FUNCTIONAL SIGNIFICANCE OF ENDOPHYTE COMMUNITIES FROM A CONIFER FOREST

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Authorization to Submit Dissertation

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Abstract

The microbiome of the conifer forest may be functionally significant in forest hosts, the forest itself, and in agricultural fields converted from forests and woodlands. To assess functional significance of the forest microbiome, we examined within-microbiome interactions, interactions of microbiomes with the host, and the effects of microbiome-host interactions in both natural and managed systems. To determine the effects of within-microbiome interactions on *Dothistroma* needle blight in *Pinus ponderos*a, we inoculated non-pathogenic endophytes from *P. ponderosa* into emerging needles of *Dothistroma*-infected trees. Four of six endophytes enabled the pathogen by increasing disease severity as much as 4.7% while one endophyte antagonized the pathogen, reducing severity of the disease by 7%, indicating the potential of non-pathogens interact with pathogens within the microbiome. We then examined the potential function of the microbiome of *Pinus ponderosa* in conspecific recruitment by inoculating *P. ponderosa* seed with endophytes of mature conspecifics prior to germination. Endophytes from *P. ponderosa* reduced germination and emergence of conspecific seed. A needle endophyte showed the strongest inhibitory effects, reducing emergence by as much as 67% indicating foliar endophytes in the litter microbiome may contribute to beta diversity of conifer forests. Finally, to determine whether the conifer microbiome might be significant to the success of winter wheat production in converted woodlands, we conducted a series of experiments to study the potential of the microbiome to mediate disease resistance and stress tolerance in hard red winter wheat. The whole microbiome of pine litter was most effective for suppression of *Fusarium culmorum*, reducing plant mortality and increasing biomass of *Fusarium*-

infected plants by over 67% compared to untreated plants. Forest endophytes significantly increased root development during vernalization of wheat seedlings. A root endophyte from the microbiome of *Pseudotsuga menziesii* var. *glauca* doubled the yield of *Fusarium*-infected wheat subjected to water stress, indicating that the microbiome of the pine woodlands may have facilitated the success of dryland wheat production in the Pacific Northwest, USA. On-going research promises to expand our knowledge and understanding of the functional significance of the conifer forest microbiome.

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Chapter 1

Finding the Forest in the Trees: an Introduction to the Forest Microbiome

A biome is an ecological community identified by geography, climate, fauna, and flora. The interactions of abiotic conditions and biotic communities are considered definitive to the ecology of a biome (Barnes *et al.* 1997). There is a growing recognition in ecological circles that the microbial components of an ecosystem play a significant role in defining the functional ecology and community structure of that system (Klironomos 2002; Packer and Clay 2000; Redman *et al.* 2002). These microbial components, that populate the soils, organic debris, animals, and plants in interactive, dynamic, and diverse communities, function as microbiomes. Like any other community, this unseen community helps to shape its environment (and is shaped in turn) via its individual members and as a whole. But, what is its function within its environment? Even as we ask the question, we are confronted with a biome as complex as it is unseen.

The Microbiome

The term “microbiome” was originally coined by Joshua Lederberg to describe the microbial communities of the human body. A microbiome is defined as “the ecological community of commensal, symbiotic, and pathogenic microorganisms” within a system (Lederberg and Mccray 2001). The human microbiome is the whole community of microbial

flora and fauna within and upon the body. This may be broken up into separate microbiomes by organ such as the microbiome of the skin or the microbiome inhabiting the digestive tract—all of which are infinitely important to human health and function. In the forest, the whole-forest microbiome may be subdivided into individual microbiomes more specific in nature, from the microbiome of a single organism (a plant) to the microbiome of a single organ (a leaf) or the microbiome of an ecological entity (the soil). Components of microbiomes may span all six kingdoms (Baynes *et al.* 2012; Chelius and Triplett 2001; Evans and Johansen 1999; Petrini 1991; Zentmyer and Mircetich 1966) or be confined to a few species (Ganley and Newcombe 2006). The microbiome of a plant as a whole organism encompasses above and below ground tissues, the rhizosphere, and surrounding soils (Bever 1994; Klironomos 2001; Wardle *et al.* 2004). This continuum is also composed of individual microbial communities that may be highly divergent from each other (Fisher *et al.* 1993; Sun *et al.* 2012) but interact along the continuum (Porras-Alfaro and Bayman 2011).

Phytopathogens gave students of ecology the earliest glimpse into the microbiomes of plants, and their importance to human survival naturally led to rigourous study and investigation (Agrios 2005). The association of non-pathogenic microbial organisms with plants has long been recognized (deBary 1866; Kamieński 1882). Debary (1866) first coined the term endophyte to describe non-pathogenic residents of plant microbiomes. Growing interest in mycorrhizal associations (Hatch 1936; Trappe 1962) coupled with the discovery of endophytic mutualisms in grasses (Bacon *et al.* 1977; Clay 1988) saw interest in the microbiomes of plant systems expand beyond research of pathogens and pathogen complexes and begin to embrace non-pathogen residents as well.

Forest Microbiomes

Bernstein and Carroll (1977) were among the earliest to examine endophytic communities in temperate forests and suggest possible mutualisms between residents of these microbiomes and their associated hosts. Investigations into the extent of plant microbiome complexes subsequently identified endophytic associations in trees of every forest type around the world (Carroll 1995). Foliar endophytes have been studied in both tropical and temperate forests (Arnold 2007; Arnold and Lutzoni 2007; Carroll and Carroll 1978; Ganley *et al.* 2004). Root endophytes have since attracted attention for study into ecology and function of the root microbiomes of forest trees (Ahlich and Seiber 1996; Jumpponen and Trappe 1998; Kernaghan 2013), and mycorrhizal and rhizobacterial associations in the microbiome of the plant rhizosphere have also been extensively investigated in forest species (Chaia *et al.* 2010; Tedersoo *et al.* 2010). Soil microbiomes in temperate forest systems have been intensively studied for their importance (Buee *et al.* 2009; Hackl *et al.* 2004; Marrow 1932). Altogether, there is growing recognition that these unseen biomes of the forest are both highly diverse and critical to the function of the larger biomes which they compliment (Talbot *et al.* 2014).

Diversity of Forest Microbiomes

Forest microbiomes represent complex communities of incredible diversity. Arnold and others (Arnold *et al.* 2000; Arnold and Lutzoni 2007) investigated the diversity of fungi in the foliar microbiomes of tropical dicots and speculated that these foliar microbiomes

might be “hotspots” for fungal biodiversity. Over a period of almost four decades, extensive surveys were conducted on species throughout temperate forests and established recognition for the vast diversity of the needle microbiomes of temperate conifers (Arnold *et al.* 2007; Carroll and Carroll 1978; Dobranic *et al.* 1995; Ganley *et al.* 2004; Johnson and Whitney 1989; Müller and Hallaksela 1998; Petrini and Carroll 1981; Stefani and Bérubé 2006) and woody dicots (Jumpponen and Jones 2009; Sieber *et al.* 1991; Sieber and Dorworth 1994; Unterseher *et al.* 2007).

Root microbiomes of temperate forest trees yield numerous microflora spanning fungal endophytes, mycorrhizae, and bacterial endophytes (Gottel *et al.* 2011; Hoff *et al.* 2004; Sieber and Grünig 2006). While much research to date has focused on mycorrhizal associations within the forest rhizosphere, non-mycorrhizal organisms are receiving greater attention (Ahlich and Sieber 1996). Although roots of a number of temperate forest species are heavily colonized by fungi of the so-called dark septate endophyte (DSE) complex (Ahlich and Sieber 1996; Grunig *et al.* 2008), Summerbell (2005) found roots of boreal forest *Picea mariana* to be colonized with a diversity of fungal endophytes. Hoff *et al.* (2004) identified a number of diverse fungal endophytes in the root microbiomes of *Pinus ponderosa* and *Pseudotsuga menziesii*. Gottell *et al.* (2011) genetically profiled the microbiome in the roots of *Populus deltoides* and found it to be rich in bacterial endophytes as well as fungal endophytes with considerable variation. However, the associated soils of the *P. deltoides* rhizosphere supported a microbiome richer in diversity than that of the roots themselves, albeit somewhat less variable (Gottell *et al.* 2011).

The divergence and variation in the community profiles of the microbiomes of the roots and the rhizosphere soils of *Populus deltoides* (Gottel *et al.* 2011) only emphasizes the incredible diversity of plant-associated microbiomes. However, the microbiome of the plant itself represents only a part of microbial diversity found associated with forest microbiomes. Forest soils abound with numerous microbes (Morrow 1932), with litter layers contributing an extensive microbiome (Baldrian *et al.* 2012). Microbes often spatially assemble into communities within the soil. Baldrian *et al.* (2012) found that the microbiome of forest soils in a temperate *Picea abies* forest diverged in composition among horizons with litter layers being dominated by fungi (predominantly decomposing fungi) and increasing bacterial diversity emerging in lower horizons. Fungi were found to be more spatially sensitive and locally abundant than bacterial taxa (Baldrian *et al.* 2012), possibly indicating the role of above-ground vegetation in formation and diversity of the soil microbiome (Wardle 2006).

The quality of forest microbiomes to be compositionally and functionally diverse extends far beyond within-microbiome or within-plant diversity. Talbot *et al.* (2014) conducted exhaustive spatial survey of pine forest soil fungi across North America and found distinct communities endemic to bioregions, revealing incredible diversity over a large spatial scale.

Potential Function and Dynamics of Forest Microbiomes

Although the function of a mere fraction of these vastly diverse plant microbiome residents is understood, substantial evidence indicates that many of these organisms play

functional roles within the microbiome, with their hosts, and in larger ecosystem dynamics (Gaiero *et al.* 2013; Rodriguez *et al.* 2009; Wardle *et al.* 2004). Conifer forest microbiomes of temperate North America represent distinct communities with potentially critical functions within the ecosystem. Many of these functions provide positive benefits within the forest system. Carroll and others (Bernstein and Carroll 1976; Carroll and Carroll 1978; Petrini and Carroll 1981) conducted extensive surveys in coniferous forests throughout the Pacific Northwest of North America and found evidence of specificity and diversity in the microbiomes of several conifers. They hypothesized that some of these organisms were mutualistically active within the plant microbiome (Carroll 1988).

Functions within the Microbiome

Numerous studies have found evidence of interactions among residents of microbiomes, ranging from synergistic to antagonistic in nature (Clay 2014; Larimer *et al.* 2012). These very interactions shape the microbiome itself. Community assemblages may be determined by the pioneering species within a microbiome. Raghavendra *et al.* (2012) identified strong exclusionary interactions among seed colonizing endophytes of the invasive forbe, *Centaurea stoebe*. The first fungus to colonize the seed was capable of excluding all others, setting the stage for seed and seedling survival and vitality (Raghavendra *et al.* 2012). Kennedy *et al.* (2009) found strong competitive interactions among ectomycorrhizal (EM) fungi colonizing pine roots and observed priority effects on the competition. These priority effects describe the phenomenon by the earliest colonizing or pioneering fungus becomes the dominant species in the microbiome (Kennedy *et al.*

2009). Mycorrhizal *Laccaria bicolor* antagonizes endophytic fungi of the dark septate endophyte (DSE) group within the root microbiomes of temperate conifers, *Pseudotsuga menziesii* and *Picea abies* (Reininger and Sieber 2012), essentially altering the community composition of the microbiome. In fact, many endophytes form communities in patterns of succession resembling successional patterns and community assembly in other ecological communities (Pan and May 2009). Patterns of succession may also be seen in decomposition of litter layers and developing organic layers (Sheu and Parkinson 1995)

In the microbiomes of forest trees, within-microbiome interactions function not only to alter microbial behavior community composition but can also impact the health, physiology, and function of the host plant. Within the foliar microbiome of *Populus* spp., fungal endophytes can interact with foliar pathogens to either benefit the tree by antagonizing the pathogen, thereby reducing severity of the disease, or enable a pathogen (Busby *et al.* 2013; Raghavendra and Newcombe 2012). Raghavendra and Newcombe (2012) identified four endophytic foliar fungi that significantly reduced severity of the foliar rust pathogen *Melampsora* x *columbiana* on hybrid poplar and *Populus trichocarpa*. By contrast, Busby *et al.* (2013) identified an endophytic species of *Penicillium* recovered from the leaves of *Populus angustifolia* that enabled the foliar pathogen *Drepanopeziza populi,* thereby increasing the severity of disease symptoms. These interactions within a plant microbiome between pathogens and non-pathogen endophytes are often specific within the complex of the host and the microbiome (Busby *et al.* 2013).

Symbiotic Functions of the Microbiome

Antagonism of non-pathogen residents of the plant microbiome toward pathogenic residents provides one of many functional benefits to the plant host. The literature now abounds with studies exploiting within-microbiome antagonism of pathogens for biological control. A number of endophytes have been found to contribute to disease resistance in forest trees. *Lophodermiun* spp. are common endophytes of the foliar microbiome of the genus *Pinus* (Ganley *et al.* 2004; Minter and Miller 1980). Minter (1981) found the non-pathogenic *L. conigenum* capable of colonizing *Pinus sylvestris* to the exclusion of the phytopathogen *L. seditiosum* from the needle microbiome. Arnold *et al.* (2003) found foliar endophyte mixtures contributed significantly to reduced leaf lesions and death from of the foliar pathogen *Phytophthora* sp. in *Theobroma cacao*. Ganley *et al.* (2008) found that mixtures of foliar endophytes from *Pinus monticola* mediated resistance in their host to the white pine blister rust, *Cronartium ribicola*. In *Populus* spp., uredinial density of *Melampsora* leaf rust was significantly reduce by single foliar endophytes recovered from *Populus trichocarpa* (Raghavendra and Newcombe 2012).

Microbiomes of forest soils and the rhizosphere also contribute to disease resistance in host plants. Disease suppressive soils have attracted attention for their significance in distribution and severity of key agricultural pathogens (Hornby 1983). Disease suppressive soils were recognized early in the 20th century (Hornby 1983), and eventually linked to the soil microbiome (Berendsen *et al.* 2012). Disease suppressive soils may develop over time and have been identified with increases in microfloral diversity and changes in community

composition (Berendsen *et al.* 2012; Sanguin *et al.* 2009). Mechanisms are poorly understood, but may include direct antagonism or exclusion of the pathogens or induction of resistance in hosts of the pathogens (Berendsen *et al.* 2012). While much of the accumulated knowledge regarding pathogen-suppressive soils applies to agricultural systems, understory microbiomes in conifer litter and forest soils are known to suppress common soil-borne pathogens (Schisler and Linderman 1984; Smith 1967; Toussoun *et al.* 1969).

Microbes within the microbiome of a plant interact not only with each other to mediate the health of the host but also with other biota that would interact with the host. Come microbes can provide their host with resistance to herbivory, both to insects and to larger herbivores (Clay 1988). For example, many forest trees show increased resistance to insect herbivory when colonized by some endophytes. The elm endophyte *Phomopsis oblonga* provides some protection against the Dutch elm pathogen, *Ceratocystis ulmi*, by disrupting reproduction of pathogen-vectoring bark beetles (Webber 1981). Carroll (1986) determined that host-specific *Rhabdocline parkeri* provided some protection to its host *Pseudotsuga menziesii* against the gall midge *Contarinia* sp. by infecting developed galls and reducing survival of the midges. Miller and others began work in the 1980's identifying conifer endophytes potentially toxic to spruce budworm (Clark *et al.* 1989). At the turn of the century, Miller *et al.* (2002) identified an endophyte, *Phialocephala scopiformis*, and its secondary metabolite rugulosin which were effective against spruce budworm *in planta*. They were later able to demonstrate that endophyte infection could remain effective against herbivory in *Picea glauca* five years after inoculation (Miller *et al.* 2008; Sumarah *et*

al. 2008). Currently, it has been determined that both the fungus and its metabolite can remain present and potentially effective within the host 20 years after inoculation (Frasz *et al.* 2014).

Non-pathogen residents of a plant microbiome not only contribute to a host's ability to resist and tolerate biotic attack but also to resist and tolerate abiotic environmental stresses. Fungal and bacterial endophytes can mediate drought-tolerance (Redman *et al.* 2001), salt tolerance (Rodriguez *et al.* 2008), thermotolerance (Baynes *et al.* 2012; Rodriguez *et al.* 2008), and cold tolerance (Gunde-Cimerman *et al.* 2003). Some endophytes may mediate resistance to a single stress while others may mediate tolerance to multiple stresses in multiple hosts. For example, a virus-enabled fungus mediates tolerance of *Dichanthelium lanuginosum* to high soil temperature of the geothermal soils on which it grows in the Yellowstone (Redman *et al.* 2002). By comparison, the generalist root endophyte *Piriformospora indica* has been found to contribute not only to growth promotion in multiple hosts but also to salt tolerance, drought resistance, flooding tolerance, and resistance to temperature stress, toxins, and heavy metals several plants (Oelmüller *et al.* 2009). Fungi and bacteria within the plant microbiome and associated soil microbiomes may also facilitate water and nutrient acquisition or stimulate growth and fecundity via greater nutrient availability or growth regulator production or stimulation (Barrow and Osuna 2002; Baynes *et al.* 2012; Gaiero *et al.* 2013; Taghavi *et al.* 2009; Tinker 1984; Xin *et al.* 2009).

Ecosystem Functions of the Microbiome

The role of the plant microbiome and the associated soil microbiome extends beyond within-microbiome interaction and interactions with the host(s) to potentially encompass ecosystem dynamics. Microbiomes of the rhizosphere and associated soils are shaped by the vegetation or plant species growing there, and in turn, they alter and shape the plant communities (Gaiero *et al.* 2013; Wardle *et al.* 2004). Pathogens of plants in many different systems are known to function as drivers of biodiversity and succession (Connell 1971; Gilbert 2002; Van der Putten and Peters 1997).

Janzen (1970) and Connell (1971) recognized pathogens, particularly soil-borne seedling pathogens, as agents for maintaining biodiversity in mixed forests by reducing seedling survival of conspecifics and thus favoring competitive establishment of heterospecifics. Pathogens from the soil microbiome of forest trees have since been recognized as agents of the Janzen-Connell hypothesis (Liu *et al.* 2012; Packer and Clay 2000). Pathogens of host plants may accumulate within the associated soil microbiome, affecting not only emerging seedlings, but also developing and mature plants (Bever 1994; Gilbert 2002; Klironomos 2002). This process is known as negative plant-soil feedback and plays a significant role in the dynamics of plant communities by regulating relative abundance of populations (Gilbert 2002; Klironomos 2002; Marion *et al.* 2014; Mordecai 2011).

Plant-soil feedbacks may be positive as well (Bever 2003; Mordecai 2011). While plants may harbor their own specific pathogens thereby regulating their own population

structure, they may also harbor generalist pathogens of a more susceptible competitor as a mechanism to successfully compete, a phenomenon known as apparent competition (Holt and Pickering 1985). When pathogens found in the microbiomes of two hosts in competition with each other which, although pathogenic to both hosts, display a greater degree of severity in one host over another, they facilitate apparent competition (Gilbert 2002; Holt and Pickering 1985; Meyer *et al.* 2014). Non-pathogens may also facilitate competitive advantage. Clay and Holah (1999) determined that endophytic fungi in *Festuca arundinacea* contributed to competitive ability of its host, increasing host dominance where infection was present.

Negative plant-soil feedbacks, while maintaining one species population in a rare state, may simultaneously facilitate the expansion of an invasive population (Klironomos 2002; Marion *et al.* 2014). Callaway *et al.* (2004) determined that while soil biota from the native range of an invasive plant contributed negative feedback for reduced biomass while soil biota from the invaded range significantly increase biomass compared to sterile soil controls. Whether suppressing recruitment of host conspecifics, facilitating competition, maintaining the status of rare species, or contributing to the invasiveness, microbiota within plant microbiomes shape the plant community and the larger biome.

Disturbance and Forest Microbiomes

Microbiomes may play a critical role in the formation and maintenance of the plant communities they inhabit, but they are highly dynamic with their environment and

responsive to changes in these communities. As these communities are changed by disturbance, significant changes can occur simultaneously in associated microbiomes. Any disturbance that alters the plant community results in the subsequent alteration of the associated soil and plant microbiomes (Gaiero *et al.* 2009; Hart *et al.* 2005; Smiley *et al.* 2013). The nature (i.e. transience vs. permanence) of changes to a microbiome may be the function of the severity of the disturbance. Some disturbances may be transient, allowing the microbiome to regenerate (Williams *et al.* 2012). Other changes are more permanent, changing not only the community assemblage but also the functionality of the microbiome (Fierer *et al.* 2013). Disturbances may be natural, such as a stand-replacing fire, or anthropogenic, such as the conversion of forest land to developed agriculture.

When a natural biome undergoes replacement by a disturbance, the microbiomes associated with the vegetation of that biome are replaced by those associated with the vegetation replacing that of the former biome. Intensive management of plant biomes such as is found in urban forestry, plantation forestry, or agroecosystems not only significantly alters plant communities but also the associated microbiomes found in foliage, roots, and soil (Hartmann *et al.* 2012; Jumpponen and Jones 2010; Matsumura and Fukuda 2013). Fierer *et al.* (2013) demonstrated significant changes in the microbiome assemblages of tallgrass prairie soils when these systems are converted to intensive agriculture.

In intensive agricultural systems where monocultures of annual crops are continuously grown, not only are the natural microbiomes of soil, rhizosphere, and plants significantly altered, but new microbiomes are assembled around the species cultivated

(Ellouze *et al.* 2013; Liang *et al.* 2012; Smiley *et al.* 2013). Negative plant-soil feedback from continuous cropping has long been recognized, and traditional agriculture around the world has combated it with crop rotation methods (Cook 1980; Smiley *et al.* 2013). However, continuous cultivation of short rotation crops only slows negative feedback development and pathogen accumulation (Cook 1980; Smiley *et al.* 2013). Changes in the structure of soil microbiomes in response to crop rotations with long-term perennials can be much more effective, indicting microbial interactions within the microbiome that lead to eventual exclusion of pathogens (Mazzola and Gu 1999). Reduced tillage may also help to restore pathogen-resistant soil microbiomes (Peters *et al.* 2003) as may applications from the microbiomes of suppressive soils (Cook and Rovira 1976).

Potential of Microbiomes in a North Idaho Mixed Conifer Forest

In the dry intermountain region of the Pacific Northwest, habitats once dominated by Palouse prairie, shrub-steppe, and open forest have been transformed to intensively cropped monocultures of short-rotation annuals such as small grains and various legumes. Although some of the most productive agricultural ground in the world is found here, the significant changes occurring over time in the microbiomes of the soils and plants bring with them potential problems for the sustainability of the system.

Adjacent to these converted agricultural lands, however, lies the mixed conifer forest of the northern Rocky Mountains. These forests contain a diversity of plant and soil types (Johnson 1995; Soil Survey Staff, NRCS 2014) and by extension may provide a diverse

reserve of microbiota (Ganley *et al.* 2004). However, these forests face a plethora of disturbance regimes—including development, wildfires, climate change, disease and insect outbreaks, and poor harvesting practices—that challenge their resilience and the resilience of their microbiomes.

Although the existence of complex plant microbiomes in temperate forest systems is well recognized, the function and extent of these microbiomes within the system is poorly understood (Talbot *et al.* 2014). Understanding the structure and function of forest microbiomes may unlock a valuable resource for maintaining and restoring functional diversity of these forests and adjacent agricultural lands. Microbiota that mediate resistance to biotic and abiotic challenges and stress might be identified, cultivated, and applied to forestry and agricultural practices.

Objectives for Researching the Function of a Forest Microbiome

The research documented in this dissertation represents in part research conducted into the extent and function of non-pathogenic fungi and bacteria from the microbiomes of three temperate conifers found in the northern Rocky Mountain mixed conifer forest of North Idaho, USA. The three primary objectives of this research are as follows:

1. To identify non-pathogenic fungi or bacteria from these microbiomes with key functional roles within the microbiome;
2. To potentially determine some of the benefits and the functions of promising non-pathogenic taxa in their host(s);

3. And to determine the potential of beneficial fungi and bacteria for use in managed forestry and agricultural systems.

Sampling Conifer Microbiomes in a North Idaho Mixed Forest

Beginning in July of 2010, in preparation for studying the microbiome of the northern Idaho temperate mixed conifer forest, root and needle samples were taken from three species of northern Rocky Mountain conifers*: Pseudotsuga menziesii* (Mirb.) Franco var. *glauca* (Beissn.) Franco, *Pinus ponderosa* Lawson and C. Lawson, and *Pinus monticola* Douglas ex D. Don. For each tree species, ten trees were sampled. Healthy, asymptomatic foliage was taken from the lower third of the crown. One hundred needles were taken from the second and third-year foliage of each tree. Root samples were extracted by trenching outward from the boll of the tree along lateral roots and excising fine laterals averaging 6 mm in diameter. Approximately 30 cm of root tissue was recovered from each tree. Only healthy, living tissue was sampled. Excised root tissue was cleaned with a vegetable brush before packing in storage bags. Tissue was processed within 72 hours following collection and stored at 4°C until processed.

Needles of all three conifer species were surface sterilized for 1 minute in 95% ethanol, 5 minutes in 6% sodium hypochlorite, and again for 30 seconds in 95% ethanol followed by rinsing in sterile distilled water (Ganley and Newcombe, 2006). Needle tissue was then plated onto 4% potato dextrose agar (PDA) to non-selectively culture endophytes

from the tissue. One hundred needles were plated from each tree of each species sampled for a total of 1000 needles per species.

Root samples were cut into sections 2 cm in length then surface sterilized in 70% ethanol for 5 minutes, 6% sodium hypochlorite for 15 or 20 minutes, and 70% ethanol for 2 minutes followed by rinsing in sterile distilled water. Root sections were plated onto modified yeast-glucose agar (see appendix).

In the summer of 2012, further sampling of the root microbiome of *Pseudotsuga menziesii* var. *glauca* was conducted along three elevation gradients in intermountain conifer forests. Root samples were collected and processed as above. However, root pieces were plated onto either 4% PDA or 25% glycerol PDA (see appendix) to select for elevation differences and xerotolerance in the microbiome.

Tissues were tested for efficacy of surface sterilization using imprint plates. Once tissues were surface sterilized, some tissue was plated onto a fresh agar plate and left for approximately 90 seconds. Tissue was then removed and the plates were sealed. One imprint plate was made for every four plates of plant tissue. Imprint plates were monitored for two weeks after plating.

Following isolation, representatives of each morphotype were subcultured. Fungal subcultures were made on 4% PDA. Cultures were kept at 4°C following subculturing. Initial identification was made to genus level for numerous isolates. Identifications to genus were determined by morphological examination. Where morphological examination failed to identify the organism, representative isolates were sequenced.

Summary of the Research

In the following chapters, we examine functions of the microbiome of temperate intermountain forest conifers in both host and ecosystem processes. In the second chapter, we discuss interactions within the needle microbiome to either antagonize or enable a foliar pathogen of *Pinus ponderosa*. In the third chapter, we visit the potential of a cryptic member of the needle microbiome of *Pinus ponderosa* to function as an agent for promoting beta diversity within mixed conifer forests. In the fourth chapter, we examine the potential of the conifer microbiomes of northern Idaho forests to restore health, resilience, and productivity to degraded agricultural biomes.

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Chapter 2

Modification of Pine Needle Blight Severity by Fungi in the Needle Microbiome

Abstract

Foliar disease is most often understood as simply the product of the interaction between host and pathogen in a particular abiotic environment. But, other microbes may increase or decrease disease severity as either pathogen enablers or antagonists; the frequency and extent of such modification in nature are largely unknown. Here, we provided an opportunity for six fungi to modify the severity of *Dothistroma* needle blight in *Pinus ponderosa* in natural forest near Priest River, Idaho, USA. Five fungi were common members of the *Pinus ponderosa* microbiome in the region, and one was isolated from co-occurring *Pseudotsuga menziesii*. Inoculum of each of the six was applied to an average of 187 newly emerging needles in the candles (new shoots) of each of ten, seven-year-old trees; control candles were treated with sterile distilled water. Disease severity per needle (i.e., lesion length / total needle length) was determined one year later following natural infection and development of *Dothistroma* needle blight in a total of 13,085 needles. Five of the six fungi significantly modified disease severity; the overall model ("inoculant", "tree", and "inoculant x tree" as predictors) explained 53% of the variation in disease severity. *Penicillium goetzii* was the sole antagonist; it reduced disease severity by nearly 7% compared to control needles. Four of the fungi (*Hormonema dematioides*, *Clonostachys rosea*, *Penicillium raistrickii*, and a culturable species of *Elytroderma*) acted as pathogen

enablers, increasing disease severity 4.7, 4.2, 3.6, and 2.5 %, respectively. Our results show that many microbes may modify expression of *Dothistroma* needle blight in nature, if given the opportunity to infect emerging needles. But, the extent of modification may go beyond the modest effects reported here for just a few members of the microbiome of the host and its forest community.

Key words: *Dothistroma septosporum*, microbiome, endophytes, *Hormonema, Elytroderma*, *Cladosporium*, *Clonostachys, Penicillium*

Introduction

Traditionally, plant disease is conceptualized as the product of the 'disease triangle': a susceptible host, a virulent pathogen, and an abiotic environment that allows for infection. The biotic environment is ignored in this view, even though scattered examples of pathogen-antagonizing and pathogen-enabling microbes from managed ecosystems are known. In managed ecosystems, antagonists and enablers are sought for applied purposes to either reduce or increase disease severity in desirable and undesirable plants, respectively.

Thus, the frequency and extent of plant disease modification in nature are essentially unknown. By frequency we refer to the percentage of disease-modifying members of a given microbiome to which the pathogen also belongs; modifiers can be either enablers or antagonists. By extent we refer to increases or decreases in disease

severity brought about by modifiers.

Pathogens are typically more common in the native range of a plant than elsewhere (Mitchell and Power 2003), because plant propagules are likely to be introduced elsewhere without non-systemic, horizontally transmitted symbionts, and most pathogens fall into this category. In other words, relatively few pathogens are systemic and vertically transmitted (Mitchell andPower 2003). But, this difference in ranges is also likely to be true of most endophytic, disease-modifying symbionts that are, by and large, also non-systemic and horizontally transmitted. Thus, the frequency and extent of disease modification should also be more common in the native than in the introduced range of a plant.

Dothistroma needle blight is a damaging foliar disease of *Pinus* subgenus *Pinus* (Barnes *et al.* 2011), although there are also reports of this disease from subgenus *Strobus* (Farr *et al.* 2014). The disease can be caused by either of two related fungi, *Dothistroma septosporum* and *Dothistroma pini*, both of which appear to be native to North America (Barnes *et al.* 2004). *Dothistroma septosporum* is the more global of the two, and it is common in *Pinus ponderosa* and *Pinus contorta* in natural forests in northern Idaho (Sinclair and Lyon 2005). Young, emerging needles of new shoots are infected in spring. Severe disease retards growth of susceptible pines in plantings in the southern hemisphere (Pas 1981), and some trees may even be killed, but disease is typically much less severe among pines within natural forest (Sinclair and Lyon 2005). Although this phenomenon has not been fully explained, modification of disease trends toward antagonism within natural forest, and the converse in plantings outside the native range. We observed this

phenomenon in recent study in which we identified four strong antagonists of *Melampsora* rust within the native range of *Populus trichocarpa* (Raghavendra and Newcombe 2013); however, in a related study we found an enabler of a necrotrophic pathogen of *Populus angustifolia* (Busby *et al.* 2013), also in its native range.

In this study we explored disease modification of *Dothistroma* needle blight in *Pinus ponderosa* via co-occurring non-pathogenic fungi in a riverside forest near Priest River, Idaho, USA. In nature, newly emerging needles of pines are relatively uncolonized by microbes (Stone *et al.* 2000). By inoculating emerging needles with putative modifiers at the same time that the pathogen itself typically infects, we increased the chances that any subsequent disease modification was due to priority effects of putative modifiers. Unlike greenhouse studies in which background infection by most naturally occurring microbes is limited, in this study we merely boosted infection by one member of the community at a key juncture when the pathogen also infects the host. In total, we determined the enabling or antagonistic, priority effects of six microbial members of the microbiome of the host and its forest community. This study was conducted under natural conditions and without exclusion of the rest of the microbial community of needles of *Pinus ponderosa*.

Materials and Methods

Study Site

Dothistroma septosporum was identified in a small stand of *Pinus ponderosa* Lawson

and C. Lawson var. *ponderosa* C. Lawson (hereafter simply *Pinus ponderosa*) along the Priest River in Bonner County, Idaho, USA. The stand lies within a low, riverside fog belt that is relatively moist for *P. ponderosa*, and the natural conifer forest includes *Thuja plicata*, *Abies grandis*, and *Pseudotsuga menziesii* var. *glauca*. *Dothistroma* was positively identified from this site on the basis of micromorphology and recovered in pure culture from infected needles of *P. ponderosa* in September 2011 by incubating the needles in a moist chamber and plating emerging conidia onto 4% potato dextrose agar (PDA).

Sampling the Microbiome of Pinus ponderosa

A number of endophytic fungi were recovered from needles and root sections of *Pinus ponderosa* during summer sampling of the host's microbiome. Asymptomatic, healthy needles were collected from trees growing in the University of Idaho Experimental Forest, Latah County, ID, USA. One hundred individual needles were sampled in the lower canopy from each of ten trees of *P. ponderosa* for a total of 1000 needles. Needles were surface sterilized by serial sterilization (1 minute in 95% ethanol, 5 minutes in 6% NaOCl, and 30 seconds in 95% ethanol) before plating onto 4% potato dextrose agar (PDA). Smaller sample groups were also taken from trees in the diseased pines that appeared to have lower disease severity. Endophytic root fungi were obtained by sampling fine lateral roots approximately 6 mm in diameter. Root pieces were cut into sections 2 cm in length then serially surface sterilized in 70% ethanol for 5 minutes, 6% sodium hypochlorite for 15 or 20 minutes, and 70% ethanol for 2 minutes followed by rinsing in sterile distilled water. Root sections were plated onto modified yeast-glucose agar. A total of 20 root sections were

plated per tree per species with 2 sections per plate. Following recovery from the plant tissues, representative cultures of fungal isolates were made and stored at 4°C on PDA.

Preparation of Inocula of Putative Disease Modifiers

Pure cultures of six selected fungi were made on 4% PDA. A single plate of a mature culture of each fungus was used to generate inocula, with the exception of three plates each of *Elytroderma* sp. and *Penicillium raistrickii* that were needed due to slower growth. Inocula were made by flooding culture plates with approximately 20 mL of sterile distilled water (SDW) and loosening the spores into suspension by passing a sterile, bent glass rod over the surface of the culture. Fragments of sterile mycelium of *Elytroderma* sp. were obtained in like manner and reduced to atomizable particles by processing with a Tissue Tearor (BioSpec Products, Bartlesville, OK, USA) while in suspension. All suspensions were brought to a volume of 250 mL with SDW and placed in sterilized hand spray bottles. A solution of 250 mL of SDW was also included as a control.

Spring 2012 Inoculations

The following spring (May 2012) developing candles were monitored until needles began to emerge from the fascicles. Once this point of development was reached, the candles were inoculated in the field with the inoculum suspensions of fungal endophyte propagules using a hand spray bottle. Controls were sprayed with sterile distilled water. A single candle was inoculated with each treatment on each of ten trees, so that each inoculant was represented on every tree. Candles were randomly selected and assigned to an inoculant. The number of needles varied per candle but were of a sufficient number for

adequate replication per inoculant per tree (an average of n=187, range=51 to 319). Treated candles were tagged according to inoculant. Trees for inoculation were selected along a contour diagonal across the pine stand, so that selected trees fell approximately at the same elevation. Since trees were from seedlings and genotypic variation could be expected and environment varied across the stand, trees were not replicated but represented blocks in the design. Inoculations were made at sunset. Inoculated candles were enclosed in polythene bags over night to retain surface moisture needed for endophyte infection. Controls were also enclosed in polyethylene bags over night. These bags were removed at sunrise the next morning.

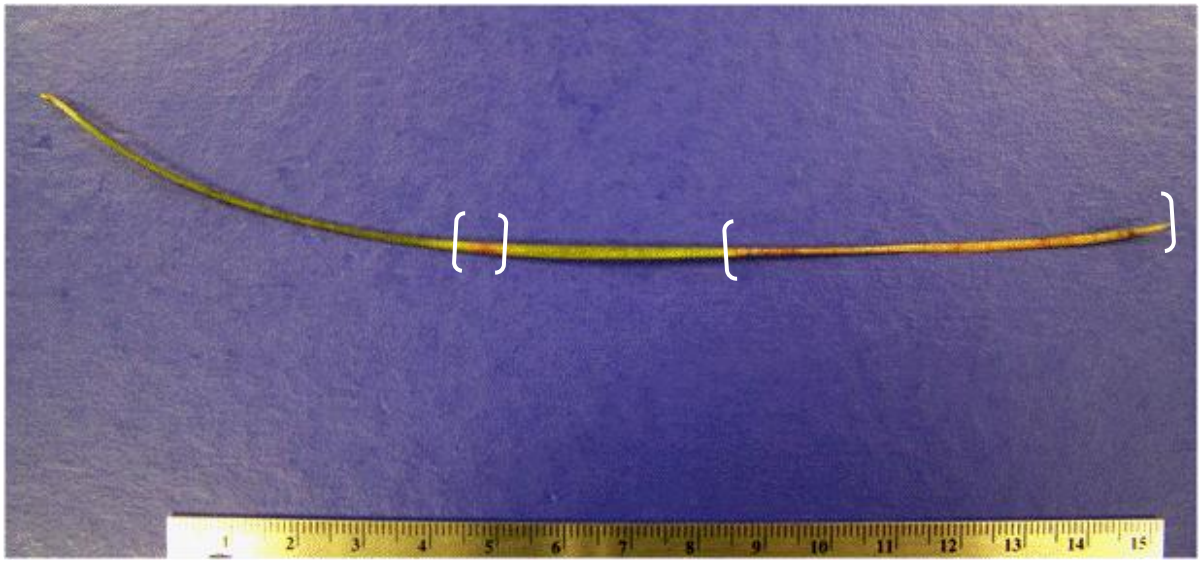
Spring 2013 Determinations of Disease Severity

In May 2013 (i.e., one year after inoculations of emerging needles), treated branches of mature needles from the 2012 growing season were removed to measure disease severity. Harvested material was kept frozen until *Dothistroma* needle blight severity could be scored. Needles were removed first from branches and then from the fascicles and measured individually. Disease severity was scored by measuring the length of each needle (L) and the length of the infected portion of the needle (D). Disease severity was computed as the ratio D/L (Figure 2.1).

Data Analysis

Disease severity was analyzed by analysis of variance for a randomized complete block design using SAS and SYSTAT. Mean disease severity ratios for the main effects of inoculant and tree were determined as were the interaction means for tree by inoculant.

Figure 2.1. Disease severity of *Dothistroma* was determined for each needle by measuring symptomatic portions of the needle (in brackets), adding them, and dividing the total length of the diseased portions by the total length of the needle. The ruler at bottom provides scale in centimeters.



Tests for normality of the data were determined by residual and normality plots and univariate procedures. Due to differences in sample population numbers of needles from individual trees and inoculants, differences between mean disease severity for the main effects of inoculant and tree were determined by least-squares (LS) mean comparisons at the 5% level.

Results

Sampling of the microbiome and selection of putative disease modifiers

Several hundred fungal morphotypes were recovered from both needle and root microbiomes of healthy *Pinus ponderosa* var. *ponderosa* and well over a thousand individual

isolates were recovered from needles alone. By far, the most common genus recovered was *Lophodermium*, an expected result (Ganley and Newcombe 2006). The next four most common genera observed among the foliar isolates included a non-pathogenic *Hormonema dematioides,* and culturable, non-pathogenic *Elytroderma, Cladosporium, and Penicillium*.

Table 2.1. Endophytes selected for testing against *Dothistroma septosporum* were recovered from the native range of the pathogen and host *Pinus ponderosa*. Except for *Clonostachys rosea* all were among the endophytes most frequently isolated from ponderosa pine. *Clonastachys* was added as a recognized hyperparasite recovered from a co-occurring host in the same forest type.

Endophyte	Rep. GenBank Accn. No.	Host	Source Tissue	Recovery Site
Cladosporium sp.	GU214630.1	*Pinus ponderosa*	Needles	University of Idaho Experimental Forest (UIEF), Latah County ID, USA
Clonostachys rosea	GU934503.1	*Pseudotsuga menziesii* var. *glauca*	Roots	Moscow, Latah County ID, USA
Elytroderma sp.	AY465437	*Pinus ponderosa*	Needles	Priest River, Bonner County ID, USA
Hormonema dematioides	AY253451.1	*Pinus ponderosa*	Needles	UIEF, Latah County ID, USA
Penicillium goetzii	JX997042.1	*Pinus ponderosa*	Roots	UIEF, Latah County ID, USA
Penicillium raistrickii	JN406592.1	*Pinus ponderosa*	Needles	UIEF, Latah County ID, USA

Penicillium was the second most common genus recovered from root tissues, and *Penicillium goetzii* was the species most commonly recovered within the genus. *Hormonema, Elytroderma*, and *Penicillium* spp. were also recovered at high frequency in *Dothistroma* infected foliage sample from the Priest River site. Given these findings, five commonly isolated fungi from the microbiome of *Pinus ponderosa*, and *Clonostachys rosea* from roots of seedlings of *Pseudotsuga menziesii* var. *glauca* (Table 2.1) were selected and employed as putative disease modifiers. *Clonostachys rosea* is a known parasite and antagonist of other fungi (Morraga-Suazo *et al.* 2011).

Modification of Disease Severity

When introduced into emerging needles, five of the six putative modifiers significantly modified disease severity of *Dothistroma* needle blight by either enabling or antagonizing the pathogen (Figure 2.2). The *P. ponderosa* root endophyte, *Penicillium goetzii,* antagonized *Dothistroma*, reducing disease severity by 6.9% compared to untreated foliage ($P < 0.0001$). However, other fungi common to the needle biome enabled the pathogen and increased disease severity. *Hormonema dematioides* increased severity significantly by 4.7% ($P < 0.0001$); and *P. raistrickii* and *Elytroderma sp.* increased disease severity by 3.6% ($P < 0.0003$) and 2.5% ($P < 0.0181$), respectively. Only *Cladosporium* sp. had no effect on disease severity ($P < 0.9996$). *Clonostachys rosea* recovered from the root microbiome of *Pseudotsuga menziesii* increased disease severity by 4.2% ($P < 0.0001$).

Overall disease severity varied significantly by tree (Table 2.2), where ‘tree’ represents the combined effects of tree genotype and micro-environment. Severity varied

significantly from tree to tree from as little as 24.1% to as much as 77%. Modifying inoculant effects were strongly significant ($P<0.0001$), as were their interactions with trees ($P<0.0001$).

Figure 2.2. Endophytes co-occurring in the native range of *Dothistroma septosporum* modified its disease severity in *Pinus ponderosa*. *Penicillium goetzii* significantly reduced disease severity, while *C. rosea*, *Elytroderma* sp., *H. dematioides*, and *P. raistrickii* significantly increased disease severity. Disease severity was measured as symptomatic needle length divided by total needle length. Error bars indicate standard error. Significance was determined at $\alpha=0.05$.

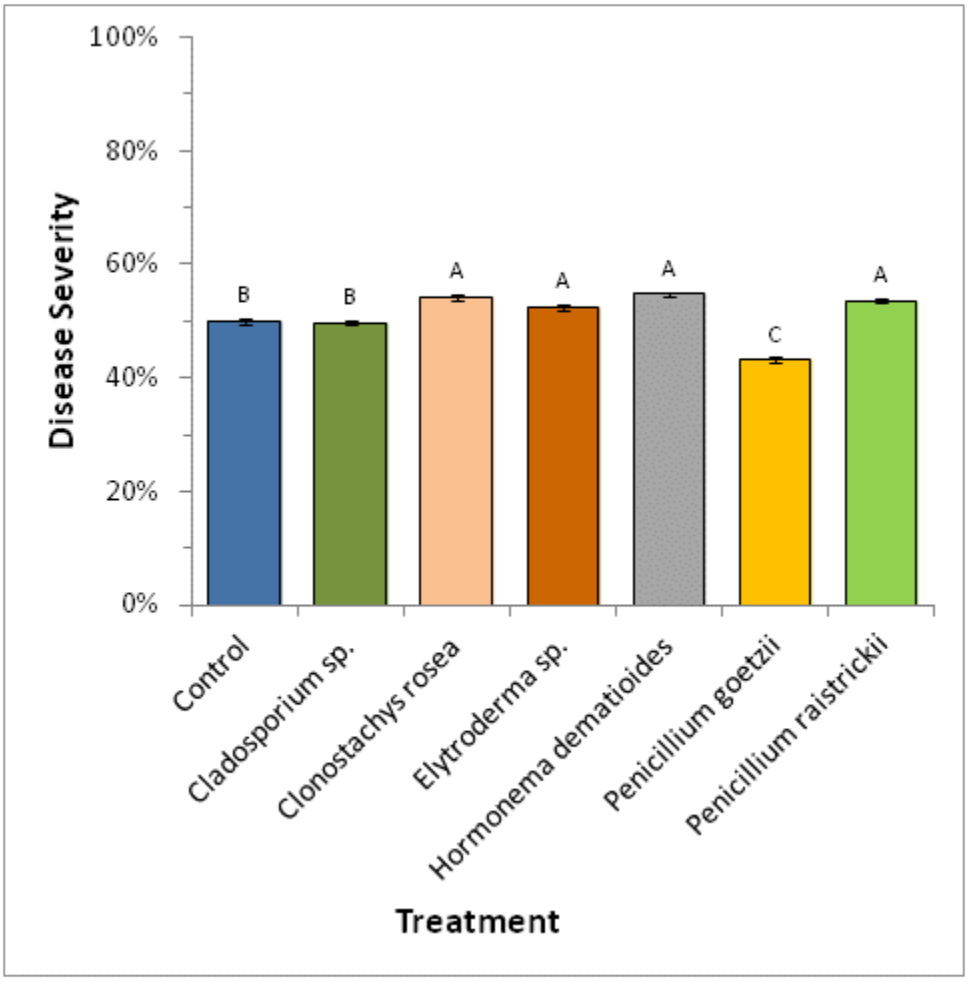


Table 2.2. ANOVA table showing significant effects of treatment with disease modifiers and trees with strong interaction. Significance was determined at α=0.05.

Source	DF	F-value	P-value
Treatment	6	50.07	<.0001
Tree	9	957.98	<.0001
Treatment*Tree	52	104.44	<.0001
Model	67	222.33	<.0001

N*	Mean DS**	Standard Deviation	Standard Error	R^2	CV***
13085	0.5108	0.2486	0.0022	0.5337	33.3274

*N represents the total number of needles sampled for all trees.
**DS=Disease severity as determined by measuring symptomatic length/total needle length.
***Coefficient of variation.

Discussion

Foliar disease occurs in the context of the foliar microbiome. In other words, foliar pathogens are just part of a highly diverse microbial community. Interaction, leading to disease modification, is likely among members of the community. And both types of disease modification (i.e., antagonism and enabling) have been observed in various natural and managed ecosystems (Table 2.3). Yet, disease modification remains a challenging phenomenon to investigate in nature.

Exclusion of all microbes is one way to determine the contribution of the entire foliar microbiome to disease modification (Arnold *et al.* 2003). But, to address questions of the frequency and extent of individual contributions, addition experiments are needed

Table 2.3. Non-pathogenic fungal members of foliar microbial communities of a number of plants are capable of antagonizing or enabling pathogenic members of that community *in vivo*. Although antagonists outnumber enablers in the literature, more studies are taking a whole-community approach to microbiomes that may reveal a comparable if not greater number of cryptic enablers.

Pathogen	Host/System/ Status	Modifier	Effect	Citation
Alternaria triticimaculans	*Triticum aestivum (Poaceae)* Agricultural/Introduced	*Aspergillus niger*	Antagonist	Perello *et al.* (2002)
		Bacillus sp.	Antagonist	
		Chaetomium globosum	Neutral	
		Cryptococcus sp.	Neutral	
		Epicoccum nigrum	Neutral	
		Fusarium moniliforme var.*anthophilum*	Antagonist	
		Nigrospora sphaerica	Antagonist	
		Paecilomyces lilacinus	Antagonist	
		Rhodotorula rubra	Neutral	
		Stemphylium sp.	Neutral	
Bipolaris sorokiniana	*Triticum aestivum (Poaceae)* Agricultural/Introduced	*Aspergillus niger*	Antagonist	Perello *et al.* (2002)
		Bacillus sp.	Antagonist	
		Chaetomium globosum	Antagonist	
		Cryptococcus sp.	Antagonist	
		Epicoccum nigrum	Neutral	
		Fusarium moniliforme var.*anthophilum*	Antagonist	
		Nigrospora sphaerica	Antagonist	
		Paecilomyces lilacinus	Antagonist	
		Rhodotorula rubra	Antagonist	
		Stemphylium sp.	Antagonist	
Cronartium ribicola	*Pinus monticola (Pinaceae)* Natural/Native	*Endophyte mixture*	Antagonist	Ganley *et al.* (2008)

Table 2.3. cont.

Pathogen	Host/System/ Status	Modifier	Effect	Citation
Drechslera tritici-repentis	*Triticum aestivum (Poaceae)* Agricultural/Introduced	*Aspergillus niger* *Bacillus* sp. *Chaetomium globosum* *Cryptococcus* sp. *Epicoccum nigrum* *Fusarium moniliforme* var.*anthophilum* *Nigrospora sphaerica* *Paecilomyces lilacinus* *Rhodotorula rubra* *Stemphylium* sp.	Antagonist Antagonist Antagonist Antagonist Antagonist Antagonist Antagonist Antagonist Antagonist Antagonist	Perello *et al.* (2002)
Drepanopeziza populi	*Populus angustifolia* Natural/Native	*Penicillium* sp. *Truncatella angustata*	Enabler Neutral	Busby *et al.* (2013)
Erysiphaceae	Various Agricultural/Introduced Natural/Native	Various endophytic and saprophytic fungi	Antagonists	Kiss (2003)
Melampsora x columbiana	*Populus sp. (Salicaceae)* Natural/Native	*Stachybotrys* sp. *Trichoderma atroviride* *Truncatella angustata* *Ulocladium atrum*	Antagonist Antagonist Antagonist Antagonist	Raghavendra and Newcombe (2013)
Phytophthora sp.	*Theobroma cacao* (Malvaceae) Natural/Native	*Colletotrichum* sp. *Fusarium* spp. *Xylaria* sp.	Antagonist Antagonist Antagonist	Arnold *et al.* (2003)

Table 2.3. cont.

Pathogen	Host/System/ Status	Modifier	Effect	Citation
Puccinia polygoniamphibii var. *tovariae*	*Fallopia japonica* (Polygonaceae) Invasive/Native	*Alternaria* sp. *Colletotrchum* sp. *Pestalotiopsis* sp. *Phoma* sp. *Phomopsis* sp.	Antagonist Antagonist Neutral Enabler Neutral	Kurose *et al.* (2012)
Puccinia recondita f. sp. tritici	*Triticum aestivum (Poaceae)* Agricultural/Introduced	*Chaetomium* sp. A *Chaetomium* sp. B *Phoma* sp.	Antagonist Antagonist Antagonist	Dingle and Mcgee (2003)
Puccinia xanthii	*Xanthium occidentale (Asteraceae)* Invasive/Introduced	*Alternaria zinniae* *Colletotrichum accutatum* *C. coccodes* *C. dematium* (a) *C. dematium* (b) *C. gloeosporioides* *C. orbiculare* *C. truncatum* *Phomopsis* sp.	Enabler Enabler Neutral Enabler Neutral Neutral Enabler Neutral Neutral	Morin *et al.* (1993a)
Puccinia xanthii	*Xanthium occidentale (Asteraceae)* Invasive/Introduced	*Colletotrichum orbiculare*	Enabler	Morin *et al.* (1993b)

Table 2.3. cont.

Pathogen	Host/System/ Status	Modifier	Effect	Citation
Sclerotinia homoeocarpa	*Festuca* sp. Agricultural/Introduced Native	*Epichloë festucae*	Antagonist	Clarke *et al.* (2006)
Septoria tritici	*Triticum aestivum (Poaceae)* Agricultural/Introduced	*Aspergillus niger*	Antagonist	Perello *et al.* (2002)
		Bacillus sp.	Antagonist	
		Chaetomium globosum	Antagonist	
		Cryptococcus sp.	Antagonist	
		Epicoccum nigrum	Antagonist	
		Fusarium moniliforme var.*anthophilum*	Antagonist	
		Nigrospora sphaerica	Antagonist	
		Paecilomyces lilacinus	Antagonist	
		Rhodotorula rubra	Antagonist	
		Stemphylium sp.	Antagonist	
Ustilago maydis	*Zea mays (Poaceae)* Agricultural/Introduced	*Fusarium verticillioides*	Antagonist	Lee *et al.* (2009)
Venturia inaequalis	*Malus domestica (Rosaceae)* Agricultural/Introduced	*Aureobasidium pullulans*	Antagonist	Andrews *et al.* (1983)
		Chaetomium globosum	Antagonist	
		Cryptococcus sp.	Antagonist	
		Flavobacterium sp.	Antagonist	
		Microsphaeropsis olivacea	Antagonist	
		Trichoderma viride	Antagonist	

(Arnold *et al.* 2003), since individual exclusions are not possible. Additions (via inoculations) of horizontally transmitted microbes are best timed to take advantage of the initial stages of community assembly (Fukami and Morin 2003; Kennedy *et al.* 2009) in freshly emerged host tissues. Inoculations reported here thus mimicked the timing of the pathogen itself and maximized the probability of interaction between the pathogen and the inoculant. That probability was also increased by temporarily increasing the population of a particular inoculant. When introduced to young needles at approximately the same time as natural infection by *Dothistroma septosporum*, five of six inoculants were able to modify subsequent disease severity. Interestingly, the sole antagonist modified disease to a greater extent than any one of the four enablers. The third possible outcome (i.e., no effect) was achieved following inoculation with *Cladosporium* sp.

If all six inoculants had been associated with the same effect (i.e., either antagonism or enabling or no effect) inferences would have been weaker. Instead, the fact that all three effects were obtained with the six inoculants allows us to infer that the interactions with *Dothistroma septosporum* were specific, even if the mechanisms of those interactions remain unknown. Possible mechanisms range from mycoparasitism to induction or suppression of host resistance. The latter two mechanisms might explain antagonism and enabling, respectively.

The relatively moist microclimate of the *Pinus ponderosa* site was essential for this study since it facilitated more severe disease (an overall mean of 53.7%) than is typical for the region, and a substantial range in severity among the ten trees (from 24.1% to 77%).

Variation in severity among the ten 'trees' may actually have been the product of tree genotype, microclimate, non-uniform inoculum of *Dothistroma septosporum*, or all three factors. Disease severity of *Dothistroma* is known to be affected by abiotic variables such as moisture, temperature, and light (Gadgil 1967; Gadgil 1977); host resistance also varies (Bradshaw 2004). In particular, severity is increased by moisture during the growing season (Gadgil 1974; Harrington and Wingfield 1998; Welsh *et al.* 2014; Wood *et al.* 2005). In any case, it was against this variable background in disease severity that modifiers produced their three effects. In other words, the effects were not contingent upon a single, uniform level of disease severity. The nature of the significant interaction between inoculants and 'trees' is not clear, because it likely hinges upon an understanding, that we have yet to develop, of the mechanism of interaction between the inoculant and the pathogen.

Just as pathogens are more common within the native ranges of their hosts (Mitchell and Power 2003), one might also expect to find higher frequencies of disease modifiers within native ranges. This hypothesis has, however, never been tested, to our knowledge. Most research into the interactions of potential disease modifiers and pathogens has focused on the selection of antagonists for the applied purpose of disease management (Table 2.3). Applied studies have been conducted in introduced ranges more often than in native ranges. To date, no study has deliberately contrasted disease modification in both native and introduced ranges. Studies have also varied substantially in sampling intensity, with more effort expended for agricultural crops of economic importance. The disparate compilation of studies (Table 2.3) show merely that both antagonism and enabling do occur in a range of diverse plants. Beyond that, no inferences may be drawn. Although we did

obtain a relatively high frequency of modification in this study, the scope of our study was too small to test the hypothesis. The modifiers identified represent only a small fraction of putative modifiers populating the microbiome of a single species of *Pinus*. We also did not conduct a parallel study in another part of the world (e.g., New Zealand) where both the pine host and *Dothistroma* are introduced.

Disease severity of *Dothistroma* needle blight already varies dramatically around the globe. In the introduced range disease severity is typically greater than in the native range (Barnes *et al.* 2008; Watt *et al.* 2009). There are two primary ways that disease modifiers from the native range might help to explain the greater severity of disease in the introduced range: 1) the absence of antagonists that were left behind in the native range, or 2) the co-introduction of enablers. A third possibility is the shift of enabling microbes from plants of the introduced range to introduced *Pinus*.

Modifiers in the native range might, or might not, act in the same way in the introduced range. If disease modification depends upon a ripple, or priority, effect initiated by the inoculant, that then stimulates some other member of the microbiome to interact proximately with the pathogen, then modifiers might be inconsistent when moved. The latter prediction assumes that communities differ substantially between ranges, or at least that proximate modifiers do. On the other hand, if disease modification depends on mycoparasitism or induced or suppressed resistance then the effects of an introduced modifier might be more predictable. Of course, mechanisms might vary among disease modifying organisms.

Conclusions

Frequently, the approach to understanding pathogens of global impact such as *Dothistroma septosporum* is limited to the disease triangle. Increases in disease severity in the introduced range are most often assumed to reflect genetic changes in host and/or pathogen, or new abiotic conditions. Interactions with non-pathogens within the microbiome are often ignored. In this study we present exploratory research on these interactions in the native range of host and pathogen where disease modifiers should be most common.

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Chapter 3

Endophytes as Pre-emergent, Janzen-Connell Agents

Abstract

Janzen-Connell (JC) agents diversify forest communities by reducing conspecific, but not heterospecific, recruitment beneath parent trees. Among the best known JC agents are microbial pathogens that attack emergent seedlings. However, the extent to which microbial JC agents may operate pre-emergence is unknown. We employed an *ex situ*, emergence assay to assess JC effects of common members of the microbiome of *Pinus ponderosa* in a northern Idaho forest. Host seeds and those of heterospecific, *Pseudotsuga menziesii* var. *glauca,* were treated with one of four inoculants prior to sowing: *Hormonema dematioides* (an endophyte from asymptomatic needles), *Pencillium goetzii* and *Pseudomonas sp*. (endophytes from healthy roots), and *Morchella snyderi,* a common resident of forest litter layers. We expected potential JC agents to negatively affect conspecific seedlings but to have no effect on heterospecifics. Pre-emergent effects were common as all three endophytes reduced emergence; post-emergent effects were absent. The strongest pre-emergence effect against *P. ponderosa* belonged to the foliar fungus, *Hormonema dematioides*. In four, separate, four-week assays H. dematioides always reduced emergence of *P. ponderosa*, whereas it had no effect on emergence of heterospecific *Pseudotsuga menziesii*. *Hormonema dematioides* reduced emergence by reducing germination. Microbes that reduce germination and emergence of conspecific but not heterospecific seeds may contribute to JC dynamics in *P. ponderosa*, and this may be

especially true for its own endophytes.

Key words: Janzen-Connell agents, *Hormonema*, seedling emergence, conspecific recruitment, biodiversity

Introduction

Janzen (1970) and Connell (1971) first proposed the concept of biotic agents driving beta diversity in plant communities by reducing natural recruitment of conspecifics beneath parent plants (Carson *et al.* 2008). In as much as Janzen-Connell (JC) agents are host-specific pathogens, they favor heterospecific recruitment (Janzen 1970; Packer and Clay 2000).

Soil-borne phytopathogens that attack emergent seedlings are recognized as the dominant JC agents in many plant communities (Bagchi *et al.* 2014; Liu *et al.* 2012; Packer and Clay 2000). These communities range from tropical rainforests (Augspurger and Wilkinson 2007) to temperate grasslands (Peterman *et al.* 2008). These pathogens are understood to accumulate in soils surrounding their host, reducing recruitment of host conspecifics and favoring competitive survival by heterospecifics (Schnitzer *et al.* 2011; Van der Putten and Peters 1997).

Most identified JC pathogens attack conspecific seedlings post-emergence (Augspurger and Wilkinson 2007; Packer and Clay 2000). Yet, pre-emergent interactions between seeds and microbes could be just as important. For plant species with persistent seed banks, failure to emerge can have significant consequences (Meyer *et al.* 2014).

However, for species without seed banks, failure to emerge may have much more serious consequences. A number of conifer species, including *Pinus ponderosa*, are known to lack persistence in seed banks (Pratt *et al.* 1984). Therefore seeds of *P. ponderosa* that fail to germinate and emerge during the first spring after deposition are not likely to emerge in subsequent springs (Bai *et al.* 2004).

Packer and Clay (2000) and Augspurger and Wilkinson (2007) identified oomycetous *Pythium* species as JC agents in both temperate and tropical forests. A number of fungal genera have also been associated with JC dynamics in tropical and temperate plant communities (Hersh *et al.* 2012; Konno *et al.* 2011; Liu *et al.* 2012), but more often, no attempt is made to isolate and identify individual JC agents (Bagchi *et al.* 2014; Beckman *et al.* 2014; Bever 1994; Van der Puttenand Peters 1997). While obvious soil pathogens, such as *Fusarium* spp. and oomycetes (Liu *et al.* 2012; Packer and Clay 2000), are readily recognized JC agents, very little of the microbial diversity associated with plants has been assessed for effects on recruitment of conspecific and heterospecific seedlings.

The plant microbiome includes both root and foliar endophytes that might interact with seeds and seedlings. This is obvious enough for roots but fallen leaves undergo a microbial succession in litter in which seeds frequently germinate (Visser and Parkinson 1975). An important point is that, initially and for some time, fallen leaves include the microbiome of living leaves (Voříšková and Baldrian 2012) which may be composed of a highly diverse assemblage of microorganisms (Arnold 2007; Carroll and Carroll 1978; Ganley *et al.* 2004). It is possible, then, that JC agents may be found among the diverse fungi

associated with both roots and leaves.

Forest biomes of northern Idaho, USA support a diverse community of species in part due to an exceptional intermountain climate and coastal disjunct populations thought to be residues of glacial refugia (Johnson 1995). *Pinus ponderosa* is an important intermountain conifer that ranges from being merely a component of diverse, mesic forest to being the dominant climax species at the dry, steppe-forest interface (Daubenmire 1966). Examples of JC dynamics from temperate forests have been identified (Johnson *et al.* 2012; Lambers *et al.* 2002; Packer and Clay 2000; Piao *et al.* 2013), and at the mesic end of the range of *P. ponderosa* where this drought-tolerant pine overlaps the range of the mesophilic *Pseudotsuga menziesii* var. *glauca*, the JC model might be expected to operate. However, are interactions primarily pre- or post-emergence? Moreover, are JC agents restricted to the community of soil residents or might they also include root and foliar endophytes?

In this study, we performed *ex situ* assays to explore possible JC effects of three endophytes common to the microbiome of *Pinus ponderosa* in a mesic, diverse forest: *Hormonema dematioides*, a fungus from asymptomatic needles, and *Penicillium goetzii* and *Pseudomonas* sp., a fungus and a bacterium, respectively, from healthy roots. In choosing the endophytes we avoided obviously pathogenic symbionts since confirmation of these as JC agents seemed almost certain. A non-endophytic representative of the soil microbiome of northern Idaho mixed conifer forest, *Morchella snyderi*, was included. We hypothesized that putative JC agents from *P. ponderosa* would reduce emergence of conspecific seedlings

but would have no effect on heterospecific seedlings of *Pseudotsuga menziesii*.

Materials and Methods

Sampling for Common Members of the Pinus Ponderosa *Microbiome*

The microbiomes of ten healthy *Pinus ponderosa* Lawson and C. Lawson var. *ponderosa* C. Lawson trees in the University of Idaho Experimental Forest, Latah County, ID, USA, were sampled in August 2010. To identify common foliar endophytes, one hundred needles were sampled from each of ten *P. ponderosa* for a total of 1000 needles. Needles were surface-sterilized (1 minute in 95% ethanol, 5 minutes in 6% NaOCl, and 30 seconds in 95% ethanol, (Ganley and Newcombe 2006) before plating onto 4% potato dextrose agar (PDA).

Root samples were extracted by trenching outward from the boll of the tree along lateral roots and excising fine laterals averaging 6 mm in diameter. Root samples were cut into sections 2 cm in length, serially surface sterilized in 70% ethanol for 5 minutes, 6% sodium hypochlorite for 15 or 20 minutes, and 70% ethanol for 2 minutes and then rinsed in sterile distilled water. A total of 200 root sections were plated onto modified yeast-glucose agar.

Tissue was processed within 72 hours following collection and stored at 4°C until processed. Following recovery from the plant tissue, fungal isolates were cultured and stored at 4°C on PDA. Prior to seed inoculations fresh cultures were made by transferring

spores to fresh 4% PDA.

Seed Sources and Pre-Treatment

Three separate emergence experiments were conducted using multiple seed provenances and two species of western conifers to determine effects of common residents of the microbiome of *Pinus ponderosa* on the emergence of both conspecific and heterospecific seedlings. *Pinus ponderosa* seed was sourced from three lots and two subspecies, and *Pseudotsuga menziesii* var. *glauca* seed from one lot was used (Table 3.1). Seed was stored at 5°C until needed. Prior to beginning the emergence studies, seed was removed from storage, surface-sterilized in 2.5% NaOCl for 8 min, and soaked in running water. After 24 hours in the running water, seed was thoroughly rinsed, wrapped in a clean paper towel, sealed in a sandwich bag, and placed in stratification at 2°C. After 4 weeks,

Table 3.1. Two northern Idaho, USA conifer species and three provenances were used to test the effects of *Pinus ponderosa* endophytes on seedling emergence.

Species (Lot #)	**Provenance**
Pseudotsugamenziesii (Mirb.) Franco var. *glauca* (Beissn.) Franco (1)	Cherry Lane Seed Orchard, Lewiston, ID, USA
Pinus ponderosa Lawson and C. Lawson subsp. *ponderosa* C. Lawson (1 and 2)	University of Idaho Experimental Forest, Flat Creek Unit, Princeton, ID, USA
Pinusponderosa Lawson and C. Lawson subsp. *scopulorum*Engelm. (3)	San Isabel National Forest, CO, USA

seed was removed from stratification and thoroughly rinsed.

Experiment 1: Extent of Pre- and Post-Emergent Effects Caused by Common Members of the Pinus Ponderosa *Microbiome*

In this first experiment, three common members of the *Pinus ponderosa* microbiome were used to inoculate seeds to detect both pre- and post-emergent effects. A fungal resident of the mixed conifer forest soil microbiome was also tested. Seeds of *P. ponderosa* were inoculated by soaking over night in suspensions of endophyte inocula at a concentration of one three-week-old plate to 50 mL of sterile distilled water (SDW). Inocula of bacteria and sporulating fungi were made by flooding three-week-old plates with sterile distilled water (SDW) and passing a sterile bent glass rod over the culture surface to loosen spores. Non-sporulating cultures were prepped by flooding plates with SDW and scraping the mycelium from the agar surface with a scalpel before macerating the mycelium in a blender. Inoculum was suspended in SDW with one plate per 50 mL SDW. Seeds for the control treatment were soaked in SDW.

Following inoculation, seed was sown in #1 Sunshine Mix® in Leach® tubes and germinated in the greenhouse at a 16-hour daylength and diurnal temperatures of 25/16°C. After 4 weeks, emergence was recorded as presence or absence (1 or 0, respectively) of a seedling per tube. Emergent plants were examined daily during the four-week period for post-emergent disease symptoms.

Experiment 2: Confirmation of Pre-Emergent Effects on Pinus Ponderosa

The second experiment was designed to confirm the pre-emergent effects of the strongest potential JC agent identified in the first experiment. Seeds of *Pinus ponderosa* were soaked overnight in a spore suspension of the potential JC agent. Inocula was made by flooding one three-week-old plate with SDW and passing a sterile bent glass rod over the culture surface to loosen spores before suspending in 50 mL of SDW for a final spore concentration of approximately $6.75x10^6$ cells/mL. Seeds for the control treatment were soaked in SDW.

Following inoculation, 600 seeds were sown in three 200-cell plug trays per treatment and incubated for 4 weeks in a greenhouse with a 16-hour daylength and diurnal temperatures of 25/16°C. After 4 weeks, emergence was recorded as presence or absence (1 or 0, respectively) of a seedling in a cell.

Experiment 3: Janzen-Connell Effects

A third emergence experiment was conducted to test for possible JC effects of the strongest putative JC agent on emergence of *Pinus ponderosa* subsp. *ponderosa* and *Pinus ponderosa* subsp. *scopulorum*, and a second conifer with overlapping range: the heterospecific *Pseudotsuga menziesii* var. *glauca*. Seeds of both species were soaked overnight in spore suspensions. Methods for inoculum preparation, seed sowing, and germination followed Experiment 2 protocol. After 4 weeks, emergence was recorded as presence or absence (1 or 0, respectively) of a seedling in a cell.

Experiment 4: Testing the Mechanism of Pre-Emergent Janzen-Connell Effects

To determine the possible JC mechanism of the strongest putative JC agent, non-emergent seeds were tested for viability following a fourth emergence experiment. Seeds of *Pinus ponderosa* subsp. *ponderosa* were cleaned and stratified following previous protocol. Two hundred seeds each were treated with either a suspension of the potential JC agent or SDW following Experiment 2 protocols. After 12 hours in the suspensions, seeds were sown in cell flats and placed in the greenhouse. Following emergence, ungerminated seeds were removed from the cells and washed. Fifty non-stratified, untreated seeds were washed to be tested as a positive control. Fifty more non-stratified, untreated seeds were boiled for 20 minutes then frozen overnight. All seeds were soaked for approximately 2 hours prior to viability treatment. Seeds were then split longitudinally in half, and the seed halves were submerged in 2,3,5-triphenyl-tetrazolium chloride (TTC) for 20 hours following protocol by Grano (1958). Ungerminated seeds testing positive for dehydrogenase in the embryo were considered viable.

Statistical Analyses

Seedling emergence and viability outcomes were analyzed by the Pearson chi-squared contingency test using SYSTAT® and R (SysStat Software Inc. 2010; Development Core Team 2013).

Results

Isolation of Common Members of the Pinus Ponderosa *Microbiome*

Of the numerous endophytes recovered from needles of trees of *Pinus ponderosa* var. *ponderosa* in a mixed northern Idaho forest, *Hormonema dematioides* was among the most common. This endophyte was recovered from all ten trees sampled and was only slightly less common than the expected *Lophodermium* species complex (Ganley *et al.* 2004) and the *Elytroderma* species complex (Ridout and Newcombe, unpublished). Both *Lophodermium* and *Elytroderma* can be strongly pathogenic so we selected the less obviously pathogenic *Hormonema dematioides* (Funk 1985) for further work.

Penicillium goetzii and a fluorescent *Pseudomonas* sp. were common endophytes recovered from root tissues. *Penicillium* spp. were only slightly less common than the dark-septate-endophyte or DSE complex that is expected in conifer roots (Grünig *et al.* 2008). *Pseudomonas* was less common, but is a wide-spread rhizobacterial taxon common in forest soils and frequently associated with plant roots (Axelrood *et al.* 2002; Lugtenberg and Kamilova 2009). The choice for further work with the first two taxa was arbitrary since none of the three are known to be strongly pathogenic.

Finally, *Morchella snyderi* was recovered from an ascocarp from soil of the mixed conifer forest. *Morchella snyderi* is a common soil resident in the mixed conifer forest understory with no known pathogenic effects. However, *Morchella* spp. can be mycorrhizal in pines (Dahlstrom *et al.* 2000).

Putative Janzen-Connell Agents

Overall, across experiments, the foliar endophyte *Hormonema dematioides* was found to reduce emergence of its host species by 14% to as much as 67% (Figures 3.1). Regardless of subspecies and area of origin for host seeds, *Hormonema*-mediated reductions in emergence of *Pinus ponderosa* were significant in all cases (Table 3.2). Effects of the two root endophytes were weaker (Figure 3.3). *Morchella snyderi* was the only non-endophyte tested, and it failed to reduce emergence ($P<0.139$, Table 3.2). Not one

Figure 3.1. *Hormonema dematioides* visibly reduced emergence of *Pinus ponderosa* germinated in seedling trays in the greenhouse, even four weeks after sowing. On the left, untreated seed emerged rapidly and consistently; treated seedlings (right) emerged more slowly and poorly.

Table 3.2. *Pinus ponderosa* var. *ponderosa* foliar endophyte *Hormonema dematioides* significantly reduced emergence of *P. ponderosa* seed four weeks after sowing. Other endophytes also reduced emergence significantly, but not to the same extent. Below are percent emergence and chi-square values comparing emergence of conifer seed treated with endophytes with that of untreated (control) seed at the P<0.05 level of significance. Data are sorted by experiment, host species, and inoculant. Percentages are shown as percent of seed sown and percent of control emergence.

Experiment	Species	Inoculant	Percent emergence	Percent of Control	Chi-square	P-value
Exp. 1	*Pinus ponderosa* var. *ponderosa*	*Hormonema dematioides*	17.2	32.7	88.827	0.000
		Morchella snyderi	42.8	82.9	2.190	0.139
		Penicillium goetzii	40.8	74.9	4.110	0.043
		Pseudomonas sp.	35.3	67.8	13.636	0.000
		Control	47.8			
Exp. 2	*Pinus ponderosa* var. *ponderosa*	*Hormonema dematioides*	55.8	86.6	9.402	0.002
		Control	64.5			
Exp. 3	*Pinus ponderosa* var. *ponderosa*	*Hormonema dematioides*	89.0	92.4	23.741	0.000
		Control	96.3			
	Pinus ponderosa var. *scopulorum*	*Hormonema dematioides*	28.5	70.1	19.629	0.000
		Control	40.7			
	Pseudotsuga menziesii var. *glauca*	*Hormonema dematioides*	94.7	101.4	0.724	0.395
		Control	93.3			
Exp. 4	*Pinus ponderosa* var. *ponderosa*	*Hormonema dematioides*	68.0	76.4	24.900	0.000
		Control	89.0			

Figure 3.3. All three endophytes tested for their effects on host emergence significantly reduced seedling emergence of *Pinus ponderosa* var. *ponderosa* four weeks after sowing. However, *Hormonema dematioides* reduced emergence by over 50% of that of other treatments.

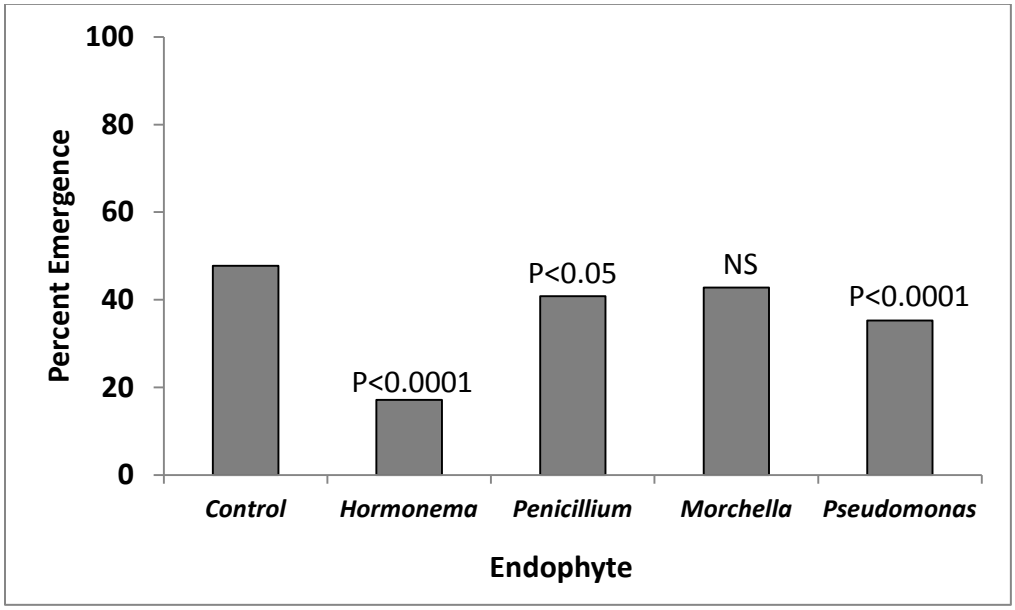
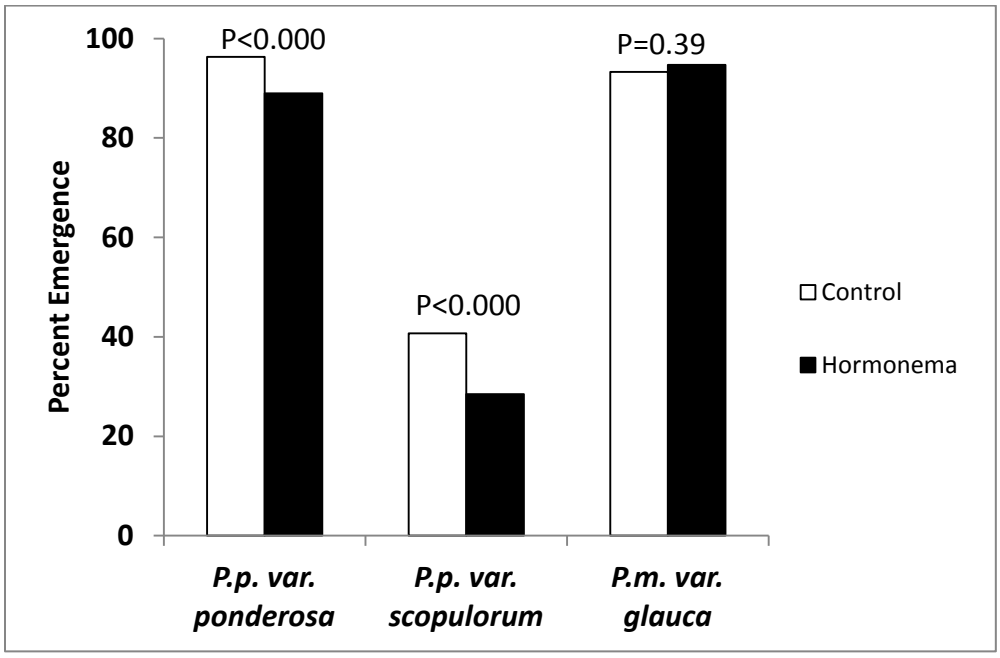


Figure 3.4. *Hormonema dematioides* significantly reduced emergence of *Pinus ponderosa* var. *ponderosa* and *P. ponderosa* var. *scopulorum* but slightly, although not significantly, increased emergence of competitor *Pseudotsuga menziesii* var. *glauca* seed inoculated with the foliar endophyte compared to untreated seed. Data were collect four weeks after sowing. Black bars show percent emergence of *Hormonema*-treated seed. White bars indicate percent emergence of control groups.



emerged seedling died in any treatment or in any experiment. Post-emergent effects were thus entirely absent.

Effects of the Strongest, Putative JC Agent on Heterospecifics and Conspecific Provenances

As expected for a JC agent, the foliar endophyte, *Hormonema dematioides*, did not reduce emergence of heterospecific *Pseudotsuga menziesii* (Figure 3.4). In the sameexperiment the *Hormonema* endophyte did reduce emergence of both provenances of conspecific *Pinus ponderosa*. In the non-local *P. ponderosa* subsp. *scopulorum* reduction of emergence was 30%. Reduction in emergence of local *P. ponderosa* subsp. *ponderosa* was less, but still highly significant ($P < 0.0001$) due to better overall emergence.

Figure 3.5. Seed halves with embryos were stained with tetrazolium chloride to determine viability of non-emergent seed four weeks after sowing. Clockwise from left is the boiled negative control, the untreated, unstratified positive control, *Hormonema*-treated seed, and the untreated seed. Staining of the embryos indicates viable embryos capable of germination under favorable conditions and physiological status.

Mechanism of Pre-Emergent Janzen-Connell Effects Is Not Reduced Viability

Although *Hormonema* significantly reduced emergence, seed viability was not significantly reduced by the fungus compared to the untreated seed (χ^2=0.017, P=0.8962) or the untreated, non-stratified positive control (χ^2=0.2986, P=0.5848). Seed viability was effectively destroyed by boiling the negative control, providing a visually effective comparison of seed viability and indicating that although the fungus reduced emergence it failed to kill seeds (Figure 3.5).

Discussion

Some of the best known Janzen-Connell agents are host-specific phytopathogens that reside in the soil and attack conspecific seedlings post-emergence (Augspurgerand Wilkinson 2007; Liu *et al.* 2012; Packer and Clay 2000).The contribution of endophytes to JC dynamics has not been systematically investigated in any plant community, and pre-emergent effects have been largely ignored. Here, in repeated experiments, a common foliar endophyte of *Pinus ponderosa* consistently delayed conspecific seed germination, and hence emergence, but the same fungus had no effect on heterospecifics. To a lesser extent the two common root endophytes acted similarly, although they were not tested with heterospecifics. These results indicate that, as drivers of beta diversity, the importance of microbial JC agents has likely been underestimated.

JC agents reduce recruitment of host conspecifics versus heterospecifics (Connell

1971; Janzen 1970). *Pseudotsuga menziesii* var*. glauca* is a primary competitor of *Pinus ponderosa* in the dry, mixed-conifer forests of the intermountain west of North America (Johnson 1995) and overlaps the range of both subspecies tested in this study along the western Rocky Mountains. *Hormonema dematioides* reduced emergence of both subspecies of *P. ponderosa* but had no effect on *P. menziesii* var. *glauca*. This specificity should enhance beta diversity (Benítez *et al.* 2013; Janzen 1970).

This initial study was limited to four microbes common to *Pinus ponderosa* (i.e., its three endophytes) and its plant community (i.e., *Morchella snyderi*) in northern Idaho. Had we used as inoculants isolates of strong pathogens such as species of *Lophodermium* and *Elytroderma* we expect that effects against conspecifics would have been even stronger than those observed for *Hormonema dematioides*, a weak pathogen (Funk 1985). Results may have also included post-emergent effects that were entirely absent here. Since the foliar microbiome of *P. ponderosa* in northern Idaho is dominated by *Lophodermium*, *Elytroderma*, and *Hormonema*, this might mean that most of this largely fungal community can contribute to JC dynamics. In this study, we also employed common root endophytes that have not been previously associated with disease in *P. ponderosa*. Yet, they too reduced emergence of *P. ponderosa.* These results indicate that future studies should not be limited to well-known soil-borne pathogens with post-emergent effects (Augspurger and Wilkinson 2007; Liu *et al.* 2012; Packer and Clay 2000).

Hormonema dematioides does not fit the conventional definition of a phytopathogenic JC agent, since its effects are pre- not post-emergence. However, H.

dematioides is a common symbiont of a host without a seed bank (Pratt *et al.* 1984; Ridout and Newcombe, unpublished). By reducing conspecific germination and emergence without killing seed (Figure 5), *Hormonema* and other endophytic putative JC agents subject the seed to a delay that reduces the capacity of an emergent seedling to compete effectively (Fenner and Thompson 2005). In addition, because little seed of *P. ponderosa* remains viable over a second winter (Bai *et al.* 2004; Pratt *et al.* 1984), the four-week delay that we observed is reduces the likelihood that the seed will remain viable and subjects the seed to potential predation (Shearer and Schmidt 1970).

The results of this study are congruent with longstanding observations of improved natural regeneration of *Pinus ponderosa* following removal of litter (Ellis and Bilderback 1991; Sackett 1984). Explanations of improved regeneration have tended to focus on abiotic factors associated with litter removal (Facelli and Pickett 1991). Biotic factors were not considered. One of the difficulties associated with testing the validity of the JC model is that of separating the effects of the abiotic environment on conspecific recruitment patterns from those contributed by biotic JC agents (Carson *et al.* 2008). By testing our hypothesis *ex situ*, we were able to demonstrate the importance of the microbiota in litter layers as a potential JC mechanism in natural regeneration and beta diversity in the mixed conifer forest.

Conclusion

Microbial Janzen-Connell agents are not fully appreciated as drivers of beta

diversity, perhaps because their definition has been confined to well-studied, post-emergent seedling pathogens. Here, we have demonstrated that highly diverse endophyte communities may harbor overlooked JC agents, and that pre-emergent interactions need to be considered. Further research is needed to determine the contributions to JC dynamics of entire endophyte communities *in situ*.

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Chapter 4

Symbiotic Benefits to Wheat from Pine Woodland-to-Wheat Conversions

Abstract

Conversion of pine woodlands and dry forest margins of the intermountain Pacific Northwest (PNW) USA to dryland wheat agriculture has been highly successful. High yields of wheat in the region have traditionally been attributed to deep soils, favorable climate, breeding, and agronomic improvements. However, microbes associated with pine forests could have initially provided symbiotic benefits to wheat. We designed a series of experiments to determine whether a forest microbiome adjacent to converted wheat land was capable of mediating resistance to disease and abiotic stress in hard red winter wheat. When wheat seedlings inoculated with the crown rot pathogen, *Fusarium culmorum*, were grown with a forest litter microbial community, survival was improved three-fold and growth increased over 60% compared to untreated controls. In the greenhouse, winter wheat exposed to drought conditions while infected with crown-rot pathogen *Fusarium culmorum* responded to inoculation with a conifer isolate, *Penicillium* WPT111A3, by doubling yield compared to untreated plants in identical conditions. *Penicillium* spp. from conifer roots also significantly increased yield by as much as 32.6% and above-ground biomass by 8.5 % in the absence of the pathogen where sufficient moisture was present. Wheat seedlings inoculated with fungi from forest conifers also developed significantly larger roots systems during vernalization at 5°C. It seems likely that wheat land in the

intermountain PNW may have reaped symbiotic benefits from the microbiomes of converted pine woodlands. These microbiomes and some of the individual microbes might still have applied value.

Key words: *Fusarium culmorum*, *Penicillium*, *Phialocephala*, *Morchella*, Litter microbiome

Introduction

Wheat is one of the top three cereals produced worldwide and among the top five agronomic crops that feed the world. According to the USDA Economic Research Service (2014), nearly 220 million hectares of wheat were grown around the world in 2013. Winter wheat, *Triticum aestivum* L., production in the Pacific Northwest (PNW) intermountain region of the USA is predominantly dryland. Deep loess soils and a Mediterranean climate have supported wheat production in this region for over a century (Burt 1981). Some of the most productive wheat land in the world, many of these fields were carved from shrublands, grassland prairie, pine woodlands, and the margins of dry intermountain forests (Black *et al.* 1998).

These replaced native systems, once dominated by perennial vegetation, host complex microbiomes of microbial flora which populate native plant species and soils. Fungi inhabiting the microbiomes of plants and their native soils can play a significant role in the function of host plants and the plant communities as a whole (Rodriguez *et al.* 2009; Kloronomos 2002). Conversion of these natural biomes to croplands in the PNW has vastly

altered the complex system of microbiomes in vegetation and soils restructuring communities and reducing natural diversity (Frier *et al.* 2013). Furthermore, continual annual cropping has lead to a build-up of undesirable pathogens and negative plant soil feedback (Cook 1980).

In these dryland cropping systems, *Fusarium culmorum* is a significant root and crown rot pathogen in wheat production. A classic negative-feedback pathogen, *F. culmorum* may be mitigated with rotational cropping, but dryland farming limits rotational effectiveness to mitigate loss from outbreaks. Not only are *Fusarium* crown and root rots costly in terms of plant survival and yield, but they thrive in dryland production and increase in virulence during periods of drought when plants are already stressed (Papendick and Cook 1974). In turn, damage to the crown and roots restricts water movement to above-ground tissues creating a spiraling cycle of disease and water stress that impacts yield.

Microbiomes of forest litter and forest soils are also known to suppress common pathogens and reduce the fecundity of *Fusarium* spp. (Schisler and Linderman 1984). Forest soils gradually reduced presence and viability of *Fusarium oxysporum* in the root zone of infected *Pinus Iambertiana* (Smith 1967). *Fusarium*-suppressive soils have long been of interest to agricultural plant pathologists (Alabouvette 1979; Burke 1954 and 1965; Scher and Baker 1980) and it is now recognized that members of these soil microbiomes mediate disease suppression.

A number of fungi occupying the internal microbiome of plants are known to mediate host resistance to pathogens (Dingle and Mcgee 2003; Ganley *et al.* 2008; Waller *et*

al. 2005). Single endophytes and rhizosphere fungi from native plant systems have been found to control soil pathogens in crops and agricultural systems (Kemf and Wolf 1989; Khasitini *et al.* 2014).

Crops suffering abiotic stress are more susceptible to soil-borne pathogens, such as *Fusarium culmorum* (Cook 1973; Gaudet and Chen 1987; Papendick and Cook 1974). Residents of natural microbiomes can mediate resistance to abiotic stress in plant species, improving plant water relations (Hubbard *et al.* 2012; Hubbard *et al.* 2013) and mitigating temperature stress (Baynes *et al.* 2012; Barka *et al.* 2006) while improving viability, growth, and fecundity (Baynes *et al.* 2012; Barka *et al.* 2006; Hubbard *et al.* 2013).

Benefits of microbiomes may be more marked when novel relationships are formed between fungi occupying the microbiomes of natural plant systems and exotic plant species (Baynes *et al.* 2012), a premise known as the enhanced mutualism hypothesis (Baynes *et al.* 2012; Richardson *et al.* 2000). This phenomenon is frequently seen in studies of other crops species, where it has been exploited for biological control (Khastini *et al.* 2014) and yield enhancement (Naveed *et al.* 2014). Baynes *et al.* (2012) found a native *Morchella* sp. functioning as an endophyte could increase growth, fecundity, and heat tolerance of *Bromus tectorum*, an invasive, exotic winter annual now naturalized throughout the dry interior United States. *Morchella* spp. are common fungi inhabiting soil and litter layers of forest systems in the intermountain PNW.

Many fungi inhabiting the microbiomes of conifers and woody shrubs in dry forest systems of the Northern Rocky Mountains, ID, USA, belong to taxonomic groups that

contain fungi either exhibiting or associated with the ability to survive xeric conditions and extreme temperatures. Species from these taxa are also associated with antibiotic metabolite production that might contribute to disease resistance. Two of the dominant taxonomic groups associated with these microbiomes are *Phialocephala* spp. and *Penicillium* spp. (Ridout and Newcombe, unpublished).

Phialocephala spp. belong to a complex of melanized fungi known as dark septate endophyte (DSE) fungi. These fungi are commonly recovered from roots of plants around the world and appear to replace mycorrhizae in cold- and/or water-stressed habitats (Mandyam and Jumpponen 2005; Newsham 2011; Newsham *et al.* 2009). Dark septate endophytes are also recoverable from above-ground tissues where their functional roles appear more obscure (Grünig *et al.*, 2009) but have been associated with anti-insectan metabolite production (Frasz *et al.* 2014, in press). They have been associated with xeric environments (Knapp *et al.* 2012) and shown to provide thermotolerance (Redman *et al.* 2002) and disease resistance to *Verticillium dahliae* in tomato (Andrade-Linares *et al.* 2011) and *Fusarium oxyspoum* f. sp. *melonis* in watermelon (Khastini *et al.* 2014).

Many *Penicillium* spp. are associated with abiotic extremes (Frisvad *et al.* 2006; Houbraken *et al.* 2011; Pitt 1973; Vyas *et al.* 2007). They have been recovered from desiccated plant matter (Pitt and Christian 1968; Unterseher *et al.* 2012) and are identifiable with xeric environments (Vyas *et al.* 2007). Members of this genus and associated genera are also capable of functioning at extreme temperatures (Frisvad *et al.* 2006; Houbraken *et al.* 2012). The genus contains a number of species producing secondary

metabolites—many of which are anti-microbial (Frisvad *et al.* 2006; Houbraken and Samson 2011).

To test the hypothesis that forest soil and root microbiomes of the intermountain PNW could mediate tolerance to a soil-borne pathogen and abiotic stress in winter wheat while increasing growth and fecundity, we conducted a series of experiments testing community effects of a pine litter microbiome from a *Pinus ponderosa* woodland and individual effects of the litter fungus *Morchella snyderi* and selected endophytes of *Penicillium* and *Phialocephala* spp. from the root microbiome of *Pseudotsuga menziesii* var. *glauca*.

First we tested a selection of these fungi with putative benefits to exotic hosts to determine whether they might mediate low temperature stress and improve development and vigor of seedlings undergoing vernalization. To determine whether drought-adapted endophytes might contribute to resistance of host plants to infection by *Fusarium culmorum* and water stress, a study was conducted in the greenhouse to test the activity of select endophytes in winter wheat against drought stress and *F. culmorum*. Finally, we used non-selective inocula from forest pine litter and litter mulches to determine the effectiveness of litter-borne forest microbiome as a community to reduced severity of *F. culmorum* in wither wheat.

Materials and Methods

Assay for Contributions of Forest Fungi to Winter Wheat Development during Vernalization

Seeds of a single line of hard red winter wheat (HRWW, University of Idaho line 306 UI-SRG) were sown in 2 cm^3 200-cell trays nested in seed flats. Seedlings were sprouted in a growth chamber at a 23/16°C diurnal temperature cycle with a 16-hr. photoperiod. Seven days after the seed was sown, fully emerged seedlings were inoculated in the crown with 1 mL of a single inoculum. Seeds received one of five inocula. These inocula included a sterile distilled water (SDW) control, *Morchella snyderi* (recovered from an ascocarp growing in litter of an intermountain conifer forest) and three endophytes recovered from the root microbiome of *Pseudotsuga menziesii* var. *glauca*: *Phialocephala* sp. BHIAR, a novel xerophilic isolate of *Penicillium* (*Penicillium* sp. *nova* WPT111A3), and the xerophilic *Penicillium goetzii*.

The four fungi chosen for this experiment were cultured on PDA. Plates containing the non-sporulating, mature cultures of *M. snyderi* and *Phialocephala* were flooded with sterile distilled water (SDW) and scraped with a sterile scalpel to remove the mycelium. Mycelia were then processed in a blender until finely fragmented before suspending in SDW. Final solutions of *M. snyderi* and *Phialocephala* inocula were brought to volume between 10^4 and 10^6 fragments per mL. Plates of the sporulating *Penicillium* species *P. goetzii* and WPT111A3 were flooded with SDW and spores were loosened by passing a sterile bent glass rod over the mycelium. Spore solutions were suspended in SDW and final inocula of *P. goetzii* and WPT111A3 were brought to volume at 10^6 to 10^7 cells per mL. The

control consisted of SDW only.

Seedlings were returned to the growth chamber following inoculation, and the temperature was reduced to a 19/16°C diurnal cycle. Four days after inoculation, growth chamber temperatures were reduced to a 5/5°C diurnal cycle, and photoperiod was reduced to a 10-hr. daylength. Plants were vernalized at this temperature and daylength for 8 weeks, at which time seedlings were removed from the growth chamber. During vernalization, seedlings were fertilized with 15-30-15 N-P-K every 2 weeks. Observations were taken before 85 seedlings were transplanted for the further testing.

To gather empirical data, a second vernalization assay was undertaken. Seeds of HRWW line 306 UI-SRG were sown in 2 cm^3 80-cell trays nested in seed flats Seedlings were sprouted in a growth chamber at a 23/16°C diurnal temperature cycle with a 16-hr. photoperiod. Seven days after the seed was sown, fully emerged seedlings were inoculated in the crown with 1 mL of a single inoculum. Five endophyte inocula were applied: *Morchella snyderi*, an isolate of *Phialocephala* BH1A recovered from foliage of *Psuedotsuga menziesii* var. *glauca*, *Phialocephala* BH1AR, *Penicillium* sp. *nova* WPT111A3, and an SDW control. Inocula were made from plate cultures as describe above. Eight trays were inoculated for each treatment. Following inoculation, four trays of each treatment were placed in a greenhouse with a 9-hr. photoperiod and a 25/19°C diurnal temperature cycle. The other four trays per treatment were returned to the growth chamber with a slightly reduced diurnal temperature cycle of 19/16°C. Four days after inoculation, growth chamber temperatures were reduced to a 5/5°C diurnal cycle and photoperiod was reduced to a 9-hr.

daylength. Seedlings at both benchtop and vernalization temperatures were fertilized every two weeks with 15-30-15 N-P-K until harvest. Seedlings in the warm greenhouse were harvested 4 weeks following inoculation to prevent stress and senescence from the small plug size. Seedlings in the growth chamber were vernalized for 12 weeks before harvesting.

All seedlings were harvested by removing them from the trays, thoroughly washing root systems to remove soil and debris, and severing above-ground biomass from root biomass at the crown. Root and above-ground tissues were dried in a drying oven at 70°C for 48 hrs. then weighed on an analytical balance for dry biomass.

Assay for Contributions of Forest Fungi to Winter Wheat Development during Vernalization Outdoor Over-wintering

To test the effects of forest fungi on wheat seedlings vernalized at natural over-wintering temperatures. Seeds of HRWW were sown in 6-in. pots containing silica sand. Seedlings were grown in sand to facilitate root harvesting. Eight days after sowing emerged seedlings were inoculatedp with one of three inocula: SDW, *Phialocphala* BH1AR, or *Penicillium* WPT111A3. Inocula were prepared as described previously. Twenty-eight seedlings were inoculated per treatment with 5 mL applied in the crown. Half the seedlings in each treatment were vernalized as previously described. The other half were acclimated to temperatures just above freezing before being placed outdoors in a straw bale enclosure to protect the vulnerable root systems in the above-ground pots. Plants were left exposed unless temperatures fell below -7°C, at which point they were covered. Plants in both treatments were fertilized weekly with 15-30-15 N-P-K. Plants were vernalized for 10 weeks

at their respective vernalization treatments. Plants were harvested for above- and below-ground biomass. Biomass was over dried at 70°C for 48 hrs. and weighed on an analytical balance.

Testing Efficacy of Forest Fungi against Drought and Fusarium culmorum *Foot Rot in Wheat*

To determine whether xerophilic and drought-adapted fungi could promote drought tolerance in winter wheat either by directly facilitating drought resistance or by suppressing crown rot pathogens, we designed a three-factor factorial experiment. Seeds of HRWW line 306 UI-SRG were sown as above in 2 cm^3 200-cell trays nested in seed flats. Seedlings were sprouted in a growth chamber at a 23/16°C diurnal temperature cycle with a 16-hr. photoperiod. Seven days after the seed was sown fully emerged seedlings were inoculated in the crown with 1 mL of a single inoculum. Inocula were prepared as previously described and consisted of five different fungal treatments: *Morchella snyderi, Penicillium goetzii, Penicillium* sp. *nova* WPT111A3, *Phialocephela* sp. BHIAR, and an SDW control. Following inoculation, seedlings were vernalized for 8 weeks as described above.

Following vernalization, seedlings were potted into 3-liter pots containing equal volumes of soilless potting mix. Plants were placed in a greenhouse at 21/15°C with a 16-hr. photoperiod. The plants were allowed to stabilize for five days, at which time half of the seedlings in each fungal treatment were inoculated with a spore suspension of *Fusarium culmorum* inoculum at a concentration of 2.9 x 10^6 cells per mL. Ten milliliters of inoculum was applied to the crown of each seedling; each seedling within the pathogen-free group received 10 mL of SDW. Plants were then given either sufficient or deficit irrigation until

harvest. Half the plants within each irrigation treatment were either inoculated with the root rot pathogen *Fusarium culmorum* or not. In order to accommodate the irrigation system design, the treatment x irrigation x pathogen factorial was arranged in a split block design with three blocks with one half of each block receiving deficit irrigation while the other half receive sufficient irrigation. Each treatment x irrigation x pathogen factorial contained 21 replicates for a total of 420 plants. Sufficient irrigation was determined at approximately 30% container capacity per irrigation event with deficit irrigation determined at 15 % container capacity. Container capacity was determined to be 1.4 L using the method described by Holstead (1983).

After 15 weeks all plants were harvested. First all seed heads were removed and counted. Seed heads were dried at 50 °C until they shattered and weighed on an analytical balance to determine yield. Tiller numbers were also counted. Vegetative biomass was severed from the roots at the crown. Above ground biomass was dried at 50 °C until dry and weighed for vegetative yield. Root balls were removed from the pots, split in half and dried at 80 °C until all moisture was removed. A relative below-ground biomass was determined by weighing the whole root ball.

Testing Forest Litter Suppression of Fusarium culmorum

Litter was collected from a pine forest understory in early spring (Idler's Rest Nature Reserve, Nature Conservancy, Moscow, ID, USA). Overstory vegetation was dominated by *Pinus ponderosa* with some *Pseudotsuga menziesii* var. *glauca*. Understory shrubs included *Holodiscus discolor* and *Symphoricarpus albus*. Subsamples of the litter were autoclaved at

120°C for 45 min. Other subsamples of non-sterile or untreated litter were collected in four 3.8 x 10^3 cm^3 storage bags and incubated at 22°C for 3 weeks.

After 3 weeks fungal growth was visible on untreated litter samples. Each bag was flushed with approximately 300 mL of SDW, thoroughly shaken to loosen spores and microbial cells, and briefly flushed a second time. This solution was filter through four layers of cheese cloth to remove larger soil particles and plant debris and brought to a total volume of 1.5L with SDW. An autoclaved control sample was made with four storage bags full of autoclaved litter. The autoclaved litter bags were flushed with SDW as above, and the solution was likewise filtered and brought to 1.5L with SDW before being autoclaved at 120°C for 20 min.

Seeds of HRWW line 306 UI-SRG were sown in 100 3-liter pots containing soilless potting mix. Twenty pots were mulched with autoclaved pine litter approximately 3 cm thick. Another 20 pots were mulched with untreated litter. Following seedling emergence, seedlings in the remaining 60 unmulched pots were inoculated in the crown with one of 3 inocula: untreated litter wash, autoclaved litter wash, or the SDW control. After 10 days, 10 plants from each of the 5 litter treatments were inoculated in the crown with 10 mL of *Fusarium culmorum* suspension as previously described. The other 10 plants of each litter treatment received 10 mL of SDW. Plants were grown for 9 weeks at a 25/19°C diurnal temperature cycle and 16 hr photoperiod. Plants were not vernalized and were harvested for vegetative biomass only.

Plants were harvested for above-ground biomass by severing vegetative growth

from the root at the crown. Above-ground tissues were dried in a drying oven at 70°C for 72 hrs. then weighed on an analytical balance for dry biomass.

Statistical Analyses

Mean biomass for vegetative development of vernalized and non-vernalized seedlings was analyzed by analysis of variance for a randomized complete block design for the main effects of treatment. Differences between means were determined by least-squares (LS) mean comparisons and Fisher's least significant difference (LSD) at the 5% level.

ANOVA and split-plot analyses were used to analyze factorial and main effects of treatments on yield, biomass, tiller, and head production of mature plants exposed to drought and *Fusarium culmorum* infection. Differences between means for the effects of treatment, irrigation, pathogen and factorial interactions were determined least-squares (LS) mean comparisons at the 5% level.

Above-ground vegetative biomass for plants in the litter experiment was analyzed for a complete randomized design. Mean biomass for the main effects of treatment were determined for both *Fusarium*-infected and *Fusarium*-free plants. Severity of *Fusarium* infection was determined by mortality and survival as binomial data (1=alive, 0= dead). Survival was analyzed by the Pearson chi-squared contingency test using R (Development Core Team 2013).

Normality of all data was determined by residual and normality plots and univariate procedures. Normal, ANOVA, and factorial analyses were conducted using either UNIVARIATE, GLM and MIXED procedures using the statistical package SAS (SAS Inst., Inc. 2007).

Results

Contributions of Forest Fungi to Winter Wheat Development During Vernalization and Outdoor Over-wintering

Seedlings undergoing vernalization had significantly greater root proliferation and

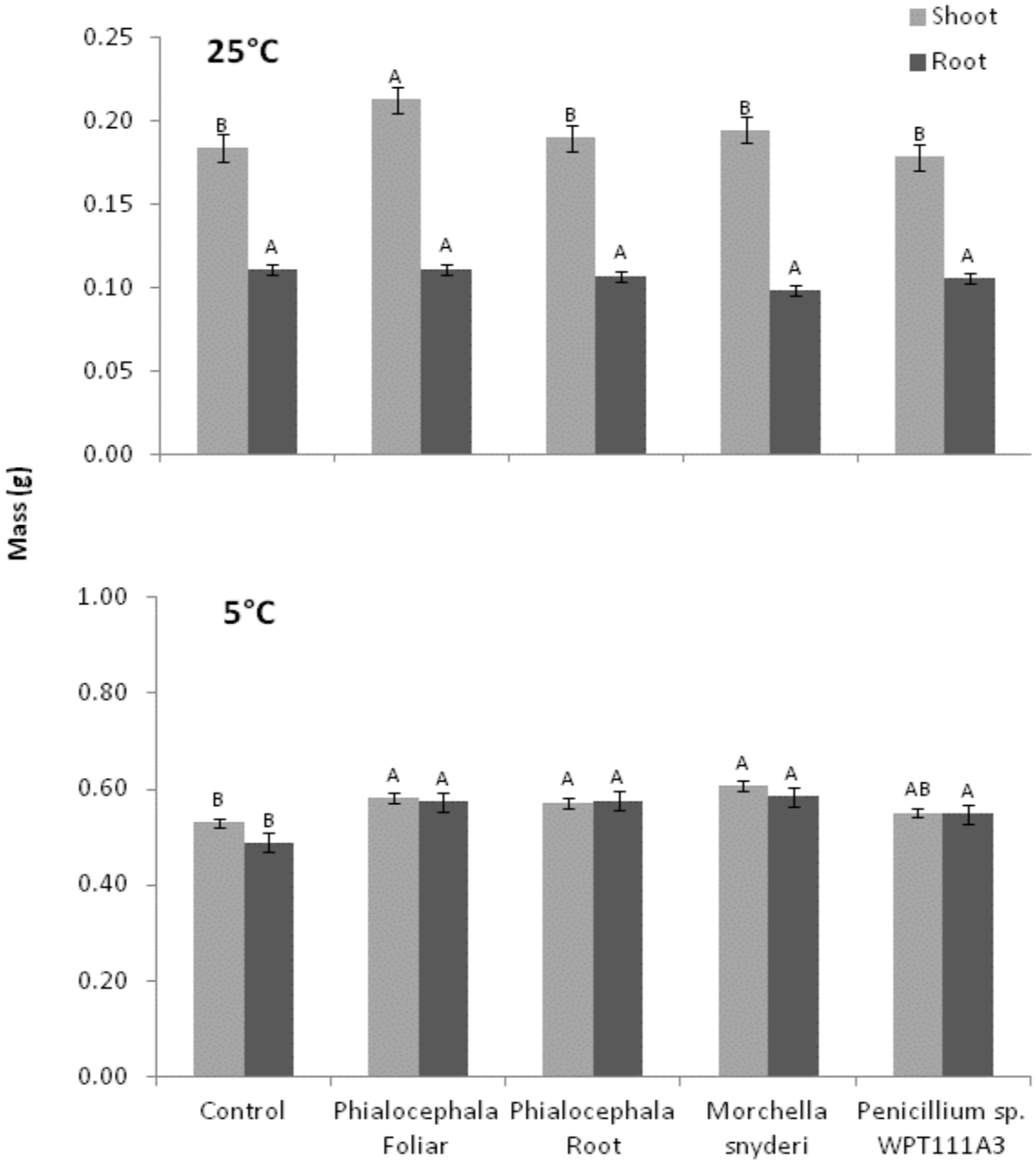
Figure 4.1. Root development during vernalization was significantly greater in winter wheat seedlings inoculated with of fungi from the forest microbiome. Left to right: Control, *Morchella snyderi*, *Phialocephala* sp., *Penicillium goetzii*, and *Penicillium* sp. WPT114. While differences in root development were visually significant at this point of development, restricted development from limited pot volumes and unnaturally severe infections of *Fusarium culmorum* from artificial inoculations possibly led to a loss of significance in root mass and equalization of treatments at the close of the experiment (see Figure 4.5).

development when previously inoculated with forest endophytes ($P<0.0001$, Figures 4.1 and 4.2). Three out of four of the endophytes tested in the second vernalization trial also significantly increase above-ground biomass ($P<0.0001$, Figure 4.2). Although *Morchella snyderi* was less visually beneficial to root development during the first vernalization trial (Figure 4.1), the fungus induced the greatest biomass production, increasing above-ground biomass by 14.7% and root biomass by 19.9% compared to untreated controls ($P<0.0001$) during the second trial (Figure 4.2). *Phialocephala* spp. from the foliar and root microbiomes of *Pseudotsuga menziesii* var. *glauca* promoted shoot development by 9.8% ($P=0.0005$) and 7.9% ($P=0.0036$), respectively, and root development by as much as 17.4% ($P=0.0002$) and 18% ($P<0.0001$), respectively, compared to untreated controls. *Penicillium* WPT111A3 stimulated a 12.3% increase in root biomass ($P=0.0061$) but failed to significantly increase above-ground biomass compared to the controls ($P=0.1315$).

By contrast, non-vernalized seedlings kept at warm temperatures were, for the most part, not significantly affected by the addition of endophytes. However, the foliar isolate of *Phialocephala* sp. significantly increased above-ground biomass by 15.7% (($P<0.0217$, Figure 4.2).

In the over-wintering trial, above- and below-ground biomass of plants grown in silica sand was similar regardless of fungal treatment ($P>0.05$). Plants vernalized at constant 5°C temperatures were larger at the close of the experiment and several of the plants vernalized outdoors died. Mortality was also not significant ($\chi=2.706$, $P=0.258$).

Figure 4.2. Seedling development responded differently to fungal inoculation at differing temperatures, being most responsive to forest fungi during the low temperature stress of vernalization. At a 25/19°C diurnal temperature cycle (top), there were no significant differences (α=0.05) in root biomass after four weeks growth. However, the foliar isolate of *Phialocephala* significantly increased above-ground biomass compared to the untreated controls. By contrast, all endophyte treatments significantly increases root biomass of seedlings grown for 12 weeks at 5°C (bottom) when compared to the control, and only *Penicillium* WPT111A3 failed to significantly increase above-ground biomass compared to the control. Error bars indicate standard error.

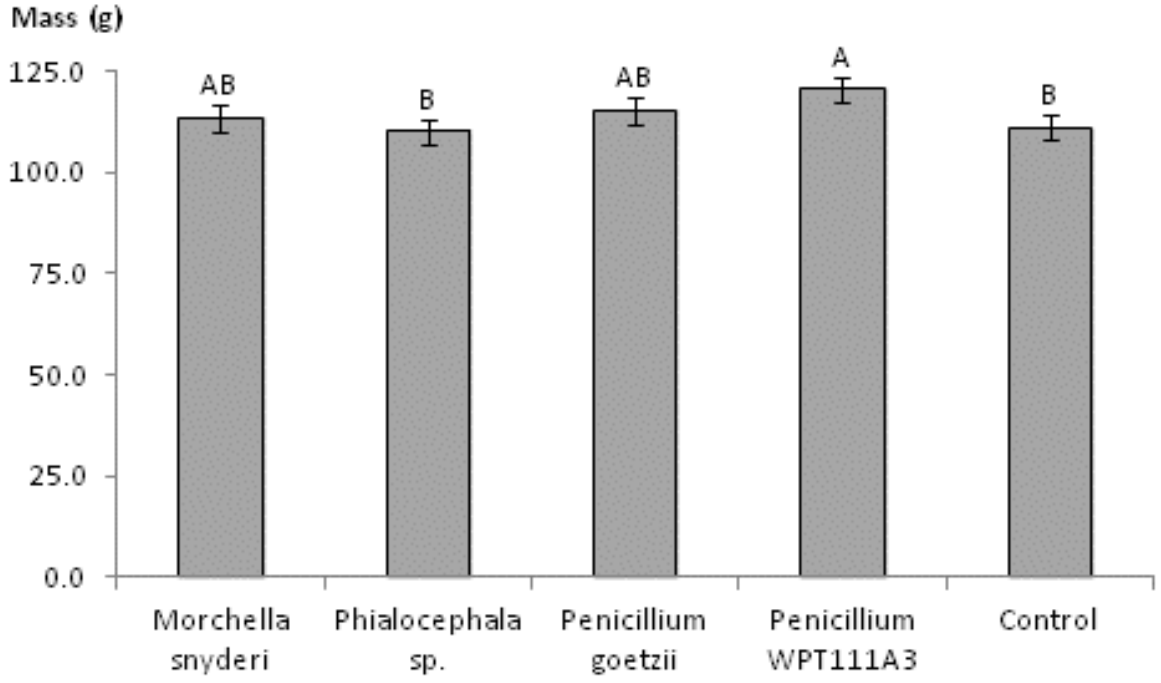


Forest Fungi Increase Winter Wheat Resistance to Fusarium culmorum *during Drought Stress*

Fusarium culmorum infection was visible in the crown of inoculated wheat plants 14 days following inoculation. Plants subjected to deficit irrigation developed the severest symptoms and developed symptoms sooner than those with sufficient water. Plants inoculated with *Phialocephala* and *Penicillium* spp. appeared to develop symptoms of pathogen infection more slowly than SDW controls and plants treated with *Morchella snyderi* (data not shown).

Total harvested biomass (Figure 4.3) and yield (Figure 4.4, top) significantly

Figure 4.3. Total above-ground biomass was significantly increased (α=0.05) by the inoculation of *Penicillium* WPT111A3 in *Fusarium*-free plants under sufficient irrigation compare to untreated plants. Error bars represent standard error.



increased (P=0.0397, P=0.0003, respectively) when plants were inoculated with drought-adapted endophyte *Penicillium* WPT111A3 in the absence of either *Fusarium culmorum* orwater deficiency compared to uninoculated plants. However, yield, head number, and

Figure 4.4. *Penicillium* sp. WPT111A3 significantly increased yield (α=0.05) of *Fusarium*-free HRWW under sufficient irrigation compared to endophyte-free controls (top) but more than doubled yield in drought-stressed plants infected with *Fusarium culmorum* compared to controls for a 256% increase (bottom). Error bars indicate standard error.

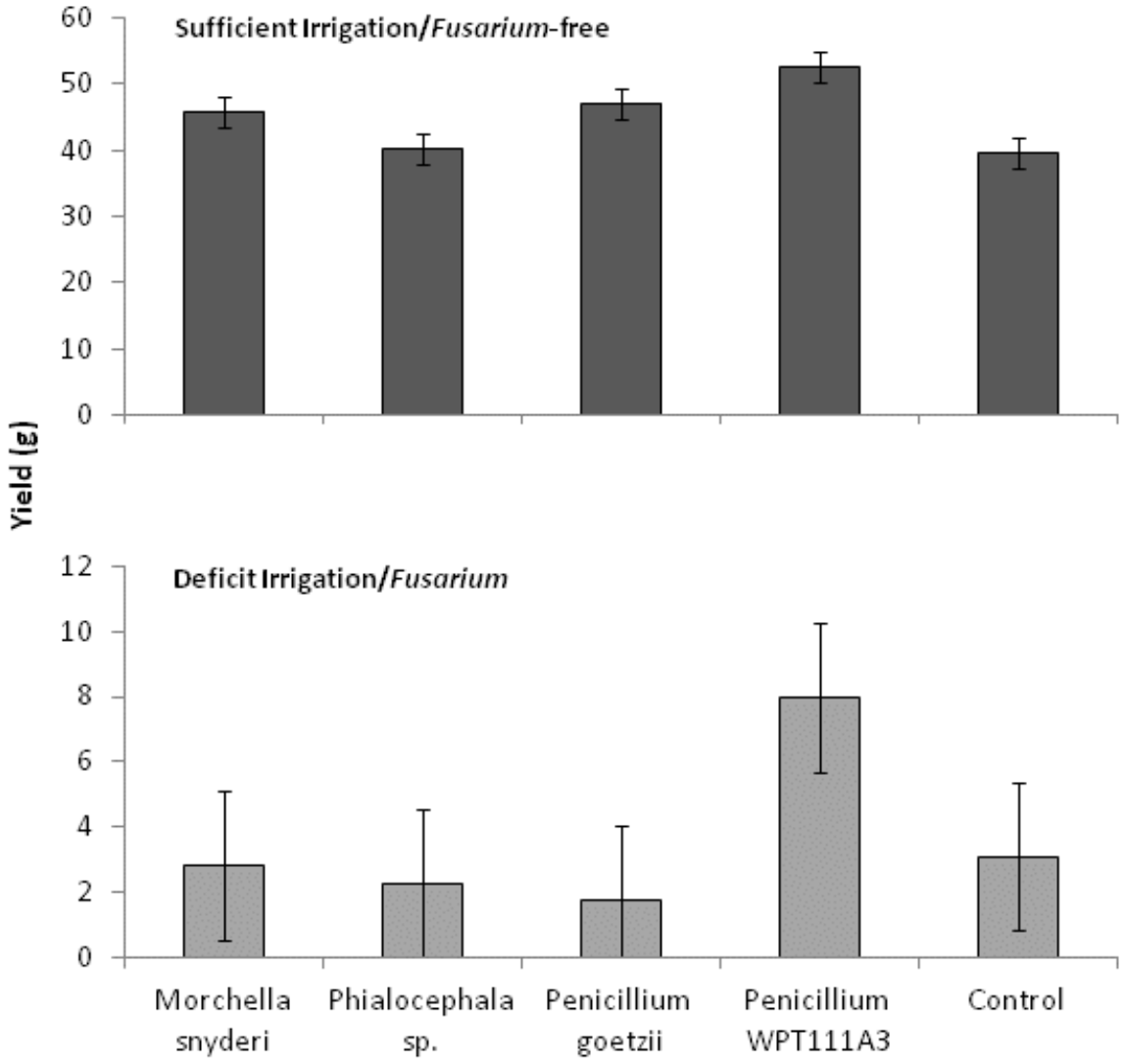
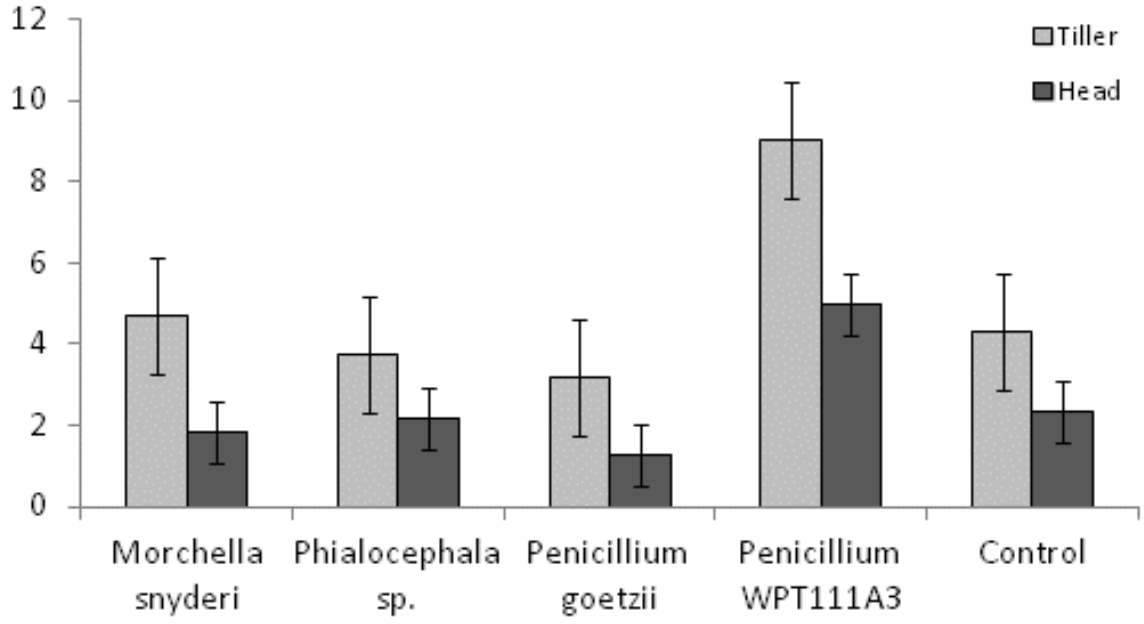


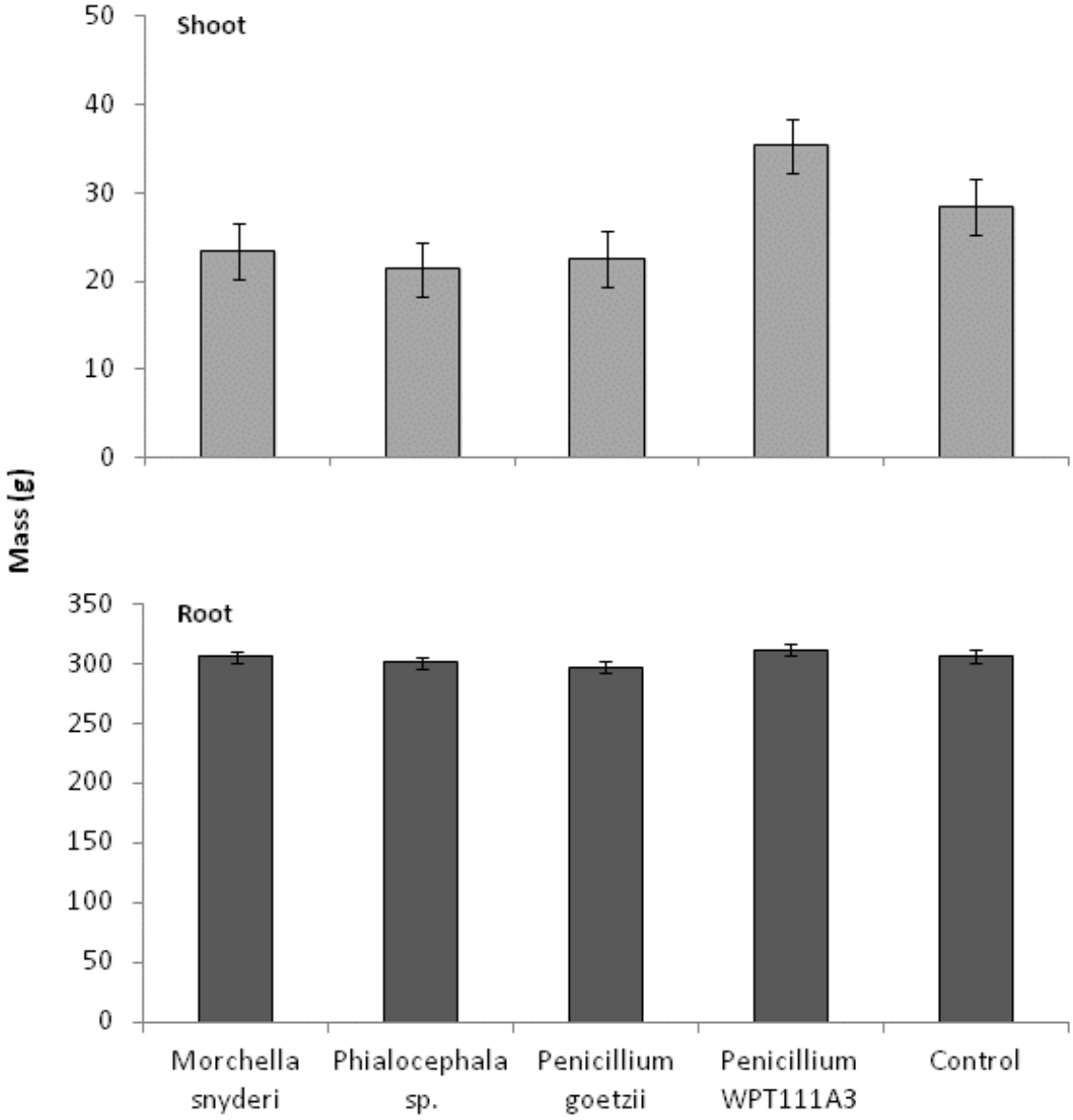
Figure 4.5. HRWW plants inoculated with *Penicillium* sp. WPT111A3 produced twice the number of tillers and double the number of heads compared to endophyte-free control plants (α=0.05) under deficit irrigation when infected with *Fusarium culmorum*. Error bars indicate standard error.



tiller number were doubled by the presence of this same endophyte when infected with *F. culmorum* under deficit irrigation ((P=0.0188, P=0.0256, P=0.0244, respectively, Figures 4.4 and 4.5). *Penicillium* WPT111A3 increased both above- and below ground biomass, but notsignificantly (P>0.05, Figure 4.6). Plants inoculated with the other three endophytes under the same pathogen and drought stress had no better growth and yield than untreated controls (P>0.05).

In the absence of *Fusarium culmorum*, both *Penicillium* WPT111A3 and *P. goetzii* increased yield significantly (P=0.0003, P=0.0285, respectively) in plants under sufficient irrigation. *Penicillium* WPT111A3 contributed to a 32.6% increase and *P. goetzii* to an 18.7% increase in yield compared to the untreated control (Figure 4.4). *Penicillium* WPT111A3 also increased above-ground vegetative biomass of *Fusarium*-free plants by 8.5% (P=0.0397)

Figure 4.6. Above-ground biomass (top) and relative root mass (bottom) were both increased under deficit irrigation when challenged with *Fusarium culmorum* if they were inoculated with *Penicillium* sp. WPT111A3. However, biomass was not significantly different from that of the control (α=0.05). Error bars indicate standard error.



compared to untreated controls under sufficient irrigation (Figure 4.3). Other fungi failed tosignificantly affect growth and yield of *Fusarium*-free plants under sufficient irrigation.

Fungal endophytes failed to benefit *Fusarium*-infected plants under sufficient

irrigation, although *Penicillium goetzii* did slightly reduce above-ground biomass and head number (P=0.0418, P=0.0256, respectively). Fungal endophytes also failed to significantly benefit *Fusarium*-free plants under deficit irrigation and actually reduced tiller number in all endophyte treatments (P>0.05) compared to the control.

As expected, significant differences between irrigation treatments and between *Fusarium*-infected and *Fusarium*-free treatments were found. The plants with sufficient irrigation infected with *Fusarium culmorum* produced 24.4% of the yield of *Fusarium*-free plants (P<0.0001), while diseases plants undergoing drought stress produced only 12.2% of the yield of drought-stressed plants that were disease-free (P<0.0001).Drought-stressed plants produced one-third of the yield of plants receiving sufficient irrigation when infected with *F. culmorum* (P<0.0001). By contrast, in the absence of *F. culmorum*, plants suffering drought-stress had 65.8% of the yield of plants receiving sufficient moisture (P<0.0001).

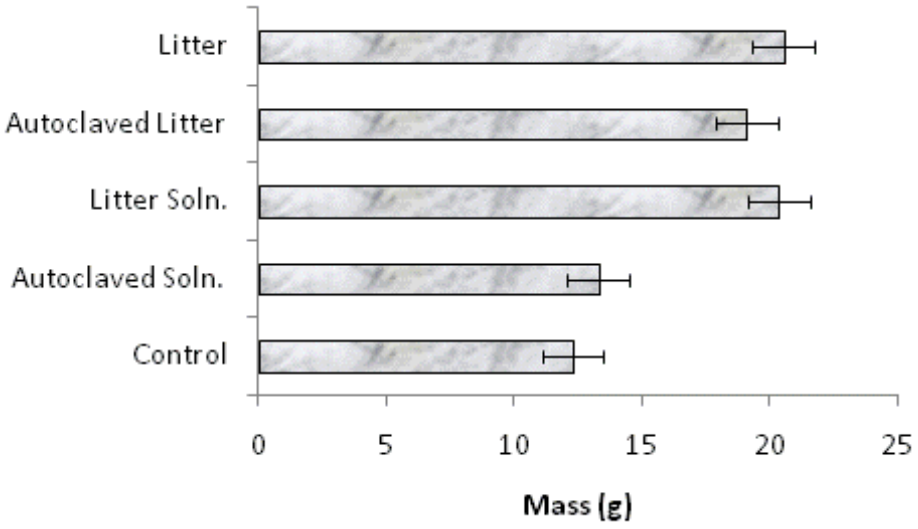
Vegetative yield, both above and below ground, reflected yield for both overall disease and irrigation treatments with significantly greater growth and yield for disease-free plants compared to diseased and sufficiently irrigated plants compared to drought-stressed (P<0.0001).

Effective Suppression of Fusarium culmorum *with Forest Litter Microbes*

Microbes in forest pine litter significantly reduced the severity of *Fusarium culmorum* in winter wheat, both by increasing survival (Figure 4.7) and above-ground vegetative biomass (Figure 4.8). Litter treatments slowed the onset of infection symptoms by seven days (data not shown). Untreated pine litter wash more than doubled survival

Figure 4.7. Winter wheat infected with *Fusarium culmorum* had a higher percentage of survival and greater vegetative yield when inoculated with a microbial solution derived from pine litter (right) than plants receiving no litter solution (controls, left).

Figure 4.8. Vegetative above-ground biomass of winter wheat plants infected with *Fusarium culmorum* was significantly greater when mulched with either non-sterile or autoclaved pine litter or when inoculated with microbial solution derived from washing non-sterile litter (α=0.05). Autoclaved treatments contained a *Bacillus* sp. that survived the autoclaving process at 120°C. The interaction of the *Bacillus* with the litter and pathogen may have lent to the significance of the autoclaved litter treatment.



compared to the control (100% vs. 40%, χ=5.95, P=0.015, Figure 4.7). Survival was not significantly improved by other litter treatments.

However, above-ground vegetative biomass was not only 65% greater (P<0.0001) for plants in the untreated litter wash compared to the control, but 67% greater (P<0.0001) for plants mulched with the untreated litter and 55% greater (P=0.0003) for plants mulched with autoclaved litter (Figure 4.8). Only the autoclaved litter wash failed to increase biomass above that of the control. *Fusarium*-free plants had 100% survival and lacked significant biomass differences among treatments (P=0.5222) with a mean (37.4 g) more than double the mean biomass (17.1 g) of *Fusarium*-infected plants.

Discussion

Microbiomes of forest soils and roots of forest plants are known to suppress pathogens and mediate resistance to abiotic stresses in host plants. By contrast, highly disturbed agricultural soils are often depauperate of microbial diversity and inhabited by soil-borne pathogens. By challenging plants treated with microbes from forest litter layers and conifer roots, we were able to confirm the hypotheses that fungal endophytes and litter microbes contribute to suppression of *F. culmorum* in hard red winter wheat (HRWW) while increasing stress tolerance and enhancing overwintering development of HRWW seedlings during vernalization.

In our study, the most effective control of *Fusarium culmorum* in HRWW was achieved with a non-selective suspension of microbes and leachate taken from untreated pine litter and associated debris. Litter mulch had similar ability but was less effective for plant survival. These treatments represented the whole microbiome of the litter layer and were more effective than single endophyte treatments.

Schisler and Linderman (1984) found collective microbiomes of forest soils more effective in suppressing *Fusarium oxysporum* than single microbes. Testing of autoclaved litter wash and autoclaved litter mulch indicated at least one bacterium, a *Bacillus* sp., survived passage through 120°C sterilization. Although this bacterium was applied in these treatments and may have slowed the onset of *Fusarium* infection symptoms it did not significantly increase either growth or survival of *Fusarium*-infected plants. The long-term effectiveness of untreated litter and litter wash indicate a community effect—that the most effective antagonism of *F. culmorum* may be achieved with an intact microbiome.

Although HRWW plants in the litter assay were not vernalized and grown to reproductive maturity, individual forest endophytes were shown to be capable of increasing yield over 30% in normal growing conditions and more than doubling yield of plants infected with *Fusarium culmorum* under drought conditions. However, infection with *F. culmorum* strongly reduced overall growth and yield.

The strength of the community effect of a pine litter microbiome against a notable soil-borne pathogen from disturbed agricultural soils once part of the pine woodland and perennial-dominated shrublands indicates the importance of the whole microbiome to

plant health and fitness. The microbiome that so effectively reduced the severity of the pathogen in the exotic crop has been replaced over time by continual annual cropping and disturbance pressures with a microbiome less resistant and less antagonistic to pathogens that are cultivated around susceptible crop species. Some measure of crop protection may perhaps be achieved by reintroducing residents of these natural microbiomes into adjacent the agricultural lands.

Individual members of forest microbiomes can provide some measure of resistance to soil-borne pathogens and abiotic stress. Moreover, by testing the efficacy of individual forest fungi to mediate both drought and disease tolerance in winter wheat we were able to identify a factorial interaction. The presence of *Penicillium* WPT111A3 significantly increased yield when both *Fusarium culmorum* and drought stress were present. Drought-stressed wheat plants are more susceptible to *F. culmorum* infection and the disease becomes more severe in times of drought (Cook 1973). At this critical interaction point *Penicillium* WPT111A3 appears to be most active. However, this *Penicillium* increased yield over 30% when both stresses were absent and only failed to increase yield when interacting with a single stress. Cook (2007) found selection of hard red wheat varieties with drought tolerance or avoidance was ineffective for resisting *F. culmorum*. This might support our results that antagonism of Penicillium against *F. culmorum* is dependent on its own response to drought stress rather than a direct interaction with the plant to reduce plant drought stress.

Increases in root biomass and fine root proliferation during vernalization indicate another potential mechanism of fungal-mediated disease resistance and drought-stress tolerance in a winter annual crop such as HRWW. *Fusarium culmorum* and related *Fusarium* crown rot pathogens thrive in during periods of drought when soils are dry and plants water stressed (Cook 1973; Papendick and Cook 1974). Improved root development during cold fall, winter, and early spring weather would provide a head start for a winter annual crop at the start of the growing season and possibly contribute to earlier maturity and thus drought avoidance and resistance to *F. culmorum*. A larger root system would also provide the plant with greater ability to extract water from drying soils as the plants move into dry summer thereby reducing drought stress and resultant susceptibility to the crown rot pathogen.

Without exception, endophytes from the forest microbiomes contributed to visually and statistically significant increases in root development of HRWW seedlings undergoing vernalization. *Penicillium* spp. have been recovered from Arctic soils (Frisvad *et al.*, 2006). *Phialocephala* and related DSE taxa have been frequently associated with cold high-elevation or arctic and boreal environments where it has been speculated that their function is to perhaps contribute to the ability of their hosts to survive and thrive while tolerating low temperature stress (Newsham *et al.* 2009). Since positive effects to above- and below-ground biomass were absent for the most part in non-vernalized seedlings, our results might confirm such a function.

Phialocephala and related DSE fungi have been frequently associated with increased nitrogen availability in plants and show greater affinity to symbiotic association with plants

where organic nitrogen is available (Newsham, 2011). Moreover, *Phialocephala* BH1AR colonizes the root hairs of its host (Figure 4.9), an ability that may indicate a symbiotic function at the root-soil interface. Likewise species of both DSE fungi and *Penicillium* are

Figure 4.9. *Phialocephala* sp. colonizing a root hair of winter wheat.

capable of solubilizing phosphate and releasing it for plant use (Kucey 1987; Mandyam and Jumpponen 2005). Since these fungi are also capable of functioning at low temperatures (Newsham *et al.* 2009; Rinu *et al.* 2012), they may facilitate uptake and assimilation of nutrients in plant hosts at temperatures normally below plant physiological function thereby facilitating continuous root development even at winter (vernalization) temperatures. The failure of endophytes to mediate growth and development in plants grown in the silica sand at low temperatures might indicated that the fungi mediated plant development via nutrient availability and assimilation not merely as a function of tolerance to cold stress.

With the exception of above-ground biomass in plants treated with *Penicillium* WPT111A3, differences in above and below-ground biomass were lost at maturity. Artificial limitations imposed on natural above- and below-ground development through restricted pot volumes possibly resulted in equalization of treatments over time. Under field conditions roots developing during the winter vernalization period would not be disturbed and would have fewer limitations on root development than those seen in the greenhouse.

In both greenhouse studies *Fusarium culmorum* was applied directly into the crown of developing seedlings at a dosage not seen in nature. This technique achieved exaggerated infection and facilitated rapid disease development for expediting the study. However, the severity of the infection from this artificial dosage challenged the plants to a degree that led to variable development, maturity, and water usage where water was applied at a constant rate. Natural levels of *F. culmorum* inoculum in field soils would result in less severe symptoms of infection.

Conclusions

Conifer forests and other plant community types adjacent to the productive wheat fields of the intermountain Pacific Northwest (PNW) host microbiomes that harbor microbes capable of contributing tolerance to disease and abiotic stresses in neighboring exotic crops. We have shown that forest microbes promote early development of over-wintering seedlings of hard red winter wheat and mediate tolerance to *Fusarium culmorum* infection and drought in controlled studies. Restoration of microbiomes to these soils could

promote resistance to pathogens and abiotic stress in crops grown in these disturbed soils. Reintroduction and maintenance of healthy microbiomes to these agricultural regions may require changes in agricultural practices and a better understanding of the ecological functions of natural microbiomes. Further study should be made in field settings, and a greater diversity of forest microbes and microbiomes might be assayed to better understand the role and potential of native microbiomes in agriculture fields that once hosted these microbiomes.

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Chapter 5

Further Research into the Conifer Forest Microbiome

Introduction

The preceding chapters of this dissertation discuss roles of the forest microbiome in ecosystem processes at the level of microbiome interactions, microbiome host interactions, and whole system processes. This research was selected for documentation in this dissertation in order to answer these specific objectives:

1. To identify non-pathogenic fungi or bacteria from a forest microbiome with key functional roles within the microbiome;
2. To determine the functional interactions of cryptic non-pathogens in their host(s);
3. And to determine the potential roles of beneficial fungi and bacteria in managed forestry and agricultural systems.

However, many more studies were conducted both to identify the functional roles of the microbiome and potential applications. Much of the early work was centered on developing rapid assays for selection by function. Other research projects developed around these assays and their results.

This chapter contains a selection of the more involved projects: ranging from observational study to negative results projects to on-going research with potential for further development and study.

University of Idaho Experimental Forest Endophyte Collection

Most of the research documented in this dissertation originated with the summer 2010 endophyte collection made from the University of Idaho Experimental Forest, Latah County, ID. Root and needle tissues were collected from ten trees each of *Pinus ponderosa, Pinus monticola,* and *Pseudotsuga menziesii* var. *glauca*. In all, over 3000 isolates and several hundred distinct morphotypes were isolated per species. Endophytes assayed in the research summarized in this chapter and recorded in the previous chapters were selected for study based on characteristics of the genera or species as described in the literature, on production of metabolites and volatiles suggestive of functional behavior, or on select physiological or morphological responses in culture. Various assays described here were also used to select fungi and bacteria for further research.

Observations and Assays into the Forest Microbiome

Endophyte Specialization in Roots or Needles

Of the many genera recovered from the needle and root microbiomes of *Pseudotsuga menziesii* var. *glauca*, *Pinus ponderosa* and *Pinus monticola,* only three genera were found in common to both needle and root communities (Table 5.1). However, among these three genera there were no species common between the microbiomes of the tissue

Table 5.1. Fungal taxa from the microbiome of intermountain conifers were found to be highly specific to the organs from which they were isolated. Genera were found to be more specific to organ than to host. Although genera marked with an asterisk were found in both tissue types, species within the genus were specific to tissue type. Only a single strain of *Phialocephala*** was recovered from both roots and needles. Presence/absence is indicated as +/-.

Taxon	*Pseudotsuga menziesii* var. *glauca*		*Pinus ponderosa*		*Pinus monticola*	
	Foliar	Root	Foliar	Root	Foliar	Root
Alternaria	-	+	-	+	-	-
Aspergillus	-	+	-	-	-	+
Aureobasidium	+	-	+	-	+	-
Cadophora	-	+	-	-	-	-
*Chaetomium**	-	-	+	+	-	-
Cladosporium	+	-	+	-	+	-
Elytroderma	-	-	+	-	+	-
Gliomastix	-	+	-	-	-	-
Gymnoascus	-	+	-	+	-	-
Hormonema	+	-	+	-	+	-
Lophodermium	-	-	+	-	+	-
Paecilomyces	-	-	-	+	-	-
*Penicillium**	+	+	+	+	-	+
*Phialocephala***	+	+	-	+	-	+
Rhabdocline	+	-	-	-	-	-
Sagenomella	-	+	-	+	-	+
Sordaria	-	+	-	-	-	-
Talaromyces	-	-	-	+	-	+
Trichoderma	-	+	-	+	-	+
Trichothecium	-	+	-	-	-	-
Ulocladium	-	-	-	+	-	-
Zygomycota	-	+	-	-	-	+

types sampled. More genera were shared among the three host species than between roots and needles. Species of the genus *Penicillium* represented the genus with the highest number of isolates from both needles and roots. This group is highly diverse with many positive characteristics for mutualisms, including drought-tolerance, thermotolerance, salt-

Table 5.2. Although Penicillium was one of the most common genera recovered from the microbiomes of the three conifers, no single species was found to be shared between root and needle microbiomes. Presence/absence is indicated as +/-.

Species	**Section**	*Pinus monticola*		*Pinus ponderosa*		*Pseudotsuga menziesii var. glauca*	
		Foliar	Root	Foliar	Root	Foliar	Root
Penicillium canescens complex; clade 1	*Canescentia*	-	-	-	+	-	+
Penicillium canescens complex; clade 2A	*Canescentia*	-	-	-	-	-	+
Penicillium canescens complex; clade 2B	*Canescentia*	-	-	-	+	-	-
Penicillium canescens complex; clade 3A	*Canescentia*	-	-	-	-	-	+
Penicillium canescens complex; clade 3B	*Canescentia*	-	+	-	-	-	-
Penicillium canescens complex; clade 4	*Canescentia*	-	-	-	+	-	-
Penicillium canescens complex; clade 5	*Canescentia*	-	-	-	-	-	+
Penicillium canescens complex; clade 7	*Canescentia*	-	-	-	-	-	+
Penicillium cf. *hordei*	*Fasiculata*	-	-	-	+	-	-
Penicillium expansum	*Penicillium*	-	-	-	-	-	+
Penicillium glabrum	*Aspergilloides*	-	-	+	-	+	-
Penicillium godlewskii	*Citrina*	-	-	-	-	-	+
Penicillium goetzii	*Chrysogena*	-	+	-	+	-	+
Penicillium griseolum	unknown (new section?)	-	+	-	+	-	+
Penicillium heteromorphus	*Exilicaulis*	-	-	-	+	-	-
Penicillium idahoense	*Cinnamopurpurea*	-	-	-	-	-	+
Penicillium raistrickii	*Ramosa*	-	-	+	-	+	-
*Penicillium soppii**	*Ramosa*	-	-	-	+	-	-
Penicillium swiecickii	*Ramosa*	-	-	-	-	-	+
Penicillium yarmokense	*Canescentia*	-	+	-	+	-	+
Penicillium sp. nov.	Unknown (new section?)	-	-	-	+	-	+
Penicillium sp. nov. *(Penicillium moldavicum)*	Not belonging to *Penicillium sensu stricto*	-	-	-	-	-	+

tolerance, anti-biotic production and anti-insecten production (Houbraken and Samson 2011). Sixty isolates were submitted for sequencing and identification to CBS-KNAW Fungal Biodiversity Centre *Penicillium* taxonomist, Jos Houbraken, and were found to be specific to tissues from which they were isolated. Although some species were common among host species they were not shared between needles and roots of the same host (Table 5.2). Although tissue specificity has been observed in microbiomes of other host species (Fisher *et al.* 1995; Kumar and Hyde 2004), the characteristic may be stronger in conifers. Evergreen leaves, such as conifer needles, are more likely to be heavily defended than deciduous leaves (Bryant *et al.* 1983), reducing the likelihood of broad, unselected colonization by non-specific microbes. This characteristic may indicate potential function within the host microbiome, allowing for inference into selection of microbes for further research.

Forest Endophytes for Mediating Resistance of Pseudotsuga menziesii *to Douglas-fir Tussock Moth*

With the help of Dr. S. Cook, an assay was conducted to examine the effects of endophyte inoculation of *Pseudotsuga menziesii* var. *glauca* seedlings on feeding and survival of Douglas-fir tussock moth (DFTM, *Orygia pseudotsugata*) caterpillars. One hundred and eighty first-year seedlings of *P. menziesii* var. *glauca* were inoculated with a total of five endophytes using two inoculation treatments per endophyte. Inoculation treatments included root inoculations made by soaking root masses in inoculum suspensions and foliar inoculations made by spraying endophyte suspensions. Endophyte treatments included the following: *Phialocephala* sp. BHIA isolated from both root and needle tissue of *P. menziesii* var. *glauca*, selected for its potential systemic effects and

possible anti-insectan production; a *Rhabdocline* sp., selected to represent the genus which contains species associated with insect inhibition; a bacterial endophyte to represent that group of organisms; a foliar endophyte with a “piney” scent with potential for producing anti-insect metabolites; and a potentially entomopathogenic *Verticillium* sp. isolated from *Populus angustifolia*. A sterile water control treatment was also applied following protocols identical to the endophyte inoculations. After 3 weeks, the seedlings were infested with tussock moth caterpillars in the second instar at 3 caterpillars per tree. Seedlings were monitored over a 3-week period before being disinfested. Individual caterpillar weights were taken for caterpillars retrieved from each seedling. Caterpillar weights lacked significance among treatments compared to the control, as did needle feeding damage. There were also no significance differences between inoculation methods.

A field-to-laboratory assay was also conducted to examine the effects of treating emerging needles with select endophytes for mediating resistance to feeding from DFTM. Expanding buds of *Pseudotsuga menziesii* var. *glauca* were inoculated with one of three endophyte treatments: *Phialocephala* sp. BHIA, a *Penicillium* sp. of the *P. canescens* complex or sterile distilled water for a control. Ten expanding buds were inoculated for each treatment. After 3 weeks, cuttings containing the new growth from the inoculated buds were removed and placed in 0.95 L jars with a single caterpillar infesting each cutting. Observations were taken on caterpillar development (cuticle shedding) and data on feeding damage, caterpillar weight gain and frass weight was collected and analyzed. Although feeding damage on endophyte-treated tissue was slightly less than that on the sterile water control, there were no significant effects of inoculation with endophytes.

Control of Fusarium *spp. in Conifer Seedlings*

Fusarium root rot is a common problem in forestry nurseries. *In agaro* competition studies were initiated to identify endophytes from *Pseudotsuga menziesii* var. *glauca* and *Pinus ponderosa* that might be antagonistic to *Fusarium* spp. recovered from infected root tissues of seedlings of both hosts. Endophytes were tested for their ability to inhibit growth of *Fusarium* spp. *in agaro*. Plates were inoculated with both *Fusarium* and endophytes of interest with competing colonies 4 cm and 2 cm apart. Sixteen *P. menziesii* var. *glauca* endophytes and 7 *P. ponderosa* isolates inhibited or over-grew *Fusarium* spp. *Pseudotsuga menziesii* var. *glauca* isolates showed the strongest antagonism. Genera with species that demonstrated antagonism to *Fusarium* spp. *in agaro* included *Trichoderma, Penicillium, Hormonema*, *Gliomastix*, and *Clonostachys*.

To determine the effectiveness of putative *Fusarium* antagonists *in planta*, two separate greenhouse experiments were conducted with seedlings of both *Pseudotsuga*

Table 5.3. Most fungal species tested for *in planta* antagonism of *Fusarium* spp. in conifer seedlings were selected from *in agaro* assays. *Morchella snyderi* was selected as a putative antagonist of *Fusarium* in sweet corn. Sterile distilled water was applied as a control.

Host	Source	Endophyte Inoculum
Pseudotsuga menziesii var. *glauca*	*P. menziesii* var. *glauca*	*Clonostachys rosea*
		Gliomastix sp.
		Trichoderma polysporum
	Morchella ascocarp	*Morchella snyderi*
Pinus ponderosa	*Pinus ponderosa*	*Hormonema dematioides*
		Penicillium goetzii
		Pseudomonas sp.
	Morchella ascocarp	*Morchella snyderi*

menziesii var. *glauca* and *Pinus ponderosa* to determine whether putative antagonist could reduce *Fusarium* severity. Seeds of *P. ponderosa* and *P. menziesii* were inoculated with 4 single endophyte treatments per host prior to sowing (Table 5.3). Following germination, *Fusarium* spp. recovered from the respective hosts was applied as a drench to half the seedlings. Seedlings were grown until mortality was observed in *Fusarium*-treated seedlings. Surviving seedlings were harvested for shoot and root biomass. *Fusarium* presence visibly reduced seedling size, compared to *Fusarium*-free controls. *Fusarium* infection significantly reduced biomass of infected seedlings compared to *Fusarium*-free seedlings. However, effects of endophyte treatments lacked significance ($P>0.05$).

UIEF Field Experiment

In May 2012, a field planting was established for long-term monitoring of the effectiveness of pre-planting inoculations of endophytes on growth improvement, out-planting success, and disease and insect resistance in *Pseudotsuga menziesii* var. *glauca, Pinus ponderosa* and *Pinus monticola*. First-year seedlings inoculated in the fall of 2011 were planted in the University of Idaho Experimental Forest (UIEF). The planting was established on the site of a harvest unit harvested in the summer of 2011. Slash burning was completed in the fall of 2011. No other site preparation was made. Six treatments were tested for each species: 4 single-endophyte treatments per species, 1 combination treatment of all 4 endophytes tested for that species, and a sterile water control (Table 5.4).

Data was collected in early fall. Data taken on *Pinus ponderosa* included survival, height, and caliper. Due to highly variable growth of the *Pinus monticola* and *Pseudotsuga menziesii* var. *glauca*, only survival data was taken. Dry summer weather resulted in high

Table 5.4. Four different endophytes per host were applied as single endophyte treatments into their hosts prior to out planting in the field. All four endophytes were combined to create a mixed inoculum that was applied to the respective host as a fifth treatment. Sterile distilled water was applied as a control.

Host	Source	Endophyte Inoculum
Pinus monticola	*Pinus monticola*	*Cladosporium* sp.
		Geomyces sp.
		Trichoderma atroviride
	Sarcosphaera ascocarp	*Sarcosphaera*
Pinus ponderosa	*Pinus ponderosa*	*Cladosporium* sp.
		Hormonema dematioides
		Trichoderma polysporum
	Morchella ascocarp	*Morchella snyderi*
Pseudotsuga menziesii var. *glauca*	*P. menziesii* var. *glauca*	*Penicillium canescens* species complex
		Phialocephala sp. BH1AR
		Trichoderma sp. 3S4
		Trichoderma polysporum

mortality.

Trends favored improved growth and survival of endophyte-inoculated seedlings, but they were not significant. Nearly 70% of *Pseudotsuga menziesii* var. *glauca* seedlings failed to survive the summer. The few survivors were planted in heavily burned microsites where competitive vegetation had been removed. Survival was not significant to treatment ($P=0.422$). Greater than 70% of both pine species survived the dry summer. However, survival for both species was not significant to treatment ($P>0.05$). Height and caliper of *Pinus ponderosa* also lacked significance ($P>0.05$).

Over-wintering survival was poor for all three conifers. Most of the *Pseudotsuga menziesii* var. *glauca* failed to survive. Browsing damage from elk reduced survival in *Pinus ponderosa*. *Pinus monticola* had the highest survival, but survival was not significant to

treatment (P=0.175). Surviving *P. monticola* is being monitored annually.

On-going research

Drought-tolerant Endophyte Project

Observations of the abundance of root endophytes belonging to genera containing putative xerophilic and thermophilic fungi led to the formulation of hypotheses that these fungi might dominate the root microbiome in xeric environments and potentially mediate drought tolerance in their hosts. To test the first hypothesis, endophyte collections from the root microbiomes of woody plants were made along elevation and precipitation gradients during the summers of 2012 and 2013. Ten different gymnosperms and 10 different angiosperms ranging from trees to shrubs were sampled (Table 5.5). Drought–tolerant

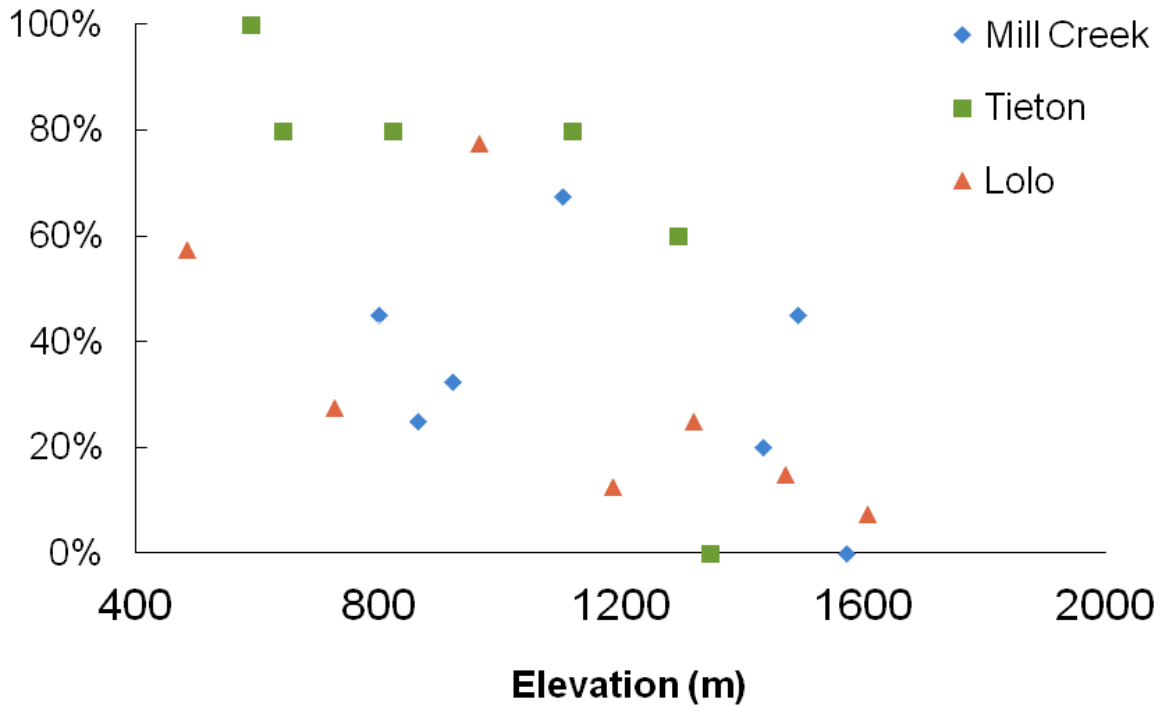
Table 5.5. The root microbiomes of 10 different gymnosperms and 10 different angiosperms were sampled by selective isolation for xerophilic fungi.

Gymnosperms	**Angiosperms**	
	Tree	Shrub
Abies lasiocarpa	*Alnus viridis ssp. sinuata*	*Artimesia tridentata*
Juniperus communis	*Malus domestica*	*Holodiscus discolor*
Juniperus occidentalis	*Prunus domestica*	*Lonicera ciliosa*
Juniperus scopulorum	*Sorbus scopulina*	*Purshia tridentata*
Picea abies		*Rosa acicularis*
Pinus ponderosa		*Symphorocarpus alba*
Pseudotsuga menziesii var. *glauca*		
Thuja plicata		
Tsuga heterophila		
Tsuga mertensiana		

endophytes were selectively isolated on a low water-potential potato dextrose agar (PDA) medium amended with glycerol at 25% v/v (see appendix). Protocols for tissue processing and isolations followed those described in Chapter 1.

While data have not yet been fully analyzed, trends indicated that higher numbers of drought-tolerant fungi were isolated from xeric sites, with as many as 100% of root sections showing xerophile colonization at the driest sites and 0% at the most mesic sites (Figure

Figure 5.1. In the summer of 2012, the root microbiome of Pseudotsuga menziesii var. glauca was sampled across three elevation gradients in three drainages: the northern Blue Mountains (Mill Creek), the central Cascade Mountains (Tieton River), and the northern Rocky Mountains (Lolo Pass). The scatter plot indicates decreasing frequency of xerophilic fungi with increasing elevation. However, microsite characteristics, such as slope or aspect, led to variability in the data.



5.1). The frequency and diversity of xerophiles recovered were higher in conifers than in angiosperms sampled. Most of the xerophilic fungi recovered belonged to the genus *Penicillium*, or to related genera including *Talaromyces* and *Aspergillus*. Other genera selectively isolated as xerophiles included *Cladosporium* spp., *Aureobasidium* spp., *Fusarium* spp., and two different Zygomycetes as well as several Ascomycetous fungi yet to be identified.

Root endophytes belonging to the artificial group known as dark septate endophytes (DSE) were also recovered. Although this group was less tolerant of the xerophile selection medium, they are frequently associated with xeric environments and temperature extremes (Mandyam and Jumpponen 2005; Newsham 2011; Newsham *et al.* 2009). Dark septate endophytes appeared to be more dominant in the root microbiome of conifers than those of angiosperms and were also more dominant at higher elevations, where they replaced selectively-isolated xerophiles. They were also among the more dominant fungi recovered from *Artemisia tridentata* in xeric shrubsteppe. This research and its analysis are ongoing.

Given the number of putative xerophiles recovered from the root microbiomes of conifers in the dry interior Pacific Northwest (PNW) forests, we hypothesized that these fungi might mediate drought tolerance in their hosts. *Morchella snyderi* was recovered as an ascocarp from the litter microbiome of one of these same forests. *Morchella* sp. was found to increase growth, fecundity, and heat tolerance in grass species (Baynes *et al.* 2012). Using the *Morchella* system as a model, we assayed the effects of endophyte symbiosis on the tolerance both a grass and an herb to water stress. *Morchella snyderi*, *Penicillium goetzii* and the DSE fungus *Phialocephala* BHIAR were inoculated into seeds of soft white winter

wheat (*Triticum aestivum*) and tomato (*Solanum lycopersicum*). Sterile distilled water was also applied for a control. After a period of growth, plants were subjected to periods of drought and revived. Before and after each drying event, the whole-plant weight for each plant was taken. Following several drought events, oven-dried biomass from each plant was analyzed. Although data for analyses of endophyte-treated wheat were not significant, *Morchella snyderi* increased root biomass of tomato by 9% ($P<0.0001$) compared to the control, indicating a possible mechanism for mediating tolerance of host plants to xeric environments or periodic drought. Results of this study were used to design the winter wheat study recorded in Chapter 4.

Within-microbiome Interactions and Differential Host Response

To determine the specificity of induced disease resistance, we conducted an assay using the facultative endophyte *Morchella snyderi* against the heteroecious rust *Melampsora* sp. in its alternate hosts, *Populus trichocarpa* and *Pseudotsuga menziesii* var. *glauca.*

Seeds of *Pseudotsuga menziesii* var. *glauca* inoculated with *Morchella snyderi* were germinated in the greenhouse. Cuttings of *Populus trichocarpa* were inoculated while rooting in sterile distilled water SDW prior to planting in the greenhouse. Controls of both hosts were treated with SDW. Seedlings and cuttings were allowed to grow in the greenhouse for several weeks before inoculation with *Melampsora.* Infection was scored as uredinial density on *P. trichocapa* and as numbers of aecia per seedling on *P. menziesii* var. *glauca.*

Morchella snyderi significantly reduced (P<0.005) uredinial density on *Populus trichocarpa* (as did 4 bacterial endophytes), but increased aecial counts in *Pseudotsuga menziesii* var. *glauca* (P=0.002), indicating a strong interaction between the host and its symbionts—both pathogen and non-pathogen. This research is collaborative and ongoing.

The Conifer Forest Microbiome and its Promise in Sweetcorn Production

Idaho ranks high in the USA as one of the top states for production of sweet corn (*Zea mays* L. var. *saccharata*) seed. *Fusarium* spp. are dominant seed-borne pathogens in sweet corn that are costly to control. In the wheat research discussed in Chapter 4, the microbiome of the pine woodland effectively reduced severity of *Fusarium culmorum* in winter wheat. We hypothesized that forest fungi might antagonize *Fusarium* spp. infecting roots, stalks, and seed of sweet corn, reducing severity of the pathogens and increasing yield. A field trial of two projects to determine the efficacy of forest fungi for control of *Fusarium* spp. in sweet corn was initiated in the summer of 2012. When applied to seed prior to sowing, *Morchella snyderi* from forest litter reduced *Fusarium* severity in the roots, and crown and increased yield by almost 40% (P<0.0001). *Morchella snyderi* applied to silk at pollination failed to effectively antagonize*Fusarium* in the developing ears, but the mycoparasitic *Clonostachys rosea*, found in both forest litter and conifer root microbiomes increased ear weight by 11% when applied to emergent silks(P=0.032).

A severe storm in the summer of 2013 compromised field trials. However, new trials have been initiated for 2014. This research is on-going, and observations from greenhouse trials indicate that forest microbiomes may have contributed to the success of agriculture in the intermountain PNW and may provide new options for control of emerging and

expanding pathogens in crops.

Conclusions

The microbiome of a forest is a dynamic entity that we are only beginning to understand. Microbes interact within that community and symbiotically with plant hosts to control interactions and responses to biotic and abiotic stimuli. These interactions shape the microbiome, may shape the forest itself, and may have historically shaped agriculture in fields converted from woodlands. Understanding the functional significance of the microbiome, both at the level of the individual microbe and the level of the community, may provide insight into new approaches to forestry, restoration, and agriculture.

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Appendix

The following media recipes are adapted from the Handbook of Microbiological Media. Yeast-glucose agar was adapted for non-selective isolations of both fungi and bacteria from root tissues. Glycerol PDA was modified to select desiccation-tolerance fungi.

Modified Yeast-Glucose Agar (YGA)

Potato dextrose agar(PDA)	39g
Yeast extract	10g
Glucose	20g
Distilled water	1L

Slowly dissolve glucose in 500 mL water over heat, stirring constantly. Add PDA and yeast extract. Bring to volume. Autoclave at 120°C for 20 minutes. pH to 5.6.

25% Glycerol PDA for Xerophile Selection (GPDA)

Potato dextrose agar(PDA)	39g
Yeast extract	3g
KH_2PO_4	20g
Glycerol	250mL
Distilled water	750mL

Dissolve PDA, yeast extract, and KH_2PO_4 in distilled water. Add glycerol and shake gently. Autoclave at 120°C for 20 minutes. pH=5.47.

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