DEVELOPMENT OF MOLECULAR ASSAYS TO DETECT THE PRESENCE AND VIABILITY OF PHYTOPHTHORA RAMORUM AND GROSMANNIA CLAVIGERA

by

Barbara Wong

B.S.F., The University of British Columbia, 2014

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

The Faculty of Graduate and Postdoctoral Studies

(Forestry)

THE UNIVERSITY OF BRITISH COLUMBIA (Vancouver)

December 2016

© Barbara Wong, 2016

ABSTRACT

In order to determine if living fungi of phytosanitary concern are present in wood or to evaluate the efficacy of treatments, the method of choice is to grow microbes in petri dishes for subsequent identification. However, some fungi are difficult or impossible to grow in cultures, and thus, to validate the effectiveness of existing and emerging wood treatments, a molecular methodology that can detect living fungi and fungus-like organisms is required. RNA-based molecular diagnostic assays were developed to detect the presence of living fungi and fungi-like organisms of phytosanitary concern. Since RNA represents the transcription of genes and can therefore only be produced by living organisms, it provides a marker to determine if an organism is alive. The assays were designed to target genes that are essential to vital processes, then used to assess their presence and abundance through real-time reverse transcription polymerase chain reaction (PCR). A stability analysis was conducted by comparing the RNA to DNA ratio over treatment time. The results illustrated that for treated samples, DNA remained stable over a period of 10 days post treatment, whereas RNA could not be detected after 24 hours for *Phytophthora ramorum* or 96 hours for *Grosmannia clavigera*. Therefore, this method provides a reliable way to evaluate viability of organisms following treatments and can have profound impacts on assessing both timber and non-timber forest products of commercial value.

PREFACE

This thesis is original, unpublished work by the author, B. Wong.

The wood inoculation experiment was a collaborative effort of I. Aron, J. Burke, A. Dale, and S. Pokorny. Log preparation, wood inoculation, sample collection and DNA/RNA extraction were primarily done by myself at FPInnovation, Vancouver, BC as well as the Forest Pathology lab at the University of British Columbia, Point Grey campus.

The pathogen viability experiment was all independent work, from assay development and data collection to data analysis. Data collection of *Phytophthora ramorum* heat treatments was conducted at the Pacific Forestry Centre, Victoria, BC. Data collection of *Grosmannia clavigera* heat treatments was conducted in the Forest Pathology lab at the University of British Columbia, Point Grey campus. The entire data analysis was done by myself, with assistance from N. Feau.

I was responsible for the manuscript composition. R. Hamelin, the supervisory author of the project, as well as N. Feau and I. Leal were involved throughout in concept formation and manuscript edits.

TABLE OF CONTENTS

ABSTRACT	ii
PREFACE	iii
TABLE OF CONTENTS	iv
LIST OF TABLES	vi
LIST OF FIGURES	viii
LIST OF ABBREVIATIONS	xi
ACKNOWLEDGMENTS	xii
DEDICATION	xiii
INTRODUCTION	1
LITERATURE REVIEW	3
Detection of microorganisms using molecular methods	3
Detection and quantification of RNA by real-time reverse-transcription PCR	4
Phytophthora ramorum, the causal agent of sudden oak death and sudden larch death	5
Background	5
Symptoms	6
Life cycle	6
Prevention	7
Grosmannia clavigera, a fungal symbiont of the mountain pine beetle	8
Background	8
Life cycle	8
Current protocols to detect Phytophthora ramorum and Grosmannia clavigera	9
Objectives	10
MATERIALS & METHODS	11
Design of Assays	11
Polymerase Chain Reaction	13

Wood Inoculation	14
Heat treatment	15
SPF Kiln-Drying schedule	
Short heat treatment	
Re-isolation of pathogen	16
RNA and DNA extractions	17
Wood samples	17
Pure cultures	17
Quantitative Polymerase Chain Reaction	18
Statistical Analysis	18
RESULTS	
Specificity of the PCR assays	20
Analysis of gene expression in infected tissues	23
Detection of Phytophthora ramorum and Grosmannia clavigera in inoculated wood	23
Use of molecular assays to assess viability of pathogens following heat treatment	25
Re-isolation of Pure Cultures	
DISCUSSION	
Detection of wood-invading microorganisms using RNA and DNA	
Assessing viability of pathogens following treatment	
Detection of pathogen in Wood	
CONCLUSION	
REFERENCES	
APPENDICES	51
Appendix A: Geneious assay development criteria	51
Appendix B: List of Isolates	52
Appendix C: Recipes	53
Appendix D: SAS script	55
Appendix E: Statistical analysis	58
Appendix F: Sensitivity standard curve	65
	v

LIST OF TABLES

Table 1: Genes and assay sequences used for detection of Phytophthora ramorum and Grosmannia
<i>clavigera</i> . Probe modification includes 5' 6-FAM [™] and 3' Iowa Black [®] FQ quencher and fluorophore12
Table 2: Phytophthora ramorum ANOVA output of the heat treatment experiment analyzed as a 2-factor
split plot using the RNA/DNA ratio. Treatment represents the non-treated pure culture samples, the
"kiln" treated samples and the "short" (1 hr @ 70oC) samples. Samples were collected at various time
points (0, 6, 12, 24, 48, 96, 168 and 240 hrs)26
Table 3: Grosmannia clavigera ANOVA output of the heat treatment experiment analyzed as a 2-factor
split plot using the RNA/DNA ratio. Treatment represents the non-treated pure culture samples, the
"kiln" treated samples and the "short" (1 hr @ 70oC) samples. Samples were collected at various time
points (0, 6, 12, 24, 48, 96, 168 and 240 hrs)26
Table 4 : Isolates of Phytophthora ramorum from each of the 4 lineages used in inoculation and heat
treatment experiment
Table 5: Mastermix recipe for polymerase chain reaction. 53
Table 5: Mastermix recipe for polymerase chain reaction.53Table 6: QuantiTect Reverse Transcription kit (QIAGEN; gDNA wipeout during cDNA synthesis).53
Table 5: Mastermix recipe for polymerase chain reaction.53Table 6: QuantiTect Reverse Transcription kit (QIAGEN; gDNA wipeout during cDNA synthesis).53Table 7: QuantiTect Reverse Transcription kit (QIAGEN; cDNA synthesis).54
Table 5: Mastermix recipe for polymerase chain reaction
Table 5: Mastermix recipe for polymerase chain reaction
Table 5: Mastermix recipe for polymerase chain reaction
Table 5: Mastermix recipe for polymerase chain reaction
Table 5: Mastermix recipe for polymerase chain reaction
Table 5: Mastermix recipe for polymerase chain reaction53Table 6: QuantiTect Reverse Transcription kit (QIAGEN; gDNA wipeout during cDNA synthesis)53Table 7: QuantiTect Reverse Transcription kit (QIAGEN; cDNA synthesis)54Table 8: RT-qPCR recipe using treatment samples54Table 9: Phytophthora ramorum test of normality using RNA/DNA CT ratio data set of all treatments(control, kiln, short). All p-values are greater than the alpha of 0.05- indicating residuals are normallydistributed59Table 10: Phytophthora ramorum ANOVA using RNA/DNA CT ratio data set of all treatments (control, kiln, short)- testing the treatment model's significance60
Table 5: Mastermix recipe for polymerase chain reaction. .53 Table 6: QuantiTect Reverse Transcription kit (QIAGEN; gDNA wipeout during cDNA synthesis). .53 Table 7: QuantiTect Reverse Transcription kit (QIAGEN; cDNA synthesis). .54 Table 8: RT-qPCR recipe using treatment samples. .54 Table 9: Phytophthora ramorum test of normality using RNA/DNA CT ratio data set of all treatments .59 (control, kiln, short). All p-values are greater than the alpha of 0.05- indicating residuals are normally .59 Table 10: Phytophthora ramorum ANOVA using RNA/DNA CT ratio data set of all treatments (control, kiln, short)- testing the treatment model's significance. .60 Table 11: Grosmannia clavigera tests of normality using RNA/DNA CT ratio data set of all treatments .60

Table 12: Grosmannia clavigera ANOVA using RNA/DNA CT ratio data set of all treatments (control, kiln,	
short)- testing the treatment model's significance63	3

LIST OF FIGURES

Figure 3: Electrophoresis gel comparing species specificity of *Phytophthora ramorum* against closely related species of clade 8 using primers targeting gene, PH178. No amplification product was observed for closely related species of *P. brassicae* (lane 1 & 2), *P. cryptogea* (lane 3 & 4), *P. drechsleri* (lane 5 & 6), *P. foliorum* (lane 7 & 8), *P. hibernalis* (lane 9 & 10), *P. porri* (lane 11 & 12), *P. primulae* (lane 13 & 14) and *P. syringae* (lane 14 & 16). *P. ramorum* isolate 5073 (lane 17) DNA was used as a positive control. 21

Figure 6: Amplification plot of *Phytophthora ramorum*, isolate PFC-5073 gDNA and *Grosmannia clavigera*, isolate KW1407 gDNA using target primer pairs and TaqMan probes. a) Amplification (yellow line) of *P. ramorum* gDNA using gDNA specific TaqMan probe, PH178_P; b) Amplification (green line) of *G. clavigera* gDNA using gDNA specific TaqMan probe, MS359_P; c) No amplification (light green line) of

Figure 8: *Grosmannia clavigera* lesions on artificial wood inoculation samples using isolate KW1407 a 28day lesion found on Lodgepole pine log #1 b 28-day lesion found on Lodgepole pine log #2......24

Figure 9: Amplification plot of infected lodgepole pine with *Grosmannia clavigera*, isolate KW1407, using target primer pairs and TaqMan probes. a) Amplification (pink line) of *G. clabigera* gDNA using gDNA specific TaqMan probe, MS359_P b) Amplification (pink line) of *G. clavigera* cDNA using cDNA specific TaqMan probe, MS359_EX c No amplification (green line) of *G. clavigera* gDNA using cDNA specific TaqMan probe, MS359_EX.

Figure 10: Real-time PCR amplification plots of 24 samples of the pure culture PFC-5073 of *Phytophthora ramorum* (NA2) after heat-treatment at 70oC for 1 hr. Plots show amplification of gene PH178 using primers targeting genomic DNA (gDNA) and complementary DNA (cDNA) and sampled at various time points following treatment.

Figure 13: Boxplot of *Grosmannia clavigera* cDNA CT values over time, targeting gene MS359 a) "Kiln" heating schedule treatment with control b) Short heat treatment (1 hr at 70oC) with control. "Kiln"

treated samples show no amplification at any time point, while "short" heat-treated samples show signaling up until 96 hrs
Figure 14 : Growth-test of <i>Phytophthora ramorum</i> isolate 5073 after kiln and heat treatments. a) No growth visible on re-isolation 28 days after kiln schedule treatment; b) No growth visible on re-isolation 28 days after short (1hr at 70°C) heat treatment
Figure 15 : Growth-test of <i>Grosmania clavigera</i> isolate KW1407after kiln and heat treatments. a) No visible growth following attempted re-isolation 28 days after kiln schedule treatment; b) Re-isolation and growth 28 days after short (30 min at 56°C) heat treatment; c) No visible growth 28 days after short (1hr at 70°C) heat treatment
Figure 16: Phytophthora ramorum residual distribution generated from SAS 9.4 using RNA/DNA CT ratiosagainst all treatments (control, kiln, short). The plot shows a fairly equal distribution
Figure 18 : Normality plot of <i>Grosmannia clavigera</i> using RNA/DNA CT ratio data set of all treatments (control, kiln, short) with histogram and boxplot
Figure 19 : <i>Grosmannia clavigera</i> plot of residual distribution using RNA/DNA CT ratio data set of all treatments (control, kiln, short). Plot shows points scattered fairly equal along the x-axis- indicating a relatively equal distribution of residuals
Figure 20 : Boxplot of <i>Grosmannia clavigera</i> cDNA/gDNA ratio by treatment (Control, Kiln, short 1hr at 70°C)
using Taqman probe, PH178_EX R ² = 0.998 Eff%= 92.332
using Taqman probe, MS359_EX. R ² = 0.981 Eff%= 100.303

LIST OF ABBREVIATIONS

cDNA	Complementary Deoxyribonucleic Acid		
CEGMA	Core Eukaryotic Genes Mapping Approach		
CFIA	Canadian Food Inspection Agency		
DBH	Diameter at Breast Height		
DNA	Deoxyribonucleic Acid		
FUNYBASE	Fungal Phylogenomic Database		
gDNA	Genomic Deoxyribonucleic Acid		
ISPM15	International Standard for Phytosanitary Measures		
MEA	Malt Extract Agar		
MPB	Mountain Pine Beetle		
mRNA	Messenger Ribonucleic Acid		
PCR	Polymerase Chain Reaction		
qPCR	Quantitative Polymerase Chain Reaction		
RNA	Ribonucleic Acid		
rRNA	Ribosomal Ribonucleic Acid		
RT-PCR	Reverse Transcription Polymerase Chain Reaction		
SPF	Spruce Pine Fir		
TBE	Tris Borate EDTA		
USDA	United States Department of Agriculture		

ACKNOWLEDGMENTS

I would first like to thank my thesis advisor, Dr. Richard Hamelin, for his continued guidance, mentorship, expertise, and support as well as inspiring me to continue my work in this field. Thank you to all the members of my M.Sc. committee: Dr. Yousry El-Kassaby, for his encouragements and being a great mentor; Dr. Nicolas Feau, for always having his door open whenever I had a question about my research or writing; and Dr. Isabel Leal for her supervision at the Pacific Forestry Centre (PFC) as well as feedback and advice.

Additional thanks to Dr. Adnan Uzunovic and Angie Dale, for their wisdom and access to the regulated lab at FPInnovations; Brett Ford, for his help at PFC; Dr. Simon Shamoun, for access to his regulated lab; Dr. Val LeMay, for her statistical expertise; Ionut Aron at MKRF for cutting down the trees for my wood inoculations; Dr. Jordan Burke and Stan Pokorny, for bringing me back a lodgepole pine from their field season; as well as every member of the Hamelin lab, Padmini Herath, Dr. Arnaud Capron and Hesther Yueh for their support. Thank you to my office mate, Marc-Antoine Leclerc, for constantly putting up with my distractions and listening to me think out loud.

Finally, special thanks to my family, who supported me throughout my years of education at UBC and for offering their unconditional love. Thank you for always lending your ears.

To my parents for their unwavering support in everything I do.

INTRODUCTION

The introduction of invasive forest pathogens into new ecosystems can have significant ecological and economic impacts (Loo, 2009; Mitchell and Power 2003). Trade is an important factor in the spread of invasive species. Canada has 348 million hectares of forestland, representing 9% of the world's forests (NRCan, 2016a; Canadian Food Inspection Agency [CFIA], 2014). Canada is an important trading nation and exports by the forestry sector contribute \$17.1 billion in net trade (NRCan, 2015). Additionally, Canada imports a large volume of manufactured products. But one negative impact of trade is that it can contribute to the risk of pest movement globally. The shipping industry uses wood to make crates and as a packing material. Several insects and some pathogens are known to spread via the wood used for shipping. To counter this threat, regulations such as the International Standard of Phytosanitary Measures no. 15 (ISPM15) have been developed to treat wood packing material and ensure that they are free of organisms that could present threats to Canadian forests (Food and Agriculture Organization [FAO], 2016). Although those treatments have been tested and proved efficacious for insects, their efficacy for pathogens has not been fully demonstrated. It is therefore crucial to assess the efficacy of such treatments to ensure that imported and exported wood products and wood packing material are pathogen-free.

Pathogens are difficult to detect in woody tissues and this complicates the assessment of treatment efficacy. Detection usually requires isolating and culturing the organisms on artificial media in Petri plates, followed by morphological identification. New methods that rely on detection of pathogen DNA directly from samples are promising and can uncover a minute amount of pathogens in a variety of material. Although this approach is very sensitive to detect the presence of a pathogen's DNA, it does not allow the differentiation of living and dead organisms because DNA can remain detectable even after a lethal treatment of microorganisms. This is an important criterion in assessing the efficacy of treatment and therefore a new method is required.

In my project, I used genomics to generate a novel approach to determine the presence of microbes and fungi in wood products and to assess their viability. I developed a method based on the knowledge that messenger RNA (the gene transcript) is more labile than the gene itself (within the

genomic DNA) and will degrade rapidly following cell death. By measuring the ratio of RNA:DNA, I can then assess the presence and viability of the targeted microorganisms. To develop the method, I targeted the amplification of genes that are common to all microorganisms and are expressed under any conditions and designed probes that can differentiate between the genes and their RNA transcripts. I demonstrate the development and efficacy of this method on two organisms that encompass the sub-Kingdoms Dikaria (the fungal associate of the mountain pine beetle *Grosmannia clavigera*) and Stramenopiles (the oomycete plant pathogen *Phytophthora ramorum*). Both of these organisms have the capacity to grow within woody tissues and therefore have the potential to be transmitted in untreated wood packing material or in woody products. This methodology will be invaluable to the industry and the regulators to evaluate the efficacy of forest product treatments to eliminate fungi and other microbes that can be found in wood products.

LITERATURE REVIEW

Detection of microorganisms using molecular methods

To evaluate the efficacy of treatments and assess the presence of living microorganisms in wood products, culture-based approaches are generally used. This requires isolating microorganisms and growing them in pure cultures on artificial media. This method has been used for several decades and has the advantage of only detecting living microorganisms; it is therefore ideally suited to assess and evaluate the effect of treatments applied to wood products. However, culture-based methods have limitations as it is now estimated that only a small fraction of the microorganisms present in natural environments can be grown on artificial media (Pace, 1997; Rappe and Giovannoni, 2003). This methodological limitation probably results in a large number of false negatives. Particularly, this is a problem when testing a treatment applied to wood: the same experimental outcome, i.e. absence of growth on a Petri dish, would be expected for an efficacious treatment and a false negative caused by the presence of an uncultured microorganism, creating a confounding experimental effect. In addition, some fungi have slow growing behaviour and rely on a complex nutrient requirement. This is the case of myco-heterotrophic fungi that are outcompeted by fast growing saprophytic species (Taylor et al, 2002). This causes another level of difficulty in interpreting results. An additional challenge of identification of microorganisms based solely on morphological characteristics is that the paucity of morphological traits often does not provide reliable identification and can lack the level of precision to distinguish closely related species (Chimento et al, 2011; Osterbauer and Trippe, 2005).

The use of molecular methods, in particular DNA amplification of universal genes using the <u>P</u>olymerase <u>C</u>hain <u>R</u>eaction (PCR), followed by amplicon sequencing and DNA barcoding (matching the unknown sequence by homology using public databases to provide identity), have become the standard in identification of fungi and oomycetes (Hamelin et al, 1996; Hamelin et al, 2000; Schoch et al, 2012). Once a species' DNA sequence is known, it becomes possible to generate specific assays for detection using PCR. Real-time-PCR, a method that uses fluorochromes for detection of target DNA during the amplification process has increasingly replaced conventional PCR as it generates highly specific assays and allows quantification of target DNA. Protocols have been developed and applied to detect several

tree pathogens, including *P. ramorum* (Bilodeau et al, 2006; Bodles et al, 2005; Hayden et al, 2006; McCartney et al, 2003; Osman and Rowhani, 2005).

Detection and quantification of RNA by real-time reverse-transcription PCR

Most molecular detection methods for plant pathogens are aimed at detecting the presence, but not the viability, of pathogens and are based on detection of the pathogen DNA. Since DNA is stable and does not rapidly degrade following cell death, these assays are not useful to assess viability of the targeted organisms and thus do not help in determining the efficacy of a treatment. The development of real-time reverse-transcription PCR has revolutionized the analysis of gene expression in living organisms. This method generates reverse complementary DNA (cDNA) from the RNA of targeted genes. The cDNA can then be quantified by using standard curves and can therefore measure the gene expression level. This method has been extremely useful in plant pathology by allowing investigators to assess pathogen gene expression during its interaction with the host. Real-time reverse-transcription PCR has the advantage of having higher sensitivity and specificity compared to conventional reversetranscription PCR and can provide precise quantification (Bustin, 2002; Gachon et al, 2004).

RNA represents the transcription product of genes and it is only produced when the organism is alive (White, 2009). RNA, unlike DNA, is labile and degrades rapidly in dead cells (Abassi et al, 2013; Pozhitkov et al, 2016). To amplify RNA, it is necessary to provide cDNA, which is the double-stranded DNA synthesized from a single stranded RNA (e.g., messenger RNA (mRNA) template in a reaction catalysed by the enzyme reverse transcriptase. cDNA can be used to measure the expression of RNA and serve as a marker of cell viability. cDNA can be differentiated from genomic DNA (gDNA) because genes in eukaryotes comprise exons that code for amino acids and are transcribed into RNA, and introns (or intervening sequences) that are spliced out in mature messenger RNA (mRNA) transcripts. Therefore, the sequence of a cDNA representing a mature gene product differs from the sequence of that gene in the genomic DNA (gDNA) by the absence of introns. An assay that differentiates the gene from the gene transcript would be optimal for assessing viability of an organism since it would allow detection and quantification of mRNA without cross-amplification of the genomic gene copy.

One challenge of this approach is that not all genes are expressed all of the time. In order to regulate and control a living organism, genes must interact with and respond to the organism's

environment. Constitutive genes are expressed regardless of the environment while induced genes are expressed under certain environmental or developmental conditions (White, 2009). Selected target genes should be constitutively expressed and transcribed continually as opposed to those expressed following an environment signal. This ensures that when no expression of a constitutive gene is detected, it is due to the death of the organism instead of the gene being turned "off" by the environment. Therefore, in order to develop markers indicative of cell viability, it is important to identify genes that are present and expressed at every stage of the organism's life cycle and under different environmental conditions; genes involved in basic metabolic processes fulfill this criterion. Housekeeping genes are responsible for basic cellular functions such as respiration, cell division or basic metabolism (White, 2009). They are conserved components of eukaryotic genomes and they are expected to be constantly expressed within living organisms, two important criteria in developing assays to assess viability in eukaryotic organisms. The development of molecular assays that target housekeeping genes in the mitochondrial genome was effective for detecting P. ramorum (Dheda et al, 2004; Jain et al, 2006; Martin et al, 2004). Molecular methods based on RNA were also used as a tool to test for viability in Xanthomonas citri (Golmohammadi et al, 2012), P. ramorum (Chimento et al, 2011) and the pinewood nematode, Bursaphelenchus xylophilus (Leal et al, 2013). RNA expression could not be detected after a pathogen had been dead for an extended period of time, while the detection of DNA was still possible (Chimento et al, 2011; Leal et al, 2013).

Phytophthora ramorum, the causal agent of sudden oak death and sudden larch death Background

Phytophthora ramorum is an aggressive plant pathogen that emerged in the mid-1990s (Grunwald et al, 2012; Werres et al, 2001). It infects a broad range of hosts and causes a variety of symptoms, including tip and leaf blight and cankers (Garbelotto et al, 2003; Swiecki and Bernhardt, 2005). This pathogen was observed on tanoak (*Lithocarpus densiflorus*) and coast live oak (*Quercus agrifolia*), causing stem cankers that lead to mortality (Davidson et al, 2002; Garbelotto et al, 2003; Rizzo et al, 2005). It has been detected in California, Oregon, Washington, parts of Europe as well as British Columbia (Grunwald et al, 2012; Meentemeyer et al, 2004). The discovery of *P. ramorum* in North American and European nurseries (Hayden et al, 2006), its ability to infect a broad range of host species

and be transmitted on horticultural plant material caused major concerns to the forest and horticultural industries and to the regulatory authorities.

Symptoms

Unlike many other *Phytophthora* species that infect their host through the roots, *P. ramorum* has the ability to infect its hosts through stems, leaves and needles (Davidson et al, 2003). As a result, this pathogen causes two main aboveground symptoms: cankers and foliage dieback. Canker formation is associated with sudden oak death and frequently results in host death, by girdling the tree stem, damaging the phloem and causing severe canopy wilting (Rizzo et al, 2002; Rizzo et al, 2005; Swiecki & Bernhardt, 2005). Other symptoms to the host foliage comprise shoot dieback and necrotic lesions on the leaves, which results in disruption of the photosynthetic processes (Grunwald et al, 2008).

Life cycle

Phytophthora ramorum produces 3 spore types in nature: sporangia, chlamydospores, and zoospores. The sexual spore stages, the oospores, have never been reported in nature. The production of these spores depends on environmental factors such as temperature and moisture content (Werres et al, 2001; Kliejuna, 2010). The sporangia are asexual propagules that assist in direct or indirect dispersal of the pathogen and mainly produced on the host surface, especially foliage (Waterhouse 1983). Sporangia are caducous and can be easily dispersed by wind or water (Davidson et al, 2002; Hansen, 2008; Judelson and Blanco, 2005). To infect the host directly, the sporangia germinate and produce germ tubes and appressoria that attach to the host surface. The pathogen mycelium can penetrate the host directly through the leaf surface by generating infection pegs that push through the host epidermis. Alternatively, the sporangia can germinate and produce mycelium that penetrates through the leaf stomates (Hardam and Hyde, 1997; Widmer, 2009). Indirect infections occur when the sporangia release zoospores, which subsequently infect the host (Hardam and Hyde, 1997; Widmer, 2009). Zoospores are motile spores that use a flagellum to move through fluids and can target the host by using chemical, electrical and physical recognition of the host (Tyler 2002; Widmer, 2009). Chlamydospores are abundantly produced within infected hosts as well as the plant debris left on soil surfaces and allow the pathogen to survive in unfavorable environmental conditions (Elliott et al, 2015; Grunwald et al, 2012).

There are 4 known asexual lineages of *P. ramorum* each named after their primary location of discovery: North American 1 (NA1), North American 2 (NA2), European 1 (EU1) and European 2 (EU2). This pathogen is a generalist that has a high frequency of infecting ornamental species, allowing spread through nursery trade (Davidson et al, 2002; Tooley, 2004). Recent discovery of the newest lineage (EU2) after an epidemic of sudden larch death occurred in the United Kingdom, demonstrates that the host range of this pathogen is very broad (Hyun and Choi, 2014; Pouke et al, 2012; Webber et al, 2010). This outbreak highlights the destructive nature of the pathogen and consequences of introductions to new ecological landscapes.

Prevention

In most countries, including Canada, where *P. ramorum* is not established in natural landscapes, management and prevention of the disease relies upon inspection, early detection, and local eradication. These actions are aiming to prevent the spread of the pathogen from nurseries, where the pathogen can be contained and eradicated, to natural forests or plantations, where control and management is very challenging. The outbreaks of *P. ramorum* in tanoak stands, nurseries and, more recently, larch plantations have been economically and environmentally devastating.

Effective preventative measures are vital to contain the disease and limit infection. This relies largely on early and accurate detection of the pathogen. Quarantine measures to restrict movement of material infected by *P. ramorum* have been established to prevent invasion into disease-free areas (Redlin et al, 2014). Nurseries undergo intensive monitoring for symptoms of the disease, followed by testing to identify positives. Nurseries that test positive for *P. ramorum* must undergo intensive removal of the plants in the affected areas (CFIA, 2014). Within the United States, the USDA has restricted movement of nursery stock from Oregon, Washington and California to prevent further spread (APHIS, 2013). Despite government efforts, new invasions have occurred in the Pacific Northwest, through nursery-to-nursery trade or naturally infected stands. In Canada, *P. ramorum* has been discovered in nurseries every year since 2003 (BC Ministry of Agriculture, 2016). However, the pathogen has so far failed to spread outside nurseries, thanks to aggressive eradication and nursery certification programs. Although *P. ramorum* is known to grow within woody tissues, it is unknown whether it is capable of

spreading via wood products. However, this possibility has not been ruled out (Davidson et al, 2002; Grunwald et al, 2012).

The Canadian Food Inspection Agency (CFIA) has assessed the risk of *P. ramorum* to Canada as medium to low (CFIA 2012). However, due to the generalist nature of the pathogen, and its recent jump to conifers in Great Britain (Lane et al, 2003), close monitoring of plant material entering or leaving Canada is warranted to prevent accidental escape that could lead to serious economic and environmental damage. One scenario of concern is that *P. ramorum* could escape from nurseries and attack one of Canada's tree species, which could affect that species' health in planted and forested areas and eventually negatively affect the export market, as well as having ecological consequences (Grunwald et al, 2012).

Grosmannia clavigera, a fungal symbiont of the mountain pine beetle

Background

The symbiotic association of beetles and fungi have been one of the leading contributors to forest devastation (Ayres and Lombarddero, 2000; Kirisits, 2004). In Canada alone, the mountain pine beetle (MPB; *Dendroctonus ponderosae*) outbreak led to the infestation of over 18.3 million hectares of forests (NRCan, 2016b). *Grosmannia clavigera* is an *Ophiostomatoid* fungal symbiont of the mountain pine beetle that contributes to the beetle's ability to attack trees and produce viable progenies. Tree mortality is a result of the joint attack of the MPB and the fungi (Lee et al, 2006; Lieutier et al, 2009; Robinson, 1962; Six, 2012; Wang et al, 2013). This fungus has a wide geographical range across western Canada (British Columbia and Western Alberta) and the United States (Washington, Oregon and California inlands to South Dakota, Colorado and New Mexico) where the main MPB hosts are found (Lim et al, 2004; Six et al, 2003; Six and Paine, 1999; Zambino and Harrington, 1992).

Life cycle

G. clavigera and *D. ponderosa* establish a mutualistic relationship. The beetle serves as a vector for the transportation of the fungal spores and provide a point of entry through the tree's bark and into the inner nutrient-rich tissue. In counterpart, the fungus aids the beetle to colonize the tree by providing nutritional supplementation, detoxification of host defense compounds, and conditioning the host-tree

tissues to favor insect development (Benz and Six, 2006; Bleiker and Six, 2007; DiGuistini et al, 2011; Paine et al, 1997; Six and Wingfield, 2011). This fungus has the ability to kill its host tree even in the absence of beetles, when inoculated at a high density, making it a critical component of the MPB epidemic (Lee et al, 2006). Though the exact mechanism of the fungi's attack on its host is not well understood, mortality is caused by nutrient blockage and the inability to allocate water within the tree. The beetle-pathogen interaction has evolved specific mechanisms that allow the colonization of healthy trees (Hesse-Orce et al, 2010; DiGuistini et al, 2011; Lah et al, 2013; Wang et al, 2013). Upon infection, *G. clavigera* produces a melanin pigment that discolors the sapwood, staining it blue (Wang et al, 2010). This alteration in color is an integral visual component of the invasion and remains in the host even after the death of the pathogen.

Current phytosanitary actions/preventions

Though these pathogens do not have a negative influence on the mechanical properties of wood (Lum et al, 2006), many countries are concerned about introducing non-native fungi. Treatment requirements (ISPM No.15) are applied to all imported wood packaging products. Canadian legislation requires wood exports to be treated to address phytosanitary concerns. ISPM15 addresses the treatment needs for wood packaging material used for international trade (FAO, 2013). All wood packaging material must be debarked to a specific size threshold and fumigated with methyl bromide (MBr) or heat-treated with a specific time-temperature schedule that achieves a minimum of 56°C for a minimum of 30 min (FAO, 2013). This minimum standard is the most common and well-accepted approach (Uzunovic and Khadempour, 2007). This criterion was shown to be effective on a majority of MPB-associated *Ophiostomatoid* fungi, including *G. clavigera* (Uzunovic and Khadempour, 2007).

Current protocols to detect *Phytophthora ramorum* and *Grosmannia clavigera*

There has been a need to develop DNA-based detection approaches for both pathogens under study in this project. DNA-based detection is part of the regulatory agency protocols for identification of *P. ramorum* (Bilodeau et al, 2006; Cook et al, 2000; Hayden et al, 2004; Hayden et al, 2006; Martin et al, 2004). DNA-based approaches are used to identify closely related species Ohiostomatoid fungi, including *G. clavigera*, that are transported by the MPB and that co-infect pines (Khadempour et al, 2010;

Khadempour et al, 2012; Roe et al, 2010; Tsui et al, 2012). However, none of those methods can be used to test viability.

Interest in assays for viability in *P. ramorum* has led to development of a method based on realtime RT-PCR using SYBR-Green, a stain (Chimento et al, 2011; Orlando et al, 1998). However, this method has some shortcomings. The use of a stain instead of an internal probe reduces its specificity. In addition, the published assay targeted a mitochondrial cytochrome oxidase subunit I (COX 1) gene. This gene does not contain introns and therefore it is impossible to discriminate between the DNA and RNA amplification products. Although the samples were treated with an RNase free DNase solution for the purification of samples and removal of genomic DNA, even a small amount of gDNA contamination could result in a false positive (Leal et al, 2013).

Objectives

The objective of my research was to develop molecular assays that can assess the abundance and viability of a fungus and an oomycete in wood products. I designed assays and conducted experiments to amplify constitutive genes and target sequence regions that comprise introns to differentiate between RNA and DNA. By measuring the abundance of RNA of constitutive genes using real-time reverse transcription PCR and calculating the ratio of RNA:DNA of these constitutive genes, I assessed pathogen viability in order to assess the efficacy of treatments designed to kill these microorganisms. I verified the usefulness of this approach by testing the viability of *P. ramorum* and *G. clavigera* following various heat treatments.

MATERIALS & METHODS

Design of Assays

The program named "PHYLORPH" (PHYLogenetic markers for ORPHans) was used to reconstruct the gene alignments with intron/exons junction for *P. ramorum*, *G. clavigera* and other closely related species (Feau et al, 2011). Conserved proteins for *P. ramorum* were identified by performing a BLAST (Basic Local Alignment Search Tool) search with a protein database (CEGMA) against several known target genomes. CEGMA (Core Eukaryotic Genes Mapping approach) is built using National Center for Biotechnology Information (NCBI) eukaryotic clusters of orthologous groups (Parra et al, 2007). The same procedure was performed on the *G. clavigera* genome using a separate protein database (FUNYBASE) against several known fungal genomes. FUNYBASE (Fungal Phylogenomic Database) is a protein database dedicated to the analysis of fungal single copy genes classified into orthologs. A total of 119 and 80 candidate genes, from *P. ramorum* and *G. clavigera*, respectively, were identified from PHYLORPH before narrowing down (Appendix A) to our single target gene through another software, Geneious.

Diagnostic assays involve the design of a DNA oligonucleotide primer pair and two corresponding probes (Table 1). Primers are used to differentiate between species while probes add another level of specificity to distinguish between gDNA and messenger RNA (mRNA). An example of the physical structure of the genes, indicating the location of the primers and probes can be found in Figure 1. The placement of the probe on the specific locations along the genetic sequence allows differentiation between gDNA and mRNA samples. The chosen nuclear gene sequence contains an intron, which is present in gDNA but is excised by splicing in the mature mRNA. The internal probes used for the detection of gDNA bind within the intron, whereas the probes used to detect the mRNA were designed to span the exon-to-exon junction and therefore should not bind the gDNA but only the cDNA. This probe, EX, was designed to anneal to the exon splice in order to ensure the exclusive amplification of reverse transcribed mRNA through the amplification of complementary DNA (cDNA; i.e. doublestranded DNA synthesized from messenger RNA) and rule out the possibility of gDNA being amplified.

Species targeted	Gene	Function	Assay name ¹²	Sequence 5' – 3'
			PH178_F	TTTAGTCGGCTCTTATCCGGCATG
	01170	Predicted membrane	PH178_R	CAGCAAGTAATAGAACAGGTTCCCCT
	PU1/0	protein	T3_PH178_EX	GCTTCAAGGAGAAAATTGCTCAGAACCA
			PH178_P	TCAGCTGGAGGATGGAGTTGACCCATGTT
P. Tumorum		218 Chorismate mutase	T2_PH218_3F	ACCGGATCAACATCAACGATCAAATCA
	PH218		PH218_3R	ACTTGCCGAAATGGATACGCTTACT
			T3_PH218_3EX	ATGACACCGCGTATGGCTCTACGG
			T2_PH218_3P	CTGCGCAGTTGTTGCTCACTTGGGGGA
G. clavigera	NAD-dependent 510- MS359 methylenetetrahydrafolate	MS359_F	AACAATGACCCTGCCGCA	
		mathylapototrahydrafolato	MS359_R	CGTCAATCGAGTCGTCGTTATTG
		MS359_EX	CTGCCAAGAGAATGGCTTCGCCTTC	
		uenyurogenase	MS359_P	CTATGGTCAATTGGCTCTATGACTCGTGA

Table 1: Genes and assay sequences used for detection of *Phytophthora ramorum* and *Grosmannia clavigera*. Probe modification includes 5' 6-FAM[™] and 3' Iowa Black[®] FQ quencher and fluorophore.

 $^{^1}$ Assay names ending with "_F" and "_R" refers to the direction of the oligonucleotides 2 Assay names ending with "_EX" and "_P" refers to the probe sequences



Figure 1: Molecular assay design to differentiate genomic DNA (gDNA) and complementary DNA (cDNA). Primers are homologous to sequences in the exons while "Probe" and "Probe EX" are designed to anneal to the intron and the exon-to-exon junction, respectively.

Using Geneious (v8.1.6) sequences from closely related species were compared. Within *Phytophthora* spp., members of clade 8 were compared: *Phytophthora lateralis*, *P. hibernalis*, *P. foliorum*, *P. syringae*, and *P. brassicae*. Assays for *Grosmannia clavigera* were designed by comparing the known transcription sequence of *Neurospora* with other closely related species: *Leptographium* spp., *Sporothrix schenckii*, *Ophiostoma montium*, *O. ulmi*, and *O. picea*. Following standard criteria (SIGMA, 2015), optimal alignment that yields high levels of accuracy and sensitivity for our assays was determined. Optimal alignments containing introns no smaller than 40 base pairs (BP) or larger than 100BP in length as well as high levels of polymorphism between species were analyzed. Total size of the amplicon for gDNA must be less than 300bp due to limitations of the real time PCR's sensitivity (Saunders and Lee, 2013). Candidate genes selected for *P. ramorum* are *PH179* (Predicted membrane protein) and *PH218* (Chorismate mutase), while the gene selected for *G. clavigera* is *MS359* (NAD-dependent 510-methylenetetrahydrafolate dehydrogenase).

Polymerase Chain Reaction

PCR was used to multiply target DNA fragments flanked by the forward and reverse primers for preliminary testing of primer specificity. The amplification products were observed by gel electrophoresis. This screening was conducted to determine if the primer pairs amplified only *P*.

ramorum and *G. clavigera*. Duplicates of gDNA from the 4 *P. ramorum* lineages and *G. clavigera* were compared with closely related species. *P. ramorum* was compared against 9 species within clade 8: *P. brassicae*, *P. cryptogea*, *P. drechsleri*, *P. foliorum*, *P. hibernalis*, *P. lateralis*, *P. porri*, *P. primulae* and *P. syringae*. Duplicate samples within the bluestain group were used for the *G. clavigera* comparisons: *Grosmannia aurea*, *L. longiclavatum*, *L. terebrantis*, *L. wingfieldi* and *Ophiostoma monitum*. The protocol for each reaction can be found in Appendix C. One percent (1%) agarose gels were used as medium for electrophoresis to separate DNA and cDNA, amplification products based on size. For identification of the PCR product, 4µl of the PCR product was mixed with 1µl of Safe Green dye for a final volume of 5µl then inserted into separate wells via dry/wet loading. The agarose gel was run with 1% TBE for 30min at 100V. Quantitative PCR was not used for the initial testing of primer specificity in order to conserve the cost of developing corresponding probes for non-specific primer sequences. After verification of primer specificity, corresponding TaqMan probes were ordered to use in stability analysis.

Wood Inoculation

Two isolates were selected from each of the four lineages of *P. ramorum* for the wood inoculation experiment (Appendix B). One isolate of *G. clavigera* was used since there is no divergent lineage in this species. All isolates of *P. ramorum* and *G. clavigera* were obtained from long term storage, plated on carrot and malt extract (MEA) media, respectively and sub-cultured to fresh plates 10 days prior to treatments. This protocol alleviates the storage lethargy effect that is often observed.

In order to stimulate live infection on the host, living trees were freshly felled and prepared for the artificial inoculation. Three different species of trees were felled on April 14th, 2015 at UBC's Malcolm Knapp Research Forest in Maple Ridge, BC: Douglas fir (*Pseudotsuga menziesii*), Japanese larch (*Larix kaempferi*) and Western hemlock (*Tsuga heterophylla*). We selected trees with a minimum diameter at breast height (DBH) of 12cm. One log of lodgepole pine (*Pinus contorta*) with the same DBH requirements was felled May 2015 near Nelson, BC. Each tree was then divided into two 1 meter-long logs and wrapped with a garbage bag at both ends to prevent drying out before transporting to FP innovation's research lab, Vancouver, BC. The lodgepole pine logs were transported and handled at the Centre of Advanced Wood Processing (CAWP), Vancouver, BC. Logs were cleaned with a wire brush to remove excess bark, moss and dirt. Each log was cut into 0.5m long bolts, resulting in 2 bolts

representing each tree species. Bolts were hosed with water and were labeled as "species-biological replicate" (eg. Douglas fir, rep 1 = D1). One isolate from each lineage of *P. ramorum* was used in duplicate to inoculate one bolt, while the second set of isolates was used on the second bolt (Appendix B). Only one isolate, KW1407, of *G. clavigera* was used. A blank agar plug was placed on each bolt as a negative control.

Heat treatment

Two heat treatments were conducted on pure cultures of *P. ramorum* and *G. clavigera*. The first heat treatment simulates an SPF kiln-drying schedule (8 hours), while the second exposes the pathogen to a high temperature for a short period of time (56°C for 30 min for *G. clavigera*; 70°C for 1 hour for *P. ramorum*). Temperatures of 56°C for 30 min are suggested by ISPM15 (FAO, 2016) and have been shown to be lethal in previous studies (Uzunovic and Khadempour, 2007). Due to the discrepancy of different lethal treatment temperatures of pure *P. ramorum* cultures (Chimento et al, 2011; Tubajika et al, 2007b), a higher temperature of 70°C for 1 hour was also added to ensure that I had a lethal treatment in my experiments.

Cultures prepared for heat treatments were grown on cellophane for efficient removal while samples used for wood inoculations were grown directly in suitable agar media (clarified V8 and MEA, respectively). I prepared three petri dishes, each containing one inoculum plug for each of the 9 heat treatment time points. For the time-course study, 1.5 ml Eppendorf tubed containing 30 mg of mycelia each were collected from pure cultures of *P. ramorum* (PFC-5073 (NA2) and *G. clavigera* (KW1407). One tube was immediately frozen to serve as the no heat treatment control.

SPF Kiln-Drying schedule

Mycelia samples were first transferred from petri plates into 0.1mL strip tubes and placed into a thermocycler. The thermocycler was used to conduct the long heat treatment to simulate the kiln-drying schedule used to treat wood under the standards used by the CFIA (Cai and Oliveira, 2011). The schedule (Figure 2) brings the samples to 70°C for a minimum duration of 60 minutes. The mycelium was sampled at 0, 6, 12, 24, 48, 96, 168 and 240 hours after treatment. Samples were transferred into 1.5 ml microtubes, submerged in liquid nitrogen then stored at -80°C.



Figure 2: Thermocycler schedule for heat treatment of pure cultures of *Phytophthora ramorum* isolate 5073, and *Grosmannia clavigera*, isolate KW1407, to simulate a kiln-drying schedule. The schedule includes a minimum of 56°C for 30min as required by ISPM15.

Short heat treatment

A laboratory oven was preheated to 70°C to incubate 24 replicate plates of *P. ramorum isolate 5073* and of *G. clavigera* isolate KW1407 for 1 hour. After the hour-long treatment, all plates were removed from the oven and set aside at room temperature before collection. Another short treatment of 56°C for 30 min was performed for *G. clavigera* on another set of 24 pure cultures of isolate KW1407. Post-treatment sampling time points were the same than for the SPF kiln-drying.

Re-isolation of pathogen

For each treatment and time point, starting at 0 hr after treatment, mycelia were taken and reisolated on three clarified V8/MEA agar plate. The remaining samples were collected at designated time points and stored for subsequent DNA and RNA extractions. Re-isolated plates were incubated in a dark growth chamber at room temperature for 28 days.

RNA and DNA extractions

Wood samples

Inoculated *P. ramorum* and *G. clavigera* wood samples were collected, immediately submerged in liquid nitrogen, and stored at -80°C. *G. clavigera* wood samples were placed in 15 ml vials with two 10 mm stainless steel balls and were again submerged in liquid nitrogen to keep all samples frozen. Vials of *G. clavigera* infected wood samples were placed in the Geno/Grinder (SPEX SamplePrep 2010) at 15,000 rpm for 30 seconds then immediately submerged back in liquid nitrogen. This process was repeated to ensure wood samples were ground into powder form. *P. ramorum* wood samples were hand-ground using a mortar and pestle. All grinding instruments and the lab bench were wiped with RNase Away to remove potential contamination. Individually, samples were placed in the mortar, immersed in liquid nitrogen then ground up into powder. All ground wood samples were then transferred into 1.5 mL microcentrifuge tubes and submerged in liquid nitrogen, and stored at -80°C until further needed.

Pure cultures

Mycelial samples of *P. ramorum* were placed in lysing matrix C (MPBiomedicals Santa Ana, CA). This lysing matrix consists of a 2 mL tube containing 1 mm silica spheres. All samples were flash frozen in liquid nitrogen before placing in a FastPrep-24 homogenizer (MPBiomedicals) at 5.5 rpm for 30 seconds for grinding then re-submerged in liquid nitrogen. This process was repeated to ensure mycelial samples were ground into powder form. For *G. clavigera* samples, mortar, pestles and lab bench were first wiped with RNase Away to remove potential RNA contamination. Samples were removed from the freezer and submerged in liquid nitrogen to inhibit RNA degradation. Individually, samples were placed in the mortar, immersed in liquid nitrogen then ground up into fine powder. Ground fungal samples were then transferred into 1.5 mL microcentrifuge tubes and submerged into a fresh container of liquid nitrogen.

Following the grinding of samples, the simultaneous extraction of DNA and RNA was preformed using the AllPrep DNA/RNA Micro kit³ (QIAGEN Inc., Valencia, CA). It is important to obtain both the DNA and RNA from the same sample in order to accurately investigate degradation over time. Samples were analyzed using a NanoDrop spectrophotometer to test the quality and quantity of the extraction. Using

³ https://www.qiagen.com/ca/resources/resourcedetail?id=379f613c-98fa-4116-a2d5-b8e7a3239b29&lang=en

the quality RNA, a cDNA synthesis was performed using the QuantiTect Reverse Transcription Kit⁴ (QIAGEN). The ratio of the RNA to DNA quantity for the targeted genes was calculated to assess the proportion of viable pathogen. For long-term storage, extracted DNA and RNA were stored in -80°C freezers. During short-term storage, DNA and cDNA samples were stored in -20°C freezers.

Quantitative Polymerase Chain Reaction

Using the TaqMan Assays, expressions of treated and untreated samples were investigated to show the assays ability to differentiate between living and dead pathogens. RT-qPCR reactions were performed in MicroAmp Optical 96-well plates. Each of the reaction was set as described in Appendix C. Two different reactions per time point were performed, differentiating in template (gDNA and cDNA) with their corresponding probes (targeting each template respectively). Samples ran under technical duplicates and biological triplicates in the same 96-well plate. Thermal cycling parameters used were 5 minutes at 95°C for enzyme activation, followed by 40 cycles of denaturation at 95°C for 30 seconds and 60 seconds of annealing/extensions at 60°C.

Statistical Analysis

The cDNA expression is evaluated by the cycle threshold (C_T), a value that reflects the fluorescence signals significantly above the background, with the threshold set when a detectable amount of amplicon has been produced. The lower the C_T , the earlier the amplicon product is detected and the more target was present at the beginning of the reaction; a final C_T value of 40 indicates that there was no detectable target in the initial reaction. The C_T values of the RT-qPCR were exported to an Excel file for statistical analysis.

Using statistical analysis software (SAS 9.4), the significance of the treatment values were tested using a 2 factor split plot, where factor A is heat treatment and factor B is time after the treatment. The two main objectives of this analysis were to determine whether there was an effect of treatment and an interaction of treatment and time. In order to address both objectives, samples were measured and

⁴ https://www.qiagen.com/ca/resources/resourcedetail?id=f0de5533-3dd1-4835-8820-1f5c088dd800&lang=en

tested for the following attributes:

- C_T value of gDNA
- C_T value of cDNA
- Ratio: C_T value of cDNA/C_T value of gDNA

RESULTS

Specificity of the PCR assays

To test the specificity of the oligonucleotide primers I conducted PCR using gDNA from pure cultures and detected the amplified DNA by electrophoresis on agarose gels. The first level of screening of the *P. ramorum* assays was conducted on *P. ramorum* and *P. lateralis* because they are the most closely related, possessing 91.4% genome identity at the nucleotide level (N. Feau, personal communication). To assess technical variation, duplicates of gDNA from *P. ramorum* and *P. lateralis* PCR samples were compared. Five out of the seven assays targeting different genes yielded amplification products detectable on the agarose gel for *P. lateralis* and were eliminated because they failed the specificity criterion. The remaining two assays, targeting gene *PH178*, a predicted membrane protein and *PH218*, a Chorismate mutase homolog were deemed specific towards *P. ramorum*. The second level of screening involved an additional eight species within the phylogenetic clade 8, to which *P. ramorum* belongs: *P. brassicae, P. cryptogea, P. drechsleri, P. foliorum, P. hibernalis, P. porri, P. primulae* and *P. syringae*. These species are considered the closest relatives of *P. ramorum*, after *P. lateralis*. Amplification occurred only for *P. ramorum* DNA, confirming that these two assays are specific to *P. ramorum* (Figure 3).

The first level of screening of the *G. clavigera* assays was conducted on *G. clavigera* and its closest relative, *L. longiclavatum* (a sister species with 97.5% identity with *G. clavigera* at the genome level; N. Feau, personal communication) and with other members of the same clade: *L. terebrantis, L. wingfieldi* and *O. montium*. All 12 assays produced amplification of non-target products detectable on the agarose gel, but the assay targeting gene *MS359*, encoding a NAD-dependent 510-methylenetetrahydrafolate dehydrogenase, was specific to the two sister species, *G. clavigera* and *L. longiclavatum* (Figure 4). Since these two sister species occupy a similar niche (mountain pine beetle galleries) and have similar biology, we decided to select the assay targeting this gene to develop the RNA assay.



Figure 3: Electrophoresis gel comparing species specificity of *Phytophthora ramorum* against closely related species of clade 8 using primers targeting gene, PH178. No amplification product was observed for closely related species of *P. brassicae* (lane 1 & 2), *P. cryptogea* (lane 3 & 4), *P. drechsleri* (lane 5 & 6), *P. foliorum* (lane 7 & 8), *P. hibernalis* (lane 9 & 10), *P. porri* (lane 11 & 12), *P. primulae* (lane 13 & 14) and *P. syringae* (lane 14 & 16). *P. ramorum isolate* 5073 (lane 17) DNA was used as a positive control.



Figure 4: Electrophoresis gel comparing species specificity of *Grosmannia clavigera* against closely related species using primers targeting gene, MS359. No amplification product was observed *for* closely related species of *G. aurea* (lane 1 & 2), *L. wingfieldi* (lane 5 & 6, *L. terebrantis* (lane 9 & 10), and *Ophiostoma montium* (lane 11 & 12). Amplification visible for *L. longiclavatum* (lane 7 & 8) and *G. clavigera* (lane 3 & 4).

We verified the presence of an intron in the DNA samples by comparing the size of the PCR products obtained by amplification of the gDNA and cDNA of the same samples. For gene PH178 a smaller amplification product was seen in the cDNA than in the gDNA, confirming the presence of the intron in the gDNA (Figure 5). No variation was observed in the size of the amplification product of *P. ramorum* (Figure 5).



Figure 5: Electrophoresis gel of primer pairs targeting gene PH178 to differentiate between gDNA and cDNA based on amplicon size difference in base pairs using *Phytophthora ramorum* pure cultures. *P. ramorum*, isolate 5073 (NA2) gDNA amplified in duplicate (lane 1 & 2); isolate 05-16848 (NA2) (lane 3, 4 & 5) cDNA and isolate 5073 (lane 6, 7 & 8) cDNA amplified in triplicate.

Since the size difference confirmed the presence of the intron, I proceeded to design Taqman probes that encompass the exon to intron junction (for gDNA) or the exon-to-exon junction for cDNA (Figure 6). To select one of the two assays that passed the screens for *P. ramorum*, I assessed assay efficiency, the ability to amplify products relative to the concentration of target samples by conducting a series of dilutions and producing a sensitivity standard curve (Appendix F). Based on this analysis, I selected the gene PH178 in *P. ramorum*, the assays with a better efficiency. Two TaqMan probes, one targeting gDNA and the other one cDNA for the gene PH178 in *P. ramorum* and for *MS359* in *G. clavigera* was used for subsequent experiments.



Figure 6: Amplification plot of *Phytophthora ramorum*, isolate PFC-5073 gDNA and *Grosmannia clavigera*, isolate KW1407 gDNA using target primer pairs and TaqMan probes. **a)** Amplification (yellow line) of *P. ramorum* gDNA using gDNA specific TaqMan probe, PH178_P; **b)** amplification (green line) of *G. clavigera* gDNA using gDNA specific TaqMan probe, MS359_P; **c**) no amplification (light green line) of *P. ramorum* gDNA using cDNA specific TaqMan probe, T3_PH178_EX; **d)** no amplification (red line) of *G. clavigera* cDNA using cDNA specific TaqMan probe, MS359_EX.

Analysis of gene expression in infected tissues

Detection of Phytophthora ramorum and Grosmannia clavigera in inoculated wood

In order to validate that the genes developed in our assays were expressed by the pathogens while growing within wood tissues conifer logs were inoculated with *P. ramorum* and *G. clavigera* and incubated during 28 days to allow development of the pathogens. Lesions were observed around all points where the microorganisms were inoculated (Figure 7 & 8) but not in the blank control. Fifty to 80 mg of wood scrapings were collected at each inoculation point from 8 logs of 4 conifer species and used for both DNA and RNA extractions. I conducted qPCR assays targeting gene PH178 for *P. ramorum* and and gene MS359 for *G. clavigera*. C_T values lower than 35, indicating presence of a PCR product, were detected for both cDNA and gDNA from all inoculated samples (Figure 9), but not for the control. There was no significant difference in C_T values when comparing the cDNA of wood and pure culture samples
of *P. ramorum* (α =0.05, *P*=0.421), but a significant difference was observed for *G. clavigera* (α =0.05, *P*<0.001). I found higher gene expression in the wood compared to pure cultures in this fungus.



Figure 7: *Phytophthora ramorum* lesions on artificial wood inoculation samples using isolate P2111 (EU2); **a)** 28 days lesion observed on Japanese larch log; **b)** 28 day lesion found on Douglas fir log; **c)** 28 day lesion found on Western hemlock log.



Figure 8: *Grosmannia clavigera* lesions on artificial wood inoculation samples using isolate KW1407; **a)** 28-day lesion found on Lodgepole pine log #1; **b)** 28-day lesion found on Lodgepole pine log #2.



Figure 9: Amplification plot of infected lodgepole pine with *Grosmannia clavigera*, isolate KW1407, using target primer pairs and TaqMan probes. **a)** Amplification (pink line) of *G. clabigera* gDNA using gDNA specific TaqMan probe, MS359_P; **b**) Amplification (pink line) of *G. clavigera* cDNA using cDNA specific TaqMan probe, MS359_EX; **c**) No amplification (green line) of *G. clavigera* gDNA using cDNA specific TaqMan probe, MS359_EX

Use of molecular assays to assess viability of pathogens following heat treatment

To further evaluate mRNA stability over time and the reliability of my approach to measure viability, I conducted experiments with pure cultures so that I could artificially induce lethal conditions. I assessed viability by measuring and comparing the mRNA to gDNA C_T value ratio as a measure of degradation that could provide an indicator of survival following treatment. Residuals plots of *P. ramorum* indicated that the relationship between the treatment and time variables was linear and data followed the normal distribution required before performing an ANOVA analysis (Appendix E). *G. clavigera* samples also met most of the assumptions required for the statistical test (Appendix E).

A significant effect of heat treatment, time points and their interaction on cRNA/gDNA ratios was revealed by the ANOVA for *P. ramorum* (Table 2). Treatment was also significant for *G. clavigera* but there was no significant change over time (α =0.05, *P* = 0.079) (Table 3). There was an interaction between treatment and time for this pathogen (α =0.05, *P* < 0.01). In *P. ramorum*, there was no

significant difference between the biological replicates (α =0.05, *P* = 0.785) while replicates were significantly different for *G. clavigera* (α =0.05, *P* < 0.05).

Table 2: *Phytophthora ramorum* ANOVA output of the heat treatment experiment analyzed as a 2-factor split plot using the RNA/DNA ratio. Treatment represents the non-treated pure culture samples, the "kiln" treated samples and the "short" (1 hr @ 70°C) samples. Samples were collected at various time points (0, 6, 12, 24, 48, 96, 168 and 240 hrs).

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Treatment	2	1.443	0. 721	72.38	<0.000 1
Rep	2	0.004	0.002	0.24	0.784
Treatment*Rep	4	0.023	0.005	0.59	0.674
Time Point	7	0.330	0.047	4.73	0.0006
Time Point* Treatment	13	0.542	0.041	4.19	0.0002

Table 3: *Grosmannia clavigera* ANOVA output of the heat treatment experiment analyzed as a 2-factor split plot using the RNA/DNA ratio. Treatment represents the non-treated pure culture samples, the "kiln" treated samples and the "short" (1 hr @ 70°C) samples. Samples were collected at various time points (0, 6, 12, 24, 48, 96, 168 and 240 hrs).

Source	DF	Type III SS	Mean Square	F value	Pr > F
Treatment	2	18.24	9.12	25.22	<0.0001
Rep	2	2.71	1.35	3.74	0.032
Treatment*Rep	4	13.89	3.47	9.60	<0.0001
Time Point	7	5.04	0.72	1.99	0.079
Time Point* Treatment	14	13.66	0.98	2.70	0.007

To demonstrate that the molecular assays developed can differentiate between dead and live *P. ramorum* and *G. clavigera*, I conducted two heat treatments on a pure culture of isolate 5072 (NA2 lineage) grown in clarified V8 and isolate KW1407 grown in MEA media, respectively. The first treatment simulates an SPF kiln-drying schedule, while the second exposes the pathogen to a lethal temperature for a short period of time (70°C for 1 hour). The mycelia were sampled at 0, 6, 12, 24, 48, 96, 168 and 240 hours after treatment and their RNA and DNA were extracted and used in qPCR experiments with the same gDNA and cDNA assays used for the inoculated wood. Amplification of gene *PH178* gDNA was

observed in all heat-treated samples of *P. ramorum* while amplification of cDNA was observed only in the control and in all samples up to 24 hrs post heat-treatment (Figure 10). No amplification of cDNA of gene *PH178* was observed 24 hrs post-treatment. Similarly, amplification of gene *MS359* DNA was observed in all heat-treated samples of *G. clavigera* while no amplification was observed after 96 hrs (Figure 11). Amplification of cDNA was not observed at any time point for kiln-treated samples (Figure 12 & 13). Clearly, DNA remained stable after heat treatment for both species, but RNA degrades at different rates following treatment for these two organisms (Figure 10 & 11). The C_T values of DNA and RNA were compared with each treatment; no significant changes in DNA content of the pathogen were observed over a period of 240 hrs but significant changes in cDNA (mRNA) content following both treatments. cDNA content did not change significantly over the course of 240 hrs for untreated samples, whereas for "kiln" and "short" (1 hr @ 70°C) heat treated samples of both *P. ramorum* and *G. clavigera*, cDNA was not detected after 24 hrs and 96 hrs, respectively (Figure 12 & 13).



Figure 10: Real-time PCR amplification plots of 24 samples of the pure culture PFC-5073 of *Phytophthora ramorum* (NA2) after heat-treatment at 70°C for 1 hr. Plots show amplification of gene *PH178* using primers targeting genomic DNA (gDNA) and complementary DNA (cDNA) and sampled at various time points following treatment.



Figure 11: Real-time PCR amplification plots of 24 samples of pure culture KW1407 of *Grosmannia clavigera*, isolate, after a heat-treatment at 70°C for 1 hr. Plots show amplification of gene *MS359* using primers targeting genomic DNA (gDNA) and complementary DNA (cDNA) and sampled at various time points following treatment.

For the short heat treatment, *P. ramorum* cDNA C_T values of less than 40 were detected up to 24 hrs with a C_T value of 29, but no amplification was detected 48 hrs post treatment (Figure 12). C_T values of less than 40 were detected for cDNA of *G. clavigera* samples up until 96 hrs post short treatment (Figure 13) with a C_T value of 32. This outcome indicates that the RNA of the targeted genes is likely degraded and thus not detectable 24 (*P. ramorum*) and 96 (*G. clavigera*) hours after treatment while DNA integrity is preserved making it still detectable by qPCR.



Figure 12: Boxplot of *Phytophthora ramorum* cDNA C_T values over time after, targeting gene *PH178*; **a**) "Kiln" heating schedule treatment with control; **b**) Short heat treatment (1 hr at 70°C) with control. No amplification was observed at any time point for the "Kiln" treated samples, while some amplification was observed for the "short" heat-treated samples up until 24 hrs.



Figure 13: Boxplot of *Grosmannia clavigera* cDNA C_T values over time, targeting gene *MS359* **a**) "Kiln" heating schedule treatment with control **b**) Short heat treatment (1 hr at 70°C) with control. "Kiln" treated samples show no amplification at any time point, while "short" heat-treated samples show amplification up to 96 hrs after treatment.

Re-isolation of Pure Cultures

Small samples of mycelia were collected after each of the heat treatments at time point 0 and plated onto a sterile clarified-V8/MEA agar Petri dish to verify whether or not the heat treatments had

been lethal. No growth was observed from any of the cultures that had been exposed to kiln and 70°C for both microorganisms 28 days post treatment (Figure 14 & 15).



Figure 14: Growth-test of *Phytophthora ramorum* isolate 5073 after kiln and heat treatments. **a)** No growth visible on re-isolation 28 days after kiln schedule treatment; **b)** No growth visible on re-isolation 28 days after short (1hr at 70°C) heat treatment.

Initially, 56°C for 30 min was used as the short heat treatment for *G. clavigera* but from the reisolation results (Figure 15), a new heat treatment (70°C for 1 hr) was used instead. The mycelium of all plates that contained re-isolated *G. clavigera* following the 56°C treatment was black coloured and covered the entire plate after a 28-day growth period (Figure 15). Scrapings from the plate were collected for DNA extractions and PCR identification confirmed the black fungal growth to be *G. clavigera*. However, 3/3 replicates of cultures from post kiln and 3/3 replicates of cultures from post 70°C treatment failed to yield colonies.



Figure 15: Growth-test of *Grosmania clavigera* isolate KW1407after kiln and heat treatments. **a)** No visible growth following attempted re-isolation 28 days after kiln schedule treatment; **b)** Re-isolation and growth 28 days after short (30 min at 56°C) heat treatment; **c** No visible growth 28 days after short (1hr at 70°C) heat treatment.

DISCUSSION

Detection of wood-invading microorganisms using RNA and DNA

Real-time PCR is increasingly becoming the method of choice for molecular detection of microorganisms and for high-throughput gene expression analysis. Currently the method used for assessing the efficacy of treatment of wood products is to culture microorganisms (Tubajika et al, 2007a). This method has the advantage of simultaneously assessing the presence of microorganisms and their viability. But it has the disadvantage of producing a high rate of false negative. This is due to the fact that some microorganisms are difficult to isolate in pure cultures (Kaeberlein et al, 2002; Nichols et al, 2008; Puspita et al, 2012). The present study was undertaken to evaluate the feasibility to develop a method that would address the weakness of the culture method by assessing the presence and viability of tree-colonizing microorganisms using a molecular detection tool. I was able to successfully meet the research objective for *P. ramorum* and *G. clavigera* by showing that RNA and the RNA/DNA ratio decreased significantly following treatments that are lethal to the microorganisms.

By designing assays with probes that overlap the exon-intron and exon-exon junctions, it was possible to differentiate between the RNA and the corresponding genomic gene copy. This allowed the calculation of the ratio of mRNA to DNA and provides a measure the presence and abundance of the microorganisms (Marter and Vivanco, 2007), as well as assess their viability. RNA-based molecular assays have proven to be successful in detecting different pathogens. For example, Aarthi et al (2012) have used assays to detect infectious bacterial endophthalmitis in humans. However, their study did not target mRNA, but instead the ribosomal RNA (rRNA), 16SrRNA gene, which is a multicopy locus typically used in phylogenetic studies. Bergin et al (2010) studied the detection of periprosthetic bacterial infections and found a strong correlation between rRNA signal and cell death. Though effective in identifying viable bacterial genomes, a drawback is rRNA do not have spliced introns and therefore this approach cannot differentiate the RNA samples from DNA. Menzel et al (2002), designed an end-primer sitting on the exon-exon junction in order to differentiate between mRNA and DNA. However, though effective as an internal control of amplifying only RNA, the assay was designed to target a gene within the host mitochondrial DNA (nad5; NADH dehydrogenase subunit 5) instead of the virus of interest.

The most closely related detection study of *P. ramorum* investigated the viability of *P. ramorum* using mycelial cultures grown in broth following a rapid lyophilisation treatment to cause cell death (Chimento et al, 2011). These investigators designed qPCR primers to amplify the cytochrome oxidase subunit I (COX 1) gene, a mitochondrial gene that does not contain introns (Martin et al, 2007). Consequently, the qPCR assay developed in this study could not differentiate between the mitochondrial and the transcribed gene copies. This raises the possibility of false positive amplification from accidental mitochondrial DNA contamination. The present study increased the specificity of the test by developing assays including a probe that binds the exon junction, allowing only the amplification of the transcript product (RNA) previously converted into cDNA. This generates a more accurate detection and eliminates potential false-positives due to DNA contamination in the RNA sample. Furthermore, the use of one primer that does not differentiate between RNA and DNA as done in Chimento et al (2011) is less species-specific compared to the current technique that uses a probe that binds to the exon junction.

Assessing viability of pathogens following treatment

In order to test whether or not the assays I developed were efficient to assess viability, it was important to determine the RNA/DNA ratios following treatments that are lethal to the pathogens. Based on the literature and some preliminary experiments, I decided to conduct heat treatments at 70°C to kill the pathogens. Other authors have used lower temperatures and reported conflicting results. Harnik et al (2004) showed that infected leaves treated for 22 hr at 55°C killed *P. ramorum* by their inability to recover the pathogen through plating. Tubajika et al (2007b) observed no growth after a heat treatment of 60°C or 65°C for 1 hr but Chimento et al (2011) states that the treatment (60°C for 1 hr) did not kill the pathogen but instead delayed its growth. Because of these discrepancies regarding the lethal temperature and duration required to kill this pathogen, I selected the treatment of 70°C for a minimum of one hour. Clearly, this temperature treatment was lethal to *P. ramorum* and *G. clavigera* as re-isolations of the microorganisms failed to produce living cultures and I conclude that these pathogens do not survive a treatment at 70°C for 1 h.

However, the temperature recommended to exporters (ISPM15) is much lower (56°C) and the time of exposure is much shorter (30 min). Treatment of 56°C for 30 min was only applied to G.

clavigera because of its ineffectiveness as a lethal temperature for *P. ramorum* (Chimento et al, 2011; Tubajika et al, 2007b). However, when I replicated this treatment for *G. clavigera*, the fungus was still able to grow on a Petri plate 28 days after re-isolation. Therefore, the 56°C for 30 min heat treatment was not lethal to the pathogen and could jeopardize the efficacy of this treatment in killing *Ophiostomatoid* fungi and Phytophthora species. Uzunovic and Khadempour (2007) stated that that this temperature-time of 56°C for 30min was sufficient to kill the majority of blue stain fungi belonging to the *Ophiostomatoid* group. No survival of *G. clavigera* was found on both wood and agar samples after 56°C for 30 min but a slight resistance was found in *O. montium*, as four out of six replicates survived this treatment (Uzunovic and Khadempour, 2007). Most kiln and heat treatment on wood products will have the core temperature exceed the ISPM15 requirement. For SPF wood products, core temperature can rise up to at least 80°C, with the majority of the schedule exceeding 56°C (Cai and Olivera, 2011). The ISPM15 standard of 56°C for 30min was not effective in killing the *G. clavigera* but proven successful in other studies. Potential pitfalls of this experiment could be the lethal temperature not reaching the mycelia for the minimum duration, a discrepancy between the temperature inside the oven and the temperature of the mycelia or difficulty with the delivery of the heat treatment.

In spite of the reduction in RNA following treatments, the RNA/DNA ratio is not an exact predictor of viability. Even 24 h (for *P. ramorum*) or 96 h (for *G. clavigera*) following the short heat treatments, I detected some RNA. However, I did not recover living cultures at any time point following this heat treatment. It is possible that the RNA is less labile than anticipated in culture and that RNA degradation takes longer than expected. All my tests were performed in Petri plates, an environment where RNA degradation could be slowed down. It is intriguing that no RNA was detected following the SPF-Kiln treatment. Possibly, the longer time of exposure (8 hr) contributes to RNA degradation for that treatment. Subsequent work should aim to determine if RNA/DNA ratios decrease faster within the wood substrate.

Detection of pathogen in Wood

DNA and RNA extraction from wood can be challenging (Asif and Cannon, 2005; Rachmayanti et al, 2006). In addition, my assays rely on the expression of genes that can be affected by environmental conditions. Therefore, following the testing of my method using pure cultures, I aimed to verify that my

35

assays worked when the microorganisms were present within woody tissues. Wood has a high content in phenolic compounds, polysaccharides and metabolites, raising some technical challenge when extracting DNA and RNA (MacRae, 2007). In my study, DNA and RNA was extracted and amplified from wood samples that were artificially inoculated with the microorganisms. The RNA, following transformation to cDNA, was successfully amplified with the assays developed in this study, confirming that the target genes of these microorganisms are expressed and can be detected in woody tissues.

Nevertheless, there were some quantitative differences in abundance (as measured by C_T values) of RNA and DNA in wood compared to pure cultures. This could be due to the stability of gene expression causing fluctuations. Gene expression can differ according to experimental conditions, including that of the host (Jain et al, 2006). By comparing the expression of genes under different conditions, then the ideal control gene should have similar expression regardless of experimental conditions, including different cell types, developmental stages, and/or sample treatment. However, no one gene has a stable expression under every experimental condition, as numerous studies reported that expression of housekeeping genes can also vary considerably with experimental conditions (Jain et al, 2006). Consequently, in order to compare gene expression, it is necessary to validate expression stability of a control gene under specific experimental conditions prior to its use for normalization. In spite of this, the present study investigates viability through the RNA to DNA C_T ratio. Since the ratio is similar within the wood and pure culture samples it provides the most reliable measure of viability. By validating the assays developed using RNA extracted directly from wood samples, I was able to demonstrate successful detection of the pathogen with the material for which these assays will be applied.

CONCLUSION

The Food and Agriculture Organization (FAO) often revises the list of suggested treatments for wood packaging material, emphasizing the importance of reducing the risk of quarantine pests associated with wood exports (FAO, 2013). Also, lists of microorganisms indicate species that can be of concern for phytosanitary regulators (FAO, 2006). P. ramorum and G. clavigera are microorganisms of phytosanitary concern in several countries that regulate them (i.e. Australia [Phytophthora], China, France, UK, etc.). The present research acts as a proof-of-concept to enhance future molecular detection methods of invasive and native pathogens of phytosanitary concern. Through this experiment I was able to verify two outcomes. First, I developed a method that can differentiate between dead and alive pathogens using qPCR assays. The location of specific probes that encompass the exon-exon junction effectively detects only the cDNA of the targeted genes, eliminating potential effects of contamination with DNA during extraction. Second, I successfully applied this approach in detecting gene expression within infected wood samples. The use of this application can evaluate efficacy more accurately for different wood treatments by directly extracting nucleic acid from treated and untreated wood. Finally, housekeeping genes were not detected by real-time reverse transcription PCR between 2-3 days after the death of P. ramorum and G. clavigera. These assays therefore provide a powerful tool to assess treatment efficacy in eliminating these microorganisms. Further investigation on a more elaborate and stringent time course should be conducted to properly monitor the RNA degradation, in particular in wood. The ability to simultaneously detect pathogens within wood samples and to test for their viability is beneficial for future commercial use as a standard for evaluating exports and imports and to assess treatment efficacy. I tested my method with infected wood samples, an important validation step that demonstrates it can be transferred outside of a laboratory setting. This will make it possible to test the efficacy of treatment of wood products and assess their impact of viability of microorganisms. Overall, this approach of RNA-based molecular assays was successful and can be adopted for the detection for viability of other pathogens of phytosanitary concern.

37

REFERENCES

- Aarthi, P., Bagyalakshmi, R., Therese, K., Malathi, J., Mahalakshmi, B., & Madhavan, H. 2012. Optimization and application of a reverse transcription polymerase chain reaction to determine the bacterial viability in infectious endophthalmitis. Current Eye Research. 37:1114-1120
- Abbasi, N., Park, Y. & Choi, S. 2013. RNA deadenylation and decay in plants. Journal of Plant Biology. 56:198-207
- Anderson, T. W. 1962. On the distribution of the two-sample Cramer-von Mises Criterion. The Annals of Mathematic Statistics. 33:1148-1159
- Anderson, T. W. & Darling, D. A. 1952. Asymptotic Theory of Certain "Goodness of Fit" Criteria Based on Stochastic Processes. Annals of Mathematics Statistics. 23:193-212

Animal and Plant Health Inspection Service (APHIS). 2015. *Phytophthora ramorum* Domestic Quarantine by Deregulating Areas in California, Oregon, and Washington. United States Department of Agriculture. Available from:
https://www.aphis.usda.gov/plant_health/plant_pest_info/pram/downloads/pdf_files/DA-2015-18.pdf

- [APHIS] Animal and Plant Health Inspection Service. 2013. Phytophthora ramorum Nursery Survey. United States Department of Agriculture. Available from: https://www.aphis.usda.gov/plant_health/plant_pest_info/pram/downloads/surveyplan/2013-SurveyManual.pdf
- Asif, M.J. & Cannon, C.H. 2005. DNA extraction from processed wood: A case study for the identification of a endangered timber species (*Gonystylus bancanus*) Plant Molecular Biology Reporter. 23: 180-187
- Ayres, M. P. & Lombardero, M. J. 2000. Assessing the consequences of global change for forest disturbance from herbivores and pathogens. Science Total Environment. 262: 263-286

- BC Ministry of Agriculture. 2016. Ramorum blight and dieback (Sudden Oak death). Government of Canada. Available from: <u>http://www2.gov.bc.ca/assets/gov/farming-natural-resources-and-industry/agriculture-and-seafood/animal-and-crops/plant-health/phu-ramorumblight-diebackss.pdf</u>
- Bilodeau, G. J., C. A. Lévesque, A. W. A. M. De Cock, C. Duchaine, S. Brière, P. Uribe, F. N. Martin, and R.
 C. Hamelin. 2007. Molecular detection of *Phytophthora ramorum* by real-time polymerase chain reaction using TaqMan, SYBR Green, and molecular beacons. Phytopathology. 97: 632-642
- Bentz, B.J., & Six, D.L. 2006. Ergosterol content of fungi associated with *Dendroctonus ponderosae* and *Dendroctonus rufipennis* (Coleoptera: Curculionidae, Scolytinae). Annals of the Entomological Society of America. 99:189–194
- Bleiker, K.P., & Six, D.L. 2007. Dietary benefits of fungal associates to an eruptive herbivore: potential implications of multiple associates on host population dynamics. Environmental Entomology. 36:1384–1396
- Bodles, W., Fossdal, C., & Woodward, S. 2005. Multiplex real-time PCR detection of pathogen colonization in bark and wood of *Picea sitchensis* clones differing in resistance to *Heterobasidion annosum*. Tree Physiology. 26:775-782
- Brasier, C. M. 2003. Sudden oak death: *Phytophthora ramorum* exhibits transatlantic differences. Mycological Research. 107:258-59
- Brasier, C., & Webber, J. 2010. Plant pathology: sudden larch death. Nature 466:824-825
- Bustin, S. A. 2002. Quantification of mRNA using real-time reverse transcription PCR RTPCR: trends and problems. Journal of Molecular Endocrinology. 29:23-29
- Canadian Food Inspection Agency. 2012. Pest Assessment Summary. Government of Canada. Available from: http://www.inspection.gc.ca/plants/plant-pests-invasive-species/diseases/sudden-oakdeath/summary/eng/1327588394994/1327589076065

- Canadian Food Inspection Agency. 2014. Sudden Oak Death- Phytopthora ramorum. Government of Canada. Available from: http://www.inspection.gc.ca/plants/plant-pests-invasivespecies/diseases/sudden-oak-death/eng/1327587864375/1327587972647
- Chimento, A., Cacciola, S. O., & Garbelotto, M. 2011. Detection of mRNA by reverse-transcription PCR as an indicator of viability in *Phytophthora ramorum*. Forest Pathology. 42:14-21
- Cooke, D. E. L., Drenth, A., Duncan, J. M., Wagels, G., & Brasier, C. M. 2000. A molecular phylogeny of *Phytophthora* and related oomycetes. Fungal Genetics and Biology. 30:17-32
- Davidson, J. M., Rizzo, D. M., & Garbelotto, M. 2002. *Phytophthora ramorum* and sudden oak death in California: II. Pathogen transmission and survival. USDA General Report 91:741–49
- Davidson, J. S., Werres, M., Garbelotto, E., Hansen, E., & Rizzo, D. 2003. Sudden oak death and associated diseases caused by *Phytophthora ramorum*. Plant Health Progress 7

DiGuistini, S., Wang, Y., Liao N. Y., et al. 2011. Genome and transcriptome analyses of the mountain pine beetle-fungal symbiont *Grosmannia clavigera*, a lodgepole pine pathogen. *Proceedings of the National Academy of Sciences* 108:2504–2509

Dheda, K., Huggest, J., Bustin, S., Johnson, M., Rook, G., & Zumla, A., 2004. Validation of housekeeping genes for normalizing RNA expression in real-time PCR. BioTechniques. 37:112-119

Dorak, M. T. 2007. Real-Time PCR. New York, NY. Taylor & Francis Group. ISBN 0-203-96731

- Elliott, M., Shamoun, S. F., & Sumampong, G. 2015. Effects of systemic and contact fungicides on life stages and symptom expression of *Phytophthora ramorum* in vitro and in planta. Crop Protection. 67:136-144
- Food and Agriculture Organization. 2006. ISPM No. 19. Guidelines on lists of regulated pests. Food and Agriculture Organization of the United Nations, Rome, Italy. 223-291 Available from: ftp://ftp.fao.org/docrep/fao/009/a0450e/a0450e01.pdf
- Food and Agriculture Organization. 2013. Regulation of wood packaging material in international trade. ISPM No. 15. Produced by the Secretariat of the International Plant Protection Convention. Food

and Agriculture Organization of the United Nations, Rome, Italy. Adopted 2013; published 2016 Available from:

https://www.ippc.int/static/media/files/publication/en/2016/06/ISPM_15_2013_En_2016-06-07.pdf

- Food and Agriculture Organization. 2016. ISPM 15: Regulation of wood packaging material in international trade. Secretariat of the International Plant Protection Convention. Food and Agriculture Organization of the United Nations, Rome, Italy. 105
- Feau, N., Decourcelle, T., Husson, C., Desprez-Loustau, M., & Dutech, C. 2011. Finding single copy genes out of sequenced genomes for multilocus phylogenetics in non-model fungi. Plos ONE 6: e18803
- Gachon, C., Mingam, A., & Charrier, B. 2004. Real-time PCR: What relevance to plant studies? Journal of Experimental Botany. 55:1445-1454
- Garbelotto, M., Davidson, J. M., Ivors, K., Maloney, P. E., Huberli, D., Koike, S. T., & Rizzo, D. M. 2003. Non-oak native plants are main hosts for sudden oak death pathogen in California. California Agriculture. 57:18-23
- Grunwald, N. E., Goss, & Press, C. 2008. *Phytophthora ramorum*: a pathogen with a remarkably wide host range causing sudden oak death on oaks and *P. ramorum* blight on woody ornamentals.
 Molecular Plant Pathology 9:729-740
- Grünwald, N., M., Garbelotto, E., Goss, K., Heungens, & S., Prospero. 2012. Emergence of the sudden oak death pathogen *Phytophthora ramorum*. Trends in Microbiology 20:131-138
- Hamelin, R. C., Berube, P., Gignac, M. & Bourassa, M. 1996. Identification of root rot fungi in nursery seedlings by nested multiplex PCR. Applied Environmental Microbiology 62:4026–4031
- Hamelin, R. C., Bourassa, M. & Jimmy, R. 2000. PCR detection of *Gremmeniella abietina*, the causal agent of Scleroderris canker of pine. Mycological Research 104:527–532
- Hansen, E. 2008. Alien forest pathogens: *Phytophthora* species are changing world forests. Boreal environment research 13:33-41

- Harnik, T., Mejia-Chang, M., Lewis, J., & Garbeletto, M. 2004. Efficacy of heat-based treatments in eliminating the recovery of the Sudden Oak Death pathogen (*Phytophthora ramorum*) from infected California Bay Laurel leaves. HortScience 39:1677-1680
- Hayden, K. J., Rizzo, D.M., Tse, J., & Garbelotto, M. 2004. Detection and quantification of *Phytophthora ramorum* from California forests using a real-time polymerase chain reaction assay.
 Phytopathology 94:1075-83
- Hayden, K., Ivors, K., Wilkinson, C., & Garbelotto, M. 2006. TaqMan chemistry for *Phytophthora ramorum* detection and quantification, with a comparison of diagnostic methods. Phytopathology 96:846-854
- Hesse-Orce, U., DiGuistini, S., Keeling, C. I., et al. 2010. Gene discovery for the bark beetle-vectored fungal tree pathogen *Grosmannia clavigera*. BMC Genomics 11:536
- Hyun, I.H., & Choi, W. 2014. Phytophthora species, new threats to the plant health in Korea. The Plant Pathology Journal. 30:331–342
- International Plant Protection Convention (IPPC). 2009. Revision of ISPM15: Regulation of wood packaging material in international trade. International Standards for Phytosanitary Measures. 1-16 Available from: https://www.ippc.int/largefiles/adopted_ISPMs_previousversions/en/ISPM_15_2009_En_2009-04-23.pdf
- Jain, M., Nijhawan, A., Tyagi, A., & Khurana, J. 2006. Validation of housekeeping genes as internal control for studying gene expression in rice by quantitative real-time PCR. Biochemical and Biophysical Research Communications. 345:646-651
- James, P. M. A., Coltman, D. W., Murray, B. W., Hamelin, R. C., & Sperling, F. A. H. 2011. Spatial genetic structure of a symbiotic beetle-fungal system: toward multi-taxa integrated landscape genetics. PLoS ONE 6:e25359
- Judelson, H., & Blanco, F. 2005. The spores of *Phytophthora*: Weapons of the plant destroyer. Natural Reviews Microbiology 3:47-58

- Kaeberlein, T., Lewis, K. & Epstein, S.S. 2002. Isolating "uncultivable" microorganisms in pure cultures in a simulated natural environment. Science. 296:1127-1129
- Khadempour, L., LeMay, V., Jack, D., Bohlmann, J. & Breuil, C. 2012. The relative abundance of mountain pine beetle fungal associates through the beetle life cycle in pine trees. Microbial Ecology.
 64:909-917
- Khadempour, L., Massoumi-Alamouti, S., Hamelin, R. C., Bohlmann, J., & Breauil, C. 2010. Target-specific
 PCR primers can detect and differentiate Ophiostomatoid fungi from microbial communities
 associated with the mountain pine beetle *Dendroctonous ponderosae*. British Mycological
 Society. 114:825-833
- Kirisits, T. 2004. Fungal Associates of European Bark Beetles with Special Emphasis on the
 Ophiostomatoid Fungi. In: Lieutier F, Day KR, Battisti A, Grégoire J-C, Evans HF, editors. Bark and
 Wood Boring Insects in Living Trees in Europe, a Synthesis. Springer Netherlands. 181–236
- Kliejunas, J. 2010. Sudden Oak Death and *Phytophthora ramorum*: A summary of the literature. 2010 edition. Gen. Tech. rep. PSW-GTR-234. Albany, CA. United States Department of Agriculture, Forest Services, Pacific Southwest Research Station, 1-181
- Lah, L., Haridas, S., Bohlmann, J., & Breuil, C. 2013. The cytochromes P450 of *Grosmannia clavigera*: Genome organization, phylogeny, and expression in response to pine host chemicals. Fungal Genetics and Biology. 50:72-81
- Lane, C.R., Beales, P.A., Hughes, K.J.D., Griffin, R.L., Munro, D., Brasier, C.M. & Webber, J.F. 2003. First outbreak of *Phytophthora ramorum* in England, on *Vibrurnum tinus*. Plant Pathology 52:414
- Leal, I., Foord, B., Allen, E., Campion, C., Rott, M. & Green, M. 2013. Development of two reverse transcription-PCR methods to detect living pinewood nematode, *Bursaphelenchus xylophilus*, in wood. Forest Pathology. 43:104-114
- Lee, S., Kim, J-J., & Breuil, C. 2006. Pathogenicity of *Leptographium longiclavatum* associated with *Dendroctonus ponderosae* to *Pinus contorta*. Canadian Journal of Forest Research 36:2864-2872

- Lieutier, F., Yart, A., & Salle, A. 2009. Stimulation of tree defenses by Ophiostomatoid fungi can explain attack success of bark beetles on conifers. Annals of Forest Science. 66:801
- Lim, Y., Alamouti, S., Kim, J., Lee, S., & Breuil, C. 2004. Multigene phylogenies of *Ophiostoma clavigerum* and closely related species from bark beetle-attacked *Pinus* in North America. FEMS Microbiology Letters. 237:89-96
- Loo, J.A. 2009. Ecological impacts of non-indigenous invasive fungi as forest pathogens. Biological Invasions. 11:81-96
- Lum, C., Byrne, T., & Casilla, R. 2006. Mechanical properties of lodgepole pine containing beetletransmitted blue stain. Forest Products Journal. 56:15-50
- MacRae, E. 2007 Extraction of Plant RNA. Methods in Molecular Biology: Protocols for Nucleic Acid Analysis by Nonradioactive Probes, 2nd Edition. Humana Press, Totowa. 353: 15-24
- Manter, D.K. & Vivanco, J.M. 2007. Use of the ITS primers, ITS1F and ITS4, to characterize fungal abundance and diversity in mixed-template samples by qPCR and length heterogeneity analysis. Journal of Microbiological Methods. 71:7-14
- Martin, F.N., Bensasson, D., Tyler, B.M., & Boore, J.L. 2007. Mitochondrial genome sequence and comparative genomics of *Phytophthora ramorum* and *P. sojae*. Current Genetics. 51:285-296
- Martin, F. N., Tooley, P. W., & Blomquist, C. 2004. Molecular Detection of *Phytophthora ramorum*, the causal agent of sudden oak death in California, and two additional species commonly recovered from diseased plant material. Phytopathology. 94: 621-631
- McCartney, H. A., Foster, S. A., Fraaije, B. A. & Ward, E. 2003. Molecular diagnostics for fungal plant pathogens. Pest Management Science. 59:129-142
- Meentemeyer, R., Rizzo, D. M., Mark, W., & Lotz, E. 2004. Mapping the risk of establishment and spread of sudden oak death in California. Forest Ecology and Management. 200:195-14
- Menzel, W., Jelkmann, W. & Maiss, E. 2002. Detection of four apple viruses by multiplex RT-PCR asays with coamplification of plant mRNA as internal control. Journal of Virological Methods. 99:81-92

- Meyers Norris Penny, Limited Liability Partnership. 2015. BC Forestry Industry: Economic Impact Study. Available from: http://www.cofi.org/wp-content/uploads/2015/01/bc_industry_impact_01-2015.pdf
- Ministry of Forest, Lands and Natural Resource Operations. 2013. Mountain Pine Beetle. Government of British Columbia. Available from https://www.for.gov.bc.ca/hfp/mountain_pine_beetle/Updated-Beetle-Facts_April2013.pdf
- Mitchell, C.E. & Power, A.G. 2003. Release of invasive plants from fungal and viral pathogens. Nature. 421:625-627
- Mock, K. E., Bentz, B. J., O'Neill E. M., Chong, J.P., Orwin, J., & Pfrender, M. E. 2007. Landscape-scale genetic variation in a forest outbreak species, the mountain pine beetle (*Dendroctonus ponderosae*). Molecular Ecology 16:553-568
- Nichols, D., Lewis, K., Orjala, J., Mo, S., Ortenberg, R., O'Connor, P., Zhao, C., Vouros, P., Kaeberlein, T. & Epstein, S.S. 2008. Short peptide induces an "uncultivable" microorganism to grow in vitro. Applied and Environmental Microbiology. 74:4889-4897
- [NRCan] Natural Resource Canada. 2015. [Online] Forest Products. Government of Canada. [Cited 2016 March 12]. Available from http://www.nrcan.gc.ca/forests/industry/13317
- [NRCan] Natural Resource Canada. 2016a. [Online] Forest Products. Government of Canada. [Cited 2016 August 5]. Available from http://www.nrcan.gc.ca/forests/report/area/17601
- [NRCan] Natural Resource Canada. 2016b. [Online] Mountain Pine Beetle (factsheet). Government of Canada. [Cited 2016 August 5]. Available from http://www.nrcan.gc.ca/forests/fire-insectsdisturbances/top-insects/13397
- Orlando, C., Pinzani, P., & Pazzagli, M. 1998. Developments in quantitative PCR. Clinical Chemistry and Laboratory Medicine. 36:255-269

- Osman, F., & Rowhani, A. 2005. Application of spotting sample preparation technique for the detection of pathogens in woody plants by RT-PCR and real-time PCR (TaqMan). Journal of Virological Methods. 133:130-136
- Osterbauer, N., & Trippe, A. 2005. Comparing diagnostic protocols for *Phytophthora ramorum* in rhododendron leaves. Online. Plant Health Progress doi:10.1094/PHP-2005-0314-01-HN.
- Pace, N.R. 1997. A Molecular View of Microbial Diversity and the Biosphere. Science. 276:734–740
- Paine, T.D., Raffa, K.F., & Harrington, T.C. 1997. Interactions among scolytid bark beetles, their associated fungi, and live host conifers. Annual Review of Entomology. 42:179–206
- Parra, G., Bradnam, K. & Korf, I. 2007. CEGMA: a pipeline to accurately annotate core genes in eukaryotic genomes. Bioinformatics. 23:1061-1067
- Plattner, A., Kim, J. J., DiGuistini, S., & Breuil, C. 2008. Variation in pathogenicity of a mountain pine beetle-associated blue-stain fungus, *Grosmannia clavigera*, on young lodgepole pine in British Columbia. Canadian Journal of Plant Pathology. 30:457–466

Pozhitkov, A. E., Neme, R., Domazet-Loso, T., Leroux, B., G., Soni, S., Tautz, D. & Noble, P.A. 2016. Thanatotranscriptome: genes activity expressed after organismal death. BioRxiv p.058305

- Puspita, I.D., Kamagata, Y., Tanaka, M., Asano, K. & Nakatsu, C.H. 2012. Are uncultivated bacteria really uncultivable. Microbes and Environments. 27:356-366
- Rachmayanti, Y., Leinemann, L., Gailing, O. & Finkeldey, R. 2006. Extraction, amplification and characterization of wood DNA from dipterocarpaceae. Plant Molecular Biology Reporter. 24:45-55
- Rappe, M.S. & Giovannoni, S.J. 2003. The uncultured microbial majority. Annual Reviews in Microbiology. 57:369-394
- Redlin, S. C., Werres, S., & Schröder, T. 2014. Invasive pathogens in plant biosecurity. Case study:
 Phytophthora ramorum Werres et al.: cause of Sudden Oak Death, ramorum leaf blight and
 ramorum dieback. In *The Handbook of Plant Biosecurity*. 593-611

- Riedel, M., Werres, S., McKeever, K., Elliott, M. & Shamoun, S.F. 2012. Histopathological investigations of the infection process and propagule development of *Phytophthora ramorum* on rhododendron leaves. Forest Phytophthoras. 2:593-611
- Ristaino, J. M., & Gumpertz. 2000. New frontiers in the study of dispersal and spatial analysis of epidemics caused by species in the genus *Phytophthora*. Annual Review of Phytopathology 38:541-576
- Rizzo, D.M., Garbelotto, M., Davidson, J.M., Slaughter, G.W. & Koike, S.T. 2002. *Phytophthora ramorum* as the cause of extensive mortality of *Quercus* spp. and *Lithocarpus densiflorus* in California. Plant Disease. 86:205–214
- 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. Annual Review Phytopathology. 43:309–335
- Roe, A.D., Rice, A.V., Bromilow, S.E., Cooke, J.K. & Sperling, F.A.H. 2010. Multilocus species identification and fungal DNA barcoding: insights from blue stain fungal symbionts of the mountain pine beetle. Molecular Ecology Resources. 10:946-959
- Saunders, N.A. & Lee, M.A. 2013. Real-Time pcr: Advanced technologies and applications. Norfolk, England: Caister Academic Press
- Schoch, C. L., Seifert, K. A., Huhndorf, S., Robert, V., Spouge, J. L., Levesque, C. A., Chen, W. & Miller, A.
 N. 2012. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. Proceedings of the National Academy of Sciences. 109:6241-6246
- SIGMA Life Science. 2015. qPCR Technical Guide. Available from: https://www.sigmaaldrich.com/content/dam/sigmaaldrich/docs/Sigma/General_Information/qpcr_technical_guide.pdf
- Six, D. L., & Paine, T. D. 1999. Allozyme diversity and gene flow in Ophiostoma clavigerum (Ophiostomatales: Ophiostomataceae), the mycangial fungus of the Jeffrey pine beetle, Dendroctonus jeffreyi. Canadian Journal of Forest Research. 29:324-331

- Six, D. L. 2003. Bark Beetle-Fungus Symbioses. In: Bourtzis, K., Miller, T. A, editors. Insect Symbioses. CRC Press. pp. 97–114
- Stephens, M. A. 1979. Tests of Fit for the Logistic Distribution Based on the Empirical Distribution Function. Biometrika 66:591-596
- Swiecki, T. J., & Bernhardt, E. 2005. Disease risk factors and disease progress in coast live oak and tanoak affected by *Phytophthora ramorum* canker (sudden oak death). Sudden Oak Death Science Symposium, 2nd. General Technical Report. 196:383-411
- Taylor, D.L., Bruns, T.D., Leake, J.R. & Read, D.J. 2002. Mycorrhizal specificity and function in mycoheterotrophic plants. Mycorrhizal Ecology. Ecological Studies, Vol. 157 (eds M.G.A.Van Der Heijden & I. Sanders). Springer-Verlag Berlin Heidelberg
- Tooley, P., Kyde, K., & Englander, L. 2004. Susceptibility of selected ericaceous ornamental host species to *Phytophthora ramorum*. Plant Disease. 88:993-999
- Tsui, C.K.M., Roe, A.D., El-Kassaby, Y.A., Rice, A.V., Alamouti, S.M., Sperling, F.A.H., Cooke, J.E.K.,
 Bohlmann, J., & Hamelin, R.C. 2012. Population structure and migration pattern of a conifer
 pathogen, *Grosmannia clavigera*, as influenced by its symbiont, the mountain pine beetle.
 Molecular Ecology. 21:71-86
- Tsui, C.K.M., Wang, B., Schoen, C.D. & Hamelin, R.C. 2013. Rapid identification and detection of pathogenic fungi by padlock probes. In: *Laboratory Protocols in Fungal Biology*, pp. 505-517.
 Springer New York.
- Tubajika, K.M., Jonawiak, J.J., Mack, R. & Hoover, K. 2007a. Efficacy of radio frequency treatment and its potential for control of sapstain and wood decay fungi on red oak, poplar, and southern yellow pine wood species. The Japan Wood Research Society. 53:258-263
- Tubajika, K.M., Singh, R., & Shelly, J.R. 2007b. Preliminary observations of heat treatment to control*Phytophthora ramorum* in infected wood species: an extended abstract. Technical report.Available from:

http://www.fs.fed.us/psw/publications/documents/psw_gtr214/psw_gtr214_477-480_tubajika.pdf

- Tyler, B. 2002. Molecular basis of recognition between *Phytophthora* pathogens and their hosts. Annual Review of Phytopathology. 40:137-167
- Uzunovic, A. & Khadempour, L. 2007. Heat Disinfestation of Mountain Pine Beetle-affected wood. Mountain Pine Beetle Initiative working paper. Canadian Forest Service. ISBN: 978-0-662-45373-4
- Van Poucke, K., Franceschini, S., Webber, J., & Brasier, C., 2012. Discovery of a fourth evolutionary lineage of *Phytopthora ramorum*: EU2. Fungal Biology. 116:1178-1191
- Wang, Y., DiGuistini, S., Wang, T. T., Bohlmann, J., & Breuil, C. 2010. Agrobacterium-mediated gene disruption using split-marker in *Grosmannia clavigera*, a mountain pine beetle associated pathogen. Current Genetics. 56:297-307
- Wang, Y., Lim, L., DiGuistini, S., Robertson, G., Bohlmann, J., & Breuil, C. 2013. A specialized ABC efflux transporter GcABC-G1 confers monoterpene resistance to *Grosmannia clavigera*, a bark beetleassociated fungal pathogen of pine trees. New Phytologist. 197:886-898
- Webber, J. F., Mullett, M., & Brasier C. 2010. Dieback and mortality of plantation Japanese larch (*Larix kaempferi*) associated with infection by *Phytophthora ramorum*. New Disease Reports. 22:2044-0588
- Werres, S., Marwitz, R., Man, In'T, Veld, W. A., De Cock, A. W. A. M., Bonants, P. J. M., et al. 2001.
 Phytophthora ramorum sp. nov., a new pathogen on Rhododendron and Viburnum. Mycological
 Research. 105:1155-64

White, R. J. 2009. Gene Transcription: Mechanisms and Control. Hoboken, New Jersey: Wiley-Blackwell

Widmer, T. L. 2009. Infective potential of sporangia and zoospores of *Phytophthora ramorum*. Plant Disease 93:30-35

Zambino, P.J. and Harrington, T.C., 1992. Correspondence of isozyme characterization with morphology in the asexual genus Leptographium and taxonomic implications. Mycologia. 84:12-25

APPENDICES

Appendix A: Geneious assay development criteria

- 1. Intron size: Between 40-100 base pairs (BP)
- 2. High levels of single nucleotide polymorphisms (SNP)
- 3. Increase difference and variation between sequences
- 4. Amplicon size (final product) of DNA fragment shorter than 210 BP
- 5. Tm of the probes should be 8°C-10°C higher than associated primer pairs

Appendix B: List of Isolates

Lineage	Isolate	Origin	Year Collected	Source
NA 1	PR_01_004	OR, USA	2001	FPInnovations
NA 1	Pram_04_2231	BC, Canada	2004	FPInnovations
NA 2	Pram_05_16845	BC, Canada	2005	FPInnovations
NA 2	Pram_07_17204	BC, Canada	2007	FPInnovations
NA 2	PFC_5073	CA, USA	2005	Pacific Forestry Center
EU 1	Pram_07_13013	BC, Canada	2007	FPInnovations
EU 1	PR_09_106	OR, USA	2009	FPInnovations
EU 2	Pram_P2111	North Ireland	2007	FPInnovations
EU 2	Pram_P2460	North Ireland	2010	FPInnovations

Table 4: Isolates of *Phytophthora ramorum* from each of the 4 lineages used in inoculation and heat treatment experiment

Appendix C: Recipes

	Per Reaction (µl)	Concentration
10x Buffer	2.5	1X
dNTP	0.5	200 µM
MgCl ₂	0.75	150 μM
Primer (Forward)	2.5	1 μΜ
Primer (Reverse)	2.5	1 µM
Template	1.0	1 ng
Taq polymerase	0.2	
Distilled Water	15.05	
Total Volume	25.0	

Table 5: Mastermix recipe for polymerase chain reaction

Table 6: QuantiTect Reverse Transcription kit (QIAGEN; gDNA wipeout during cDNA synthesis)

	Per Reaction (µl)
gDNA Wipeout Buffer, 7x	2.0
Template RNA	Variable (up to 1 µg)
RNase-free water	Variable
Total Volume	14.0

Table 7. Quantifect Reverse franscription kit (QIAGEN, CDNA synthesis	Table 2	7: QuantiTect	Reverse Tran	scription kit	(QIAGEN: cDNA	synthesis
---	---------	---------------	---------------------	---------------	---------------	-----------

	Per Reaction (µl)
Quantiscript Reverse-transcriptase	1.0
Quantiscript RT Buffer, 5x	4.0
RT Primer Mix	1.0
Template RNA (gDNA elimination reaction)	14.0
Total Volume	20.0

Table 8: RT-qPCR recipe using treatment samples

	1 Reaction (μl)	Concentration
2X Master Mix	5.0	1X
Primer (Forward)	0.4	400 nM
Primer (Reverse)	0.4	400 nM
Taqman Probe	2.0	200 nM
cDNA Template	2.2	2.2 ng
Total Volume	10.0	

Appendix D: SAS script

* change DATAFILE to the location of desired dataset; **PROC IMPORT** out=salix DATAFILE="E:\sas pr mar28.xlsx" DBMS=EXCEL REPLACE; SHEET="Sheet1\$"; GETNAMES=YES; MIXED=NO; SCANTEXT=YES; USEDATA=YES; SCANTIME=YES; run; options ls=70 ps=50 pageno=1; DATA salix2; SET salix; * set up labels for treatments for plotting; IF (Treatment ='control') AND (Time ='0') THEN treatment='control-0'; IF (Treatment ='control') AND (Time ='6') THEN treatment=' control -6'; IF (Treatment ='control') AND (Time ='12') THEN treatment=' control -12'; IF (Treatment_='control') AND (Time ='24') THEN treatment=' control -24'; IF (Treatment ='control') AND (Time ='48') THEN treatment=' control -48'; IF (Treatment ='control') AND (Time ='96') THEN treatment=' control -96'; IF (Treatment ='Hshort') AND (Time ='168') THEN treatment=' control -168'; IF (Treatment ='Hshort') AND (Time ='240') THEN treatment=' control -240'; IF (Treatment ='Hshort') AND (Time ='0') THEN treatment=' Hshort -0'; IF (Treatment ='Hshort') AND (Time ='6') THEN treatment=' Hshort -6'; IF (Treatment ='Hshort') AND (Time ='12') THEN treatment=' Hshort -12'; IF (Treatment ='Hshort') AND (Time ='24') THEN treatment=' Hshort -24'; IF (Treatment ='Hshort') AND (Time ='48') THEN treatment=' Hshort -48'; IF (Treatment ='Hshort') AND (Time ='96') THEN treatment=' Hshort -96'; IF (Treatment ='Hshort') AND (Time ='168')

```
THEN treatment=' Hshort -168';
IF (Treatment ='Hshort') AND (Time ='240')
THEN treatment='Hshort -240';
IF (Treatment_='Hkiln') AND (Time ='0')
THEN treatment='Hkiln-0';
IF (Treatment ='Hkiln') AND (Time ='6')
THEN treatment='Hkiln-6';
IF (Treatment ='Hkiln') AND (Time ='12')
THEN treatment='Hkiln-12';
IF (Treatment ='Hkiln') AND (Time ='24')
THEN treatment='Hkiln-24';
IF (Treatment ='Hkiln') AND (Time ='48')
THEN treatment='Hkiln-48';
IF (Treatment ='Hkiln') AND (Time ='96')
THEN treatment='Hkiln-96';
IF (Treatment ='Hkiln') AND (Time ='168')
 THEN treatment='Hkiln-168';
IF (Treatment ='Hkiln') AND (Time ='240')
THEN treatment='Hkiln-240';
RUN;
* get boxplots and means by site;
PROC SORT data=salix2;
BY treatment;
RUN;
PROC MEANS data=salix2;
VAR Ratio ;
BY treatment;
RUN;
PROC BOXPLOT data=salix2;
PLOT Ratio_*Treatment_ / boxstyle = schematic;
LABEL Ratio_ = 'Ratio';
LABEL Treatment = 'Treatment ';
RUN; QUIT;
* get boxplots and means by treatment;
PROC SORT data=salix2;
BY Treatment ;
RUN;
PROC MEANS data=salix2;
VAR Ratio ;
BY Treatment ;
RUN;
PROC BOXPLOT data=salix2;
PLOT Ratio *treatment / boxstyle = schematic;
LABEL Ratio = 'Ratio ';
LABEL Treatment = 'treatment';
RUN; QUIT;
```

```
* original biomass data;
PROC GLM data=salix2;
TITLE 'Ratio_';
CLASS Treatment_ Rep_ Time_;
MODEL Ratio_=Treatment_ Rep_ Treatment_*Rep_
Time_ Time_*Treatment_;
RANDOM Treatment_ Treatment_*Rep_;
TEST h=Treatment_ e=Time_*Treatment_;
LSMEANS Time_ Time_*Treatment_/tdiff pdiff;
LSMEANS Treatment_/e=Time_*Treatment_ tdiff pdiff;
OUTPUT OUT=glmout1 PREDICTED=predict1 RESIDUAL=resid1;
RUN; QUIT;
```

```
* residual plot;
AXIS1 LABEL = ('Residuals');
AXIS2 LABEL = ('Predicted Biomass');
PROC GPLOT data=glmout1;
PLOT resid1*predict1 / vaxis=axis1 haxis=axis2 vref=0;
RUN;
```

```
* normality tests and normal probability plots;
PROC UNIVARIATE data=glmout1 plot normal;
VAR resid1;
LABEL resid1 = 'Residual';
RUN;
```

Appendix E: Statistical analysis

The residual plots of *P. ramorum* indicated that the relationship between the treatment and time variables was linear; the residuals were balanced between the negative and positive values along the x-axis (Figure 16). The assumption of equal variance appears to be met, as the width of values scattered is fairly consistent across the x-axis (Figure 16). It is worth noting that the scatter is slightly sparser within the middle third of the diagram (x-axis), indicating that there may be some variability in the residuals for different values of x. The assumption of normal distribution is also met, as the plotted values on the normality plot (Figure 17) appears to be closely following the hypothetical line. Values from the normality test (Table 9) show that all p-values to be greater than an alpha of 0.05 which accepts the H_0 of the residuals being normally distributed.



Figure 16: *Phytophthora ramorum* residual distribution generated from SAS 9.4 using RNA/DNA C_T ratios against all treatments (control, kiln, short). The plot shows a fairly equal distribution



Figure 17: Distribution and normality plot for residuals for Phytophthora ramorum

Table 9: Phytophthora ramorum test of normality using RNA/DNA CT ratio data set of all treatments (control, ki	iln,
short). All p-values are greater than the alpha of 0.05- indicating residuals are normally distributed	

Test	Statistic			P-value
Shapiro-Wilk	W	0.986205	Pr < W	0.6483
Kolmogorov- Smirnov	D	0.063744	Pr > D	> 0.1500
Cramer-von Mises	W-Sq	0.042096	Pr > W-Sq	> 0.2500
Anderson-Darling	A-Sq	0.270798	Pr > A-Sq	> 0.2500

For the coefficient of multiple determination, R^2 , the value is 0.8546, meaning that 85.4% of the variation in the C_T ratios were accounted for by the regression. This indicated a moderately good fit. An F-test of the model indicated that the model is significant. The calculated F-test of 8.40 has a p-value of less than 0.0001, further validating the significance of the model (Table 10).
Table 10: *Phytophthora ramorum* ANOVA using RNA/DNA C_T ratio data set of all treatments (control, kiln, short)-testing the treatment model's significance

Source	DF	SS	MS	F Value	P-value
Model	28	2.34393108	0.08371182	8.40	<0.0001
Error	40	0.39878929	0.00996973		
Corrected Total	68	2.74272037			

The statistical analysis of *G. clavigera* was not optimal and did not show a normal distribution with the data both from the residual plot (Figure 18) and the tests of normality (Table 11). The data points did not accurately align with the curve of predicted values and there appears to be a horizontal plateau of values across the middle (Figure 18). The curve begins at below the normal line, bends to vaguely follow it, then end above indicates long tails- suggesting there are more variance that expected in a normal distribution. Looking at the normality tests (Table 11), none of the p-values were greater than the alpha of 0.05, failing to reject the H_o and indicating the residuals are not normally distributed. However, when looking at the histogram of residuals generated a bell-shaped curve is produced, with a fairly balanced taper on either sides (Figure 18). This shape illustrates a "normal" distribution. The associated box and whisker plot (Figure 18), showed outliers at the upper and lower limits which could potentially be the reason for the increase in variance of the data.



Figure 18: Normality plot of *Grosmannia clavigera* using RNA/DNA C_T ratio data set of all treatments (control, kiln, short) with histogram and boxplot.

Table 11: *Grosmannia clavigera* tests of normality using RNA/DNA C_T ratio data set of all treatments (control, kiln, short).

Test	Statistic			P-value
Shapiro-Wilk	W	0.986205	Pr < W	0.0025
Kolmogorov- Smirnov	D	0.063744	Pr > D	<0.0100
Cramer-von Mises	W-Sq	0.042096	Pr > W-Sq	<0.0050
Anderson-Darling	A-Sq	0.270798	Pr > A-Sq	<0.0050

The residual distribution of *G. clavigera* (Figure 19), illustrated a relatively linear relationship of treatment and time, as the residuals are balanced between the negative and positive values along the x-axis. The scatter width of the values appear was fairly consistent across the x-axis (Figure 19) and there does not appear to be taper or skew in any direction. Though not all assumptions were met for the statistical analysis, it was very close to the favourable condition and therefore, the output was still analyzed to investigate potential trends. The model is significant with a calculated F-value of 5.10 and a p-value of <0.0001 (Table 12). For the coefficient of multiple determination, R^2 , the value is 0.778952, meaning that 77.9% of the variation in the C_T ratios were accounted for by the regression. This indicated a moderately good fit.



Figure 19: *Grosmannia clavigera* plot of residual distribution using RNA/DNA CT ratio data set of all treatments (control, kiln, short). Plot shows points scattered fairly equal along the x-axis- indicating a relatively equal distribution of residuals.

Table 12: *Grosmannia clavigera* ANOVA using RNA/DNA CT ratio data set of all treatments (control, kiln, short)-testing the treatment model's significance.

Source	DF	SS	MS	F Value	P-value
Model	29	53.53939768	1.84618613	5.10	<0.0001
Error	42	15.19322318	0.36174341		
Corrected Total	71	68.73262085			

Statistical Improvement

The *G. clavigera* dataset of RNA to DNA C_T ratios was not able to perfectly fit the requirements of normal distribution in order to perform an analysis of variance (ANOVA). The residual plots and normality tests failed to show a linear relationship between the treatment and time variables, but met all other assumptions. Though the histogram of residuals followed a symmetrical bell-shape, the residual plot showed a plateau in the middle before bending to follow (Figure 17). This suggests more variance than expected in the normal distribution. The test most likely picked up the little variance of some samples and the great difference between that and other treatments. Seeing that the sample size is small, the test for goodness of fit is more likely to fail to reject when it matters. The ratio shows large variation in values for both Kiln and short treatment, but little fluctuation with the control samples was observed (Figure 20). If the sample size is increased, there will be a decrease in variation; however, processing an increased amount of samples will involve substantially more time and resources.



Figure 20: Boxplot of Grosmannia clavigera cDNA/gDNA ratio by treatment (Control, Kiln, short 1hr at 70°C)

The experiment illustrated a significant effect of treatment on samples as well as a significant treatment-time interaction. When looking to investigate the interaction of treatment at each time point, it was difficult to find significant values. The statistical analysis could be improved on with a larger sample size. The present study has 3 biological replicates within each treatment-time point to compare, decreasing the power of the test. The F-value of treatment is 72.38 for P. ramorum and 25.22 for G. clavigera, while the F-value of time is 4.73 and 1.99, respectively. When comparing that to the treatment-time interaction F-values of 4.19 and 2.70, there is a noticeably lower value, indicating a weaker interaction. Though the time points have a significant interaction on expression, it is comparatively lower than treatment. The sample size must be increased in order to improve power and show the specific interactions between different treatments and time points. An improvement involves increasing the number of RNA samples extracted for each biological replicate. Though having screened and quantified the quality and quantity of the extraction, the use of a single RNA extraction as a representation of the entire biological replicate can mask potential contamination and technical errors, alongside decreasing the soundness of statistical analyses. This fault is further enhanced by only using 3 samples per treatment-time. By increasing the sampling size and replicates, there is a better insurance of reliable data.

Appendix F: Sensitivity standard curve



Figure 21: Standard curve produced by serial dilution (1:10) of *Phytophthora ramorum*, isolate 5073 using Taqman probe, PH178_EX R²= 0.998 Eff%= 92.332



Figure 22: Standard curve produced by serial dilution (1:10) of *Grosmannia clavigera*, isolate KW1407 using Taqman probe, MS359_EX. R²= 0.981 Eff%= 100.303