179=0.25	0.617	F1,230=0.24	0.627	F1,263=0.15	0.701
Inoculaiton	F1,4175=0.02	0.878	F1,230=0.75	0.387	F1,263=2.07	0.151
Seed Source	F1,2037=5.66	**0.017**	F1,259=2.73	0.100	F1,263=5.47	**0.020**
Disinfection x Inoculaiton	F1,4174=0.15	0.695	F1,230=0.84	0.360	F1,263=0.60	**4.39E-01**
Disinfection x Seed Source	F1,4181=2.35	0.126	F1,230=6.71	**0.010**	F1,263=0.06	0.804
Inoculation x Seed Source	F1,4175=6.82	**0.009**	F1,230=4.14	**0.043**	F1,263=1.01	0.316
Disinfection x Inoculation x Seed Source	F1,4174=0.03	0.856	F1,230=2.15	0.144	F1,263=0.19	0.667
Replicate Plot	X21=0.38	**2.2E-16**	X21=1.74	**2.2E-16**	X21=5.67	**1.73E-02**



Fusarium Abundance in Crowns and Seeds

The *Fusarium* specific IGS gene copy number abundance obtained via qPCR was 18 times higher in maize seeds sourced from OF2 than OF1 ($t_{3.66}$=0.002, p=0.002).

However, plants grown from these seeds did not significantly differ in terms of copy number abundance in their crowns or their harvested seeds, as a mixed linear model of IGS-Fus abundance indicated no significant effects of Farm or SeedSource as factors (Table 2). Crown rot severity was not correlated with the abundance of *Fusarium* in crown tissue determined by qPCR, as inferred by a likelihood ratio test comparing the full model with IGS-Fus copy number as a covariate, to the null linear mixed model (X_2=0.123, P=0.94).

Across all farms, the biocontrol inoculant treatment reduced the *Fusarium*-specific gene copy abundance in crowns by 37% (Table 2 P=0.051). There was also a significant Disinfection x Inoculation effect on *Fusarium* abundance in crowns. The abundance of *Fusarium* in seeds harvested from this experiment was mediated by a Farm x Inoculation effect, where Inoculation reduced seedborne *Fusarium* at OF1, increased it at OF2, and had a marginal effect on seedborne *Fusarium* at OSU.

Table 2: Linear mixed model results of *Fusarium* abundance in crown tissue samples determined by qPCR, across all farms and at OSU Field Lab. Significant (or marginally significant) P-values are in bold. Plots were random terms in the model.

		Crowns		Seeds	
	Model Term	F or X2 statistic	P-value	F or X2 statistic	P-value
All Farms	R^2	0.23		0.17	
	Farm	F2,52=0.15	0.862	F2,13=0.15	0.863
	Disinfection	F1,52=1.43	0.237	F1,41=0.22	0.644
	Inoculation	F1,52=3.98	**0.051**	F1,41=0.15	0.700
	SeedSource	F1,52=0.53	0.469	F1,40=1.36	0.250
	Farm:Disinfection	F2,52=2.60	0.084	F2,41=0.42	0.659
	Farm:Inoculation	F2,52=1.22	0.303	F2,41=4.27	**0.021**
	Disinfection:Inoculation	F1,52=4.63	**0.036**	F1,41=0.58	0.452
	Farm:Disinfection:Inoculation	F2,52=0.33	0.717	F2,41=1.15	0.328
	Plot	X21	1.000	X21	0.097
OSU	R^2	0.17		0.14	
	Disinfection	F1,54=0.87	0.355	F1,53=0.00	0.995
	Inoculation	F1,53=4.06	**0.049**	F1,53=0.03	0.865
	SeedSource	F1,55=2.45	0.124	F1,53=1.68	0.201
	Disinfection:Inoculation	F1,53=5.38	**0.024**	F1,53=0.04	0.845
	Disinfection:SeedSource	F1,55=0.96	0.332	F1,53=0.00	0.997
	Inoculation:SeedSource	F1,54=0.92	0.343	F1,53=7.09	**0.010**
	Disinfection:Inoculation:SeedSource	F1,53=1.06	0.309	F1,53=0.64	0.426
	Plot	X21	0.617	X21	1.000

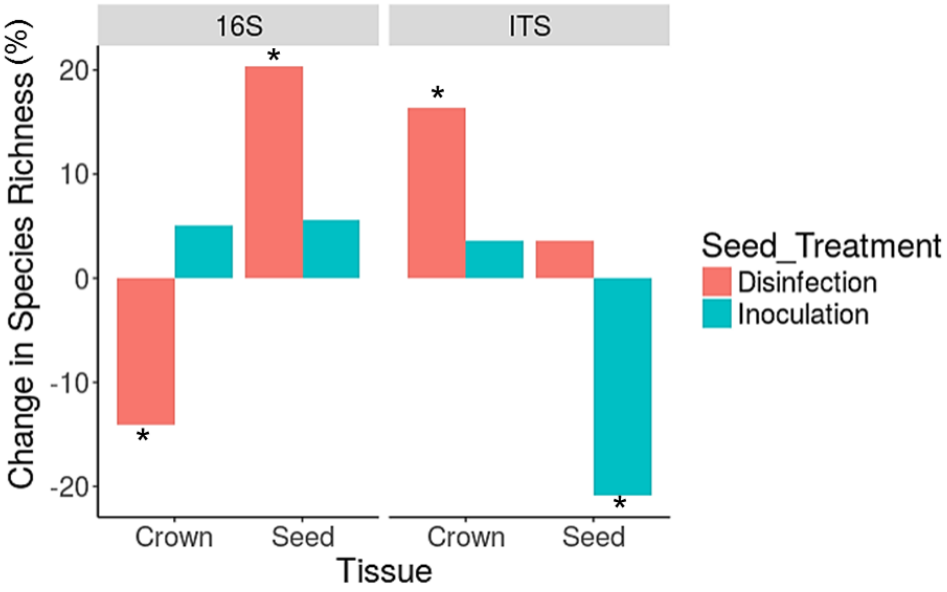
Endophyte Alpha and Beta Diversity

Based upon rarefied counts of amplicon sequence variants (ASVs) produced by Illumina sequencing, we found bacterial endophytes were generally more diverse than fungal endophytes in maize crowns and seeds. An average crown sample contained 335 ASVs of bacterial 16S, and 31 ASVs of fungal ITS. Seed samples, which each represented a pool of 20 seeds for each DNA sample, averaged 60 and 29 ASVs of bacteria and fungi, respectively. Crown tissue was generally more species rich than seed tissue, particularly in the case of bacterial endophytes, which were over 10 fold more speciose in crowns versus seed. Fungal crowns were generally undersampled in terms of Illumina sequencing depth. When rarefying to control for sampling depth, crown tissues contained over twice the number of fungal endophyte taxa than seeds. Soil samples

contained 845 bacterial ASVs and 290 fungal ASVs. Based on 2D NMDS ordination of Bray-Curtis dissimilarity measure between samples, bacterial and fungal endophyte communities in seeds were most distinct from soil microbial communities, with crown endophyte communities falling in between seed and soil in ordination space.

Seed disinfection and inoculation treatments differentially affected bacterial and fungal endophyte communities found in crowns and harvested seeds (Figure 8). Across all farms, disinfection of seeds generally resulted in a 14% reduction in bacterial species richness in crowns, and a 20% increase in bacterial species recovered from seeds at harvest. Bacterial endophyte richness was not affected by the bacterial inoculum treatment. In contrast, fungal endophyte richness was 15% greater in crown tissues of plants grown from seeds that had been disinfected. Inoculation of seeds with the biocontrol bacterium prior to planting was associated with a 21% reduction in fungal species richness in harvested seeds, but we determined no significant effect on crown fungal endophyte diversity.

Figure 8: Seed treatment effects on rarefied species richness of 16S (bacterial) and ITS (fungal) ASVs, indicated by percent change from the control. Statistically significant changes, determined by contrasts of linear mixed models are indicated with an asterisk.



Bacterial endophyte communities in crown and seeds varied widely with respect to the farms from which they were grown (Table 3). Although the composition of crown bacterial endophyte communities did not differ with respect to either seed treatment, the the biocontrol inoculant strongly affected bacterial endophytes that were found in seeds at harvest, an effect that was also mediated by Farm.

The composition of fungal endophyte communities of crowns did not significantly vary between farms, but the seed-associated fungal endophyte community composition strongly differed with respect to farm (Table 3). The disinfection treatment was associated with a moderate shift in crown fungal endophyte community composition, and a farm-specific shift in seedborne fungal endophyte community composition. The biocontrol inoculant did not significantly alter Bray Curtis or PCA-CLR measures of fungal endophyte community composition in crowns, but did affect the composition of seed-associated fungal endophytes.

Table 3: Summary of linear mixed models effects on endophyte community measures in crowns and seeds. Each column contains either an R^2 value or F or X^2 statistic and its p-value. Significant p-values (<0.05) are in bold.

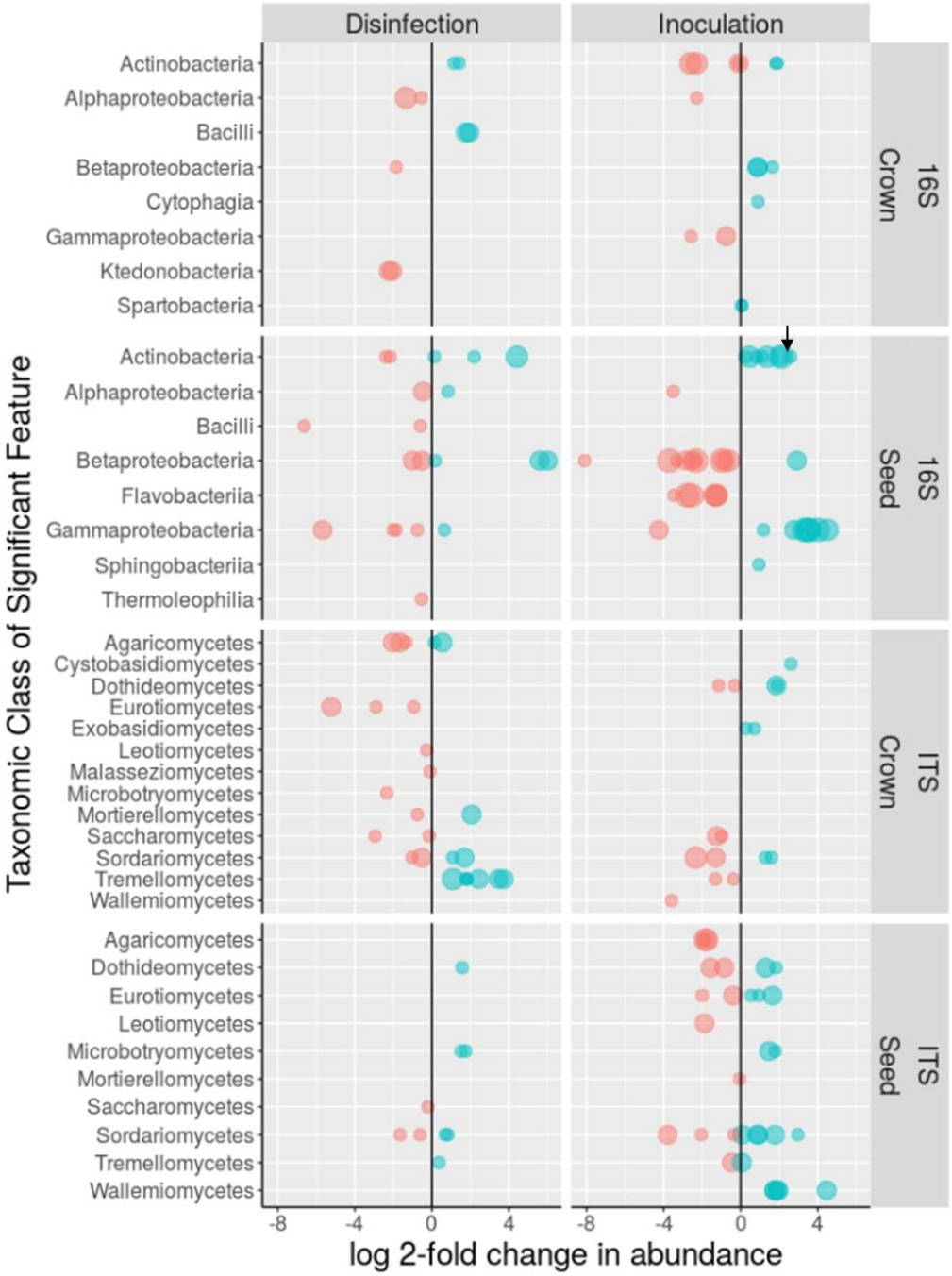
		Crown Endophytes				Seed Endophytes			
	Terms in Model	Rarefied ASV Richness	Bray- Curtis PC1	Bray-Curtis PC2	CLR PC1	CLR PC2	Rarefied ASV Richness	Bray- Curtis PC1	Bray-Curtis PC2	CLR PC1	CLR PC2
16S Bacteria	R2	0.20	0.79	0.13	0.74	0.18	0.37	0.77	0.50	0.87	0.68
	Farm	F2,52=0.67 0.518	**F2,52=83.37 2.2E-16**	F2,52=1.56 0.220	**F2,52=59.62 3.49E-14**	F2,52=1.60 0.212	F2,50=3.54 0.037	**F2,10=29.63 5.72E-05**	**F2,50=14.90 8.41E-06**	**F2,50=27.53 8.67E-09**	**F2,50=12.46 4.06E-05**
	Disinfection	**F1,52=4.34 0.042**	F1,52=1.45 0.233	F1,52=2.53 0.117	F1,52=0.11 0.742	F1,52=3.68 0.061	**F1,50=7.28 0.010**	F1,41=0.37 0.547	F1,50=0.00 0.975	F1,50=0.41 0.526	F1,50=0.25 0.619
	Inoculation	F1,52=0.60 0.441	F1,52=0.20 0.659	F1,52=0.00 0.999	F1,52=0.14 0.708	F1,52=0.11 0.747	F1,50=0.64 0.429	**F1,41=18.37 1.09E-04**	F1,50=0.73 0.396	**F1,50=17.64 1.10E-04**	**F1,50=5.97 0.018**
	SeedSource	F1,52=0.13 0.723	F1,52=0.08 0.773	F1,52=0.01 0.939	F1,52=0.12 0.731	F1,52=0.01 0.906	**F1,50=4.20 0.046**	F1,42=0.87 0.357	F1,50=0.02 0.892	F1,50=1.14 0.290	F1,50=0.18 0.670
	SeqDepth	F1,52=0.90 0.348	F1,52=0.03 0.858	F1,52=0.09 0.762	F1,52=0.27 0.608	F1,52=1.08 0.304	F1,50=5.09 0.028	**F1,50=6.54 0.014**	F1,50=1.12 0.295	**F1,50=171.11 2.2E-16**	**F1,50=59.99 4.13E-10**
	Farm:Disinfection	F2,52=1.18 0.317	F2,52=0.50 0.607	F2,52=1.38 0.259	F2,52=0.13 0.877	F2,52=2.14 0.127	F2,50=1.33 0.273	**F2,41=5.09 0.011**	F2,50=1.53 0.227	F2,50=4.13 0.022	F2,50=2.22 0.119
	Farm:Inoculation	F2,52=1.24 0.297	**F2,52=3.17 0.050**	F2,52=0.70 0.501	F2,52=1.17 0.319	F2,52=0.79 0.461	F2,50=1.29 0.284	**F2,42=24.69 8.31E-08**	F2,50=0.51 0.602	**F2,50=14.16 1.34E-05**	**F2,50=12.36 4.36E-05**
	Disinfection:Inoculation	F1,52=0.12 0.735	**F1,52=4.65 0.036**	F1,52=0.02 0.898	F1,52=1.67 0.201	F1,52=0.63 0.433	F1,50=0.96 0.331	F1,41=0.21 0.646	F1,50=1.87 0.177	F1,50=0.00 0.989	F1,50=0.91 0.346
	Farm:Disinfection:Inoculation	F2,52=2.57 0.086	F2,52=0.97 0.387	F2,52=0.15 0.862	F2,52=0.46 0.634	F2,52=0.27 0.762	F2,50=0.51 0.602	F2,41=1.66 0.202	F2,50=0.04 0.958	F2,50=1.56 0.219	F2,50=0.83 0.441
	Plot	X21=0.00 1.000	X21=0.00 1.000	X21=0.00 1.000	X21=0.00 1.000	X21=0.00 1.000	X21=0.00 1.000	X21=0.19 0.662	X21=0.00 1.000	X21=0.00 1.000	X21=0.00 1.000
ITS Fungi	R2	0.47	0.35	0.42	0.81	0.28	0.51	0.52	0.53	0.78	0.53
	Farm	F2,8=0.20 0.822	F2,13=2.36 0.135	F2,9=2.31 0.153	F2,14=1.07 0.369	F2,52=2.37 0.104	F2,16=2.56 0.109	**F2,12=11.07 0.002**	**F2,49=12.31 4.67E-05**	F1,42=0.14 0.715	**F2,14=17.69 1.42E-04**
	Disinfection	**F1,35=4.13 0.050**	F1,41=1.20 0.280	F1,40=0.98 0.329	F1,44=0.68 0.415	**F1,52=9.03 0.004**	F1,42=0.10 0.752	F1,40=0.12 0.729	F1,49=0.06 0.814	F1,42=0.14 0.715	F1,41=0.12 0.728
	Inoculation	F1,33=0.24 0.630	F1,41=2.24 0.143	F1,38=0.00 0.949	F1,43=0.62 0.437	F1,52=0.26 0.612	**F1,42=4.33 0.044**	**F1,41=4.56 0.039**	F1,49=0.00 0.967	**F1,43=6.89 0.012**	F1,41=0.63 0.433
	SeedSource	F1,34=0.37 0.547	F1,42=0.35 0.557	F1,40=2.62 0.114	F1,45=1.29 0.262	F1,52=0.01 0.933	F1,42=0.00 0.950	F1,40=0.42 0.518	F1,49=0.28 0.601	F1,43=0.01 0.903	F1,41=0.20 0.661
	SeqDepth	**F1,45=27.38 4.17E-06**	**F1,49=21.09 3.07E-05**	**F1,52=4.94 0.031**	**F1,52=199.11 2.2E-16**	**F1,52=5.68 0.021**	**F1,47=48.11 1.00E-08**	F1,48=0.15 0.698	**F1,49=14.76 3.52E-04**	**F1,47=73.69 3.45E-11**	**F1,48=28.13 2.80E-06**
	SeqRun	**F1,46=5.55 0.023**	F1,47=3.19 0.080	**F1,49=23.82 1.17E-05**	**F1,51=4.46 0.040**	F1,52=2.82 0.099	**F1,48=4.16 0.047**	F1,46=0.42 0.518	F1,49=0.51 0.480	F1,47=0.31 0.581	F1,47=0.12 0.727
	Farm:Disinfection	F2,34=1.86 0.172	F2,41=0.64 0.533	F2,38=0.69 0.507	F2,43=1.36 0.266	F2,52=2.33 0.108	F2,42=2.69 0.080	F2,40=2.34 0.109	**F2,49=4.74 0.013**	F2,42=1.86 0.168	**F2,41=3.66 0.034**
	Farm:Inoculation	F2,33=0.22 0.801	F2,40=0.75 0.478	F2,37=0.45 0.639	F2,42=0.31 0.733	F2,52=0.11 0.895	F2,42=0.39 0.682	F2,40=0.05 0.950	F2,49=1.36 0.265	F2,42=2.47 0.097	F2,41=0.19 0.829
	Disinfection:Inoculation	F1,38=1.29 0.262	F1,43=1.34 0.254	F1,41=0.99 0.326	F1,45=0.00 0.976	F1,52=0.93 0.340	F1,42=0.00 0.954	F1,40=0.07 0.788	F1,49=0.21 0.650	F1,42=0.00 0.955	F1,41=0.06 0.801
	Farm:Disinfection:Inoculation	F2,36=0.01 0.992	F2,42=0.78 0.467	F2,40=2.00 0.149	F2,44=0.25 0.780	F2,52=0.03 0.972	F2,42=0.45 0.642	F2,41=0.87 0.428	F2,49=1.05 0.356	F2,43=1.10 0.343	F2,42=0.07 0.934
	Plot	X21=0.44 0.508	X21=2.43 0.119	X21=0.31 0.576	X21=0.03 0.866	X21=0.00 1.000	X21=2.20 0.138	X21=3.53 0.060	X21=0.00 1.000	X21=0.00 0.994	X21=2.68 0.101

Unequal Response to Seed Treatments among Fungal and Bacterial Endophyte Taxa

We found numerous bacterial and fungal endophyte taxa that consistently differed with respect to disinfection and inoculation seed treatments, using a combination of generalized linear models, random forest feature selection techniques, sparse partial least squares linear discriminant analysis (sPLS-DA) and ALDEx2 differential expression. For further analysis, we retained all taxa that were independently confirmed by two or more methods (Figure 9). Seed treatments affected bacterial and fungal endophytes differently depending on the taxonomic designation and tissue from which they originated. For example, disinfection of seeds disproportionately increased crown-associated bacterial endophytes belonging to the taxonomic classes of Bacilli and Actinobacteria, while reducing Alpha- and Beta-proteobacteria and Ktedonobacteria. Fungal endophtyes within the Eurotiomycete, Malasseziomycete and Saccharomycete classes generally were lower abundance in crown tissue following seed disinfection, while Tremellomycete populations were enriched (Figure 9).

Of all of the endophyte responses to seed treatments, the seed-associated bacterial endophyte response to the Inoculation treatment was the most pronounced. The bacterial inoculant resulted in a notable increase in endophytes in the Actinobacteria and Gammaproteobacteria classes and a reduction in endophyte taxa within the Betaproteobacteria and Flavobacteria classes. One of the bacterial species significantly enriched in seeds of inoculated plants was found to be *Rhodococcus erythropolis*, one of the species of Actinobacteria present in the biocontrol bacterial inoculant.

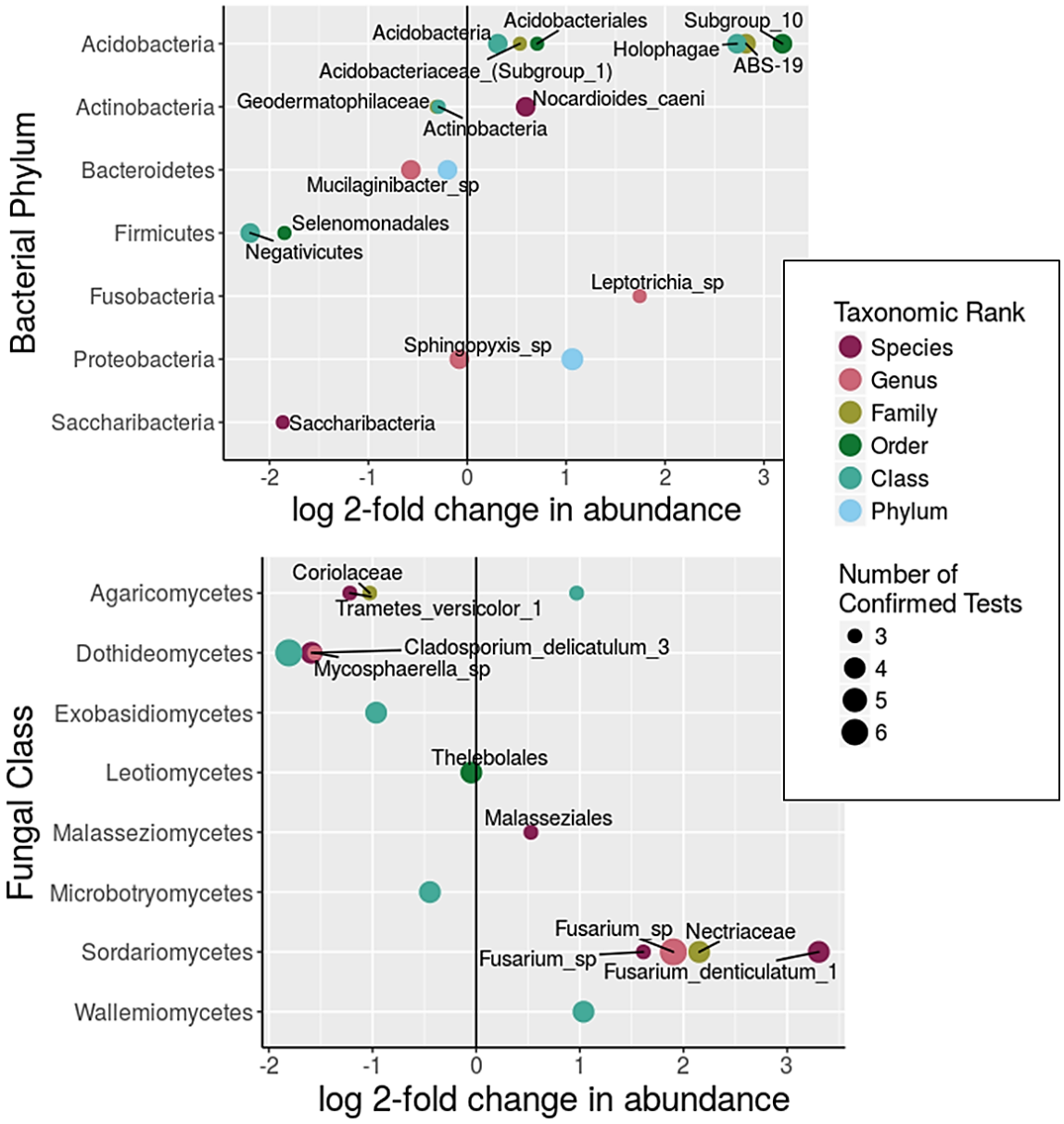
Figure 9. Summary of taxonomic features significantly affected by seed treatments using six feature selection methods, organized by taxonomic class and plotted based on log2 fold change in abundance as determined by *DESeq2*. Each point represents significant taxonomic ranks (Order, Family, Genus, or ASV) falling within the listed taxonomic class. Point color is based on the direction of effect, and point size corresponds to the number of feature selection methods confirming that taxon, from 3 to 6. The arrow indicates the *Rhodococcus erythropolis* ASV, one of the species present in the inoculum.



Taxa Associated with Crown Rot Index

We discovered several bacterial and fungal crown-associated endophyte ASVs that significantly differed with respect to Crown Rot Index residuals extracted from REML linear mixed models that accounted for Farm, Seed Source, and Disinfection and Inoculation seed treatments (Figure 10). Crown tissue samples scoring a greater Crown Rot Index than model predictions contained disproportionately more *Fusarium* spp, and bacterial taxa of Acidobacteria and Proteobacteria (Figure 10). Fungal endophyte classes associated with a low Crown Rot Index included Dothideomycetes, Exobasidiomycetes, and Microbotrymycetes. Bacterial endophytes of Actinobacteria and Negativicutes were also proportionately more abundant in crowns with a lower disease score. Examining lower taxonomic levels, the bacterial genera *Mucilaginibacter* and *Sphingopyxis* and fungal genera *Cladosporium* and *Mycosphaerella* were significantly more abundant in healthier crowns (Figure 10).

Figure 10. Bacterial and fungal endophyte taxa positively and negatively associated with Crown Rot Index residuals from full REML linear mixed models. Taxa disproportionately occurring in crowns with a lesser degree of crown rot than predicted by the model are on the left side of the x-axis, and taxa associated with increased crown rot are on the right side of the x-axis.



DISCUSSION

Context Dependent Response to Seed Treatments

Complex plant traits such as yield and disease tolerance are mediated by many interacting factors, including genotype, environment, and plant microbiome. Antimicrobial and biocontrol seed treatments are intended to decrease disease symptoms and increase yield by targeting plant pathogens. However, seed treatments will have non-target effects on microorganisms of the plant microbiome, which may result in unpredictable feedback effects on plant traits. In this experiment, we found that our seed treatments resulted in differential phenotypic responses in maize, depending on the farm on which the plants were grown, or where the seeds had been grown in the previous year, i.e., Seed Source (Figure 7A,C.). Endophyte communities in crowns and seeds differed significantly with respect to farm, with the exception of crown fungal endophytes, suggesting that divergent endophyte community structure across sites may have played a role in context-dependent observations of plant disease and harvest measures. Field site appears to be a key factor that determines endophyte communities in other studies, such as leaves and roots of *Boechera stricta* (Wagner et al., 2016) and seeds of *Phaseolus vulgaris* L (Klaedtke et al., 2015).

As hypothesized, we found a significant interaction effect between Disinfection and Inoculation seed treatments, in which the biocontrol inoculant was only consistently effective at reducing the incidence of crown rot following disinfection of the seed (Figure 7A,B). We predicted this to be the case based on the assumption that seed disinfection would first remove seedborne microbes from the seed that may interfere with the establishment and proliferation of the 8 strains of bacteria composing the inoculant. This

result, combined with significant interactions between Inoculation and Seed Source (Figure 7C), suggests that seedborne microbes may play a significant role in mediating the effect of seed inoculants, and thus may be a contributing factor to the relatively high inconsistency of biological seed treatments in agriculture. Disinfecting seeds prior to inoculation may offer more consistent results.

Differential Responses of Crown and Seed Endophyte Communities

Although seed treatments did not have any consistent impacts on maize crown rot or yield in this study, the maize-associated endophyte populations experienced general shifts in diversity patterns in response to Disinfection and Inoculation, regardless of farm or seed source (Figure 9). Following the seed disinfection treatment, bacterial endophyte ASV richness was generally diminished in crowns, with no measurable effects on community composition, while fungal richness increased, with a coincident shift in community composition (Table 3). It is possible that disinfection of seeds may have created a more favorable environment in maize crowns for soilborne opportunistic fungi, but not bacterial endophytes. In contrast, disinfection of seeds resulted in a 20% enrichment of bacterial ASVs in the subsequent generation of seeds, although we detected no significant effects on bacterial community composition. Fungal species richness in harvested seeds was not affected by seed disinfection, but fungal taxa were significantly depleted in seeds grown from plants receiving the bacterial biocontrol inoculant, suggesting the biocontrol inoculant may be antagonistic to other endophytic fungi besides its intended target *Fusarium* spp, such as fungal endophytes of Leotiomycetes and the more distant Basidiomycete class of Agaricomycetes (Figure 9).

Although bacterial species richness was generally not affected by the bacterial

inoculant, seed endophytes harvested from plants receiving the inoculant had a markedly different bacterial community composition. These seeds were disproportionately enriched in the Gammaproteobacteria genus *Stenotrophomonas*, and several members of the Actinobacteria class, including endophytes from the genera of *Microbacterium*, *Clavibacter*, and *Rhodococcus*. The *Rhodococcus* sp. amplicon sequence variant most closely matched to the species *Rhodococcus erythropolis*, in the NCBI nBLAST database, which is one of the species in the 8-strain biocontrol inoculant, MicroAF, used in this study. Although impossible to confirm at the strain-level based on the 16S rDNA V5-V7 region, we report this as a possible case of vertical transmission of a biological control inoculant, and warrants more research. Microbial inoculants often provide inconsistent benefits to plants due to their inability to persist in the environment, and vertical transmission through seed is one promising way to maintain beneficial microbes in a crop production system. For example, Mitter et al. (Mitter et al., 2017) developed a system to introduce a *Paraburkholderia* bacterium to maize, wheat, soy, and pepper seeds by inoculating flowers, creating a seedborne heritable symbiont that enhanced the growth rate of these crops.

Here, we show that biological seed inoculants can significantly shift the plant endophyte microbiome in crown, and harvested seeds, an effect that may be inherited across plant generations. There are few studies that have examined interactions between biological inoculants and plant microbiomes, but those that have typically observe a significant inoculant effect. For example, Conn and Franco (Conn & Franco, 2004) found that commercial inoculants severely reduced actinobacterial diversity of wheat roots by 50%, and in chamomile plants, bacterial inoculants have been shown to shift rhizosphere

community structure and diversity (Schmidt et al., 2014). Our study suggests that inoculants may have a stronger effect on plant endophytic microbes than antimicrobial seed treatments. Although we did not assess fungicides or other antimicrobial chemicals, we show that biological, non-chemical seed treatments that are typically classified as organic, can strongly shift plant microbiomes. This inoculant-mediated disruption of plant microbiomes merits future research regarding the implications for plant health, and may need to be addressed in future decisions about organic standards.

Fusarium as a Predictor of Crown Rot, and Possible Biocontrol Candidates

Fungal pathogens of the genus *Fusarium* are believed to be a primary cause of crown rot in the Willamette Valley, OR, the location of this study (Miller & Ocamb, 2009). Although the genus contains non-pathogenic and beneficial species (Rodriguez Estrada, Jonkers, Kistler, & May, 2012), we hypothesized that Crown Rot Index would be correlated with prevalence of *Fusarium* spp. in crown tissue measured via qPCR analysis. However, this association was not found, after correcting for effects of farms and seed treatments. In contrast to qPCR methods, using feature selection methods of Illumina sequencing data, we determined a high positive association between Crown Rot Index residuals and ASVs of the genus *Fusarium* (Figure 10). Next-generation sequencing datasets are inherently compositional, as opposed to qPCR, which is based on absolute abundances. It may be that the relative abundance of *Fusarium*, rather than absolute abundance, is a better predictor of crown rot. Next-generation sequencing may provide more accurate results than qPCR-based methods, which are difficult to optimize for environmental samples, and run the risk of quantifying of non-target DNA.

We found several bacterial and fungal taxa that were negatively associated with

the Crown Rot Index residuals. While we caution that these relationships can be due to any number of mechanisms associated with disease, we assume that taxa negatively associated with crown rot are putative biocontrol candidates. Negatively associated bacteria included ASVs from the genera *Mucilaginibacter* (Bacterioidetes), and *Sphingopyxis* (Alphaproteobacteria), and the little understood candidate phylum of Saccharibacteria. Fungal genera of *Cladosporium*, *Mycosphaerella* and *Trametes* were proportionally more abundant in healther crowns. Of these three genera, *Cladosporium* is best known for biological control. The *Cladosporium_delicatulm_3* ASV biocontrol candidate DNA sequence also exactly matches *Cladosporium cladosporioides*, which has been used for biological control of apple scab (Köhl, Scheer, Holb, Masny, & Molhoek, 2014), and has shown some efficacy for control of *Fusarium* in wheat and maize (Luongo et al., 2005). However, the genus contains known plant pathogens (Collemare et al., 2014).

Towards Predictive Models that Include Endophytes as Mediators of Plant Traits

Due to the context-dependency inherent in complex plant traits such as disease resistance, it is imperative to search for generalizable patterns across multiple environments that may better explain the observed variation in traits. Similar to genome-wide-association studies for detection of important genes, we can detect taxonomic features in the plant microbiome that correlate with the trait of interest. To account for natural variation in taxa across environments, and with respect to experimental treatments, we utilized residuals from the full REML linear mixed model designed to predict plant measures, focusing on Crown Rot Index. Positive residuals represent samples that contained a greater Crown Rot Index than predicted by the model, and

samples with negative residuals had lower Crown Rot Index than predicted by the model. By partitioning residuals into these two categorical variables, we were able to utilize a broader range of methods to discover discriminant taxa, including methods originally designed for transcriptomic analysis, such as sPLS-DA implemented by mixOmics, and the ANOVA-like discriminant analysis implemented in ALDEx2. Microbiome data is complex to analyze, due to variation in sequencing depth, the high probability for nonlinear interactions between different taxa, and the fact that it is compositional in nature. Instead of choosing a single method for discovering important taxonomic features, we chose to integrate the results of multiple models, minimizing the risk of false assumptions and false positives. Random forest machine learning methods in particular do not require any assumptions of normality and are robust against interactions between predictor variables (Beck & Foster, 2014). These methods may be particularly useful for discovery of important endophytes that mediate plant traits, and prospecting for beneficial plant endophytes.

Seed-Associated Microbes as a Tool for Plant Microbiome Research

Seed fungal and bacterial endophyte communities generally responded to seed treatments more significantly than crown endophyte communities, particularly in the case of bacteria (Table 3). This may be because crown endophytes are physically closer to the species-rich soil environment, so there is more variation in response from sample to sample. Crown endophyte communities in maize are likely to be dynamic over the course of the growing season, and may differ within a field in a given sampling day due to natural variation in maturity rate between plants. Indeed, plant age has been found to be a very significant determinant of microbiome composition in aboveground and

belowground plant tissue (Wagner et al., 2016).

In contrast to crowns in this study, and other crop plant tissues which may change significantly over a growing season, seeds represent a stable, integrated sample of the plant microbiome across a growing season. We demonstrated that seedborne microbial communities shifted strongly with respect to site and agronomic treatment, and thus may be used as standard indicators of the composition of a plant microbiome in a given growing season. Seedborne microbes represent not only a measure of the past, but they are also potentially a heritable component of the plant microbiome. We found circumstantial evidence that seedborne bacteria and fungi can have a significant influence on plant traits. The source of seed was a significant determinant of a plant's response to disinfection or inoculation seed treatment, in terms of both disease susceptibility and yield (Figure 7). Furthermore, disinfecting seeds typically enhanced the effect of the microbial inoculant.

Here, we present strong evidence that seed disinfection and particularly inoculation treatments affect the composition and diversity of bacterial and fungal endophytes during a growing season, and within the subsequent generation of seeds. It remains to be determined whether these changes to seedborne microorganisms will have cascading effects on plant traits across plant generations. Chapters II and III represented experimental manipulations of seedborne endophytes, to delineate their relative importance across plants in the greenhouse and farms in the Willamette Valley. In contrast, Chapter IV is an observational study to understand the general patterns of bacterial and fungal seedborne endophytes across many different maize varieties and environments.

CHAPTER IV

ENGAGING WITH SEED SAVING FARMERS TO DETERMINE BROADSCALE PATTERNS OF SEEDBORNE ENDOPHYTES IN MAIZE

INTRODUCTION

The plant microbiome mediates agronomically important traits in crops, such as yield, nutrient uptake, pest and pathogen resistance, and drought tolerance (Berg, Rybakova, Grube, & Köberl, 2016). Given these potential benefits, there has been considerable effort to research and develop practices that utilize plant-associated microbes to improve crop performance. For example, inoculants of root-nodulating nitrogen-fixing bacteria such as *Rhizobia* spp. have been utilized successfully for over a century to enhance the nitrogen fixation ability of legume crops (Catroux, Hartmann, & Revellin, 2001). Increasingly, other microbial products are marketed to farmers and gardeners, including *Bacillus* spp., *Trichoderma* spp., and mycorrhizae, which have been shown to improve plant growth or serve as a biological control for pest or pathogens (Jefwa et al., 2014). Additionally, growers are continuously experimenting with soil management methods, soil amendments, and foliar sprays which likely function in part by altering the plant microbiome (Scheuerell & Mahaffee, 2002). However, often the benefits of microbial products are highly contingent across different crop varieties and environments (Hawkes & Connor, 2017). Unlike the relatively predictable chemistry of organic and chemical nutrient amendments, the underlying microbial ecology of plant-associated microbes is far more complex and context-dependent (Berg, 2009; Trabelsi & Mhamdi, 2013). In the case of plant microbiome management, the use of microorganisms

is outpacing our general scientific understanding of their effects on the plant microbiome.

Scientists have begun to characterize the microbiome of a number of crops, in various distinct microbial habitats in the plant host, including the rhizosphere, phyllosphere, and endosphere, i.e., the root-soil interface, the foliar surface, and plant interior, respectively (Berendsen, Pieterse, & Bakker, 2012; Hardoim et al., 2008; Partida-Martínez & Heil, 2011; Whipps, Hand, Pink, & Bending, 2008). Each microbial habitat type contains its own distinct microbial community, for reasons that are not yet clear (Lundberg et al., 2012). However, recent studies suggest that the structure of the plant microbiome is determined by a combination of neutral processes, such as the stochastic loss of microorganisms and dispersal from the environment and other host individuals, and non-neutral, selective processes, such as plant recruitment, defenses, and interactions with other members of the plant microbiome. For example, several recent studies have concluded that a combination of plant host genotype, environment, and genotype x environment (GxE) interactions influence microbiome composition (Adam, Bernhart, M??ller, Winkler, & Berg, 2016; Bouffaud, Poirier, Muller, & Moënne-Loccoz, 2014; Peiffer et al., 2013). Further complicating our understanding of the plant microbiome in agriculture is the influence of a broad range of agricultural practices that may interact with plant, microbiome, and environment in complex ways (Busby et al., 2017).

Seedborne Endophytes as a Vehicle for Studying the Plant Microbiome

Endophytes are bacteria or fungi that reside inside the plant (i.e., in the endosphere) without causing apparent disease symptoms (Stone et al., 2000). Endophytism appears to be ubiquitous in plants, likely since plants were first evolving to

live in terrestrial habitats (Rodriguez & Redman, 2008). The role of endophytes in plants is still little understood, but fungal and bacterial endophytes have been implicated in mediation of pathogen resistance, stress tolerance, nutrient acquisition, growth promotion, and immune regulation (Hardoim et al., 2008; Rodriguez et al., 2009). Fungal and bacterial endophytes can be highly diverse in plants, and any given endophyte can contribute positively, negatively or neutrally to plant fitness depending on the environmental context (Davitt et al., 2011). Some endophytes (such as some members of the genus *Fusarium*) can become latent pathogens during plant stress (Carroll, 1988). Endophytes are found in all tissues of the plant, including roots, stems, leaves, flowers, fruit and seeds.

Seeds contain bacteria and fungi that have entered the embryo, endosperm, or seed coat at some point during its development (Shade et al., 2017). Seeds may be colonized via airborne microbes that enter the pollen tube or permeate seed coats (Truyens et al., 2014). Endophytes of mature plants are also very common in seeds, as they may enter the ovary via the plant interior. Importantly, bacteria and fungi may be inherited via seeds, which may affect the health and functioning of plants across generations (Schardl et al., 2004). By treating the surface of the seed with disinfectants, we can study putative endophytes in the seed. Most of what we know about seed-borne microbes is based upon studies of fungal and bacterial seedborne pathogens and mycotoxigenic species such as *Aspergillis* and *Fusarium* spp. that produce aflatoxins and trichothecenes in grains, and seedborne fungal *Neotyphodium* of grasses that are toxic to livestock (Munkvold et al., 1997; Schardl et al., 2004). Seeds can contain dozens of bacterial and fungal endophytes species, and it is not clear how efficiently and

consistently these diverse seedborne endophytes transmit across plant generations, nor the degree to which they can affect plant fitness. However, seedborne endophytes do represent to some degree both indicator organisms of the plant microbiome of the previous generation, and also potentially inherited, non-plant-genetic components that affect plant fitness in subsequent generations. As microbial DNA in seeds is likely to be relatively static and well preserved, seeds offer us an excellent opportunity to sample plant-associated microbial communities and characterize them through next-generation sequencing.

Seed Savers as Citizen Scientists

Farmers and gardeners who save seed are intimately familiar with the plants, soil, and agricultural practices that produced the seed. Therefore, seed savers can provide valuable information for each seed sample. Furthermore, seed savers may be invested in the knowledge gained by studying microbes present in their seeds, personally and for public good. For example, the invisible buildup of seedborne pathogens such as *Fusarium* in maize is a common concern for seed savers and seed producers alike (Munkvold et al., 1997).

To acquire better understanding of broadscale patterns of seedborne bacterial and fungal endophytes, I recruited seed savers to submit seeds for determination of bacterial and fungal seed endophyte communities using next-generation Illumina sequencing methods. In exchange, farmers would receive information on the identity of seedborne fungi and bacteria present in their seeds. I limited the seed samples to corn, particularly flint, flour and dent corn types. As seed samples came through voluntary participation by farmers, there was no underlying experimental design. However, given this donor-driven

sampling of seeds, I sought to accomplish the following aims:

1. Determine the relative importance of "genotype" (i.e., corn variety and corn type) versus environment, (i.e., seed grower) in determining the diversity and composition of seed-associated bacteria and fungi.
2. Delineate how climatic factors correlate with alpha and beta diversity measures of bacteria and fungi in the seed.
3. Examine the composition and dynamics of the most common microbial taxa that are present in seed samples
4. Assess the efficacy of this approach as a model for future citizen science research projects for the study of plant-associated endophytes.

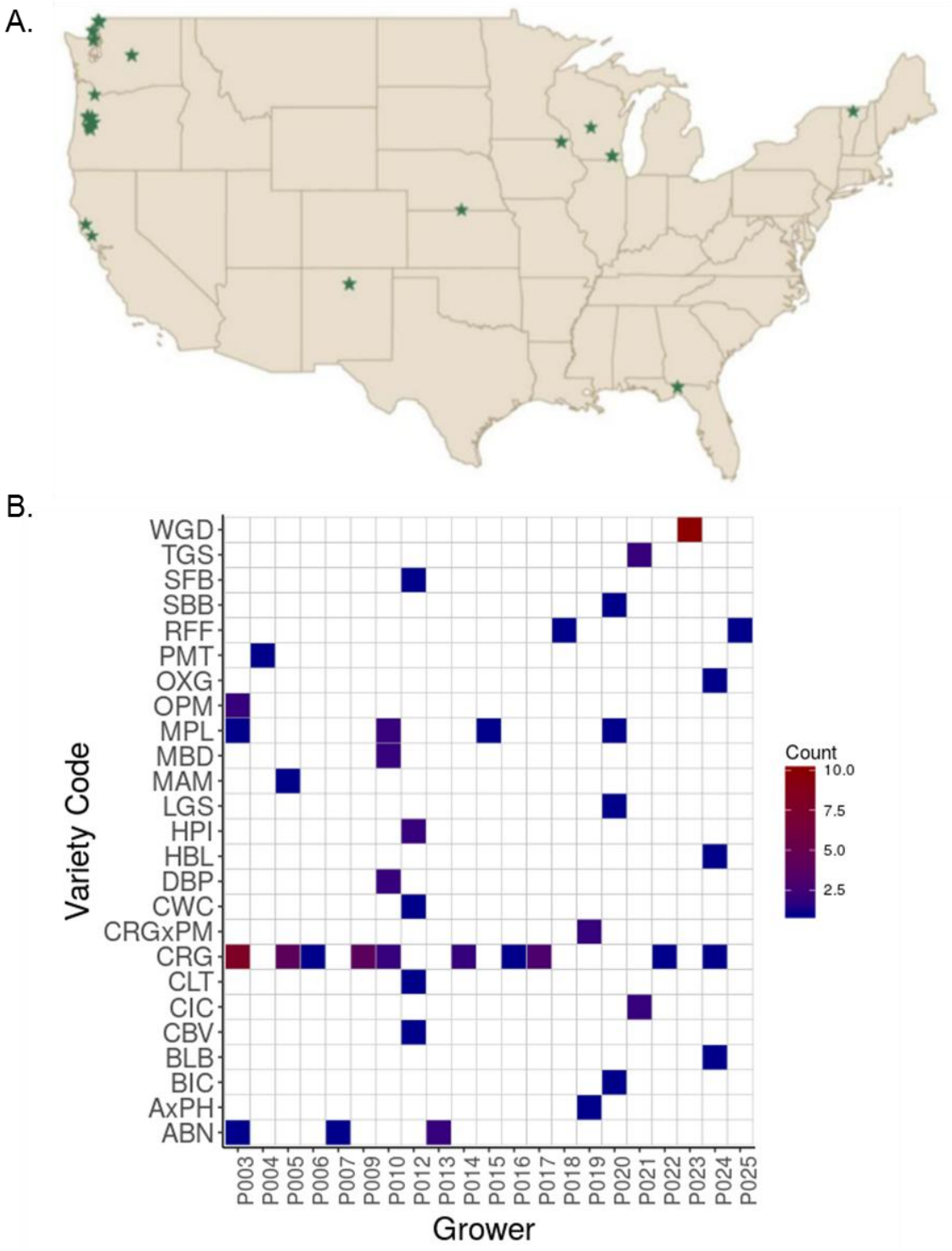
MATERIALS AND METHODS

Seed Saver Recruitment and Sample Collection

I recruited seed savers at the Organicology Conference (2013, Portland, OR) and Organic Seed Growers Conference (2014, Corvallis, OR), both conferences organized by the Organic Seed Alliance Nonprofit advocacy group. I presented a poster at each conference and used a signup sheet to recruit participants in the Community Research Network (CoRN) seedborne endophyte trial. Additionally, I worked with a local seed company, Adaptive Seeds (Brownsville, OR), who included my project information on their website. I also recruited seed growers locally (Eugene, OR area), advertising through word-of-mouth during social engagements with growers. I maintained my own website to provide background information, and a place where people could register online (www.microbialinheritance.org, later changed to www.seedmicrobes.org).

I informed potential project participants that they could participate by sending at least 20 seeds of each variety harvested in a particular year as a representative sample of all seeds in their seed lot. I accepted only open-pollinated corn varieties. I did not set a limit on the number of samples growers could send, but I limited the project to flint, flour and dent corn varieties to maintain a consistent set, and so I was likely to have more replicate varieties within each type. However, popcorn and sweetcorn samples were processed if submitted to me. I also sought from each participant sample information including the variety, year, location grown, and where the seeds had been grown. Farmers were asked this basic information when they registered to submit samples on my website. However, not all farmers registered when submitting samples. I told project participants that in exchange for providing me seeds, I would provide them information on the identity of the bacteria and fungi in each of their seed samples. I followed IRB protocol to remove any individual identifiers from the dataset. Seed sample location was most heavily weighted in the Pacific Northwest, where the recruiting occurred (Figure 11A). Overall, I recruited 21 growers, who sent in 71 seed samples, to which I added some from my field experiments (Chapter III), for a total of 83 seed samples (Figure 11B).

Figure 11. Extent of seed sampling effort. Seed samples were concentrated in the Pacific Northwest region (A). The contingency table (B) illustrates that some varieties (e.g., CRG, Cascade Ruby-Gold) were grown by multiple growers, and some growers sent in multiple varieties.

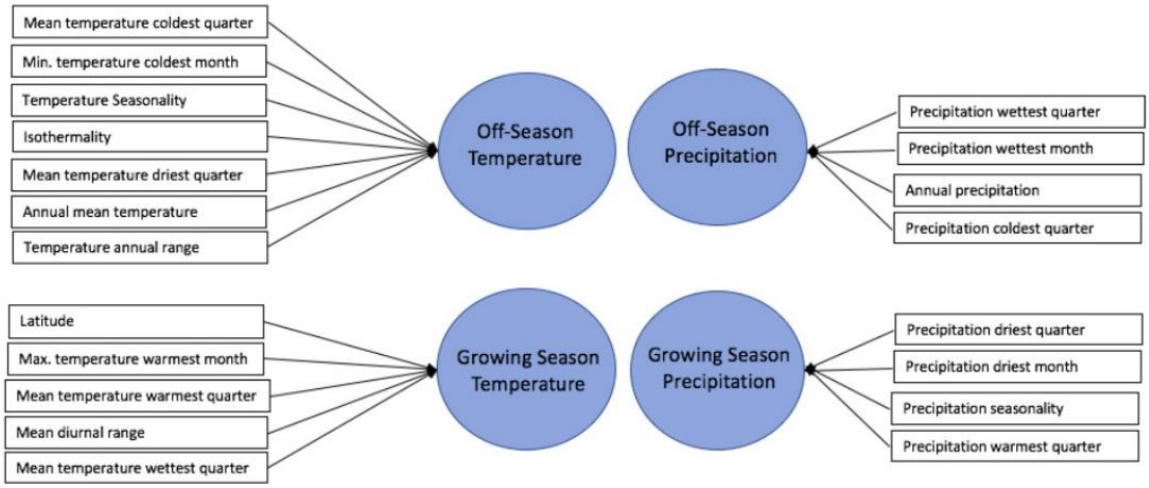


I also recruited project participants for follow-up interviews to better understand how farm management practices are related to the community composition of seed-associated microbes. These interviews were conducted by undergraduate researchers Adrian Robins (2015) and Kate Jaffe (2016), as part of their undergraduate theses (Jaffe, 2017; Robins, 2015). They interviewed a total of 16 participants, asking questions about their tillage practices, fertility methods and rates, and motivations for their practices. While this interview data was not used for this study, it is summarized in their theses.

Climate Data

In addition to interviews, Kate Jaffe assembled climate data using the locations of all of the seed samples. She utilized an online, open-source database called WorldClim (Worldclim.org) to access climate variables for each of the seed samples. The 19 "bioclimactic" variables of this database encompass average climate between the years 1960-2000, within a ~1km^2 resolution. They are called "bioclimactic" because they are intended to be specific to biological and seasonal patterns. She used exploratory factor analysis using the *fa* function with a "varimax" rotation option of the *psych* package in R. Through this, Kate effectively created four latent factors that distilled 19 climatic variables into four independent variables, Off-Season Temperature, Off-Season Precipitation, Growing Season Temperature, and Growing Season Precipitation (Figure 12; Jaffe, 2017).

Figure 12. Selected climactic variables using exploratory feature analysis. Twenty bioclimactic variables are reduced to four independent bioclimactic variables. From Jaffe (2017).



Seed sample processing and DNA extraction

Seeds were collected primarily by mail shipment over the years 2013-2015, and stored at -20°C before processing by myself and undergraduate lab assistants. We processed 20 seeds from each seed submission, first surface-sterilizing (Johnston-Monje & Raizada, 2011) and then extracting DNA. We surface-sterilized seeds in 0.1% Tween detergent, 3% sodium hypochlorite and 95% ethanol by submerging them in the liquid within falcon tubes and placing them in a rotary shaker as follows: 10 minutes in detergent, 10 minutes in bleach (twice), and 10 minutes in ethanol, completely replacing the solution each time, and then finally three washes in sterile distilled water. Seeds were allowed to dry before grinding them in pre-sterilized ceramic burr grinders. We extracted DNA using a modified protocol and the DNeasy PowerPlant Pro kit (MoBio Laboratories, Carlsbad, CA, USA). We amplified DNA barcode regions from the bacterial 16S rDNA V5-V7 regions (799F/1193R; 402 bp) and fungal DNA from the

ITS1 region (~280 bp) using Illumina (Illumina, San Diego, CA, USA) compatible primers (See Table S2) and used the MiSeq v3 Illumina kit for sequencing. See Chapter II Materials and Methods for a detailed DNA extraction and Illumina library preparation protocol.

Processing and Statistical Analysis of Illumina Sequences

Fungal (ITS) and bacterial (16S) amplicon sequences from Illumina MiSeq were quality-filtered, merged and processed using a custom pipeline that utilizes the DADA2 package (Callahan et al., 2016) in R (R Core Team, 2017) The DADA2 package estimates sequencing error of Illumina sequencing runs to deduce which DNA sequences are true biological sequences, and which are artifacts of error caused by sequencing. The developers of DADA2 call these amplicon sequence variants, (ASVs), a term which I will use throughout this paper to refer to putative bacterial or fungal taxa that each of these variants represents. I filtered our chimeric sequences and assigned taxonomy using the DADA2 package, as described in Chapter II.

I generated and analyzed microbial community metrics for each DNA sample using a combination of methods in R. First I calculated species richness and other alpha diversity measures using the *add_alpha_diversity* function in the package *phyloseq* (McMurdie & Holmes, 2013). To account for variation in sequencing depth across samples, I iteratively subsampled 50 random 16S or ITS sequences of each seed sample and assessed the mean ASV richness over 100 iterations. I also accounted for variable sampling depth by adding log-read abundance as a factor in linear models, described below. For ITS amplicon sequence analysis, I also used a variable representing the relative proportion of reads in each sample from two consecutive sequencing runs, to

account for variation in sequencing runs.

To analyze microbial community composition, I first transformed the ASV sequence abundances across samples to account for the mean-variance relationship due to variable sequencing depth, using two methods. In the first method, I normalized raw ASV counts using a variance-stabilizing transformation (VST) in the DESeq package, which adjusts count data by fitting each taxon to a mean-variance curve (Love et al., 2014). In the alternative method, I normalized taxa counts to relative abundances of the total sequence count in each sample (total sum scaling, TSS), and then adjusted relative abundances to their centered log-ratio (CLR), which effectively transforms the relative abundance data for use in Euclidean space (Lê Cao et al., 2016). I used the Bray-Curtis dissimilarity metric to estimate ASV compositional differences between each pair of samples. The distance-dissimilarity matrix was then ordinated in 2-dimensional space using principal components analysis (PCoA) in the *phyloseq::ordinate* function. CLR-transformed data was used directly in PCoA biplots using the *pca* function in the mixOmics package (Le Cao et al., 2017). Each axis in the PCoA biplots represent a separate, orthogonal measure of the relative community similarity between samples.

Microbial diversity metrics were modeled against predictor variables using linear models, with the *lm* and *drop1* functions of the *stats* package in R. Statistical significance of each predictor variable was assessed using the likelihood ratio test (LRT), which computes a X^2 statistic of a the full model against a null model without the predictor term. To assess the relative importance and statistical confidence of corn variety or type (e.g., flint or dent) versus the farm on microbial diversity metrics, we compared the p-value and relative variance explained by each method.

Taxonomic Features Positively or Negatively Associated with *Fusarium*

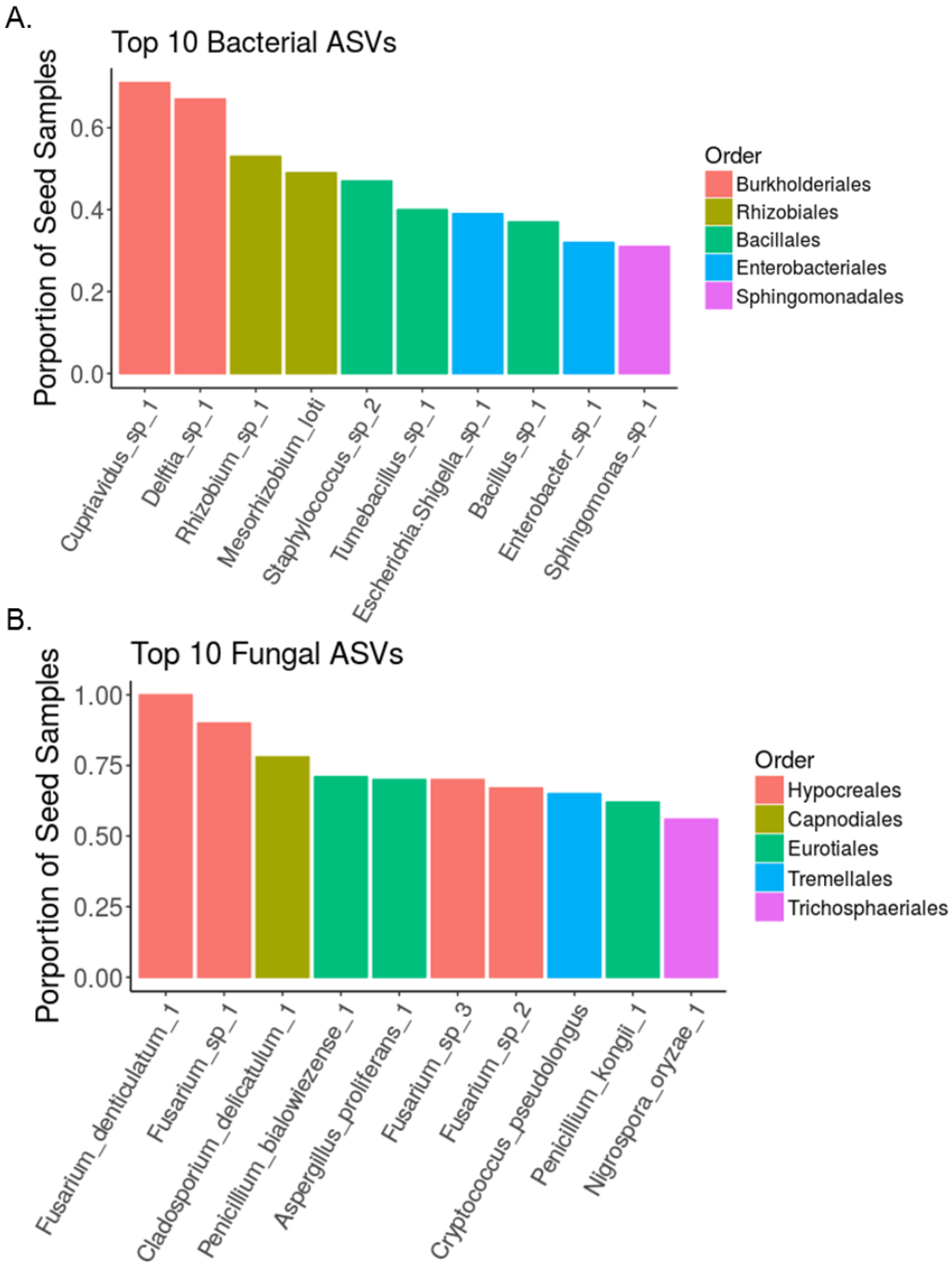
To determine which seedborne endophytes have positive or negative associations with *Fusarium*, we used the sum relative abundance of all ASVs assigned to the genus *Fusarium*, and converted it to a categorical variable, in which samples containing less than 50% of *Fusarium* were "low *Fusarium*" and samples containing greater than 50% *Fusarium* were "high *Fusarium*". We used six feature selection methods, and searched for taxa that were most consistently selected across all tests. In the first method, we used the *dds* and *DESeq* functions of the *DESeq* package to stabilize the mean-variance relationship of raw sequence counts before performing regression. Additionally, we used random forest feature selection methods on CLR-transformed relative abundance counts using the R package *randomForest* (Liaw & Wiener, 2002). The random forest algorithm uses decision trees on randomly subsampled predictor variables to determine the taxonomic features that best predict the response variables of interest. To maximize certainty in this method, we utilized three different random forest feature selection techniques, the standard *out-of-bag* method *(randomForest* package) where we selected the top 5 features, *recursive feature elimination* implemented through the *caret* package (Wing et al., 2017), and an *all-relevant-features* feature selection method implemented in package *Boruta* (Kursa & Rudnicki, 2010). Lastly, using CLR-transformed data, we also utilized ANOVA-like differential expression analysis (*ALDEx2* package (Fernandes et al., 2013)) and sparse partial least squares discriminant analysis (sPLS-DA) implemented in the *mixOmics* package (Le Cao et al., 2017).

RESULTS

Most Common Seedborne Endophytes

The top ten most common bacterial endophyte taxa were found across 47% of all samples, while the ten most common fungal endophyte taxa occurred in 73% of all samples (Figure 13). The most common bacterial endophytes, *Cupravidus* sp. and *Delftia* sp., are both classified in the taxonomic order Burkholderiales, followed by *Rhizobium* and *Mesorhizobium loti* sp. in the Rhizobiales order. *Fusarium* spp. represented 4 of the top 10 fungal ASVs detected in seeds, and the top-occurring ASV classified as *Fusarium_denticulatum_1* was detected in 100% of seed samples investigated.

Figure 13. Top occurring bacterial and fungal ASVs across all seed samples.



Endophyte Richness in Seeds

Overall, richness of bacterial (16S) amplicon sequence variants (ASVs) averaged 43 ASVs across all seed samples, compared to a mean richness of 25 fungal ASVs. When

accounting for sequence abundance variation, bacterial seedborne endophyte ASVs were twice as abundant as fungal seedborne endophyte ASVs (F_{1122}=30.89, P<0.001). Bacterial ASV richness differed significantly across seed growers and corn varieties, and was negatively associated with Off Season Temperature (Table 4). Most variance in bacterial ASV richness was explained by Seed Grower. Fungal ASV richness was explained primarily by Sequence Counts, i.e., the number of DNA sequences recovered in each sample. Seed Grower explained a significant proportion of variation in fungal amplicon sequence variation, when also accounting for Corn Type as a factor (Table 4).

Corn lineage versus seed grower in determining endophyte community composition

Variation in seedborne bacterial endophyte community composition was best explained by Seed Grower in linear models with Corn Type included (Table 4). Corn Variety consistently explained marginally more of the variation in bacterial endophyte communities than Seed Grower in models including both terms. Bacterial endophyte communities found in seeds significantly differed with respect to Off Season Temperature and Off Season Precipitation patterns (Table 4). Seed-associated fungal endophyte community structure was explained by both Corn Type (Table 4) and Corn Variety with more statistical confidence than Seed Grower. Fungal endophyte communities found in seeds were not associated with any climatic variables in this study. Linear models of fungal and bacterial endophyte community measures were both strongly affected by sequence abundance in each sample.

Table 4. Linear models of bacterial and fungal seedborne endophyte community richness and diversity across seed samples. P-values are based on X^2 likelihood test of full and reduced models, and variance is based on proportion of Type III sum of squares. Significant p-values are indicated in bold.

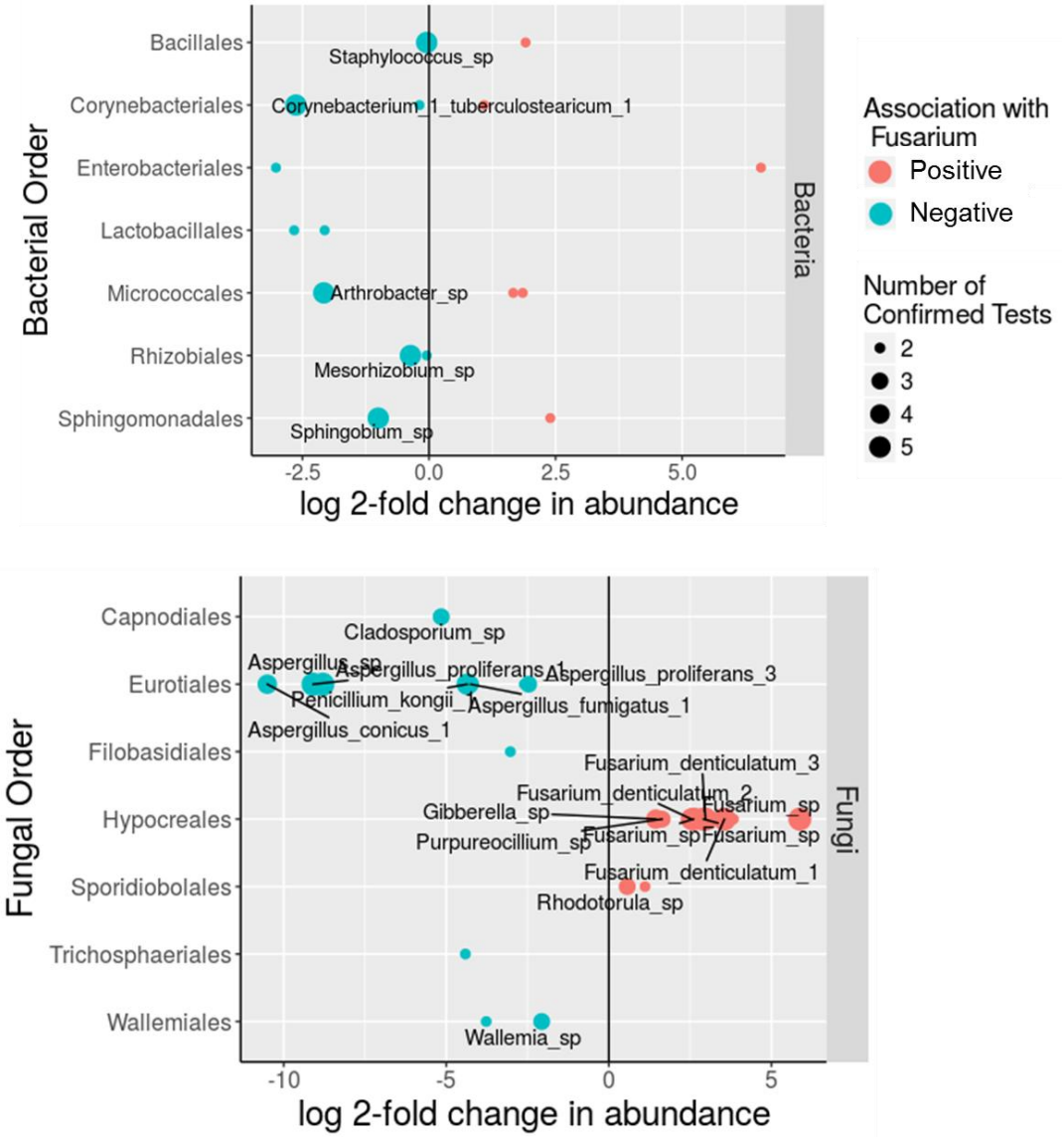
	Rarefied Richness		Bray PC1		Bray PC2		CLR PC1		CLR PC2	
	P-Value (Chisq)	Explained Var. (%)	P-Value (Chisq)	Explained Var. (%)	P-Value (Chisq)	Explained Var. (%)	P-Value (Chisq)	Explained Var. (%)	P-Value (Chisq)	Explained Var. (%)
Bacteria (16S)										
Corn Type vs. Seed Grower										
Seed Grower	0.056	31	**0.007**	37	0.436	22	**0.030**	3	**0.033**	34
Corn Type	0.054	8	0.672	2	0.439	4	0.101	1	0.188	5
Sequence Counts	0.424	1	**0.006**	6	**0.020**	5	**0.000**	91	0.416	1
Corn Variety vs. Seed Grower										
Seed Grower	**0.000**	30	0.270	17	**0.019**	18	**0.000**	3	0.094	21
Corn Variety	**0.014**	25	0.895	10	**0.000**	39	**0.001**	3	0.686	14
Sequence Counts	0.160	1	**0.009**	6	**0.024**	3	**0.000**	90	0.606	0
Climate Factors										
Off Season Temp.	**0.014**	8	0.220	2	0.965	0	**0.009**	0	0.300	1
Off Season Precip.	0.091	4	**0.003**	11	0.123	3	**0.033**	0	**0.004**	11
Growing Season Temp.	0.842	0	0.700	0	0.354	1	0.533	0	0.241	2
Growing Season Precip.	0.396	1	0.823	0	0.399	1	0.059	0	0.819	0
Sequence Counts	0.532	0	**0.007**	8	**0.040**	5	**0.000**	95	0.259	2
Fungi (ITS)										
Corn Type vs. Seed Grower										
Seed Grower	**0.039**	22	0.407	17	**0.003**	29	0.436	14	0.168	22
Corn Type	0.123	4	**0.007**	10	**0.000**	15	**0.004**	9	**0.001**	14
Sequence Counts	**0.000**	32	**0.000**	21	**0.001**	6	**0.000**	33	**0.000**	10
Sequncing Run	0.333	0	0.703	0	**0.000**	10	0.561	0	0.344	1
Corn Variety vs. Seed Grower										
Seed Grower	0.255	11	0.342	10	**0.000**	27	0.328	9	0.296	10
Corn Variety	0.286	14	**0.000**	34	**0.000**	43	**0.004**	25	**0.000**	44
Sequence Counts	**0.000**	33	**0.000**	16	**0.001**	4	**0.000**	27	**0.000**	9
Sequncing Run	0.373	0	0.272	1	**0.008**	2	0.273	1	0.547	0
Climate Factors Model										
Off Season Temp.	0.069	2	0.652	0	0.106	3	0.411	0	0.996	0
Off Season Precip.	0.895	0	0.930	0	0.791	0	0.566	0	0.846	0
Growing Season Temp.	0.483	0	0.230	1	0.165	2	0.547	0	0.542	0
Growing Season Precip.	0.093	2	0.318	1	0.197	2	0.591	0	0.486	0
Sequence Counts	**0.000**	52	**0.000**	35	**0.001**	12	**0.000**	45	**0.000**	21
Sequncing Run	0.866	0	0.816	0	0.060	4	0.605	0	**0.049**	4

Taxa Positively and Negatively Associated with *Fusarium* in Seeds

Using a range of feature selection methods, we identified bacterial and fungal ASVs and higher taxonomic classifications that were consistently associated with the abundance of *Fusarium* in seed samples (Figure 14). Significant discriminating bacterial features were generally negatively associated with the relative abundance of *Fusarium* across all seed samples, and bacterial ASVs belonging to the *Arthrobacter*, *Corynebacterium*, *Mesorhizobium*, *Sphigobium*, and *Staphlococcus* genera were

negatively associated with abundances of the dominant seedborne fungus (Figure 14). A number of fungal endophytes of the Eurotiales, including *Aspergillis* and *Penicillium* were negatively associated with *Fusarium*. Furthermore, *Cladosporium* and *Wallemia* were proportionately more abundant in samples with a lower percentage of *Fusarium* ASV sequences (Figure 14).

Figure 14. Seedborne endophyte ASVs and genera positively and negatively associated with seedborne *Fusarium* proportional abundance, organized into taxonomic rank of Order. The log2 fold change in abundance represents the difference in abundance of taxa between seeds with low (<50%) relative abundance of *Fusarium* and seeds with high relative (>50%) abundance of *Fusarium*.



DISCUSSION

Seedborne Endophyte Species Richness Differs with Respect to Seed Saver

Species richness of bacterial and fungal endophyte species significantly differed with respect to each project participant, based on linear models predicting the abundance of fungal and bacterial amplicon sequence variants (ASVs) in each seed sample. Any number of environmental factors may be the cause for this observation, including measured and unmeasured climate or biogeographical effects and farm management strategies. Seedborne bacterial ASV richness was affected by Seed Grower with greater statistical confidence and explained variance than seedborne fungal ASV richness, suggesting that farming environment or farming practices may have a significant impact on the diversity of seedborne bacteria. This observation, in combination with the fact that bacterial ASV richness differed significantly with respect to Off Season Temperature, indicates that broadscale biogeographical and climate factors may significantly determine richness of seedborne bacterial endophytes in bacteria. It remains to be determined whether seedborne ASV richness is an indicator of the ASV richness of the plant microbiome that produced it, or if it is affected by other environmental factors.

Bacterial and Fungal Endophyte Communities Differ with Respect to Genetic and Environmental Factors

We did not genotype seed samples, so we chose to use corn variety and type as proxies for corn genetics. The corn variety names used in this study, all open-pollinated varieties, represent diverse, interbreeding populations that are maintained by seed savers to contain a suite of traits specific to each variety. However, we can expect significant genetic variation across each open-pollinated variety due to genetic drift, contaminating

pollen, and recurrent mass selection in different environments. Generally speaking, dent, flint, flour, sweetcorn and popcorns types differ in genetics to some degree. Popcorn is genetically isolated from the other types due to gametophytic incompatibility factors during pollination, and flint corn diverged significantly from other varieties for over 1000 years due to adaptation to higher latitudes (Doebley, Goodman, & Stuber, 1986). However, interbreeding between types is common, and a single gene can determine whether a corn variety is floury versus flint, or sugary versus starchy (Brown & Harrah, 2016). Thus, the composition of the kernel, e.g., whether it is starchy (flour) or fatty (flint), may play a more significant role in determining seedborne endophytes than general genetic differences when considering corn type.

Bacterial endophyte community structure in seed samples was most consistently explained by environmental factors (i.e., Seed Grower and climate variables), rather than factors based on corn genetics, i.e., Type and Variety. In contrast, fungal endophyte community was most significantly predicted by Corn Variety and Corn Type, with minor associations with seed grower, and no associations with climate variables. The comparatively high correlation between seedborne fungal endophyte community composition and host plant classification may imply that plant host genetics play an important role in determining the composition of fungal endophyte communities. This observation may additionally imply that fungi generally exhibit higher rates of vertical transmission across plant generations than bacteria, and are able to be maintained in different plant lineages. For example, efficient vertical transmission of fungal endophytes is well documented in the *Epichloë*/*Neotyphodium* literature, and selective forces such as herbivore pressure can dramatically increase the vertical transmission efficiency of

Neotyphodium fungi in wild grass populations (Clay et al., 2005). However, these relationships between plant host type and fungal endophyte community can also be explained by seed habitat preference, which would not necessitate genetically determined host selection or efficient vertical transmission efficiency. For example, maturing flour and sweet corns seeds have a higher content of starch and sugars, compared to dent corn, and especially flint and popcorn varieties, which contain a higher lipid and protein content. These characteristics alone could result in fungal endophyte community composition varying significantly with plant host type.

Common Seedborne Endophytes

This survey of seedborne endophytes across diverse environments and corn varieties indicates that certain bacterial and fungal genera are more consistently seed-associated than others. The top four bacterial endophytes recovered from seeds are from bacterial genera that have the ability to fix atmospheric nitrogen. We cannot verify that the *Cupravidus* and *Delftia* ASVs have the nitrogen-fixing trait, although both genera are associated with enhancing plant growth, and the *Delftia* ASV has an exact DNA sequence match to *Delftia tsuruhatensis*, which has N-fixing ability (Han et al., 2005). *Rhizobia* sp. and *Mesorhizobium loti* are commonly known to form root nodules in legumes and to fix nitrogen. Corn has the ability to acquire a significant proportion of its nitrogen via nitrogen-fixing endophytes (Montañez et al., 2008), and some bacterial endophytes cultured from maize seeds are able to grow on nitrogen-free media (Johnston-Monje & Raizada, 2011), suggesting that beneficial nitrogen-fixing bacteria may commonly be transmitted in seeds across plant generations.

This research confirms other findings that *Fusarium* is a highly important

seedborne fungal endophyte in maize. The work of Saunders and colleagues demonstrated that *Fusarium* spp. are able to detoxify important plant defense compounds in corn, allowing these species to be nearly ubiquitous endophytes of corn (Saunders & Kohn, 2008, 2009). Although this genus is infamously implicated as a potent plant pathogen and producer of mycotoxins in grains, endophytes of this genus have also been demonstrated to be beneficial for plants, e.g., by protecting against the *Ustilago maydis* pathogen (Lee et al., 2009) or by increasing abiotic stress tolerance (Rusty J. Rodriguez et al., 2008). However, the high prevalence of *Fusarium* in these samples suggests that more monitoring should be done to manage *Fusarium* in these crops, and to make sure that grains have safe levels of mycotoxins. Most of the observed common fungal endophytes of maize are categorized as molds, including species of *Fusarium*, *Penicillium* and *Aspergillis*. Although *Aspergillis flavus* is associated with toxic aflatoxin production in maize kernels, other species may have beneficial effects; for example, some *Penicillium* spp. have been shown to have biocontrol abilities (Waqas et al., 2015). Further research should address the potential roles of common seedborne bacterial and functional endophytes in plant health.

Potential Biological Control Candidates for *Fusarium*

The 100% prevalence of *Fusarium* across seed samples allowed us to assess positively and negatively associated taxa using the entire dataset. Positive or negative associations do not necessarily imply direct microbe-microbe relationships, as taxa can be correlated due to host genetic or environmental factors. Additionally, each seed sample is a composite sample of 20 seeds, and thus these are average associations across multiple seeds. Nevertheless, this approach may be used to identify potential biocontrol

antagonists of *Fusarium*. Using this approach, we found that the majority of significant bacterial taxa were negatively associated with *Fusarium*. It is possible that *Fusarium* is generally antagonistic to bacteria. For example, *Fusarium* spp. produce fusaric acid, which is known to have antimicrobial properties against bacterial endophytes (Bacon et al., 2004). Alternatively, *Fusarium* spp. may have an opposite environmental or host-genetic preference than a number of bacterial species.

Negatively associated fungi may be in direct competition with *Fusarium* for the same niche within the seed. Indeed, fungal species within *Aspergillis, Penicillium*, and *Cladosporium* are associated with ear rot and mycotoxin production, similar to *Fusarium* (Ismaiel & Papenbrock, 2015). However, *Cladosporium* is also considered a putative biological control agent (Luongo et al., 2005) against *Fusarium* in wheat and maize. *Wallemia* fungal endophytes were also more prevalent win samples with less *Fusarium*. This basidiomycete fungus is known to tolerate dry environments, such as the seed, though its ecological role as an endophyte is unknown (Jančič et al., 2015)

Bacteria from the genera of *Arthrobacter, Corynebacterium, Mesorhizobium*, and *Sphingobium* each had a significant negative association with *Fusarium* abundance (Figure 14). In Chapter II, we found that a seed-associated *Arthrobacter ilicis* was antagonistic to *Fusarium* in culture and on maize seedlings. This finding suggests that species from the genus *Arthrobacter* may be a promising seedborne bacterium for the control of *Fusarium*. Growth-promoting strains of *Mesorhizobium* bacteria have effectively been used as biological control of *Fusarium oxysporium* in chickpea (Das, Rajawat, Saxena, & Prasanna, 2017). Furthermore, *Sphingobium* has been used as biological control of corky root disease in lettuce (Bruggen, Francis, & Jochimsen, 2014).

Future association studies, combined with targeted culturing of biocontrol candidates may allow us to produce seedborne endophyte inoculants that help us manage *Fusarium* abundance in seeds and in our crops.

A Model for Future Endophyte Research

Thanks to the participation of seed growers, we were able to contribute to the general understanding of the nature of seedborne endophytes. This study suggests that bacterial endophyte community composition may be driven more by environmental factors, and fungal endophyte community composition may be driven more by plant genetic factors. Furthermore, we were able to find common endophytes across diverse cultivars and seed savers. Seedborne *Fusarium* remains a troubling concern in maize, due to its potential for producing mycotoxins and reducing yields. We can use broad association studies like this to find potential biocontrol candidates against *Fusarium.*

This research project is not finished; future goals include delivering these research findings and personalized reports to the project participants, with the goal of showing each participant where their samples fit into the entire sample database. Using these methods, seed growers can be informed about the extent to which *Fusarium* and other potentially harmful bacteria and fungi may dominate their seed samples. Ideally, growers can contribute metadata to samples to improve our understanding of which factors predict diversity patterns of seedborne bacteria and fungi.

In future work, this research design would be improved by more comprehensive data collection involved with each sample, including factors that relate to the farm management history, fertility and irrigation regimes that may affect the composition of seedborne endophyte communities. Additionally, this research would benefit from on-

farm measures including local climate data and soil quality. As the database grows, so will the statistical certainty of its conclusions. This study may serve as a model for future microbiome businesses or private or state-funded microbiome research initiatives.

CONCLUSION OF CHAPTERS

Chapters II, III, and IV encompassed observations on the nature of seedborne endophytes across a continuum of spatial and temporal scales. In each scale, we observed how seedborne endophytes interacted with various factors associated with that scale, including the soil microbiome, seed treatments, farms, and climate patterns. Across all scales, it is clear that seedborne endophytes are significantly influenced by a combination of interacting factors relating to plant host, environment, and agricultural practices such as seed treatments. Moreover, seedborne endophytes can have a substantial impact on plant host traits, and mediate the effects of microbial inoculants. These results have significant implications both for theoretical and applied aspects of microbial ecology. We have found that seedborne endophytes have varying degrees of vertical transmission efficiency and benefit for the plant, from pathogenic to potentially positive, including the possibility of disease suppression and nitrogen-fixing ability. It remains to be determined whether we can influence the ecology of seedborne endophytes for the benefit of agriculture, specifically in maize grown the Willamette Valley, and generally in other plants and regions around the world.

APPENDIX

Table S1. Bacterial endophytes isolated from various maize tissues. Some were screened for growth ability in maize phytotoxin BOA. Those isolates that matched 16S rDNA amplicon sequences from culture-independent Illumina MiSeq sequencing efforts are indicated by the tissue in which they were found.

Isolate_ID	Isolate species	Substrate	Growth in 0.5 mg/mL BOA (% of control)	MiSeq exact sequence match
M45a1	*Achromobacter xylosoxidans*	cob		
M80	*Agrobacterium tumefaciens*	cob		crown, seed
M97	*Arthrobacter ilicis*	seed	83%	
M119	*Azospirillum lipoferum*	cob		
M105	*Azospirillum oryzae*	crown		
M82	*Bacillus aryabatthai, megaterium*	seed		
Mx3	*Bacillus megaterium*	cob	49%	
M120	*Bacillus subtilis*	seed	112%	crown, seed
M90	*Bacillus subtilis*	seed	74%	
M65	*Bacillus subtilis/amyloliquefaciens*	cob	5%	
M87	*Bacillus spp.*	seed	92%	
M131	*Bacillus toyonensis*	seed		
M45b	*Burkholderia cenocepacia/metallica*	crown		crown, seed
M125	*Cedecea davisae*	seed		crown, seed
M126	*Cedecea davisae*	seed		
M51	*Chryseobacterium culicis*	crown		crown, seed
M77	*Curtobacterium plantarum*	cob		crown, seed
M99	*Curtobacterium plantarum*	cob		crown, seed
M135	*Curtobacterium plantarum*	seed		crown, seed
M138	*Curtobacterium plantarum*	seed		
M211	*Curtobacterium plantarum*	seed		
M61	*Enterobacter ludwigii*	cob		crown, seed
Mx5	*Enterobacter ludwigii*	cob		
M104	*Enterobacter ludwigii*	crown		crown, seed
M129	*Enterobacter ludwigii*	seed		crown, seed
M130	*Enterobacter ludwigii*	seed		crown, seed
M209a	*Hafnia paralvei*	seed		
M212	*Methylobacterium brachiatum*	seed		
M71a	*Methylobacterium mesophilicum/brachiatum*	seed		
Mx4	*Microbacterium spp.*	cob		crown, seed
M69	*Microbacterium testaceum*	cob		crown, seed
Mx8	*Mucilaginibacter angelicae/gynuensis*	cob		
M73	*Novosphingobium resinovorum/barchaimii*	crown		
M192	*Paenibacillus rhizosphaerae/cineris*	seed		
M43	*Pantoea agglomerans*	cob		
M112	*Pantoea agglomerans*	seed		
M113	*Pantoea agglomerans*	seed		
M127	*Pantoea agglomerans*	seed	39%	
M134	*Pantoea agglomerans*	seed		
J4	*Pantoea agglomerans/vagans/anthophila*	seed		

Table S1. (continued)

Isolate_ID	Isolate species	Substrate	Growth in 0.5 mg/mL BOA (% of control)	MiSeq exact sequence match
J17	*Pantoea ananatis*	seed		
J18	*Pantoea ananatis*	seed		
M204	*Pantoea ananatis*	seed		
M210	*Pantoea ananatis*	seed		
M132b	*Pantoea vagans/anthophila*	seed		crown, seed
M102	*Pseudomonas baetica*	cob		crown, seed
M206	*Pseudomonas baetica/jessenii*	seed		
M203a	*Pseudomonas brassicacearum/migulae*	seed		crown, seed
M84b	*Pseudomonas fluorescens*	cob	10%	
M122	*Pseudomonas graminis*	seed		
M128	*Pseudomonas graminis*	seed		
M93	*Pseudomonas graminis*	seed		
M194	*Pseudomonas lurida;marginalis;poae*	seed		
M123	*Pseudomonas migulae*	seed		
M198	*Pseudomonas poae*	seed		crown, seed
M198	*Pseudomonas poae*	seed		
M74	*Rhizobium galegae*	cob		
M94	*Rhizobium galegae/huautlense*	seed	12%	crown, seed
M199a	*Serratia liquefaciens/grimesii*	seed		
M213	*Serratia liquefaciens;quinivorans*	seed	45%	
M6	*Sphingobacterium spp.*	stalk		
M37	*Sphingomonas aquatilis*	stalk		
M34	*Sphingomonas koreensis*	cob		
M58	*Sphingomonas koreensis*	crown		
M83	*Sphingomonas pituitosa*	crown		
M60b	*Sphingomonas pituitosa*	stalk		
M8	*Sphingomonas pituitosa*	stalk		crown
M15	*Sphingomonas pituitosa*	stalk		
M30	*Sphingomonas pituitosa*	stalk		crown
M29	*Sphingomonas trueperi/azotifigens*	crown		crown, seed
M79	*Stenotrophomonas maltophilia*	cob		

Table S2. Illumina sequencing primers used in this dissertation.

Nextera Variable-Length 2-step PCR Primers- Used in first Illumina sequencing run					
PCR-1: Variable length, amplicon specific					
	Nextera tag		Variable Length Spacer		Amplicon specific primer
16S 799F	CGTCGGCAGCGTCAGATGTGTATAAGAGACAG	+	A/TC/CAC/ACAA /TGCAA	+	AACMGGATTAGATACCCKG
16S 1193R	CTCGTGGGCTCGGAGATGTGTATAAGAGACAG	+	A/TC/CAC/ACAA /TGCAA	+	ACGTCATCCCCACCTTCC
ITS1F	CGTCGGCAGCGTCAGATGTGTATAAGAGACAG	+	A/TC/CAC/ACAA /TGCAA	+	TAGAGGAAGTAAAAGTCGTAA
ITS2	CTCGTGGGCTCGGAGATGTGTATAAGAGACAG	+	A/TC/CAC/ACAA /TGCAA	+	TTYRCTRCGTTCTTCATC
PCR-2: Barcode and Sequence adapter					
	Illumina compatible 5' end		8-bp barcode		Overhang with PCR-1 Primer
Forward	AATGATACGGCGACCACCGAGATCTACAC	+	NNNNNNNN	+	TCGTCGGCAGCGTC
Reverse	CAAGCAGAAGACGGCATACGAGAT	+	NNNNNNNN	+	GTCTCGTGGGCTCGG
Single-Step ITS1 Primer - Used in 2nd Illumina sequencing run					
	One-step Illumina primer for ITS1		8-bp barcode		Amplicon specific primer
ITS1F	AATGATACGGCGACCACCGAGATCTACAC	+	NNNNNNNN	+	TATGGTAATTGTCTTGGTCATTTAGAGGAAGTAA
ITS2	CAAGCAGAAGACGGCATACGAGAT	+	NNNNNNNN	+	AGTCAGTCAGCCGCTGCGTTCTTCATCGATGC

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