MOLECULAR DIVERSITY OF FOLIAR Fungal Endophytes in Relation to Defense Strategies and Disease in Whitebark Pine

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MOLECULAR DIVERSITY OF FOLIAR FUNGAL ENDOPHYTES IN RELATION TO DEFENSE STRATEGIES AND DISEASE IN WHITEBARK PINE

By

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Thesis

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Molecular Diversity of Foliar Fungal Endophytes in Relation to Defense Strategies and Disease in Whitebark Pine

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Abstract

An invasive fungal pathogen, *Cronartium ribicola* (the causative agent of white pine blister rust) infects and kills whitebark pine (*Pinus albicaulis*) throughout the western US. Blister rust has decreased whitebark pine populations by over 90% in some areas. Whitebark pine, a keystone species, has been proposed for listing under the Endangered Species Act in the U.S., and the loss of this conifer is predicted to have severe impacts on forest composition and function in high elevations. Hundreds of asymptomatic fungal species live inside whitebark pine tissue, and recent studies suggest that these fungi can influence the frequency and severity of pathogens such as *C. ribicola*. I used molecular methods to characterize the fungal community in whitebark pine seedlings from 21 half-sibling seed families, sourced throughout the Pacific Northwest, and grown in a common garden. I characterized endophyte communities before and after experimental inoculation with blister rust and compared community composition in susceptible vs. resistant seedlings. I also explored the defensive chemistry of these same seedlings in relation to the fungal community and overall disease severity. Seed family was the biggest driver of endophyte community composition in our common garden, but I also observed shifts in fungal communities in response to blister rust infection. Seed family identity also influenced defensive chemistry, with terpene concentration differing in resistant and susceptible seedlings. In addition, both defensive chemistry and endophyte community were correlated with characteristics of disease severity. Endophyte communities and defensive chemistry in whitebark pine that can predict disease severity may act as biomarkers of disease resistance for future breeding programs. These results suggest that the resistance to white pine blister rust observed in natural whitebark pine populations may be a combination of genetics, endophytes, and terpene composition in needle tissue, where initial interactions between the pathogen endophytes, the host take place.
Preface

This research investigates the relationships between foliar fungal endophytes, pathogens and tree defensive chemistry in 20 whitebark pine seedling families that were experimentally infected with white pine blister rust.

In Chapter one entitled "Endophytes, pathogens and host physiological response: An introduction and literature review," I introduce the fungal pathogen Cronartium ribicola, the causal agent of the disease white pine blister rust, and explain its effect on whitebark pine, an important species in high elevation ecosystems. I also describe how, within the needle tissue of whitebark pine, foliar endophytes, pathogens and tree defensive chemistry interact and that these interactions can determine disease severity and microbial community composition in infected trees.

In Chapter 2 entitled, "Molecular diversity of foliar fungal endophytes in relation to defense strategies and disease in whitebark pine," I characterize endophyte communities in whitebark pine seedlings that were experimentally infected with white pine blister rust in a common garden. I explore how blister rust infection influences these endophyte communities. I also analyze tree defensive chemistry within the same seedlings in relation to disease resistance and endophyte community composition in needle tissue, where initial interactions between pathogens, endophytes and hosts take place. Endophyte community composition shifted in response to blister rust infection, and was similar in both resistant and susceptible seedlings. The strongest driver of endophyte community composition in infected seedlings in the common garden was seed family. Defensive chemistry was also strongly correlated to seed family, with higher levels of some compounds expressed in resistant trees. Individual fungal endophytes and terpenes had both negative and positive correlations with disease severity. These results highlight the complex relationship between host, foliar microbiome, and tree defensive chemistry, with linkages to host genetics and disease resistance.
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CHAPTER 1. ENDOPHYTES, PATHOGENS AND HOST
PHYSIOLOGICAL RESPONSE: AN INTRODUCTION AND
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Introduction

Invasive pathogens affect plant communities in North America on a continental scale (Loo 2009). Pathogens that cause disease in North American tree species can be particularly detrimental to forest ecosystems. Since the early 1900s, invasive pathogens of trees have seen a dramatic rise in both species richness and abundance, and in less than a century, entire tree species have been nearly eliminated from the landscape (Loo 2009). The chestnut blight, caused by an invasive fungal pathogen, *Cryphonectria parasitica*, has decimated the American chestnut, which was once a dominant tree in the Eastern United States (Schlarbaum *et al*., 1998). The butternut canker fungus (*Sirococcus clavigignenti-juglandacearum*) infects walnut trees and causes branch and stem cankers, and has caused extensive tree mortality since the 1980s (Parks *et al*., 2013). Other invasive fungal pathogens including those causing Dutch elm disease and American beech bark disease have also altered the composition of native North American forests (Schlarbaum *et al*., 1998; Loo 2009). Understanding plant defenses to pathogens has been a central focus in plant pathology and ecology for decades (Ingham 1972; Schlarbaum *et al*., 1998). In addition to plant defenses many abiotic and biotic factors have been proposed as primary or contributing drivers of disease spread and severity (Burdon & Chilvers 1982; Delledonne *et al*., 1998; Brown 2002; Keeling & Bohlmann 2006).

*White pine blister rust*

*Cronartium ribicola* J.C. Fisch is an invasive fungal pathogen that causes the disease, white pine blister rust, in five-needle pine trees. It was introduced separately into eastern and western North America around 1900 on white pines imported from European nurseries (Kinloch *et al*., 1998). White pine blister rust disease manifests as bark cankers on stems and trunks, as well as necrotic spotting on needles, branch dieback, and eventually death of the tree (Patton & Johnson 1970).

Like all rust pathogens (Basidiomycota; Pucciniales), *C. ribicola* rust is an obligate biotrophic fungus requiring multiple living hosts to complete its life cycle. It is a heteroecious pathogen, meaning that it alternates between two hosts: *Ribes* species and to a lesser extent *Pedicularis* and *Castilleja* as its primary hosts, and five-needle pines as its secondary hosts (McDonald *et al*., 2006). On *Ribes*, *C. ribicola* forms urediniospores during summer that can
disperse through the air and infect other nearby *Ribes* (Liu *et al*., 2015). In late summer and early fall, tubular structures called telia form on the underside of *Ribes* leaves (Figure 1a). Basidiospores are subsequently produced and dispersed through the air to infect pine hosts. Germinated basidiospores colonize five-needle pines through small openings in the needles called stomata (Liu *et al*., 2015). Initial colonization of white pines produces distinct yellow spots on pine needles that are easily observed (Figure 1c). Mycelium then grows through needles into branches of susceptible trees, into the main stem (Patton & Johnson 1970). After colonizing the main stem, the fungus forms cankers that eventually girdle and kill the tree (Figure 1b, Campbell & Antos 2000).

*C. ribicola* infect all nine five-needle pine species native to North America at varying levels of severity (Sniezko *et al*., 2008, Tomback & Achuff 2010), but is especially virulent to whitebark pine (*Pinus albicaulis*). White pine blister rust has spread across nearly the entire range of whitebark pine in the United States (Schwandt *et al*., 2010), and in some areas, whitebark pine has decreased to less than 10% of its natural population due to infection (Kendall & Arno 1990; Keane & Arno 1993). In addition, studies suggest that whitebark pine trees infected with *C. ribicola* may be more attractive to mountain pine beetle (Jules *et al*., 2016; Six & Adams 2007), increasing the rate of mortality.

**Whitebark pine**

Whitebark pine provides habitat, nutrients and structure for high elevation ecosystems from British Columbia to the Sierra Nevada and Western Wyoming (Tombback & Achuff 2010). It can survive harsh condition at high elevations, and once established, whitebark pine trees can provide stability and cover, supporting the establishment of later successional species (Resler & Tombback 2008). Whitebark pine reduces erosion caused by high winds and spring runoff and increases snowpack retention (Keane & Arno 1996). It also supports soil and litter accumulation, facilitating in the growth and establishment of other conifers (Callaway 1998; Resler & Tombback 2008). Whitebark pine also provides seeds high in protein and other nutrients for grizzly bears, Clark's nutcrackers and other animals (Tombback 1982, Hoff *et al*., 2001; Tombback & Kendall 2001). As Clark's nutcracker relies on whitebark for food, whitebark also relies largely on Clark's nutcrackers for seed dispersal (Tombback 1982).
Whitebark pine is undergoing rapid decline due to mountain pine beetle and white pine blister rust (Keane & Arno 1996). Whitebark pine is a federally listed endangered species in Canada under the Species at Risk Act (SARA) (Government of Canada 2012), and was proposed for listing under the Endangered Species Act (ESA) in the United States (US Fish and Wildlife Service 2011). Whitebark pine has also been classified as Endangered on the latest IUCN Red List of Threatened Species (Mahalovich & Stritch 2013). The loss or extensive range reduction of this species is predicted to have severe impacts on forest composition and distribution in high elevation ecosystems (Hoff et al., 2001). However, natural resistance has been documented in some populations (Sniezko et al., 2011).

Plant defensive chemistry

To defend against pathogens and herbivores many conifers produce high quantities of organic compounds known as terpenes (Trapp & Croteau 2001, Karst et. al., 2015). The vast majority of terpenes are secondary compounds, meaning they are not required for growth, but instead are involved in communication and tree defense (Gershenzon & Dudareva 2007). Conifers produce some terpenes continuously, as a form of constitutive resistance to repel, kill or contain invaders such as pathogens or insects (Bonello et al., 2006; Keeling & Bohlmann 2006). Terpenes in trees may also be produced as a form of induced resistance, where compounds are synthesized or up-regulated in response to specific stressors. Constitutive terpenes may be the first line of defense to inhibit initial growth of pathogenic fungi that infect conifer species (Bridges 1987; Michelozzi et al. 1990; Evenesen et al., 2000; Lombardero et al., 2006). Studies have shown that slow growing, high-elevation conifer species like whitebark pine tend to invest more in constitutive defenses and less in inducible defenses as part of a defense trade-off, as they cannot easily replace damaged tissues (Moreira et al., 2014). In whitebark pine, terpenes may play a significant role in limiting infection by C. ribicola (Mirov 1961; Richardson et al., 2014; Richardson et al., 2015).

Precursors required for terpene production are the same for angiosperms and gymnosperms and evidence suggests that genes involved in terpene synthase evolved from a common ancestor (Trapp & Croteau 2001). First, isopentenyl diphosphate (IPP) is synthesized by one of two pathways in the cytosol of plant cells (Trapp & Croteau 2001). From IPP, geranayl diphosphate (GPP), farnesyl diphosphate (FPP) and geranylgeranyl diphosphate
(GGPP) are formed as the immediate precursors to monoterpenes, sesquiterpenes and
diterpenes, respectively. Oleoresin, a defensive substance in conifers that accumulates in resin
ducts located in stem and needle tissue (Wu & Hu, 1997) contains all three of these classes of

The relative concentrations of terpenes, and how terpenes in trees respond to pathogens
and herbivores show high heritability in conifers (Baradat & Yazdani 1988, Sampedro et al.,
2010). In a study of 17 half-sibling families of Pinus pinaster, Sampedro et al., (2010) found
that genetic variation was the main source of phenotypic variation of foliar terpene profiles in
seedlings exposed to methyl jasmonate, a hormone analog used to imitate herbivory and
artificially induce terpene production in pines. However, individual terpenes have varying levels
of heritability (Zhang et al., 2016).

**Fungal endophytes**

Endophytes are microorganisms that live within plants without creating visible symptoms of
infection. They can influence plant performance in a variety of ways including increased growth
(Bullington & Larkin 2015), pathogen resistance (Arnold et al., 2003, Ganley et al., 2008),
nutrient uptake (Rahman & Saiga 2005; Yang et al., 2014), drought resistance (Bae et al.,
2009), and herbivore defense (Cheplick & Clay 1988; Zhang et al., 2011). Endophytes can vary
from mutualists to latent pathogens and saprophytes (Carroll 1988, Rodriguez et al., 2009).
They can inhibit pathogen infection and spread in pines, and an increasing number of studies
demonstrate their potential as biocontrols in controlling disease (Berube et al., 1998; Ganley et
al., 2008; Rideout & Newcombe 2015). Endophytes are ubiquitous in plant tissues (Carroll
1988, Rodriguez et al., 2009) and can affect pathogens in many ways including through
mycoparasitism (Evans et al., 2001) and competition (Ganley et al., 2008). For example,
endophytes have been shown to colonize pseudostroma (stroma-like supportive tissue) of a pod
rot pathogen, inhibiting the growth of that pathogen (Evans et al., 2001). Endophytes can also
produce defensive compounds of their own that inhibit or kill pathogens (Mousa & Raizada
2013). Some endophytes in the genus, *Trichoderma* are capable of both predation and
parasitism of pathogenic fungi and upregulate genes involved in nutrient acquisition and
production of antimicrobials when interacting with plant pathogenic fungi (Atanasova et al.,
2013). In these ways, endophytes can act as a mode of defense or add to defenses that already
exist in plants. For example, Arnold et al., (2003) found that endophytes limited pathogen damage in a tropical tree and that endophyte-mediated protection from pathogens was significantly greater in mature leaves, which are less equipped with natural host defenses.

More than one thousand endophytic species have been recovered in white pines (Bullington & Larkin et al., 2015) and some have shown potential to decrease white pine blister rust severity. Berube et al., (1998) tested 63 fungal endophytes and found 7 isolates that decreased symptoms of blister rust in eastern white pine. In that study, some endophytes reduced needle spots on one-year-old seedlings up to 98%. In another study, endophytes decreased the number of needle spots and stem cankers, and increased the survival rate of white pines infected with blister rust (Ganley et al., 2008). However, in these studies only endophytes that could be grown from healthy needles and into culture were considered, and with no knowledge of their natural relative abundance in resistant vs. susceptible trees.

Host-mediated interactions

Abiotic and biotic interactions shape the microbial community composition within hosts. In particular, the chemical environment within plant tissues can influence the community composition of microbes that inhabit that tissue (Bailey et al., 2005). Defensive compounds in plant tissues can act as a filter between host trees and microbial colonizers, as well as affect the outcome of interactions between microbial colonizers themselves. For example, Arnold et al. (2003) found that endophyte communities in tropical forests were host species-specific and outcomes of fungal-fungal interactions depended on species-specific host leaf chemistry. In the same study, endophytes were also found to reduce damage to trees due to pathogens. These findings indicate that host chemistry largely determines endophyte community composition by promoting some species while inhibiting others.

Competition among microbes, once established, may also affect community composition and structure. Plant chemistry may then mediate interspecific interactions inside the plant tissue. In turn, endophytic colonization may also have important implications on the relative concentration of defensive compounds within trees (Mucciarelli et al., 2007). Endophytes can induce chemical responses in plants (Mousa & Raizada 2013), and fungal endophytes in conifers can produce their own antimicrobial compounds as well (Strobel et al., 2011; Stierle & Stierle 2015). These microbial mediated changes in chemistry likely also affect community
composition. For example, in one study, foliar fungal endophytes isolated from eastern white pine (*Pinus strobus*) produced compounds toxic to at least two rust pathogens (Sumarah *et al.*, 2010) and in a second study it was found that some endophytes produce compounds that were toxic to white pine blister rust, specifically (Sumarah *et al.*, 2015). Together, these findings highlight the importance of host, pathogen and endophyte interactions.

*Breeding programs*

Whitebark pine breeding programs selectively breed five-needle pines for genetic resistance to white pine blister rust. These programs often select seeds from trees that grow in areas of high blister rust severity, but remain healthy, despite repeated exposure to the pathogen. Dorena Genetic Resource Center (DGRC) in Cottage Grove, OR has bred five-needle pines for over half a century to identify families and parent trees that exhibit characteristics associated with heritable pathogen resistance (Sniezko *et al.*, 2014). At the DGRC, researchers screen seedling families (half-siblings or open-pollinated seeds collected from the same parent tree) for resistance to blister rust. Seeds are collected from surviving trees throughout the Pacific Northwest, germinated and the resulting seedlings grown in a greenhouse for 1-2 years before being experimentally inoculated with white pine blister rust spores collected from known *Ribes* populations. All inoculations occur in a single location and trees are subsequently planted into the same common garden. Seedlings are then monitored for many years and surveyed periodically for characteristics of disease resistance.

Seed collections made specifically for resistance testing of whitebark pine began in the 1990's and the first resistance screening of seedling families occurred in 2002 (Sniezko *et al.*, 2008b). Major genes for resistance against white pine blister rust exist in at least four five-needle pine species including *P. lamberitana* (sugar pine), *P. monticola* (Western white pine) *P. strobiformis* (southwestern white pine), and *P. flexilis* (limber pine) (Schoettle *et al.*, 2014). Evidence for genetic resistance of whitebark pine to blister rust has been documented (Sniezko *et al.*, 2011), but no major genes for resistance have been identified to date. The resistance observed in some whitebark pine may be, at least partially, related to tree chemical defense profiles and endophyte communities.
Objectives

In this study, I looked at the foliar fungal endophyte community composition and chemical profiles of whitebark pine seedlings inoculated with white pine blister rust in a common garden. I investigated foliar fungal endophytes of whitebark pine before and after inoculation with blister rust to determine the effect of the pathogen on the endophyte community. I also explored differences in fungal communities between resistant and susceptible seedlings to look for those endophytes most likely to play a role in blister rust resistance. In addition, I tested for correlations between tree defensive compounds and disease severity characteristics. I hypothesized that endophyte communities would respond to blister rust infection, and that the presence of some endophyte species would correlate with disease resistance. I also hypothesized that there would exist a negative relationship between terpene concentrations of hosts and disease severity. With these data, I have begun to elucidate the complex relationships between resistant phenotypes, host chemistry, and fungal endophyte communities of whitebark pine.

The specific goals of my research were to:

1) **Compare the fungal endophyte communities in whitebark pine seedlings before and after inoculation with *C. ribicola* and determine how the communities respond to infection.**

2) **Explore differences in fungal endophyte communities between resistant and susceptible whitebark pine seedlings after exposure to white pine blister rust to determine if abundance of specific endophyte species correlates with blister rust resistance.**

3) **Investigate whether correlations exist between tree defensive chemistry and characteristics of disease severity.**

4) **Determine the relationship between resistance phenotypes, terpene production and fungal endophyte community in needles of whitebark pine.**

Broader implications and gaps in knowledge

White pines host a diverse community of fungal endophytes, and to gain a better understanding of endophyte community composition and its influence on disease, we must first get a clear
picture of the endophyte species present. Next generation sequencing (NGS) methods enable us to gain a more comprehensive understanding of fungal richness and community composition in the environment than allowed through traditional culture-based methods or older sequencing methods such as Sanger sequencing. Recent NGS technology has enabled researchers to perform large-scale investigations into entire microbial populations of almost any system, and has been used extensively to study the microbial diversity of soils (Delmont et al., 2012), fungal root symbionts (Lekberg et al., 2011) and foliar endophytes of deciduous trees (Jumpponen & Jones, 2009). To date, the fungal internal transcribed spacer (ITS) region of the ribosome encoding genes has the highest probability of successful identification for the broadest range of fungi (Schoch et al., 2012). As such, ITS can be used as a barcode to identify fungal groups by matching DNA sequences to a previously curated fungal database, such as UNITE (http://unite.ut.ee; Abarenkov et al., 2010; Kõljalg et al., 2013). This allows for taxonomy assignment in environmental samples down to the species level in some cases. With a broader more inclusive perspective on fungal endophyte biodiversity, we will better understand the interspecific interactions that take place within five needle pines and their influence on subsequent ecological processes. Despite the growing body of evidence on endophytes and their influence on conifers and disease, little is known about fungal community composition within trees. By using molecular methods to explore patterns of endophyte community composition in both resistant and susceptible seedlings exposed to disease, we can identify precisely those species likely to have a more positive effect on tree health.

Endophytes are important producers of antimicrobial secondary compounds and have previously been shown to inhibit C. ribicola infection in other five-needle pines (Berube et al., 1998; Ganley et al., 2008). Investigations into which fungi associate with resistant trees could lead to a better understanding of whitebark pine resistance mechanisms and enhance future breeding efforts to ensure the successful restoration of this species. Concurrent comparisons of chemical profiles in resistant and susceptible seedlings will shed light onto the chemical environment of hosts needle tissues, where initial interactions between the host, pathogens and endophytes take place. Whitebark pine is a keystone five-needle pine in decline, and together this knowledge can inform breeding practices and restoration efforts throughout the Pacific Northwest. To our knowledge, no study to date has explored foliar defensive chemistry in
whitebark pine populations in response to blister rust infection, let alone in relation to the foliar fungal endophyte communities in conifers.
Figure 1. a) Telia of *C. ribicola* on the bottom of infected *Ribes* leaves. b) Orange aecia of *C. ribicola* forming cankers on branch and stem tissue of whitebark pine. c) Yellow spots on needle tissue of whitebark pine caused by *C. ribicola* infection. (Photo credit: Lorinda Bullington (a,b) Richard Sniezko (c)).
Chapter 2: Molecular diversity of foliar fungal endophytes in relation to defense strategies and disease in whitebark pine
Introduction

*Cronartium ribicola* J.C. Fisch is an invasive fungal pathogen that causes the disease, white pine blister rust, in five-needle pine trees native to North America. *C. ribicola* causes bark cankers on stems and trunks, as well as necrotic spotting on needles, branch dieback, and eventually death (Patton & Johnson 1970). *C. ribicola* spores colonize pines through small openings in the needles called stomata (Liu *et al*., 2015). Mycelium then grows through needles and branches of susceptible trees, into the main stem where *C. ribicola* eventually girdles and kills the tree (Patton & Johnson 1970; Campbell & Antos 2000). White pine blister rust has spread to nearly the entire range of whitebark pine in the United States (Schwandt *et al*., 2010), and in some areas, whitebark pine has decreased to less than 10% of its natural population largely due to *C. ribicola* infection (Kendall & Arno 1990; Keane & Arno 1993). The loss of whitebark pine is predicted to have severe impacts on forest composition and distribution in high elevation ecosystems (Hoff *et al*., 2001), but some resistance to *C. ribicola* has already been documented in natural whitebark pine populations (Sniezko *et al*., 2011).

Understanding tree defense strategies in response to pathogens and herbivores has been a central focus in forest ecology for decades (Fowler and Lawton 1985; Schlarbaum *et al*., 1998), and various abiotic and biotic factors have been proposed as primary drivers of disease spread and severity, as well as resistance, in these systems (Burdon & Chilvers 1982; Delledonne *et al*., 1998; Brown 2002; Keeling & Bohlmann 2006). Microorganisms that live ubiquitously within plant tissues without causing any visible signs or symptoms of infection, referred to as endophytes, (Carroll 1988, Rodriguez *et al*., 2009) may contribute to tree defense strategies. Endophytes can inhibit pathogen infection and spread in pines, and an increasing number of studies demonstrate their potential as biocontrols in reducing disease (Berube *et al*., 1998; Ganley *et al*., 2008; Rideout & Newcombe 2015). Endophytes co-occur in trees along with fungal pathogens and have ample opportunity to influence disease through many processes. Some endophytes can parasitize plant pathogens, resulting in stunted growth and death (Evans *et al*., 2001; Atanasova *et al*., 2013), and they often act as a complementary or even a substitutive layer of resistance for plants. For example, Arnold *et al*., (2003) found that endophytes limit pathogen damage in a tropical tree and that endophyte-mediated protection
from pathogens was significantly greater in mature leaves, which are less equipped with natural host defenses.

As a natural defense against stressors such as pathogens, herbivores, or insects, many conifers produce organic compounds known as terpenes (Trapp & Croteau 2001, Karst et al., 2015). Conifers continuously produce some terpenes as a form of constitutive resistance to kill or contain invaders as they attack, or to repel herbivores (Bonello et al., 2006; Keeling & Bohlmann 2006). Terpenes in trees may also be produced as a form of induced resistance, where compounds are synthesized or up-regulated in response to specific stressors. Terpenes may be an important first line of defense to inhibit initial growth of pathogenic fungi that infect conifer species (Bridges 1987; Michelozzi et al. 1990; Evenesen et al., 2000; Lombardero et al., 2006), and studies have shown that slow growing, high-elevation conifer species like whitebark pine tend to invest more in constitutive defenses and less in inducible defenses as part of a defense trade-off, as they cannot easily replace damaged tissues (Moreira et al., 2014).

Both abiotic and biotic interactions shape the microbial community composition within host plants (Figure 1). The chemical environment within plant tissues influences the composition of microbes, both endophytes and pathogens that inhabit that tissue (Bailey et al., 2005). Specifically, plant defensive compounds can act as a filter between host trees and microbial colonizers (Figure 1F & 1C), as well as effect the outcome of interactions between microbial colonizers (Figure 1G). For example, Arnold et al. (2003) found that endophyte communities in tropical forests were host-specific, and that outcomes of fungal-fungal interactions depended on specific host leaf chemistry. Endophytes in this study were also shown to reduce pathogen damage in trees (Figure 1B). These findings suggest that host chemistry affects the endophyte community by promoting colonization of some species while inhibiting others. Pathogens that can tolerate initial host defensive chemistry and are able to colonize the plant must compete with co-occurring endophytic colonizers. Plant chemistry may then mediate interactions inside the plant, as part of the tree's overall defensive strategy (Figure 2G). Together, this highlights the importance of host, pathogen and endophyte relationships.

In turn, endophytic colonization may also have important implications on the relative concentrations of defensive compounds within trees (Figure 1E, Mucciarelli et al., 2007). Endophytes can induce chemical responses in plants (Mousa & Raizada 2013), and fungal endophytes in conifers can produce their own antimicrobial compounds as well (Strobel et al.,
In one example, foliar fungal endophytes isolated from eastern white pine (Pinus strobus) produced compounds toxic to at least two rust pathogens (Sumarah et al., 2010) and follow-up testing showed compounds that were toxic to C. ribicola specifically (Sumarah et al., 2015).

More than one thousand endophytic species have been recovered in white pines (Bullington and Larkin 2015), and some have already shown potential to decrease white pine blister rust (Berube et al., 1998; Ganley et al., 2008). Despite the growing body of evidence on endophytes and their influence on conifers and disease, little is known about the factors that shape fungal community composition within trees. By using molecular methods to explore patterns of endophytic diversity in both resistant and susceptible seedlings exposed to disease, we can identify precisely those fungal species likely to have a more positive effect on tree health. Further knowledge about the fungal endophyte community that co-infects whitebark pine along with white pine blister rust will help to elucidate the interactions that take place in needle tissues at time of infection. Investigations into which fungi associate with resistant trees could lead to a better understanding of whitebark pine resistance mechanisms and enhance future breeding efforts to ensure the successful restoration of this species. Concurrent comparisons of terpene profiles in resistant and susceptible seedlings will shed light onto the chemical environment of host needle tissues, where initial interactions between host, pathogens and endophytes take place. Tree chemical defense profiles and endophyte communities associated with healthy trees in these studies could greatly contribute to what is known of the mechanisms underlying disease resistance in whitebark pine.

Whitebark pine is a keystone five-needle pine in decline, and together this knowledge will enhance breeding practices and restoration efforts throughout the Pacific Northwest. No study to date has explored foliar defensive chemistry in whitebark pine populations in response to blister rust infection, let alone in relation to the foliar fungal endophyte communities in conifers. In this study I investigate the relationship between whitebark pine (Pinus albicaulis) and the pathogen causing white pine blister rust in a common garden, and the fungal endophytes that co-infect whitebark pine along with C. ribicola. In addition I analyzed tree defensive chemistry in needle tissue, where the initial interactions between host, endophytes and pathogens take place. I characterized foliar fungal endophytes of whitebark pine before and after inoculation with blister rust to determine the effect of the pathogen on the endophyte
community (Figure 1A). I also explored differences in fungal communities between resistant and susceptible seedlings to look for those endophytes most likely to aid in blister rust resistance (Figure 1B). In addition, I determined the relationships between tree defensive compounds and disease severity characteristics (Figure 1C). I hypothesized that we would see a response of endophyte communities to blister rust infection, and observe individual endophytes that correlate with disease resistance. I also hypothesized that I would see a negative relationship between terpene concentrations of hosts and disease severity. With this data, I hope to elucidate the complex relationships between resistant phenotypes, host chemistry, and fungal endophyte communities of whitebark pine to assist future breeding efforts.

**Methods**

*Data collection*

The Dorena Genetic Resource Center (DGRC) in Cottage Grove, OR has bred five-needle pines to some extent for over half a century to identify families and parent trees that exhibit genetic characteristics associated with blister rust resistance (Sniezko *et al.*, 2014). Researchers screen seedling families (half-siblings or seeds collected from the same parent tree) collected from areas of known high blister rust severity to look for natural resistance to rust infection. Seeds are collected from naturally occurring, healthy, surviving trees and grown in a greenhouse for 1-2 years before being experimentally inoculated with white pine blister rust spores collected from nearby *Ribes* populations. For this experiment, seed families were sourced throughout the Pacific Northwest (Figure 2) and stored at the DGRC. A total of 131 seedlings belonging to 20 whitebark pine seed families (6-10 individual seedlings per family), plus a subset of 10 seedlings belonging to two of the same families that served as controls (Appendix I, Table 1) were stratified and sown directly into cone containers in accordance with standard DGRC protocols (Riley *et al.*, 2007). Seedlings were germinated and maintained in an unheated, open greenhouse and subjected to regular watering and fertilization. After two years, seedlings were placed in an inoculation chamber and saturated with white pine blister rust spores with the exception of the 10 seedlings that served as controls. After inoculation, all seedlings, including controls, were out-planted into a common garden.

Needles were collected from seedlings two times. The first collection occurred
immediately prior to blister rust inoculation on September 11, 2014. We collected both primary and secondary needles from each seedling. We then pooled needle tissue within family to ensure sufficient sample volume. This resulted in a total of 22 pooled samples before blister rust inoculation. Phenotypic characteristics associated with quantitative resistance to blister rust were assessed at 8 months (inspection 1) and 14 months (inspection 2) after inoculation. Inspection 1 included a total count of needle spots per seedling. Data recorded at inspection 2 included the number of cankers on the entire seedling and number of cankers on the main stem. Overall disease severity was also measured. Severity ranged from 0-9, with '0' representing seedlings with no symptoms and '9' representing seedlings dead from blister rust (Sniezko et al., 2014). Approximately one month before inspection 2 we collected needle tissue from the same seedlings as sampled before. In this sample each seedling represented one replicate and needles were pooled within seedling for a total of 141 individual seedlings sampled after inoculation. On each seedling we again collected both primary and secondary needles. Samples were kept on ice or at -20 °C until processing.

Terpene Analysis
To develop profiles of terpene concentrations in each seedling at inspection 2, a subsample of needle tissue was used from the needle samples taken from the 141 individual seedlings. The needle tissue was ground in liquid nitrogen, weighed and sent on dry ice to the University of Alberta for terpene analysis. Extraction and analysis of terpenes were performed as described in Erbilgin et al., (2014) and Karst et al., (2015). Briefly, 100.00 +/- 2.86 mg of ground needle tissue was extracted twice using 0.01% tridecane in 0.5ml dichloromethane. Extracts (1 µl) were injected in an Agilent 7890A Gas Chromatograph (Agilent Technologies, Santa Clara, CA, USA) with an HP Innowax (Agilent Technologies) column (Erbilgin et al., 2014). Peaks were identified using the following standards: borneol, ρ-cymene α-humulene, α-terpinene, α-terpineol, 3-carene, terpinolene, (-)-α-pinene, (+)-α-pinene, racemic a-pinene, (-)-β-pinene, (S)-(-)-limonene, (R)-(+)limonene, myrcene, bornyl acetate, and β-phellandrene. The quantity of chemicals was calculated using response curves generated from analyses of a dilution sequence of known quantities of standards. Calibration with these standards allowed for analysis of quantitative differences among treatments. For some compounds (terpineol acetate, elemene, cadinene, and germacrene) no standards were available so peak area was compared for
qualitative and quantitative differences among samples; however, concentrations between these compounds and all other compounds could not be compared.

*Fungal endophyte community characterization*

The remaining needle tissue from individual seedlings, and the 22 pooled samples collected before blister rust inoculation, were washed and surface sterilized in 70% ethanol for 1 min, 6% sodium hypochlorite for 5 min, and then washed again in 70% ethanol for 1 min (Larkin *et al*., 2012). To verify sterilization efficacy, surface-sterilized needles were imprinted on malt extract agar and monitored for fungal growth. No evidence of contamination was observed. Surface sterilized tissue was freeze-dried using a Labconco Freezone benchtop freeze dry system (Labconco, Kansas City, MO, USA). Dried needle tissue was then macerated to a fine powder using a 1600 MiniG® tissue homogenizer & cell lyser (Spex SamplePrep, Metuchen, NJ, USA).

Genomic DNA was extracted from ground needle tissue using a modified Cetyl trimethylammonium bromide (CTAB) extraction protocol (Larkin *et al*., 2012; Bullington & Larkin 2015). Fungal DNA was amplified directly from pine needle tissue and prepared for Illumina sequencing using a two-step PCR protocol to first amplify our target region and then attach unique identifiers to DNA from each sample. The ITS2 region was initially amplified using a mix of forward fungal primers flITS7 (Ihrmark *et al*., 2012) and fl ITS7o (Kohout *et al*., 2014) and the reverse primer ITS4 (White *et al*., 1990). To reduce problems related to low sequence diversity at the conservative regions where primers bind (Fadrosh *et al*., 2014), we increased sequence variability of our amplicon libraries by using a heterogeneity spacer region (0-6 nucleotides) between target primers and 22 bp Fluidigm universal tags (Fluidigm Inc. San Francisco, CA, USA) in our target primer complex for PCR1. The universal tags CS1 and CS2 were added to the forward and reverse primer complexes, respectively. This generated seven unique forward and seven unique reverse target primer complexes. Reactions were carried out in 12.5 μL reaction volumes containing 1 μL of template, 20 pmol of each primer in 1X GoTaq® Green Master Mix [(Green GoTaq® Reaction Buffer, 200 μM dATP, 200 μM dGTP, 200 μM dCTP, 200 μM dTTP and 1.5 mM MgCl₂) Promega, USA]. Each reaction was performed in a Techne TC-4000 thermocycler (Bibby Scientific, Burlington, USA) under the following conditions: 3 min at 94 °C followed by 35 cycles of 60 s at 95 °C, 40 s at 54 °C, 40 s at 70 °C and a final extension step at 68 °C for 7 min before storage at 4 °C.
To confirm the presence of our target amplicon, all reactions were analyzed by 1.5% agarose gel electrophoresis using a 100 bp ladder (O’GeneRuler DNA Ladder, Thermo Scientific, USA). In the second PCR reaction, we flanked PCR1 amplicons with barcodes and Illumina flowcell adapters to create unique identifiers for target DNA in each sample. PCR2 primer complexes consisted of the same Fluidigm tags (CS1 or CS2) as PCR1, 8 bp Illumina Nextera barcodes (Illumina Inc., San Diego, CA, USA), and Illumina adapters. PCR2 was carried out in 25 µL reaction volumes containing 1 µL of template, 20 pmol of each primer in 1X GoTaq® Green Master Mix (Promega, USA). Each reaction was performed in a Techne TC-4000 thermocycler (Bibby Scientific, Burlington, USA) under the following conditions: 95 °C for 1 min; 10 cycles of 95 °C for 30 sec, 60 °C for 30 sec, 68 °C for 1 min; and 68 °C for 5 min. Samples were pooled based on band intensities in a 1.5% agarose gel electrophoresis of PCR 2 product. Sequencing was done at the Institute for Bioinformatics and Evolutionary Studies (iBEST) genomics resources core at the University of Idaho (http://www.ibest.uidaho.edu/; Moscow, ID, USA). Amplicon libraries were sequenced using 2 x 300 paired-end reads on an Illumina MiSeq sequencing platform (Illumina Inc., San Diego, CA, USA).

Initial bioinformatics analyses were conducted using “quantitative insights into microbial ecology” (QIIME version 1.9.1; Caporaso et al., 2010). Paired reads were assembled using fastq-join (Aronesty, 2013) with a minimum overlap of 20 bp and allowing a maximum mismatch of 10% within the region of overlap. We followed primary quality filtering parameters as recommended from Bokulich et al., (2013) with the exception of the minimum acceptable Phred quality score, which we adjusted to 27 due to the high quality of our reads. All trimmed and quality filtered sequences were clustered using the QIIME implementation of Sortmerna (Kopylova et al., 2012). Operational taxonomic units (OTUs) were delineated at 97% pairwise similarity, which is within an acceptable range for fungal ITS species divergence (Nilsson et al., 2008; Tedersoo et al., 2010). The most abundant sequence in each cluster was designated as the representative sequence. Chimera checking was performed on all sequences using USEARCH 6.1 chimera checking software in QIIME (Edgar, 2010). Taxonomic identification was determined using the QIIME based Sumaclust taxonomy assigner (Mercier et al., 2013) and the UNITE fungal ITS sequence database (http://unite.ut.ee; Abarenkov et al., 2010; Kõljalg et al., 2013). All taxonomic designations refer to assignments based on a minimum pairwise similarity of 97% to sequences within the UNITE fungal database with 90%
sequence coverage, unless otherwise noted. All OTUs that were represented by less than 0.001% of total sequences (16 sequences) or present in fewer than two seedlings were removed to avoid potential PCR and sequencing artifacts.

**Data analysis**
The relationships between terpenes, endophytes and disease phenotype of the same individual seedlings were analyzed using R (version 3.3.1, R Core Team 2016) with lme4, vegan, mvabund and afex packages (Bates et al., 2015; Oksanen et al., 2017; Wang et al., 2016; Singmann et al., 2017) unless otherwise noted. Significance was inferred at \( P < 0.05 \) for all tests.

To assess the relationship between tree defensive chemistry and *C. ribicola* in inoculated whitebark pine seedlings, we used a generalized linear mixed model (glmer, Bates et al., 2015) with poisson error distribution and a log-link function, and all models were checked for overdispersion. We used this analysis as data were obtained from seedlings belonging to the same seed family, which violates the assumption of sampling independence. We considered disease severity as the response, concentrations of each terpene as a fixed factor in individual models and the interaction between terpene and seed family as a random effect. Statistical significance was assessed using parametric bootstrapping and 999 permutations (mixed function, Singmann et al., 2017). Only individual terpenes with available standards were considered in total terpene and total monoterpene analyses. Pearson correlations were conducted between individual terpene concentrations and relative abundances of endophytic OTUs. Trace compounds were not included in these analyses.

For all community analyses, we rarefied sequencing depth to 900 sequences per seedling to. Seedlings with fewer than 900 sequences were excluded from further analyses for a total of 16 pooled samples before inoculation and 123 inoculated seedlings and 9 control seedlings. Sequence and OTU data were used to estimate foliar fungal species richness within seedlings and diversity indices (evenness, Fisher's alpha) in all samples using the vegan community ecology package (Oksanen et al., 2017). Diversity was visualized using box and whisker plots, and analyses were performed using a one-way ANOVA followed by Tukey's HSD to assess post hoc contrasts.

To elucidate patterns in fungal community composition in all seedlings before and after inoculation as well as in seedling group (resistance, susceptible and control seedlings), we used
non-metric multidimensional scaling (NMDS) based on Bray-Curtis distances of rarefied relative abundances of OTUs (metaMDS function, Oksanen et al., 2017). To identify main factors influencing fungal communities, we tested disease phenotypes, seed family identity, latitude, longitude and region for significant correlations with NMDS ordinations using the envfit function. Envfít fits vectors of both continuous variables and centroids of class level variables onto ordinations (Oksanen et al., 2017). Significance was assessed with 999 random permutations. To provide additional support to significant results of NMDS and envfit in testing for differences between fungal communities we also performed permutational multivariate analysis of variance using the adonis function with 999 permutations on Bray-Curtis distances. This function was also used to test for differences in terpene profiles between seedling groups. These analyses were all performed using the vegan community ecology package (Oksanen et al., 2017).

Kruskal-Wallis tests were performed on rarefied data for individual OTUs to look for differences in average individual OTU abundances between resistant and susceptible seedlings (those that developed cankers and those that did not), as well as before and after inoculation with a Bonferroni correction for multiple comparisons. For these analyses, QIIME 1.9.1 was used (Caporaso et al., 2010).

**Results**

*Disease development*

Of the 131 seedlings inoculated, all presented needle spots at inspections 1 and 2. A total of 100 seedlings from 20 families developed cankers on stems or branches by inspection 2, and were considered 'susceptible' to blister rust (Appendix I, Table 1). Susceptible seedlings developed 5.4 total cankers on average, ranging from 1-12 cankers per seedling. The remaining 31 seedlings from 11 families appeared resistant to blister rust, developing zero cankers on stems or branches at time of needle collection. Overall disease severity differed between seed families in seedlings inoculated with blister rust (F = 3.82, P < 0.0001). The most resistant seed families were CA-62 collected from North Cascades National Park in Region 1, CO-121 from Colville National Forest in Region 1 and CL-72 from Crater Lake National Park in Region 4. Only 2 out of 7 seedlings in each of these families had developed cankers at inspection 2. The ten seedlings
that were never inoculated and served as controls did not present any symptoms of blister rust infection at any point during this study.

**Terpenes and disease in whitebark pine**

In the seedlings sampled one year after inoculation with *C. ribicola*, we detected 23 total terpenes including 17 monoterpenes (β-phellandrene, p-cymene, γ-terpinene, terpinolene, sabinene hydrate, 4-allylanisole, bornyl acetate, borneol, 3-carene, myrcene, (-)-α-pinene, (+)-α-pinene, (-)-β-pinene, (+)-β-pinene, (-)-limonene, (+)-limonene, terpineol acetate), and 6 sesquiterpenes (β-caryophyllene, humelene, germacrene, γ-Cadinene, Cubebene, γ-Elemene) in needle tissue of whitebark pine seedlings. Considering only those terpenes where concentrations could be calculated from available standards, the most abundant terpenes in whitebark pine were 3-Carene, followed by (-)-α-pinene and β-phellandrene.

Overall terpene composition showed significant genetic variation (*Adonis*, $R^2 = 0.332$, $P < 0.001$), indicating high heritability of foliar terpene profiles of whitebark pine. Terpene profiles also differed between seedling groups (resistant, susceptible and control seedlings, *Adonis*, $R^2 = 0.047$, $P = 0.01$). Accounting for genetic variation, generalized linear mixed models (GLLMs) showed variation of individual terpenoid compounds in relation to overall disease severity (Table 1). As predicted, resistant seedlings contained higher concentrations of multiple terpenes, including (+)-limonene, (-)-α-pinene, total monoterpenes and total terpenoid compounds recovered from needle tissues (Figure 3). Pearson's correlations between phenotypic characteristics of blister rust infection and these terpenes showed that (+)-limonene had a negative correlation with disease severity at time of collection ($R^2 = -0.171$, $P = 0.049$), but a positive correlation with the number of needle spots at inspection one ($R^2 = 0.248$, $P = 0.004$).

**Fungal endophytes**

After quality filtering and demultiplexing, 1,631,451 total sequences remained for a total of 1415 OTUs in all seedlings and 1348 OTUs in seedlings sampled at inspection 2. No single OTU was recovered from all trees. The most abundant OTUs were found in at least 75% of seedlings and most closely matched to *Cladosporium exasperatum*, unidentified Ascomycota, *Paraphoma* sp., *Gibberella tricincta*, and *Pleosporacea* sp. We observed little difference in the fungal endophyte communities between resistant and susceptible seedlings (*Adonis*, $R^2 = 0.009$, ...
P= 0.087), but infection with *C. ribicola* affected fungal endophyte community composition. Comparing endophyte communities in seedlings before and after *C. ribicola* inoculation and control seedlings, seedling group explained most of the variation in endophyte community composition (*envfit*, $R^2 = 0.093$, $P < 0.001$; *Adonis*, $R^2 = 0.027$, $P < 0.001$). Endophyte communities in susceptible and resistant seedlings were more similar to each other than to control seedlings that were never inoculated (Figure 4). Endophyte communities in control seedlings most closely resembled endophyte communities in seedlings before inoculation, indicating a direct effect of inoculation on the endophyte community. In addition, eighteen individual OTUs showed significant variation between seedlings before and after inoculation, and controls (Appendix I, Table 1). All of these endophytes decreased in abundance after inoculation with blister rust, but many maintained high abundances in control seedlings that were never inoculated.

Overall, measures of evenness (Pielou’s $J'$ evenness; $J' = H'/H'_{max}$), rarefied richness and Fisher's Alpha ($N(I-x)/x$) of fungal communities also differed in seedlings before blister rust inoculation compared to susceptible seedlings after inoculation (Figure 5, $P = 0.003$, $P = 0.006$, and $P = 0.005$, respectively). Specifically, richness and Fisher's alpha decreased more in susceptible seedlings than resistant seedlings or seedlings never inoculated, but evenness in susceptible seedlings was higher than in any other group.

Looking only at resistant and susceptible seedlings inoculated with white pine blister rust, we saw correlations between abundant endophytes and blister rust disease characteristics. OTUs associated with *Lophodermium indianum* and *Paraphoma* spp., negatively correlated with overall disease severity of inoculated seedlings (df = 121; $R^2 = -0.216$, $P = 0.017$; $R^2 = -0.207$, $P = 0.021$, respectively). The second most abundant OTU belonging to *Ascomycota* sp. was recovered from 102 seedlings and was positively correlated with both 3-carene and number of needle spots (df = 121, $R^2 = 0.222$, $P = 0.013$; $R^2 = 0.182$, $P = 0.044$, respectively). *Metarhizium anisolpliae* was recovered from 88 seedlings and its presence was negatively correlated with both the number of needle spots and the number of cankers on whitebark seedlings (df = 121; $R^2 = -0.233$, $P = 0.009$; $R^2 = -0.191$, $P = 0.034$, respectively). Another OTU belonging to *Helotiales* was recovered from 81 seedlings and was negatively correlated with 3-carene (df = 121, $R^2 = -0.187$, $P = 0.038$).

Seedling family (seedlings with the same maternal parent) was the greatest driver of
overall fungal endophyte composition in inoculated seedlings \((envfit, R^2 = 0.270, P = 0.004; Adonis, R^2 = 0.160, P = 0.046)\). Seed family region (Figure 2) also influenced fungal endophytes (Figure 6, \(envfit, R^2 = 0.070, P = 0.030; Adonis, R^2 = 0.101, P = 0.054\)), but effects of latitude and longitude were not significant. These results were reflective of the genetic variation of whitebark pine trees from the same or nearby regions as shown by Liu et al., (2016), with similar north to south trends in genetic structure as we see in endophyte community structure. In Liu et al 2016, seed families explained 27% of the genetic variation in whitebark pine populations.

**Discussion**

Host genotype, endophyte community and terpenes appeared interconnected within individual seedlings, with implications for blister rust severity. We saw an influence of blister rust infection on both terpene and endophytic profiles of whitebark pine seedlings. Subsequent correlative relationships of endophytes, terpenes and resulting disease severity suggest that interactions that take place inside needle tissue relate to the resulting pathology of blister rust infection. The response of both terpene and endophytic profiles to blister rust infection was also related to host genotype.

*Defensive strategies correspond with disease outcome*

Fewer stem infections, latent stem infections, fewer bark reactions and higher survival are all phenotypic characteristics of seedlings or seed families exhibiting partial resistance to blister rust (Sniezko et al., 2014). Little research exists on the underlying mechanisms of these resistant traits in whitebark pine, but our study shows at they may be related to levels of individual terpenes and the genes involved in production of secondary defense compounds found in needle tissue. (+)-Limonene was positively correlated with the number of needle spots, but negatively correlated with overall disease severity later on, suggesting that initial infection severity (i.e., needle spotting) may induce production of (+)-limonene, inhibiting disease spread. Limonene is a common terpene in nature and is known to inhibit fungal growth (Duetz et al., 2002). It also is an important compound in the resistance of Italian Stone pine (\(Pinus pinea\)) and Alleppo pine (\(Pinus halepensis\)) to insect herbivores (Mita et al., 2002). Concentrations of (-)-\(\alpha\)-pinene were higher in trees exposed to \(C.\) ribicola and highest in resistant seedlings than in any
other group (Figure 3). A recent study by Burke and Carrol (2016) suggested that elevated levels of α-pinene might increase attack success and aggregation by mountain pine beetle. Those results, combined with the current study support observations by Six and Adams (2007) and Jules et al., (2016) that mountain pine beetles appear to be more attracted to trees infected by blister rust disease, although in the former study, the healthiest trees were avoided. The implications of these observations should be considered in future management efforts in whitebark pine, especially if levels of α-pinene remain elevated in some resistant trees as a form of acquired resistance to blister rust disease. These results provide insight into the defensive strategies of whitebark seedlings and illustrate the need for further studies to determine the genes involved in terpene synthesis in whitebark pine needle tissue, to better understand the molecular mechanisms associated with genetic resistance.

As in Sampedro et al., (2008), we saw correlations between seed family and the terpene profile of individual seedlings (Adonis, $R^2 = 0.332$, $P < 0.001$). Due to the strong heritability of terpene profiles, and because we did not analyze terpenes in seed families both before and after inoculation, we can only speculate whether terpene concentrations reported here represent constitutive or induced levels. We are also hesitant to use control seedlings as a baseline, as they only represent two seed families, one of which has exhibited high resistance to blister rust when inoculated in other trials. However, sampling terpenes in the same seedlings both before and after inoculation with blister rust would still only yield correlative results, as it is not known whether terpenes directly inhibit white pine blister rust or are a byproduct of some other defensive mechanism.

We did not detect fungal DNA matching the ITS2 region of white pine blister rust in needle tissue of any seedlings sampled in this study. We do not believe that this was due to methods, as we have detected blister rust in needle tissue of other five needle pines one year after inoculation using the same methods. It is more likely that blister rust was undetectable because it had grown into branch and stem tissues or succumbed to tree defenses in needles, or both. Differences seen in terpene concentrations, particularly increased concentrations of some terpenes in resistant vs. susceptible seedlings are perhaps even more striking then, as they persist even after the pathogen has been eliminated from needle tissues. This may indicate a form of acquired resistance in whitebark, or an increase in resistance to blister rust after exposure to the pathogen (Kloepper et al., 1992; Stitcher et al., 1997). Alternatively, resistant
trees may already maintain elevated levels of terpenes before exposure to the pathogen as a constitutive defense. However, lower concentrations of individual terpenes in susceptible seedlings may also be a result of the infection itself, inhibiting or reducing tree defenses.

_Endophyte communities correlate with disease characteristics and host defensive chemistry_

In accordance with our hypothesis that we would see a response of endophyte communities to blister rust infection, endophyte communities in control seedlings at inspection 2 clustered more closely to pre-inoculation than to post-inoculation endophyte communities, indicating that colonization by the _C. ribicola_ altered the foliar endophyte community composition of whitebark pine (Figure 4). We believe the differences seen in endophyte communities are likely due to an induced response of the host to infection, as supported by subsequent differences seen in host defensive chemistry within needle tissues because of pathogen infection. After exposure to the blister rust pathogen we observed fewer fungal species in susceptible seedlings compared to the same seedlings before inoculation (Figure 5). In addition, 3-Carene, the most abundant terpene in whitebark seedlings, showed both positive and negative correlations with abundant endophytic species, demonstrating a relationship between seedling chemistry and endophyte community composition. Altered host chemistry due to disease may filter the endophytic community, reducing low abundant species that are already less tolerant of the host chemical environment. This may explain the increase in species evenness we also see in susceptible seedlings. We also saw a significant reduction in the abundance of many individual fungal species after blister rust inoculation (Appendix I, Table 2).

We also observed differences in foliar fungal endophyte communities of whitebark pine in relation to seed family (Figure 6). Because the seedlings grow in a common garden and are exposed to the same airborne fungal propagules, this suggests that host genotype filters the locally available fungal propagules and structures fungal endophyte communities. Liu et al., (2016) sampled many of the same whitebark pine populations as the current study and reported geographical trends in genetic structure in whitebark pine throughout the Pacific Northwest, similar to the observed fungal community variation in this study. Fungal communities grouped by region, from north to south, as did genetic variation of the whitebark pine populations sampled by Liu et al (2016). Seed family was the main factor affecting endophyte communities, indicating a stronger influence of the host than of the blister rust pathogen on endophyte
community composition in whitebark pine seedlings ($envfit$, $R^2 = 0.270$, $p = 0.004$; $Adonis$, $R^2 = 0.160$, $P = 0.046$).

In this study we see that the structure and composition of endophytes may largely be a product of host filtering, with conspecific hosts supporting significantly different fungal communities based on host genetics and environment. Endophytes that help inhibit blister rust infection must first show compatibility with the host's defensive chemistry at an individual tree level, which may contribute to the variability in endophyte-host assemblages within a host species. Those endophytes able to colonize multiple whitebark pine seed families, and also having negative correlations with disease characteristics, may offer some protection against blister rust and be good candidates for inoculations in future restoration efforts. Of the abundant OTUs tested, multiple fungal endophytes were correlated with disease characteristics and terpene concentrations in whitebark pine. Specifically, the presence of *Metarhizium anisopliae* was negatively correlated with both the number of needle spots and cankers on individual seedlings. *M. anisopliae* is a well-studied entomopathogenic fungus that has recently been found as an endophyte in plants (Barelli *et al.*, 2015; Jaber & Ownley 2017). However, to our knowledge, this is the first report of *M. anisopliae* in foliar tissue of conifers. *Metarhizanium* is in the family Clavicipitaceae, which contains many well-known fungal plant mutualists (Rodriguez *et al.*, 2009). Some *Metarhizanium* can produce secondary compounds toxic to both insects and other microbes and have been widely demonstrated as effective biocontrols for pathogens of various hosts (Keyser *et al.*, 2015, Barelli *et al.*, 2015). We did not recover *M. anisopliae* more often from resistant than susceptible seedlings, but less cankered seedlings were associated with higher abundances of this fungus. This suggests that endophytes may act as complementary defenses in conifers; higher abundances of this beneficial fungus may inhibit blister rust, but many other factors, including terpenes, contribute to resistance or susceptibility. Both *Lophodermium indianum* and *Paraphoma* spp. were negatively correlated with overall disease severity. *Lophodermium* belongs to the family Rhytismataceae, whose members are common in other white pines (Ganley *et al.*, 2004), and have shown potential in reducing symptoms of white pine blister rust in previous studies (Ganley *et al.*, 2008). These and our results suggest that these endophytes may have potential as biocontrol agents to protect whitebark pine from *C. ribicola*. Changes in the fungal community after inoculation with *C. ribicola* and correlations of highly abundant endophytes with terpenes and disease
characteristics, suggest that interspecific interactions inside host tissues may play an important role in mediating host defenses.

We detected little variation in the endophyte community as a whole between resistant and susceptible trees, which may be partly due to the pool of available colonizers at the DGRC. Naturally occurring whitebark pine are not found near the vicinity of the common garden, and the surrounding habitat at Dorena is much different than whitebark pine habitat, which is restricted to high elevation ecosystems. Many of the fungal species that most influence blister rust severity in whitebark pine may be absent from the surrounding environment at DGRC. However, whitebark pine genotypes supported specific endophytic community phenotypes within needle tissue, and previous studies have shown evidence of genetically correlated resistance in natural whitebark pine populations (Sniezko et al., 2011, Retzlaff et al., 2016). Future studies of endophyte communities in naturally occurring, resistant whitebark pine exposed to blister rust may be more informative as to which endophytic species or clades are likely to increase resistance to the pathogen.

Future applications
In summary, the results of this study highlight the ecological relationships between tree genotype, terpenes and foliar endophytes with implications for white pine blister rust resistance. We saw marginal differences in endophyte communities between resistant and susceptible seedlings and multiple fungal endophytes showed negative correlations with disease severity in individual seedlings. Blister rust infection influenced both terpene and endophytic profiles of whitebark pine seedlings, and subsequent correlations of endophytes and terpenes with resulting disease severity suggest that interactions that take place inside needle tissue may influence disease outcome. The response of both terpene and endophytic profiles to infection was additionally related to host genotype, which may indicate a multi-faceted form of heritable resistance in whitebark pine. These findings suggest that the genetic resistance seen in natural populations may be a combination of both endophytes and the composition of terpenes in needle tissue, where initial interactions between endophytes, the host and C. ribicola take place.

Next generation sequencing technologies now allow us to look at microbial communities within plants more efficiently and with a high degree of resolution. To supplement NGS studies, direct manipulations of fungal endophyte communities in a natural setting (Bullington & Larkin
2015) as well as investigations into the production of secondary compounds (e.g. terpenes) by the most abundant fungal taxa, will help us to better understand the ecological roles of specific fungi and the mechanisms underlying their effects on host plants. In addition, the inoculation of nursery seedlings with beneficial fungal endophytes before out-planting into high-rust areas may increase tree survival after exposure to C. ribicola, and improve the success of future restoration efforts.

The initial severity of symptoms in trees exposed to white pine blister rust is indicative of future survival (Sniezko et al., 2014), and terpene profiles that can predict disease severity may act as biomarkers for disease resistance. Quantifying terpene concentrations in whitebark pine exposed to blister rust demonstrates the potential physiological mechanisms of disease resistance, where higher or lower concentrations of certain terpenes may indicate a resistant phenotype. Five-needle pines remain an ecologically and culturally important species across the Rocky Mountain Range in both the USA and Canada, and foliar levels of defensive compounds may indicate preferred parent trees as seed sources for replanting forests in areas threatened by white pine blister rust.

References


Zhang XX, Li CJ, Nan ZB, Matthew C. 2011. Neotyphodium endophyte increases Achnatherum inebrians (drunken horse grass) resistance to herbivores and seed predators. Weed Res. 52, 70e78.

Figure 1. Hypothesized theoretical framework of potential interactions among host chemistry, blister rust and fungal endophytes. Host imposed biotic and abiotic interactions shape microbial communities in host plants. Host plants are first exposed to a large diverse pool of microbes in the environment, including both pathogens and endophytes. Host chemistry downsizes the pool of microbes by inhibiting initial colonization (C & F). Subsequent fungal-fungal interactions that take place inside the plant after initial colonization further reduce the number of successful colonizers (A & B). In addition, host chemistry can mediate these interactions (G), and both endophytes and pathogens can in turn induce a chemical response in the host (D & E). Together these interactions determine the realized microbial species assemblage of the host.
Figure 2. Map of seed family source locations in the Pacific Northwest grouped by region from north to south.
Figure 3. Concentrations of individual terpenes in resistant and susceptible *Pinus albicaulis* seedlings after infection with *C. ribicola*.
Figure 4. Results of non-metric multidimensional scaling (NMDS) analysis of foliar fungal endophyte communities in seedlings inoculated with blister rust that were susceptible to blister rust disease (red circles) or resistant (blue circles), control seedlings that were never inoculated (triangle), and all seedlings before inoculation (diamond). Shapes indicate centroids and error bars represent standard error (stress = 16.93, n=132).
Figure 5. Rarefied richness, Fisher's alpha and evenness measurements of *Pinus albicaulis* seedlings before inoculation with *Cronartium ribicola* spores compared with susceptible and resistant seedlings after infection and control seedlings that were never inoculated.
Figure 6. NMDS of fungal endophyte communities in *Pinus albicaulis* seedlings after inoculation with *C. ribicola*. Shapes represent centroids for seed family regions and error bars represent standard errors (stress = 17.95, n = 123)
Table 1. Summary of generalized linear mixed models (GLLMs) for white pine blister rust disease severity, showing the effects of defensive compounds in 123 *P. albicaulis* seedlings. Bold *P* values are significant.

<table>
<thead>
<tr>
<th>Compound</th>
<th>χ²</th>
<th>P-value</th>
<th>Compound</th>
<th>χ²</th>
<th>P-value</th>
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<td>germacrene</td>
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<td>humulene</td>
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<td>0.71</td>
<td>β-caryophyllene</td>
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<td><strong>0.04</strong></td>
<td>γ-cadinene</td>
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<td>0.54</td>
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Appendix A

Table 1. Information on seed families used in this study including coordinates of parent trees and the number of seedlings from each family that developed cankers (susceptible seedlings). Control seed families are in bold.

<table>
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<th>Seed Family</th>
<th>State</th>
<th>Forest</th>
<th>Region</th>
<th>Latitude</th>
<th>Longitude</th>
<th># seedlings</th>
<th># susceptible</th>
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Total: 141 100
Table 2. Raw differential abundances of individual OTUs between *Pinus albicaulis* seedlings before inoculation, after inoculation and those not inoculated (controls).

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<tr>
<th>Taxa</th>
<th>Bonferonni p-value</th>
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<th>After Infection</th>
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<tr>
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