Symbionts of Woody Plants in the Pacific Northwest

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AUTHORIZATION TO SUBMIT THESIS

This thesis of Shannon J. Fraser, submitted for the degree of Master of Science with a Major in Natural Resources and titled, "Symbionts of Woody Plants in the Pacific Northwest," has been reviewed in final form. Permission, as indicated by the signatures and dates below, is now granted to submit final copies to the College of Graduate Studies for approval.

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ABSTRACT

Microorganisms form endosymbiotic relationships with hosts. Better understanding of fungal microbial communities is important to science as these microorganisms affect larger ecosystems. This thesis explores fungal symbionts in woody trees, mainly black cottonwood, in the Pacific Northwest.

In the first chapter, we report a lichen *Xanthoria parietina* in Idaho for the first time. Non-native lichens have the ability to decrease native lichen diversity through displacement of local species. This coastal lichen has is now found in inland cities but is restricted to urban areas.

Next, we found the causal agent of an unknown leaf blight along the Yakima River, Washington. With multiple surveys and greenhouse assays, we see causal agent is an endophyte that may cause disease only under stress.

Lastly, we clarify the ambiguity of functional roles of endophytes in multiple hosts. Sampling shows multiple incidences of endophytes as pathogens in other hosts and a greenhouse assay confirms this.

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DEDICATION

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CHAPTER 1

Introduction of the Microbiome

Symbioses

Many plants today have symbiotic relationships with another organism. In this thesis, we are going to talk about lichens and foliar fungi particularly. A lichen can be two or three organisms consisting of a mycobiont and photobiont. The photobiont can be an alga, cyanobacteria, or both. Lichens are an essential part of the ecosystem and valuable to society for many reasons (McCune and Geiser, 2009). As is typical for lichens, the distribution of *X. parietina* depends on environmental variables such as bark pH, air quality, and distance to a water source (McCune and Geiser, 2009). Lichens are good bioindicators of air quality as species are either sensitive or tolerant to pollution.

The lichen *Xanthoria parietina* has a widespread distribution in Asia, Africa, Australia, and Northern Europe. Records of this species in the United States have been limited to coastal areas, hence its common name, "the maritime sunburst lichen" (Brodo et. al., 2007). This study will report the distribution of *X. parietina*.

Varying Roles of Pathogens and Endophytes in Different Hosts

A microbiome is an array of ecosystems whose communities of microorganisms have adapted to reside in that environmental niche. Communities of microorganisms include a diverse and ubiquitous spread of species from fungi, to bacteria, to nematodes. In some plants, such as grasses, the microbiome is more explored and the microorganisms' ecological roles are more understood. The microbiome of trees, specifically poplar, is poorly understood in comparison (Bálint et. al., 2013). The microbiome may be considered small but its impact is colossal and diverse.

Plant pathogens, diseases which cause harm to a specific host, cause an array of negative effects on communities from mortality, reduced fecundity, drive host population dynamics, and affect the overall structure and composition of natural plant communities (Gilbert, 2002). While they cause damage to plants, they also benefit the community by helping to maintain plant species diversity, facilitate successional processes, and improve the genetic diversity and structure of host populations (Gilbert, 2002). Whether it is severe

damage or inconspicuous damage, disease on poplar will be present in stands (Newcombe et. al., 2001).

Along with pathogens, studies have demonstrated the array of effects endophytes can have with pathogens, including suppression of disease microbes (Busby et al., 2015). An endophyte is an endosymbiont, such as a fungus or bacterium, which lives symbiotically within a host without causing disease symptoms (Stone et al., 2000). The functional importance of these microorganisms needs to be better understood to comprehend the complex mechanical workings occurring within microbiomes. Foliar microbiomes within *Populus trichocarpa* Torr. & A. Gray, also known as poplar or black cottonwood, need to be researched to understand their perviousness to disease.

Populus trichocarpa is the foundational species of natural, riparian communities throughout the Pacific Northwest (PNW), and the global model for woody plant genomics (Hinckley et. al., 1998). Fast-growing hybrids of *P. trichocarpa* are exploited commercially in plantations from which pulp, solid wood and energy products are produced (Hinckley et. al., 1998). One way to help maintain natural stands and plantations of poplar, is to explore the interactions fungal leaf endophytes with pathogens, and evaluate their role in plant disease.

Lastly, in addition to pathogens and endophytes, there are also spillover pathogens. Pathogen spillover is when a pathogen, that is known to specialize on a susceptible plant, is found in another, more tolerant host often without causing any symptoms of disease (Power and Mitchell, 2004). The spillover pathogen is referred to as endophytic within this tolerant host because of its asymptomatic presence, yet the host can hold a reservoir population of pathogenic propagules that can transmit the disease to the susceptible host (Beckstead, 2010). It is important we identify and try to understand why spillover pathogens are within plant tissues to know what interactions are occurring within the community and if unnatural areas are affecting natural stands of poplar, and possibly the future of the community composition there. Nonetheless, these three groups, pathogens, endophytes, and spillover pathogens, need to be better understood to comprehend the complex mechanical workings occurring within microbiomes which influence larger biomes.

A Poplar Pathogen: *Valsa sordida*

Valsa sordida Nitschke is a severe pathogen of poplar and to a range of angiosperm hosts, but in its history it has only caused infection on stems or branches as a canker (Bier,

1961; Kristen et al., 2014; Kepley & Jacobi, 2000). Known as blackstem disease, *Cytospora* canker, or *Valsa* canker, *V. sordida* is an opportunistic fungi that typically infect trees that are distressed. Cankers appear as slightly sunken, often elongate discolored areas. At first infection, the bark turns orange or brown and becomes black, hence the common name blackstem, and appears roughly pimpled (Callan, 1998). *Valsa* can cause girdling of stems, killing the plant above the canker. Cankers of *V. sordida* were first recorded and reviewed in North America and Europe by Schreiner (1931).

Bier (1961) found that the turgidity of bark were indicators of disease susceptibility for canker pathogens. In all of his pathogens and hosts, no canker occurred at a relative turgidity higher than 80% (Bier, 1961). Those studies were followed up by research proving this moisture content level alongside observations showing that cuttings did not produce as much suberin as those with higher moisture content (Butin, 1955).

Factors including moisture content, region of the shoot, age of shoot, temperature, relative humidity, and soil moisture content was studied by Bloomberg (1962a) on *P. trichocarpa* and two of its hybrids. He found that the critical bark moisture deficit for infection was lowest in *P. trichocarpa* and that under drought the canker would continue to grow rapidly, girdle the plant, and kill it (Bloomberg, 1962a). However, once the girdled plants were watered regularly they put out new shoots and a year later there was healthy shoot growth and no further advance of the canker (Bloomberg, 1962a). After addressing those environmental factors on the disease, Bloomberg (1962b) researched the interaction of the disease on its host. He found that hybrids had 50% wider piths, 30% wider vessels, longer phloem rays, wider sieve tube zones, thicker periderms with 40% more phellem cells and fibers covering 50% more area in the bark (Bloomberg, 1962b). He also, as previously found, documented hybrids with a higher moisture content level that led to their greater resistance in canker infection compared to *P. trichocarpa* (Bloomberg, 1962b).

Bloomberg and Farris (1963) furthered moisture related research and showed that cuttings grew faster when at a 33% saturation rate and the cell counts, sizes and deposition of tannins and lignification differed depending on moisture. During these canker studies, *P. trichocarpa* was compared to the hybrids. Compared to the hybrids, they observed more lignified cells, lighter deposition of tannins, disorganized cells that appeared loosely arranged, and cells that were about 40% larger (Bloomberg and Farris, 1963). Although the *P. trihcocarpa* had larger cells, the hyrbids had more cells in a zone, even when the zones were

the same widths (Bloomberg and Farris, 1963). Their lack of discovery in suberization, led to their inference of that process as a secondary stage in arresting canker growth, compared to the primary tannin deposition. (Bloomberg and Farris, 1963). In all, their work resulted in showing the significance of cell structure and tannin deposition as resistance factors for the invading hyphae of *V. sordida*.

More work was done on the histopathology of the canker disease in 1983 to show a detailed analysis of the infection of cankers' invasion in the xylem by their hyphae and necrosis of the cambium (Biggs and Davis). The canker disease invades nonsuberized tissue and causes necrophylactic periderm with redifferentiation of new phellogen and cambium (Biggs and Davis, 1983).

Next, more research was done analyzing environmental factors effecting *V. sordida* in greenhouses, labs, and field assays by studying trees under drought, flooding, or defoliation on *Populus* (Guyon et al., 1996). Studies agreed again that a negative water potential, in this study peak canker expansion at -1.6MPa, was associated with canker growth (Guyon et al., 1996). The stress of drought and defoliation both increased the size of cankers significantly, while flooded trees did not increase canker growth but still had some mortality (Guyon et al., 1996). This was the first paper to address defoliation as a stress and consider management of trees while considering water levels, stem and foliar diseases, and insect damage.

A paper by Kepley and Jacobi (2000) addressed how *V. sordida* caused cankers on multiple hardwoods, but the disease was dependent on the combination of the fungal isolate and tree species. The canker disease of *V. sordida* is ubiquitous on hardwoods, one that is managed in nurseries, and is of growing concern for native poplar. In Colorado, foresters have seen mortality increase in areas by percentages larger than 50% between 2005 and 2006 for aspen dominated forest (Worrall et al., 2008). There are multiple factors to which blame this mortality including *V. sordida*, aspen bark beetles, poplar borer, bronze poplar borer, and predisposing factors such as climate and acute drought (Worrall et al., 2008).

In conclusion *V. sordida* is a damaging canker pathogen. The mechanisms of *Valsa* canker infection have been studied and researchers concluded that environmental factors largely effected its presence. Pathogenesis has been seen exacerbated by stresses like extreme weather or pathogens can attack healthy hosts. Invasion of less pathogenic species, such as *Armillaria gallica*, can be brought nearly entirely dependent on host stress (Rishbeth, 1982). A range of studies have shown that fungi can exist for years within healthy xylem of various

broadleaved trees as latent invaders or endophytes until damage to the host, such as drought, that allows them to extend by pathogenic growth within the sapwood and into the overlying bark (Bassett and Fenn, 1984; Carroll, 1988; Chapela, 1988; and Hendry, 1993). For *V. sordida*, saturation rate was a main factor that effected the size of cankers, structure and composition of cells. This relation to environmental factors, primarily drought, shows how climate can impact its disease.

Climate

Climate change is occurring globally and altering climates for disease. With climate change, it is of ever increasing importance to understand the interactions of the microbiome with its larger system. The geographic range of trees and fungi are delineated by factors such as temperature, moisture, elevation, and wind (Lonsdale and Gibbs, 1996). Species will only occur in an area if these factors create a suitable habitat for its reproduction and dispersal. Pathogens occur in ranges where the habitat is suitable and where there is a potential host. Climate change will alter disease incidence and severity by its alteration on the environment (Lonsdale and Gibbs, 1996). We know that diseases are sensitive to environmental change. For example, *Melampsora allii-populina*, also known as rust, is sensitive to temperature fluctuation and is actually more thermophilic than its closely related species *M. larici-populina* at some stages in its life cycle (Somda and Pinon, 1981). Changes are all speculative until we see them occur, but we do know facts about fungal spores in general needing wetter and low humidity areas to reproduce. This tells us that in cool-temperature maritime areas, diseases may be more present in the early spring although dispersal of disease could be lower in a drier summer (Lonsdale and Gibbs, 1996). Changes in regions of snow pact latitudes to rainier climates could also alter disease significantly with a warmer and rainier winter. Increased temperature during the summer with drought could be expected to shift the distributions of fungi northward towards a range of potential hosts (Lonsdale and Gibbs, 1996). Applying the mechanism of most diseases, forests that will have more frequent disease in drier and hotter summers which will increase tree mortality, which will open the canopy to increase wind exposure and cause more stress to the remaining trees (Lonsdale and Gibbs, 1996).

Endophytes as Pathogens in Other Hosts

Plant disease studies have researched pathogens in single host species to show diseases' host range. Yet, as we begin to understand pathogens more, we see that pathogens occur in multiple host systems (Dobson and Foufopoulos, 2001; Holt et al., 2003; Power and Mitchell, 2004; Woolhouse et al., 2001). Pathogens occur in hosts and cause disease, but pathogens also occur in hosts as endophytes and do not cause disease. The latter is referred to as a spillover pathogen.

It is difficult to define microorganisms into clear categories. Pathogens cause disease symptoms in a single host (Hammond-Kosack, 1996) or multiple hosts (Woolhouse et al., 2001). Historically, endophytes were latent saprotrophs which did not affect hosts (Ainsworth, 1971; De Barry, 1879; De Barry, 1866; Ellis 1972, Schultz et al. 1999). Today, endophytes are considered to be endosymbionts which live symbiotically within a host without causing disease symptoms (Carroll, 1988; Stone et al., 2004). Historically, spillover pathogens occur in tolerant hosts without causing disease symptoms. This study defines this, 'tolerant host,' hereafter as a, 'spillover host.' A tolerant host refers to a host which is infected by a pathogen and its fitness effected as it has developed a resistance to the pathogen (Horns and Hood, 2012), whereas a spillover host simply holds a reservoir population of pathogenic propagules. These propagules can then transmit the disease to a susceptible host (Beckstead, 2010) and cause disease (Dazak; Power and Mitchell, 2004). Susceptible hosts impose a higher disease burden upon vulnerable competitors and play a large role in apparent competition (Horns and Hood, 2012), but little is understood about interactions within the host range of spillover pathogens. While spillover pathogens are well studied in host systems involving humans and domesticated animals or plants (Power and Mitchell, 2004), a huge amount of work needs done to improve the understanding of spillover pathogens, especially in natural plant communities. The dynamics of pathogens in multiple systems are significant for conservation of the natural landscape, production of agricultural crops, and human health.

Many molecular studies represent spillover pathogens in community analysis, but studies do not show the ecological role or the significance in the occurrence of spillover pathogens within hosts. A justification for this shortcoming in research is probably due to the difficulty in isolating some fungi (Schulz, 1993) and furthermore proving pathogenicity through Koch's Postulates (Ganley et al., 2004). For example multiple *Botrytis* species were found in knapweed (*Centaurea stoebe*) during an endophyte community analysis (Shipunov et

al., 2008) but *Botrytis* has never been recorded as a pathogen of knapweed (Farr et al., 2016). Species in the genus *Botrytis* are known pathogens which cause mold on many hosts. However, in the study by the *Botrytis* was residing as an endophyte, and the study concluded that the species found had evolved from parasites (Shipunov et al., 2008).

Many studies have explored plant tissues' microbial communities (Arnold, 2000; Arnold, 2007; Busby et al. 2015; Ganley et al., 2004; Lamit, 2014) as fungal microbes exist ubiquitously in leaves, stems and roots (Rodriguez et al. 2009). Leaves are a commonly studied tissue (Arnold, 2007; Busby et al., 2013a; Busby et al., 2013b; Busby et al., 2015) due to the abundance of diversity of the microbial community in foliage compared to other plant tissue. The abundance and diversity of microbes in leaves could be attributed to the optimal defense theory which assumes that defenses are both beneficial and costly depending on the presence of predators, parasites, or pathogens. Studies have shown that higher foliar nitrogen percentages will increase photosynthesis and growth rates (Reich et al. 1997, Wright et al. 2004) which will benefit the plant in the absence of pathogens. In the presence of herbivory in grasses, plants will contribute some of its nitrogen to endophytes, at a cost, and maintain a lower foliar nitrogen percentage to remain less palatable (Sullivan et al. 2007). Cost of microbes in leaves has also shown to be beneficial when pests are present in which endophytes decrease mechanical damage and can decrease insect reproduction (Bultman et al. 2004). Microbes are also modifiers of disease severity and can be beneficial in the presence of pathogens (Freeman and Rodriguez 1993; Arnold et al. 2003, Busby et al. 2013). Therefore it seems that microbes will reside in leaves at a cost to the plant, depending on the plant species and presence of defense.

Severe or inconspicuous damage from disease will be present in poplar, *Populus trichocarpa* Torr. & A. Gray (Newcombe et. al., 2001). It is because of this disease presence that the fungal foliar microbial community was studied in poplar (Busby et. al. 2015). Understanding the microbiome of poplar is important as it is a foundational species of riparian communities and hybrids of poplar are grown in plantations for biofuels (Ceulemans et al. 1992). While Busby et. al (2015) focused on the diversity of endophytes, this study focuses on spillover pathogens within poplar.

In short, the microbe community is complex. Along with pathogens and endophytes, a plant can have microbes that host a reservoir population of other microbes which can cause disease on other hosts (Power and Mitchell, 2004). There have been numerous studies in

agronomy that show examples of species in natural habitats that serve as reservoir populations to agricultural lands (Wisler and Norris, 2005). Bias of pathogen spillover is apparent in agricultural studies while less attention has been given to how spillover pathogens effect natural areas (Power and Mitchell, 2004). It is important that we identify, try to understand why spillover pathogens are within plant tissues, and analyze what possible interactions they are having within the microbiome. There are many examples where habitat modification to managed, unnatural areas, has led to spillover pathogens into new natural directions and the effects have been largely underestimated (Blizter et. al., 2012). It is important to know if unnatural areas, such as agricultural lands, are affecting natural stands of poplar, and possibly the future of the community composition there. Disease movement from semi-natural habitats to domesticated plants and animals has been recorded (Daszak et al., 2000). A study found that 77% of pathogen infecting mammalian livestock and 90% of pathogens infecting domestic carnivores were pathogen generalist (Cleaveland et al., 2001). However, not all spillover pathogens are damaging. Many have been looked to use for biological controls and some studies have focused on management of unnatural areas that include an extra field habitat to promote spillover of beneficial pathogens (Pell et. al, 2008). *Fusarium culmorum,* is a pathogen which has never been found as a pathogen of poplar, but my work illustrates its significance as a spillover pathogen.

A Grass Pathogen: *Fusarium culmorum*

Fusarium culmorum (W.G. Sm.) Sacc. is an aggressive disease of grasses in agriculture and in the wild (Urban et al., 2002). It is the cause of ear blight disease on cereal crops worldwide and in Urban's study (2002) they found it infected tobacco, tomato, soybean, and *Arabidopsis* Heynh. It causes brown, necrotic lesions at the base of culms in grasses and eventually mortality in seedlings as well as the ability to cause cereal ears to turn light brown with a mass of pink conidia on the ear tissue (Urban et al., 2002; Kang and Buchenauer, 2000). There are many species of *Fusarium* and every species is different. *Fusarium culmorum* was found to have lower incidence of infection with optimum temperatures of 18°C and 26.5°C and infection is associated with cooler, wet, humid conditions (Xu and Nicholson, 2009).

Fusarium species are well adapted for invasion. The genus known to be able to produce the toxine fusicoccin, which can open plant stomata for plant invasion and reverse the

fighting effects of syringomycin (Duffy et al., 2003). *Fusarium* also can produce multifunctional metabolites that offer protection from abiotic environmental stress or produce secondary metabolites that are toxic to plants and animals fusaric acid, trichothecenes, fumonisins or enniatins (Roncero et al., 2003). Mechanisms in general of how pathogens infiltrate their hosts are variable. The evolution of the plant immune response has accumulated a highly effective defense system that can make it resistant to microbial pathogens, but pathogens have also evolved to fight these defense systems. Plants are constantly exposed to microbes and pathogens can invade plants by wounds or natural openings. Pathogens invade plants by some means of suppression of PAMP-triggered immunity (PTI) that interfers with recognition at the plasma membrane or by secreting effector proteins in the plant cytosol that alter resistance signaling of resistance responses (Chisholm et al., 2006). The mechanics of the root-infecting pathogen *Fusarium* Link include: sensing stimuli from the plant and responding with morphological or biochemical changes, breaking down the cell wall by the secretion of cell wall-degrading enzymes (CWDEs), detoxifying the plants secondary metabolites with antimicrobial activity, produce phytotoxins as virulence determinants, and then maintaining the ability for the *Fusarium* to reproduce (Roncero et al., 2003). Many questions remain that question how these compounds facilitate fungal interactions within host plants.

Research is still going into how to use biological controls against *F. culmorum,* as well as the potential use of *F. culmorum* against invasive grasses ((Xu and Nicholson, 2009; Dooley and Beckstead, 2010). Spread of spillover pathogens could be used beneficially to decrease the invasive species cheatgrass (*Bromus tectorum*) and North Africa grass (*Ventanata dubia*), as bioncontrols have been used on those grasses (Dooley and Beckstead, 2010). More research is studying the use of biocontrols in ecosystems, but we do have research on plant pathogens that has taken place for many years showing a variety of effects they have on systems including: the Janzen-Connell hypothesis, plant-soil feedbacks, competition-defense trade-offs, escape of invasive plants from their enemies, and epidemic-driven community shifts (Mordecai, 2011).

CHAPTER 2

Xanthoria parietina in the Inland Pacific Northwest

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Introduction

Xanthoria parietina (L.) Th. Fr. is a lichen found primarily on woody plants. In North America it has been reported from coastal areas from Newfoundland to Pennsylvania along the Atlantic coast, and from California to the Pacific Northwest along the Pacific coast (Brodo et al, 2001). It has also been reported from a small part of the Gulf coast in Texas. In *Macrolichens of the Pacific Northwest*, the lichen's range within the region is described as west of the Cascades, from the Willamette Valley to the Puget Trough (McCune and Geiser, 2009). More recently, it has been reported in an inland site in western Montana (McCune et al., 2014), and that stimulated our surveys.

As is typical for lichens, the distribution of *X. parietina* depends on environmental variables such as bark pH, air quality, and distance to a water source. Due to their sensitivity to pollution, lichens are known indicators of air quality. Surveys for *X. parietina* must, of course, distinguish it from similar, yellowish orange lichens on woody plants, and in the inland Pacific Northwest the similar species are *Xanthomendoza hasseana, Xanthomendoza fulva,* and *Xanthoria polycarpa.*

Xanthoria parietina is a eutroph which colonizes woody plants in areas with high levels of nitrogen deposition. This lichen is typically found in areas with nitrogen deposition levels between 4.5 and 8 kg/ha/yr, which illustrates its tolerance for nitrogenous pollutants (McCune and Geiser, 2009). In addition, *X. parietina* has been found in areas polluted with 15-30 ppb of sulfur dioxide annually which illustrates its intermediate tolerance to this pollutant (McCune and Geiser, 2009). Both nitrogen and sulfur dioxide are products of industrial and automobile pollution that can accumulate in the environment (Alejo, et. al., 2013). Thus, increasing amounts of air pollution could increase rates of colonization and growth of *X. parietina* in new regions, including inland areas. Some lichens, known as oligotrophs or mesotrophs such as *Alectoria and Usnea*, exhibit greater sensitivity to

pollutants and/or lower growth rates in polluted areas (McCune and Geiser, 2009). Such pollution sensitive lichens could eventually be displaced by *X. parietina* (McCune and Geiser, 2009; Gadson, et. al., 2010; Silberstein, et. al., 1996; Brodo, et. al., 2007). *Xanthoria parietina* also has a second advantage over pollution-sensitive lichens: a relatively large thallus (to 8-10 cm) with wide (1-7 mm) lobes (Figure 2.1) (McCune and Geiser, 2009). It seems plausible to hypothesize that a relatively large, pollution-tolerant lichen like *X. parietina* will eventually displace smaller, sensitive competitors on woody plants in more polluted areas like cities (Gadson, et. al., 2010). Recently, *X. parietina* has been monitored in coastal areas by the volunteer activities of people connected with iNaturalist (2009-2015: Alien Lichens of the Pacific Northwest, iNaturalist).

Figure 2.1. Thalli of *X. parietina* collected in the University of Idaho Arboretum, Moscow, Idaho.

Having confirmed *X. parietina* for the first time in the University of Idaho Arboretum in Moscow, ID, we hypothesized that *X. parietina* was introduced on woody plants purchased from coastal nurseries, and that it would therefore be more abundant in the Arboretum than in natural areas outside Moscow. We thus surveyed woody plant species that were well represented both in the UI Arboretum and also in natural forest on Moscow Mountain, and further surveyed inland Northwest cities other than Moscow, and planting stock in a coastal nursery.

Materials and Methods

During a survey of lichens on woody plants in the University of Idaho Arboretum, *X. parietina* was discovered. Specimens were collected, and sent to Dr. Bruce McCune for confirmation and then deposition in the Oregon State University herbarium. To test the hypothesis that *X. parietina* was introduced on planting stock from coastal nurseries, additional surveys were performed. First, to confirm that coastal nurseries have planting stock that is already colonized by *X. parietina* we inspected stock for sale in a nursery in Sherwood, OR southwest of Portland. Secondly, we surveyed other inland cities in which coastal nursery stock is commonly planted: Coeur d'Alene ID, Boise ID, Pullman WA, and Spokane WA. Thirdly, we surveyed naturally occurring, woody plants in and around Moscow.

Surveys in and around Moscow were limited to native, angiosperm trees of Idaho, as *X. parietina* is most often found on woody angiosperms and we wanted to analyze the same woody species within the UI Arboretum and in wild stands (McCune and Geiser, 2009). In and around Moscow there were seven sample sites. Three sites had planted trees: East City Park (46° 43' 59.54"N, 116° 59' 24.47"W), Latah County Fairgrounds (46° 43' 26.36"N, 116° 58' 56.55"W), and the UI Arboretum (46° 43' 8.68"N, 117° 0' 58.33"W). Two sites had wild trees: Idler's Rest
(46° 48' 1.13"N, 116° 57' 9.92"W) and the SouthSide of Moscow Mountain (46° 47' 42.41"N, 116° 52' 33.86"W). At two sites the status of either planted or wild trees was not clear: the edge of an agricultural field near Idler's Rest (46° 47' 14.74"N, 116° 57' 56.32"W) and Heron's Hideout (46° 43' 25.98"N, 116° 58' 43.78"W). Nine species were surveyed for *X. parietina* in the UI Arboretum and at least one other site: *Acer glabrum* Torr., *Alnus tenuifolia* Nutt.,

Amelanchier alnifolia (Nutt.) Nutt. ex M. Roem., *Artemisia tridentata* Nutt., *Crataegus douglasii* Lindl., *Holodiscus discolor* (Pursh) Maxim., *Populus trichocarpa* Torr. & A. Gray, *Prunus virginiana* L., and *Sambucus nigra* L. In the Arboretum there were five individual trees of three additional species: *Populus deltoides* W. Bartram ex Marshall, *Populus tremuloides* Michx., and *Purshia tridentata* (Pursh) DC; these three were not found in the six other sites and were not included in table 2.1. In all there were 49 and 66 individual woody plants in planted and wild sites, respectively. Surveys were performed by the first author. Each tree was observed for one minute for the presence or absence of *X. parietina* and the three other nitrogenous lichens (*Xanthomendoza fulva* (Hoffm.) Søchting, Kärnefelt & S. Kondr., *Xanthomendoza hasseana* (Rasanen) Sochting, Karenfelt & S. Kondratyuk, and *Xanthoria polycarpa* (Hoffm.)
Rieber).

Results

Xanthoria parietina was found on all 12 woody plant species surveyed in the UI Arboretum. Of the 35 individual Arboretum plants surveyed, 25 were positive for *X. parietina.* In contrast, only one other plant of the other 159 surveyed was positive for *X. parietina* (Table 2.1). That one was an *Acer glabrum* individual in East City Park. In wild or natural sites outside the city of Moscow, *X. parietina* was not found. The lichen was found in urban areas of the inland Northwest other than Moscow: Coeur d'Alene and Boise (Roger Rosentreter, personal communication), in Idaho; Pullman and Spokane, in Washington. In each of these additional inland cities, the local pattern of distribution was as in Moscow: *X. parietina* was only within city limits and commonly in parks on hardwood ornamental trees that were planted.

Table 2.1. Surveys for *Xanthoria parietina* and any one of three similar species (*Xanthomendoza fulva, Xanthomendoza hasseana*, and *Xanthoria polycarpa*), were conducted in seven sites in and around Moscow, Idaho. For each tree species, each column shows the total trees present with any of the three similar lichen species (column 1), *X. parietina* (shaded column 2), and the total trees surveyed at each site (column 3). All woody species in the Arboretum and East City Park sites were planted; all those surveyed in the Idler's Rest and South-Side sites were wild.

Planted, wild, or ambiguous trees	Location	*Acer glabrum*			*Alnus tenuifolia*			*Amelanchier alnifolia*			*Artemesia tridentata*			*Crataegus douglasii*			*Holodiscus discolor*			*Populus trichocarpa*			*Prunus virginiana*			*Sambucus nigra*			Totals		
Planted	Arboretum	3	2	5	1	1	1	5	4	5	1	1	1	3	2	4	7	3	7	2	2	2	3	3	3	2	2	2	22	20	30
Planted	East City Park	4	1	6																									4	1	6
Planted	Latah County Fairgrounds																			8	0	8							8	0	8
Wild	Idlers Rest	6	0	14				3	0	3				0	0	2	0	0	8				6	0	7				15	0	34
Wild	South side Moscow Mtn				7	0	10	5	0	7	2	0	6				0	0	4							5	0	5	19	0	32
Ambiguous	Agricultural field near Idlers Rest													0	0	5				6	0	6							6	0	11
Ambiguous	Heron's Hideout				5	0	6	5	0	5				6	0	6	4	0	4	10	0	12							30	0	33
Totals		13	3	25	13	1	17	18	4	20	3	1	7	9	2	17	11	3	23	26	2	28	9	3	10	7	2	7	104	21	154

In keeping with our hypothesis, *X. parietina* was found on nursery stock near Sherwood, Oregon (Figure 2.2). The lichen was abundant on larger trees in 3-10 gallon pots. In Moscow, the other three species of yellowish orange, nitrophilous lichens on trees (*X. polycarpa*, *X. fulva*, and *X. hasseana*) were found on trees both inside and outside the city on all nine species that were surveyed both in planted, urban sites and in sites outside the city of Moscow. On trees sampled within the Arboretum, the other orange nitrophiles were found at an incidence of 90%. On trees sampled outside of Moscow, where *X. parietina* was not found at all, the other orange nitrophiles were found at 52% incidence.

Figure 2.2. *Xanthoria parietina* on a potted *Styrax japonicus* Siebold & Zucc. in a tree nursery near Sherwood, Oregon.

Discussion

The findings are consistent with the hypothesis that *X. parietina* can be introduced to inland areas if propagules are transported on nursery stock from its coastal range. The movement of nursery stock was suggested by Brodo and Lindblom (2007, 1997) as a dispersal method for *X. parietina*. Apart from scattered urban occurrences in inland Idaho, Montana, and eastern Washington, it is clear that *X. parietina* is still primarily coastal within the PNW. In addition to published records, a total of 87 unpublished observations of *X. parietina* (2009-2015: Alien Lichens of the Pacific Northwest, iNaturalist) have confirmed a predominantly coastal distribution. It is likely that *X. parietina* already occurs in other population centers of the inland PNW that we did not survey. More studies are needed to

determine where *X. parietina* occurs within the inland PNW, and whether those populations persist in the long term.

In coastal areas, *X. parietina* can dominate and displace or exclude other nitrophiles (McCune, 2003). In contrast, on UI Arboretum trees its incidence was typically codominant with other nitrophiles. Figure 2.3 shows an exception with *X. parietina* by itself on *P. tremuloides*. Dominance by *X. parietina* and displacement of the native species might eventually become commonplace in Moscow, but it is not obvious at the present time.

Figure 2.3. *Xanthoria parietina* (e.g. circled in red) dominating the lichen community on *P. tremuloides* near a drainage ditch at the Arboretum.

The finding that other nitrophilous lichens decreased in abundance with distance from urban centers and their nitrogen sources was not surprising as that has been researched before (Rogers, et al. 2009; Ra, et al., 2004; Geiser and Neitlich 2006). Competition among nitrophilous tree lichens (Figs. 2.2-2.5) might be influenced not only by nitrogen deposition and microclimate but also by direct interaction among competitors (Velthof, et. al., 2009).

Figure 2.4. Thalli of *Xanthomendoza hasseana* collected in the University of Idaho Arboretum, Moscow, Idaho.

Figure 2.5. Thalli of *Xanthomendoza fulva* collected in the University of Idaho Arboretum, Moscow,

Figure 2.6. Thalli of *Xanthoria polycarpa* collected in a riparian area on the edge of Moscow, Idaho.

We do not know when *X. parietina* first appeared in Moscow, but the UI Arboretum began to be developed in the late 1970s. *Xanthoria parietina* was most commonly found on trees planted during the mid-1990s and later, but the lichen was found on older trees as well. It tended to be at a higher density on the younger trees. The lichen has been found on trees planted within the last three years within a park of the Boise River Greenbelt, whereas the lichen is still absent on the native *P. trichocarpa* near it along the river (Roger Rosentreter, personal communication).

The question of naturalization of *X. parietina* is an important one. At present, there is no evidence for it, as naturalization would only be confirmed by spread of *X. parietina*. Monitoring of the spread of *X. parietina* from planted to local trees is needed since reproduction and spread without human aid is the essence of naturalization. Lichen species diversity in the inland PNW might be at risk if *X.*

parietina does eventually naturalize and behave as it is known to do in coastal areas (Brodo, 2007; Gadson, et. al., 2010; Silberstein, et. al., 1996).

CHAPTER 3

Valsa leaf blight of *Populus trichocarpa*

Introduction

Populus trichocarpa, or western black cottonwood, is common in riparian plant communities throughout the Pacific Northwest (PNW). Its diseases have been well studied west of the crest of the Cascades (Newcombe 1996, Newcombe et al. 2010, Newcombe et al. 2001), in coordination with the development of this model woody plant (Bradshaw 2001, Stanton et al. 2010, Tuskan et al. 2006). Relative to west-side populations, east-side cottonwood populations and their diseases have been neglected. We have, however, recently shown that foliar fungi of *P. trichocarpa* differ east and west of the mountains (Busby et al. 2015a). In that study, a leaf blight was first observed along the Yakima River that drains the eastern slopes of the central Cascades of Washington. Affected trees exhibited leaf blight that was reminiscent of Venturia leaf blight caused by *Venturia inopina* (Newcombe 2003), in that apical and marginal lesions tended to spread to leaf midribs and then down them towards petioles. However, the lesions were unlike Venturia leaf blight in exhibiting concentric patterns within lesions; the asexual state of *V. inopina* was also absent. A third reason to doubt that it was Venturia leaf blight is that the latter has only been confirmed by the senior author west of the Cascades but not east.

Here, we set out to determine the causal agent of the Yakima leaf blight. First, we attempted to complete Koch's Postulates for a Yakima fungus that was associated with leaf blight. Second, we conducted field surveys in five west- and eight east-side populations to determine the distribution of the blight and the wider presence of the putative pathogen in leaves (as revealed by both culture-based and DNA sequencing).

Materials and Methods

Sampling of blighted leaves and putative pathogen isolation.

In June 2014, 45 blighted leaves were collected along the Yakima River from Union Gap northward to Easton, Washington. Leaves were surface-sterilized for two minutes in 1% hypochlorite (NaOCl) solution, followed by two rinses in sterile deionized water (SDW) for 1 min each (Raghavendra & Newcombe 2013). Leaves were then put into petri dishes

comprising five incubation treatments: i) potato-dextrose agar (Difco PDA), ii) PDA with an autoclaved leaf, iii) 1% glycerol agar, iv) charcoal agar, and v) sterile, zippered sandwich bags with a moistened, sterile paper towel. Dishes were stored in lab at room temperature (22°C) with no supplemental lighting.

Identification of putative pathogen.

First, the fungus that commonly developed from blighted leaves was identified on the basis of morphology (Sutton 1980). Secondly, fungal genomic DNA was extracted from an isolate obtained from a blighted leaf (YK24A) using a Qiagen DNEasy plant mini kit (Qiagen Inc., Valencia, CA) according to the manufacturer's directions. Ten mg of mycelia, pycnidia, and conidia were removed from PDA then added to the prepared tubes. The ITS ribosomal region was amplified in polymerase chain reaction (PCR) with the enzyme PrimeSTAR GXL DNA Polymerase and ITS1F and LR3 primers, following the manufacturer's standard protocol. Sanger sequencing followed using forward and reverse sequencing for each sample with an ABI PRISM 3730 Genetic Analyzer.

Inoculation assays.

First in planta assay. Seeds of *P. trichocarpa* were collected from the Yakima River in early July 2014. They were germinated in 4-inch pots in the greenhouse and ten seedlings were transplanted to 1-gallon pots after eight weeks of growth. Plants grew for four months before inoculation. Unhealthy leaves were removed from the plants 48 hours prior to inoculation so that healthy leaves only were subsequently inoculated. Inoculum was made with 3 week old isolates as described earlier. Ten plates of *V. sordida* (YK23A) were used, and 100 ml of SDW added for dilution, to create 150 ml of inoculum.Viability of inoculum was checked by spraying inoculum on PDA. Five control plants were treated with SDW, and five plants were inoculated with an isolate (YK23A) from blighted leaves.

Both surfaces of every leaf of each of the five inoculated plants were sprayed with inoculum and then enclosed in an incubation chamber (1 m x 1 m frame covered in plastic) for 30 hours. Stems were also sprayed. Control plants were treated the same except that they were sprayed with SDW. Following inoculation plants were returned to the greenhouse bench. Four weeks post-inoculation leaves were detached and photographed. In the treatment from blighted leaves, nearly every leaf from each plant was processed for reisolation of *V. sordida*. Control

leaves were processed as well from each plant.. First, half of the leaves from each treatment were surface-sterilized for two mins in 1% hypochlorite (NaOCl) solution, followed by two rinses in SDW for one min each; the other half were not surface-sterilized. Then, leaves were placed into one of the three reisolation treatments: i) PDA, ii) 25% glycerol agar, and iii) sterilized zippered sandwich bags enclosing sterile, moist paper towels.

Second in planta assay. Nine-month-old plants from the Yakima River were used for the second assay. Again, there were two treatments: i) a control (SDW) and ii) inoculation with the same isolate (YK23A) as used in the first *in planta* assay. Inoculated plants were incubated in moist conditions for only twelve hours instead of 30, as in the first assay. Inoculum viability plates were as before. Three weeks after the inoculation, symptomatic leaves were detached, photographed, sterilized, and plated on PDA as described above.

Fungal communities in asymptomatic leaves in 2013 and 2014.

In October 2014 and 2015, leaves of *P. trichocarpa* along ten rivers were sampled by Busby et al. (2015) (Table 3.1). The community was evaluated by conducting molecular studies with next-generation sequencing (NGS) to illustrate the abundance and distribution of foliar fungi (Busby et al. 2015). We are using the dataset to evaluate the abundance and distribution of *Valsa sordida.* Procedures for both years were followed as explained in Busby et al. (2015). At each site, nine asymptomatic leaves of each of six trees were detached. Six were moist-incubated and three were processed for NGS. Operational taxonomic units (OTUs) were selected if identified as *V. sordida* with at least 98% identity and coverage to *V. sordida* (GenBank). Any matching OTU sequences were aligned with the Sanger-sequenced isolate from a blighted leaf (YK24A) using NCBI BLAST. For each river site in both years, the percent incidence in moistened incubation chambers, and the percent molecular sequences of the matching OTU found out of its total OTU sequences were graphed in RStudio (2015) to illustrate the abundance of a single endophyte at riparian sites.

Table 3.1. Riparian populations west and east of the Cascades that were surveyed for leaf blight incidence and fungal communities in asymptomatic leaves in both 2013 and 2014 used in this study from the Busby et at. (2015) data. Asymptomatic leaves were sampled systematically: one site/river; six trees/site; nine leaves/tree. Of each set of nine asymptomatic leaves per tree, six were moist-incubated for morphology-based determinations and three were processed for NGS.

River sites	Location	Annual Rainfall ($cm\ yr^{-1}$)
West-side		
Skagit	48.51, -121.90	180
Snoqualmie	47.53, -121.81	157
Dosewallips	47.70, -122.90	137
Carbon	47.10, -122.16	112
Nisqually	46.85, -122.36	112
East-side		
Tieton	46.67, -121.04	66
Kettle	48.82,-118.17	46
Little Salmon	45.23, -116.33	43
Clearwater	46.44,-116.91	33
Yakima	46.92, -120.51	25

Survey for Valsa leaf blight.

In July 2015, the Yakima and three, additional east-side rivers (the Entiat, Methow, and Wenatchee) were surveyed for Valsa leaf blight incidence. One minute was spent looking for blight in each tree. Symptomatic leaves were detached from all blighted trees and moist-incubated for confirmation of *V. sordida*.

Results

Sampling of Yakima leaf blight and putative pathogen isolation.

Blighted branches were scattered across the crowns of affected trees. Nearly all the leaves on affected branches showed symptoms (Fig. 3.1A). *Venturia inopina* was not isolated in any of the four incubation treatments (PDA, PDA with an autoclaved leaf, 1% glycerol agar, and charcoal agar). But there was a high-frequency isolate of a particular cultural morphology that was then considered to be the putative pathogen in need of identification and confirmation, via Koch's Postulates.

Figure 3.1. (A) Characteristic field symptoms of *Valsa* leaf blight along the Yakima River. Field symptoms that are also associated with *V. sordida* are (B) browning along the Methow River, (C) spotting along the Methow River, (D) lesions along the Wenatchee River, and (E) midrib and petiole blackening of leaves along the Yakima.

Confirmation of pathogen identity.

Valsa sordida was isolated from every symptomatic leaf based on pycnidial and conidial morphology; conidia of all isolates were hyaline, aseptate, allantoid and 5 by 1 µm.

Sanger-sequenced isolate YK24A was a 98 % match (98 % coverage) to *V. sordida* (GenBank accession AB188679.1).

Pathogenicity tests with inoculation assays.

First in planta assay. Five days post-inoculation, the *V. sordida* treatment from blighted leaves (YK23A) caused spotting on the leaves of all the inoculated plants. By the tenth day, gray necrotic spots were present on nearly every leaf of every inoculated plant (ca. 250 leaves), but lesions characteristic to field symptoms were absent. After the detached leaves were moist-incubated for 14 days, characteristic field symptoms were observed. *Valsa sordida* was reisolated from every symptomatic, inoculated leaf. In surface-sterilized treatments *V. sordida* was the only fungus re-isolated. Control plants remained asymptomatic. After incubation, *Penicillium* species grew on some control leaves. After the detached leaves were moist-incubated for 14 days, characteristic field symptoms were seen. *Valsa sordida* was reisolated from every symptomatic, inoculated leaf. In surface-sterilized treatments *V. sordida* was the only microbe to be re-isolated. Control plants remained asymptomatic. After incubation, *Penicillium* species grew on some control leaves.

Second in planta assay. Twelve days post-inoculation, the *V. sordida* treatment (YK23A) caused spotting on a total of 55 of the inoculated leaves in total from 4 plants. This was out of the 8 plants (ca. 400-500 leaves) inoculated with *V. sordida.* Not all plants bore symptomatic leaves. After the leaves were detached from the plants and incubated for five weeks, seven of the 55 developed characteristic field symptoms. No lesions formed on the control plants.

Fungal communities in asymptomatic leaves in 2013 and 2014.

Moistened incubation chambers. Pycnidia and conidia of *Valsa sordida* formed on asymptomatic, incubated leaves of east-side populations only in both 2013 and 2014 (Fig. 3.2). Wetter, west-side populations did not yield *V. sordida*, and the two wettest east-side populations, (Tieton and Kettle) yielded little to no *V. sordida* (Fig. 3.2). In 2013, the highest east-side percentage was found in the driest site, the Yakima population (75%). In 2014, the Yakima was again highest at 27.78%.

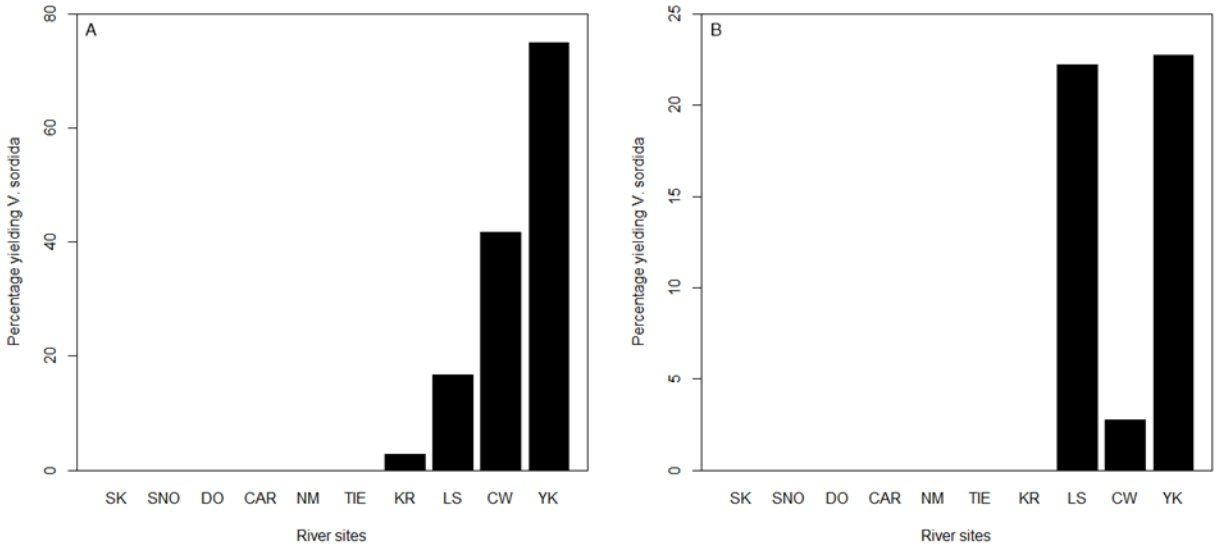

Figure 3.2. Percentage of asymptomatic, moist-incubated leaves in which *Valsa sordida* developed (each riparian site: six leaves per each of six trees, 36 leaves total): (A) in 2013 and (B) in 2014 using Busby et al. (2015) data. Sampling from wettest to driest of five west-side (SK, SNO, DO, CAR, and NM) and five east-side (TIE, KR, LS, CW, and YK) sites revealed an exclusively east-side distribution.

Next-generation sequencing. One OTU matched (99.6%) the Sanger-sequenced isolate of *V. sordida* (YK24A); it was also a 100% match to accession JX978237.1 of *V. sordida* in GenBank. This OTU of *V. sordida* was present in asymptomatic leaves from eight and nine of ten sites in 2013 and 2014, respectively. Of the total sequences reads (6,165,560) excluding singletons (Busby et al. 2015), 0.02% of the sequence reads (1164) was *V. sordida* in 2013; and of the total (3,765,240) sequences reads, 0.09% of the sequence reads (3086) was *V. sordida* in 2014. In 2013 and 2014, of only the *V. sordida* sequences 84.62% and 99.3% of sequence reads were from east-side populations, respectively (Fig. 3.3). Of only the *V. sordida* sequences in 2013, the Yakima (53.78%) and the Clearwater River (29.04%) comprised of the most sequence reads. In 2014, relative abundance in these two sites had shifted: the Clearwater (92.06%) and the Yakima (4.15%).

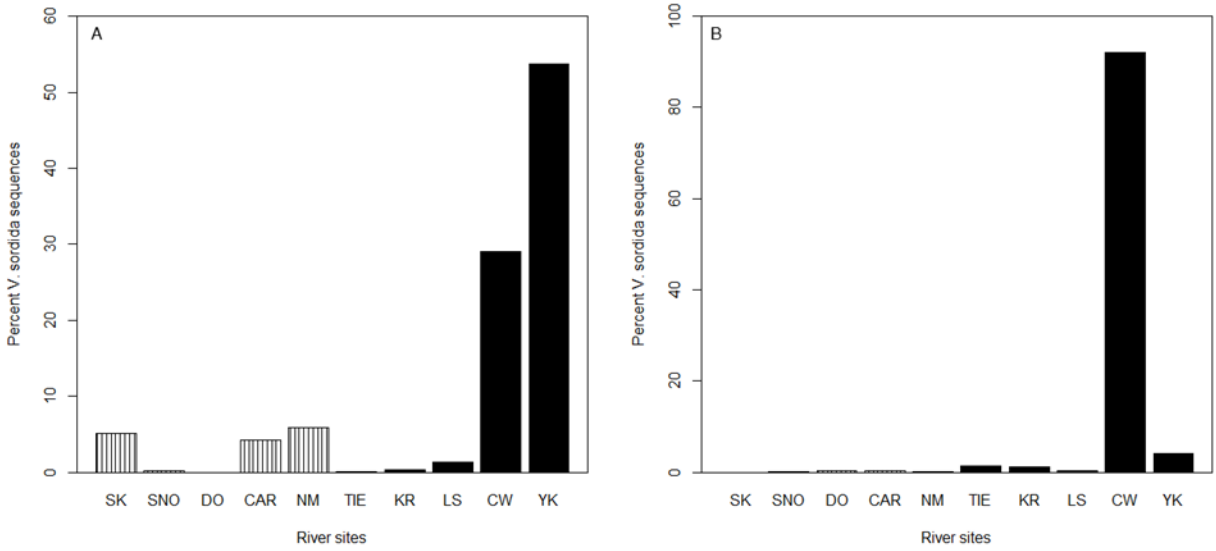

Figure 3.3. Percentage of *Valsa sordida* sequences per site of total *V. sordida* sequence reads overall (three leaves per each of six trees, 18 leaves total, in each riparian site): **(A)** in 2013 and **(B)** in 2014 using Busby et al. (2015) data. Sampling from wettest to driest of five west-side (striped bars: SK, SNO, DO, CAR, and NM) and five east-side (filled bars: TIE, KR, LS, CW, and YK) populations, showing east-side dominance overall.

Of the total sequence reads from 2013, 0.26% were two *Venturia* OTUs; and no *Venturia* OTUs were found in 2014 (Busby et al. 2015). The more abundant of the sequence reads (15, 540) was much more common on the west-side and comprised of 96% of all sequence reads for this common *Venturia* OTU. In contrast, there was little evidence for this *Venturia* in east-side populations where the leaf blight in question was observed. A second, less common, *Venturia* OTU showed the same pattern. Of 796 total sequence reads, 97% of this second *Venturia* OTU were from west-side populations.

2015 survey for Valsa leaf blight.

Of Yakima leaves with symptoms typical of Valsa leaf blight (Fig. 3.1A), *V. sordida* was isolated from 92%. Scattered tree mortality was observed but it could not be attributed to Valsa leaf blight alone since other factors could have contributed. Atypical symptoms of what may also be Valsa leaf blight were observed along the Entiat, Methow, and Wenatchee rivers (Fig. 3.1B-3.1E), three additional east-side rivers. Atypical leaf blight was most commonly observed from Pehastin to Monitor along the Wenatchee River. Along the Entiat River two site close to Entiat were observed. Along the Methow River leaf blight occurred

from Pateros to Winthrop; it was most common in two sites which had been burned recently. In total, isolation frequencies of *V. sordida* in these atypical blighted leaves were: 90% (Wenatchee), 13% (Entiat), and 79% (Methow).

Discussion

Although the appearance of the Yakima River leaf blight initially suggested *Venturia inopina* as the pathogen, our subsequent findings show that *Valsa sordida* is the cause of the leaf blight. *Valsa sordida* was consistently associated with the pathogen and pure cultures caused symptoms after inoculation of healthy trees in the greenhouse. *Valsa sordida* commonly causes stem cankers of species of *Populus*, *Salix*, and more than 30 other genera (Farr et al. n.d.), but this is the first report, to our knowledge, of Valsa leaf blight of any plant.

Weak pathogens exhibit long latent periods during which their presence in asymptomatic tissues may be detected either by isolation in culture or DNA sequencing. Weak pathogens then occasionally cause disease, contingent upon environmental circumstances. Koch's Postulates may therefore be difficult to prove for such pathogens (Fisher et al. 1994; Chapela & Boddy 1988; Priest and Goodfellow 2012; Stanosz et al. 2001). In particular, forest trees seem to host large numbers of endophytic microbes that are frequently described as weak or latent pathogens in the absence of proof of Koch's Postulates. For example, *Dermea tetrasperma* is a pathogen associated with cankers and dieback of many coniferous trees in the Pacific Northwest and also a needle endophyte (Funk 1985). Additionally, *Apioplagiostoma populi* (Smith Ostry and Anderson 2002) is said to cause bronze leaf disease even though the full proof of Koch's Postulates is not possible given its unculturability.

Our findings suggest that *V. sordida* fits the definition of a weak foliar pathogen. *Valsa sordida* as a stem pathogen is associated with what is called 'Cytospora canker' which has also always been a weak pathogen. *Valsa sordida* is known to appear with stem cankers only when trees are stressed. Most often the stress is a lack of water (Biggs et al. 1983, Bloomberg 1962, Bloomberg and Farris 1963, Kaczynski and Cooper 2013, Kaczynski et al. 2014). Thus, Cytospora cankers typically expand with decreasing water potential (Guyon et al. 1996). In *Alnus*, epidemics of Cytospora canker have also been associated with summer heat (Worrall et al. 2010).

At this point, Valsa leaf blight is restricted to east-side populations in the rain shadow of the Cascades. There are several possible explanations for this trend such as: i) climatic

gradients, ii) other microorganisms in the community, or iii) host genetics. Not only is average annual precipitation greatly reduced in east-side sites (Table 3.1), but east-side populations also experience greater summer heat. Asymptomatic presence of *V. sordida* also followed this trend of east-side dominance seen in the leaf-blight surveys. When asymptomatic leaves were moist-incubated, *V. sordida* was common in east-side populations but never seen in west-side counterparts. Only one culture of *V. sordida* was Sanger sequenced, but the morphology of *V. sordida* is distinct for identification. NGS data from the Busby et al. (2015) study shows *V. sordida* was detected in asymptomatic leaves of west-side populations, but incidence was very low each year relative to that of the east side (Fig. 3.2). Not only does the abiotic environment differ but the microbial communities of leaves of east- and west-side populations differ substantially (Busby et al. 2015a). Other members of these communities could influence the colonization of leaves by *V. sordida*, as disease modification is common in nature (Busby et al. 2015b). Host genetic resistance to *V. sordida* is unlikely to be the factor that explains these differences, although resistance to Cytospora canker has been reported to differ between *P. trichocarpa* and its interspecific hybrids (Bloomberg and Farris 1963).

The field symptoms (Fig. 3.1A) and our inoculation experiment link *V. sordida* to this particular manifestation of what we are calling Valsa leaf blight. However, we also observed atypical symptoms (Fig. 3.1 B-3.1E) of what may be the same disease along other east-side rivers. The Methow population was interesting because there was high incidence of these atypical symptoms of *Valsa* leaf blight in trees close to recent burns, again suggesting a possible environmental correlate of a form, at least, of Valsa leaf blight.

Leaves inoculated with *V. sordida* showed symptoms but stems did not, when they were also inoculated. It is possible that *V. sordida* colonized stems but that stem latent periods are longer than leaf latent periods or that environmental triggering for leaf and stem disease differs. It is also possible that the population of *V. sordida* that causes leaf blight is specialized; we did not compare leaf populations to stem populations of the fungus in this study.

NGS studies are sometimes regarded as descriptive only (Jansson and Prosser 2013). It is thus interesting to note that in this study we productively combined the NGS approach with traditional methodologies. Use of the latter allowed us to determine the etiology of the disease whereas the former showed that the poplar leaf blight niche is occupied by different pathogens adapted to either the west-side (*Venturia inopina*) or the east (*V. sordida*). Overall, our

findings here again illustrate the importance to poplar leaf microbiomes of the Cascade Range (Busby et al. 2015a).

CHAPTER 4

Functional roles of pathogens of other plants influence riparian plant communities

Introduction

Disease plays a critical role in plant community ecology and can be more significant in the establishment and survival of plants than the ability of plants to outcompete other plants for resources (Borer et al. 2007). By mediating competitive interactions among host species, specialized pathogens can regulate plant community dynamics (e.g., increasing beta diversity via Janzen-Connell dynamics (Benitez et al 2013; Comita et al 2014; Mordecai 2011) and increasing alpha diversity via coevolutionary arms races (Thompson and Burdon 1992). However, not all pathogens are highly specialized on theirs hosts, with large effects on host fitness and a narrow host range. Some pathogens can be found across a broad range of plants without causing symptoms of disease, and therefore acting as endophytes. The role of these pathogens of other plants are not understood in all the hosts they reside in.

Some fungi can switch between pathogenic and endophytic states depending on environmental conditions and on the host (Comby et al. 2016; Schulz and Boyle, 2005; Sieber, 2007; Malcolm et al., 2013). A pathogen that is known to cause disease on a host can be found in another host as an endophyte without causing obvious disease (Power and Mitchell 2004; Wisler and Norris, 2005). Then it still can be transmitted back to a disease host and cause symptoms (Beckstead, 2010). While the role of an obligate pathogen with a narrow host range has been well researched in ecological theory, only recently have we begun to explore endophyte communities (Arnold, 2000; Arnold, 2007; Busby et al. 2015; Ganley et al., 2004; Lamit, 2014).

It is important to understand the roles of microbes within the community because the role of pathogens and endophytes could potentially help endophytic hosts in a form of apparent competition (Newcombe et al. 2009). Since functional roles of a microbe vary in different hosts, the pathogen's presence can influence the community composition. While in the endophytic host alone, a pathogen of another plant may appear to be neutral, however it

may in fact benefit the endophytic host when transmitted to other plants in the community on which it may cause disease and negatively effect, thereby reducing potential competition

In this study, we enumerate some of the pathogens of other plants that infect asymptomatic leaves of poplar. From these pathogens of other plants, we take one such spillover pathogen and subject it to functional tests. We determine whether it causes obvious disease in a known disease host and also whether it remains endophytic in its endophytic host, in order to understand what may be occurring in regards to competition in plant communities. Then we analyze data from the USDA Fungal Database to broaden our scope of the hosts which this pathogen of another plant may be found functioning as an endophyte or pathogen.

Materials and Methods

NGS data. Busby et al. (2015a) sampled poplar stands along 10 rivers in the Cascade Mountains in order to use next-generation sequencing (NGS) to illustrate the abundance and distribution of foliar fungi in 2013. We used this dataset to determine the frequency of endophytes in poplar trees that are known pathogens in other species. At each site, 6 trees were haphazardly chosen for asymptomatic leaf sampling. Each tree had 9 leaves, between LPI 3-5, detached for a total of 54 leaves per site for next-generation sequencing (NGS) and for placement in moistincubation chambers. See Busby et al. (2015) for more details.

Fungal isolate. In another study, Busby (unpublished) used culture-based methods to characterize the fungal leaf endophtyes of P. trichocarpa from the same poplar sites surveyed in Busby et al. 2015. Leaves were surface-sterilized for two minutes in 1% hypochlorite (NaOCl) solution, followed by two rinses in sterile deionized water (SDW) for 1 minute each (Raghavendra & Newcombe 2013) and then placed leaves on a moist, sterile towel in sterile zippered sandwich bag. Four weeks post-incubation, the presence or absence of endophytes was recorded. Endophytes were identified morphologically to the genus level, and later the ITS region was sequenced using ITS1F and LR3 primers to obtain a species-level identification (Busby et al., unpublished). We used one particular isolate from this effort, *Fusarium culmorum*, a known pathogen of wheat, in our inoculation experiment.

Pathogenicity test of endophyte in its disease host.

Plant material. We sowed three seeds per four inch pot of Sunshine Professional Growing Mix #1 (Sun Gro Horticulture, Sacramento, CA) in a greenhouse for both poplar and wheat seeds. The greenhouse was kept under 16 hour natural and fluorescent light with a monitored temperature of 18°C (daytime) and 15°C (nighttime). The poplar seed came from one single tree in Moscow, Idaho and the wheat seed came from one hard red winter wheat cultivar (University of Idaho line 306 UI-SRG, Lot:1209 Moscow HRWW5). For each species, there was a total of 90 plants. We thinned seedlings out to one seedling per pot.

Greenhouse inoculation assay. For each plant species, we applied three inoculation treatments to 30 plants twelve days after planting. The treatments consisted of i) a wheat pathogen isolate (FCW) used for the positive control, ii) the endophyte isolate (FCP) collected from a poplar leaf in a moistincubation chamber, and iii) a negative control treatment of SDW. The endophyte isolate (FCP) was collected from a poplar leaf in 2012 (SNO 11-1) (Busby et al., unpublished) . The wheat pathogen isolate (FCW) was collected from a lesion on a wheat blade, isolated, and sequenced (Washington State University/ USDA ARS Pullman cereal pathogen collections). We placed each of the isolates onto PDA two weeks before the inoculations for optimal growth. The morning of the inoculation, we opened isolates in a laminar flow hood and poured SDW into the plates. We used a scalpel to remove the mycelium and conidia. We placed the solution in a Tissue Tearor^{TM} to homogenize the mycelium and conidia. We did hemacytometer counts and made viability plates. The FCW inoculum had a spore density of 100,000 spores/ml and the FCP inoculum had 1000 spores/ml.

At the greenhouse, we inoculated plants by pippetting 10 ml of each treatment along the base of each plant and into the soil. After all the plants were inoculated, we moved the pots into a completely randomized pattern amongst each other on the bench.

Due to a limited amount of space in the drying oven, we removed the wheat and poplar from the greenhouse at different times; but each species was processed completely within six hours. Forty-seven days post-inoculation, we photographed all of the wheat plants, cut culms at the base of the plant, placed each plant's above-ground biomass into a labelled manila envelope, and then transported samples to the drying oven.

We placed all the samples into the same drying oven and dried for 48 hours at 60°C. We weighed samples every 12-24 hours until the samples no longer loss any mass. We removed samples from the oven for weighing the next day. We used the same procedure for the poplar plants but we cut the poplar plants 51 days post-inoculation and samples remained in the oven for 70 hours.

Statistical analysis. We used the dry above-ground biomass of each plant for statistical analysis. We analyzed the weight of each plant rather than quantify the lesions formed from *Fusarium culmorum* as each plant produced variable numbers of culms. An F-test was used to evaluate the statistical significance of the treatments' above-ground biomass, with each pot as a replicate (n = 30). We used a linear ANOVA for analysis in RStudio (RStudio Team 2015).

Demonstrating a host range. Using this study and the USDA Fungal Database, we created a figure for the endophyte and pathogen *Fusarium culmorum* in order to illustrate how a microbe may have a wide host range. In the USDA Fungal Database, we went through all of the hosts of *F. culmorum*, categorized them by family, and graphed how commonly *F. culmorum* was found in each family based on how many species were infected per family using RStudio (RStudio Team 2015).

Results

Next-Generation Sequencing. Endophyte sequence reads from NGS showed many specific cases of spillover pathogens that matched with sequences in GenBank of a known disease pathogen. Different OTU sequence reads matched to 21 sequences of pathogens causing disease in hosts of agricultural crops (Table 3.1), one sequence of an endophyte of wheat in *Gnomoniopsis idaeicola* (Table 4.1), and 34 sequences of pathogens in hosts of natural, wild plant communities (Table 4.2).

Table 4.1. Endophyte sequences residing in poplar leaves (column 1) resulting from NGS matches to molecular sequences in GenBank of pathogen studies in agricultural host plants (columns 2-5). The total OTU sequence reads (column 6) represents the abundance of an endophyte's sequences from poplar foliage.

Pathogen of other plant	Disease host plant	Disease symptoms	Location	Accession number from GenBank	Total OTU sequences
Ramularia vizellae	*Brassica* crops	Leaf spot	The Netherlands	EU019285	51,043
Monilinia sp.	*Malus*	Fruit rot	Japan	AB693917	42,128
Neofabraea malicortis	*Malus* and *Pyrus*	Stem canker	The Netherlands	AF141161	30,625
Microcyclospora tardicrescens	*Malus domestica*	Sooty blotch	Slovenia	GU570541	25,323
Phoma macrostoma	*Malus domestica*	Fruit rot	Switzerland	HQ166389	4,210
Phaeosphaeria pontiformis	*Triticum aestivum*	Leaf blight	Sweden	KC989090	469
Phoma macrostoma	*Lens esculenta*	Fruit rot	Canada	DQ474091	468
Cladosporium cladosporioides	*Vitis vinifera*	Fruit rot	Chile	EU622927	428
Diaporthe eres	*Vitis vinifera*	Dieback	California, USA	KF017914	372
Microcyclospora pomicola	*Malus domestica*	Sooty blotch	Germany	GU570539.1	222
Diaporthe cf. nobilis	*Malus pumila*	Dieback	New Zealand	KC343149.1	151
Gnomoniopsis idaeicola	*Actinidia deliciosa*	Spillover pathogen in wheat	France	KT692597	68
Devriesia pseudoamericana	*Malus domestica*	Sooty blotch	Germany	GU570527	62
Phaeomoniella zymoides	*Prunus salicina*	Wood necrosis	South Africa	GQ154600	45
Pyrenophora tritici-repentis	*Triticum*	Tan spot	Japan	AM887495	39

Table 4.2. Endophyte sequences residing in poplar leaves (column 1) resulting from NGS matches to molecular sequences in GenBank of pathogen studies in wild, natural host plants (columns 2-5). The total OTU sequence reads (column 6) represents the abundance of an endophyte's sequences from poplar foliage.

Pathogen of other plant	Disease host plant	Disease symptoms	Location	Accession number from GenBank	Total OTU sequences reads
Ramularia pratensis	*Rumex crispus*	Leaf spot	South Korea	KF251223	139,536
Botrytis cinerea	*Picea abies*	Root rot	Canada	KF859924	66,618
Ramularia eucalypti	*Eucalyptus*	Leaf spot	Australia	EF394862	39,172
Fusarium proliferatum	*Pinus*	Pitch canker	The Netherlands	KM231816	19,980
Fusarium avenaceum	*Phragmites australis*	Leaf blight	New Jersey, USA	KT827258	18,810
Ramularia eucalypti	*Corymbia grandifolia*	Leaf spot	Italy	EF394861	15,955
Colletotrichum phormii	*Phormium*	Leaf blight	New Zealand	DQ286142.1	13,641
Sydowia polyspora	*Pinus mugo*	Needle blight	Lithuania	GQ412724	12,521
Elytroderma deformans	*Pinus ponderosa*	Needle blight	Montana, USA	AF203469	8,259
Ramularia eucalypti	*Eucalyptus*	Leaf spot	The Netherlands	KF251221	3,634
Neostagonospora caricis	*Carex*	Leaf blight	The Netherlands	KF251163	3,346
Taphrina carpini	*Carpinus betulus*	Witches' broom	Slovakia	AF492085	2,666
Curvularia trifolii	*Leucospermum*	Leaf spot	Australia	JN712459	2,091
Cryptodiaporthe pulchella	*Salix lucida*	Dieback	Maryland, USA	GU367061	1,808
Devriesia fraseriae	*Melaleuca*	Leaf spot	The Netherlands	HQ599602	1,717
Toxicocladosporium strelitziae	*Strelitzia reginae*	Floral lesions	South Africa	JX069874	1,193
Pilidium acerinum	*Aesculus hippocastanum*	Leaf blight	The Netherlands	NR_119500	1,041
Xenostigmina zilleri	*Acer macrophyllum*	Leaf spot	Canada	FJ839639	983
Mycosphaerella fragariae	*Platanus*	Leaf blight	South Korea	GU214691	830
Ilyonectria radicicola	*Pinus sylvestris*	Root rot	Sweden	KF156312	676

Table 4.2. Continued**.**

Pathogen of other plant	Disease host plant	Disease symptoms	Location	Accession number from GenBank	Total OTU sequences reads
Taphrina alni	*Alnus incana*	Tongues on female catkins	Austria	AF492076	630
Taphrina communis	*Prunus americana*	Plum pockets and leaf curl	USA	AF492086	619
Neosetophoma samarorum	*Urtica dioica*	Fruit rot	The Netherlands	KF251162	576
Ciborinia camelliae	*Hepatica*	Root rot	Japan	AB516659	547
Phoma sp.	*Rosa rugosa*	Root rot	Lithuania	KF646102	421
Boeremia exigua var. heteromorpha	*Nerium oleander*	Leaf spot & dieback	United Kingdom	JX467690	390
Drechslera dematioidea	*Poaceae* grasses	Leaf spot	British Columbia, Canada	JN712466	364
Diplodina microsperma	*Protea*	Leaf spot	New Zealand	JN712461	334
Diaporthe viticola	*Fraxinus excelsior*	Leaf spot	The Netherlands	KC343230	327
Phoma herbarum	*Rosa multiflora*	Leaf spot	The Netherlands	KF251212.1	255
Knufia cryptophialidica	*Populus balsamifera*	Stem canker	Alberta, Canada	JN040501.1	253
Plectosphaerella sp.	*Alnus glutinosa*	Stem canker	Latvia	JF340251.1	165
Plagiostoma barriae	*Acer macrophyllum*	Anthracnose	Washington, USA	EU254997.1	127
Strumella sp.	*Alnus incana*	Stem canker	Latvia	GU062276	111
Taphrina weisneri	*Prunus fruticosa*	Witches' broom and leaf curl	Portugal	AF492126.1	102
Rhizosphaera pseudotsugae	*Pseudotsuga menziesii var. menziesii*	Needle cast	Germany	EU700369	93
Diaporthe sp.	*Actinidia*	Leaf spot	New Zealand	KC145848	90
Teratosphaeria knoxdaviesii	*Protea*	Leaf spot	South Africa	EU707866.1	79
Catenulostroma hermanusense	*Phaenocoma prolifera*	Leaf bract lesions	South Africa	JF499833	64
Phaeosphaeria nodorum	*Lolium perenne*	Blotch	Denmark	KF251177	44
Septoria cretae	*Nerium oleander*	Leaf spot	Greece	KF251233.1	42

Pathogenicity test of spillover pathogen in its susceptible host.

Greenhouse inoculation assay. Both *F. culmorum* isolates (FCP and FCW) caused significant effects on the growth of the above-ground biomass with mortality in wheat (Fig. 4.1). Ten days after the inoculation, leaves showed symptomatic browning. The first lesion appeared

on the twelfth day. The control remained healthy in wheat (Fig. 4.1). There was no negative effect in poplar.

Figure 4.1. In the greenhouse assay from 2015, **(A)** the *Fusarium culmorum* isolate from wheat, FCW (left), and endophytic *F. culmorum* from poplar, FCP (right), caused mortality in wheat with the control (center) unharmed; while **(B)** all 3 inoculation treatments do not cause any damage to poplar.

Statistics from the above-ground biomass measurements confirmed observations of treatments. The average weight of the wheat control was 7.70 grams, compared to both significantly lighter *F. culmorum* treatments ($p = 2.803 \times 10^{-5}$). The average weight of the FCW and FCP isolates were 6.22 and 6.35 grams, respectively (Fig. 4.2). On average, the FCW and the FCP treatments were 21.26% and 19.22% lighter than the control, respectively. All three inoculation treatments (FCW, CTRL, FCP) had no effect in the dry, above-ground biomass of poplar growth ($p = 0.674$) (Fig. 4.2).

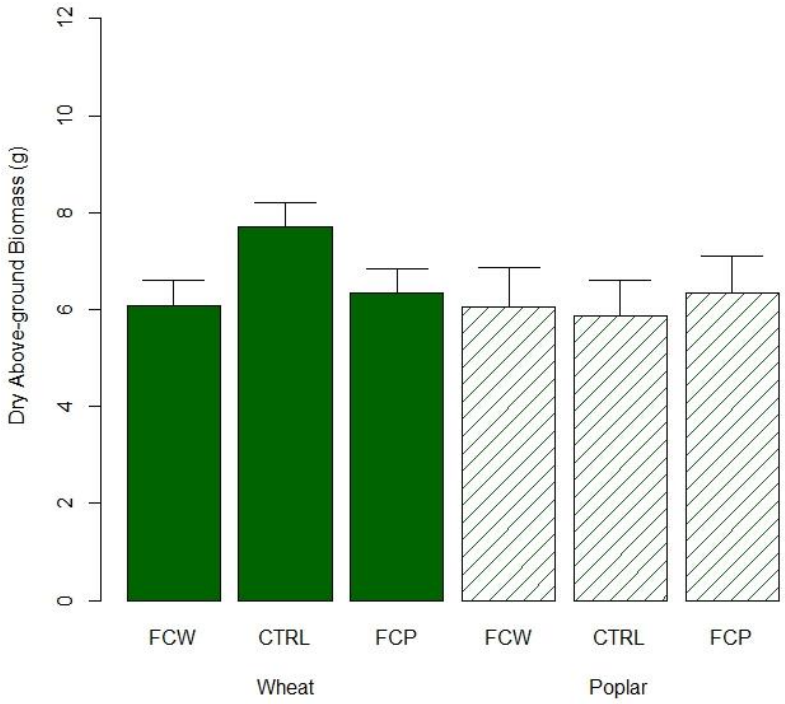

Figure 4.2. Mean weights of above-ground biomass from greenhouse plants in 2015 shows both the pathogenic (FCW) and endophytic (FCP) isolates of *Fusarium culmorum* significantly reduce wheat plant growth (green) compared to the control (CTRL) ($p = 2.80 \times 10^{-5}$) with standard error bars; while there is no effect in the poplar plant growth (stripes) ($p = 0.674$).

Demonstrating a host range. The USDA fungal database shows that *F. culmorum* has been found on 133 different hosts in 23 total families (Farr et al. 2016). This study adds Salicaceae as an endophytic host of *F. culmorum* (Fig. 4.3).

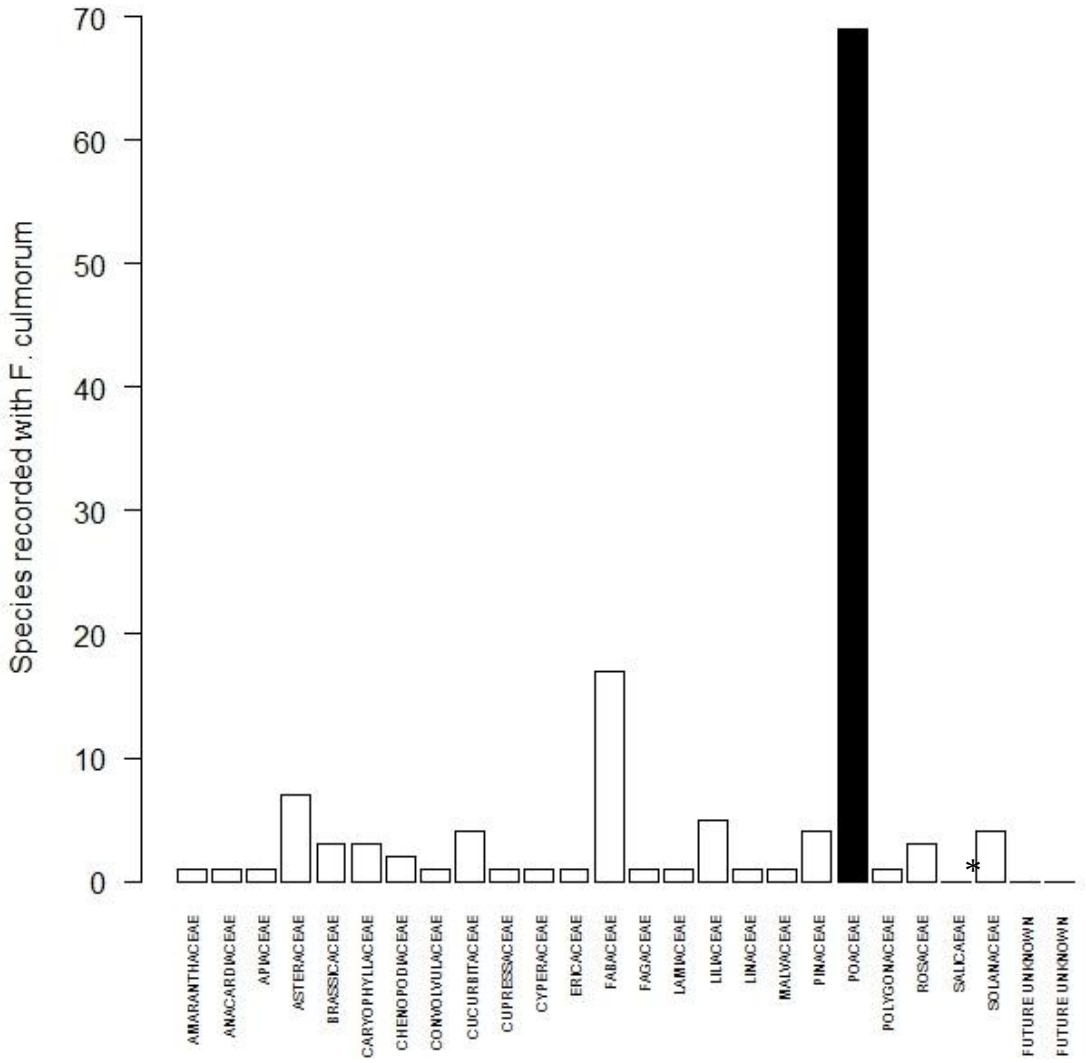

Figure 4.3. *Fusarium culmorum* appears to have a wide host range from USDA SMML records, yet it is seen in nature as a pathogen primarily on plants of Poaceae. Our study (*) adds Salicaceae to the list of 'host families' even though no negative effects of inoculation with *F. culmorum* were seen in *P. trichocarpa.* There are also other families in which F. culmorum may be a pathogen (Asparagaceae) and our point here is not to be comprehensive but to make a point about Poaceae, for one, and then our finding.

Discussion

Many so-called generalist pathogens (e.g., *Fusarium culmorum*) are pathogens in only some of the plant species from which they have been reported; in others they are present as endophytes (Fig. 4.3). With unequal effects on different species of plants in communities

they are likely to be important drivers of 'apparent competition' among species (Cobey and Lipsitch 2013; Orrock and Witter 2010), wherein apparent competition refers to the survivorship and growth of plant species over another. Endophytes that are actually pathogens of competing plants could appear to be neutral if they are only considered in relation to their host (Fig. 4.4A). However, considered in light of competitive interactions among plants they could have net positive or negative effects on their host at the community level. For example *F. culmorum* is a known pathogen of grasses, but is seen in many other host families as an endophyte (Farr et al. 2016). If poplar holds a reservoir of *F. culmorum* propagules, the pathogen may be able to infect grasses, as grasses also grow in the same riparian communities, and actually benefit the poplar (Fig. 4.4B), possibly through reduced competition.Competition experiments could be used to test this hypothesis.

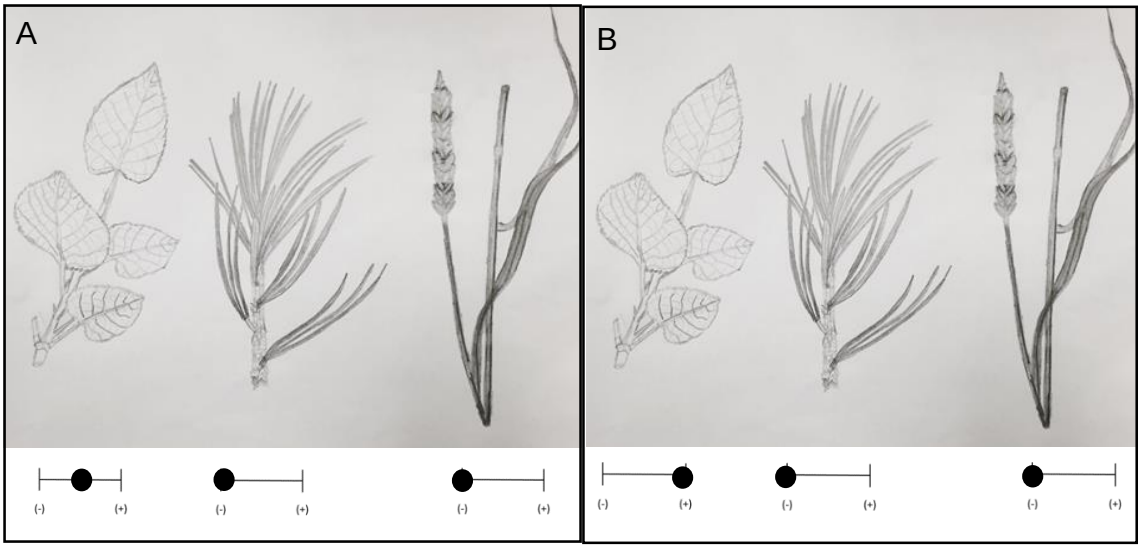

Figure 4.4. The functional roles of an endophyte in poplar (left) of both *Elytroderma dermormans* and *Fusarium culmorum,* while the effect of both pathogens *E. dermormans* (center) and *F. culmorum* (right) is clearly negative on both hosts. **(A)** While the endophytes do not seem to be harming the poplar host, **(B)** the poplar could or could not be benefiting from the negative effects of the pathogens on the other hosts in a form of apparent competition.

With the NGS methodology we were able to see the full fungal community, including many pathogens of other plants. However, testing the functional role of all these pathogens of other plants was beyond the scope of our study. We demonstrated a negative effect on another plant with *F. culmorum* on wheat and it seems likely that many of the OTUs

(Tables 4.1 and 4.2) are pathogens in the region as many of them infect plant species in the region that do compete with poplar.

Another aspect of this same question is raised by the 59 OTUs of putative pathogens of 20 plant families (Tables 4.1 and 4.2) that were found in this limited survey of poplar leaves. Outside the bounds of a pathogen's host range, non-host resistance is supposed to keep out pathogens of other plants (Schulze-Lefert and Pastruga 2011). Endophytes have been found to benefit a host by enabling growth or antagonizing disease. Yet the cost, benefit relationship of how pathogens of other plants effect their endophytic host has not been well-researched. In this study we saw the functional role of a microorganism in an endosymbiotic relationship can range from either negative effects, neutral effects, or beneficial effects on different hosts in the community. Overall, our results indicate that the roles of plant pathogens and endophytes should be considered in the context of community interactions instead of simply in single host systems.

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