AN ABSTRACT OF THE THESIS OF

Joseph M. Hulbert for the degree of Master of Science in Wood Science and Botany and Plant Pathology presented on September 19, 2014

Title: Phytosanitation of Douglas-fir (*Pseudotsuga menziesii*) Logs to Mitigate the Risk of Artificially Disseminating *Phytophthora ramorum* Within the Global Trade of Timber

Abstract approved:

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*Phytophthora ramorum* is a plant pathogen that was accidentally introduced to the United States. This invasive microorganism has killed over a million trees in California and Oregon, and continues to spread to new areas. Diseased areas have been nationally quarantined to prevent further spread and more than ninety countries include the organism on regulated pest lists or mention it in their legislation. Legislation and quarantines can slow the spread, but it will also be important to develop methods for eliminating the presence of this organism on various plant materials. Among the most important would be woody tissues, notably logs exported from infested areas. This study examined the potential for *P. ramorum* to colonize Douglas-fir logs and the ability of disodium octaborate tetrahydrate (DOT), a water diffusible fungicide, to control *P. ramorum* in Douglas-fir logs.

*Phytophthora ramorum* was capable of growing into Douglas-fir sapwood but the results were inconsistent. DOT levels above 0.1% Boric Acid Equivalent (BAE) were inhibitory to *P. ramorum* in a boron amended media bioassay, while levels above 1.5% BAE were lethal, indicating that boron has the potential to be an effective mitigation measure. Boron was capable of diffusing through bark and into the sapwood of the Douglas-fir log sections and diffusion was improved slightly through inclusion of glycol. While further research is needed to confirm that *P. ramorum* can grow into Douglas-fir sapwood, the results indicate that boron diffusion might be a useful method for mitigating the risk of spreading this pathogen on logs.

# Phytosanitation of Douglas-fir (*Pseudotsuga menziesii*) Logs to Mitigate the Risk of Artificially Disseminating *Phytophthora ramorum* Within the Global Trade of Timber

by
Joseph M. Hulbert

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Master of Science

Presented September 19, 2014
Commencement June, 2015

Master of Science thesis of Joseph M. Hulbert presented on September 19, 2014

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

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Joseph M. Hulbert, Author

ACKNOWLEDGMENTS

The completion of this work was made possible by financial support from the Guistina Foundation and the 2012 Giustina Innovative Grants Program in the College of Forestry.

I am sincerely grateful for the opportunity to complete a Masters of Science at Oregon State University. Dr. Jeffrey Morrell and Dr. Everett Hansen granted me a second chance and I appreciate the risk taken. I attribute my success to their life changing guidance and unique knowledge. It is an honor to have joined the lineages of Dr. Hansen and Dr. Morrell because they have genuinely made history. Together, Dr. Morrell and Dr. Hansen equate to more than 75 years of research from which I am fortunate to have had as a resource. The impacts they have made during their careers are inspiring and it is because of individuals like them that I strive to make an impact on this planet.

I will forever feel bound to Dr. Morrell. I appreciate his patience and tolerance. His support has allowed me to become much more than a 'master of science'. With his understanding I was able to discover my passion for science communication and my enjoyment of outreach and engagement. The work presented in this thesis is a result of his initiative.

I would also like to acknowledge several individuals who have made important impacts on my life during the recent years: Zhian Kamvar, my close friend and radio show co-host, has balanced my arrogant ambitions with precision, caution, reliability, and consistency. Justin Shaffer, a friend and role model, who continuously provided encouragement during my application to graduate programs. Sarah Navarro for setting the bar in Botany and Plant Pathology and keeping Everett on his toes. My dear dad, for involving me in his research projects growing up and the many Kansas summers spent in his cornfield. Nik Grünwald, Chris Mundt, Jeff Stone, Dave Shaw and Camille and Michael Freitag for their support and advice regarding my next move and future career decisions. Most importantly, my girlfriend, Michelle Agne for providing her support and love during much of my self-discovery and recent accomplishments.

Finally, the work presented in this thesis could not have been completed without help from the following individuals: Teresa Sawyer in the Scanning Electron Microscopy Facility for her interest and masterful use of the equipment. Ariel Muldoon in the College of Forestry for keeping our heads above the deep sea of statistics. Wendy Sutton for always keeping a positive outlook. Paul Reeser, for his precision and ability to see positive results and meaning in my seemingly failed attempts to inoculate wood pieces. I would also like to acknowledge the help of my committee members: Peter Bottomley, and Seri Robinson.

## CONTRIBUTION OF AUTHORS

Dr. Jeffrey Morrell and Dr. Everett Hansen contributed to the editing of this entire document.

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“Each imported pathogen is therefore an uncontrolled, potentially dangerous, open-ended experiment in evolution”, (Brasier, 2008a)

# PHYTOSANITATION OF DOUGLAS-FIR (*PSEUDOTSUGA MENZIESII*) LOGS TO MITIGATE THE RISK OF ARTIFICIALLY DISSEMINATING *PHYTOPHTHORA RAMORUM* WITHIN THE GLOBAL TRADE OF TIMBER.

# CHAPTER 1: INTRODUCTION

Thirty one percent of our global terrestrial systems are forest lands (FAO, 2010a). Half of the forested lands in the world are located in the Russian Federation, Brazil, Canada, the United States of America, and China. Thirty percent of forests in the world are primarily used for production of forest products (FAO, 2010a). Forested lands provide valuable products such as timber, fuel wood, fiber, and non-wood forest products, contributing to the livelihood of many rural communities. These lands are sources of invaluable aesthetics, recreational uses and wildlife habitat. They provide vital ecosystem services by protecting watersheds, maintaining biodiversity, sequestering carbon, and preserving social and cultural values (FAO, 2009). Unfortunately, these values are at risk in many areas of the world from introductions of nonindigenous forest pests that may seriously threaten our forests. About 0.3 percent of the total forested area in the world is reported as affected by forest diseases annually (FAO, 2009). This statistic is expected to climb because of increasing global trade that provide more opportunities for pest movement, coupled with climate change that improves ecosystem suitability and increases the likelihood of pest establishment (Dale et al., 2001; FAO, 2010a). The establishment of nonindigenous forest pathogens can change entire landscapes and consequently threaten the values forests provide.

## Globalization

Trade has increased during the past 25 years between the United States and other regions of the world (Aukema et al., 2010). The number of live plants imported in the United States has increased 500% in the past 43 years (Liebhold et al., 2012). Increases in globalization and world trade are directly related to the increase of exotic and invasive plant pathogen introductions around the world (Aukema et al., 2010; Boyd et al., 2013; Brasier, 2008b; Liebhold et al., 2012; Santini et al., 2013; Stack et al., 2010). Fortunately, not all introductions result in pathogen establishment and very few pathogens actually become invasive (Aukema et al., 2010). Even still, there are an abundance of alien pathogens established around the world; in the United States, Pimentel et al. (2000) noted more than 20 known nonindigenous forest pathogens and Aukema et al. (2010)

reported at least 16. A recent collaborative study involving 20 European nations compiled the historical data of invasive forest pathogens and suggested that 60 species are currently established in Europe (Santini et al., 2013). The presence and abundance of these organisms is concerning when considering the impacts an individual species can have on societies, ecosystems, countries, and the entire planet.

**Invasive Forest Pathogens**

Forest pathogens have historically influenced ecosystems by promoting genetic diversity, influencing species distributions, maintaining carbon and nutrient cycles, driving succession, and influencing forest structure (Castello et al., 1995; Hansen and Goheen, 2000; Loo, 2008). Loo (2008) suggested that native pathogenic species have generally contributed to the stabilization of forest systems, and Castello *et al*. (1995) asserted that pathogens regulate patterns and processes in forest ecosystems. Today, exotic forest pathogens are substantially affecting forest community composition and biodiversity by nearly driving individual species to extinction. It has been suggested that *Phytophthora ramorum* (Werres et al., 2001) will likely cause extinction of mature tanoak trees in parts of Oregon and California (Cobb et al., 2012). This would be similar to the action of *Cryphonectria parasitica*, which virtually eliminated mature trees of American chestnut (*Castanea dentata*) from their natural landscapes (Brasier, 2008b; Loo, 2008). Native forest pathogens also influence forest ecosystems, but managers are faced with the introduction and establishment of new forest pathogens that may have catastrophic and long lasting impacts on natural plant systems (Loo, 2008). The development of the field of forest pathology can largely be attributed to management of introduced pathogens. The field has been stimulated by the introductions of pathogens that cause chestnut blight and white pine blister rust (Boyce, 1961). Additional diseases caused by introduced microorganisms include beech bark disease, larch canker, Dutch elm disease, dogwood anthracnose and Port-Orford cedar root disease.

Exotic and invasive forest pathogen introductions can cause catastrophic losses to natural biota. Two notable forest diseases that have provided challenges to forest pathologists for nearly a century are Dutch elm disease and chestnut blight. Dutch elm

disease is a vascular wilt that spreads through the vascular system of elms (*Ulmus* spp.) and is caused by *Ophiostoma ulmi* and *O. novo-ulmi. O. ulmi* was initially introduced into North America with wood boring insect vectors in the genus *Scolytus* (Gibbs, 1978). Historically, this disease has been driven by two different pathogen outbreaks; the second pandemic, caused by *O. novo-ulmi,* is estimated to have killed between 30 and 50 million elms in the United Kingdom alone (Brasier, 2008b). Chestnut blight was introduced to the US around 1910 and had killed most of the mature American chestnut trees throughout its range within 30 years (Brasier, 2008b; Liebhold et al., 2012). The introduction of these diseases fundamentally altered ecosystems as entire tree species have been practically eliminated from the landscape (Loo, 2008). The ability of alien organisms to impact ecosystems and society is bolstered by the potential for sexual recombination or hybridization. Brasier (2001, 2008b) suggested that the second pandemic of the Dutch elm disease resulted from the hybridization between *Ophiostoma novo-ulmi* and *O. ulmi*, which allowed unilateral gene flow from one species to another while it spread across North America. Another product of hybridization, *Phytophthora alni* subsp. *alni,* is killing native alders across Europe (Brasier, 2001, 2008b; Brasier et al., 2004; Santini et al., 2013). *Phytophthora alni* originated from the hybridization of *P. cambivora* and *P. fragariae* (Brasier et al., 2004). Exotic pathogen introduction events provide organisms with novel sexual recombination opportunities that may alter pathogenicity, host ranges, or abiotic and biotic tolerances. Studying these historical introductions and the evolution of forest pathogens can provide insights to help prevent future introductions.

**Introduction Pathways**

Expanding international trade means that pathogen introductions are likely to continue and the pathways from which they are introduced need to be addressed and abated. Introduction pathways refer to the means by which pathogens are introduced into a new ecosystem. The majority of the introductions in the last few decades have been attributed to trade in live plant materials (Aukema et al., 2010; Brasier, 2008b; Liebhold et al., 2012). Traded plant parts are ideal sources for long-distance transport of pathogens

(Harnik et al., 2004). Chestnut blight, dogwood anthracnose, pitch canker, white pine blister rust and poplar leaf rust are all examples of diseases caused by pathogens introduced via nursery stock (Britton, 2004). One study estimated that 47% and 19% of the economically significant pathogens that have historically been introduced into the United States arrived on live plants and timber logs, respectively (Liebhold et al., 2012). In a study focusing on Europe, the authors suggest that 57% of the introductions were from the live plant trade and 10% were from wood material (Santini et al., 2013). Aukema et al. (2010) recently noted increased introduction frequencies of wood-borers as an insect group and several wood boring insects are associated with pathogenic fungi. Five of the 16 forest pathogens noted in the Aukema et al. (2010) study are vectored by insects. *Ophiostoma novo-ulmi* is an example of a pathogen that was introduced through trade of timber between the US and Europe (Boyce, 1961; Brasier, 2001; Liebhold et al., 2012). Another example is the redbay ambrosia beetle, *Xyleborus glabratus*—the insect vector for the laurel wilt pathogen, *Raffaelea lauricola*. These wood boring insect vectored pathogens pose a high risk for introduction via the timber product trade markets or in wood packaging material. Additional exotic pathogen introductions from trading wood products may be rare, but still account for 10-19% of all introductions. Pathogens and pests contained within logs are exceptional challenges to biosecurity because they are well protected and, thus, particularly difficult to detect or treat.

**United States Roundwood Production and Trade**

In 2010, 98 percent of the timber harvested in western Oregon was coniferous (Oregon Forest Resource Institute, 2012, See Appendix). Major commercial conifer species on the west coast of the United States, include, but are not limited to, Douglas-fir (*Pseudotsuga menziesii*), ponderosa pine (*Pinus ponderosa*), coast redwood (*Sequoia sempervirens*), sugar pine (*Pinus lambertiana*), incense-cedar (*Calocedrus decurrens*), western hemlock (*Tsuga heterophylla*), sitka spruce (*Picea sitchensis*), and multiple true fir species (*Abies* spp.) (Morgan et al., 2012; Oregon Forest Resource Institute, 2012; Washington State Department of Natural Resources, 2013). Between 2000 and 2013, the United States produced the most coniferous roundwood in the world (FAO, 2014).

During that time period, the United States was second to the Russian Federation in quantities exported. While the quantities of roundwood produced between 2006-2009 steadily decreased with the US recession, the annual US quantities exported were relatively static until 2012. In 2012, the annual quantity of coniferous roundwood exported from the United States increased by 162 percent (from 7.53 $m^3$ to 12.27 million $m^3$) and then by another 119 percent in 2013 to 14.65 million $m^3$ (FAO, 2014). With these increases in 2012 and 2013, the United States surpassed the quantities of coniferous roundwood exported by the Russian Federation, as did New Zealand. In 2013, New Zealand exported more coniferous roundwood (16.64 million $m^3$) than any other country in the world. This market for coniferous roundwood represents a substantial proportion of revenue for the major producing countries. In 2013, the United States generated 2.44 billion US dollars from exported coniferous roundwood (FAO, 2014). In decreasing order, the top five importers were China, Japan, Canada, Republic of Korea, and Italy (Comtrade, 2014).

In 2011, more than 2 billion board feet of logs were exported from ports on the West Coast of the United States (Xiaoping and Warren, 2012). Washington, Oregon, California, and Alaska Customs Districts exported 678.5, 965.4 48.6, and 275.1 million board feet of logs in 2011, respectively. Oregon Customs Districts exported the greatest volume during 2011. In 2010, Douglas-fir accounted for 73.6 percent of the timber harvested in Western Oregon (Oregon Forest Resource Institute, 2012, See Appendix). Of the logs exported in 2011 from Oregon and Washington Customs Districts to all countries, more than 60 percent (~1 billion board feet) were Douglas-fir (Xiaoping and Warren, 2012).

Forest product markets are sensitive to changes in regulation and importing/exporting partnerships between countries can be challenged once accidental establishment of invasive organisms occurs. Organisms that are considered innocuous in native regions may have the potential to become serious problems when introduced into new environments with susceptible hosts. Forest ecosystems may suffer considerable losses if these pathogens are introduced via the trade of timber. The global trade of timber

products is a significant pathway for forest pathogen introductions and developing methods to mitigate the risk of dissemination is critical (Liebhold et al., 2012; Santini et al., 2013). To date, very little research has been conducted to determine whether *Phytophthora* species are capable of being transported on conifer or hardwood timber logs. However, Vannini et al. (2012) suggested wood product trading should be considered a common and high-risk introduction pathway after alien fungi were discovered on wood utensils shipped to Italy. This study further illustrates the difficulty in monitoring the hidden movement of fungi and stresses the importance of recognizing timber product markets as a means of pathogen introductions.

**Introduction to Phytophthora Species**

The name *Phytophthora,* meaning: "plant destroyer", was first coined by Anton de Bary in 1876, when he introduced the genus for the potato late blight pathogen, *Phytophthora infestans* (Erwin et al., 1983). *Phytophthora* species have several features that distinguish them from many other pathogenic fungi: 1) they produce motile biflagulate zoospores; 2) can produce several sporangia on single sporangiaphores; 3) their cell walls are composed primarily of β-glucans and cellulose, rather than chitin; 4) a single oospore is normally formed within each oogonium; 5) *Phytophthora* spp. have diploid life cycles with meiosis occurring in gametangia; and 6) they are unable to synthesize sterols, necessary for sporulation, requiring exogenous supplies (Erwin et al., 1983). Species within the genus can be distinguished by morphological characteristics associated with the primary structures (Table 1.4). *P. ramorum* is characterized morphologically by abundant production of chlamydospores and elongate, ellipsoid, caducous sporangia with short pedicels (Werres et al., 2001). Sporangia, chlamydospores, and zoospores are disseminated long distances by waterways or soil translocation (Edmonds et al., 2011; Fichtner et al., 2007, 2009).

Oomycete pathogens in the genus *Phytophthora* include some of the most destructive plant pathogens in the world and 100-500 species are estimated to still be unknown (Brasier, 2008b, 2009). *Phytophthora ramorum* is the causal organism of sudden oak death and is known to infect over 100 different hosts (USDA APHIS, 2013).

Another species, *P. cinnamomi* is found in forests all over the world, and is considered one of the top five environmental threats facing Australia (Hansen, 2008); it can infect over 3000 plant species (Brasier, 2008b) and has affected both forest ecosystems and agricultural production (Newhook and Podger, 1972; Weste and Marks, 1987). About half of the forest diseases in the United Kingdom are caused by introduced *Phytophthora* species (Brasier, 2012). Table 1.3 summarizes some of the major *Phytophthora* species affecting forest systems.

**Sudden Oak Death**

Sudden oak death is a disease of oaks and tanoaks in forests of Oregon and California. The presence of the disease is environmentally and economically damaging. *P. ramorum* has killed over a million trees in California in the last decade (Parke et al., 2007) and was first observed in coastal forests of California in the mid-1990s (Rizzo and Garbelotto, 2003; Rizzo et al., 2002, 2005). It was first observed causing sudden oak death in four species of oaks in California (Rizzo et al., 2002). Reports of tanoaks (*Notholithocarpus densiflorus*) and coast live oaks (*Quercus agrifolia*) dying across private and public lands in California stimulated interest by disturbance ecologists of all specialties. Initial investigations pointed to stress factors coupled with secondary or opportunistic organisms (Kliejunas, 2010). The causal organism, *P. ramorum*, was not identified as the cause of sudden oak death until 2000 (Garbelotto et al., 2001; Rizzo et al., 2002, 2005). *P. ramorum* had been previously identified in Europe in the early 1990s for causing decline in rhododendrons (Werres et al., 2001).

**Phytophthora Species and Timber Material**

Many species of *Phytophthora* are recognized because of the severe foliar damage they cause. However, foliage and fine branches are generally left behind in the trade of timber materials, possibly providing a false sense of security. *P. ramorum* creates lethal bole cankers in several species of the Fagaceae (oaks), potentially contaminating and colonizing timber material other than foliage. Similarly, several species of *Phytophthora* are known to attack living tissues in the cambium and phloem of hardwood trees, causing

stem necrosis (Ahumada et al., 2012; Brown and Brasier, 2007). For example, the bole tissues in numerous *Quercus* species have been identified as susceptible to *P. ramorum* (Ahumada et al., 2012; Brown and Brasier, 2007; Hansen et al., 2005; Moralejo et al., 2007), and *P. kernoviae* has also been isolated from the xylem and bark of European beech (*Fagus sylvatica)* (Brown and Brasier, 2007). There has been little concern in North America because these species are not primary commercial species. However, pathogens of the genus *Phytophthora* are exceptionally well suited to spread within the trade of plant material because of their ability to cause asymptomatic infection (Denman et al., 2006). In addition, microorganisms are generally difficult to detect because of latent periods in their lifecycle as well as cryptic signs and symptoms of their presence (Aukema et al., 2010; Brasier, 2012; Liebhold et al., 2012). *P. ramorum* threatens an oak product market in California that averaged almost $50 million annually between 1996 and 2000 (Kliejunas, 2010). In a study about the removal and use of infected material, *P. ramorum* was successfully isolated from 39 of the 864 (~4%) specimens of unprocessed tanoak and coast live oak wood (Shelly *et al*. 2005). In addition, *P. ramorum* isolations were successful from both rainwater runoff of infested wood material and the dust from processing equipment. Although *P. ramorum* was recovered from a relatively low percentage of specimens, wood material is considered a pathway for exotic organisms by many regulating countries. The degree of risk for accidentally disseminating *P. ramorum* within the trade of wood material remains unclear and inconsistent within separate risk assessments. Wood and wood products are considered high risks for dispersal potential in the USDA risk analysis (Cave et al., 2008), but susceptible wood from the USA or Canada is believed to be a low, or very low risk pathway of entry to Europe by the EU Sixth Framework Project RAPRA, respectively (Sansford et al., 2009). However, the EU Sixth Framework Project RAPRA does rate susceptible isolated bark from the USA as a medium risk pathway of entry to Europe. Despite the inconsistencies between the assessments in the two countries, some risk is still recognized by both agencies.

In a study to investigate species susceptibility and assess the accuracy of artificial inoculation techniques, Hansen et al. (2005) determined that inoculating logs with *P. ramorum* provided a realistic test of susceptibility. The study exposed 49 western forest trees and shrubs to *P. ramorum* to artificial inoculation by removing the bark with a cork borer and placing inoculum plugs directly onto the cambium. Susceptibility was determined by measuring lesion area within the cambium. *P. ramorum* caused the greatest lesion areas in tanoak, chinquapin, Port-Orford cedar, sitka spruce and Douglas fir, in decreasing order. Douglas-fir had the fifth largest lesions of the 19 species tested. The results suggest that *P. ramorum* can contaminate and survive on the cambial tissues of many species including some that are important commercially (Hansen et al., 2005).

Brown and Brasier (2007) investigated the ability of several *Phytophthora* species to colonize xylem tissues. Sapwood discoloration has commonly been observed with bole infections from many species of *Phytophthora*, but this study was the first to attempt to isolate *Phytophthora* species from the discolored xylem tissues. Xylem tissue discoloration has generally been attributed to the reaction of the host to colonization and discoloration may represent xylem dysfunction (Brown and Brasier, 2007). In some host-pathogen interactions, the organism is assumed to be absent without observation of discoloration (Ahumada et al., 2012). Results of this study indicated that *P. ramorum* could colonize xylem tissues of six broadleaf species (Table 1.1) with other *Phytophthora* species capable of colonizing two additional tree species. The ability to regularly isolate *Phytophthora* spp. from xylem tissues brings to question current phytosanitation procedures. Protocols and disease control efforts aimed at preventing the global spread of *Phytophthora* spp. should consider xylem tissues as a potential pathway.

Parke et al. (2007) identified a strong association between *P. ramorum* infection and xylem discoloration in naturally infected tanoak trees. Rizzo et al. (2002) had previously observed discolored tissues up to 3 cm into the sapwood of tanoak and attributed it to *P. ramorum* infection. Using light microscopy, the authors observed *P. ramorum* hyphae in vessels, ray parenchyma, and fiber tracheids of discolored tanoak xylem tissues. Chlamydospores within xylem vessels and hyphae within the bark were

also observed (Parke et al., 2007). *P. ramorum* spread longitudinally and radially throughout the xylem tissues and it was recovered at a radial depth of up to 4 cm. These results were similar to other observations of discoloration of tanoak in California (Parke et al., 2007; Rizzo et al., 2002). The study suggested that radial spread occurs through the ray cells while longitudinal spread occurs through the xylem vessels.

To investigate whether inoculum placement influenced xylem tissue colonization, Parke et al. (2007) inoculated uninfected, freshly cut tanoak logs. They placed inoculum on the bark, cambium, and xylem. Eight weeks after inoculation, *P. ramorum* had colonized sapwood tissues in all of the inoculated log sections regardless of inoculum placement; indicating that *P. ramorum* was capable of colonizing tanoak logs in general and could grow into the sapwood without direct exposure. Several other studies have alluded to the risk of spreading *Phytophthora* species through movement of timber material (Brown and Brasier, 2007; Cave et al., 2008; Shelly et al., 2006).

The ability of *P. pluvialis* and *P. kernoviae* to contaminate and colonize radiata pine logs was investigated in New Zealand by artificially applying zoospores and oospores of both species to bark sections of *Pinus radiata* (Hood et al., 2014). The study was designed to determine how long zoospores and oospores can survive on, or contaminate bark, and if zoospores and oospores of either species can colonize the bark or sapwood. The authors concluded that accidentally transporting either *Phytophthora* species on exported logs was unlikely. Reisolations of oospores and zoospores applied to bark surfaces under multiple artificial inoculation scenarios were minimal and percent recovery decreased quickly with time (Hood et al., 2014). Neither species colonized the sapwood or bark of *P. radiata*. The results indicated that there was low risk of accidentally spreading either *Phytophthora* species on New Zealand radiata pine logs. *Pinus radiata* is widely grown commercially throughout world and the ability to support *Phytophthora* species would be a major quarantine concern.

Radiata pine is also a major plantation species in Chile, where it may be infected by *P. pinifolia,* which causes a shoot and needle disease known as Daño Foliar de Pino (Ahumada et al., 2012). The risk of exporting *P. pinifolia* on radiata pine material from

Chile was investigated on green sawn lumber and lumber from infected trees. Both artificial and natural inoculation techniques were used to test whether the materials were suitable for colonization. Artificial inoculation was conducted using zoospore suspensions and actively growing mycelium on agar plugs. *P. pinifolia* failed to survive or colonize the green lumber specimens. The results of the study provided evidence that *P. pinifolia* does not occur in the wood of naturally infected trees or artificially inoculated lumber pieces. These findings are consistent with the New Zealand results, suggesting that radiata pine wood may be resistant to *Phytophthora* species colonization, or at least to foliar pathogens such as *P. pluvialis* and *P. pinifolia*. Furthermore, there is consistent evidence of a low risk of spreading these *Phytophthora* species on radiata pine logs or other wood material (Ahumada et al., 2012; Hood et al., 2014). However, it is difficult to extend these results to additional tree or *Phytophthora* species. *Pinus halepensis* and *P. pinea* were susceptible to *P. ramorum* inoculation of the inner bark, but *P. pinaster*, *P. nigra*, and *P. sylvestris* were resistant to slightly susceptible (Moralejo et al., 2007). Research has shown logs of several pine species to be resistant to *P. ramorum* colonization while numerous European hardwoods were infected (Brown and Brasier, 2007; Moralejo et al., 2007).

The risk of spreading *Phytophthora* species through the timber trade depends on the timber species being exported. This is indirectly supported by the host list for *P. ramorum* where unprocessed wood products are regulated for only a few host species (Table 1.2). *P. kernoviae* is also known to infect the xylem of several hardwood species (Brown and Brasier, 2007), but attempts to inoculate the sapwood and bark of *Pinus radiata* were unsuccessful (Hood et al., 2014). Additional research regarding the susceptibility of various timber species to combinations of *Phytophthora* species would help inform countries which timber and *Phytophthora* species pose the greatest risk.

Moralejo et al. (2007) used methods developed by Brasier and Kirk (2001) to test the susceptibility of several major Mediterranean tree species to *P. ramorum*, *P. cinnamomi* and a few other *Phytophthora* species by inoculating the inner bark of freshly cut log sections. The goal of the study was to evaluate the risk posed to Spain and

Portugal's forest habitats by assessing the capacity of *Phytophthora* species to invade stems of the dominant tree species. The log inoculation method provided a good estimate of tree response to invasion through the cambial zone and many species were susceptible to colonization.

**Regulations and Management**

Regulatory agencies have received much criticism for implementing managment strategies that involve regulatory lists of proven harmful organisms (Liebhold et al., 2012). Organisms that are currently unknown to science will perpetually limit this management strategy and illustrate the need for alternative measures. Unfortunately, several studies have demonstrated that despite current regulatory measures in the US or Europe, nonindigenous forest pathogens continue to become established (Aukema 2010, Brasier 2008, Vannini 2012). Over 70% of infested plant material is estimated to have passed through plant shipment inspections in the United States during 2009 (Liebhold et al. 2012). Multiple sources have expressed a need for inspection improvements (Liebhold et al. 2012, Aukema et al. 2010, Brasier 2008). Liebhold et al. (2012) and Brasier (2008) both agree that molecular technology will improve detection, but they recognize that it may not make a difference because of the immense amount of material being traded. In addition, regulating agencies such as APHIS are cautious to make changes to the system because of the profound economic impacts it could have on the plant-based market.

Britton (2004) suggested that pest exclusion was the cheapest way to prevent biological pollution. If establishment of introduced microorganisms cannot be prevented, eradication may be the best option. "Fight them on the beaches or let the new order begin" (Hal Mooney, Stanford University (Hansen, 2008)), was the basis for action in Oregon with the initiation of the eradication program for sudden oak death. *Phytophthora* species are difficult to control because of their different spore forms, ranging from motile zoospores to thick-walled oopsores, that may require different management techniques (Erwin et al., 1983). Boyce (1961) also recommended immediate action to eradicate or control introduced pathogens to prevent valuable timber species from being destroyed.

Once a nonindigenous microorganism is introduced and becomes established, there are limited options for control. Preventing the initial introduction is the best management method. This is often the objective of establishing legislation that prevents the importation of infected material. Introductions can have confounding economic impacts on plant-related industries through the implementation of regulatory actions, policies, and quarantines (Aukema et al., 2010; Liebhold et al., 2012). Policies requiring additional inspections or pesticide applications may increase production costs, and the viability of business may be limited with regulations restricting logs or other timber products (Aukema et al., 2010). After the discovery of *P. ramorum* in California, numerous countries adopted emergency measures to require phytosanitation or prohibit importation of material from Oregon and California (Frankel 2008).

Currently, wood products are regulated for 11 hardwood species in the United States (7 CFR 301.92) (Table 1.2). Unmanufactured wood and wood products are considered regulated articles, while bark chips and mulch are considered restricted articles requiring more stringent transportation permits. Federally, interstate transport of regulated wood material (Table 1.2) from a quarantined area requires a phytosanitary certificate confirming that the material has been debarked or is in compliance with 7 CFR 305 and the PPQ Treatment manual (USDA 2012). Wood products from non-bole hosts are not regulated except that material is required to be free of soil, needles, foliage, and plant debris. Wood products from bole hosts (Table 1.2) are subject to federal regulation 7 CFR 301.92. In Oregon, tanoak material is required to be harvested from “disease free areas” (>400 m from nearest infection) and cannot be harvested from within the generally infested area (ORS 603-051-1230). Most regulations are imposed on material from quarantined areas. After ten years of aggressive management, Oregon has shifted its objectives from eradication to containment, reflecting the State’s commitment and investment for maintaining forest health. It has been estimated that regulatory action will cost an additional $50 million per year to the California Douglas-fir and redwood industries (Kliejunas 2010). In the eastern US, *P. ramorum* is speculated to threaten more than $30 billion in commercial timber production in hardwood forests (Kliejunas 2010).

In addition, the international trade of timber material may be at risk indirectly through the loss of timber markets. Canada, Europe, Australia, and South Korea have already implemented quarantines for plant material from California and Oregon (Rizzo and Garbelotto 2003).

Wood products of several known hosts are also regulated articles in Canada and the US, although there are some differences between the countries (Table 1.2). Canada regulates unprocessed wood products from red oak (*Quercus rubra*) material that originated in a regulated area, while the US does not regulate wood products from red oak. Conversely, the US regulates unprocessed wood material from Campthor trees (*Cinnamomum camphora)* and Canada does not (Government of Canada, 2010).

Mitigating the threats to the world's forests requires concerted international action. Collaboration to develop and establish functional sanitary standards is key to preventing the global movement of forest pathogens. Fortunately, these measures are being developed by the International Plant Protection Convention (IPPC) in the form of International Standards for Phytosanitary Measures (ISPMS) (FAO, 2010a; IPPC, 2009).

**Phytosanitation**

Historical practices to mitigate the spread of forest pests through the timber trade included visual inspections, debarking, topical biocides, diffusible biocides, fumigation, kiln-drying, heating, and irradiation (He et al., 1997; Morrell, 1995). These processes, however, would have to be applied within any quarantine area, most likely on full logs.

*Heat Treatments for Biological Control*

Colonization of wood substrates by microorganisms can occur when conditions are suitable for spore germination. Isolating wood substrates, specifically freshly cut logs, from spore contact is impractical (Shmulsky and Jones, 2011). To prevent infection, much effort has been dedicated to developing methods to promote unfavorable conditions for microorganism colonization. The major hurdle created by *P. ramorum*, is ensuring material leaving the quarantine area is not in a state suitable for *P. ramorum* development. This could possibly be accomplished with heat treatments. Fungal

colonization of wood products requires moist conditions. Therefore, one of the best ways to prevent infection is to use elevated temperature treatments and keep wood dry (Shmulsky and Jones, 2011).

Heat treatments are widely used globally, especially for limiting the risk of pest movement of pallets and other solid wood packing (FAO 2014). They may be the most reliable methods for minimizing the risk of transporting unwanted organisms, but the method is viewed by the industry as costly and time consuming (He et al., 1997). Heat treatments have been used to control fungi in wood products for decades. In the beginning of the 21st century, North America and the European Union agreed to use heat treatments to prevent transmission of the pine wood nematode (*Bursaphelenchus xylophilus*) (Uzunovic et al., 2008). In 2002, the International Plant Protection Consortium (IPPC) published ISPM 15—guidelines for heat-treating wood packaging material (IPPC, 2009). By 2009, the document had become an international standard and has been widely adopted for wood products. The scope of ISPM 15 is to standardize methods to reduce the risk of introduction and dissemination of quarantine pests in wood packing material made from raw wood (IPPC, 2009). Heat treatments are now required for wood packaging material to meet international phytosanitary shipping regulations by IPPC signatory nations.

It has been suggested that the temperatures reached during kiln-drying lumber would likely be lethal to *P. ramorum* (Tubajika et al., 2007). Tubajika et al (2007) studied the relationship between temperature, debarking, and *P. ramorum* survivability. Due to difficulty of proving presence of active *P. ramorum*, the results were inconclusive. However, *P. ramorum* was isolated from one sample treated at 56°C for 30 minutes, suggesting that the current IPPC standard may not be adequate for killing *P. ramorum* in wood (Tubajika et al., 2007). This study presented inconclusive evidence that the time/temperature relationship required by the IPPC would be suitable for eliminating *P. ramorum* from firewood. Additional research investigating the required time and temperature to eliminate *P. ramorum* from wood material would be useful for landowners within the quarantine interested in selling firewood.

Although heat is widely used as a mitigation measure for global commerce in wood, it is difficult to apply with large logs because long heating periods are required to reach the required temperatures (Morrell, 1995). Heat treatment of chips or other processed wood may be feasible within the quarantine area, however, the rate of heat transfer in wood is extremely slow, particularly in log form, making this difficult to accomplish in the field. A final negative to using heat would be an inability to verify proper treatment since there would be no evidence of heating (Morrell, 1995). This problem is also a concern for ISPM heat treatments of solid wood packaging and could be addressed by regular assessment of materials to ensure that *P. ramorum* was not present.

Despite the implementation difficulties, several studies have shown that ISPM No. 15 protocol is not effective for all microorganisms (Ramsfield et al., 2010; Tubajika et al., 2007; Uzunovic et al., 2008). Until the efficacy of the current heat treatment protocol for eliminating *P. ramorum* from unprocessed logs is confirmed, alternative control methods need to be considered.

*Borate Applications for Biological Control*

Use of borates for wood preservation has recently grown because of interest in reducing the use of heavy metals in wood preservatives, such as chromated copper arsenate (CCA) (Caldeira, 2010). Many preservative formulations that were previously widely accepted have recently been identified to cause adverse effects on human health or the environment and have become subject to greater scrutiny. Controlling wood deterioration using less toxic chemicals has been a constant challenge for wood preservation industries. Not only does treated wood in service raise concern, but disposal can cause serious environmental problems (Ahn et al., 2010). Developing alternative methods based on natural products with little or no toxicity is difficult because the wood must resist many different types of organisms over long periods.

There are several compounds that incorporate elemental boron in commercial products for protecting against insect and fungal deterioration. Boron is an attractive

biocide because of its broad spectrum efficacy and low acute mammalian toxicity (Caldeira, 2010). Borates are also capable of diffusion after application. However, much hesitation has existed in the wood preservation realm because it is difficult to immobilize, or fix, boron once it is impregnated into the wood (Caldeira, 2010). There is a tradeoff with the applicability of borates; boron-containing compounds are attractive because of their ability to diffuse; yet they are also highly susceptible to leaching.

Although borates are susceptible to leaching in environments where treated wood products are exposed to moisture, borates are attractive as wood protectants because of their low cost (Ahn et al., 2010) and their resistance to biological degradation compared to organic preservatives. Historically, borates have been used to prevent deterioration by termites (Ayrilmis et al., 2005; Jorge et al., 2004), wood infesting beetles (Robinson and Barlow, 1993), mold and stain fungi (Kartal et al. 2005), and annosum root rot, *Heterobasidion annosum* (Garbelotto and Gonthier, 2013; Pratt and Quill, 1996). Much higher loadings of borates have also been used to improve the fire performance of wood (Ayrilmis et al., 2005).

Many wood preservation studies have investigated the efficacy of borates against organisms that damage wood products. Disodium octaborate tetrahydrate (DOT) and boric acid treatments protected oriented strand board (OSB) panels against both brown- and white-rot fungi (Ayrilmis et al., 2005). Both sodium tetraborate decahydrate (borax) and DOT are widely used in practical forestry to control infection from *H. annosum* (Garbelotto and Gonthier, 2013). However, the drawbacks generally limit borate use to dry environments such as indoor construction.

Outside the realm of wood preservation, many fruit growers have also investigated the efficacy of borates for control of post harvest losses due to fruit deteriorating organisms. Application of boric acid has been shown to reduce incidence of green mold of citrus caused by *Penicillium digitatum* (Smilanick and Sorenson, 2001), and can reduce the severity of eutypa dieback caused by *Eutypa lata* in grapevines (Rolshausen and Gubler, 2005). Potassium tetraborate strongly inhibited spore germination, germ tube elongation, and mycelial spread of *Botrytis cinerea* on grapes

(Qin et al., 2010). The same authors determined that boron inhibited the growth of *Penicillium expansum* due to the loss of membrane integrity and the leakage of cellular constituents (Qin et al., 2010).

Boric acid has also been promoted as a safe chemical for controlling many surface borne potato tuber diseases (Arora, 2005). Three percent boric acid treatments are considered non-toxic and are a common treatment for surface sterilization in tubers (Basu and Das, 2003). Boric acid treatments have also been recommended to control black scruf (*Rhizoctonia solani*), common scab (*Streptomyces scabies*), dry rots (*Fusarium spp.)* and soft rots (*Erwinia spp.)* of potatoes (Arora, 2005).

Boron activity against *Phytophthora* species has also been investigated in Israel. Researchers hypothesized that boron could reduce the severity of late blight caused by *P. infestans* (Frenkel et al., 2010). They tested their hypothesis with field experiments conducted to determine if sub-phytotoxic levels (700 mg $L^{-1}$) of boric acid applied to potato foliage affected the intensity of late blight epidemics. They observed late blight suppression in trials where boron was combined with low levels of fungicide, however, the authors attributed some of the success to an alternative mode of action than lethality to *P. infestans* propagules. *In vitro* experiments suggested that boron was a relatively weak fungicide and that its effective dosage was 256.4 mg $L^{-1}$, 6400-fold greater than the dosage for the fungicide chlorothalonil (Frenkel et al., 2010). The application rates of boron were limited to levels that were not phytotoxic to tomato plants, and even though boric acid was applied at levels much greater than the effective dose (700 mg $L^{-1}$), the highest levels detected within the leaves were 132 mg boron $kg^{-1}$ dry weight. Increasing the boron concentrations within the leaves in controlled experiments did not decrease the lesion size caused by *P. infestans,* suggesting that the boric acid treatments were not directly fungicidal. The authors speculated that boron might actually induce systemic acquired resistance against *P. infestans* because the effects were not linearly related to boron concentration. However, the authors concluded that boron, in sub-phytotoxic concentrations, restricted late blight development. This study may be the most thorough investigation of the activity of boron against *Phytophthora* sp. to this date.

An earlier study investigated zoospore production of *P. cinnamomi* and *P. drechsleri* when subjected to 3 concentrations of DOT (Halsall, 1977). None of the concentrations ($10^{-5}$–$10^{-7}$ M) of DOT reduced the quantity of zoospores produced. However, 200 ppm borax totally inhibited both sporangial and zoospore germination in a leaf blight caused by *P. colocasiae* in taro plants (Misra et al., 2007). This suggests that borate effects are limited to germination prevention, or more simply, that activity varies between species of *Phytophthora*.

The mechanisms of boron action are poorly understood. Qin et al. (2010) attributed the control of *Botrytis cinerea* to cell membrane disruption resulting in the loss of cytoplasmic materials (proteins and carbohydrates) from the hyphae. Spore germination, germ tube elongation, and mycelial spread were all inhibited by boron (Qin et al., 2010). They also observed transcriptomic effects against *Penicillium expansum* in suppression of the expression antioxidant enzyme resulting in oxidative stress (Qin et al., 2010). Alternatively, borates are used preventatively for the control of *H. annosum* on freshly cut stumps because of their direct effects on fungal metabolism (Garbelotto and Gonthier, 2013).

The actual methods of borate application to wood materials can vary. Dipping wood pieces into tanks of boron solutions has been a common method of application. However, Morrell and Lebow (1992) have shown greater penetration depths can be reached with pressure treatment. Generally, the method of application is determined by the practicality of treating the desired quantity of material. Treating whole logs with dip treatments may be more practical than pressure treatments, especially in remote locations where equipment is not readily available. Vapor and liquid boron pressure treatments have been compared as well (Tsunoda, 2001) but would be difficult to apply in a field environment. Heating the borate solutions before application may improve diffusion rates and penetration depths (He et al., 1997; McQuire and Goudie, 1972).

Boron diffusion varies between species (Lebow and Morrell, 1989) and may also depend on the preparation of the material (Ra et al., 2001). Generally, boron will penetrate farther in green wood than it will dry wood (Lebow and Morrell, 1989). Lebow

and Morrell (1989) also observed greater DOT penetration depths in incised wood compared to wood which was not incised. Wood is an anisotropic material, meaning diffusion rates will differ depending on the direction in relation to the grain. Ra et al. (2001) observed faster diffusion rates in the longitudinal direction of southern pine (*Pinus* spp.), followed by radial and then tangential directions. Diffusion rates are also influenced by temperature and moisture content (Morrell and Freitag, 1995). Penetration depth and retention are further influenced by diffusion storage duration, combinations of seasoning, and treatment duration and type.

He et al. (1997) investigated the potential of using heated DOT to diffuse into peeled Douglas-fir logs. Boron was observed up to 10cm into the sapwood, but the authors did not recommend using the treatment as a sole means for pest control (He et al., 1997). This study demonstrated the ability of DOT to diffuse through the sapwood of Douglas-fir. In cases where debarking logs is less practical, borates may need to diffuse through the bark. Rates of diffusion through intact bark have not been extensively investigated. Much of the literature on this topic has tested material with bark removed (He et al., 1997), or in processed lumber (Lebow and Morrell, 1989; Morrell and Lebow, 1992). Cassens and Schmidt (1998) found bark and outer sapwood penetration by DOT of hickory (*Carya cordiformis*) rounds with intact bark. This is the only evidence borates can diffuse through bark, and it is difficult to determine whether the same results would be attained with Douglas-fir because of differences in bark thickness and chemistry.

Borate treatments combined with other chemicals have demonstrated greater activity against fungi. Temiz et al. (2008) observed greater decay resistance in wood treated with both tall oil and boric acid than with either component alone. Certain additives can enhance efficacy and uptake when combined with biocides (Singh et al., 2005). Glycol has been shown to help improve boron diffusion into wood (Freitag and Morrell, 2002; Gezer et al., 1999). However, addition of glycol did not support the increased costs of using glycol rather than water in Douglas-fir sapwood wafers (Freitag and Morrell, 2002).

**Purpose and Objectives**

The effect of sudden oak death is an example of the consequences of introducing nonindigenous microorganisms. The global trade of plant material presents an unpredictable risk to natural systems around the world because of the potential to accidentally introduce invasive microorganisms. Mitigating this risk requires identification of possible introduction pathways and determination of effective control methods. The ability of Douglas-fir to host *P. ramorum* provides an opportunity to proactively investigate a potential introduction pathway and develop novel methods of control. The following chapters seek to identify if moving Douglas-fir timber material is a potential pathway for *P. ramorum* dissemination, and whether boron diffusion can be an effective method of control in Douglas-fir log material.

**Tables and Figures**

Table 1.1: Tree species with observations of *Phytophthora ramorum* in xylem tissues.

| Common Name | Latin Name |
|---|---|
| Balsam fir[1] | *Abies balsamea* |
| Tamarack[1] | *Larix laricina* |
| Douglas-fir[2] | *Pseudotsuga mensiesii* |
| Tanoak[3,4] | *Notholithocarpus densiflorus* |
| Sycamore maple[5] | *Acer pseudoplatanus* |
| European beech[5] | *Fagus sylvatica* |
| Japanese evergreen oak[5] | *Quercus acuta* |
| European turkey oak[5] | *Quercus cerris* |
| Cornish oak[5] | *Quercus petrea* |
| N/A[5] | *Schima argenta* |
| Sugar maple[1] | *Acer saccharum* |
| Yellow birch[1] | *Betula alleghaniensis* |
| White ash[1] | *Fraxinus americana* |
| Northern red oak[1] | *Quercus rubra* |

Footnotes indicate source: [1](Simard et al., 2010) [2](McKeever, 2010) [3](Collins and Parke, 2008) [4](Parke et al., 2007) [5](Brown and Brasier, 2007)

Table 1.2: Tree species with wood products that are regulated for *Phytophthora ramorum*.

| Common Name | Latin Name |
|---|---|
| Planetree maple | *Acer pseudoplatanus* |
| Horse chestnut | *Aesculus hippocastanum* |
| Camphor tree | *Cinnamomum camphora* |
| European beech | *Fagus sylvatica* |
| Tanoak | *Notholithocarpus densiflorus* |
| Coast live oak | *Quercus agrifolia* |
| European turkey oak | *Quercus cerris* |
| Canyon live oak | *Quercus chrysolepis* |
| Southern red oak | *Quercus falcata* |
| California black oak | *Quercus kelloggii* |
| Shreve's oak | *Quercus parvula var. shrevei* |

Source: USDA APHIS List of Regulated Hosts and Plants Proven or Associated with *Phytophthora ramorum*, August 2013. Available here: http://www.aphis.usda.gov/plant_health/plant_pest_info/pram/downloads/pdf_files/usdaprlist.pdf

Table 1.3: Notable *Phytophthora* species affecting forest systems globally

| Species | Brief Description |
|---|---|
| *P. cinnamomi* | *Phytophthora cinnamomi* Rands is the causal organism of a widespread epidemic in the jarrah eucalyptus forests of western Austrailia (Newhook and Podger, 1972; Weste and Marks, 1987). *P. cinnamomi* has had impacts throughout the world and has recently been identified killing oak trees in Europe, Argentina, Chile, Canada, the United States, and Mexico (Agrios, 2005). *P. cinnamomi* is primarily a soil-borne pathogen that attacks roots. The species was first described in 1922 by Rands in Sumatra (Erwin et al., 1983). By the 1980s, *P. cinnamomi* was known to infect nearly 1,000 hosts (Erwin et al., 1983). Then by 2008, the host list had increased to more than 3,000 species (Brasier, 2008b; Hansen, 2008). It is now recognized as one of the most ubiquitous, destructive, and widely distributed plant pathogens in the genus *Phytophthora*. |
| *P. lateralis* | *Phytophthora lateralis* is the causal organism of Port Orford-cedar root rot. The pathogen has killed large numbers of Port-Orford cedar and a few yew trees throughout Oregon. (Hansen et al., 2000; Jules et al., 2002). *P. lateralis* was first described in 1942 by Tucker and Milbrath (Erwin et al., 1983) and it is closely related to *P. ramorum* (Hansen, 2008). Nursery industries have also been affected because the principal host, Port Orford cedar, is also it is a highly valued horticultural commodity with more than 100 variant individuals in trade (Hansen, 2008; Zobel et al., 1985). |

| | |
|---|---|
| *P. ramorum* | *Phytophthora ramorum* (Werres et al. 2001) is the microorganism responsible for sudden oak death. The organism was accidentally introduced into the US through the nursery trade. The origin of this organism remains unknown. Plants purchased from an infected nursery planted in urban oak woodland interfaces likely transmitted *P. ramorum* into the forest. Now, two decades later, the oomycete has spread nearly throughout the entire distribution of tanoak. *P. ramorum* is unusual because of its enormous host range, causing damage to hardwood and softwood trees and shurbs (Edmonds et al., 2011; USDA APHIS, 2013). Simultaneously to its introduction into the United States, *P. ramorum* was discovered causing dieback of rhododendrons in Europe (Werres et al., 2001). *P. ramorum* was also spread into nurseries across Europe (Moralejo et al., 2007) and is currently causing epidemics in Japanese larch (*Larix kaempferi*) plantations in great Britain (Brasier and Webber, 2010). |
| *P. pluvialis* | *Phytophthora pluvialis* is a recently described species that has been recovered from baited streams, soil and canopy drip in southwestern Oregon's mixed conifer-tanoak forests (Reeser et al., 2013). The species has partially deciduous sporangia and is capable of spreading aerially. The only other area *P. pluvialis* has been recovered is New Zealand, where it is known to cause red needle cast of radiata pine, *Pinus radiata* (Dick et al., 2014; Hood et al., 2014). Although this species is not known to cause mortality in forest systems, it is discussed because it is included in some of the tests conducted for this thesis. Reeser et al. (2013) suggests *P. pluvialis* may be endemic to Oregon and therefore this species may represent a threat to new systems. |
| *P. kernoviae* | *Phytophthora kernoviae* is known for causing bleeding stem lesions on *Fagus sylvatica* and necrosis of *Rhododendron ponticum* (Brasier et al., 2005). P. kernoviae was first discovered in 2003 in southwest England (Brasier et al., 2005). However, the organism has been present in New Zealand for at least 60 years and is occasionally associated with red needle cast of radiata pine (Dick et al., 2014; Hood et al., 2014). The pathogen is adapted for aerial dispersal and has a wide host range (Denman et al., 2006). It has also been shown colonize the sapwood of *F. sylvatica* (Brown and Brasier, 2007). |

Table 1.4: Brief descriptions of the primary *Phytophthora ramorum* structures.

| Structure | Description of Morphological Characteristics |
|---|---|
| Chlamydospores | Chlamydospores are globose and mostly thin-walled spores of *P. ramorum* that grow intercalary, terminally, and occasionally lateraly from hyphae (Werres et al., 2001). They are capable of germinating with sporangiophores or hyphae. The diameter of *P. ramorum* chlamydospores can range from 20-91μm with averages between 46.4-60.1μm. Chlamydospores can be transported with soil and plant debris (Fichtner et al., 2007, 2009). They can persist for long periods in soil (Shishkoff, 2007) and may have led to the recovery of *P. ramorum* five to six years after eradication treatments in Oregon (Goheen et al., 2007). Chlamydospores are the most persistent, long lived asexual structure produced by *Phytophthora* species (Tooley et al., 2014). |
| Zoospores | Zoospores are produced and differentiated within the sporganium before they are released through the apex. Zoospores have a tinsel and a whiplash flagellum (Erwin et al., 1983), allowing motility. These spores have the ability to synthesize a cyst cell wall within minutes of encystment (Erwin et al., 1983). Zoospores are known to have chemotactic abilities that allow them to reliably find host material in aqueous environments. |
| Sporangia | Sporangia are produced singly or in clusters from sporangiophores. Sporangia are semi-papillate, ellipsoid, and elongated (Werres et al., 2001). Sporangia are caducous, meaning they can be shed as dispersal structures by wind or water tension. These structures are generally short lived. Sporangia may also release zoospores. |
| Oospores | Oospores are formed in sexual reproduction, with the fertilization of an oogonium by an antheridium. Oospores are thick walled structures that can germinate to give rise to sporangia or hyphae. These structures serve as persistent resting spores for many *Phytophthora* species (Hood et al., 2014). |

# CHAPTER 2: ABILITY OF *PHYTOPHTHORA RAMORUM* AND *PHYTOPHTHORA PLUVIALIS* TO COLONIZE DOUGLAS-FIR LOG MATERIAL

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In preparation for submission to Phytopathology, a publication of the American Phytopathological Society.

**Abstract**

The potential to disseminate *Phytophthora ramorum* and *P. pluvialis* through the trade of Douglas-fir logs was investigated. Multiple methods of inoculation were attempted to infer if either species could colonize Douglas-fir log material. This assessment introduces novel methods for determining susceptibility of woody tissues to colonization by *Phytophthora* species. Four studies were conducted to evaluate the ability of *P. ramorum* and *P. pluvialis* to colonize material collected from Douglas-fir logs. Three isolates of *P. ramorum* were used. Only two of the studies included an isolate of *P. pluvialis*. Because of difficulties replicating results, each study utilized different methods to attempt to colonize log material. The most effective method for *P. ramorum* inoculation involved soaking the material for 24-48 hours in a zoospore suspension of the 7904 isolate. Collectively, the studies suggest there is a limited risk of accidentally transporting *P. ramorum* or *P. pluvialis* propagules through the transport of Douglas-fir sapwood material. However, the results were not consistent across all of the studies, and in some cases, could not be replicated.

**Introduction**

*Phytophthora* species are exceptionally well suited to spread through international trade of plant material because they produce resistant resting spores (chlamydospores). They are often benign in their native range because they have coevolved with the associated plant community (Brasier, 2012; Hansen, 2008); however, introduction of these organisms to new environments with susceptible hosts can cause dramatic epidemics (Hansen 2008). To date, little research has been conducted to determine whether *Phytophthora* species are capable of being transported on conifer or hardwood logs. Many species of *Phytophthora* are recognized because of the severe foliar damage they cause. However, foliage and fine branches are generally left behind in trade of timber materials, possibly providing a false sense of security. *P. ramorum* creates lethal bole cankers in several species of the Fagaceae (oaks), potentially contaminating and colonizing timber material other than foliage. Similarly, several species of *Phytophthora*

are known to attack living tissues in the cambium and phloem of hardwood trees, causing stem necrosis (Ahumada et al., 2012; Brown and Brasier, 2007).

*P. ramorum* has been shown to colonize the cambial tissues of many species including some that are commercially important, such as Douglas-fir (Hansen et al., 2005). Brown and Brasier (2007) investigated the ability of several *Phytophthora* species to colonize xylem tissues. Their results indicated that *P. ramorum* could colonize xylem tissues of six broadleaf species. Using microscopy, Parke et al. (2007) observed *P. ramorum* hyphae in vessels, ray parenchyma, and fiber tracheids of discolored tanoak xylem tissues. Chlamydospores within xylem vessels and hyphae within the bark were both observed (Parke et al., 2007). *P. ramorum* spread longitudinally and radially throughout the xylem tissues and it was recovered at a radial depth of up to 4 cm. The study suggested that radial spread occurred through the ray cells while longitudinal spread occurred through the xylem vessels. The ability to regularly isolate *Phytophthora* spp. from xylem tissues brings to question current phytosanitation procedures. Protocols and disease control efforts aimed at preventing the global spread of *Phytophthora* spp. need to consider xylem tissues as a potential pathway.

The ability of *P. pluvialis* and *P. kernoviae* to contaminate and colonize radiata pine logs was investigated in New Zealand by artificially applying zoospores and oospores of both species to bark sections of *Pinus radiata* (Hood et al., 2014). The study was designed to determine how long zoospores and oopsores can survive on, or contaminate bark, and if zoospores and oospores of either species can colonize the bark or sapwood. The authors concluded that accidentally transporting either *Phytophthora* species on exported logs was unlikely. Reisolations of oospores and zoospores applied to bark surfaces under multiple artificial inoculation scenarios were minimal and percent recovery decreased quickly with time (Hood et al., 2014). Neither species colonized the sapwood or bark of *Pinus radiata*. The results indicated that there was low risk of accidentally spreading either *Phytophthora* species on New Zealand radiata pine logs. *Pinus radiata* is widely grown commercially throughout world and the ability to support *Phytophthora* species would be a major quarantine concern. Additional research

regarding the susceptibility of various timber species to numerous *Phytophthora* species would help inform countries which timber and *Phytophthora* species combinations pose the greatest risk.

*P. pluvialis* is another species of concern because it has been shown to cause red needle cast of *Pinus radiata* in New Zealand (Dick et al., 2014; Hood et al., 2014). Symptoms of the disease were first recognized in New Zealand in 2008, but the causal organism was not described until 2013 (Dick et al., 2014; Reeser et al., 2013). The first isolate of *P. pluvialis* was recovered in Oregon in 2002, the only other place *P. pluviailis* has been found (Reeser et al., 2013). Preliminary investigation into the diversity of isolates in Oregon and New Zealand suggest that *P. pluviailis* may have been introduced to New Zealand (Dick et al., 2014). Although it is speculated that the population levels would have taken a few seasons to build up to levels sufficient to cause an epidemic (Dick et al., 2014), it is possible the trade of timber between Oregon and New Zealand was the vector for the introduction. In 2007, the US exported 1,763,513 kg of untreated coniferous logs or poles (Commodity #440320) into New Zealand (Comtrade, 2014, See Appendix), while New Zealand reported importing 20,904 kg of the same commodity in 2006 (Comtrade, 2014, See Appendix). Determining whether those logs were exported from the area where *P. pluvialis* occurs in western Oregon would require a considerable amount of investigation and would be of minimal value because the organism has already become established. However, it may provide insight into whether untreated log imports are an introduction pathway.

Ideally, evaluating the risk of *Phytopthhora* sp. introduction on a given woody material would consider zoospores and sporangia as well as chlamydospores and viable hyphae, but sporangia can be difficult to reliably produce. Mycelia are more easily maintained and consistently prepared, and present an aggressive colonization threat. In this report, we describe the potential for *P. ramorum* and *P. pluvialis* to colonize Douglas-fir through four separate studies using zoospore suspensions or actively growing mycelial agar plugs for inoculation. Douglas-fir sapwood colonization was investigated using wafers cut from the sapwood, sawn blocks containing sapwood, cambial and bark

tissues, and intact log sections. Colonization was determined using microscopy or reisolation. The overall objective of this assessment was to determine if *P. ramorum* or *P. pluvialis* could colonize Douglas-fir tissues.

Four studies were conducted to assess the suitability of Douglas-fir log material as a substrate for *P. ramorum* or *P. pluvialis* growth (Table 2.1). The objective of the first study was to assess whether zoospores could colonize sapwood. The objective of the second study was to determine which log material tissues were susceptible to colonization. In the third study, the objectives were to assess if sapwood could be colonized by actively growing mycelia in agar plugs, and if *P. ramorum* structures could be identified within wood cells. Finally, the objective of the fourth study was to evaluate a method to infect intact logs using actively growing mycelia in agar plugs.

**Materials and Methods**

In the first two studies, 1 x 0.5 x 3 cm Douglas-fir sapwood wafers were inoculated with zoospore suspensions. Wafers were cut from the sapwood of green untreated log sections and frozen for several years until use. The third and fourth studies used actively growing cultures in agar plugs to inoculate Douglas-fir sapwood wafers and freshly cut, green Douglas-fir log sections.

*P. ramorum* and *P. pluvialis* isolates used in the studies were originally recovered from infested areas of western Oregon (Table 2.1). Isolates were obtained from E.M. Hansen, Oregon State University. The *P. ramorum* isolates were of the North American (NA-1) genotype (Personal Communication with Everett Hansen). Prior to use, the isolates were stored on agar plugs in vials of sterile water at room temperature and then maintained on corn meal agar (CMA) until needed. Zoospore suspensions were generated for three isolates of *P. ramorum* (7904, 9488, 4313) and one isolate of *P. pluvialis* (LC-9-2-020508). Suspensions were created by a technique hereafter referred to as flooding. For each isolate, twenty 9 mm diameter agar plugs were taken from the actively growing margins of 7-20 day old cultures growing on 1/3 V8 media and placed into empty petri plates. Roughly 5 plates per isolate were created. Each plate was flooded with ~25 ml of

unfiltered water from Oak Creek, Corvallis, Oregon, to induce sporangial production. Enough water was added to barely cover the surface of the agar plugs. The Oak Creek water was replaced daily for 3-5 days until large quantities of sporangia were produced on hyphae protruding from the agar surface. Once sufficient sporangia were observed, the plates were chilled at 4 C for one hour to encourage zoospore release and returned to room temperature for 1-2 hours. Zoospores were then quantified using a hemocytometer. Zoospore inoculum viability was confirmed by placing 1 mL aliquots onto Petri dishes containing *Phytophthora* selective agar CARP (Hansen et al., 2005) and incubated under the same conditions as the inoculated specimens to serve as positive controls.

*Study 1: Zoospore inoculation and surface sterilization of Douglas-fir sapwood wafers*

Four well plate trials were completed for the zoospore inoculation and surface sterilization of Douglas-fir sapwood wafers study (Figure 2.1; Table 2.2). Wafers were removed from the freezer storage and thawed at room temperature before inoculation. Each Douglas-fir sapwood wafer was inoculated by adding a 1 mL aliquot of zoospore suspension into a well plate containing the wafers (Figure 2.1). Entire well plates were dedicated to each of the three *P. ramorum* isolates, one *P. pluvialis* isolate, and a control. However, *P. pluvialis* was not included in the first two trials. One mL aliquots of non-sterile de-ionized (DI) water were added to well plates containing control specimens. Well Plate Trials 1 and 2, and Trials 3 and 4, were exact replicates, but Trials 3 and 4 differed from Trials 1 and 2. In the first two trials, wafers were left in zoospore suspensions or DI water for 24-48 hours at 20 C in the dark before incubation (Figure 2.1). Specimens were then placed on a wire mesh surface several centimeters above moist paper towels in a polystyrene storage container and incubated for 3-5 weeks at 20 C (Figure 2.1). Only *P. ramorum* isolate 7904 was used in the second trial. Wafers in Well Plate Trials 3 and 4 were inoculated in the same manner as the first two trials; however, the specimens were allowed to remain in the zoospore suspension during incubation. Specimens were left standing upright in the well plates containing zoospore suspension, placed in a polystyrene storage container, and incubated for four weeks at 20 C in the dark.

After incubation, specimens were randomly assigned to three immersion treatments: 10% bleach (900 mL de-ionized water; 100 mL household bleach) for 30 seconds, 5% bleach (950 mL de-ionized water; 50 mL household bleach) for 60 seconds, or no treatment. These treatments were designed to eliminate any surface propagules and thus to demonstrate that if the test organisms were recovered following surface disinfestation they had actually colonized the wood. In the third and fourth trials, the efficacy of the surface sterilization techniques was tested immediately after immersion. Prior to incubation, 10 wafers were exposed to bleach treatments (5 each), split longitudinally into thirds, and placed onto Petri dishes containing CARP medium. Petri dishes were evaluated after two weeks for the presence of actively growing hyphae and served as a measure of the efficacy of surface sterilization treatments to eliminate zoospores contaminating the surface.

Specimens in all four trials were surface sterilized in bulk by removing them from incubation on the wire mesh (Trial 1 and 2) or the well plates (Trial 3 and 4) and adding them to a beaker containing the bleach solutions for the allotted amount of time (30 or 60 seconds). Specimens were weighed down during surface sterilization treatment to ensure full submersion. Specimens that were not treated were processed directly from the crispers containing the wire mesh or well plates. Each specimen was split longitudinally into three separate pieces and placed onto the CARP medium and incubated at 20 C in the dark for 2-4 weeks. Plates were then evaluated for presence of characteristic *Phytophthora* hyphal branching, chlamydospores or oospores, hyphal swelling, and growth into the medium.

In Well Plate Trials 3 and 4, four wafers were removed from each well plate 30 minutes after adding the zoospore suspensions. The wafers were immediately treated with one of the two bleach surface sterilizing treatments. Two wafers that had been inoculated with each isolate of *P. ramorum*, two wafers inoculated with *P. pluvialis*, and two wafers that served as controls received each surface sterilizing bleach treatment. Wafers were then plated onto CARP and later checked to evaluate the efficacy of the surface sterilizing treatments against *Phytophthora* spp. zoospores present on the surface.

*Study 2: Zoospore inoculation of Douglas-fir sapwood blocks containing bark*

Four trials were performed in this study (Table 2.2). In the first two, freshly cut Douglas-fir sapwood blocks containing bark were dipped into zoospore suspensions of *P. ramorum*, *P. pluvialis,* or a water control (Figure 2.2). In both trials, zoospore suspensions for three isolates of *P. ramorum* were tested. Each specimen was dipped halfway into the suspension for 1-3 seconds. Controls were dipped in non-sterile DI water. Blocks were dipped horizontally so that the bark, cambium, phloem, and sapwood were all equally in contact with the zoospore suspension. After inoculation, specimens were placed on a mesh rack above a moist paper towel in a polystyrene storage container and incubated for two weeks in the dark at 20 C. DI water was added to the crispers three times over the incubation period to limit drying. The first two trials will hereafter be referred to as the Dip Trial 1 and Dip Trial 2. Specimens inoculated in Dip Trial 1 were incubated in total darkness while specimens in Dip Trial 2 were partly covered in a 12-hour light cycle.

In the third and fourth trials, Douglas-fir sapwood blocks containing bark were soaked in zoospore suspensions for 24 hours, here on referred to as Soak Trial 1 and Soak Trial 2 (Figure 2.2). The blocks were placed standing horizontally in a container so that the bark, cambium, phloem, and sapwood were all equally submerged in 5-10 mm of zoospore suspensions of *P. ramorum*, *P. pluvialis*, or a water control. Zoospore suspensions for three isolates of *P. ramorum* were tested in Soak Trial 2, but the *P. ramorum* zoospore suspensions were mixed together for Soak Trial 1. Specimens were placed on a mesh rack above a moist paper towel in a crisper box after soaking for 24 hours and incubated for two weeks in the dark at 20 C. No water was added to the crispers during the incubation period for the Soak Trials.

At the end of the incubation period in all four trials, the blocks were split into three sections and placed into petri dishes containing CARP media. Blocks were split in a manner so that there was a section containing only bark, a second section containing bark, cambium, phloem and sapwood, and a third section containing only sapwood. After an additional two weeks of incubating in the selective media, the plates were evaluated

for presence of *Phytophthora* structures and the section that the growth originated from was recorded. Presence of other fungi and their origins were also recorded in Soak Trials 1 and 2.

*Study 3: Microscopy of Douglas-fir sapwood wafers inoculated with agar plugs*

In the third study, two trials were conducted to investigate the histology of *P. ramorum* in Douglas-fir sapwood, Microscopy Trial 1 and Microscopy Trial 2 (Table 2.2). In both trials, Douglas-fir sapwood wafers (10 x 5 x 30 mm) were inoculated using 8 mm diameter mycelial plugs of *P. ramorum* grown on CMA medium. Plugs were taken from the actively growing edges of 14-20 day old cultures, and placed directly onto the surface of the sapwood wafers that were then wrapped in 2 layers of cheesecloth saturated with DI water. Agar plugs from media without *P. ramorum* were added to wafers and processed in the same way to serve as controls. Plugs were placed inoculum side up. In the first trial, the cheesecloth was placed directly on top of a single plug, on top of the wafer. In the second trial, three plugs were placed on top of the wafers, but the cheesecloth was placed beneath the wafer. Next, the sapwood wafer, agar plug(s), and cheesecloth were wrapped into single pieces of foil incubated at 20 C (Figure 2.5). The wafers used in Microscopy Trials 1 and 2 had the same dimensions, but were cut so that the agar plugs were placed on a tangential (Trial 1) or radial face (Trial 2) (Figure 2.3).

In both microscopy trials, twenty inoculated wafers (five per isolate of *P. ramorum* and controls) were randomly selected for processing. Wafers were inoculated nine months prior to processing in Microscopy Trial 1 and 3 months prior in Microscopy Trial 2. Additionally, wafers used in Microscopy Trial 1 were processed in late May, and the wafers from Microscopy Trial 2 were processed in mid July to mid August. Each wafer was unwrapped and wiped with a paper towel before processing. The wafers were cut into several pieces to expose areas representing each of the three faces (radial, tangential and transverse sections). Small cuts were then made using a coarse double-sided blade to remove previously unexposed slivers of each face. These slivers were then placed directly onto a microscope slide and covered with a coverslip. No slivers were collected from the original exposed surface. General dimensions of the slivers were 2-5

mm wide by 4-8 mm long. Up to 15 slivers (5 per face) were collected on a microscope slide per sample. Each slide represented one wafer. Deionized water was added to a few of the samples after adding the cover slip and left to sit at room temperature for 1-2 days before evaluation. Remaining wafer material was placed into petri dishes containing CARP to determine if *P. ramorum* was present and still viable. Recovery percent was recorded for the group of plates representing each isolate or control.

Slivers were examined using a light microscope without any staining. DI water was added to the microscope slides at the time of observation for *P. ramorum* structures. A slide was recorded to have *P. ramorum* if structures that resembled chlamydospores were observed. Chlamydospores were counted if more than one was observed within a slide. Material from the two slides with the most chlamydospores were processed to be further examined using scanning electron microscopy.

Scanning electron microscopy was completed using an FEI QUANTA 3D dual beam SEM/FIB instrument located in the OSU Electron Microscope Facility. Samples were fixed in 2.5% glutaraldehyde and 1% paraformaldehyde for 1 hour, rinsed in 0.1M sodium cacodylate buffer for 15 min, post fixation stained with $OsO_4$ for 1 hour, dehydrated through a graded series of acetone, and finally sputter coated with gold/palladium to improve electrical conductivity for more efficient imaging.

DNA was extracted from slivers on three preliminary slides where chlamydospore structures were observed to determine the chlamydospores were in fact *P. ramorum*. Extracted DNA was used for polymerase chain reaction (PCR) amplification of the internal transcribed spacer (ITS) region of rDNA. Diagnostic primers and methods developed for *P. lateralis* were used to confirm the presence of *P. ramorum* as previously described (Winton and Hansen, 2001). The multiplexed reaction included an internal check on the procedure by including universal control primers based on sequence fragments common to plants, protists, and true fungi. This procedure was shown to detect *P. lateralis* in cedar stem tissue (Winton and Hansen, 2001).

*Study 4: Log inoculations using agar plugs*

Only one trial was conducted for this study. Log inoculations were attempted using methods similar to Hansen et al. (2005) and Parke et al. (2007). Both studies attempted to inoculate logs with agar plugs containing actively growing *P. ramorum* cultures. Parke et al. (2007) used a drill to create 6 mm holes at three different depths in tanoak logs. Hansen et al. (2005) used a cork borer to remove only the bark, exposing the cambial tissues of numerous tree species, including Douglas-fir. In our study, a 1.5 cm cork borer was used to remove the bark, and a 9 mm hole was drilled through the cambium into the xylem of nine 30 cm long green Douglas-fir logs collected from the McDonald Dunn Forest outside of Corvallis, Oregon. Logs were cut and stored in covered crates at 4 C for one month before inoculation. Two holes were made in each log (Figure 2.4). One was inoculated using 8 mm CMA plugs containing actively growing *P. ramorum* isolate 7904, and the other hole contained sterile CMA plugs used as controls. In both cases, three agar plugs were stacked to fill the holes and maintain contact with both the xylem and the cambium. Each set of plugs was covered by the original bark piece previously removed with the cork borer, covered with moist cheese cloth and then foil, and held in place with duct tape (Figure 2.4). The logs were enclosed in clear plastic bags and incubated at 20 C. Logs in our study were inoculated in late July and incubated almost four months before being removed and split straight down the center to divide the inoculation points in half. Tissue near the inoculation points was cultured using CARP and incubated for an additional two weeks. Cambial, sapwood, and phloem tissues were all cultured. Cambial tissues were taken at the advancing margin of cambium discoloration, if it existed.

**Results**

*Study 1: Zoospore inoculation and surface sterilization of Douglas-fir sapwood wafers*

Four Well Plate Trials were conducted to assess the ability of *P. ramorum* zoospores to colonize or survive on Douglas-fir sapwood wafers (Table 2.2). Wafers were surface treated with household bleach to limit the risk of culturing *Phytophthora* propagules as surface contaminates. No *Phytophthora* species were recovered from any of the control wafers in any trial (Table 2.3). *P. ramorum* isolates 9488 and 4313, and *P.*

*pluvialis* were not recovered from the wafers in any of the trials in this study. Only *P. ramorum* isolate 7904 was reisolated after inoculation, incubation, and surface treatment (Table 2.3). Isolate 7904 was recovered in Well Plate Trials 1, 2, and 4, although recovery was much greater in Well Plate Trials 1 and 2, compared to Well Plate Trial 4. *P. ramorum* isolate 7904 was reisolated from 20 out of 20 (100%) wafers in Well Plate Trial 1 without a surface sterilization treatment (Table 2.3). Isolate 7904 was recovered from 88.9% and 80% of the wafers treated with 5% household bleach and 10% household bleach, respectively. Similar recoveries were observed in Trial 2 for *P. ramorum* isolate 7904. *P. ramorum* was reisolated from 20 of the 22 wafers (95.5%) without a surface sterilization treatment. Treating the blocks with 5% and 10% bleach reduced the recovery to 81.8% and 70%, respectively. In Well Plate Trial 4, *P. ramorum* isolate 7904 was only recovered from 1 of 6 (16.7%) wafers that were not surface sterilized. Neither *P. ramorum* or *P. pluvialis* were reisolated from any of the wafers in Trial 3. *P. ramorum* was not reisolated from any of the wafers removed immediately after inoculation and treated with the bleach concentrations indicating that surface sterilizing was effective on propagules.

Bacterial growth was observed in the majority of the plates evaluated for Well Plate Trials 3 and 4. Growth was generally observed within a couple mm of the wood piece and surrounded the wafer pieces uniformly. Although it was not quantified, bacterial growth was also present in the control plates, but appeared to be more abundant in plates containing wafers inoculated with zoospore suspensions. Filamentous hyphae from fungi other than *Phytophthora* species were commonly observed, although not quantified. There was no observed difference in abundance or occurrence of the other filamentous fungi between controls and inoculated wafers.

*Study 2: Zoospore inoculation of Douglas-fir sapwood blocks containing bark*

Two Dip Trials and two Soak Trials were conducted in this study to assess the ability of *P. ramorum* and *P. pluvialis* to colonize or survive on Douglas-fir bark, cambial tissues, and sapwood (Table 2.2). *Phytophthora* sp. was not recovered from any of the

sections on blocks that were dipped or soaked in DI water to serve as controls in all four trials (Table 2.4, 2.5, and 2.6).

*P. ramorum* was not recovered from any of the blocks inoculated with zoospores from isolates 9488 or 4313 in the Dip Trials and was only recovered from 2 of the 10 blocks inoculated with isolate 7904 in Dip Trial 1 (Table 2.4). The colonies on one of the *P. ramorum* positive plates originated from both sapwood and cambial tissue sections. The colony from the other *P. ramorum* positive plate originated from only the cambial tissue section. *P. pluvialis* was also recovered from 3 of the 17 wood blocks in Dip Trial 1. These colonies originated from the sapwood sections on two of the *P. pluvialis* positive plates and the cambial tissue section in the third *P. pluvialis* positive plate. None of the *Phytophthora* sp. colonies originated from the bark sections. Although 5 of the 57 total blocks that were inoculated in trial one were positive for *Phytophthora* sp., the results were not replicated in the second trial (Table 2.4).

Results from Soak Trial 1 were similar to Dip Trial 2 where no *Phytophthora* species were reisolated from any of the blocks. However, *Phytophthora* species were recovered from several blocks in Soak Trial 2. In total, *P. ramorum* was recovered from 6 of the 37 blocks inoculated with a *P. ramorum* isolate in Soak Trial 2 (Table 2.6). *P. ramorum* was recovered from at least one block from each isolate and originated from the sapwood section in every instance. *P. pluvialis* was recovered more often than *P. ramorum* in Soak Trial 2, from 4 out of 12 (33.3%) inoculated blocks. Three of the *P. pluvialis* cultures originated from sapwood sections but one originated from both the sapwood section and the cambial section (Table 2.6).

Despite using selective media, filamentous fungi other than *Phytophthora* spp. grew from the bark sections in nearly every petri plate. Other fungi were commonly observed growing from the bark and cambial tissue sections, but less frequently from the sapwood sections. The frequencies (recovery percentage) of reisolating fungi other than *Phytophthora* spp. were quantified in the Soak Trials (Table 2.5 and 2.6). Other fungi were recovered in between 97-100% and 91-100% of the petri plates in Soak Trial 1, and Soak Trial 2, respectively. Other fungi grew from 91-100% of the bark sections each trial

(Table 2.5 and 2.6). In Soak Trial 1, no other fungi grew from the sapwood sections for the control blocks but were recovered from 50-55% of the sapwood sections in blocks inoculated with *P. ramorum* or *P. pluvialis* (Table 2.5). In Soak Trial 2, other fungi were only recovered from 15-33% of the sapwood sections.

*Study 3: Microscopy of Douglas-fir sapwood wafers inoculated with agar plugs*

No discoloration was observed within the Douglas-fir sapwood wafers inoculated with *P. ramorum.* Mycelium and chlamydospores were occasionally observed growing across the surface of some wafers and pieces of cheesecloth in Microscopy Trial 1. In these cases, chlamydospore densities were greatest near the inoculum plug but were also observed on adjacent sides of wafers. Microscopy revealed the presence of *P. ramorum* structures growing inside of wood cells in Microscopy Trials 1 and 2. Microscopy samples were recorded positive for *P. ramorum* if structures resembling chlamydospores were observed (Figures 2.6-2.12), but not if only hyphae were observed. However, the presence of non-septate hyphae that could be *Phytophthora* spp. was noted. Structures resembling chlamydospores were frequently observed with non-septate hyphae. Figures 2.7-2.12 are micrographs of *P. ramorum* structures growing in the cells of Douglas-fir wafers. Chlamydospores were distinguished from tyloses based on size and morphology. Structures resembling chlamydospores were not observed in any of the control wafers. *P. ramorum* structures were not observed in resin canals. Structures within ray cells primarily consisted of only hyphae. Chlamydospores and hyphae were frequently observed growing throughout tracheid cells in Microscopy Trial 1. Multiple chlamydospores were occasionally observed along the length of the same tracheid. Hyphae from filamentous fungi were also observed in many cells of the sapwood wafers of both control and inoculated cells. Resin was also frequently observed in both controls and inoculated sapwood pieces. *P. ramorum* structures were observed in 11 of the 15 microscopy samples inoculated with *P. ramorum* in the first trial. *P. ramorum* structures were not observed in any of the controls. *P. ramorum* structures were observed in wafers inoculated with each of the isolates between 60-80 percent of the time in the Microscopy Trial 1 (Table 2.7). *P. ramorum* structures were infrequently observed in the second trial.

*P. ramorum* structures were observed in 1 of 5 samples from the 7904 isolate microscopy samples and none of the others in Microscopy Trial 2 (Table 2.7).

Multiple micrographs of possible *P. ramorum* chlamydospores were observed using the scanning electron microscope (Figures 2.14-2.16). All three SEM micrographs display chlamydospores present in the tracheid cells of the Douglas-fir sapwood slivers.

*P. ramorum* did not grow from any of the remaining wafer material plated in the Microscopy Trial 1 (Table 2.7). Three cultures in Microscopy Trial 2 were positive for *P. ramorum,* but *P. ramorum* was only identified in one of the microscopy samples. The Multiplex PCR analysis failed to confirm the presence of *P. ramorum* DNA in any of the slivers evaluated in preliminary studies. PCR also failed to amplify the universal control primers included to detect plant tissue.

*Study 4: Log inoculations using agar plugs*

*P. ramorum* was not recovered from any of the artificially inoculated Douglas-fir log sections (Table 2.8). Diamond shaped areas of discolored cambial tissues were observed in some of the sections that were inoculated. However, *P. ramorum* was not isolated from the margin of the discoloration in those cases. No discoloration within the sapwood was observed. No cambial discoloration was observed on the sections set up as negative controls. *P. ramorum* was not isolated from the control sections of the logs.

**Discussion**

*Study 1: Zoospore inoculation and surface sterilization of Douglas-fir sapwood wafers*

*P. ramorum* colonized some of Douglas-fir sapwood wafers used in this study. Wafers were cut from a single Douglas-fir tree and frozen before use. Although we recognize the variation within Douglas-fir cell structure is limited, extending these results to broader populations Douglas-fir should be done with caution. The single tree used in this study does not represent the entire population of Douglas-fir trees in the West Coast of the United States. The overall health of the individual tree processed for this study is unknown, and whether the abundance of tyloses or resins present in the wood cells was

typical for Douglas-fir cannot be discussed. Further, artificial inoculum levels were used in this study and may not represent the levels experienced in situ.

The success of *P. ramorum* isolate 7904 in colonizing the Douglas-fir wafers when the other two isolates could not may suggest there was variation in pathogenicity between the isolates. This was not tested statistically because only one isolate had any percent recovery in the Well Plate Trials (Table 2.3). The experience with the cultures maintained in the laboratory revealed that isolate 7904 typically grew faster than the other two isolates. Isolate 9488 grew slightly slower than 7904 and Isolate 4313 appeared to grow even slower. These differences in growth rates may have contributed to the differences in colonization success. The pathogenicity of each isolate may differ on other substrates since each of the *P. ramorum* isolates was isolated from a different host (Table 2.1). Hansen et al. (2005) observed no consistent differences in lesion area among the isolates on cambial tissues of logs that were artificially inoculated. However, their study used different isolates of *P. ramorum* NA-1 lineage (Hansen et al., 2005). Alternatively, Manter et al. (2010) experienced similar differences with two of the isolates in a study that did use the same isolates. Isolate 4313 produced the smallest lesion area and the least sporangia on artificially inoculated *Rhododendron catawbiense* leaves when compared to four other NA-1 lineage isolates (Manter et al., 2010). Isolate 9488 produced the second largest lesion area, but the third most sporangia of the five NA-1 isolates (Manter et al., 2010). These behaviors are consistent with the differences observed in our tests. It is difficult to suggest which isolate may be the best representative of the NA-1 lineage of *P. ramorum.*

The general decrease in percent recovery with increased bleach concentration suggests that some cultures in the non-treated samples were a result of *P. ramorum* propagule survival on the surface, rather than actual colonization of the material (Table 2.3). This could be further assessed using microscopy. The surface sterilization treatments were generally conservative because of our concern about bleach diffusing into the wafers, but the treatment did affect recovery in both Well Plate Trials 1 and 2. In the later trials, wafers were immediately surface sterilized after 30 minutes in the well

plates with the zoospores to determine whether the treatments were effective at eliminating zoospores on the surface. Nothing grew out of the wafers that were immediately treated, but neither did it grow out of wafers that were incubated in Well Plate Trials 3 and 4. *P. ramorum* was reisolated from one wafer inoculated with 7904 and not treated before plating in Well Plate Trial 4.

The inability to culture *Phytophthora* spp. in many of the wafers does not completely eliminate the possibility that *Phytophthora* sp. is present in the wood cells. Chlamydospore germination is not guaranteed and there are many factors that contribute to their germination (Tooley et al., 2014). For instance, Tooley et al. (2014) determined that the optimal temperature for germination of the EU1 clonal lineage of *P. ramorum* was 20 C. However, temperature does not explain the differences in culturing between trials because the plates were incubated in the same manner for each trial, species, isolate and treatment group. On the other hand, the optimal temperature for growth of *P. pluvialis* is between 15-20 C and may be less than *P. ramorum* (Reeser et al., 2013; Werres et al., 2001). This may have contributed to the inability to culture *P. pluvialis* from any of the wafers in Trial 3 and 4.

The inoculation methods in Well Plate Trials 1 and 2 differed from Trials 3 and 4. Although inoculum zoospore concentrations were higher in Trials 3 and 4, percent recovery was substantially lower. The primary difference in the trial methods was the duration for which the wafers were exposed to the inoculum zoospore suspension. Wafers in Trials 1 and 2 were only submerged in zoospore suspensions for 24-48 hours. In Trials 3 and 4, the wafers were left submerged in the suspensions for several weeks. Therefore, a possible explanation for the difference in recovery percent for Trials 1 and 2 compared to Trials 3 and 4, is that the wood cells of the wafers in the third and fourth trials became completely saturated with water thereby limiting oxygen and preventing the establishment of the *Phytophthora* species.

In general, the zoospore method of inoculation using well plates and exposing the wafers to inoculum for a limited 24-48 hours appeared to be an effective method for

assessing the ability of *P. ramorum* to colonize wood material. Further replication of the experiments would be necessary to confirm this claim.

The differences in bacterial growth abundance between the inoculated wafers and the controls could be attributed to the use of creek water within the zoospore suspensions. The presence of bacteria or other filamentous fungi could have contributed to the inability to culture the *Phytophthora* species from the wafers because of competition for space and nutrients. Alternatively, the inoculation and exposure conditions made it difficult to exclude other organisms. The effects of these organisms may have influenced the ability of the *Phytophthora* isolates to colonize the substrates. There is evidence that some filamentous fungi (e.g. *Trichoderma* spp.) are antagonistic to *P. ramorum* and may have inhibited the *Phytophthora* species. However, *Trichoderma* spp. were not specifically observed growing in the plates.

*Study 2: Zoospore inoculation of Douglas-fir sapwood blocks containing bark*

The ability of *P. ramorum* and *P. pluvialis* to colonize Douglas-fir sapwood was assessed in the first study. However, in order to apply the results, one must assume that *P. ramorum* will readily have access to Douglas-fir sapwood. In this study, the objective was to compare the ability of *Phytophthora* spp. zoospores to colonize or survive on the bark, cambial tissues, and sapwood of Douglas-fir blocks. This study may best represent the actual conditions experienced in the field because it is probable that the bark of logs may be the most exposed component of Douglas-fir logs in the field.

The results indicate that *P. ramorum* or *P. pluvialis* did not survive on, or colonize, the bark of the Douglas-fir blocks used in this study. It has been suggested that Douglas-fir bark may inhibit *P. ramorum* growth. McKeever (2010) found the addition of raw bark to a rhododendron leaf assay reduced the ability to bait *P. ramorum* from water. Our results do not confirm nor deny the earlier findings, but rather suggest that *P. ramorum* can still colonize the cambial or sapwood tissues if accessible and that it may be less likely to colonize bark tissues.

In Dip Trial 1, colonies from both *P. ramorum* isolate 7904 cultures and one of the *P. pluvialis* cultures originated from the cambial tissue sections. However, these sections contained a few mm of sapwood and bark on either side of the cambial tissues. Therefore, it is reasonable to interpret it as sapwood infection in plates where *Phytophthora spp.* colonies grew from both cambial sections and sapwood sections. Alternatively, the premise where the *Phytophthora* spp. colony originated from only the cambial sections, may suggest that cambial tissues were the most susceptible substrate.

*P. ramorum* and *P. pluvialis* were both recovered from blocks used in Dip Trial 1 and Soak Trial 2. *P. pluvialis* was recovered from 17.65% of the blocks when dipped in zoospore suspensions used in Dip Trial 1, and from 33.3% of the blocks that were soaked for 24 hours in zoospore suspensions used in Soak Trial 2. These results suggest that *P. pluvialis* and *P. ramorum* can survive on the sapwood of Douglas-fir. Further, these results suggest there is a potential risk of transporting these organisms with the movement of green sapwood material from western Oregon's primary commercial species, Douglas-fir.

Differences in *Phytophthora* spp. recovery between Soak Trial 1 and Soak Trial 2 may be explained by the multiple factors that were not controlled between experiments. For example, the delay between zoospore release and inoculation may have played a role. It is possible that many of the zoospores had encysted prior to inoculation in Soak Trial 1. Production of zoospores was an inconsistent and variable process that typically depended on the behavior of each isolate. For example, observations regarding the abundance of sporangia generally influenced the decision on timing. Often the decision to move forward with the inoculation was not made until zoospores were quantified with a hemacytometer, and additional time passed before inoculation. Controlling for the timing could have improved the consistency of the trials, but this is difficult to do when using different isolates, and especially different species, of *Phytophthora*. In addition, the use of creek water to facilitate sporangial production added much variation into the process. The creek water was allowed to sit, and essentially incubate, at room temperature during the intervals between trials. This may have resulted in changes in the abundance of other

organisms. In general, controlling for the variation in creek water used would likely have improved the consistency of the trials. This may have been possible by filtering the water to reduce the amount of propagules from other organisms, by freezing for storage, or by collecting fresh water before each trial—taking into account seasonal variation.

Filamentous fungi were abundant in the bark and cambial sections of the wood blocks used in this study. *Trichoderma* spp. were commonly observed, suggesting the potential for an antagonistic relationship preventing *Phytophthora* species from colonizing or surviving on the wood blocks used in this study. Isolates of *Trichoderma asperellum* have been shown to have mycoparaistic activity against *P. ramorum* (Widmer, 2014). The frequency of *Trichoderma* spp. on Douglas-fir bark is unknown, making it difficult to use the presence of these fungi to account for the limited ability of *P. ramorum* colonization of the samples. The treatment of the log sections used for this study may have contributed to the abundance of fungal contaminants and may not represent real abundance in Douglas-fir forests. Freshly cut Douglas-fir logs may be more prone to colonization by *Phytophthora* species than the Douglas-fir blocks and wafers used in these studies because of reduced exposure to antagonistic fungi.

The differences between the *P. pluvialis* results presented here and those by Hood et al. (2014) can most likely be attributed to the difference in host material susceptibility. Logs of several species of pine have been identified as resistant to *P. ramorum* colonization, while a number of other conifer species were colonized (Moralejo et al., 2007). Alternatively, differences may have arisen from variation within the methods. As seen within this assessment, the results can be inconsistent, and practically reversed, depending on the methods selected. For this reason, it is important to use several different methods before drawing conclusions based on a small number of tests.

The method used in this study may fairly represent the conditions for the risk of *Phytophthora* spp. survival on Douglas-fir log material. However, the zoospore concentrations utilized in this study were attained artificially and may not represent natural concentrations experienced in the field. In addition, the sapwood and cambial tissue sections were directly exposed to zoospore suspensions in a manner that may not

represent a realistic exposure risk. On the other hand, the wood blocks in the Dip Trials were only dipped in the suspension for a few seconds, while zoospores in the field may persist on the wood material for several days to weeks while logs remain in the infected area. Therefore, the Soak Trials may be the most natural method of inoculation.

The frequency of reisolating *P. pluvialis* from the sapwood of Douglas-fir pieces in Dip Trial 1 and Soak Trial 2 may be relevant to the possible introduction of this species to New Zealand from Oregon. The export of untreated coniferous logs from the US to New Zealand (Comtrade, 2014) coupled with the results of this study provide a possible story of the introduction and further highlight the risks created by moving plant material around the world. If this is the means of the introduction of *P. pluvialis* into New Zealand, then these results indicate that the same risk is possible with *P. ramorum*, since our study showed comparable percent recoveries of the species.

*Study 3: Microscopy of Douglas-fir sapwood wafers inoculated with agar plugs*

None of the structures observed microscopically could be proven as *P. ramorum*. Multiplex PCR analysis of DNA from three preliminary microscopy samples where *P. ramorum* had been observed were not successful possibly because of the small amount of material used. The mass of the slivers used in the DNA extraction was close to 4 mg and this may have been too little for detection. Alternatively, extractives in the wood might have interfered with extraction and amplification. The universal control primers had previously been amplified with the Multiplex PCR procedure when using Port Orford cedar bark (Winton and Hansen, 2001), but the primers hadn't been tested with sapwood tissues.

Culturing was also attempted to confirm the presence of *P. ramorum* in the remaining wafer material after prepping slides, but *P. ramorum* did not grow from any of the wafer material used in Microscopy Trial 1 and only grew from a few wafers in Microscopy Trial 2 (Table 2.7). *P. ramorum* was actually reisolated in more residual wafer material plates than were observed with microscopy in the second trial. Positive cultures where *P. ramorum* structures were not observed within wood cells using light

microscopy suggests the samples were merely surface contaminated or that the Microscopy method was insufficient to detect *P. ramorum* presence on every wafer. Alternatively, observing chlamydospore structures with light microscopy in culture negative samples, as in Microscopy Trial 1, suggests that visual inspection may detect non-viable structures or that chlamydospore germination did not occur. Another alternative is that negative cultures may actually suggest that the structures were *P. ramorum*, since cultures were attempted on *Phytophthora* spp. selective medium. Without question, the most probable reason that *P. ramorum* was not reisolated from any of the residual wafer material in Microscopy Trial 1 was the long delay between inoculation and isolation. Thus, although *P. ramorum* could grow into the cells of the Douglas-fir wafers in Microscopy Trial 1, the organism could not survive on, or germinate from, the material after 9 months. In Microscopy Trial 2, *P. ramorum* was still viable on Douglas-fir sapwood 3 months after it had been inoculated. However, it is possible that there was carry over of *P. ramorum* in agar from the initial agar plug on the surface of the residual wafer material because no surface sterilization was attempted.

Structures were recorded as *P. ramorum* if they satisfied morphological criteria consistent with *Phytophthora* spp. and were distinguishable from structures in negative controls. None of the structures thought to be *P. ramorum* were observed in any of the control wafer samples. Figure 2.3 was included to illustrate structures that were not recorded as *P. ramorum* because they did not fit the characteristic criteria for *Phytophthora* spp. and they were present in wafers which were inoculated and those that were not (controls). Figure 2.14 has both *P. ramorum* characteristic structures and unknown structures. Figures 2.7-2.12 represent structures that were recorded as *P. ramorum* using light microscopy.

Explanations for the difference in recovery between the trials include but are not limited to factors that were not controlled such as the quantity of water added to the cheesecloth, the initial agar medium quantities, the seasonality and timing of inoculation and processing, the room temperatures during processing, and the sizes of the cheese

cloth used. All agar plugs were the same size, however, age and depth of agar were not controlled and may have contributed to the variability.

*Study 4: Log inoculations using agar plugs*

While it has been suggested that similar methods can be utilized to estimate susceptibility of tree species, this method may be better suited for assessing the risk of accidentally transporting *Phytophthora* species in wood material. Several studies have suggested colonization of cambial tissues by *Phytophthora* species represents a risk of establishment within forest habitats (Brasier and Kirk, 2001; Hansen et al., 2005; Moralejo et al., 2007). Hansen et al. (2005) showed that *P. ramorum* can create small cambial lesions in Douglas-fir logs. However, it is well known that Douglas-fir trees are not at risk of mortality from *P. ramorum* in their natural monoculture forests. Instead, the results of their study suggested that Douglas-fir cambium can serve as a substrate for *P. ramorum* survival and may act as a carrier for exotic microorganisms.

Although *P. ramorum* was not recovered from any isolation made in logs in this study, symptoms were expressed that are consistent with other literature. Only one trial was attempted and culturing was the only diagnostic technique utilized. It is possible that the material had dried out too much prior to inoculation, or that the time of year was unsutiable for *P. ramorum* survival on logs. Hansen et al. (2005) and Brown and Brasier (2007) noticed variation within tests depending on the time of year. Douglas-fir had larger cambial lesions than tanoak in the log inoculation test in January, but not any other time of year (Hansen et al., 2005). Logs in our study were inoculated in late July. Differences in the methods could have also contributed to the outcome. A drill was used to create a hole into the xylem by Parke et al. (2007) with tanoak, but not by Hansen et al. (2005) or Brown and Brasier (2007). It is possible that the drilling caused damage, closed off the cells or changed the material with heat, and thereby prevented *P. ramorum* from growing into the cells.

**Conclusion**

The results of the four studies were limited to the wood wafers used within the trials. Because the wood wafers were taken from only two Douglas-fir trees and were not randomly selected, the results of these studies probably do not fairly represent the greater population of Douglas-fir trees in Oregon, but they do provide information on possible relationships between *P. ramorum* and Douglas-fir. The results indicate that the material used in the four studies infrequently served as a substrate for *P. ramorum* or *P. pluvialis* growth or survival, but implies that there may be a risk associated with moving Douglas-fir log material from infected areas. We recommend implementing careful strategies to avoid contact between *P. ramorum* spores and green Douglas-fir sapwood material. Limiting the log time at the landing and proximity to infected hosts would further alleviate the risk of accidental dissemination.

**Tables and Figures**

Table 2.1: Source of *P. ramorum* and *P. pluvialis* isolates.

| Species | Isolate | Date Isolated | Sample Type | Host | Coordinates | Isolated By |
|---|---|---|---|---|---|---|
| *P. ramorum* | 7904 | 3/3/12 | Small stems, twigs and Foliage | *Umbellularia californica* | 42.11849, -124.19735 | Joseph M. Hulbert |
| *P. ramorum* | 9488 | 7/7/06 | Cambium/inner bark lesion | *Notholithocarpus densiflorus* | 42.08961, -124.32284 | Oregon Department of Forestry |
| *P. ramorum* | 4313 | 11/21/02 | NA | *Rhododendron* sp. | NA | Oregon Department of Forestry |
| *P. pluvialis* | LC-9-2-020508 | 2/5/08 | Baited Canopy Rain Trap | NA | 42.11185, -124.25657 | Oregon Department of Forestry |

Table 2.2: Summary of studies and trials discussed in Chapter 2.

| Study | Description | Trial | Sample Size | Inoculation Method | Results Table |
|---|---|---|---|---|---|
| 1 | Zoospore inoculation and surface sterilization of Douglas-fir sapwood wafers | Well Plate Trial 1 | 229 | Zoospore soak, 24 hour | 2.2 |
| | | Well Plate Trial 2 | 93 | Zoospore soak, 24 hour | 2.2 |
| | | Well Plate Trial 3 | 90 | Zoospore soak, multiple weeks | 2.2 |
| | | Well Plate Trial 4 | 90 | Zoospore soak, multiple weeks | 2.2 |
| 2 | Zoospore inoculation of Douglas-fir sapwood blocks containing bark | Dip Trial 1 | 57 | Zoospore Dip | 2.3 |
| | | Dip Trial 2 | 51 | Zoospore Dip | 2.3 |
| | | Soak Trial 1 | 82 | Zoospore soak, 24 hour | 2.4 |
| | | Soak Trial 2 | 61 | Zoospore soak, 24 hour | 2.5 |
| 3 | Microscopy of Douglas-fir sapwood wafers inoculated with agar plugs | Microscopy Trial 1 | 20 | Agar plug | 2.6 |
| | | Microscopy Trial 2 | 20 | Agar plug | 2.6 |
| 4 | Log inoculations using agar plugs | Log Trial 1 | 9 | Agar plug | 2.7 |

Table 2.3: Percent recovery of *P. ramorum* or *P. pluvialis* from Douglas-fir sapwood wafers inoculated with zoospores.

| Species/ Isolate | Trial and Treatment | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Zoospore Conc. (per mL) | Well Plate Trial 1 | | | Zoospore Conc. (per mL) | Well Plate Trial 2 | | | Zoospore Conc. (per mL) | Well Plate Trial 3 | | | Zoospore Conc. (per mL) | Well Plate Trial 4 | | |
| | | None | 5% | 10% | | None | 5% | 10% | | None | 5% | 10% | | None | 5% | 10% |
| **7904** | $7 \times 10^3$ | 100% [83.2-100%] (20) | 88.9% [65.3-98.6%] (18) | 80% [59.3-93.2%] (25) | $7 \times 10^3$ | 95.5% [77.2-99.9%] (22) | 81.8% [59.7-94.8%] (22) | 70% [45.7-88.1%] (20) | $1.4 \times 10^4$ | 0% (6) | 0% (6) | 0% (6) | $1.0 \times 10^4$ | 16.7% [0.4-64.1%] (6) | 0% (6) | 0% (6) |
| **9488** | $6 \times 10^3$ | 0% (20) | 0% (22) | 0% (21) | NA | NA | NA | NA | $1.7 \times 10^4$ | 0% (6) | 0% (6) | 0% (6) | $1.6 \times 10^4$ | 0% (6) | 0% (6) | 0% (6) |
| **4313** | $1 \times 10^4$ | 0% (22) | 0% (24) | 0% (18) | NA | NA | NA | NA | $1.7 \times 10^4$ | 0% (6) | 0% (6) | 0% (6) | $1.5 \times 10^4$ | 0% (6) | 0% (6) | 0% (6) |
| ***P. pluvialis*** | NA | NA | NA | NA | NA | NA | NA | NA | $1.0 \times 10^4$ | 0% (6) | 0% (6) | 0% (6) | $2.2 \times 10^4$ | 0% (6) | 0% (6) | 0% (6) |
| **Control** | NA | 0% (9) | 0% (10) | 0% (9) | NA | 0% (9) | 0% (11) | 0% (9) | NA | 0% (6) | 0% (6) | 0% (6) | NA | 0% (6) | 0% (6) | 0% (6) |

Numbers in brackets represent exact binomial 95% confidence intervals. Numbers in parenthesis represent the number of replicates. None = No treatment; 5% = 5% Bleach, 60 Seconds; 10% = 10% Bleach, 30 Seconds

Table 2.4: Percent recovery of *P. ramorum* or *P. pluvialis* from Douglas-fir sapwood pieces containing bark inoculated by dipping in zoospore suspensions.

| Species/ Isolate | Douglas-fir Blocks Containing Bark Dip Trial 1[a] | | | Douglas-fir Blocks Containing Bark Dip Trial 2[a] | | |
|---|---|---|---|---|---|---|
| | Zoospore Conc. (per mL) | Recovery Percentage | Recovery Origin[1] | Zoospore Conc. (per mL) | Recovery Percentage | Recovery Origin[1] |
| **7904** | $1.4 \times 10^4$ | 20% [2.5- 55.6%] (10) | Sapwood and Cambium | $1.0 \times 10^4$ | 0% (10) | NA |
| **9488** | $1.7 \times 10^4$ | 0% (10) | NA | $1.6 \times 10^4$ | 0% (10) | NA |
| **4313** | $1.7 \times 10^4$ | 0% (10) | NA | $1.5 \times 10^4$ | 0% (10) | NA |
| ***P. pluvialis*** | $1.0 \times 10^4$ | 17.65% [3.8-43.4%] (17) | Sapwood and Cambium | $2.2 \times 10^4$ | 0% (11) | NA |
| **Control** | NA | 0% (10) | NA | NA | 0% (10) | NA |

[a]Values – brackets represent exact binomial 95% confidence intervals, parentheses represent the number of samples examined. [1]Each block was split into 3 sections prior to culturing, recovery origin refers to the piece where the *Phytophthora sp*. culture originated.

Table 2.5: Percent recovery of *P. ramorum* or *P. pluvialis* from Douglas-fir sapwood pieces containing bark inoculated by soaking in zoospore suspensions for 24 hours.

| Species | Soak Trial 1[a] | | | | |
|---|---|---|---|---|---|
| | *Phytophthora sp.* | | | Other Fungi[1] | |
| | Zoospore Concentration (per mL) | Recovery Percentage | Recovery Origin[2] | Recovery Percentage | Recovery Origin[2] |
| ***P. ramorum*** | 1 x $10^3$ | 0% (36) | NA | 97.2% (36) | 55.6% (Sapwood), 97.2% (Bark), 91.6% (Cambium) |
| ***P. pluvialis*** | 1 x $10^3$ | 0% (36) | NA | 100% (36) | 50% (Sapwood), 100% (Bark), 100% (Cambium) |
| **Control** | NA | 0% (10) | NA | 100% (10) | 0% (Sapwood), 100% (Bark), 50% (Cambium) |

[a]Values – parentheses represent the number of samples examined. [1]Other fungi is an indicator of whether or not other fungi were growing in the plates. [2]Each block was split into 3 sections prior to culturing, recovery origin refers to the section where the *Phytophthor*a sp. or orther fungi cultures originated; percentages are based on totals, some plates may have had origins on all three sections.

Table 2.6: Percent recovery of *P. ramorum* or *P. pluvialis* from Douglas-fir sapwood pieces containing bark inoculated by soaking in zoospore suspensions for 24 hours.

| Species/ Isolate | Soak Trial 2[a] | | | | |
|---|---|---|---|---|---|
| | *Phytophthora sp.* | | | Other Fungi[1] | |
| | Zoospore Concentration (per mL) | Recovery Percentage | Recovery Origin[2] | Recovery Percentage | Recovery Origin[2] |
| **7904** | $< 1 \times 10^3$ | 15.4% [1.9-45.4%] (13) | 15.4% (Sapwood) | 100% (13) | 15.4% (Sapwood), 100% (Bark), 53.9% (Cambium) |
| **9488** | $1 \times 10^3$ | 8.3% [0.2-36.3%] (12) | 8.3% (Sapwood) | 100% (12) | 25% (Sapwood), 100% (Bark), 58.3% (Cambium) |
| **4313** | $4 \times 10^3$ | 25% [5.5-57.2%] (12) | 25% (Sapwood) | 100% (12) | 25% (Sapwood), 91.7% (Bark), 50% (Cambium) |
| ***P. pluvialis*** | $2 \times 10^3$ | 33.3% [9.9-65.1] (12) | 33.3% (Sapwood), 8.3% (Cambium) | 100% (12) | 25% (Sapwood), 100% (Bark), 66.7% (Cambium) |
| **Control** | NA | 0% (12) | NA | 91.7% (12) | 33.3% (Sapwood), 91.7% (Bark), 66.7% (Cambium) |

[a]Values – brackets represent exact binomial 95% confidence intervals, parentheses represent the number of samples examined [1]Other fungi is an indicator of whether or not other fungi were growing in the plates. [2]Each block was split into three sections prior to culturing, recovery origin refers to the piece where the *Phytophthor*a sp. or other fungi culture originated; percentages are based on totals, some plates may have had origins on all three sections.

Table 2.7: Incidence of *P. ramorum* on inoculated Douglas-fir sapwood wafers as determined by microscopic examination.

| Isolate | Microscopy Trial 1 | | | Microscopy Trial 2 | | |
|---|---|---|---|---|---|---|
| | Structural Observation Percentage | Average No. Chlamydospores[1] | Recovery Percentage | Structural Observation Percentage | Average No. Chlamydospores[1] | Recovery Percentage |
| 7904 | 80% | 2 | 0% | 20% | 0.4 | 40% |
| 9488 | 80% | 9.4 | 0% | 0% | 0 | 0% |
| 4313 | 60% | 6.2 | 0% | 0% | 0 | 20% |
| Control | 0% | 0 | 0% | 0% | 0 | 0% |

[1]Values represent observations on 5 samples per isolate.

Table 2.8: Incidence of *P. ramorum* in cambium, phloem, or sapwood of Douglas-fir log sections inoculated with *P. ramorum*.

| Inoculum | Section[1] | Isolation Attempts | Percent Recovery[2] |
|---|---|---|---|
| *P. ramorum* | Cambium and Phloem | 9 | 0% |
| *P. ramorum* | Sapwood | 10 | 0% |
| Control | Cambium and Phloem | 9 | 0% |
| Control | Sapwood | 11 | 0% |

[1]Section refers to the area that was isolated from. [2]Percent recovery is calculated from the number of isolations made.

Figure 2.1: a) Well plates containing Douglas-fir sapwood wafers and 1 ml of zoospore suspension (left) and b) wafers on a wire mesh surface several centimeters above moist paper towel in a polystyrene storage container prior to incubation (right).

Figure 2.2: Douglas-fir blocks containing bark inoculated by dipping (left) or soaking (right) in zoospore suspensions.

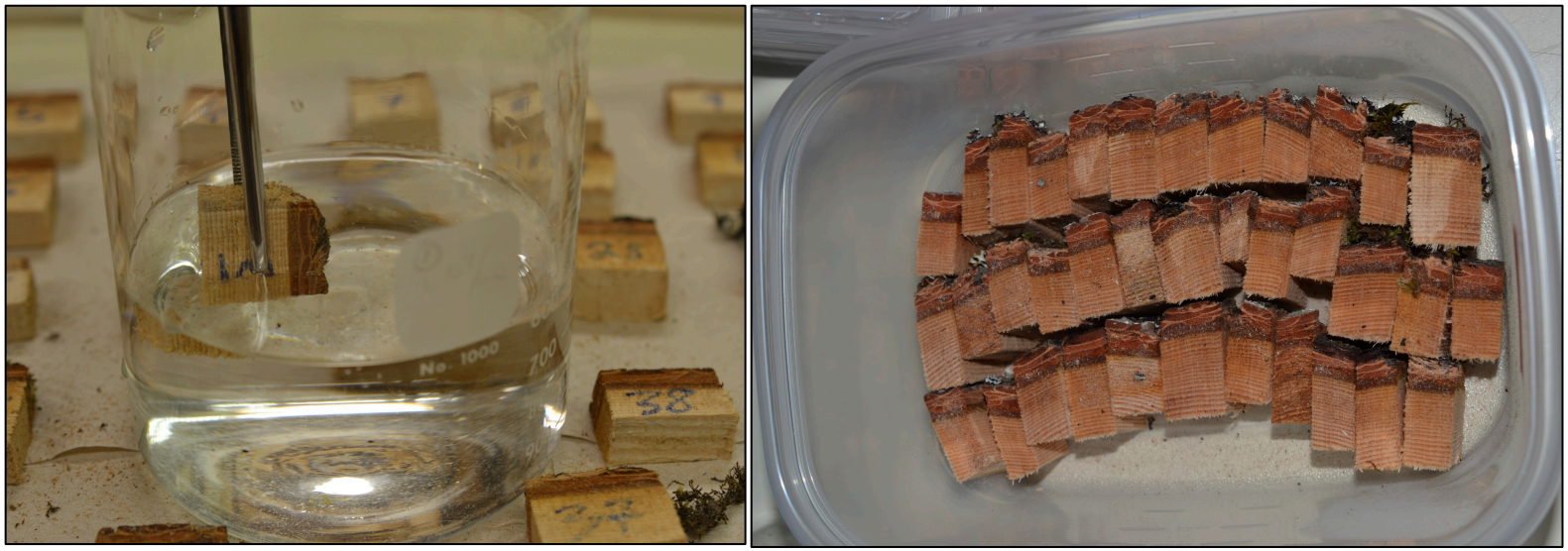


Figure 2.3: Agar plug placed on tangential (left) and radial (right) faces of Douglas-fir sapwood wafers used in Microscopy Trials 1 and 2, respectively. Images taken before (right) and after (left) incubation.

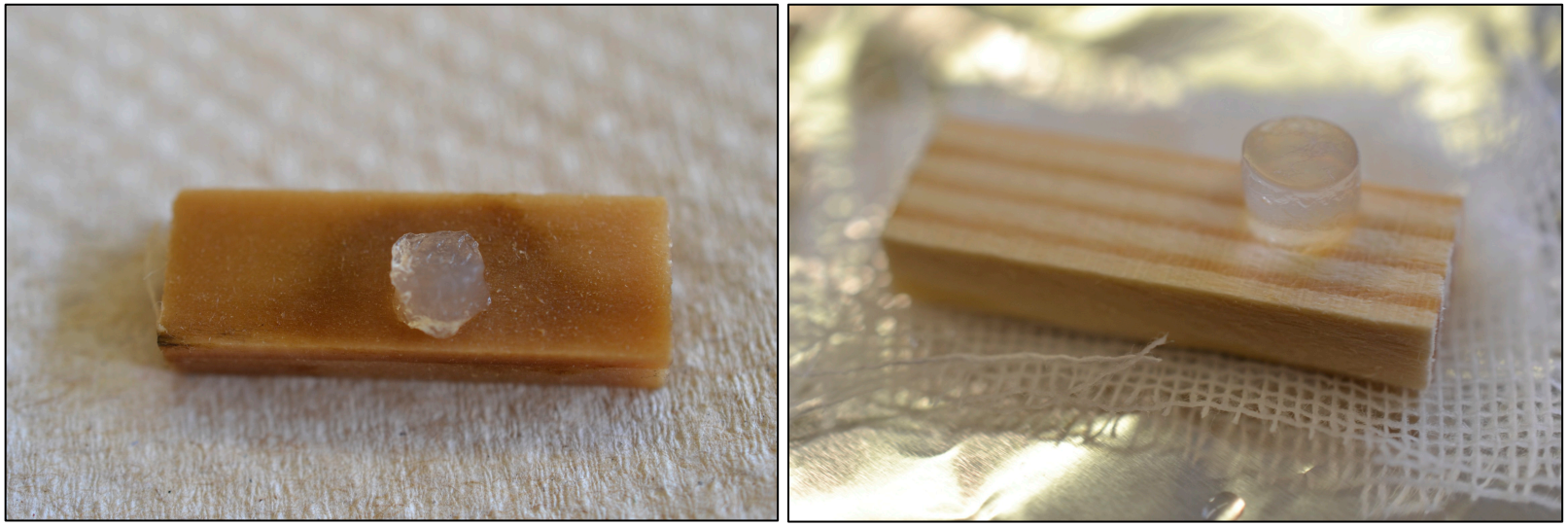


Figure 2.4: Post incubation images of Log sections with bark pieces removed using a cork borer (left) and after *P. ramorum* and control agar plugs had been inserted and logs were prepped for incubation (right).

Figure 2.5: Wrapping a Douglas-fir sapwood wafer with a *P. ramorum* agar plug and moist cheesecloth into foil for Microscopy Trials 1 and 2.

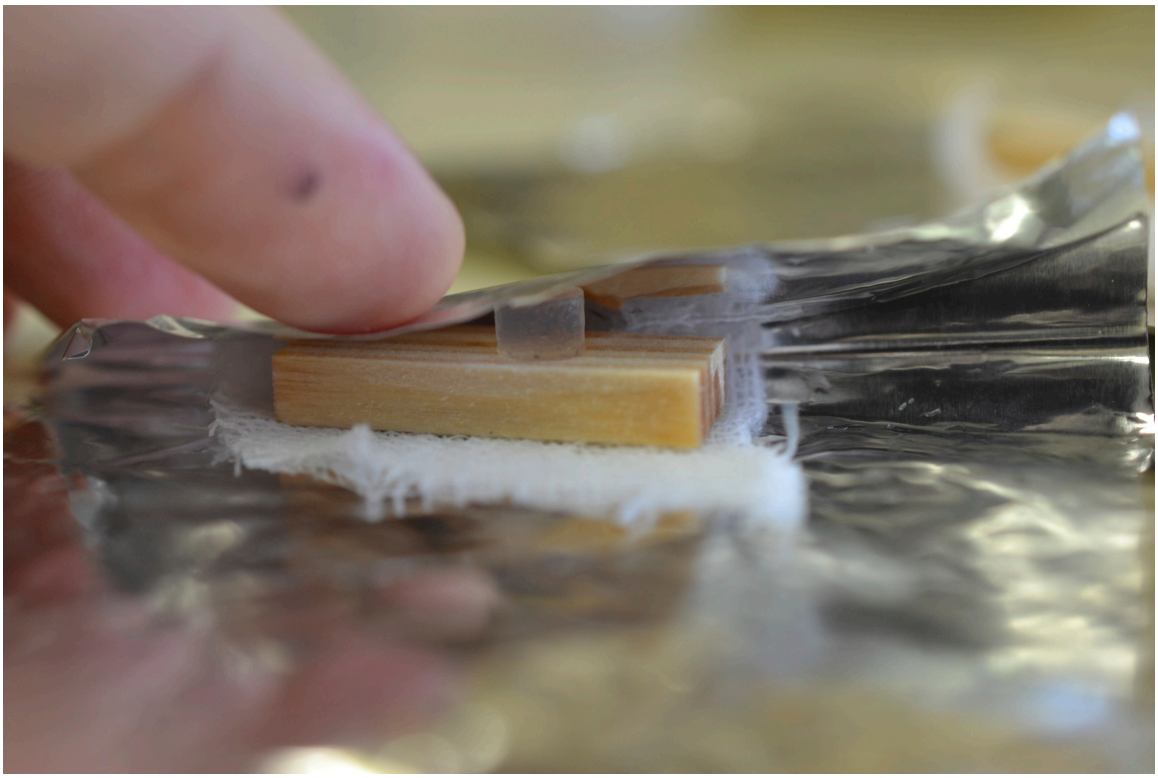


Figure 2.6: Micrograph of *P. ramorum* isolate 7904 growing on V8 media captured with a light microscope using a 10 x objective showing hyphae and chlamydospores.

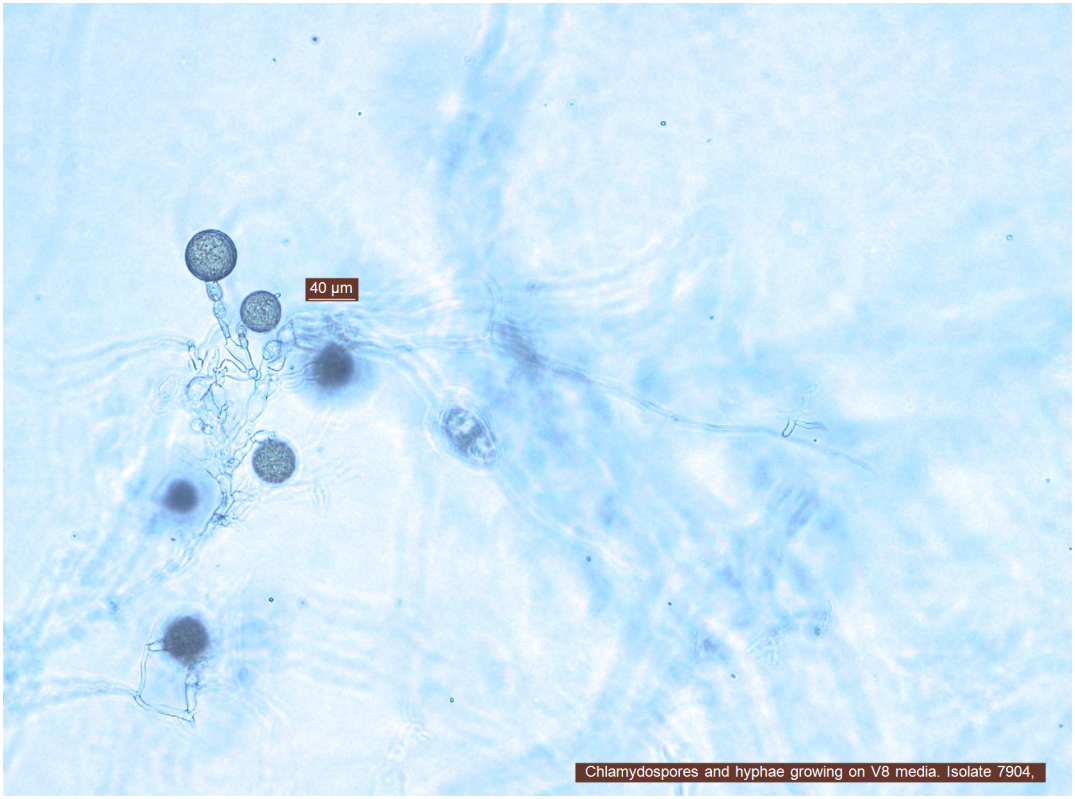


Figure 2.7: Light micrograph showing two *P. ramorum* chlamydospores and hyphae from isolate 4313 growing in a Douglas-fir tracheid cell. No stain was used.

Figure 2.8: Light micrograph showing *P. ramorum* chlamydospore and hyphae from isolate 4313 growing in a Douglas-fir tracheid cell. No stain was used.

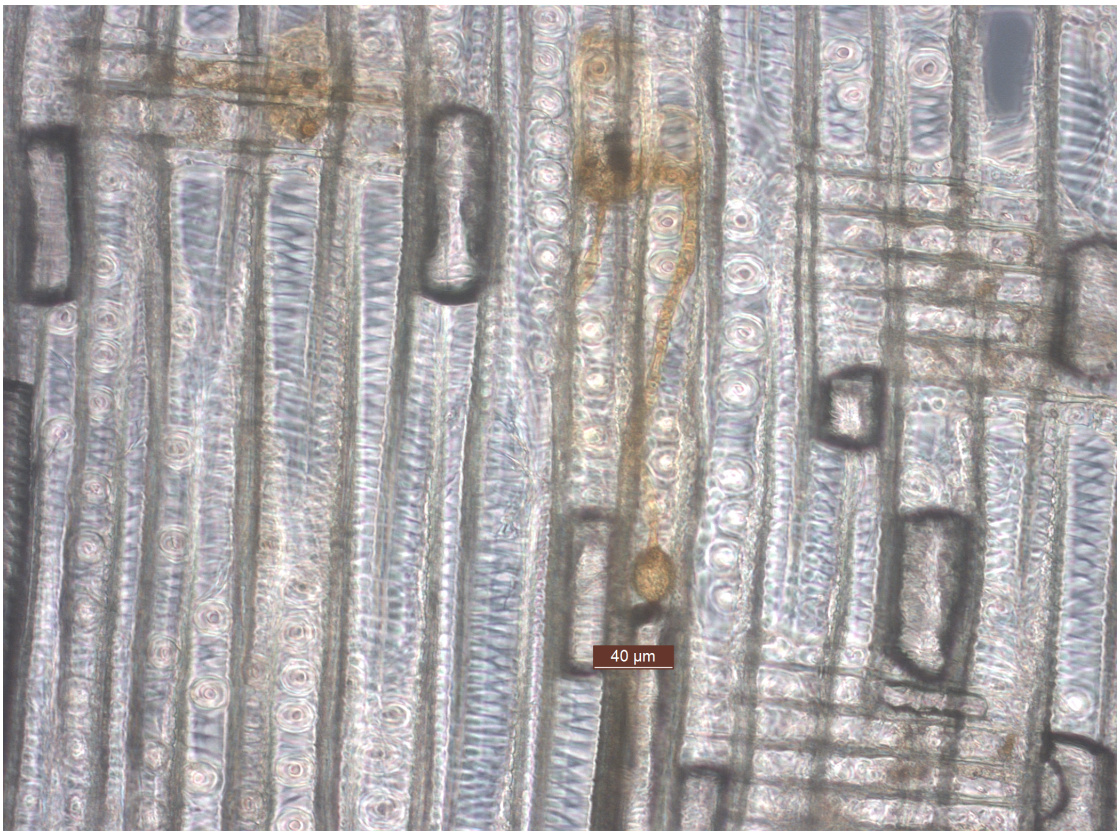


Figure 2.9: Light micrograph showing *P. ramorum* chlamydospore from isolate 7904 growing in a Douglas-fir tracheid cell. No stain was used.

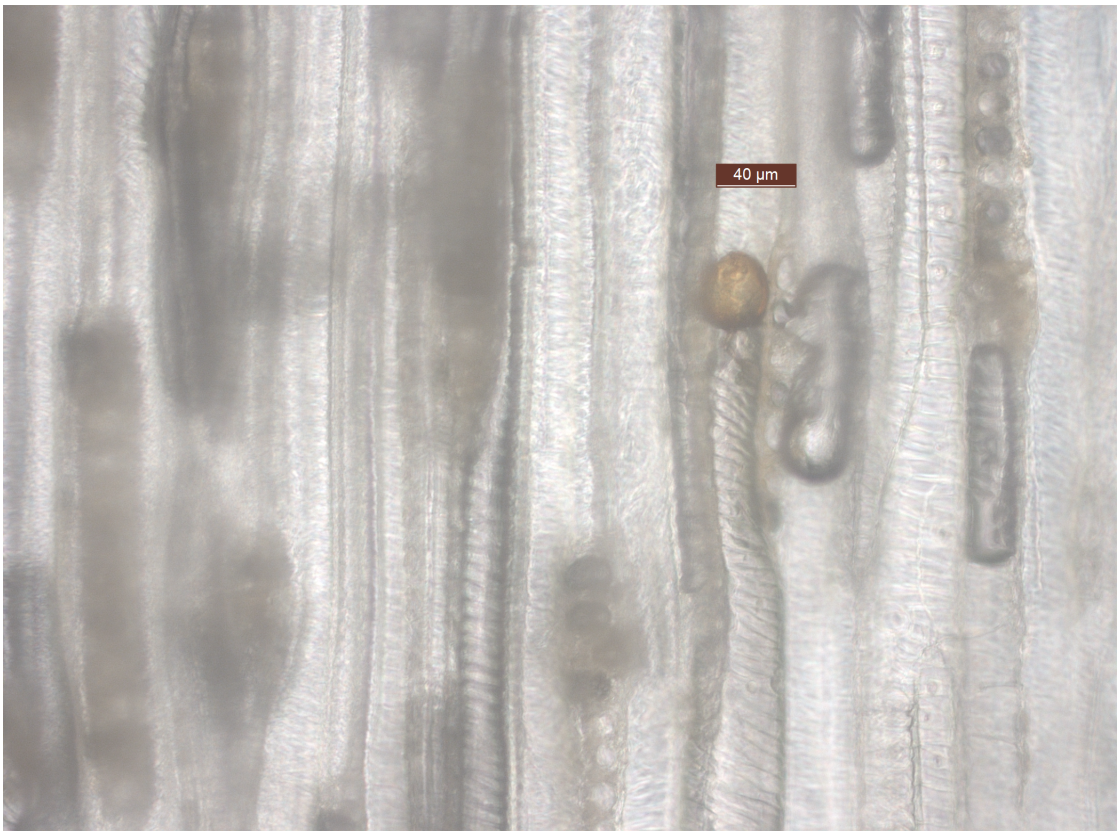


Figure 2.10: Light micrograph showing three *P. ramorum* chlamydospores and hyphae from isolate 9488 growing in two adjacent Douglas-fir tracheid cells. No stain was used.

Figure 2.11: Light micrograph showing four *P. ramorum* chlamydospores from isolate 9488 growing in Douglas-fir tracheid cells. No stain was used.

Figure 2.12: Light micrograph showing *P. ramorum* chlamydospore from isolate 9488 growing in a Douglas-fir tracheid cell. No stain was used.

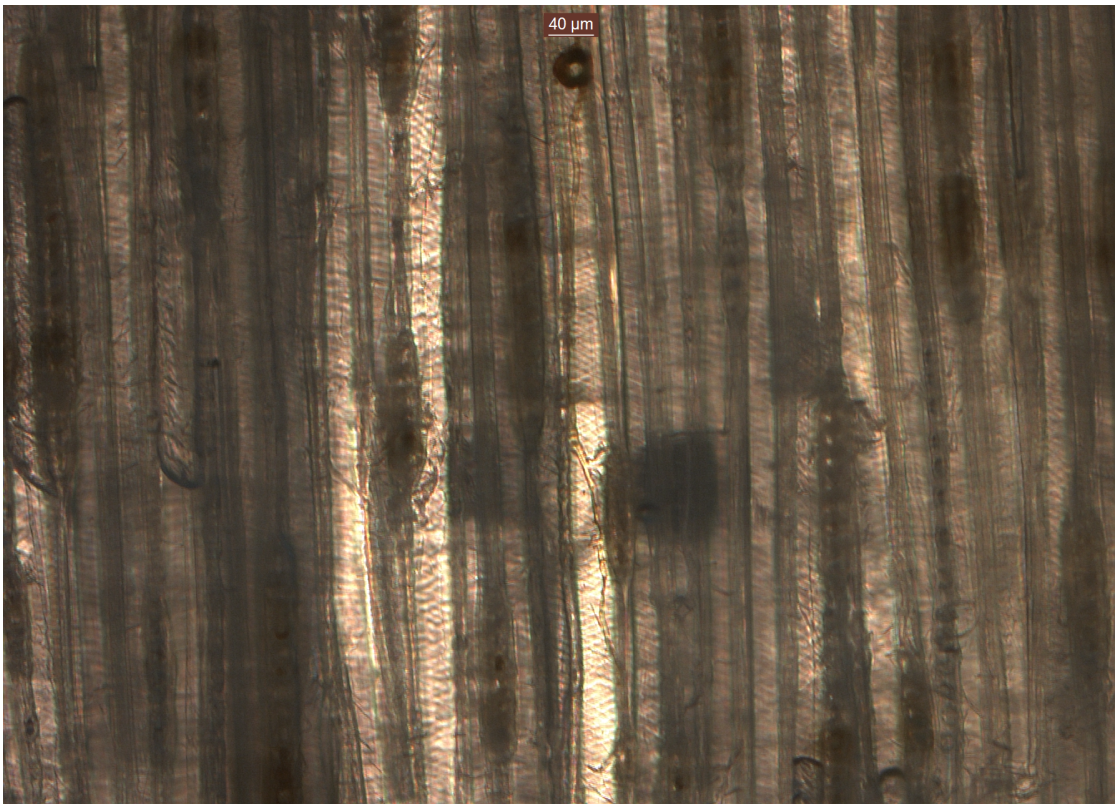


Figure 2.13: Light micrograph showing contamination in tracheid cell lumen from a wafer inoculated with isolate 4313. Similar structures were observed in control wafers.

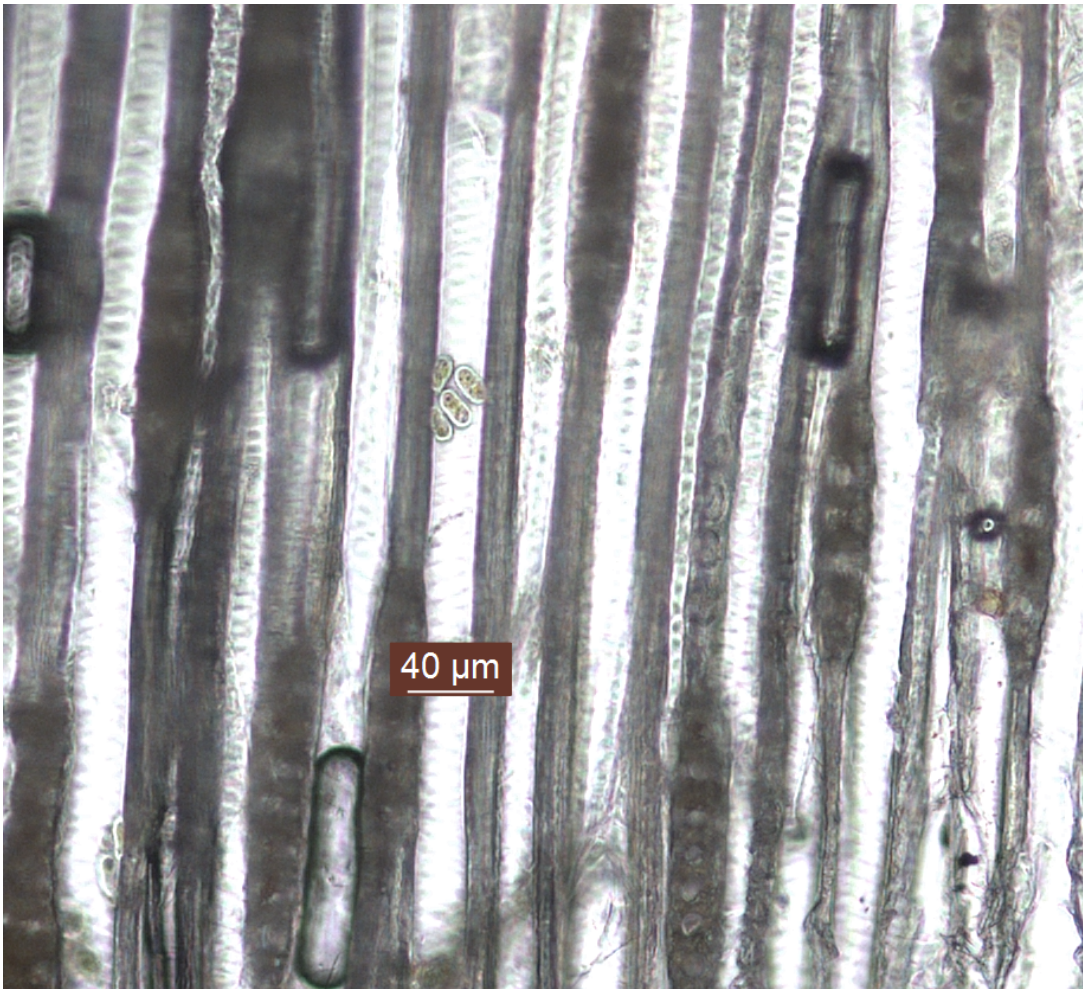


Figure 2.14: SEM micrograph of hyphae and a possible *P. ramorum* chlamydospore in the tracheid of a Douglas-fir sapwood wafer.

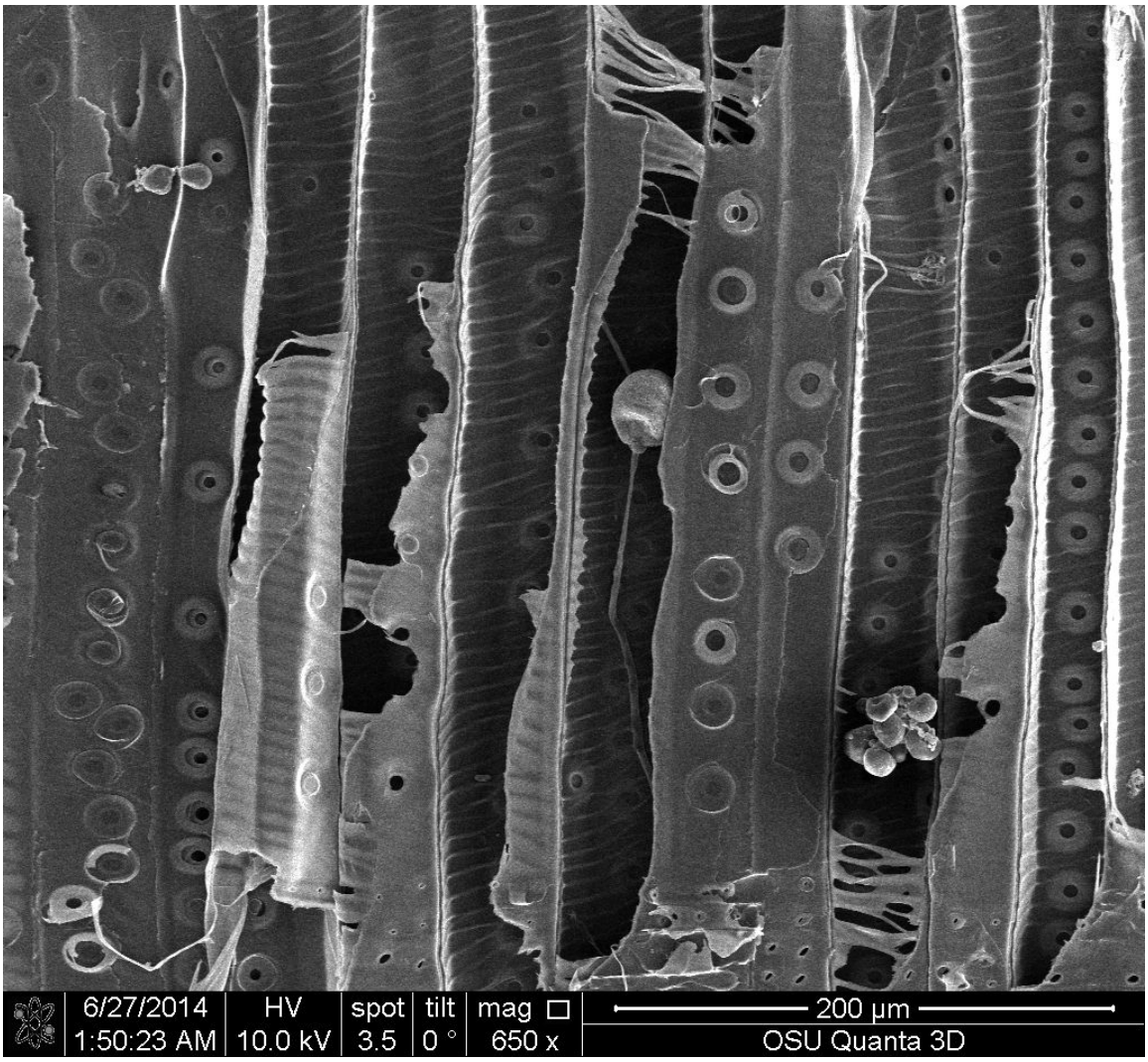


Figure 2.15: SEM micrograph of a possible *P. ramorum* chlamydospore in the tracheid of a Douglas-fir sapwood wafer.

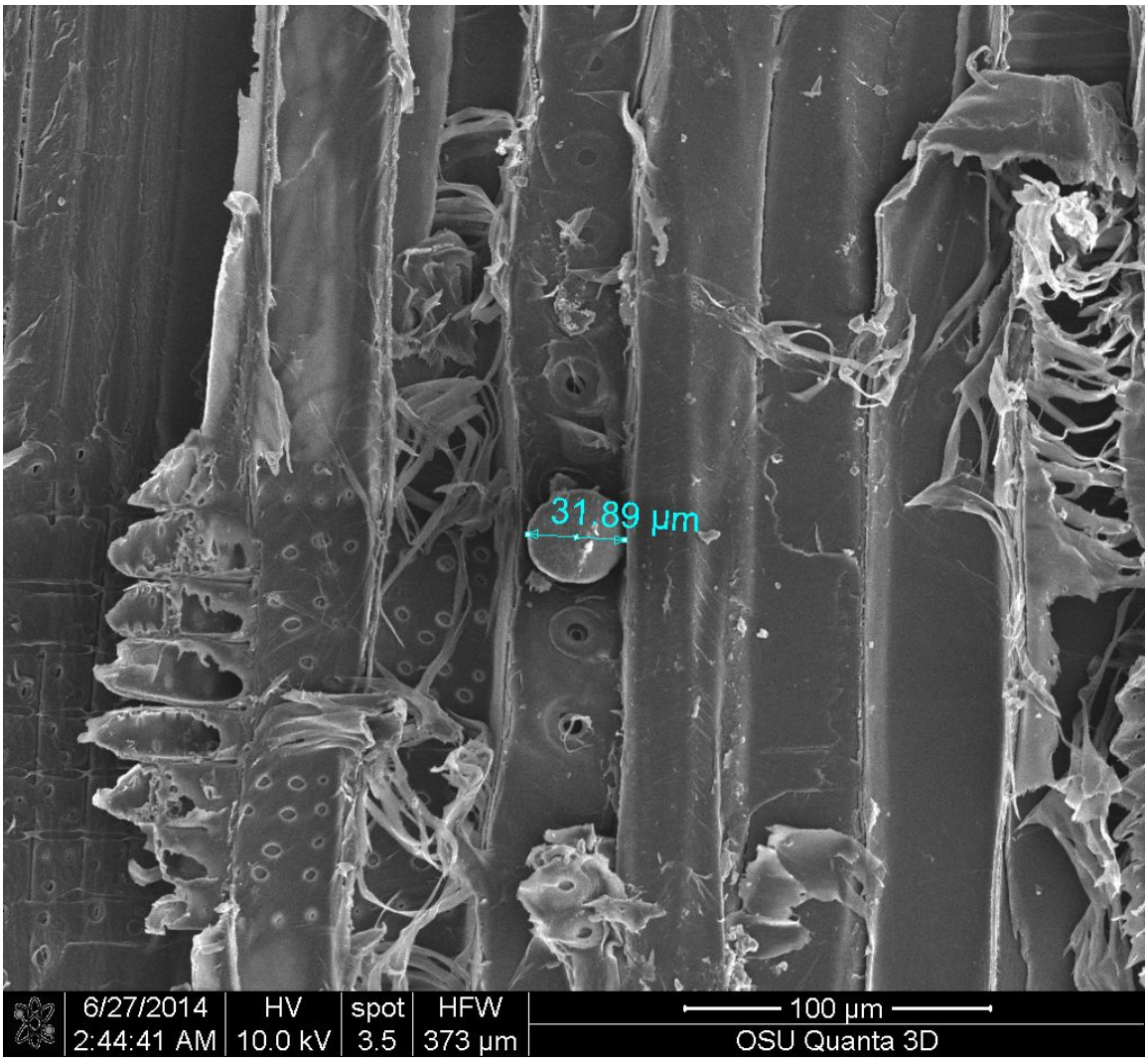
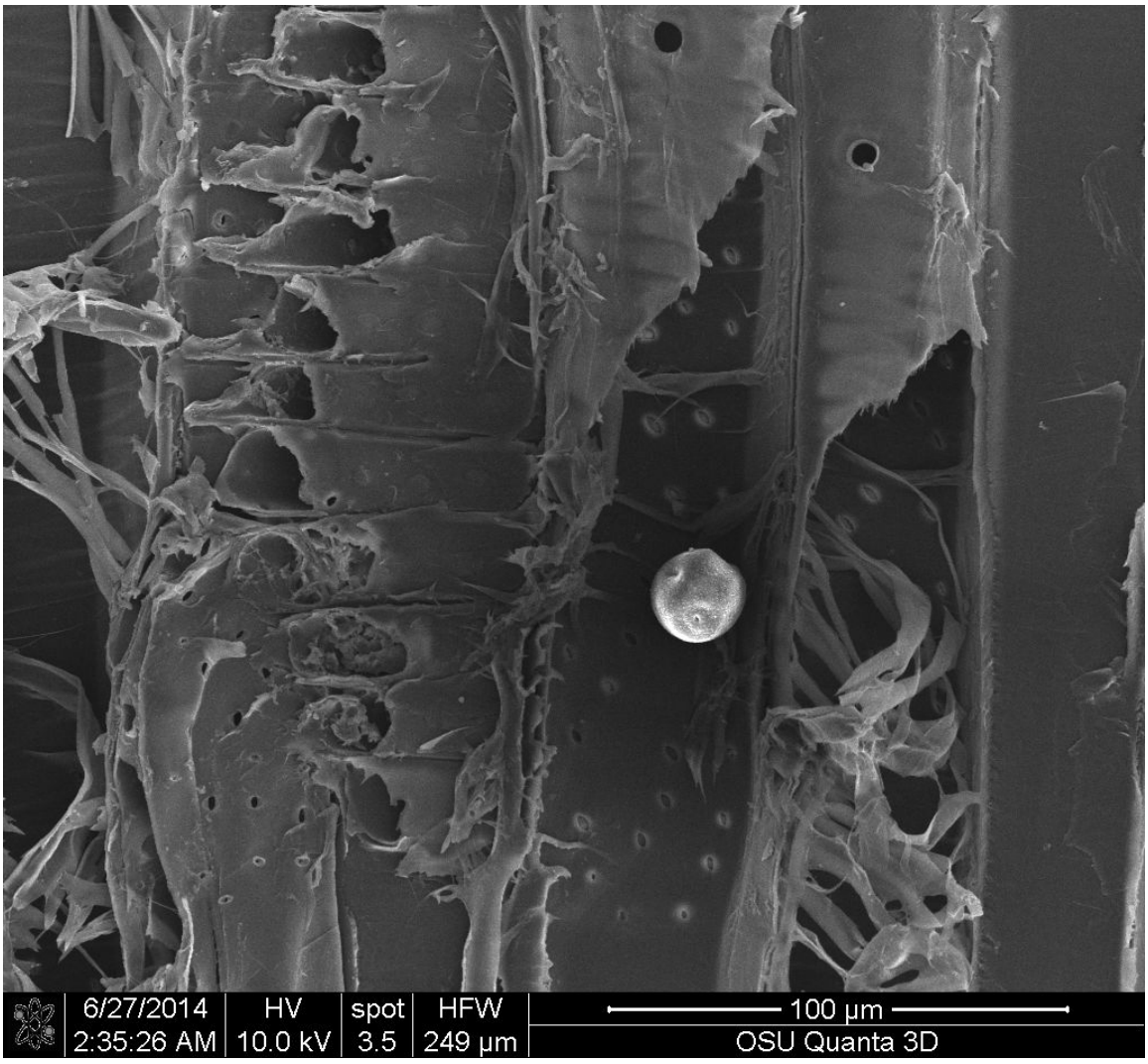


Figure 2.16: SEM micrograph of a possible *P. ramorum* chlamydospore in the tracheid of a Douglas-fir sapwood wafer.



# CHAPTER 3: THE EFFICACY OF BORON FOR PHYTOSANITATION OF DOUGLAS-FIR LOGS TO MITIGATE THE RISK OF ACCIDENTALLY DISSEMINATING *PHYTOPHTHORA RAMORUM*

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In preparation for submission to Biological Invasions or Outlooks on Pest Management.

**Abstract**

The potential to use boron for the phytosanitation of Douglas-fir logs originating from *Phytophthora ramorum* infected areas was investigated. The effects of boron were examined on three isolates of *P. ramorum* in a chemical amended medium bioassay and by treating inoculated tanoak sapwood blocks. Borates significantly inhibited growth of *P. ramorum* in petri dish tests at concentrations of 0.5 to 5 percent BAE w/v and concentrations above 1.5 percent BAE w/v were lethal. Reisolation of *P. ramorum* from inoculated tanoak sapwood blocks was infrequent and only occurred in untreated blocks. Attempts to reisolate *P. ramorum* from sapwood blocks treated with borates or borates amended with glycol were unsuccessful at all concentration levels. However, no attempt was made to determine whether the effect was lethal or inhibitory in the tanoak blocks. The results suggest that boron may be an effective biocide for mitigating the risk of spreading *P. ramorum* in logs removed from infected areas.

**Introduction**

*Phytophthora ramorum* Werres, De Cock & Man in't Veld has been found to colonize the sapwood of fourteen tree species (Table 3.1). Currently, wood products are regulated for eleven hardwood species (Table 3.2), known as bole hosts, in the United States (7 CFR 301.92). Wood products from non-bole hosts are not regulated except that material is required to be free of soil, needles, foliage, and plant debris. Wood products from bole hosts are subject to federal regulation. In Oregon, tanoak material is required to be harvested from "disease free areas" (>400 m from nearest infection) and tanoak material harvested from within the generally infested area cannot be moved outside of the quarantined area (ORS 603-051-1230, Oregon Department of Agriculture, 2012). While tanoak is not a major commercial species, other species in these forests are major sources of income for local landowners. Among the most important is Douglas-fir. In 2010, Douglas-fir accounted for 73.6 percent of the timber harvested in Western Oregon (Oregon Forest Resource Institute, 2012, See Appendix). Of the logs exported in 2011 from Oregon and Washington Customs Districts to all countries, 60.9 percent (~1 billion board feet) were Douglas-fir (Xiaoping and Warren, 2012). Although Douglas-fir has not

been reported to be a major bole host, there is preliminary evidence suggesting that xylem tissues can be colonized (McKeever, 2010). Continued expansion of quarantined areas and additional research identifying bole hosts may lead to further restrictions on movement of many timber species.

One approach to dealing with the quarantine issue is to develop methods for mitigating the risk of pathogen spread on logs, similar to phytosanitation methods applied to solid wood packaging material. These processes, however, would have to be applied within any quarantine area, most likely on full logs. There are a wide array of potential mitigation processes that could be applied to minimize the risk of *P. ramorum* spread including heat treatment, fumigation, debarking, and biocidal treatments (Morrell, 1995).

Heat treatments are used globally, especially for movement of pallets and other solid wood packing (FAO, 2010b; IPPC, 2009), and they are considered the most reliable method for minimizing the risk of transporting unwanted organisms. However, the method is viewed by the industry as costly and time consuming (He et al., 1997). The rate of heat transfer in wood is extremely slow, particularly in log form, making this difficult to accomplish in the field. Heat treatment might be suitable if materials could be partially processed on site to reduce volumes requiring heating; however, it would be difficult to accomplish on whole materials. An additional negative to using heat would be an inability to verify proper treatment since there would be no evidence of heating. The efficacy of the ISPM heat treatment method against *P. ramorum* has not been validated. *P. ramorum* has been described as "highly heat tolerant" (Harnik et al., 2004) and preliminary evidence has suggested the ISPM heat treatment method may not be adequate (Tubajika et al., 2007).

Fumigation has long been used for mitigation of pests in solid wood. Typically, methyl bromide has been used for this purpose because it has the ability to move through solid wood, but then dissipates rapidly from the wood (Schmidt, 1982). However, methyl bromide has also been implicated in ozone depletion and there is a global effort to phase out use of this chemical. Methyl bromide is also difficult to handle and would probably

be difficult to use in a field situation, although it might be a functional approach if there were special facilities within a quarantine area.

Debarking is a recommend phytosanitary method occasionally used when exporting whole logs (FAO, 2010b). The method prevents delivery of many insects and microorganisms capable of colonizing living cambial tissues, inhabiting the phloem, or contaminating the bark surfaces. Debarking may be effective at controlling the spread of *P. ramorum* if the organism was limited to these tissues. Hansen et al. (2005) determined that *P. ramorum* was capable of colonizing cambial tissues of Douglas-fir, but they did not attempt to inoculate xylem tissues. The depth that *P. ramorum* can grow into the sapwood of Douglas-fir remains undetermined, but is assumed to be deeper than debarking could control. Brown and Brasier (2007) recommended removing the outer 3 cm of sapwood because of successful *P. ramorum* isolations at depths of 25 mm in several artificially inoculated hardwood species. This may be insufficient since *P. ramorum* has also been isolated up to 4 cm into the sapwood of naturally infected tanoak logs (Parke et al., 2007). The results of these studies cannot be extended to Douglas-fir logs, but debarking would not be an effective means of preventing the spread if *P. ramorum* behaves similarly.

Chemical treatments are currently not specified in ISPM 15 or other quarantine measures, but might be suitable if it were possible to deliver chemicals to the wood surface at levels that could move through the bark to affect *P. ramorum* within the sapwood. Most chemicals lack the ability to diffuse through wet wood, however, boron compounds are unique in their ability to diffuse through wet wood and affect both fungi and insects established within. Boron is an attractive biocide because of its broad-spectrum efficacy and low acute mammalian toxicity (Caldeira, 2010). Boron dip/diffusion treatments have long been used in New Zealand to protect framing lumber from insect attack and, more recently have been used in the United States to protect lumber used in residential structures from fungal and insect attack. The primary drawback of boron as a wood preservative is that its solubility and diffusion capability make it difficult to immobilize, or fix, once it is impregnated into the wood (Caldeira,

2010). However, this limitation is not an issue for one-time sanitation purposes. Boron has exceptional activity against many fungi and insects and it is one of the few biocides that can be applied directly to wood in inhabited structures. Boron has the ability to diffuse with moisture through most wood species and can be applied using relatively simple dipping processes, thereby reducing the need for large capital investment for infrastructure. The effects of borates have been investigated on a few *Phytophthora* species. Boric acid is lethal to *P. infestans* (Frenkel et al., 2010) and borax inhibited germination of *P. colocasiae* (Misra et al., 2007). However, borax did not affect zoospore production for *P. cinnamomi* or *P. drechsleri* (Halsall, 1977). The efficacy of borates against *P. ramorum* has not been investigated.

While boron might be a useful tool for mitigating *P. ramorum*, there are no data on the ability of this chemical to affect the organism. In this assessment, we examined the effects of boron on three isolates of *P. ramorum* in two studies. The objective of the first study was to identify concentrations that were lethal to *P. ramorum* in a boron amended media bioassay, and the objective of the second study was to test those levels in tanoak sapwood material that was inoculated.

**Materials and Methods**

*P. ramorum* isolates used in the following studies were originally recovered from diseased areas of western Oregon (Table 3.3). Isolates were obtained from E.M. Hansen, Oregon State University. The isolates were of the North American (NA-1) genotype (Personal Communication with Everett Hansen). Prior to use, the isolates were stored long term on agar plugs in vials of water at room temperature. After removal from storage, three isolates of *P. ramorum* (Isolate # 4313, 7904 and 9488) were maintained on corn meal agar (CMA) until needed. Small plugs (8 mm) were cut from the actively growing edge of the culture and used to inoculate test petri plates or tanoak wood blocks.

*Study 1: Boron Amended Media Bioassay*

The ability of boron to affect growth of *P. ramorum* was assessed using petri dishes containing CMA amended with disodium octaborate tetrahydrate (DOT). Four

trials were conducted in the first study, Petri Dish Trials 1-4 (See Table 3.4). DOT was dissolved in distilled water to produce a concentrated solution that could be added to molten agar to produce the desired chemical concentrations. After cooling, a single agar plug cut from an actively growing culture of the desired isolate was placed at the center of the plate. The plates were incubated in the dark at 20° C for 4 weeks. Each chemical concentration/isolate was replicated on 5-7 plates in the Petri Dish Trial 1and 6-8 in Petri Dish Trial 2. The ability of DOT to affect *P. ramorum* growth was assessed by measuring radial growth (nearest mm) in Petri Dish Trials 1 and 2. Radial growth was measured from the outer edge of the plug to the edge of the culture. Four measurements were made along two pre-drawn axes for each plate. Plugs from cultures that failed to grow were removed and placed on CMA that contained no biocide to determine if the effect was inhibitory or lethal in Petri Dish Trial 1. The concentrations of DOT tested in Petri Dish Trials 1 and 2 were: 0.1, 0.5, 1.0, 3.0 and 5.0% boric acid equivalent (BAE) w/v.

Petri Dish Trials 1 and 2 were designed to investigate which DOT concentrations inhibited *P. ramorum* growth. Petri Dish Trials 3 and 4 were conducted to determine which DOT concentrations were lethal rather than inhibitory. In each of these trials, actively growing *P. ramorum* plugs were exposed to DOT by placing plugs onto petri dishes containing media amended with DOT. Plugs where *P. ramorum* had not grown were then moved onto CMA plates without DOT. The concentrations of DOT evaluated in Petri Dish Trial 3 were the same as Petri Dish Trials 1 and 2, but the concentrations in Petri Dish Trial 4 were: 1, 1.5, 2.0, 2.5 and 3.0 percent BAE w/v (See Table 3.3). In Petri Dish Trial 3, two *P. ramorum* plugs were placed onto 4-6 plates amended with boron (2 plugs per plate). Both plugs were replated from the original plates after different lengths of exposure to investigate whether exposure length reduced recovery. Percent recovery was also recorded for the original plates if *P. ramorum* grew (e.g. if one of the four original DOT containing plates had growth, there was a 25% recovery). In Petri Dish Trial 4, plugs were replated and evaluated twice.

Statistical analysis was conducted on the radial growth measurement data collected in Petri Dish Trials 1 and 2. Measurements were averaged for each plate to

avoid pseudoreplication. Welch's Two Sample t-tests were used to test for differences in mean radial growth between the control plates and the plates amended with boron. All statistical analysis was conducted in R version 3.0.2. The session information can be found in the appendix.

*Study 2: Effect of Borate Treatment on P. ramorum Survival in Tanoak Sapwood*

A study was conducted to assess the efficacy of boron against *P. ramorum* growing in tanoak sapwood blocks. Tanoak sapwood blocks (3 x 1 x 1 cm) were cut from freshly cut log sections of a single tree. The blocks were frozen until needed. Blocks were randomly assigned to be inoculated with an isolate of *P. ramorum*. Two actively growing plugs of a given isolate were placed on each block, bottom side down, and covered with a two-layer-thick piece of cheesecloth that was soaked with deionized water. The whole block was wrapped in foil to retain moisture (See Appendix Figure 11). Controls were prepared using the same methods except that plugs were not inoculated with *P. ramorum*. Tanoak Block Trials 1, 2, 3, and 4 contained 87, 80, 40, and 40 inoculated blocks, respectively (Table 3.9). Blocks were then incubated at 20 C in the dark for 1-4 months before treatment to allow *P. ramorum* to colonize the substrate.

Treatment solutions were designed to produce specific wt/wt concentrations of borate in the tanoak sapwood blocks. Initial moisture contents were determined by oven drying and weighing selected blocks so that boron concentration could be developed on a wood mass basis. Five blocks were immersed in deionized water for 20 minutes and weighed to determine potential solution uptake. This uptake was used to determine the borate solution concentration needed to produce a given retention. Average moisture content of the tanoak sapwood blocks after the inoculation process was 96 percent. The treatment solution was ultimately delivered by immersing colonized blocks and drawing a vacuum (80 kpa) over the solution to improve uptake. The average dry weight of the tanoak sapwood blocks was 3.96 g and the average uptake after the inoculation process was 0.314 g. Table 3.9 provides a summary of the treatment solution concentrations, the target concentrations, the duration of each step in the inoculation and treatment processes, and the actual concentrations reached.

Treatments to reach the target concentrations were conducted with the same method of determining uptake. However, uptake measurements were only conducted once and the intervals before actual treatments ranged from one to four weeks. Two borate formulations were used in three of the trials, DOT and DOT plus glycol. Disodium octaborate tetrahydrate (DOT) (Rio Tinto, Denver, Colorado) is a crystalline material that must be diluted in water while the DOT plus glycol formulation is a solution made up of 40.6% DOT plus 11.9% polyethylene glycol and 47.5% monoethylene glycol (Boracare®, Nisus, Knoxville TN). DOT plus glycol was not included as a treatment solution in Tanoak Block Trial 4. In each trial, tanoak sapwood blocks were randomly assigned to a given concentration and borate formulation with an effort to balance the number of replicates for each isolate. Blocks were submerged in their assigned treatment solution for 20 minutes under a vacuum, wrapped back up in foil, and incubated at 20 C with a 12-hr light cycle. Control treatments used the same methods but blocks were submerged in deionized (DI) water rather than a borate treatment solution.

The borates were allowed to diffuse for 21-39 days, then blocks were removed from growth chamber, unwrapped from foil, split into quarters, and plated into *Phytophthora* spp. selective CARP (Hansen et al., 2005) media. One control block was set aside from each treatment for chemical analysis to determine the actual treatment retention. Table 3.8 summarizes the Tanoak Block Trials. Areas of media on the petri dish were removed so quartered blocks could be submerged into the media. After 18 days, the plates were examined for *P. ramorum* growth, which was distinguished from other species based on morphological features such as hyphal branching, non-septate hyphae, production of chlamydospores or sporangia, growth into the bottom of the plate, and hyphal swelling. Control blocks set aside for chemical analysis were oven dried overnight (54 C), ground to pass a 20-mesh screen, and then extracted with boiling water. The resulting extract was then analyzed for boron concentration by the Azomethine H method (American Wood-Preservers' Association, 2012a). Results were recorded in percent BAE wt/wt.

**Results**

*Study 1: Boron Amended Media Bioassay*

Results from Petri Plate Trial 1 and 2 indicated that addition of 0.1% BAE DOT to the agar decreased radial growth on all three *P. ramorum* isolates to only 4−15% of growth on non-amended plates (Table 3.5; Figure 3.1). There was strong evidence that the mean radial growth for the control plates was different than the plates amended with 0.1% BAE DOT (Welch's Two Sample t-test, $t_{37}$=14.75, p-value < 2.2e-16). Further increases in boron concentration completely inhibited growth. There was strong evidence that the radial growth for the control plates was different than zero (One sample t-test, $t_{35}$=16.35, p-value < 2.2e-16). Replating of the original agar plugs on non-amended CMA in Petri Dish Trial 3 revealed that 0.5 and 1.0% BAE concentrations were fungistatic, but concentrations above 3.0% BAE were lethal (Table 3.6). Petri Dish Trial 4 was performed to more closely refine the levels of boron required and revealed that boron was fungicidal at or above 1.5% (Table 3.7). Table 3.8 summarizes which concentrations were inhibitory or lethal in Petri Dish Trials 1 and 2.

*Study 2: Effect of Borate Treatment on P. ramorum Survival in Tanoak Sapwood*

All concentrations of the borates tested inhibited *P. ramorum* growth. In all four trials, *P. ramorum* was only recovered from blocks treated with DI water (control treatment), while it was not recovered from any blocks treated with borate solutions (Tables 3.10-3.13). In Tanoak Block Trials 1 and 4, *P. ramorum* was recovered from one block inoculated with isolate 7904 and treated with DI water. In Tanoak Block Trial 2, *P. ramorum* was recovered from 6 of the 12 blocks inoculated with *P. ramorum* and treated with DI water. In Tanoak Block Trial 3, *P. ramorum* was reisolated from one block inoculated with isolate 9488 and treated with DI water.

Chemical analysis of the control blocks revealed that measured boron concentrations were higher than target concentrations. Blocks treated with borate solutions to target concentrations of 0.25% BAE had a mean% BAE of 0.47 and ranged from 0.42-0.62% BAE (Figure 3.2; Tables 3.10-3.13). The mean measured treatment

concentration reached for 0.5, 1.0, and 1.5% BAE concentrations were 1.28, 2.02 and 2.29% BAE, respectively.

**Discussion**

*Study 1: Boron Amended Media Bioassay*

Petri dish tests provide a simple method for rapidly evaluating multiple concentrations of a range of chemicals. In the boron amended media bioassay, DOT was effective against *P. ramorum* at concentrations within the range that can be solubilized in water and applied to wood. These results suggest that DOT treatments may be a viable control option to prevent the spread of sudden oak death. All concentrations of DOT were active against *P. ramorum,* but only a few were lethal.

*Study 2: Effect of Borate Treatment on P. ramorum Survival in Tanoak Sapwood*

Concentrations determined to be lethal to *P. ramorum* in the boron amended media bioassay also appeared to affect survival of *P. ramorum* in the inoculated tanoak sapwood blocks. Whether the concentrations were lethal or just inhibitory was not investigated in the inoculated tanoak block treatment study. It is possible that the DOT remaining in the tanoak blocks diffused into the agar and continued to inhibit growth. Replating the tanoak blocks onto another set of non-amended petri plates would have clarified this possibility. Recovery after treatment with DI water suggested that submerging the blocks in treatment solution under a vacuum did not affect survival of *P. ramorum.* However, it may have contributed to the low recovery percentages. Thus, it would have been valuable to include attempts to reisolate from blocks not subjected to treatments. Nevertheless, *P. ramorum* was recovered from control treated blocks inoculated with *P. ramorum* but not from any of the blocks treated with either borate formulation. Since there was no difference in *P. ramorum* recovery between the treatment concentrations or borate formulations, further research will be needed to evaluate the minimum lethal concentration in tanoak sapwood and the best formulations to reach the minimum lethal concentration at the maximum depth *P. ramorum* can grow into the sapwood. Regardless, the lethal concentrations in the bioassay and those that were

inhibitory in the inoculated block treatment could easily be achieved under field conditions and suggest that boron may be an effective mitigation method.

The low recovery percent of *P. ramorum* from tanoak sapwood poses a challenge for assessing efficacy. Wood blocks are less nutritious than *Phytophthora* spp. selective CMA media, and this may result in organisms that are less fit and therefore more susceptible to lower boron concentrations than in the boron amended media bioassay. It is not possible to determine if the media affected results because the lowest concentration of boron tested in *P. ramorum* inoculated wood blocks was 0.42% BAE. While petri dishes present a fairly artificial environment, the results suggest that further trials to evaluate both the ability of borate formulations to affect *P. ramorum* and diffuse into commercial logs are worthwhile.

The chemical analysis of the control blocks used in the treatment of inoculated tanoak blocks study revealed that measured (actual) concentrations of boron were greater than the target levels (Tables 3.9-3.13). The measured concentrations in many of the treated blocks were more than double the target concentration. This may have resulted from underestimating the amount of boron solution uptake. Allowing the blocks to dry out further may have increased the amount up uptake. In addition, uptakes were measured from blocks used in Trial 3.3.2 and only represented uptakes after the longest incubation time tested, 151 days (Table 3.9). The method used for chemical analysis also does not distinguish from boron present on the surface of the wood blocks or boron within the sapwood blocks. The detection of boron in the control blocks that had control treatments suggested that there were background levels of boron in the wood or that the methods of chemical analysis have some carry over of boron from sample to sample.

The scope of inference for this study is limited to the three isolates of *P. ramorum* used, and the tanoak sapwood material used in this study does not represent all tanoak material, and especially not other species, because the material used in this study came from a single tree that was not randomly selected. Diffusion of boron into tanoak logs that were naturally infested with *P. ramorum* prior to being cut would likely be different. Parke et al. (2007) showed that tanoak trees infected with *P. ramorum* increased the

production of vessel clogging tyloses and no effort to quantify or qualify the condition of the tanoak sapwood blocks was made during this study. The application of these results is also limited to exposed xylem tissues that are directly inoculated with agar plugs in controlled environments.

Although these results suggest *P. ramorum* can be controlled, additional research will be necessary to determine the applicability in logs. Further research should evaluate the maximum depth that DOT can diffuse, coupled with the maximum depth *P. ramorum* is capable of growing into the xylem of logs. It is also critical to determine whether the DOT concentration that developed where *P. ramorum* growth occurs is above the minimum lethal concentration.

**Conclusions**

Boron had the ability to inhibit and kill *P. ramorum* in petri plate tests. Levels above 1.5% BAE were lethal to *P. ramorum* in the petri plate tests. Levels above 0.42% BAE were inhibitory to *P. ramorum* in the treated inoculated tanoak sapwood block study. The highest concentration reached within the tanoak sapwood blocks used in this study was 2.29% BAE. While both of these studies present artificial environments for *P. ramorum*, the investigation warrants further research on the application of common wood preservatives for control of quarantined pests. Currently supported mitigation methods are difficult to apply to the large quantities of material in commerce and this research supports a novel method to control *Phytophthora* species in wood material.

**Tables and Figures**

Table 3.1: Tree species with observations of *Phytophthora ramorum* in xylem tissues.

| Common Name | Latin Name |
|---|---|
| Balsam fir[1] | *Abies balsamea* |
| Tamarack[1] | *Larix laricina* |
| Douglas-fir[2] | *Pseudotsuga mensiesii* |
| Tanoak[3,4] | *Notholithocarpus densiflorus* |
| Sycamore maple[5] | *Acer pseudoplatanus* |
| European beech[5] | *Fagus sylvatica* |
| Japanese evergreen oak[5] | *Quercus acuta* |
| European turkey oak[5] | *Quercus cerris* |
| Cornish oak[5] | *Quercus petrea* |
| N/A[5] | *Schima argenta* |
| Sugar maple[1] | *Acer saccharum* |
| Yellow birch[1] | *Betula alleghaniensis* |
| White ash[1] | *Fraxinus americana* |
| Northern red oak[1] | *Quercus rubra* |

Footnotes indicate source: [1](Simard et al., 2010) [2](McKeever, 2010) [3](Collins and Parke, 2008) [4](Parke et al., 2007) [5](Brown and Brasier, 2007)

Table 3.2: Tree species with wood products that are regulated for *Phytophthora ramorum* by the United States Department of Agriculture, Animal and Plant Health Inspection Service.

| **Common Name** | **Latin Name** |
|---|---|
| Planetree maple | *Acer pseudoplatanus* |
| Horse chestnut | *Aesculus hippocastanum* |
| Camphor tree | *Cinnamomum camphora* |
| European beech | *Fagus sylvatica* |
| Tanoak | *Notholithocarpus densiflorus* |
| Coast live oak | *Quercus agrifolia* |
| European turkey oak | *Quercus cerris* |
| Canyon live oak | *Quercus chrysolepis* |
| Southern red oak | *Quercus falcata* |
| California black oak | *Quercus kelloggii* |
| Shreve's oak | *Quercus parvula var. shrevei* |

Source: USDA APHIS List of Regulated Hosts and Plants Proven or Associated with *Phytophthora ramorum*, August 2013. Available here: http://www.aphis.usda.gov/plant_health/plant_pest_info/pram/downloads/pdf_files/usdaprlist.pdf

Table 3.3: Source of *P. ramorum* isolates.

| Isolate | Date Isolated | Sample Type | Host | Coordinates | Isolated By |
|---|---|---|---|---|---|
| 7904 | 3/3/12 | Small stems, twigs and Foliage | *Umbellularia californica* | 42.11849, -124.19735 | Joseph M. Hulbert |
| 9488 | 7/7/06 | Cambium/inner bark lesion | *Notholithocarpus densiflorus* | 42.08961, -124.32284 | Oregon Department of Forestry |
| 4313 | 11/21/02 | NA | *Rhododendron* sp. | NA | Oregon Department of Forestry |

Table 3.4: Summary of the studies and trials conducted in Chapter 3.

| Study | Description | Trials | Boron Levels Tested (% BAE) | Formulations | Replated | Results Table |
|---|---|---|---|---|---|---|
| 1 | Boron Amended Media Bioassay | Petri Plate Trial 1 | 0.1, 0.5, 1.0, 3.0, 5.0 | DOT | No | 3.4 |
| | | Petri Plate Trial 2 | 0.1, 0.5, 1.0, 3.0, 5.0 | DOT | No | 3.4 |
| | | Petri Plate Trial 3 | 0.1, 0.5, 1.0, 3.0, 5.0 | DOT | Yes | 3.5 |
| | | Petri Plate Trial 4 | 1.0, 1.5, 2.0, 2.5, 3.0 | DOT | Yes | 3.6 |
| 2 | Effect of Borate Treatment on *P. ramorum* Survival in Tanoak Sapwood | Tanoak Block Trial 1 | 0, 0.25, 1.0 | DOT & DOT/Glycol | No | 3.9 |
| | | Tanoak Block Trial 2 | 0, 0.25, 0.5, 1.0 | DOT & DOT/Glycol | No | 3.10 |
| | | Tanoak Block Trial 3 | 0, 0.25, 1.0 | DOT & DOT/Glycol | No | 3.11 |
| | | Tanoak Block Trial 4 | 0, 0.25, 0.5, 1.0, 1.5 | DOT | No | 3.12 |

Table 3.5: Mean average radial growth measurements of *P. ramorum* in Petri Dish Trials 1 and 2 evaluating the effect of DOT amendment on radial growth.

| Isolate | DOT Concentration (%BAE, wt/v) | Petri Dish Trial 1 | | Petri Dish Trial 2 | |
|---|---|---|---|---|---|
| | | Replicates | Mean Radial Growth (mm) | Replicates | Mean Radial Growth (mm) |
| 4313 | 0 (Control) | 6 | 14.2 (0.6) | 6 | 13.6 (0.7) |
| 4313 | 0.1 | 6 | 1.5 (0.3) | 4 | 0.3 (0.1) |
| 4313 | 0.5 | 4 | 0 | 4 | 0 |
| 4313 | 1 | 5 | 0 | 4 | 0 |
| 4313 | 3 | 5 | 0 | 6 | 0 |
| 4313 | 5 | 5 | 0 | 6 | 0 |
| 7904 | 0 (Control) | 7 | 23.6 (0.3) | 5 | 9.9 (0.8) |
| 7904 | 0.1 | 5 | 1.7 (0.2) | 3 | 1.3 (0.4) |
| 7904 | 0.5 | 7 | 0 | 5 | 0 |
| 7904 | 1 | 6 | 0 | 5 | 0 |
| 7904 | 3 | 7 | 0 | 6 | 0 |
| 7904 | 5 | 6 | 0 | 6 | 0 |
| 9488 | 0 (Control) | 7 | 23.6 (0.8) | 5 | 11.3 (0.6) |
| 9488 | 0.1 | 6 | 2.5 (0.5) | 5 | 0.6 (0.1) |
| 9488 | 0.5 | 5 | 0 | 5 | 0 |
| 9488 | 1 | 7 | 0 | 5 | 0 |
| 9488 | 3 | 5 | 0 | 6 | 0 |
| 9488 | 5 | 7 | 0 | 6 | 0 |

Values represent means of four measurements on each of 3 to 7 replicate plates per isolate. Values in parentheses are the standard errors for the means.

Table 3.6: Effect of prolonged exposure to boron amended media on percent recovery of *P. ramorum* from Petri Dish Trial 3.

| Isolate | Concentration (%BAE, wt/v) | Recovery (%)[1] | | | | |
|---|---|---|---|---|---|---|
| | | Number of days on original plates before *P. ramorum* was replated onto non-amended media | | | | |
| | | Original DOT Amended Plates | 13 Days | 21 Days | 39 Days | 60 Days |
| 4313 | 0.1 | 60 (5) | 100 | NR | NR | NR |
| 4313 | 0.5 | 0 (5) | 100 | 0 | 0 | 0 (1) |
| 4313 | 1 | 0 (4) | 0 | 0 | 0 (1) | 0 (1) |
| 4313 | 3 | 0 (6) | 0 | 0 | 0 | NA |
| 4313 | 5 | 0 (6) | 0 | 0 | 0 | 0 (1) |
| 7904 | 0.1 | 80 (5) | 100 | NR | NR | NR |
| 7904 | 0.5 | 0 (5) | 100 | 100 | 0 | 0 |
| 7904 | 1 | 0 (5) | 100 | 50 | 0 | 0 |
| 7904 | 3 | 0 (6) | 0 | 0 | 0 | 0 (1) |
| 7904 | 5 | 0 (6) | 0 | 0 | 0 | 0 |
| 9488 | 0.1 | 60 (5) | 100 | NR | NR | NR |
| 9488 | 0.5 | 0 (5) | 100 | 100 | 50 | 0 (1) |
| 9488 | 1 | 0 (5) | 100 | 100 | 0 | 0 |
| 9488 | 3 | 0 (6) | 0 | 0 | 0 | 0 (1) |
| 9488 | 5 | 0 (6) | 0 | 0 | 0 | 0 |

[1]All numbers are percent of cultures that grew out of attempted cultures. n=2 unless specified otherwise in parentheses. NR = not replated. Cultures were not replated if growth was observed after first replating period.

Table 3.7: Effect of exposure to boron amended media on percent recovery of *P. ramorum* in Petri Dish Trial 4.

| Isolate | Concentration (% BAE, wt/v) | Original Plates | | After First Resub | | After Second Resub | |
|---|---|---|---|---|---|---|---|
| | | Recovery Percent | Replicates | Recovery Percent | Replicates | Recovery Percent | Replicates |
| 4313 | 0 (Control) | 100 | 6 | 100 | 6 | 100 | 6 |
| 4313 | 1.0 | 0 | 7 | 0 | 7 | 0 | 6 |
| 4313 | 1.5 | 0 | 7 | 0 | 7 | 0 | 7 |
| 4313 | 2.0 | 0 | 7 | 0 | 7 | 0 | 7 |
| 4313 | 2.5 | 0 | 7 | 0 | 7 | NA | NA |
| 4313 | 3.0 | 0 | 7 | 0 | 7 | 0 | 6 |
| 7904 | 0 (Control) | 100 | 6 | 100 | 6 | 100 | 6 |
| 7904 | 1.0 | 0 | 6 | 60 | 5 | 100 | 5 |
| 7904 | 1.5 | 0 | 7 | 0 | 7 | 0 | 7 |
| 7904 | 2.0 | 0 | 7 | 0 | 6 | 0 | 4 |
| 7904 | 2.5 | 0 | 7 | 0 | 7 | NA | NA |
| 7904 | 3.0 | 0 | 7 | 0 | 7 | 0 | 7 |
| 9488 | 0 (Control) | 100 | 6 | 100 | 6 | 100 | 6 |
| 9488 | 1.0 | 0 | 7 | 0 | 6 | 0 | 7 |
| 9488 | 1.5 | 0 | 7 | 0 | 7 | 0 | 7 |
| 9488 | 2.0 | 0 | 7 | 0 | 7 | 0 | 7 |
| 9488 | 2.5 | 0 | 8 | 0 | 7 | 0 | 3 |
| 9488 | 3.0 | 0 | 7 | 0 | 7 | 0 | 7 |

Table 3.8: Fungistatic or fungicidal effects of boron exposure in *Phytophthora* spp. selective media on *P. ramorum* in petri dish Trials 1 and 2.

| DOT Concentration (% BAE, w/v) | Petri Dish Trial 3 | | Petri Dish Trial 4 | | Trials |
|---|---|---|---|---|---|
| | Fungistatic | Fungicidal | Fungistatic | Fungicidal | |
| 0.1 | N | N | | | 1 |
| 0.5 | Y | N | | | 1 |
| 1.0 | Y | N | Y | N | 2 |
| 1.5 | | | Y | Y | 1 |
| 2.0 | | | Y | Y | 1 |
| 2.5 | | | Y | Y | 1 |
| 3.0 | Y | Y | Y | Y | 2 |
| 5.0 | Y | Y | | | 1 |

Table 3.9: Effect of DOT or DOT/glycol on survival of P. ramorum in tanoak blocks.

| Tanoak Block Trial | *P. ramorum* Incubation Time (Days) | Diffusion Time (Days) | Time in Culture (Days) | Formulation | Treatment Solution Concentration | Boron Retention (% BAE, wt/wt) | |
|---|---|---|---|---|---|---|---|
| | | | | | | Target | Measured |
| Trial 1 | 138 | 39 | 18 | DI | 0 | 0.00 | 0.10 |
| Trial 1 | 138 | 39 | 18 | DOT | 3.15 | 0.25 | 0.42 |
| Trial 1 | 138 | 39 | 18 | DOT | 12.62 | 1.00 | 1.57 |
| Trial 1 | 138 | 39 | 18 | DOT/glycol | 3.15 | 0.25 | 0.42 |
| Trial 1 | 138 | 39 | 18 | DOT/glycol | 12.62 | 1.00 | 1.94 |
| Trial 2 | 151 | 33 | 18 | DI | 0.00 | 0.00 | 0.09 |
| Trial 2 | 151 | 33 | 18 | DOT | 3.15 | 0.25 | 0.43 |
| Trial 2 | 151 | 33 | 18 | DOT | 6.31 | 0.50 | 0.70 |
| Trial 2 | 151 | 33 | 18 | DOT | 12.62 | 1.00 | 2.26 |
| Trial 2 | 151 | 33 | 18 | DOT/glycol | 3.15 | 0.25 | 0.49 |
| Trial 2 | 151 | 33 | 18 | DOT/glycol | 6.31 | 0.50 | 1.42 |
| Trial 2 | 151 | 33 | 18 | DOT/glycol | 12.62 | 1.00 | 2.41 |
| Trial 3 | 151 | 33 | 18 | DI | 0.00 | 0.00 | 0.09 |
| Trial 3 | 123 | 31 | 18 | DOT | 3.15 | 0.25 | 0.51 |
| Trial 3 | 123 | 31 | 18 | DOT | 12.62 | 1.00 | 2.14 |
| Trial 3 | 123 | 31 | 18 | DOT/glycol | 3.15 | 0.25 | 0.62 |
| Trial 3 | 123 | 31 | 18 | DOT/glycol | 12.62 | 1.00 | 2.33 |
| Trial 4 | 123 | 31 | 18 | DI | 0.00 | 0.00 | 0.11 |
| Trial 4 | 108 | 21 | 18 | DOT | 3.15 | 0.25 | 0.47 |
| Trial 4 | 108 | 21 | 18 | DOT | 6.31 | 0.50 | 0.68 |
| Trial 4 | 108 | 21 | 18 | DOT | 12.62 | 1.00 | 2.21 |
| Trial 4 | 108 | 21 | 18 | DOT | 18.93 | 1.50 | 2.29 |

Table 3.10: Effect of exposure to disodium octaborate tetrahydate (DOT) alone or amended with glycol on survival of *P. ramorum* in tanoak sapwood blocks used in Tanoak Block Trial 1

| **Treatment** | **Boron Retention (%BAE wt/wt)** | | ***P. ramorum* Recovery (%)** | | | |
|---|---|---|---|---|---|---|
| | **Target** | **Measured** | **Isolate 4313** | **Isolate 7904** | **Isolate 9488** | **Control** |
| DOT | 0.25 | 0.42 | 0 (n=3) | 0 (n=4) | 0 (n=4) | 0 (n=2) |
| | 1 | 1.57 | 0 (n=6) | 0 (n=5) | 0 (n=5) | 0 (n=3) |
| DOT/glycol | 0.25 | 0.42 | 0 (n=4) | 0 (n=4) | 0 (n=4) | 0 (n=2) |
| | 1 | 1.94 | 0 (n=4) | 0 (n=5) | 0 (n=5) | 0 (n=3) |
| Deionized Water | 0 | 0.1 | 0 (n=5) | 20 (n=5) | 0 (n=5) | 0 (n=3) |

Table 3.11: Effect of exposure to disodium octaborate tetrahydate (DOT) alone or amended with glycol on survival of *P. ramorum* in tanoak sapwood blocks used in Tanoak Block Trial 2

| Treatment | Boron Retention (%BAE wt/wt) | | *P. ramorum* Recovery (%) | |
|---|---|---|---|---|
| | Target | Measured | *P. ramorum* | Control |
| DOT | 0.25 | 0.43 | 0 (n=7) | 0 (n=1) |
| | 0.5 | 0.7 | 0 (n=7) | 0 (n=1) |
| | 1 | 2.26 | 0 (n=7) | 0 (n=2) |
| DOT/glycol | 0.25 | 0.49 | 0 (n=7) | 0 (n=1) |
| | 0.5 | 1.83 | 0 (n=14) | 0 (n=2) |
| | 1 | 2.41 | 0 (n=7) | 0 (n=2) |
| Deionized Water | 0 | 0.09 | 50 (n=12) | 0 (n=2) |

Table 3.12: Effect of exposure to disodium octaborate tetrahydate (DOT) alone or amended with glycol on survival of *P. ramorum* in tanoak sapwood blocks used in Tanoak Block Trial 3

| **Treatment** | **Boron Retention (%BAE wt/wt)** | | ***P. ramorum* Recovery (%)** | | | |
|---|---|---|---|---|---|---|
| | **Target** | **Measured** | **Isolate 4313** | **Isolate 7904** | **Isolate 9488** | **Control** |
| DOT | 0.25 | 0.51 | 0 (n=2) | 0 (n=2) | 0 (n=2) | 0 (n=1) |
| | 1 | 2.14 | 0 (n=2) | 0 (n=2) | 0 (n=2) | 0 (n=1) |
| DOT/glycol | 0.25 | 0.62 | 0 (n=2) | 0 (n=2) | 0 (n=2) | 0 (n=1) |
| | 1 | 2.33 | 0 (n=2) | 0 (n=2) | 0 (n=2) | 0 (n=1) |
| Deionized Water | 0 | 0.09 | 0 (n=2) | 0 (n=2) | 50 (n=2) | 0 (n=1) |

Table 3.13: Effect of exposure to disodium octaborate tetrahydate (DOT) alone or amended with glycol on survival of *P. ramorum* in tanoak sapwood blocks used in Tanoak Block Trial 4

| Treatment | Boron Retention (%BAE wt/wt) | | *P. ramorum* Recovery (%) | | | |
|---|---|---|---|---|---|---|
| | Target | Measured | Isolate 4313 | Isolate 7904 | Isolate 9488 | Control |
| DOT | 0.25 | 0.47 | 0 (n=2) | 0 (n=2) | 0 (n=2) | 0 (n=1) |
| | 0.5 | 0.68 | 0 (n=2) | 0 (n=2) | 0 (n=2) | 0 (n=1) |
| | 1 | 2.21 | 0 (n=2) | 0 (n=2) | 0 (n=2) | 0 (n=1) |
| | 1.5 | 2.29 | 0 (n=2) | 0 (n=2) | 0 (n=2) | 0 (n=1) |
| Deionized Water | 0 | 0.11 | 0 (n=2) | 50 (n=2) | 0 (n=2) | 0 (n=1) |

Figure 3.1 Radial growth of three *P. ramorum* isolates exposed to difference concentrations of borate (DOT) amended media.

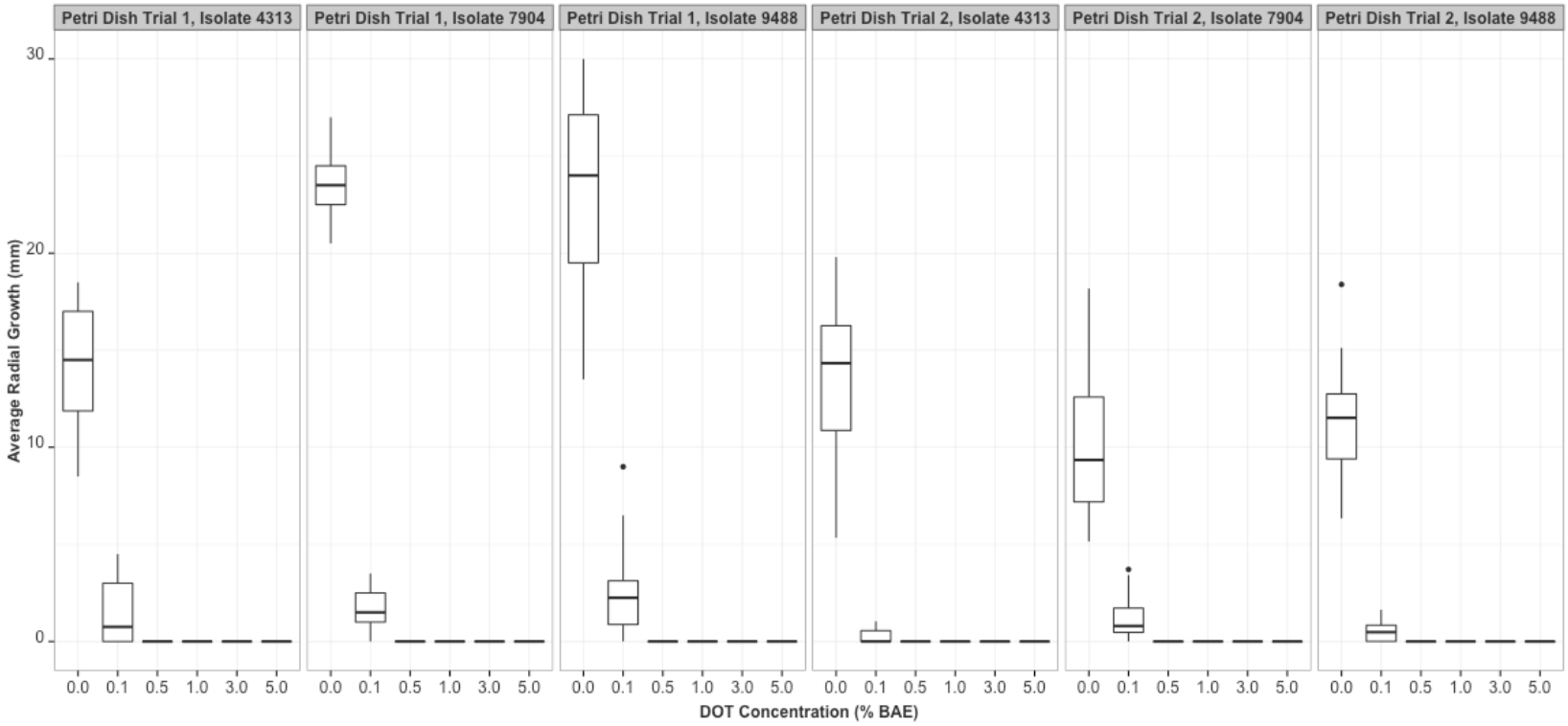
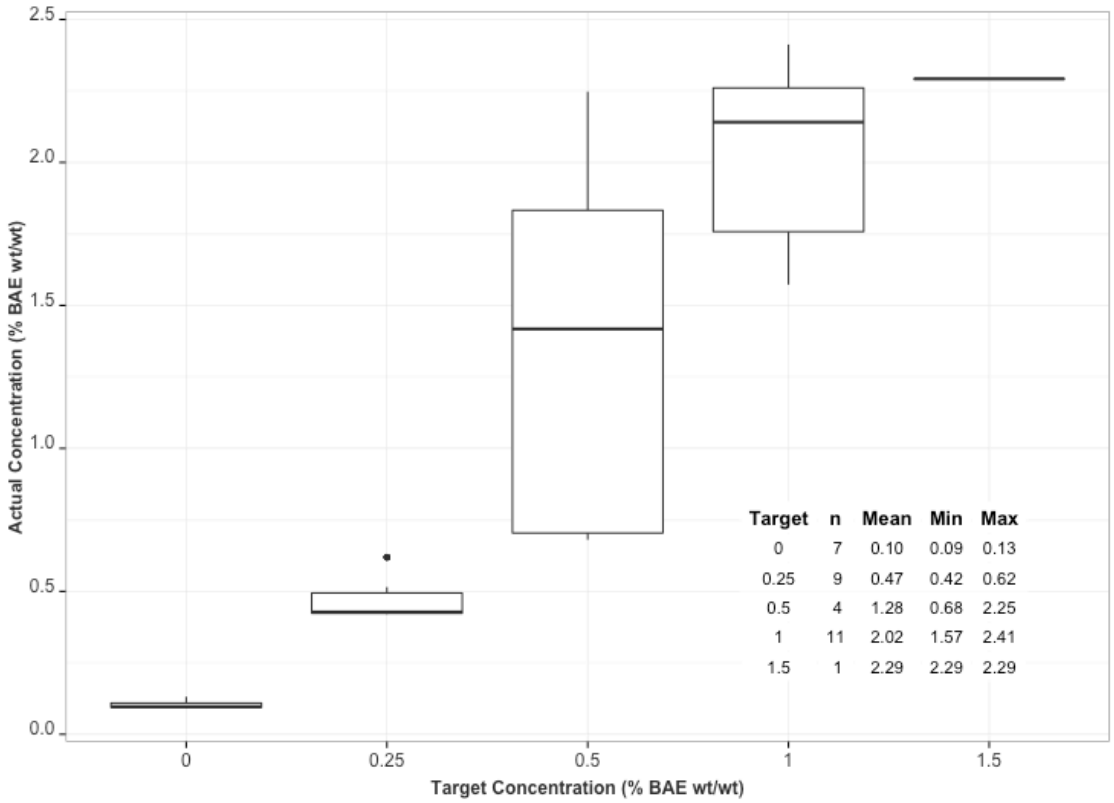


Figure 3.2: Boxplot of actual (measured) vs target boron concentrations in control blocks used to assess the effect of boron on *P. ramorum*.



# CHAPTER 4: THE ABILITY OF DISODIUM OCTABORATE TETRAHYDRATE TO DIFFUSE INTO DOUGLAS-FIR LOGS SECTIONS CONTAINING INTACT BARK

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In preparation for submission to Forest Products Journal

**Abstract**

This study explored alternative methods for sanitizing logs colonized by *Phytophthora ramorum*, the causal organism of Sudden Oak Death. Two borate formulations were applied directly to the bark surface of Douglas-fir logs. Boron levels in the wood were determined with a chemical assay and analyzed with a linear mixed model. Borate solutions with and without glycol diffused through the bark and into the sapwood. Bark thickness did not affect the amount of boron movement. Treatment with boron plus glycol resulted in greater diffusion than treatment with boron alone when including bark samples in the comparison. Boron concentrations in the sapwood were between 16 and 27% of those present in the bark and decreased with distance inward from the surface. The results indicate that boron diffusion can occur through bark and applications to logs may be a viable method for mitigating the presence of pest organisms.

**Introduction**

Forests provide a diverse array of valuable services, products and cultural values. One risk to our forests is the increased introduction of novel forest pests. Exotic forest pests are frequently introduced into new environments, but many do not become established because of unfavorable environmental conditions, preexisting niche fulfillment, competition and general unsupportive biological relationships (Aukema et al., 2010; Williamson, 2001). However, the impacts of climate change may increase the likelihood of pest establishment (FAO, 2010a). Very little propagative material (e.g. one spore) of an introduced species need survive and germinate on host material to start an epidemic. History is filled with examples of such outbreaks including the American chestnut blight outbreak, Dutch elm disease or sudden oak death.

Thirty percent of forests in the world are primarily used for production of forest products (FAO, 2010a). In 2010, ninety-eight percent of the species harvested in western Oregon were coniferous species (Oregon Forest Resource Institute, 2012, See Appendix).

Between 2000 and 2013, the United States produced the most coniferous roundwood in the world (FAO, 2014). In 2012, the annual quantity of coniferous roundwood exported from the United States increased by 162 percent (from 7.53 $m^3$ to 12.27 million $m^3$) and then by another 119 percent in 2013 to 14.65 million $m^3$ (FAO, 2014). In 2010, Douglas-fir accounted for 73.6 percent of the timber harvested in Western Oregon (Oregon Forest Resource Institute, 2012, See Appendix). Over 60 percent (~1 billion board feet) of logs exported in 2011 from Oregon and Washington Customs Districts to all countries were Douglas-fir (Xiaoping and Warren, 2012). Douglas-fir logs are an important component of the western United States export markets and their availability may become limited by the continued introduction of exotic and invasive forest pests.

Continued expansion of efforts to contain *P. ramorum*, the pathogen responsible for sudden oak death, ramorum blight and shoot dieback could eventually affect the ability to move coniferous logs in both the western U.S. and the world. While many of the species affected by *P. ramorum* are not commercially important, other host species that are major sources of income for local landowners are also affected. Among the most important is Douglas-fir. Douglas-fir is not currently recognized as a bole host, so unmanufactured wood and wood products are not regulated except that the material is required to be free of soil, needles, foliage, and plant debris. However, recognizing the importance of Douglas-fir to western North America, the extremely small threshold for starting a new outbreak, and the sensitive international concern, it is prudent to develop methods for mitigating the risk of spreading *P. ramorum* on Douglas-fir logs before they are needed.

Heat treatments are widely used globally, especially for movement of pallets and other solid wood packing (FAO, 2010b; IPPC, 2009). Heating is the most reliable method for minimizing the risk of transporting unwanted organisms, but the method is viewed by the industry as costly and time consuming (He et al., 1997). However, *P. ramorum* has been described as “highly heat tolerant” (Harnik et al., 2004), and may have the potential to survive elevated temperatures in the chlamydospore state (Tooley et al., 2008). Preliminary studies suggest that the time and temperature relationship required by the

IPPC would be effective for eliminating *P. ramorum* from firewood but the data are not definitive (Tubajika et al., 2007).

Although heat is widely used as a mitigation measure for global commerce in wood, it is difficult with large logs because long heating periods are required to reach the required internal temperatures. Heat treatment of chips or other processed wood may be feasible within the quarantine area, however, the rate of heat transfer in wood is extremely slow, particularly in log form, making this difficult to accomplish in the field. Alternative methods to heat treatment include debarking logs before exportation. Debarking is a common phytosanitation practice since many fungi can colonize and contaminate bark substrates but only a few can survive in xylem tissues. However, *P. ramorum* has the potential to colonize more deeply into the sapwood making it necessary to develop methods that sanitize farther into logs to meet regulations and mitigate the risk of the accidental spread. Brown and Brasier (2007) demonstrated that *Phytophthora* species were active and viable up to 25 mm into the xylem in several hardwood species and suggested that removing the outer 3 cm of sapwood would be required to mitigate the risk (Brown and Brasier, 2007). Currently there are no proven methods for elimination of *P. ramorum* in woody substrates (Sansford et al., 2009).

One possible mitigation strategy would be to treat logs at landings in the forest with fungitoxic compounds capable of diffusion through the bark and into the sapwood to a depth sufficient to kill *P. ramorum*. Boron is one possible candidate for this purpose. Borates have been widely used to prevent biological deterioration of wood structures. Schauwecker and Morrell (2008) examined the ability of pressure treatment with borates to eliminate wood boring insects from lumber and found that borates were not lethal to larvae but adults did not emerge after treatment. Nevertheless, the activity of borates will be different for insects, fungi, and Oomycetes. Borates may also be useful for controlling *P. ramorum*, but there are no data to support this premise.

Borate formulations have been used for control of fungi in wood preservation, agricultural applications, and forest pest establishment prevention. Borates have been shown to be effective against a variety of fungi (Arora, 2005; Ayrilmis et al., 2005;

Garbelotto and Gonthier, 2013; Kartal et al., 2005; Pratt and Quill, 1996; Qin et al., 2010; Rolshausen and Gubler, 2005; Smilanick and Sorenson, 2001). This broad applicability warrants investigation of use for phytosanitation of potential pathways of accidental pest introductions.

One of the primary questions concerning potential borate use would be its ability to diffuse through bark at levels sufficient to arrest or kill the target organisms. Bark is a potent barrier to moisture movement and might prove equally resistant to boron diffusion. The primary objective of this study was to determine whether boron could diffuse through Douglas-fir bark and into the sapwood. Douglas-fir was chosen as a model coniferous species because of its commercial importance. Secondary objectives were to answer the following questions:

-Does increasing the initial concentration of borate solution applied to the bark increase the amount of boron diffused into the sapwood?

-Is there a difference in diffusion when glycol is added to the DOT (e.g. is there greater diffusion with BORACARE® than DOT)?

-Is there an effect of bark thickness on boron diffusion into sapwood?

**Materials and Methods**

*Sample Preparation*

Two trials were conducted. In each trial, 27-31 cm lengths of Douglas-fir logs were collected from the McDonald Dunn forest located near Corvallis, Oregon. Logs used in each trial came from separate trees felled for this study. Twelve log sections were used in Trial 1 and sixteen in Trial 2. The log diameters from Trial 1 ranged from 17.2-19.9 cm and 18.7 to 24.2 cm in Trial 2. Bark thickness was measured at three places on each log and averaged. Treatment solution was delivered to the bark by attaching 1.75 cm lengths of 2.5 cm diameter PVC to the bark surface using an adhesive. In Trial 1, the PVC was adheared to the bark surface by melting Histowax on the bark around the base of the PVC. Molten Histowax solidified between the outer surface of the bark and the PVC to seal the connection and limit lateral boron movement on the bark surface. In Trial 2, the PVC sections were attached to half of the log sections (eight) using Histowax and

the others were attached using household boron-free caulking. Three PVC wells were added to each log in both trials. In the first trial, 62.55 g of DOT was added to 500 ml of heated deionized water to create a fifteen percent BAE (boric acid equivalent) solution of DOT. In the second trial, DOT and DOT plus glycol formulations were tested. Both solutions were mixed to produce a sixteen percent BAE solution. Ten mL of the treatment solution was added to each PVC well on each log in each study (See Figure 4.5). Treatment solutions were allowed to diffuse for 1 hour, then poured off, taking care to avoid spilling solution on bark outside the well. Half of the log sections in the second trial were randomly assigned to the DOT formulation, and the other half were randomly assigned to the DOT/glycol formulation. The boron plus glycol contained 40.6% disodium octobrate tetrahydrate plus 11.9% polyethylene glycol and 47.5% monoethylene glycol prior to dilution (Boracare®, Nisus, Knoxville TN).

In both trials, the logs were stored at 4 C for one month before boron movement was assessed. Each log was cut in half longitudinally, cut into 3 sections to separate the PVC wells, and subsequently cut into four or three subsections, for Trial 1 and Trial 2, respectively (See Figure 4.6). Each subsection was then cut into three samples corresponding to the bark, outer sapwood, or inner sapwood. Cambium and phloem were included with bark samples. Frequently, 1-2 mm of sapwood was included to ensure that all of the cambium was with the bark samples and that the sapwood samples were exclusively sapwood. Outer sapwood was composed of the subsequent outer 1 cm and inner sapwood included the area between 1-2 cm deep into the sapwood. All cuts were made from inside to outside to limit the potential for carrying boron on the saw blade from the surface.

*Chemical Assay*

Douglas-fir samples were oven dried overnight (54 C), ground to pass a 20-mesh screen, then extracted with boiling water. The resulting extract was then analyzed for boron concentration by the Azomethine H method (American Wood-Preservers' Association, 2012b). Results were recorded in percent BAE and PPM. Only samples from subsections that were directly under PVC wells were assayed. Two subsections

from logs in Trial 1 were assayed because four subsections were cut from each section rather than three in Trial 2. In this case, the middle two subsections from each section were used because they were directly under the PVC wells. A total of 204 samples from Trial 1 and 144 samples from Trial 2 were assayed.

*Statistical Analysis*

A Welch's t-test was used to test for differences in the mean percent BAE between the logs treated with the DOT formulation in the first and second trials. This test was also used to examine differences between the two formulations used in Trial 2. Welch's t-test was selected because it is robust to unequal variance as well as non-normality when sample sizes are large. To avoid pseudoreplication in our comparisons, all of the samples were averaged to reach a single value for each log.

One objective of this study was to test for differences in boron diffusion into the sapwood between the two boron formulations. Therefore, formulation was the primary treatment variable of interest, which was applied on the log level. However, the study was designed in such a way that we expected to have variation in diffusion due to random log-to-log differences and random section-to-section differences. For instance, we expected measurements of diffusion within a log to be more alike than measurements between logs since measurements within a log were not independent of each other. We also expected measurements within a log to be different than measurements within another log. Logs were the largest physical unit in this study and were considered a blocking variable, where the actual measurements were nested within the sections and logs. Subsection was considered a fixed effect because only the section directly under the PVC well was included in the assay in the second trial. Measurements were made on the lowest level (3 samples per subsection, with 3-6 subsections per log). Accounting for the unexplained log-level and section-level variation actually helped detect differences among groups, if they really existed. Diffusion into the sapwood was the response variable of interest so further analysis was conducted to test for diffusion differences in the sapwood for both of the formulations. The effects of bark thickness and differences between borate formulations in the sapwood samples were assessed by fitting a mixed

model where logs and sections were considered random effects and the formulation and adhesive type were considered fixed effects. We also included a term for the interaction between formulation and adhesive type and tested whether there was evidence of an interaction. The model was fit to measurements from the sapwood samples used in Trial 2.

The following mathematical model was used to describe the response:

$$\boldsymbol{Y_{ijk} = \beta_0 + \beta_1 Idot_{ijkl} + \beta_2 Iwax_{ijkl} + \beta_3 Idepth_{ijkl} + \beta_4 thick_{ijkl} + \beta_5 (Idot_{*} Iwax)_{ijkl} + p_l + p_k + \varepsilon_{ijkl}}$$

where

$Y_{ijk}$ is the mean percent BAE of the sapwood samples in the *ith* formulation with the *jth* adhesive in the *kth* section of the *lth* log and, i= DOT or DOT plus glycol; j= Histowax or household boron-free caulking; k=bottom, middle or top section, l=1,2,3,4,…16.

$\beta_0$ is the mean percent BAE of <1 cm deep sapwood samples treated with the DOT plus glycol formulation using the household boron-free caulking adhesive.

$\beta_1$ is the additive effect of the DOT formulation on the mean percent BAE of the sapwood samples.

$\beta_2$ is the additive effect of using the Histowax adhesive on the mean percent BAE of the sapwood samples.

$\beta_3$ is the additive effect of increasing the depth into the sapwood on the mean percent BAE of the sapwood samples.

$\beta_4$ is the change of the mean percent BAE of the sapwood samples with a 1 unit change in bark thickness.

$\beta_5$ is the additive effect of using both Histowax as an adhesive and using the treatment formulation DOT alone on the mean percent BAE of the sapwood samples.

*thick* is the bark thickness of the *ith* formulation with the *jth* adhesive in the k*th* section of the *lth* log.

*Idot* is 1 when the treatment formulation is DOT alone and 0 otherwise,

*Iwax* is 1 when the adhesive is Histowax and 0 otherwise,

*Idepth* is 1 when the sapwood piece is from 1-2 cm deep into the sapwood and 0 otherwise.

$p_l$ is the random effect of each individual log on the mean percent BAE of the sapwood samples, $p_l \sim N(0, \sigma_p^2)$ and $Cov(p_l,p_{l'})=0$

$p_k$ is the random effect of the log section on the mean percent BAE of the sapwood samples, $p_k \sim N(0, \sigma_p^2)$ and $Cov(p_k,p_{k'})=0$

$\varepsilon_{ijkl}$ is the random error term for the *ith* treatment formulation and the *jth* adhesive in the *kth* section of the *lth* log and $\underline{\varepsilon_{ij}} \sim MVN(\underline{0}, \Sigma)$. $\Sigma$ is the covariance matrix that describes the relationship between sections within each log. The variances differ depending on the formulation of the measurement.

All statistical analysis was conducted in R version 3.0.2. The session information can be found in the appendix.

**Results**

Boron was capable of diffusing into bark and was also detected in the sapwood of some samples. Median percent BAE in the bark samples was highest in the group treated with DOT plus glycol in Trial 2 (Figure 4.1). Bark samples from sections treated with DOT had higher median boron levels in the second trial than the first trial. Differences in median percent BAE were less distinct in the sapwood samples but the median percent BAE for sapwood samples in both trials and treatment formulations were generally reduced with distance from the surface (Figure 4.2).

There was strong evidence that the mean percent DOT diffused into the logs in Trial 1 was different than levels in Trial 2, when all of the samples were averaged together for each log (Welch's Two Sample t-test, $t_{11}$= -3.9979, p-value = 0.002032). The mean DOT level in the logs of Trial 1 was 0.27% BAE compared with 0.42% BAE in Trial 2 (Table 4.1). With 95% confidence, the mean DOT level in the logs used in Trial 1 was between 0.07 and 0.23% BAE lower than the mean DOT level in logs used in Trial 2.

There was also evidence that the mean DOT content in the sapwood of logs in Trial 1 differed from Trial 2, when all of the sapwood samples were averaged together for each log (Welch's Two Sample t-test, $t_{13}$= -3.8155, p-value = 0.002066). The mean DOT

content in sapwood of logs in Trial 1 was 0.07% BAE while that in Trial 2 was 0.1% BAE, indicating a difference of 0.03% BAE between the Trials (Table 4.2). With 95% confidence, the mean DOT content in the sapwood of logs used in Trial 1 was between 0.01 and 0.04% BAE lower than the content in logs of Trial 2.

The differences between Trials 1 and 2 in terms of formulation preclude comparison. There was evidence of a difference when all of the samples were averaged for each log section in Trial 2 (Welch's Two Sample t-test, $t_{14}$= -4.2529, p-value= 0.0008084). The mean boron levels in samples treated with DOT and DOT/glycol in Trial 2 were 0.42 and 0.62% BAE, respectively, indicating an estimated difference of 0.2% BAE (Table 4.1). With 95% confidence, the mean boron levels in samples treated with the DOT formulation was between 0.1 to 0.31% BAE lower than those treated with DOT/glycol.

There was no evidence of differences between the two formulations when only the sapwood samples were averaged for each log (Welch's Two Sample t-test, $t_{10}$= -0.3389, p-value=0.742). This lack of differences in sapwood retention between the different formulations in Trial 2 led to further investigation using a linear mixed model. Possible interactions between the type of adhesive used to secure the PVC wells and the formulation were also investigated. There was indication that the relationship between formulations depended on the type of adhesive used (Figure 4.3). Therefore, an interaction term was included in our model. Assumptions of the linear mixed model were verified using the residual and fitted values from the model. The model was refit to model the variance heterogeneity between formulations after we explored the residuals between formulations and noticed that the residuals for logs treated with DOT were less variable than the residuals for logs treated with DOT/glycol (Figure 4.4). The assumptions of the model were adequately met (See Appendix).

Although there was an indication of an interaction when exploring the data graphically (Figure 4.3), no interaction was detected statistically (F-test$_{1,11}$; F = 0.2705, p-value = 0.6133). The model also revealed that there was no effect of formulation on boron content in the sapwood in Trial 2, even after accounting for sample depth,

adhesive, bark thickness and possible interactions between adhesive and formulation (F-test$_{1,11}$; F=0.1249, p-value=0.7305). Additional parameters in the model were analyzed to address secondary objectives. Average bark thickness did not affect the mean boron content in the sapwood samples after accounting for sample depth, adhesive, formulation, and the possible interaction between adhesive and formulation (F-test$_{1,11}$; F=0.3521, p-value=0.5649). Conversely, there was evidence that sample depth (<1cm or 1-2cm deep) affected the mean boron content (F-test$_{1,47}$; F=24.1847, p-value=<0.0001). It was estimated that the mean boron content for samples 1-2 cm deep into the sapwood were 0.019% BAE lower than those near the surface. With 95% confidence, the estimated difference is between 0.011 and 0.027% BAE less for sapwood samples 1-2 cm deep compared to samples taken from less than 1 cm deep into the sapwood.

**Discussion**

Prior to this study, the only evidence that borates could diffuse through bark was presented by Cassens and Schmidt (1998). They observed bark and outer sapwood penetration by DOT in hickory (*Carya cordiformis*) rounds with intact bark. Boron also diffused through the bark and into the sapwood of the log sections used in this study. Our results were consistent with their study using hickory and may indicate that boron can diffuse through the bark of other tree species.

Mean boron content differed drastically between bark and sapwood samples (Table 4.3). Bark samples included cambial and phloem tissues, as well as surface materials such as lichens and bryophytes that may account for some of the stored boron. Sapwood samples were exclusively xylem tissues. Much higher variation was observed in the chemical analysis of bark samples compared to sapwood samples (Table 4.3). Differences between bark and sapwood samples were not tested statistically because of clear differences. The mean boron content of sapwood samples treated with DOT/glycol in Trial 2 was only 16% of the mean for the bark samples (Tables 4.1 & 4.2). A higher bark:sapwood ratio was observed in Trial 1, where sapwood samples contained only 26% as much boron as the bark samples (Tables 4.1 & 4.2). The stark differences between the bark and sapwood samples may be a result of the limited treatment solution quantities

added to the PVC wells. It is possible the 10 mL of treatment solutions was only enough to saturate the bark. Additionally, boron was only allowed to diffuse inward for one month. Increased diffusion times may result in increased diffusion into the sapwood. Further, moisture levels within the logs were not controlled, and the logs may have dried out prior to the end of the diffusion period, limiting diffusion, which requires moist wood. Although logs were stored at 4 C during the diffusion period, moisture content was not controlled and the logs were not treated immediately after harvest. Controlling for moisture content may have improved our ability to detect differences in the sapwood between formulations and trials. However, moisture change would also occur in the field, making these trials more representative of actual practice. Morrell and Freitag (1995) observed differences in diffusion when controlling for moisture content and Lebow and Morrell (1989) suggested that greater diffusion can be achieved with higher moisture contents.

It is possible that boron did not diffuse into the sapwood and that the small amounts observed in the sapwood were carried over during the processing. Although no controls were used in this study to determine whether there was carry over from sample to sample during processing, the cutting was done so that samples were cut from the inside (where any boron levels would be low) towards the bark (where boron concentrations should be higher). The logs were also processed consistently so that any contamination would have been consistent across both formulations and trials and a difference could still be detected.

Attempts to meet the secondary objectives of this study were conducted with two types of statistical analyses, Two-sample t-tests and a linear mixed model. Averaging the sections and the samples to the log level met the assumption of independence for the Welch's Two Sample t-test. However, the t-test may not have been appropriate for comparing the logs once all of the samples were averaged together because the sample sizes were reduced to 8-12 logs.

Welch's Two Sample t-tests detected differences between logs treated with DOT in the two trials. Although the initial DOT treatment solution concentrations in the two

trials were similar (15 and 16% BAE), there was evidence that the mean boron contents for log samples in the first trial was lower than those in the second trial. While we detected a difference in diffusion, we did not test the effect of the initial treatment solution concentration parameter. Increasing the initial treatment solution concentration may have increased the amount of boron available to diffuse but the differences should be minimal. Determining whether the increased diffusion was a result of the increased initial concentrations would require a separate linear mixed model to tease out the effects of the initial treatment solution concentrations. A difference was detected when limiting the analysis to sapwood samples in the logs treated with DOT in the first and second trials. This may suggest that the same variation existed between Trials 1 and 2 at the sapwood and bark levels.

The differences between logs treated with DOT in Trial 1 and Trial 2 prevented the inclusion of Trial 1 data in the comparison between formulations. Using logs from Trial 2 only, a two sample analysis with a Welch's t-test revealed there was greater diffusion in the logs treated with DOT/glycol compared to logs treated with only DOT. However, differences could not be detected between formulations when limiting the analysis to sapwood samples only. Because a difference could not be detected on the log level, a linear mixed model was created to test for differences in the sapwood between the formulations. Accounting for the unexplained log-level and section-level variation actually helped detect differences among groups. However, no effect of formulation was detected. The failure to detect a formulation effect on the mean boron content of the sapwood samples in the linear mixed model was consistent with the results of the Welch's Two Sample t-tests for the logs when all sapwood samples were averaged. Boron diffusion was greater in bark samples treated with DOT plus glycol but no difference was detected within the sapwood. These results are consistent with Freitag and Morrell (2002), who observed slight enhancements of boron diffusion with glycol at certain moisture contents. However, they concluded that the enhancement was not worth the increased costs of the DOT/glycol. In our study, DOT/glycol provided increases in diffusion between 0.1 to 0.31% BAE when averaging all of the samples into the logs.

This could be substantial if the minimum inhibitory concentrations to target pest organisms were as low as 0.25% BAE.

Despite preliminary graphical investigation suggesting interactions between the formulations and the adhesives used in Trial 2 (Figure 4.3), there was no evidence of an interaction. Since the initial graphical exploration only concerned the mean boron content for the groups of sapwood samples in each formulation-adhesive combination, the figure must not have represented the variation of each group.

Although the parameters for formulation, bark thickness, and the interaction did not affect the mean when using a linear mixed model, sample depth did have an effect. Boron content decreased with increasing sample depth. An estimated decrease between 0.011 and 0.027% BAE was associated with increased depth. These results are consistent with Morrell and Freitag (1995), who observed adequate levels for control within the first 5mm of Douglas-fir lumber but not deeper. This decrease with depth may pose a challenge to phytosanitation objectives if the target pests are present more than a few cm into the xylem.

The results suggest that boron diffusion may be an appropriate method for mitigating the risk of accidentally spreading quarantined microorganisms present near the bark surface. The methods used in this study were preliminary and similar methods would not be effective in the field. Different application methods may improve boron diffusion depth and the resulting retentions. Dipping entire logs into borate baths would ensure that treatment solution quantities were not limited. Additionally, boron delivery into the sapwood may be easier if logs lose sections of bark during harvest.

**Conclusions**

Borates were capable of diffusing through Douglas-fir bark and into the sapwood. The addition of glycol further enhanced boron movement in bark. The results suggest that boron diffusion has potential as a mitigation measure for limiting the spread of *P. ramorum.*

**Tables and Figures**

Figure 4.1: Boxplot displaying median boron content in the bark samples of Douglas-fir log sections treated with DOT alone or with glycol.

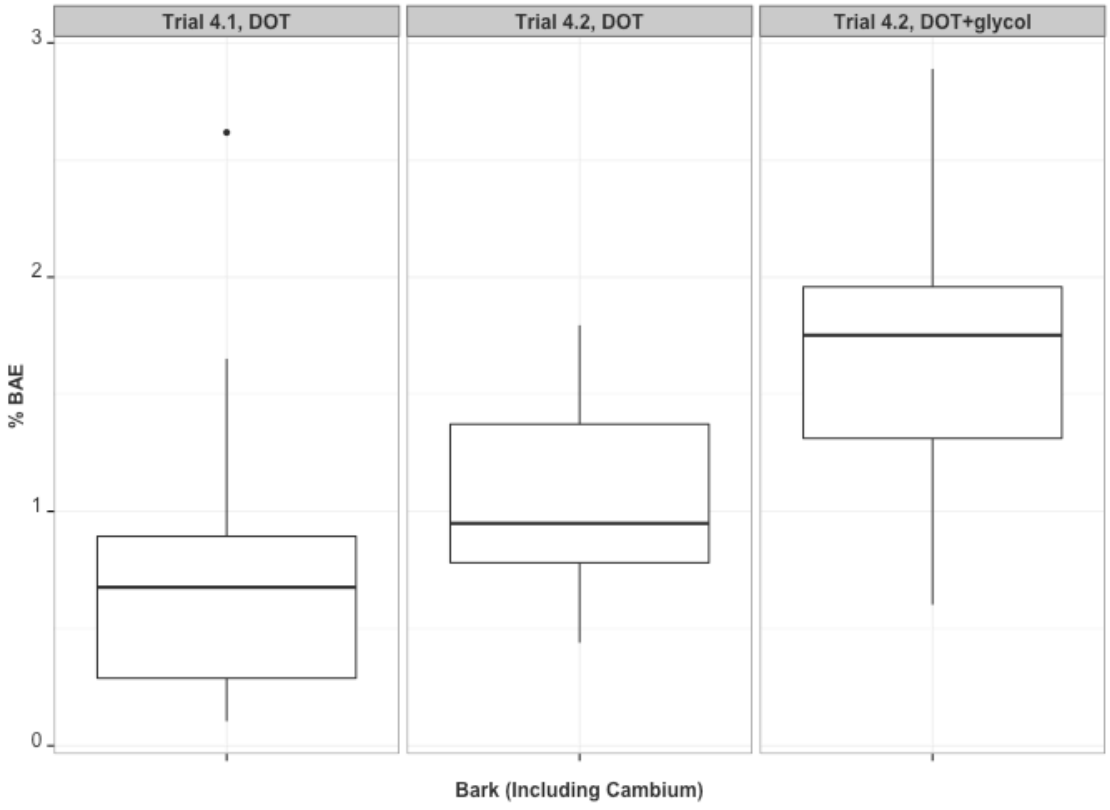

Figure 4.2: Boxplot displaying boron content in the sapwood samples of Douglas-fir log sections treated with DOT alone or with glycol.

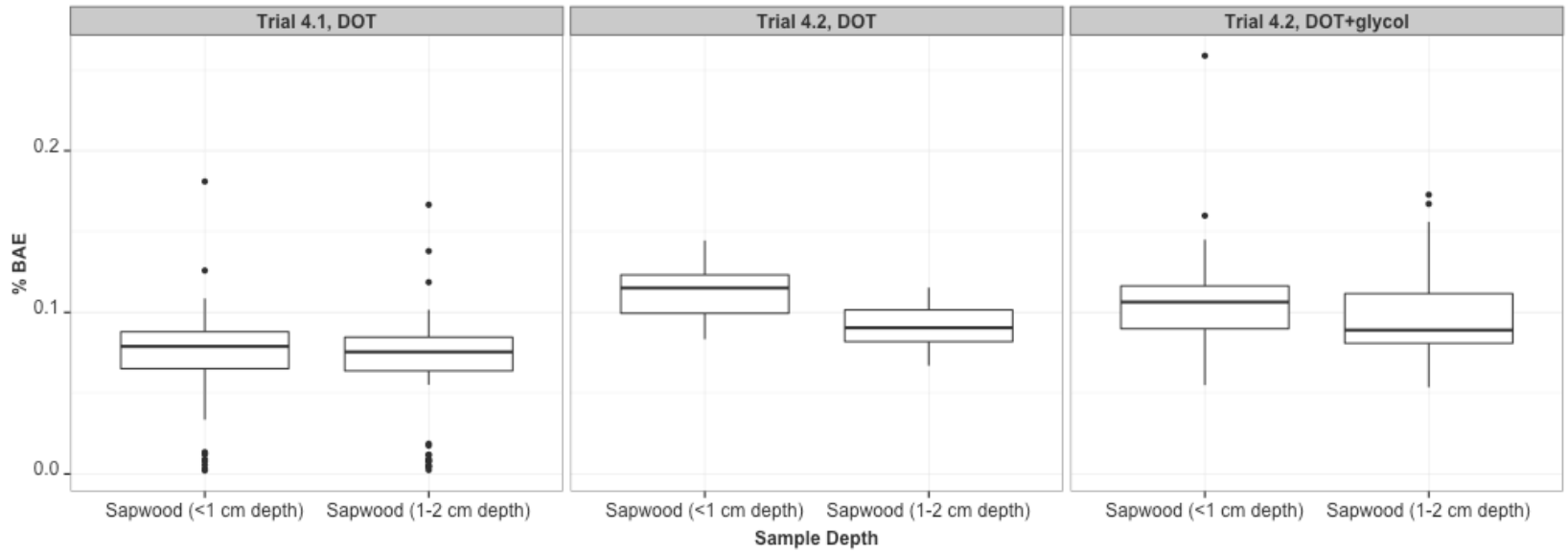

Figure 4.3: Interaction plot illustrating possible interactions between PVC adhesion method and the boron formulation for diffusion into the sapwood of samples used within Trial 2. Points represent mean boron content for the group of sapwood samples that fit the combination of formulation and adhesive.

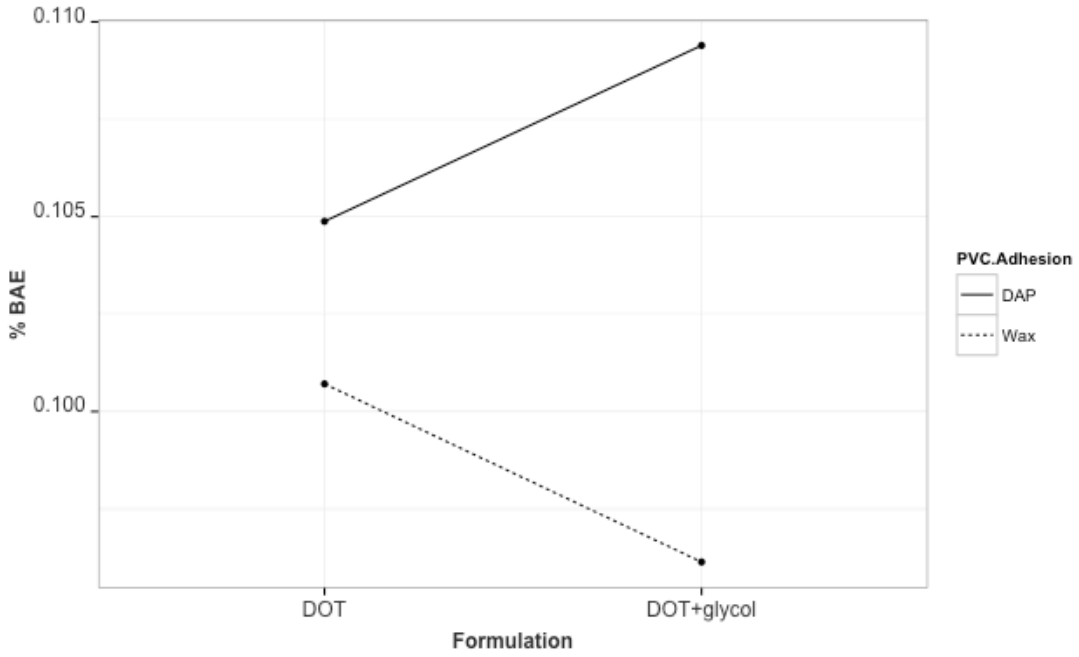

Figure 4.4: Residual plot for the linear mixed model justifying the use of different variances among the levels of formulations. Residuals for samples treated with DOT were less variable than residuals for samples treated with DOT plus glycol.

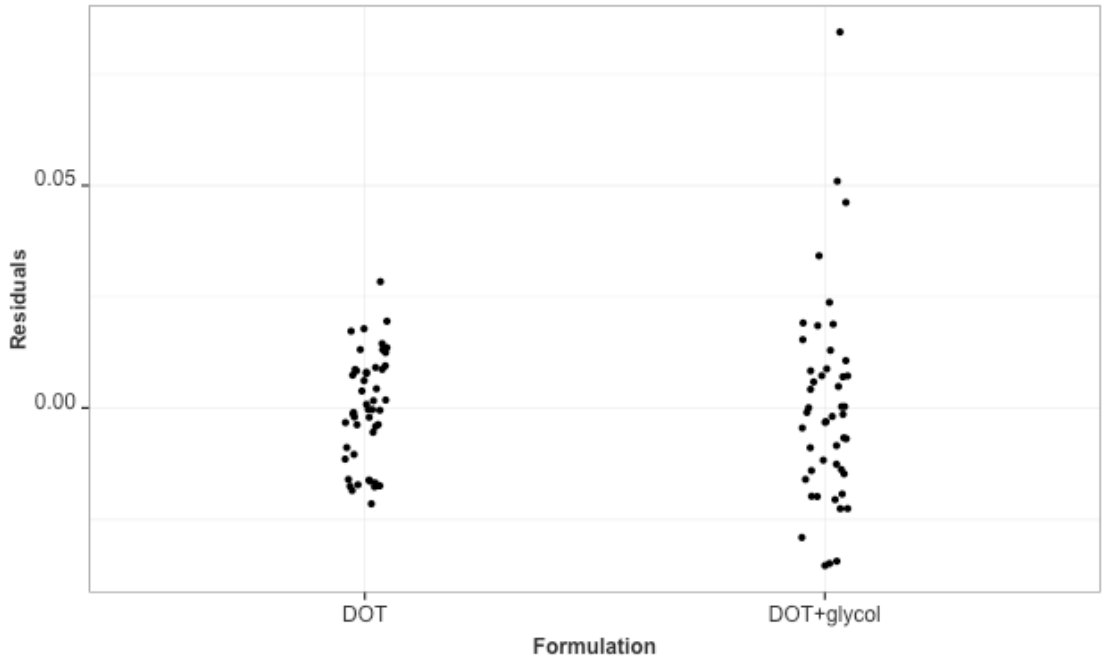

Figure 4.5: Example of Douglas-fir with borate solution in PVC wells attached with Histowax from Trial 1 of Chapter 4.

Figure 4.6: Douglas-fir samples after processing into sections and subsections in Trial 1, PVC included indicating the source of DOT.

Table 4.1: Mean boron content (% BAE) in logs treated with DOT in Trials 1 and 4.

| Trial | Formulation | n | Mean | SE |
|---|---|---|---|---|
| Trial 1 | DOT | 12 | 0.27 | 0.02 |
| Trial 2 | DOT | 8 | 0.42 | 0.03 |
| Trial 2 | DOT/glycol | 8 | 0.62 | 0.03 |

n represents the number of treated logs used in each trial for each formulation. Log-level percent BAE values were averaged across all sections and all samples to avoid psuedoreplication when making comparisons.

Table 4.1: Mean boron content (% BAE) in the sapwood of logs in Trials 1 and 2.

| Trial | Formulation | n | Mean | SE |
|---|---|---|---|---|
| Trial 1 | DOT | 12 | 0.07 | 0.01 |
| Trial 2 | DOT | 8 | 0.1 | 0 |
| Trial 2 | BORACARE | 8 | 0.1 | 0.01 |

n represents the number of treated logs used in each trial for each formulation. Log-level percent BAE values were averaged across all sections to avoid psuedoreplication when making comparisons, but only sapwood samples were included in this summary.

Table 4.3: Mean boron content (% BAE) in sections cut from the bark and sapwood of Douglar-fir log sections treated with DOT alone or with glycol.

| Trial | Formulation | Sample.Type | n | Mean | SE | Min | Max |
|---|---|---|---|---|---|---|---|
| Trial 1 | DOT | Bark,Cambium,Phloem | 67 | 0.68 | 0.06 | 0.1 | 2.62 |
| Trial 1 | DOT | Sapwood (<1 cm depth) | 68 | 0.07 | 0 | 0 | 0.18 |
| Trial 1 | DOT | Sapwood (1-2 cm depth) | 69 | 0.07 | 0 | 0 | 0.17 |
| Trial 2 | DOT | Bark,Cambium,Phloem | 24 | 1.06 | 0.08 | 0.44 | 1.79 |
| Trial 2 | DOT | Sapwood (<1 cm depth) | 24 | 0.11 | 0 | 0.08 | 0.14 |
| Trial 2 | DOT | Sapwood (1-2 cm depth) | 24 | 0.09 | 0 | 0.07 | 0.12 |
| Trial 2 | DOT/glycol | Bark,Cambium,Phloem | 24 | 1.66 | 0.12 | 0.60 | 2.89 |
| Trial 2 | DOT/glycol | Sapwood (<1 cm depth) | 24 | 0.11 | 0.01 | 0.05 | 0.26 |
| Trial 2 | DOT/glycol | Sapwood (1-2 cm depth) | 24 | 0.10 | 0.01 | 0.05 | 0.17 |

# CHAPTER 5: GENERAL CONCLUSIONS

The results presented in chapter 2 suggested there might be a risk of accidentally disseminating propagules of *Phytophthora ramorum* through the trade of Douglas-fir timber materials. Chapter 3 presented a possible solution and Chapter 4 presented a possible application. The results of Chapter 3 suggested that levels of boron above 0.1% BAE were inhibitory to *P. ramorum* and that levels above 1.5% BAE were lethal in a bioassay. In tanoak material, levels as low as 0.42% BAE were shown to be inhibitory. Application of borates to the surface of logs resulted in diffusion through the bark and into the sapwood of the log sections used in this study. Although the levels in the sapwood were below the levels determined to be lethal in the petri dish trials, *P. ramorum* may be easier to control in substrates that are less nutritious than the *Phytophthora* spp. selective media used in the bioassay. Additionally, boron delivery into the sapwood may be easier if logs lose sections of bark during harvest. Further investigation of the minimum inhibitory levels of borates against *P. ramorum* in wood substrates would bolster the implication of this method. Determining the levels sufficient to prevent zoospore or sporangial germination may inform the acceptability of lower levels in naturally unexposed tissues such as the sapwood. It would also be beneficial to know the threshold required to eliminate chlamydospores. Determining this threshold could be accomplished using studies that directly expose isolated chlamydospores. Furthermore, the ultimate acceptance of boron diffusion as a means of *P. ramorum* phytosanitation would require investigation of the depth that *P. ramorum* can grow into the sapwood. The results of our study indicate that boron has the potential to be an effective mitigation measure against pests that are present near the surface of the logs but the depth that would require treatment is still unknown.

Several methods were developed and executed to investigate if there is a risk of accidentally transporting *P. ramorum* when moving Douglas-fir logs. Many methods were implemented and altered because of the poor recoveries. The poor recovery of *P. ramorum* in the Tanoak Block, Well Plate, Dip, Soak, and Microscopy Trials suggested that the methods of inoculation were unreliable for testing the pathogenicity or the

treatability of *P. ramorum* on wood material. We believe that the poor recovery does not reflect susceptibility of Douglas-fir or tanoak material, but rather, that the methods were ineffective at producing reliable results. In general, methods that soaked Douglas-fir sapwood samples in zoospore suspensions for 24 hours provided the greatest recoveries. Refining and implementing the methods used in this thesis could inform the international community which species pose a risk for accidental dissemination on wood material and potentially prevent outbreaks similar to sudden oak death. These methods could be used to test methods of detection and develop phytosanitary standards for target organisms. While these studies present artificial environments and applications, the investigation warrants further research of the application of common wood preservatives for control of quarantined pests.

Preventative measures requiring phytosanitation are essential for protecting forest health, however, standardized methods are limited and difficult to develop for achieving broad-spectrum efficacy. Developing methods for specific target organisms may provide additional shielding against the global dissemination of species within the same genus. The immense losses caused by *P. ramorum*, *P. infestans*, *P. cinnamomi*, *P. lateralis* and *P. kernoviae* reinforce the need for standardizing phytosanitation measures that are effective against organisms in the genus *Phytophthora*. These species will continue to threaten our biosecurity until methods of containment are developed.

# BIBLIOGRAPHY

Agrios, G.N. (2005). Plant Pathology (30 Corporate Drive, Suite 400, Burlington, MA 01803, USA: Elsevier Academic Press).

Ahn, S.H., Oh, S.C., Choi, I., Han, G., Jeong, H., Kim, K., Yoon, Y., and Yang, I. (2010). Environmentally friendly wood preservatives formulated with enzymatic-hydrolyzed okara, copper and/or boron salts. J. Hazard. Mater. *178(1)*, 604–611.

Ahumada, R., Rotella, A., Slippers, B., and Wingfield, M. (2012). Potential of *Phytophthora pinifolia* to spread via sawn green lumber: a preliminary investigation. South. For. J. For. Sci. *74(4)*, 211–216.

American Wood-Protecting Association (2012a). Standard method of determining penetration of preservatives and fire retardants. Method 1. Method for determining boron-containing preservatives and fire retardants. Standard A3-97. In Book of Standards, (Birmingham, AL), p. 210.

American Wood-Protecting Association (2012b). Analysis of waterborne preservatives and fire-retardant formulations. Method 16. Determination of boron in treated wood using azomethine or carminic acid. Standard A2-98. In Book of Standards, (Birmingham, AL), pp. 207–209.

Arora, R.K. (2005). Efficacy of boric acid spray for control of black scurf in unwashed and washed potato tubers. Potato J. *32(3)*, 183–184.

Aukema, J.E., McCullough, D.G., Holle, B.V., Liebhold, A.M., Britton, K., and Frankel, S.J. (2010). Historical accumulation of nonindigenous forest pests in the continental United States. BioScience *60(11)*, 886–897.

Ayrilmis, N., Kartal, S.N., Laufenberg, T.L., Winandy, J.E., and White, R.H. (2005). Physical and mechanical properties and fire, decay, and termite resistance of treated oriented strandboard. For. Prod. J. *55(5)*, 74–81.

Basu, A., and Das, S. (2003). Integrated management of potato (*Solanum tuberosum*) disease in Hoogly area of West Bengal. Indian J. Agric. Sci. *73 (12)*, 649–651.

Boyce, J.S. (1961). Forest pathology (McGraw-Hill, New york, NY).

Boyd, I.L., Freer-Smith, P.H., Gilligan, C.A., and Godfray, H.C.J. (2013). The consequence of tree pests and diseases for ecosystem services. Science *342* (6160):1235773.

Brasier, C.M. (2001). Rapid evolution of introduced plant pathogens via interspecific hybridization. BioScience *51(2)*, 123–133.

Brasier, C.M. (2008a). The biosecurity threat to the UK and global environment from international trade in plants. Plant Pathol. *57(5)*, 792–808.

Brasier, C. (2009). *Phytophthora* biodiversity: how many *Phytophthora* species are there? In Proceedings of the Fourth Meeting of IUFRO Working Party, (Albany, CA, USA: Goheen EM, Frankel SJ), pp. 101–115.

Brasier, C.M. (2012). Plant imports, *Phytophthoras*, and forest degradation. In Frankel, S.J.; Kliejunas, J.T.; Palmieri, K.M.; Alexander, J.M. tech. coords. 2013. Proceedings of the Sudden Oak Death Fifth Science Symposium. Gen. Tech. Rep. PSW-GTR-243. Albany, CA: U.S. Department of Agriculture, Forest Service, Pacific Southwest Research Station. 169 p.

Brasier, C.M., and Kirk, S.A. (2001). Comparative aggressiveness of standard and variant hybrid alder *phytophthoras*, *Phytophthora cambivora* and other *Phytophthora* species on bark of *Alnus, Quercus* and other woody hosts. Plant Pathol. *50 (2)*, 218–229.

Brasier, C., and Webber, J. (2010). Plant pathology: Sudden larch death. Nature *466 (7308)*, 824–825.

Brasier, C.M., Kirk, S.A., Delcan, J., Cooke, D.E.L., Jung, T., and Man In't Veld, W.A. (2004). *Phytophthora alni* sp. nov. and its variants: designation of emerging heteroploid hybrid pathogens spreading on Alnus trees. Mycol. Res. *108 (10)*, 1172–1184.

Brasier, C.M., Beales, P.A., Kirk, S.A., Denman, S., and Rose, J. (2005). *Phytophthora kernoviae* sp. nov., an invasive pathogen causing bleeding stem lesions on forest trees and foliar necrosis of ornamentals in the UK. Mycol. Res. *109 (8)*, 853–859.

Britton, K.O. (2004). Biological pollution: an emerging global menace. American Phytopathological Society (APS Press).

Brown, A.V., and Brasier, C.M. (2007). Colonization of tree xylem by *Phytophthora ramorum, P. kernoviae* and other *Phytophthora* species. Plant Pathol. *56 (2)*, 227–241.

Caldeira, F. (2010). Boron in wood preservation: A review in its physico-chemical aspects. Silva Lusit. *18 (2)*, 179–196.

Cassens, D.L., and Schmidt, E.L. (1998). Use of borate compounds to prevent insect damage to furniture hickory rounds with the bark intact. For. Prod. J. *48 (3)*, 36–37.

Castello, J.D., Leopold, D.J., and Smallidge, P.J. (1995). Pathogens, patterns, and processes in forest ecosystems. BioScience *45 (1)*, 16–24.

Cave, G.L., Randall-Schadel, E.B., and Redlin, S.C. (2008). Risk analysis for *Phytophthora ramorum* Werres, de Cock & Man in't Veld, causal agent of Sudden Oak Death, Ramorum leaf blight, and Ramorum dieback (Raleigh, NC 27606: United States

Department of Agriculture, Animal and Plant Health Inspection Service, Plant Protection and Quarantine, Center for Plant Health Science and Technology, Plant Epidemiology and Risk Analysis Laboratory (USDA, APHIS, PPQ, CPHST, PERAL)).

Cobb, R.C., Filipe, J.A.N., Meentemeyer, R.K., Gilligan, C.A., and Rizzo, D.M. (2012). Ecosystem transformation by emerging infectious disease: loss of large tanoak from California forests. J. Ecol. *100 (3)*, 712–722.

Collins, B., and Parke, J. (2008). Spatial and temporal aspects of tylosis formation in tanoak inoculated with *Phytophthora ramorum*. In Proceedings of the Sudden Oak Death Third Science Symposium (U.S. Department of Agriculture, Forest Service, Pacific Southwest Research Station), p. 335.

Comtrade (2014). United Nations Department of Economic and Social Affaris Statistic Division Trade Statistics. New York, NY.

Dale, V.H., Joyce, L.A., McNulty, S., Neilson, R.P., Ayres, M., Flannagin, M.D., Hanson, P.J., Irland, L.C., Lugo, A.E., Peterson, C.J., et al. (2001). Climate change and forest disturbances. BioScience *51 (9)*, 723–734.

Denman, S., Kirk, S., Whybrow, A., Orton, E., and Webber, J.F. (2006). *Phytophthora kernoviae* and *P. ramorum*: host susceptibility and sporulation potential on foliage of susceptible trees1. EPPO Bull. *36 (2)*, 373–376.

Dick, M.A., Williams, N.M., Bader, M.K.-F., Gardner, J.F., and Bulman, L.S. (2014). Pathogenicity of *Phytophthora pluvialis* to *Pinus radiata* and its relation with red needle cast disease in New Zealand. N. Z. J. For. Sci. *44 (1)*, 1–12.

Edmonds, R.L., Agee, J.K., and Gara, R.I. (2011). Forest Health and Protection (Waveland Press, Long Grove, IL).

Erwin, D.C., Bartnicki-García, S., Tsao, P.H., and Society, A.P. (1983). Phytophthora: its biology, taxonomy, ecology, and pathology. American Phytopathological Society (APS Press).

FAO (2009). Global review of forest pests and diseases (Food and Agriculture Organization of the United Nations) Rome, Italy.

FAO (2010a). Global Forest Resource Assessment (Food and Agriculture Organization of the United Nations) Rome, Italy.

FAO (2010b). Guide to implementation of phytosanitary standards in forestry (Food and Agriculture Organization of the United Nations) Rome, Italy.

FAO (2014). Food and Agriculture Organization of the United Nations Statistic Division (Rome, Italy).

Fichtner, E.J., Lynch, S.C., and Rizzo, D.M. (2007). Detection, Distribution, Sporulation, and Survival of *Phytophthora ramorum* in a California Redwood-Tanoak Forest Soil. Phytopathology *97 (10)*, 1366–1375.

Fichtner, E.J., Lynch, S.C., and Rizzo, D.M. (2009). Survival, dispersal, and potential soil-mediated suppression of *Phytophthora ramorum* in a California redwood-tanoak forest. Phytopathology *99 (5)*, 608–619.

Freitag, C.M., and Morrell, J.J. (2002). Effect of glycol on movement of borate from fused borate rods. For. Prod. J. *52 (6)*, 68–74.

Frenkel, O., Yermiyahu, U., Forbes, G.A., Fry, W.E., and Shtienberg, D. (2010). Restriction of potato and tomato late blight development by sub-phytotoxic concentrations of boron: Boron and late blight of potato and tomato. Plant Pathol. *59 (4)*, 626–633.

Garbelotto, M., and Gonthier, P. (2013). Biology, epidemiology, and control of *Heterobasidion* species worldwide. Annu. Rev. Phytopathol. *51 (1)*, 39–59.

Garbelotto, M., Svihra, P., and Rizzo, D.M. (2001). New pests and diseases: sudden oak death syndrome fells 3 oak species. Calif. Agric. *55 (1)*, 9–19.

Gezer, E., Michael, J., and Morrell, J. (1999). Effects of glycol on leachability and efficacy of boron wood preservatives. Wood Fiber Sci. *31 (2)*, 136–142.

Gibbs, J.N. (1978). Intercontinental epidemiology of Dutch elm disease. Annu. Rev. Phytopathol. *16 (1)*, 287–307.

Goheen, E.M., Hansen, E.M., Kanaskie, A., Sutton, W., and Reeser, P. (2007). Persistence of *Phytophthora ramorum* after eradication treatments in Oregon tanoak forests. In Proceedings of the Fourth Meeting of IUFRO Working Party S07.02.09, (Monterey, California), pp. 173–176.

Government of Canada, C.F.I.A. (2010). Appendix 1- Plants Regulated for *Phytophthora ramorum* (Sudden Oak Death), Ottawa, Ontario.

Halsall, D.M. (1977). Effects of certain cations on the formation and infectivity of *Phytophthora* zoospores. 2. Effects of copper, boron, cobalt, manganese, molybdenum, and zinc ions. Can. J. Microbiol. *23 (8)*, 1002–1010.

Hansen, E.M. (2008). Alien forest pathogens: *Phytophthora* species are changing world forests. Boreal Environ. Res. *13*, 33–41.

Hansen, E.M., and Goheen, E.M. (2000). *Phellinus Weirii* and other native root pathogens as determinants of forest structure and process in western North America. Annu. Rev. Phytopathol. *38 (1)*, 515–539.

Hansen, E.M., Goheen, D.J., Jules, E.S., and Ullian, B. (2000). Managing Port-Orford-cedar and the introduced pathogen *Phytophthora lateralis*. Plant Dis. *84 (1)*, 4–14.

Hansen, E.M., Parke, J.L., and Sutton, W. (2005). Susceptibility of Oregon Forest Trees and shrubs to *Phytophthora ramorum*: A comparison of artificial inoculation and natural infection. Plant Dis. *89 (4)*, 63–70.

Harnik, T.Y., Mejia-Chang, M., Lewis, J., and Garbelotto, M. (2004). Efficacy of heat-based teatments in eliminating the recovery of the sudden oak death pathogen (*Phytophthora ramorum*) from infected california bay laurel leaves. HortScience *39 (7)*, 1677–1680.

He, W., Simonsen, W.J., Chen, H., and Morrell, J.J. (1997). Evaluation of the efficacy of selected thermal boron treatments in eliminating pests in freshly peeled Douglas-fir logs. For. Prod. J. *47 (3)*, 66–70.

Hood, I.A., Williams, N.M., Dick, M.A., Arhipova, N., Kimberley, M.O., Scott, P.M., and Gardner, J.F. (2014). Decline in vitality of propagules of *Phytophthora pluvialis* and *Phytophthora kernoviae* and their inability to contaminate or colonize bark and sapwood in *Pinus radiata* export log simulation studies. N. Z. J. For. Sci. *44 (1)*, 1–13.

IPPC (2009). ISPM15: Regulation of wood packaging material in international trade. International Plant Protection Convention, (Food and Agriculture Organization of the United Nations) Rome, Italy.

Jorge, F.C., Nunes, L., and Botelho, C. (2004). Boron in wood preservation. problems, challenges and proposed solutions. J. Fac. Sci. Tech., University Fernando Pessoa (1): 3-15.

Jules, E.S., Kauffman, M.J., Ritts, W.D., and Carroll, A.L. (2002). Spread of an invasive pathogen over a variable landscape: a nonnative root rot on port orford cedar. Ecology *83 (11)*, 3167–3181.

Kartal, S.N., Shinoda, K., and Imamura, Y. (2005). Laboratory evaluation of boron-containing quaternary ammonia compound, didecyl dimethyl ammonium tetrafluoroborate (DBF) for inhibition of mold and stain fungi. Holz Als Roh- Werkst. *63 (1)*, 73–77.

Kliejunas, J.T. (2010). Sudden oak death and *Phytophthora ramorum*: a summary of the literature, 2010 edition. Gen. Tech. Rep. PSW-GTR-234. Albany, CA: U.S. Department of Agriculture, Forest Service, Pacific Southwest Research Station. 181 p

Lebow, S.T., and Morrell, J.J. (1989). Penetration of boron in Douglas-fir and western hemlock lumber. For. Prod. J. *39 (1)*, 67–70.

Liebhold, A.M., Brockerhoff, E.G., Garrett, L.J., Parke, J.L., and Britton, K.O. (2012). Live plant imports: the major pathway for forest insect and pathogen invasions of the US. Front. Ecol. Environ. *10 (3)*, 135–143.

Loo, J.A. (2008). Ecological impacts of non-indigenous invasive fungi as forest pathogens. Biol. Invasions *11 (1)*, 81–96.

Manter, D.K., Kolodny, E.H., Hansen, E.M., and Parke, J.L. (2010). Virulence, sporulation, and elicitin production in three clonal lineages of *Phytophthora ramorum*. Physiol. Mol. Plant Pathol. *74 (5)*, 317–322.

McKeever, K.M. (2010). Characterizing Douglas-fir tissue colonization the "sudden oak death" pathogen, *Phytophthora ramorum*. MS Thesis. Washington State University.

McQuire, A.J., and Goudie, K.A. (1972). Accelerated boron diffusion treatment of timber. N. Z. J. For. Sci 2 (2), 165-187.

Misra, R.S., Maheswari, S.K., Sriram, S., Mishra, A.K., and Sahu A. K. (2007). Effect of borax for the control of *Phytophthora* leaf blight of taro (*Colocasia esculenta* (L.) Schott). J. Root Crops *33 (1)*, 49–52.

Moralejo, E., García-Muñoz, J.A., and Descals, E. (2007). Log susceptibility of Iberian trees species to *Phytophthora ramorum.* In Proceedings of the Sudden Oak Death Third Science Symposium, pp. 5–9.

Morgan, T.A., Brandt, J.P., Songster, K.E., Keegan III, C.E., and Christensen, G.A. (2012). California's forest products industry and timber harvest, 2006 (Pacific Northwest Research Station: United States Department of Agriculture Forest Service).

Morrell, J.J. (1995). Importation of unprocessed logs into North America: A review of pest mitigation procedures and their efficacy. For. Prod. J. *45 (9)*, 41–50.

Morrell, J.J., and Freitag, C.M. (1995). Effect of wood moisture content on diffusion of boron-based biocides through Douglas-fir and western hemlock lumber. For. Prod. J. *45 (3)*, 51–55.

Morrell, J.J., and Lebow, S.T. (1992). Borate treatment of seasoned western hemlock and Douglas-fir lumber. For. Prod. J. *41 (1)*, 27–29.

Newhook, F.J., and Podger, F.D. (1972). The role of *Phytophthora cinnamomi* in Australian and New Zealand forests. Annu. Rev. Phytopathol. *10 (1)*, 299–326.

Oregon Forest Resource Institute (2012). The 2012 Forest Report: An Economic Assessment of Oregon's Forest and Wood Products Manufacturing Sector. Portland, OR.

Parke, J.L., Oh, E., Voelker, S., Hansen, E.M., Buckles, G., and Lachenbruch, B. (2007). *Phytophthora ramorum* colonizes tanoak xylem and is associated with reduced stem water transport. Phytopathology *97 (12)*, 1558–1567.

Pimentel, D., Lach, L., Zuniga, R., and Morrison, D. (2000). Environmental and economic costs of nonindigenous species in the United States. BioScience *50 (1)*, 53–65.

Pratt, J.E., and Quill, K. (1996). A trial of disodium octaborate tetrahydrate for the control of *Heterobasidion annosum*. Eur. J. For. Pathol. *26 (6)*, 297–305.

Qin, G., Zong, Y., Chen, Q., Hua, D., and Tian, S. (2010). Inhibitory effect of boron against *Botrytis cinerea* on table grapes and its possible mechanisms of action. Int. J. Food Microbiol. *138 (1)*, 145–150.

Ra, J., Barnes, H., and Conners, T. (2001). Determination of boron diffusion coefficients in wood. Wood Fiber Sci. *33 (1)*, 90–103.

Ramsfield, T.D., Ball, R.D., Gardner, J.F., and Dick, M.A. (2010). Temperature and time combinations required to cause mortality of a range of fungi colonizing wood. Can. J. Plant Pathol. *32 (3)*, 368–375.

Reeser, P., Sutton, W., and Hansen, E.M. (2013). *Phytophthora pluvialis*, a new species from mixed tanoak-Douglas-fir forests of western Oregon, U.S.A. North Am. Fungi 8, 1–8.

Rizzo, D.M., and Garbelotto, M. (2003). Sudden oak death: endangering California and Oregon forest ecosystems. Front. Ecol. Environ. *1 (4)*, 197–204.

Rizzo, D.M., Garbelotto, M., Davidson, J.M., Slaughter, G.W., and Koike, S.T. (2002). *Phytophthora ramorum* as the cause of extensive mortality of *Quercus* spp. and *Lithocarpus densiflorus* in California. Plant Dis. *86 (3)*, 205–214.

Rizzo, D.M., Garbelotto, M., and Hansen, E.M. (2005). *Phytophthora ramorum*: integrative research and management of an emerging pathogen in California and Oregon forests. Annu. Rev. Phytopathol. *43 (1)*, 309–335.

Robinson, W.H., and Barlow, R.A. (1993). Diffusion of disodium octaborate tetrahydrate into southern yellow pine to control wood-infesting beetles. In *Wildey KB & WH Robinson.* Proceedings of the First International Conference on Urban Pests (BPCC Wheatons Ltd), Exeter, England.

Rolshausen, P.E., and Gubler, W.D. (2005). Use of boron for the control of Eutypa dieback of grapevines. Plant Dis. *89 (7)*, 734–738.

Sansford, C.E., Inman, A.J., Baker, R., Brasier, C.M., Frankel, S.J., de Gruyter, J., Husson, C., Kehlenbeck, H., Kessel, G., Moralejo, E., et al. (2009). Report on the risk of

entry, establishment, spread and socio-economic loss and environmental impact and the appropriate level of management for *Phytophthora ramorum* for the EU. (EU Sixth Framework Project RAPRA) http://rapra.csl.gov.uk/.

Santini, A., Ghelardini, L., De Pace, C., Desprez-Loustau, M.L., Capretti, P., Chandelier, A., Cech, T., Chira, D., Diamandis, S., Gaitniekis, T., et al. (2013). Biogeographical patterns and determinants of invasion by forest pathogens in Europe. New Phytol. *197 (1)*, 238–250.

Schauwecker, C.F.M., J. J., and MORRELL, J.J. (2008). Ability of pressure treatment with wood preservatives to kill or limit emergence of invasive insects using *Arhopalus productus* as a model species. For. Prod. J. *58 (10)*, 56-60.

Schmidt, E.L. (1982). Methyl bromide eradication of the oak wilt fungus from lumber. Int. J. Wood Preserv. *2 (3)*, 123–126.

Shelly, J., Singh, R., Langford, C., and Mason, T. (2006). Understanding the disposal and utilization options for *Phytophthora ramorum* infested wood. In: Frankel, Susan J.; Shea, Patrick J.; and Haverty, Michael I., tech. coords. Proceedings of the sudden oak death second science symposium: the state of our knowledge. Gen. Tech. Rep. PSW-GTR-196. Albany, CA: Pacific Southwest Research Station, Forest Service, U.S. Department of Agriculture: 467-482.

Shishkoff, N. (2007). Persistence of *Phytophthora ramorum* in soil mix and roots of nursery ornamentals. Plant Dis. *91 (10)*, 1245–1249.

Shmulsky, R., and Jones, P.D. (2011). Durability and protection. In Forest Products and Wood Science An Introduction, (Wiley-Blackwell), pp. 229–252.

Simard, M., Brière, S.C., Watson, A.K., and Rioux, D. (2010). Stem Susceptibility of six eastern Canadian tree species to *Phytophthora ramorum*. Canadian Forest Service, Laurentian Forestry Centre. In Phytopathology, (Quebec City, Canada), p. S197.

Singh, R., Richmond Field Station, B., Mason, T., and Consultants, T.S.S. (2005). Removal and utilization of high risk sudden oak death host material. California Department of Forestry and Fire Protection. Agreement No. 8CA01257.

Smilanick, J.L., and Sorenson, D. (2001). Control of postharvest decay of citrus fruit with calcium polysulfide. Postharvest Biol. Technol. *21 (2)*, 157–168.

Stack, J.P., Suffert, F., and Gullino, M.L. (2010). Bioterrorism: A threat to plant biosecurity? In: The Role of Plant Pathology in Food Safety and Food Security, R.N. Strange, and M.L. Gullino, eds. (Springer Netherlands), pp. 115–132.

Temiz, A., Alfredsen, G., Eikenes, M., and Terzıev, N. (2008). Decay resistance of wood treated with boric acid and tall oil derivates. Bioresour. Technol. *99 (7)*, 2102–2106.

Tooley, P.W., Browning, M., and Berner, D. (2008). Recovery of *Phytophthora ramorum* following exposure to temperature extremes. Plant Dis. *92 (3)*, 431–437.

Tooley, P.W., Browning, M., and Leighty, R.M. (2014). The effect of temperature on germination of chlamydospores of *Phytophthora ramorum*. Mycologia *106 (3)*, 424–430.

Tsunoda, K. (2001). Preservative properties of vapor-boron-treated wood and wood-based composites. J. Wood Sci. *47 (2)*, 149–153.

Tubajika, K., Singh, R., and Shelly, J. (2007). Preliminary observations of heat Treatment to control *Phytophthora ramorum* in infected wood species: An extended abstract. In: Proceedings of the Sudden Oak Death Third Science Symposium, Santa Rosa, CA, pp. 5–9.

USDA APHIS (2013). List of Regulated Hosts and Plants Proven or Associated with *Phytophthora ramorum* (http://www.aphis.usda.gov/plant_health/plant_pest_info/pram/downloads/pdf_files/usdaprlist.pdf).

Uzunovic, A., Khadempour, L., and Leung, K. (2008). Heat disinfestation of decay fungi found in post-mountain pine beetle wood (Pacific Forestry Centre) Saanich, BC, Canada.

Vannini, A., Franceschini, S., and Vettraino, A.M. (2012). Manufactured wood trade to Europe: a potential uninspected carrier of alien fungi. Biol. Invasions *14*, 1991–1997.

Washington State Department of Natural Resources (2013). 2012 Washington Timber Harvest Report. Olympia, WA.

Werres, S., Marwitz, R., Man In't veld, W.A., De Cock, A.W.A.M., Bonants, P.J.M., De Weerdt, M., Themann, K., Ilieva, E., and Baayen, R.P. (2001). *Phytophthora ramorum* sp. nov., a new pathogen on Rhododendron and Viburnum. Mycol. Res. *105 (10)*, 1155–1165.

Weste, G., and Marks, G.C. (1987). The biology of *Phytophthora cinnamomi* in Australasian forests. Annu. Rev. Phytopathol. *25 (1)*, 207–229.

Widmer, T.L. (2014). Screening *Trichoderma* species for biological control activity against *Phytophthora ramorum* in soil. Biol. Control. 79. 43-48.

Williamson, M. (2001). Can the impacts of invasive plants be predicted? In: Plant Invasions: Species Ecology and Ecosystem Management (Backhuys, Netherlands), pp. 11–19.

Winton, L.M., and Hansen, E.M. (2001). Molecular diagnosis of *Phytophthora lateralis* in trees, water, and foliage baits using multiplex polymerase chain reaction. For. Pathol. *31 (5)*, 275–283.

Xiaoping, Z., and Warren, D.D. (2012). Production, Prices, Employment, and trade in Northwest Forest Industries, All Quarters 2011 Resour. Bull. PNW-RB-264. (Pacific Northwest Research Station: United States Department of Agriculture Forest Service).

Zobel, D.B., Roth, L.F., and Hawk, G.M. (1985). Ecology, pathology, and management of Port-Orford-Cedar (Chamaecyparis lawsoniana). US Dep. Agric. For. Serv. Pac. Northwest For. Range Exp. Stn. *Gen. Tech. Rep. PNW-184*, 161.

# APPENDIX

# LIST OF APPENDIX FIGURES

# LIST OF APPENDIX TABLES

**Session information for statistical analysis of radial growth data in Chapter 2, Study 1 using R.**

R version 3.0.2 (2013-09-25)
Platform: x86_64-apple-darwin10.8.0 (64-bit)

locale:
[1] en_US.UTF-8/en_US.UTF-8/en_US.UTF-8/C/en_US.UTF-8/en_US.UTF-8

attached base packages:
[1] stats graphics grDevices utils datasets methods base

other attached packages:
[1] ggplot2_0.9.3.1 plyr_1.8

loaded via a namespace (and not attached):
 [1] colorspace_1.2-4 dichromat_2.0-0 digest_0.6.4 grid_3.0.2
 [5] gtable_0.1.2 labeling_0.2 MASS_7.3-29 munsell_0.4.2
 [9] proto_0.3-10 RColorBrewer_1.0-5 reshape2_1.2.2 scales_0.2.3
[13] stringr_0.6.2 tools_3.0.2

**Session information for statistical analysis of boron diffusion data in Chapter 3 using R.**

```
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Platform: x86_64-apple-darwin10.8.0 (64-bit)

locale:
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attached base packages:
[1] stats     graphics  grDevices utils     datasets  methods   base

other attached packages:
[1] nlme_3.1-111    plyr_1.8        ggplot2_0.9.3.1

loaded via a namespace (and not attached):
 [1] colorspace_1.2-4   dichromat_2.0-0    digest_0.6.4       grid_3.0.2
 [5] gtable_0.1.2       labeling_0.2       lattice_0.20-23    MASS_7.3-29
 [9] munsell_0.4.2      proto_0.3-10       RColorBrewer_1.0-5 reshape2_1.2.2
[13] scales_0.2.3       stringr_0.6.2      tools_3.0.2
```
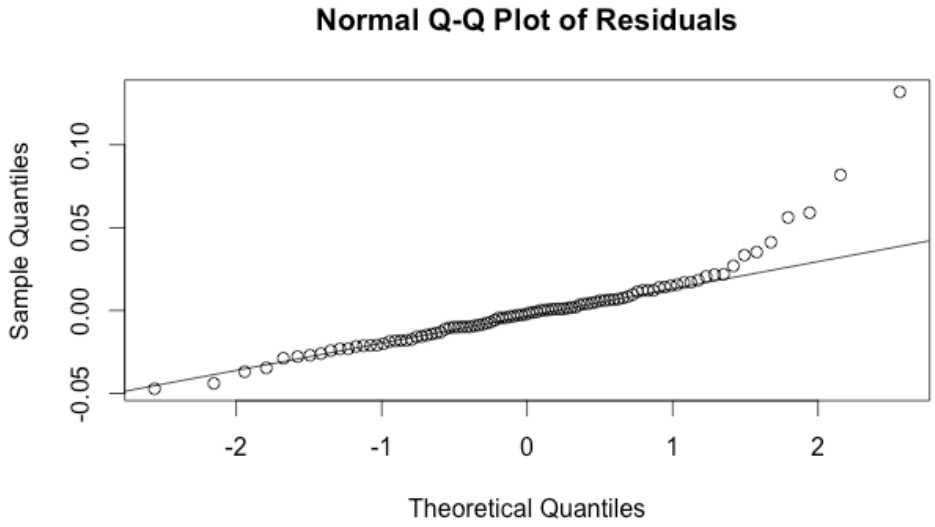
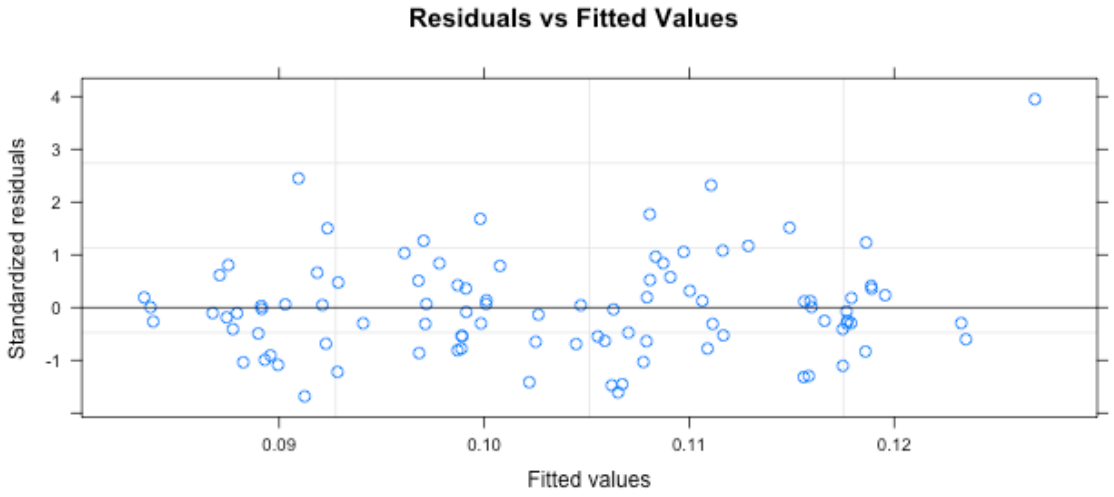
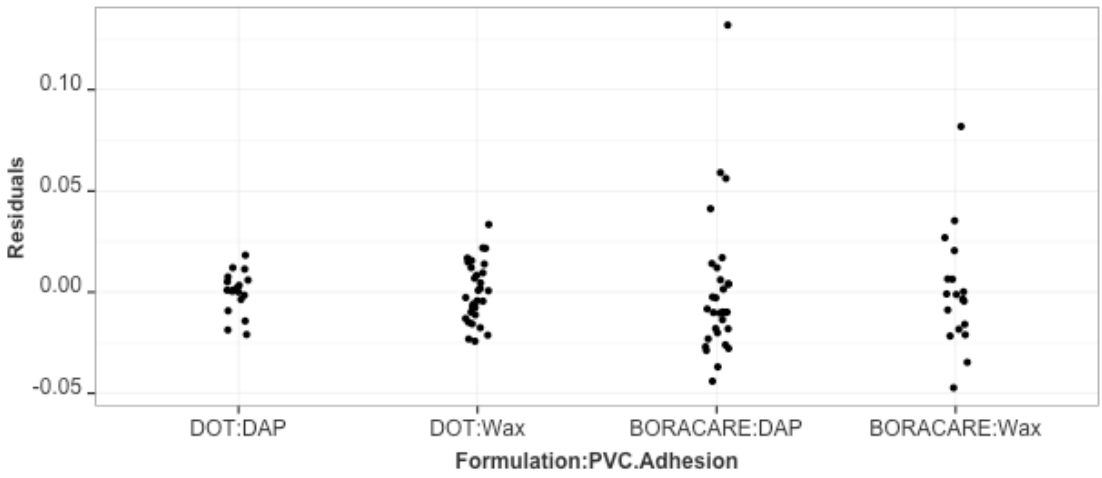

Appendix Figure 1: Normal quantile-quantile plot of residuals indicating the normality assumption of the model used in Chapter 4 was adequately met.



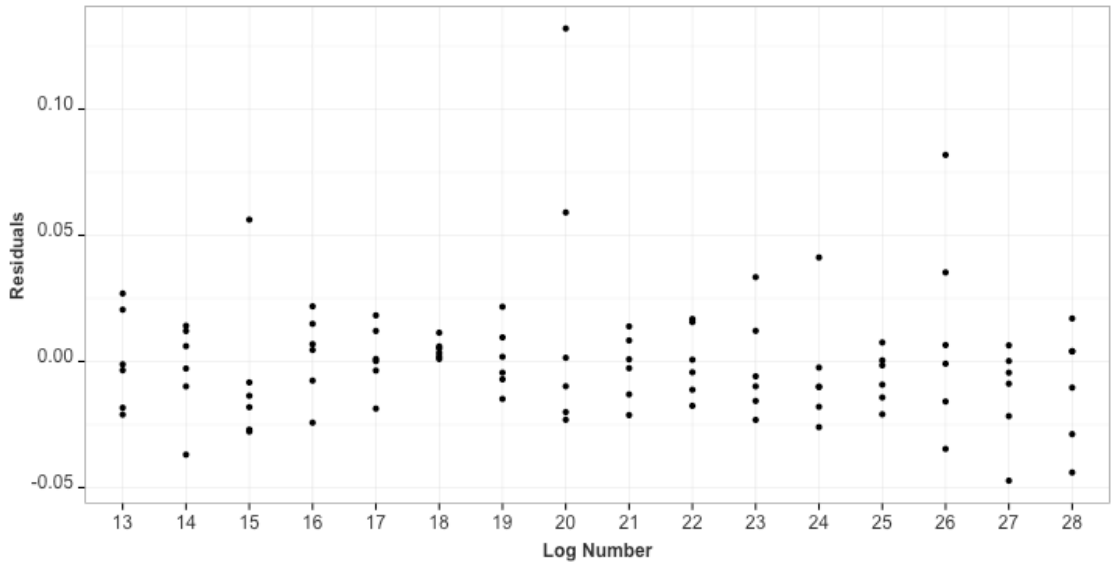
Appendix Figure 2: Residual vs Fitted values from indicating the constant spread assumption of the model used in Chapter 4 was adequately met.



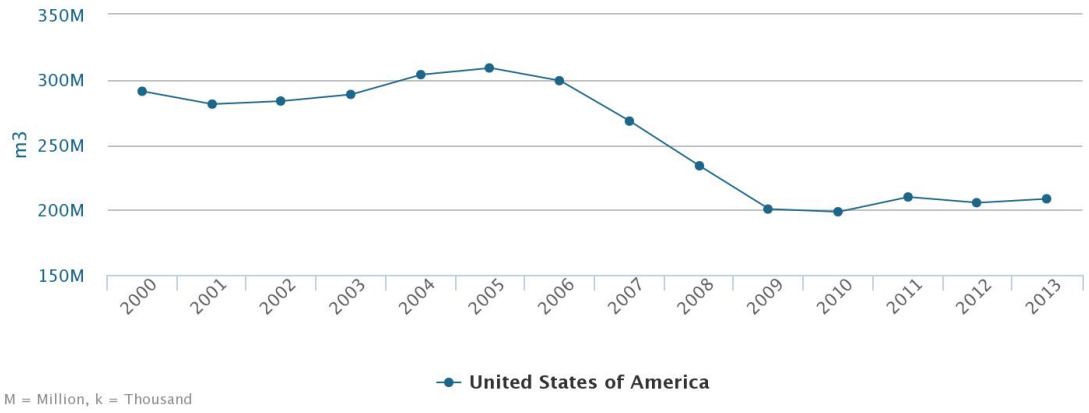
Appendix Figure 3: Residual plot for the model used in Chapter 4 showing residual variance for each treatment and each adhesive. Boracare®=DOT plus glycol.



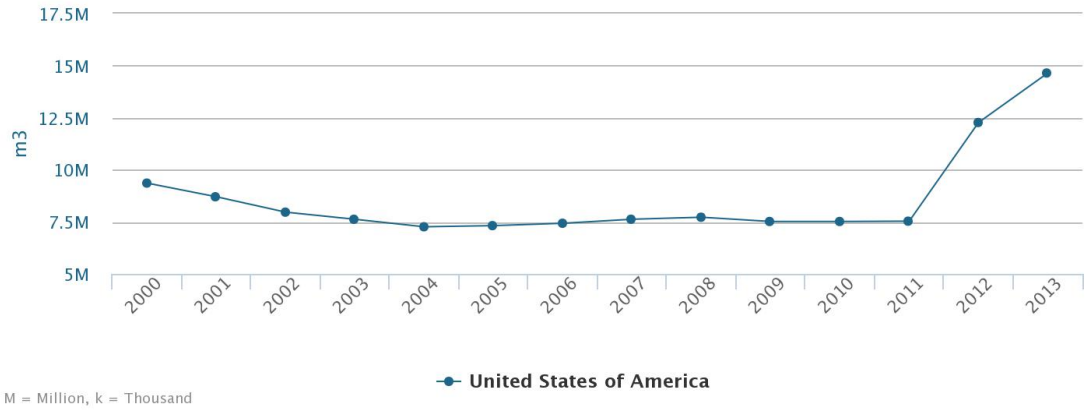
Appendix Figure 4: Residual variances for each log used in Trial 2 from the model used in Chapter 4.



Appendix Figure 5: Production of coniferous roundwood in the United States from 2000-2013 (FAO, 2014). Complimentary to Appendix Table 3.

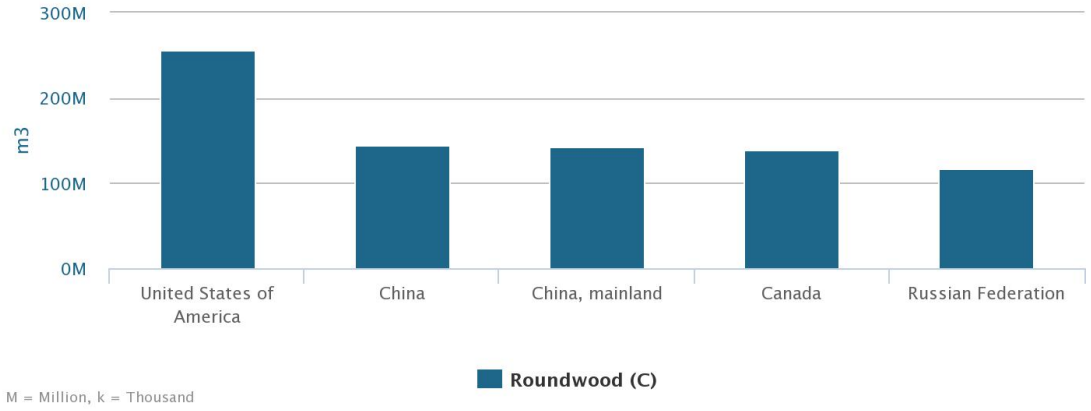


Appendix Figure 6: Coniferous roundwood exported from the United States from 2000-2013 (FAO, 2014). Complimentary to Appendix Table 4.



Appendix Figure 7: Top 5 coniferous roundwood exporting countries between 2000-2013(FAO, 2014). Values represent the average over the 13 years. Complimentary to Appendix Table 1.

Appendix Figure 8: Top 5 countries for coniferous roundwood produced between 2000-2013 (FAO, 2014). Values represent the average over the 13 years. Complimentary to Appendix Table 2.



Appendix Table 1: Top 5 coniferous roundwood exporting countries between 2000-2013(FAO, 2014). Values represent the average over the 13 years. Complimentary to Appendix Figure 7.

| Year | Item | Value | Unit |
|---|---|---|---|
| Russian Federation | Roundwood (C) | 2.45E+07 | m3 |
| United States of America | Roundwood (C) | 8605112.714 | m3 |
| New Zealand | Roundwood (C) | 8546353.143 | m3 |
| Canada | Roundwood (C) | 4144826.429 | m3 |
| Germany | Roundwood (C) | 3848936.357 | m3 |

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Appendix Table 2: Top 5 countries for coniferous roundwood produced between 2000-2013 (FAO, 2014). Values represent the average over the 13 years. Complimentary to Appendix Figure 8.

| Year | Item | Value | Unit |
|---|---|---|---|
| United States of America | Roundwood (C) | 2.56E+08 | m3 |
| China | Roundwood (C) | 1.44E+08 | m3 |
| China, mainland | Roundwood (C) | 1.43E+08 | m3 |
| Canada | Roundwood (C) | 1.38E+08 | m3 |
| Russian Federation | Roundwood (C) | 1.16E+08 | m3 |

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Appendix Table 3: Production of coniferous roundwood in the United States from 2000-2013 (FAO, 2014). Complimentary to Appendix Figure 5.

| Year | Area | Value | Unit |
|---|---|---|---|
| 2000 | United States of America | 2.91E+08 | m3 |
| 2001 | United States of America | 2.81E+08 | m3 |
| 2002 | United States of America | 2.84E+08 | m3 |
| 2003 | United States of America | 2.89E+08 | m3 |
| 2004 | United States of America | 3.04E+08 | m3 |
| 2005 | United States of America | 3.09E+08 | m3 |
| 2006 | United States of America | 2.99E+08 | m3 |
| 2007 | United States of America | 2.68E+08 | m3 |
| 2008 | United States of America | 2.34E+08 | m3 |
| 2009 | United States of America | 2.01E+08 | m3 |
| 2010 | United States of America | 1.98E+08 | m3 |
| 2011 | United States of America | 2.10E+08 | m3 |
| 2012 | United States of America | 2.05E+08 | m3 |
| 2013 | United States of America | 2.08E+08 | m3 |

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Appendix Table 4: Coniferous roundwood exported from the United States from 2000-2013 (FAO, 2014). Complimentary to Appendix Figure 6.

| Year | Area | Value | Unit |
|---|---|---|---|
| 2000 | United States of America | 9358000 | m3 |
| 2001 | United States of America | 8702102 | m3 |
| 2002 | United States of America | 7963426 | m3 |
| 2003 | United States of America | 7620944 | m3 |
| 2004 | United States of America | 7261033 | m3 |
| 2005 | United States of America | 7316464 | m3 |
| 2006 | United States of America | 7429474 | m3 |
| 2007 | United States of America | 7623730 | m3 |
| 2008 | United States of America | 7720166 | m3 |
| 2009 | United States of America | 7515341 | m3 |
| 2010 | United States of America | 7515662 | m3 |
| 2011 | United States of America | 7530236 | m3 |
| 2012 | United States of America | 1.23E+07 | m3 |
| 2013 | United States of America | 1.46E+07 | m3 |

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Appendix Table 5: 2010 Western Oregon Species Harvested in Thousand Board Feet. Source: http://www.oregon.gov/ODF/Pages/state_forests/frp/Charts.aspx

| Type | Species | BLM | USFS | Native-American | Private and Other Public | State | Total Volume | Percent of Total |
|---|---|---|---|---|---|---|---|---|
| **Hardwood** | Alder | 489 | 62 | 88.824 | n/a* | 12179 | 12818.824 | 1.90 |
| | Madrone | 0 | 0 | 0 | n/a* | 0 | 0 | 0.00 |
| | Maple | 530 | 0 | 0.74 | n/a* | 354 | 884.74 | 0.13 |
| | Other Hardwood | 0 | 0 | 0 | n/a* | 472 | 472 | 0.07 |
| | Total | 1019 | 62 | 89.564 | n/a* | 13005 | 14175.564 | 2.10 |
| **Softwood** | Cedar | 718.47 | 362 | 0 | n/a* | 319 | 1399.47 | 0.21 |
| | Douglas Fir | 104049.4 | 150345 | 13234.61 | n/a* | 229561 | 497190.01 | 73.62 |
| | Hemlock | 11666.43 | 15084 | 83.76 | n/a* | 28109 | 54943.19 | 8.14 |
| | Other Softwood | 0 | 2333 | 0 | n/a* | 7222 | 9555 | 1.41 |
| | Pine | 847.26 | 54388 | 97 | n/a* | 15 | 55347.26 | 8.20 |
| | Spruce | 0 | 0 | 3.48 | n/a* | 1433 | 1436.48 | 0.21 |
| | True Fir | 7422.84 | 31672 | 210 | n/a* | 1952 | 41256.84 | 6.11 |
| | Total | 124704.4 | 254184 | 13628.85 | n/a* | 268611 | 661128.25 | 97.90 |
| | All Total | 125723.4 | 254246 | 13718.414 | n/a* | 281616 | 675303.814 | |

Appendix Table 6: Trade flows between New Zealand and the United States (Comtrade, 2014).

| Year | Trade Flow | Reporter | Partner | Commodity Code | Qty Unit | Qty | Netweight (kg) | Trade Value (US$) |
|---|---|---|---|---|---|---|---|---|
| 2013 | Import | New Zealand | USA | 440320 | Volume in cubic meters | 9 | 17376 | 14786 |
| 2009 | Import | New Zealand | USA | 440320 | Volume in cubic meters | 8 | 3402 | 9859 |
| 2010 | Import | New Zealand | USA | 440320 | Volume in cubic meters | 5 | | 249 |
| 2010 | Export | New Zealand | USA | 440320 | Volume in cubic meters | 55 | 453630 | 42660 |
| 2011 | Import | New Zealand | USA | 440320 | Volume in cubic meters | 12 | 22919 | 17662 |
| 2002 | Export | New Zealand | USA | 440320 | No Quantity | | 24300033 | 1904062 |
| 2003 | Export | New Zealand | USA | 440320 | No Quantity | | 7761511 | 540480 |
| 2004 | Import | New Zealand | USA | 440320 | Volume in cubic meters | 24 | 8165 | 5366 |
| 2005 | Export | New Zealand | USA | 440320 | Volume in cubic meters | 85 | 52210 | 52292 |
| 2006 | Import | New Zealand | USA | 440320 | Volume in cubic meters | 26 | 20904 | 11841 |
| 1996 | Import | New Zealand | USA | 440320 | Volume in litres | 50015 | 35000 | 20525 |
| 1996 | Export | New Zealand | USA | 440320 | Volume in litres | 54076218 | 37842000 | 7164384 |
| 1997 | Export | New Zealand | USA | 440320 | Volume in litres | 37569268 | 26290600 | 4332064 |
| 1998 | Export | New Zealand | USA | 440320 | Volume in litres | 44857220 | 31390636 | 3405478 |
| 1999 | Export | New Zealand | USA | 440320 | Volume in litres | 53720257 | 37592902 | 2448020 |
| 2000 | Export | New Zealand | USA | 440320 | Volume in cubic meters | 35953 | 35909647 | 2789460 |
| 2001 | Export | New Zealand | USA | 440320 | No Quantity | | 10489950 | 660246 |
| 1989 | Import | New Zealand | USA | 440320 | Volume in litres | 190057 | 133000 | 163771 |
| 1989 | Export | New Zealand | USA | 440320 | Volume in litres | 68022 | 47601 | 32819 |
| 1990 | Import | New Zealand | USA | 440320 | Volume in litres | 68022 | 47601 | 44276 |
| 1990 | Export | New Zealand | USA | 440320 | Volume in litres | 137038 | 95898 | 92442 |
| 1992 | Import | New Zealand | USA | 440320 | Volume in litres | 29009 | 20300 | 30823 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 1993 | Import | New Zealand | USA | 440320 | Volume in litres | 198061 | 138601 | 50379 |
| 1993 | Export | New Zealand | USA | 440320 | Volume in litres | 25966788 | 18171300 | 3912808 |
| 1994 | Import | New Zealand | USA | 440320 | Volume in litres | 999 | 699 | 4640 |
| 1994 | Export | New Zealand | USA | 440320 | Volume in litres | 112318686 | 78599500 | 13918748 |
| 1995 | Export | New Zealand | USA | 440320 | Volume in litres | 62348699 | 43631000 | 6617417 |

Appendix Table 7: Trade flows between the US and New Zealand, reported by the US (Comtrade, 2014).

| Year | Trade Flow | Reporter | Partner | Commodity Code | Qty Unit | Qty | Netweight (kg) | Trade Value (US$) |
|---|---|---|---|---|---|---|---|---|
| 2013 | Import | USA | New Zealand | 440320 | Volume in cubic meters | 11 | 65445 | 9641 |
| 2013 | Export | USA | New Zealand | 440320 | Volume in cubic meters | 221 | 644267 | 63581 |
| 2007 | Import | USA | New Zealand | 440320 | Volume in cubic meters | 322 | | 136687 |
| 2007 | Export | USA | New Zealand | 440320 | Volume in cubic meters | 791 | 1763513 | 174805 |
| 2008 | Import | USA | New Zealand | 440320 | Volume in cubic meters | 89 | 183611 | 25575 |
| 2010 | Import | USA | New Zealand | 440320 | Volume in cubic meters | 65 | | 15851 |
| 2010 | Export | USA | New Zealand | 440320 | Volume in cubic meters | 189 | 349588 | 32876 |
| 2011 | Export | USA | New Zealand | 440320 | Volume in cubic meters | 18097 | 23514446 | 2372120 |
| 2011 | Re-Export | USA | New Zealand | 440320 | Volume in cubic meters | 75 | 129749 | 13089 |
| 2002 | Import | USA | New Zealand | 440320 | Volume in cubic meters | 12967 | | 1756431 |
| 2002 | Export | USA | New Zealand | 440320 | Volume in cubic meters | 382 | | 47217 |
| 2003 | Import | USA | New Zealand | 440320 | Volume in cubic meters | 12955 | | 1822848 |
| 2004 | Import | USA | New Zealand | 440320 | Volume in cubic meters | 500 | | 167328 |
| 2005 | Import | USA | New Zealand | 440320 | Volume in cubic meters | 296 | | 91647 |
| 2005 | Export | USA | New Zealand | 440320 | Volume in cubic meters | 39 | | 35000 |
| 2006 | Import | USA | New Zealand | 440320 | Volume in cubic meters | 300 | | 83328 |
| 1996 | Import | USA | New Zealand | 440320 | Volume in litres | 54455332 | 38107300 | 8015250 |
| 1997 | Import | USA | New Zealand | 440320 | No Quantity | 0 | 0 | 8892634 |
| 1998 | Import | USA | New Zealand | 440320 | Volume in litres | 36857052 | 25792199 | 6020408 |
| 1998 | Export | USA | New Zealand | 440320 | Volume in litres | 33011 | 23101 | 14760 |
| 1999 | Import | USA | New Zealand | 440320 | Volume in litres | 36071817 | 25242699 | 4542986 |
| 1999 | Export | USA | New Zealand | 440320 | Volume in litres | 336785 | 235679 | 34582 |
| 2000 | Import | USA | New Zealand | 440320 | Volume in cubic meters | 25832 | | 3377100 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 2000 | Export | USA | New Zealand | 440320 | Volume in cubic meters | 51 | | 5536 |
| 2001 | Import | USA | New Zealand | 440320 | Volume in cubic meters | 10459 | | 1123575 |
| 2001 | Export | USA | New Zealand | 440320 | Volume in cubic meters | 127 | | 2583 |
| 1991 | Import | USA | New Zealand | 440320 | No Quantity | 0 | 0 | 210199 |
| 1991 | Export | USA | New Zealand | 440320 | No Quantity | 0 | 0 | 19350 |
| 1992 | Import | USA | New Zealand | 440320 | No Quantity | 0 | 0 | 1245093 |
| 1992 | Export | USA | New Zealand | 440320 | No Quantity | 0 | 0 | 53337 |
| 1993 | Import | USA | New Zealand | 440320 | No Quantity | 0 | 0 | 6152531 |
| 1993 | Export | USA | New Zealand | 440320 | No Quantity | 0 | 0 | 28850 |
| 1994 | Import | USA | New Zealand | 440320 | No Quantity | 0 | 0 | 15545947 |
| 1995 | Import | USA | New Zealand | 440320 | Volume in litres | 62136636 | 43482600 | 9110923 |