Detection of the Oak Wilt Pathogen in Sapwood of Northern Oak Species Using PCR Technology

A THESIS
SUBMITTED TO THE FACULTY OF
UNIVERSITY OF MINNESOTA
BY

ANNA MARIE YANG

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF MASTER OF SCIENCE

Jennifer Juzwik, advisor

June 2015

Acknowledgements

This project would not have been completed without the help and guidance from numerous people. Many thanks are due to Dr. Jennifer Juzwik, who has supported me through this entire project. Her expertise in oak wilt is absolutely unparalleled. I would also like to thank my committee members, Dr. Deborah Samac, for spending so much time helping to troubleshoot my real-time PCR reactions and Dr. Gary Johnson, for giving me the encouragement and tools to share my research with field professionals. I also thank Dr. Dimitre Mollov for sparking my interest in plant disease diagnostics and for giving me exposure to the University of Minnesota Plant Disease Clinic.

Funding for this project and for my graduate education came from the Diversity of Views and Experiences (DOVE) Fellowship, College of Food, Agriculture, and Natural Resources Science (CFANS) Fellowship, USDA Forest Service Special Technology Development Program NA-2012-05, and the Minnesota Turf and Grounds Foundation.

The beta-testing of these protocols would not exist without all of the help received from the University of Minnesota Plant Disease Clinic and University of Wisconsin-Madison Plant Disease Diagnostic Clinic. Brett, Jennifer, Brian, Ann, and Sean all contributed countless hours to help with this project.

I would also like to thank Paul Castillo for the invaluable field assistance; I would have been lost without it. To all of my friends and peers in the U.S. Forest Service Greenhouse Annex, you are all truly incredible scientists and I would not have stayed afloat without your encouragement. Much credit is also due to the many cities that volunteered time and trees for this project.

Finally, I would like to thank my family and friends, especially Paul, for being there when I needed support, being flexible when my schedule was chaotic, and being ready to celebrate when it was all over. I love you all to the moon and back.

Thesis Abstract

Early and accurate diagnosis of oak wilt, caused by *Ceratocystis fagacearum*, is important when a disease control action is planned. When laboratory diagnosis is needed, standard isolation protocols that are used rely on high quality samples and require up to 14 days for incubation. New methods, involving the use of polymerase chain reaction (PCR) have the potential to offer faster and more reliable plant disease diagnostics. The purpose of this thesis was to 1) compare results from use of a nested PCR and a real-time PCR protocol for detection of *C. fagacearum* in sapwood drill shavings to results using published isolation methods and 2) develop standard branch sampling and branch segment processing guidelines for oak wilt diagnostics using the PCR protocols, and 3) test and evaluate the use of the protocols in operational plant disease diagnostic clinics. All assays were performed on samples obtained from *Quercus rubra/Q. ellipsoidalis* (red oak), *Q. macrocarpa* (bur oak), and *Q. alba* (white oak) trees in Minnesota. Drill shavings were collected from actively wilting oak trees as well as from tissues taken the year following branch or whole tree death.

Between the assays tested (nested PCR, real-time PCR, and isolation), the nested PCR assay resulted in the highest estimated probability of *C. fagacearum* detection for branch segments sampled from actively wilting oak trees. For bur and white oak branches that had been dead for ≥ 1 year**,** the pathogen was detected using nested PCR (55 and 87% of branch segments, respectively) and real-time PCR (19%, white oak only), while isolation was not able to detect the pathogen in any branch segments. All three methods were able to detect the fungus in main stem samples of red oaks with a streaking

cambium; however, the molecular protocols resulted in more frequent pathogen detection. Only the molecular assays detected the pathogen in sapwood samples underlying the remnants of *C. fagacearum* sporulation mats on main stem samples of red oak trees. The fungus was not detected by either technique in branch segments obtained from healthy oaks (controls).

Inter-laboratory testing of the nested PCR and real-time PCR protocols supported the previously described differences in assay reliability. Overall, the comparison of traditional isolation and nested PCR by the University of Minnesota Plant Disease Clinic resulted in the same number of trees testing positive for the pathogen by both detection methods. A comparison of isolation methods and real-time PCR was completed by the University of Wisconsin-Madison Plant Disease Diagnostic Clinic, which resulted in a higher rate of detection using isolation methods.

TABLE OF CONTENTS

List of Tables

List of Figures

Chapter 1. Introduction and review of oak wilt, caused by *Ceratocystis fagacearum,* and disease detection methods

Anna M. Yang

Oak as a Resource and its Distribution in the United States

Oak trees in the genus *Quercus* (Family Fagaceae) have a long history as highly valued trees. There are numerous applications in which oak trees and their products have been used by humans, including shipbuilding, furniture making, tanning, cooperage for beverages, and use as landscape trees (Johnson et al. 2002). They also hold high ecological value, serving as a habitat and food source for wildlife populations.

Globally, there are roughly 400 species of oak, approximately 70 of which are found within the United States (Johnson et al. 2002). Oak species are widely distributed in the United States and occupy a significant portion of oak-hickory, oak-pine, and oak-gum-cypress forests. The North Central Region of the United States (Indiana, Illinois, Iowa, Michigan, Minnesota, Missouri, and Wisconsin) has 77 million acres of total forested land and 14% of the national timberland. Within those 77 million acres, oak-hickory forests contribute to 29% of the total forested land, making it the most abundant forest type (Shifley and Sullivan 2002). A 2013 inventory conducted by the U.S. Forest Service, Forest Inventory and Analysis (FIA) program at the Northern Research Station, estimated that Minnesota has around 2.2 million forested acres in which oak species are a major component (Miles et al. 2014).

Minnesota is home to species in the red oak group (section *Lobatae*) and white oak group (section *Quercus*). The most common species are northern red oak (*Quercus rubra*), northern pin oak (*Quercus ellipsoidalis*), bur oak (*Quercus macrocarpa*), and white oak (*Quercus alba*).

Oak wilt, caused by the fungus *Ceratocystis fagacearum*, is a destructive disease of oaks that threatens the health of oak trees in the United States. The disease has become established in the southern half of Minnesota, particularly in the Minneapolis – St. Paul metropolitan area. As of 2015, oak wilt continues to be confirmed in new counties in the state. Oak trees are ecologically, economically, and aesthetically valuable, thus their loss due to oak wilt is of significant concern. The disease was first observed in the late 1800's in Wisconsin, but the causal agent was unknown. Investigations into the biology and detection of *C. fagacearum* have been ongoing since the cause of disease was identified in the early 1940's and successful management for the disease has been developed.

Oak Wilt

Disease distribution. Oak wilt is common in the Midwest, but its range stretches to New York and Texas. It is currently known to exist in 24 states (Juzwik et al. 2011). It commonly occurs in the North Central Region of the United States. A 2015 survey map released by the Department of Natural Resources indicated that the disease is currently present in 25 counties in Minnesota. Factors such as the wounding of healthy oaks from urbanization and wind damage have contributed to the progressive spread of *C. fagacearum* in counties surrounding the Minneapolis – St. Paul Metropolitan area (Juzwik and Schmidt 2002). Other common components contributing to the current spread of the pathogen in Minnesota are soil type, stand density, and movement of infected firewood.

Host susceptibility. *C. fagacearum* has been shown to infect 33 *Quercus* species, either under natural conditions or by artificial inoculation (Tainter and Baker 1996). Other Fagaceae species are also susceptible to the pathogen, including three species of *Castanea*, one species of *Castanopsis* (Chinquapin), and one species of *Lithocarpus* (Tainter and Baker 1996). Multiple oak species exist in most regions where the pathogen is found. Oak wilt is most severe in members of the red oak group, while species of the white oak group display some natural resistance to the disease development (Gibbs and French 1980). Within the white oak group, *Q. macrocarpa* has moderate resistance to disease progression and *Q. alba* is highly resistant, often surviving several years before tree mortality (Juzwik et al. 2011).

Pathogen biology. The oak wilt fungus produces two types of spores: conidia and ascospores. Conidia, produced by the asexual or imperfect stage, were the first spore type to be discovered in infected oak trees by Henry (1944). Cultures were described as having gray to olive-green mycelium with occasional patches of tan, septate hyphae with conidiophores bearing endogenously produced conidia. The fungus was subsequently named *Chalara quercina.* The fungus was renamed *Endoconidiophora fagacearum* by Bretz (1952) when perithecia containing ascospores, produced by the sexual or perfect stage, of the fungus were discovered. The sexual structures described were black, flask-shaped perithecia which were produced by pairing different strains of the fungus in culture (Bretz 1952). Perithecia contain asci from which ascospores are exuded through the ostiole of a perithecium in a sticky mass. Production of perithecia led to recognition

of the heterothallic nature of the fungus. Both spore types are commonly produced on fungal spore mats that develop underneath the bark of infected red oak trees. In 1956, following a taxonomic re-evaluation, the fungus was renamed *Ceratocystis fagacearum* (Bretz) Hunt (Hunt 1956).

Disease symptoms. Crown symptoms that develop from infection by the pathogen are most dramatic on members of the red oak group. Mature leaves on an infected red oak may appear dull green or water-soaked followed by the development of bronzing starting at the leaf margin. Progressive bronzing or browning occurs from margin to mid-rib of the leaves. These wilt symptoms develop in the upper crown of the tree and progress downward and inward, often producing full crown wilt within a few weeks after initial infection. Leaf abscission in both green and symptomatic leaves during the growing season is common. Dense, short-lived sucker sprouts may develop on the bole of infected trees, but quickly become symptomatic. Foliar symptoms of the disease on red oak are often accompanied by the presence of a blue-grey to brown discoloration in the outer sapwood rings. Sapwood discoloration may or may not be present and can vary in color and appearance, making it unsuitable to use alone for oak wilt diagnosis. Once infected, red oak trees do not recover from the disease. Occasionally, red oak trees may become infected late in the season, allowing them to survive over the winter. In these scenarios the trees eventually succumb to death the following spring or summer (Bretz 1951; MacDonald and Hindal 1981, Juzwik et al. 2011; Tainter and Baker 1996).

Contrasting the full crown symptoms observed on red oak species, white oak species have varying degrees of resistance to oak wilt. In general, leaf discoloration more closely resembles fall coloration and leaves can remain on the tree following branch death. Trees exhibit more localized symptoms with a scattered dieback in one or more branches over the course of a growing season. Symptoms may manifest as wilted foliage on the tips of several branches or as entire branch death. In *Q. macrocarpa*, progressive branch death can occur over one to several years before the entire crown has wilted. The most resistant species, *Q. alba,* can survive with an infection for multiple years and, in some cases, disease progression may cease. Dark discoloration in the outer sapwood is much more distinct in infected white oak species than in species of the red oak group and vascular discoloration can be easily seen in a cross-section of an infected branch (Bretz 1951; Juzwik et al. 2011; Tainter and Baker 1996).

The development of oak wilt symptoms has been well studied and documented. However, a number of other pests and pathogens may produce symptoms that may be mistaken for oak wilt. Oak anthracnose, caused by *Apiognomonia quercina*, can produce brown lesions on the leaves and cause premature leaf drop. Although not currently reported in Minnesota, bacterial leaf scorch (*Xylella fastidiosa*) produces foliar symptoms on both red and white oak species that are close in appearance to oak wilt. Symptoms may include leaf browning and branch mortality. Unlike oak wilt, bacterial leaf scorch does not lead to rapid tree mortality. Infestation by two-lined chestnut borer, *Agrilus bilineatus*, can occur on stressed oak trees, causing single or scattered branch mortality. Two-lined chestnut borer is often one of several biotic and abiotic factors associated with

oak decline. Agents such as drought, defoliation, fungal pathogens of the stem or root (other than *C. fagacearum*), and wood boring insects may all contribute to oak decline (Juzwik and Schmidt 2000; O'Brien et al. 2000). The recent discovery of bur oak blight in Minnesota has caused further confusion in the diagnosis of oak wilt. *Tubakia iowensis*, the causal agent of bur oak blight, results in the production of large areas of chlorosis and necrosis on the leaves. Branches bearing symptomatic leaves can be scattered or the entire crown may be affected, often times giving the tree a wilted appearance (Pokorny and Harrington 2011). The combined occurrence of these biotic and abiotic disorders places an importance on the accurate identification of oak wilt, particularly in scenarios where effective management is available and justified.

Disease cycle. The oak wilt fungus is spread to healthy trees by two methods: belowground transmission through grafted roots of healthy and nearby infected trees, and aboveground transmission via insect vectors (Gibbs and French 1980; Juzwik et al. 1985; MacDonald and Hindal 1981). Belowground spread of the fungus is responsible for the majority of trees killed by the disease; however, overland transmission of the fungus via vectoring beetles is usually required for the initiation of new infection centers.

The passive movement of spores through grafted roots of a healthy and an infected tree is possible because of the continuous xylem that exists between the two trees (Gibbs and French 1980). The pathogen spread through this connection results in the progressive outward expansion of infection centers. In Minnesota, the majority of oak-wilt trees deaths are associated with this type of disease transmission. The extent to

which root grafting occurs in a stand depends on numerous site conditions, such as the soil type and the composition of oak species present (Bruhn et al. 1991). Intraspecific root grafting is much more common than interspecific root grafting (Bruhn et al. 1991). For example, northern pin oak trees are much more likely to form root graft connection with other northern pin oak trees as opposed to bur oak trees. Additionally, members of the red oak group produce root grafts more frequently than members of the white oak group. Although root grafts between species of the white and red oak groups can occur, they are rare. In Minnesota, deep sandy soils favor root grafting. On such soils, radial expansion of disease centers ranges from 7.6 to 12 m per year (Juzwik et al. 2011).

Nitidulid beetles have been found to be most responsible for aboveground transmission of oak wilt in Minnesota (Gibbs and French 1980). The predominant vectors of overland spread in Minnesota are *Carpophilus sayi* and *Colopterus truncatus* (Juzwik et al. 2004). Nitidulid beetles are attracted to the fruity volatiles emitted by fungal mats and the insects acquire both conidia and ascospores when feeding on the mats. The beetles are also attracted to fresh wounds on healthy oak trees and will deposit *C. fagacearum* spores as they move across surfaces of xylem-exposing wounds. Xylem-penetrating injuries sustained from severe weather, pruning, and construction aid in the overland transmission of the fungus. Other insects, such as the oak bark beetle (*Pseudopityophthorus minutissimus*), are considered to be a minor vector species of *C. fagacearum*.

After the fungus has entered the healthy tree, either by root graft connections or insect dispersal, it spreads through the xylem vessels of the outer sapwood. This spread

occurs quickly in red oak species. During this time, the fungus is spread through the tree as conidia that are carried by the transpiration system of the tree (Jacobi and MacDonald 1980). Host trees produce tyloses and gums in response to the presence of the pathogen. These plug vessels and cause the tree to wilt. Once a tree is moribund, fungal hyphae colonize adjacent parenchyma cells where they will continue to grow intercellularly and intracellularly (Tainter and Baker 1996). While *C. fagacearum* also produces toxins that may damage host tissue, tree mortality is the result of occlusions (tyloses and gums) in the water conducting vessels of the xylem (MacDonald and Hindal 1981; Tainter and Baker 1996). *C. fagacearum* has been isolated from the xylem of roots, trunk, branches, twigs, and petioles of infected oak trees (Gibbs and French 1980). The development of tyloses and rate at which they are produced varies by the host species (Jacobi and MacDonald 1980). Differences in water transport patterns between red and white oak species may contribute to the observed differences in symptoms and host susceptibility to the pathogen (Kowalski and Winget 1963).

Following full wilt and death of the tree, the cambium turns brown and the fungus continues to grow extensively in the xylem vessels. The fungus may eventually produce sporulation mats on the cambium for up to a year following tree death. Peak mat formation occurs during the spring and fall, depending on when the tree wilted and the moisture content of the tree (Tainter and Baker 1996). Sporulation mats are characterized by a grey to olive-green layer of mycelium, conidiophores, and opposing elongated pressure pads that form against each other, one on the sapwood and one on the inner bark (Juzwik et al. 2011). The formation of raised pressure pads often exert enough pressure

to separate the bark from the wood and result in vertical cracks in the bark, allowing the fruity aroma of the fungus to attract sap beetles (Gibbs and French 1980). The presence of sporulation mats is considered to be the most diagnostic sign of an oak wilt infection; however, they are not produced on all infected oak trees. In general, members of the red oak group most frequently produce sporulation mats, but mats are known to occasionally occur on bur oak species and rarely on *Q. alba* (Cones 1967; Nair and Kuntz 1963). Furthermore, sporulation mats will not be produced on all wilted red oak trees. The formation of oak wilt mats are dependent on moisture content of the sapwood and require 37 - 55% moisture to be induced (Tainter and Baker 1996). Temperature and precipitation also influence the production and longevity of mats. The formation of spore mats is the final component of pathogen growth in the tree and they are quickly degraded or outcompeted by other microorganisms (Gibbs and French 1980).

Disease Management

Many methods of controlling the spread of the oak wilt pathogen have been investigated. Successful management protocols aim to interrupt the movement of the pathogen during specific stages of the disease cycle, both above and belowground. The removal of inoculum sources (i.e. sanitation) is key to the prevention of aboveground spread of the fungus. In Minnesota, the cambial conditions of symptomatic red oak trees are re-examined in the fall and winter following full crown wilt. Red oaks that exhibit complete crown with are generally considered to be potential spore producing trees (i.e. they may produce oak wilt mats the following spring). In Minnesota, potential spore

producing trees should be removed and properly disposed of prior to April 1st to prevent spread of the fungus via vectoring beetles (Juzwik et al. 2011). Effective disposal methods include chipping, burning, or cutting logs into firewood, covering with plastic and sealing the edges (O'Brien et al. 2000). In the latter case, plastic tarps should remain through the subsequent growing season.

Another method of preventing overland disease spread is the avoidance of tree wounding during high-risk periods. Guidelines that discourage pruning between the months of April and June have been published for Minnesota (French and Juzwik 2002). The high-risk period in Minnesota coincides with peak spore mat formation and peak insect vector flight periods (Ambourn et al. 2005). The low risk period for Minnesota is considered to be mid-July through October and the safe period is considered to be November through March (French and Juzwik 2002).

Prevention of the belowground spread of oak wilt is accomplished through the mechanical disruption of connected root systems. In Minnesota, a vibratory plow with a 152.4 cm long bade is commonly used for this purpose. Location of plow lines are designed to contain the pathogen to diseased trees and trees that may have the pathogen but have not yet started to exhibit symptoms. (Bruhn et al. 1991). The blade is inserted in the soil and pulled along the marked path. A model for the placement of plow lines has been developed for Minnesota (French and Steinstra 1975). The model utilizes both primary and secondary plow lines to limit transmission of the fungus. Primary plow lines are put in place two (or more) healthy trees away from the actively wilting tree(s). Secondary plow lines are placed between wilted trees and adjacent trees with

asymptomatic crowns. Mathematical models for the placement of plow lines have also been developed. These take into account soil type, tree diameters, rate of pathogen movement via grafts, and distance between trees (Bruhn et al. 1991).

Oak wilt can also be managed in a preventative manner with the application of propiconazole, a systemic fungicide that is applied to the root crown. In Minnesota, propiconazole is commonly used as a preventative treatment for healthy red oaks that are within root grafting distance to red oak trees that are infected with *C. fagacearum.* This is especially common in situations where the disruption of connected root systems is not possible (Blaedow et al. 2010). Injections of propiconazole have been found to prevent disease development for a minimum of 24 months in red oak species with infected root systems before requiring further applications (Blaedow et al. 2010). Propiconazole may be used as either a preventative or therapeutic treatment in white oak species. A five-year study investigated the use of propiconazole as a preventative treatment to protect apparently healthy white oak trees that were within root grafting distance from nearby infected with oak trees (Eggers et al. 2005). Only one tree developed wilt symptoms (<10% of the crown) over the five years of the study. The same study evaluated the therapeutic use of the fungicide in infected white oak trees (5-50% crown wilt) and found that treatment was able to arrest development of symptoms. Although the use of propiconazole is able to arrest development of the disease, it is not able to eradicate the fungus from the tree and re-treatments are necessary to continue disease suppression (Blaedow et al. 2010).

Like most plant diseases, effective management often depends on the integrated use of multiple tools. Successful oak wilt control programs must be tailored to fit the conditions and requirements of individual sites.

Oak Wilt Detection

Current methods for disease diagnosis. Effective oak wilt management is entirely dependent on the early and accurate diagnosis of the disease. The presence of sporulation mats is considered to be a definitive sign of the disease, but they can be difficult to detect if produced at all. The ephemeral nature of spore mats and variability in their production adds to detection difficulties. Deteriorated spore mats often leave a dark-grey to black, oval-shaped stain on the outer sapwood that is difficult to discern from other discolorations on moribund oak trees. Isolation of *C. fagacearcum* in culture in a diagnostic laboratory is a common practice when an official confirmation of the disease is required. Protocols for sample collection and fungus isolation have been published (Pokorny 1999). Tree health professionals are advised to collect samples from up to three partially wilted branches and to look for sapwood discoloration. Branches are recommended to be at least 0.39 cm (1 inch) in diameter and cut into 15 to 20 cm (6 to 8 inch) lengths. Bole samples may also be taken when branches are too high for sampling. Samples must be kept cool during transport to the diagnostic laboratory. To ensure sample quality, it is highly recommended that samples arrive as soon as possible to the diagnostic laboratory.

Once symptomatic samples have reached the laboratory, the diagnostician will attempt to isolate *C. fagacearum* from the branch or stem samples. Branches are surface

sterilized by spraying with 95% ethanol and then flaming. The bark is removed with a sterilized knife to expose the outer sapwood. Small pieces (approximately 0.6 cm) of discolored sapwood are removed from the branch using a sterile wood gouge or knife. With a sterile forceps, the wood chips are plated on to acidified potato dextrose agar (APDA) petri dishes. The petri dishes are incubated at ambient room temperature and lighting to allow for fungal growth. Successful growth of the fungus generally requires 5-14 days of incubation. The fungus is identified by the gray-green color of the mycelium and the characteristic fruity odor that is emitted. Common microscopic characteristics of conidiophore morphology and size and shape of endoconidia are also important in isolate identification (Barnett 1953). Plates are usually held for a maximum of 14 days before being determined negative. Subcultures are required when multiple fungi grow out of the plated wood chips.

Limitations to current method. Successful isolation of the fungus from symptomatic xylem tissues has numerous limitations related to sample quality. *C. fagacearum* is a poor saprophyte and is quickly replaced in the sapwood by secondary microorganisms following tree death. Thus, the fungus can no longer be cultured the year following tree death. Improper storage of sampled material can also death the fungus and lead to a false-negative report. The likelihood of culturing the fungus decreases when samples are stored at high temperatures. A 1953 examination on the effect time of and temperature on oak wilt isolation found that small diameter branches (under 2.5 cm) that are stored at 28°C and above for more than six days are unlikely to result in *C.*

fagacearum cultures (Bretz and Morison 1953). Branches of varying diameter stored for three or more days at 35°C will also lead to a false negative result. The same study also found that in partially wilted (not dead) branches, the growth of contaminating microorganisms increased as the time between sampling and culturing increased. Furthermore, it has been noted that seasonal drought can influence the ability to isolate the fungus from sapwood (Hudelson, B., *personal communication*). Oak wilt laboratory diagnostic service generally ceases in mid-September, but the cutoff date varies depending on weather conditions (Flynn, J., *personal communication*).

Molecular methods for pathogen detection. Traditionally, fungal plant diseases have been detected by the interpretation of visual symptoms and culture-based methods of laboratory identification (McCartney et al. 2003). Methods of diagnosis can be time consuming and are subject to factors that may lead to false-negative results. The advent of polymerase chain reaction (PCR) during the mid-1980's dramatically changed the manner in which plant pathogens are studied and detected. PCR is a method for synthesizing millions of copies of DNA fragments of interest within a few hours and now exists in multiple variations, including nested PCR and real-time PCR. The ribosomal DNA (rDNA) internal transcribed spacer region (ITS) has been established as a useful region for detection of and/or distinguishing among fungal species (Schoch et al. 2011). The use of molecular diagnostics for the detection of *C. fagacearum* could decrease the time between sample collection and diagnosis. Furthermore, PCR-based diagnostics

could allow for successful identification of pathogen DNA in situations where fungal spores are no longer viable and cannot be obtained in culture.

Nested PCR is a modification to conventional PCR that involves two consecutive rounds of DNA amplification. Wu et al. (2011) utilized a nested PCR design for the amplification of *C. fagacearum* from artificially inoculated, sterile wood strips (3 x 1 x 0.2 cm). The first round of PCR employed the universal fungal primers (ITS1 and ITS4), while the second round used primers that were specifically developed for the amplification of *C. fagacearum* (CF01 and CF02). Products of the nested PCR protocol were visualized on an ethidium bromide-stained gel following the second round of amplification. The protocol was designed for international regulatory use; however, further development could lead to a diagnostic tool for *C. fagacearum* detection in sapwood of suspect trees.

Real-time PCR offers a faster alternative to conventional PCR by removing the need for post-processing steps. There are multiple methods of real-time PCR technology, most of which are either amplicon sequence non-specific or amplicon sequence specific (Schena et al. 2004). Amplicon sequence non-specific methods are protocols that utilize dyes that fluoresce when bound to double stranded DNA (e.g. SYBR Green 1). Amplicon sequence specific protocols are characterized by the use of oligonucleotide probes labeled with a fluorophore and a quencher (Schena et al. 2004). When both the fluorophore and quencher remain attached to the probe, all fluorescence emitted by the fluorophore is transferred to the quencher. During amplification, the probe binds to its target sequence and the fluorophore is cleaved during Taq polymerase activity. Because of this, the level

of fluorescence emitted from the reaction is proportional to the number of amplicons produced. Detection and quantification of target DNA is monitored using the threshold cycle (Ct), which occurs during the exponential stage of DNA amplification. Amplification curves of experimental samples can be compared to that of known standards to calculate DNA concentration or copy number of experimental samples.

There are a number of published studies utilizing real-time PCR protocols for detection and quantification of plant pathogens in plant material (Kurdyla et al. 2011; Samuelian et al. 2011; Sidkar et al. 2014; Wu et al. 2011). A primer and probe set has recently been developed for the detection of *C. fagacearum* and has been tested on spore suspensions from a pure culture, purified DNA, and symptomatic sapwood chips from live oak and Monterey oak trees in Texas (Kurdyla et al. 2011). The upper-Midwest states could benefit from the improved speed and specificity that this real-time PCR protocol offers.

The nested PCR and real-time PCR protocols have the potential to alleviate many of the issues with oak with diagnostics that are related to sample quality. Samples that have been exposed to heat may no longer have viable fungus remaining, however fungal DNA is likely still present. Similarly, PCR methods should allow for the discrimination between *C. fagacearum* and other contaminating fungi in samples taken from dead branches.

Thesis Objectives

The basic protocol development for PCR-based detection of *C. fagacearum* has been accomplished (Kurdyla and Appel 2011; Wu et al. 2011), yet the technology has not been tested and/or modified for use by operational diagnostic clinics in the upper-Midwest states. The primary goal outlined in this thesis was to test and evaluate the nested PCR (Chapter 2) and real-time PCR (Chapter 3) protocols for the detection of *C. fagacearum* in 1) the sapwood of actively wilting oak trees in Minnesota, and 2) the sapwood of trees one year after death in the same locations. If the methods proved reliable and accurate, the second objective (Chapter 4) was to develop oak wilt sampling and processing guidelines to complement current diagnostic standards by plant disease diagnostic laboratories. Beta testing of the guidelines by operational diagnostic laboratories would provide feedback on accuracy, practicality, and feasibility of the protocols. Testing the new guidelines would also help to determine if molecular protocols could decrease the time between the submission of samples and detection of the pathogen.

Chapter 2. Use of nested PCR for the detection of the oak wilt pathogen, *Ceratocystis fagacearum*, in the sapwood of diseased northern *Quercus* species

Anna M. Yang

Introduction

Oak wilt, caused by the fungus *Ceratocystis fagacearum* (Bretz) J. Hunt, is an important disease of oak in the eastern USA and in Texas. Epidemics are currently ongoing in portions of Lake States and Texas (Juzwik et al. 2011). If left unmanaged, oak wilt has the ability to dramatically alter both urban and natural ecosystems (Appel 1995). Oak (*Quercus*) species differ in their susceptibility to the disease. Members of the white oak group (section *Quercus*), such as *Q. macrocarpa* and *Q. alba*, are moderate to low in susceptibility with symptoms ranging from scattered branch dieback in the crown to single limb or stem fork death (Juzwik et al. 2011). In contrast, members of the red oak species (section *Lobatae*) succumb within months to one year following infection by *C. fagacearum*. Although this variation in susceptibility to disease development exists, no *Quercus* species is immune to the disease.

The rapid development of leaf bronzing (from margins to midrib), first in the upper crown and then throughout the crown, of red oak species are classic visual symptoms. However, these symptoms may be confused with damage from bacterial leaf scorch (*Xylella fastidiosa*) and from rapid infestation by the two-lined chestnut borer (*Agrilus bilineatus*) (Gould et al. 2005; Haack and Acciavatti 1992). The inconsistent nature of symptoms within species of the white oak group is even more problematic in making a diagnosis based on crown symptoms. Insect pests, abiotic factors, and other pathogens may cause similar looking crown symptoms in white oak species (Juzwik et al. 2011). In scenarios where management is justified, an accurate and timely diagnosis is

critical. Thus, laboratory confirmation of a tentative field diagnosis is commonly justified.

Guidelines for collecting appropriate branch and main stem samples from suspect oaks in the northern states have been published (Pokorny 1999). Currently, processing of samples in a diagnostic laboratory involves standard pathogen isolation methods (Pokorny 1999). A 10 to 14 day incubation period is commonly needed before characteristic macroscopic and microscopic features are evident. Isolation success from a pathogen-colonized sample is highly dependent on sample quality. Failed isolation attempts from degraded samples result in a false-negative report. Dry branch samples and samples that were over-heated prior to laboratory submission are examples of poor quality material from which the pathogen is unlikely to be isolated. Because secondary microorganisms invade the sapwood following branch or whole tree wilt and replace *C. fagacearum* (a poor saprophyte), the fungus is generally not isolated from the outer sapwood the year following tissue death.

In general, molecular techniques using polymerase chain reaction (PCR) are faster and more sensitive methods than pathogen isolation for laboratory diagnosis of plant pathogens (McCartney et al. 2003; Schena et al. 2004). Wu et al. (2010) published a molecular assay for *C. fagacearum* using species-specific primers based on the ribosomal DNA (rDNA) internal transcribed spacer region (ITS) in a nested PCR protocol. The rDNA ITS region has been widely used for fungal identification due to its high level of similarity within species and the high level of variation between species (Schoch et al. 2011). The developed primers allowed for successful amplification of *C. fagacearum*

DNA that had been extracted from artificially inoculated, sterile wood strips (3 x 1 x 0.2 cm). Direct extraction of various fungal DNA from xylem tissues of tree species *in situ* also have been published (Linder et al. 2011; Guglielmo et al. 2007, Guglielmo et al. 2010). The application of these techniques to detect *C. fagacearum* DNA in diseased northern oak species offers the potential to dramatically improve diagnostic methods that currently rely on fungus viability.

The aim of this study was to evaluate a nested PCR protocol for the detection of *C. fagacearum* in the sapwood of actively wilting oak trees as well as from tissues taken the year following branch or whole tree death. If the method proved accurate and reliable, guidelines for their use could be developed to complement the current diagnostic standards used by plant disease diagnostic laboratories in the regions. This report details results for nested PCR versus standard isolation methods for four oak species commonly affected by *C. fagacearum* in the Upper Midwest. A preliminary report has been published (Yang, Juzwik, and Mollov 2014).

Materials and Methods

Sampling sites and protocols. In 2012, seven locations with actively wilting red (*Quercus rubra* or *Q. ellopsoidalis*), bur (*Q. macrocarpa*), and/or white oaks (*Q. alba*) were selected within the Minneapolis-St. Paul, MN metropolitan area. Sampling sites were located within the communities of Apple Valley, Arden Hills, Eagan, Minnetonka, North Branch, Oakdale, and St. Paul. Trees that had previously tested positive for oak wilt were located with the aid of city foresters and commercial arborists. Within each

community, one to three sites were chosen for sampling. Three 5.0 to 7.6 cm diameter branches were collected following published guidelines (Pokorny 1999) from the crowns of nine red, eight bur, and eight white oak trees with wilt symptoms between mid-July and late August. Four 20.3 to 30.5 cm long subsections were cut from each branch, sealed in plastic bags, and stored on ice for transport to the laboratory. At the laboratory, all samples were stored at 4˚C until they were further processed. In addition, three branches were collected from the crowns of two apparently healthy trees with no wilt symptoms of each species to serve as controls. Control trees were sampled in the same manner as symptomatic trees.

In 2013, multiple locations in the Minneapolis-St. Paul, MN metropolitan area were again selected for sampling. The locations included the communities of Apple Valley, North Branch, Forest Lake (Carlos Avery Wildlife Management Area), East Bethel (Cedar Creek Ecosystem Science Reserve), and St. Paul. Dead branches were sampled in July and August of 2013 from three bur and four white oak trees with crown dieback. The sampled branches were taken from the same wilting trees used in the previous year and were estimated to have died between mid August and early September 2012. The branches were processed in the same manner as the branches sampled in 2012. Three dead branches were sampled from the crowns of two non-oak wilt affected bur and white oak trees for use as controls. Control trees were sampled from the communities of St. Paul (Battle Creek Regional Park) and Rogers (Crow-Hassen Park Reserve). Based on bark crack symptoms and visible mycelium, three fresh oak wilt mats on stems of each of seven red oaks with crowns that had completely wilted the previous

summer were marked in early June of 2013. In late August 2013, sapwood samples approximately 20 cm x 17 cm were cut with a chainsaw from the dark scars on the outer sapwood. The scars were underneath the highly deteriorated remains of the oak wilt mat. For the other red oaks, the bark was removed to expose a 7 to 10 x 7 to 10 cm area of the outer sapwood (= sapwood window). Vascular staining characteristic of *C. fagacearum* colonization was found in the cambium of four trees while chestnut-brown colored cambium was chosen on two trees. The sapwood beneath the discolored cambium was sampled using the same process as for the mat scars. Controls were obtained by removing three bark squares to expose cambium on the stem of two non-oak wilt affected but dead red oaks in Rogers, MN. All sapwood samples were sealed in a plastic bag, stored on ice, and transported to the laboratory. The samples were stored in the laboratory at 4˚C until processed.

Processing using standard isolation protocol. All branch and stem samples were processed using published isolation protocols (Pokorny 1999). Samples were surface-sterilized by spraying the sample with 95% ethanol and briefly flaming. The bark was removed with a sterile drawknife to reveal any outer sapwood discoloration. Four small (0.4 cm^2) pieces of stained sapwood were removed with a flame sterilized wood gouge and forceps. The pieces were placed on acidified (5 mL of 20% lactic acid per 1 L) potato dextrose agar (APDA) in 100 mm deep Petri plates. The plates were incubated at room temperature (~24˚C) under ambient lighting and checked regularly for the presence of the fungus. Plates were held for a maximum of

14 days. Plated wood chips that yielded mixed fungal species were sub-cultured until pure cultures were obtained. Pure cultures of *Ceratocystis fagacearum* were identified by the presence of gray to olive-green colored colonies and characteristic fruity odor.

Processing using nested PCR protocol. Drill shavings were obtained from each branch and stem sample using methods of Guglielmo et al. (2010) with the following modifications. Previously exposed, but undisturbed portions of branch and stem segments from which wood pieces had been obtained for the standard isolation protocol were sampled for the molecular assay. Shavings were created by drilling in and out of the sapwood several times using a bleach sterilized (10% sodium hypochlorite, from a commercial bleach product) 3 mm diameter drill bit. The conical end of a 1.5 mL microcentrifuge tube was removed to create a "microfunnel." The shavings adhering to the drill bit were dislodged in the microfunnel. When the microfunnel was full, shavings were pushed through the funnel into a 2 mL microcentrifuge tube (DNAse, RNAse, and pyrogen free). The procedure was repeated at three to five locations on the sample until shavings accumulated to the 1 mL (1 cc) line. The resulting drill shavings were stored at -20˚C for no longer than three months when DNA was extracted.

Drill shavings in each tube were mixed using a sterile metal spatula and 0.18 to 0.20 g of drill shavings were transferred to a new sterile 2 mL microcentrifuge tube containing two metal beads (4.5 mm diameter). The metal beads had been sterilized in 10% sodium hypochlorite for five minutes followed by a rinse with molecular grade water. Lysis A buffer (1.4 mL) in the commercially available QIAmp DNA Stool Kit (Qiagen, Venlo, Netherlands) was added to each tube. Each sample was homogenized by

vortexing for 60 seconds. DNA was then extracted from samples using a QIAmp DNA Stool Kit according to the manufacturer's instructions.

Amplification of fungal DNA was accomplished using a nested PCR protocol. The first round of PCR was completed with the general fungal primer pair ITS1-F (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'). Each 25 µL reaction contained 12.5 µL of GoTaq Green (Promega), 0.5 µL of primer ITS1F (10 µM), 0.5 µL of primer ITS4 (10 µM), 2.0 µL of genomic DNA, and 9.5 µL of molecular grade water. The second round of PCR was completed with *C. fagacearum* specific primers, CF01 (5'-GGCGACTTCTTTCTT-3') and CF02 (5'-AAGGCTTGAGTGGTGAAA-3'), developed by Wu et al. (2011). Each 25 µL reaction contained 12.5 µL of GoTaq Green, 0.5 µL of primer CF01 (10 µM), 0.5 µL of primer CF01 (10 µM), 1.0 µL of product from the first round of PCR, and 10.5 µL of molecular grade water. DNA amplification for both first and second rounds of PCR was conducted on a Mastercycler ® (Eppendorf AG, Hamburg, Germany) as follows: 94˚C for 2 minutes, 35 cycles of 94˚C for 30 seconds, 52˚C for 30 seconds, 72˚C for 2 minutes, and a final extension of 72˚C for 7 minutes. PCR products were held at 18˚C until visualized on a 2% agarose gel. Negative controls lacking template DNA were used in each experiment to test for contamination. Products from the second round of PCR were visualized on a 2% agarose gel with an expected amplicon size of 280 bp.

Sequencing reactions of second round PCR products were performed following the BigDye terminator protocol (Applied Biosystems, Life Technologies, Waltham, MA) with primer CF02. Sequencing products were cleaned with CleanSeq (Agencourt, Brea,

CA) magnetic beads following the manufacturer's protocol and read using an ABI 3730xl DNA Sequencer (Applied Biosystems, Life Technologies, Waltham, MA) at Functional Biosciences (Madison, WI).

Sequences were examined using Sequencher® (version 5.2 sequence analysis software, Gene Codes Corporation, Ann Arbor, MI, USA) and Four Peaks (version 1.7.1 Nucleobytes B.V. Gerberastraat 117 1431 RA Aalsmeer, Amsterdam, The Netherlands). Sequence quality was evaluated and appropriate trimming was performed. *C. fagacearum* DNA sequences were identified using BLASTn (98% identity for sequences, >200 bp) to closely matching fungal accessions in the GenBank nucleotide sequence database.

Statistical analyses. A logistic-normal mixed model analysis (Agresti 2002) was used to identify differences in detection methods and to obtain predicted probabilities of detecting the fungus from actively wilting branch segments using traditional isolation and nested PCR. The logistic-normal mixed model has the form

$$\text{L1: } Y_{ijkl} \sim Bernoulli\ (P_{ijkl})$$

$$Logit\left[P\left(Y_{ijkl}=1\right)\right] = \mu + S_i + D_j + SxD_{ij} + \alpha_k + \beta_{l(k)}$$

$$\text{L2: } \alpha_k \sim N(0, \sigma^2_{tree}),\ \beta_{l(k)} \sim N(0, \sigma^2_{branch}),$$

where P is the probability of detecting the fungus, μ is the overall mean, S is the oak species variable, D is the diagnostic method variable, α is the error associated with tree, and β is the error associated with branch. The model was run as a hierarchical generalized linear mixed model with PROC GLIMMIX of SAS (SAS/STAT® Institute, version 9.3 of the SAS system for Windows platform). Numerator and denominator degrees of freedom

were calculated by the Kenward-Roger's approximation within PROC GLIMMIX of SAS. The probability of detecting the fungus from each species using either isolation methods or nested PCR was calculated using the logit transformation:

$$f(X) = \frac{\exp(X)}{1 + \exp(X)}$$

An exact Wilcoxon rank sum test was used to compare the probabilities of disease detection in one-year dead bur and white oak trees using the two diagnostic methods across species. A paired Wilcoxon ranked sums test was also used to compare the probabilities of disease detection between diagnostic methods. Multiple p-value corrections were performed using the Bonferroni-Holm adjustment for the one year-dead bur and white oak samples and also for the red oak main stem samples.

McNemar's test (Agresti 2002) was used to determine whether or not the marginal probabilities of pathogen detection in red oak bark windows and mat scars were the same between the two detection methods used. All calculations with McNemar's test were performed using R (version 3.0.2, R Core Team).

$$H_0: P_{isolation\ detection} = P_{nested\ PCR\ detection}$$

$$H_1: P_{isolation\ detection} \neq P_{nested\ PCR\ detection}$$

Sensitivity testing in spiked drill shavings. A serial dilution series using drill shavings taken from known *C. fagacearum* colonized sapwood was created to document the sensitivity of the DNA extraction and nested PCR method for the molecular detection of *C. fagacearum* in diseased sapwood.

Approximately 0.08 g of drill shavings from actively wilting red oak stained sapwood of a diseased tree was collected and combined with 0.08 g of drill shavings from the sapwood of a healthy red oak. The mixture was homogenized by vortexing for 30 seconds. Approximately 0.08 g of the mixture was removed to serve as a test sample and the remaining 0.08 g was again diluted with an equal amount of shavings from healthy sapwood. The dilution process was repeated until a series of 12 1:1 dilutions were created. DNA was extracted and nested PCR was performed as described above. Samples with clear gel electrophoresis band visibility were determined to be positive. The process was also completed with drill shavings from infected red oak sapwood of one-year dead tissue. The shavings were diluted in drill shavings from decaying sapwood of a dead, but *C. fagacearum* free red oak.

Results

***C. fagacearum* detection by standard isolation**. For current-year wilting red oak trees, 97% of subsamples were positive using standard isolation techniques (Table 2.1). For bur oak, 64% of subsamples were positive for the fungus using standard isolation. The fungus was only detected in 23% of the wilting white oak subsamples using standard isolation techniques. All of the branches from wilting red oaks were positive for the fungus. For bur oak, the pathogen was isolated from 17 of 24 branches. In white oak, only 8 of the 24 branches were positive using standard isolation techniques. When results were compiled on a tree level basis, *C. fagacearum* was detected by traditional isolation in all nine red oak trees that were sampled. In bur oak, the fungus was detected in seven

out of the eight symptomatic trees. Only six out of the nine symptomatic white oak trees yielded a positive result. The fungus was not detected in any of the healthy control trees.

C. fagacearum was not isolated from dead bur and white oak branches that had completely wilted the previous year. In addition, the fungus was not cultured from any of the one-year dead non-oak wilt control trees.

In red oak stems that were sampled approximately one year after wilt of the entire tree, the fungus was isolated from 11 of the 18 main stem window subsamples from the trees with either a streaked cambium or uniform brown-colored cambium. The fungus was not isolated from any subsamples of the sapwood underlying the remnants of deteriorated sporulation mats on six trees. Finally, the fungus was not isolated from any of the non-oak wilt affected control trees.

***C. fagacearum* detection by nested PCR.** In actively wilting red oak trees, 96% of the subsamples from nine trees were positive for *C. fagacearum* using the nested PCR protocol (Table 2.2). In bur oak, the fungus was detected in 92% of the trees. In actively wilting white oak trees, 93% of subsamples were positive for the fungus using the nested PCR protocol. When the data were compiled on a branch basis, all of the branches sampled from red, bur, and white oak trees were positive for the fungus. In each of the species, at least one segment from each branch sampled was positive. Lastly, when the data were compiled on a tree basis, each actively wilting tree assayed was determined to be positive for *C. fagacearum*.

In bur oak, the fungus was detected in 55% of branch segments, while in white oak it was detected in 86% of branch segments. In both species, at least one segment from each branch sampled was positive for the fungus.

In main stem samples of red oak taken approximately ten months after crowns had completely wilted, nested PCR detected the pathogen in all sapwood windows removed from trees with either uniform brown-colored cambium or a streaked cambium (Table 2.3). Nested PCR also detected the fungus in 19 of the 21 subsamples of the sapwood underlying the remnants of sporulation mats on seven trees.

Comparison of detection by assay methods and species. Overall, *C. fagacearum* was detected more frequently in branch segments using the nested PCR protocol versus the isolation protocol for the actively wilting trees (Figure 2.1). In the red oaks, there was no difference in detection at the branch or tree level between the two diagnostic methods. The pathogen was more frequently detected using the nested PCR protocol in bur and white oak at both the branch and tree level. Differences ($P \leq 0.0005$) were found for species, diagnostic method, and species x diagnostic method for the data contained within actively wilting red, bur, and white oak trees based on logistic-normal mixed model analysis of branch segment data (Table 2.4).

Pathogen detection rates for bur and white oak branch segments differed by diagnostic method within each species (Adj. $P < 0.001$ for both; Tukey-Kramer means comparison). Results for both isolation and nested PCR were similar for red oak branch segments (Adj. $P = 0.7348$). Based on the logistic-normal mixed model, the estimated

probabilities of detecting the fungus in bur and white oak branch segments with nested PCR was calculated to be 95%, whereas predicted probabilities of success using tradition isolation for bur and white oak were 63% and 19%, respectively (Table 2.5). For red oak branch segments, the predicted probability of *C. fagacearum* detection was >95% for both diagnostic methods.

For the ≥1-year-old dead bur and white oak branches, the proportion of segments in which the pathogen was detected was similar for both species using the traditional isolation method (W = 54, Adj. P = 1.0, Exact Wilcoxon rank sum test), but differed with the molecular test (W = 96, Adj. P = 0.0033) (Figure 2.2). For the same dead branch material, the proportion of bur oak segments and white oak segments in which *C. fagacearum* was found differed by detection method for each species (W = 0, Adj. P = 0.0117 for bur and W = 0, Adj. P = 0.0014 for white, Exact Wilcoxon rank sign test). P-values stated for all one-year dead bur and white oak results were calculated using the Bonferroni-Holm P-value adjustment method.

For sapwood from red oak main stem windows of trees ≥ 10 months dead, similar probabilities of fungus detection were found using traditional isolation and nested PCR (McNemar's $X^2 = 5.1429$, Adj. P = 0.07) (Figure 2.3). Probability of pathogen detection in sapwood sampled beneath mat scars on dead red oaks, however, did differ by diagnostic method (McNemar's $X^2 = 17.0526$, Adj. P = 0.0001) as the fungus was not detected from any mat scars using traditional isolation.

Nested PCR detection sensitivity in drill shavings. DNA of the pathogen was detected in mixtures of *C. fagacearum* colonized (actively wilting branch): non-*C. fagacearum* colonized (healthy branch) oak sapwood shavings of red oak to the 1:64 dilution level (Table 2.6). For mixtures of sapwood shavings from ≥ 1-year dead branches and shavings from *C. fagacearum*-free, but dead and decayed branches, DNA of the pathogen was detected to the 1:8 dilution.

Discussion

An improved and more accurate PCR-based diagnostic method for detecting *C. fagacearum* from currently wilting oak trees and from one-year dead branches of *Q. macrocarpa* and *Q. alba* was compared to standard isolation protocols. Although similar results were obtained from currently wilting branches and stained sapwood from the main stem of wilted red oaks with both isolation and nested PCR protocols, the latter offers the potential to verify pathogen presence in older infected red oaks by sampling sapwood areas stained by deteriorated mats (mat scars). Until now, it has been widely accepted that the detection of *C. fagacearum* in dead or moribund trees via isolation was not possible as the fungus is no longer viable. Based on an extensive investigation, this is the first report of detection of *C. fagacearum* from dead tissues of infected trees.

There are numerous advantages associated with the use of this new diagnostic protocol. A high priority was placed on the development of a diagnostic tool that could be easily adapted by plant disease diagnostic clinics that routinely process suspect oak wilt samples. The process of obtaining sapwood drill shavings may be easily substituted into

the current oak wilt laboratory processing guidelines. The nested PCR protocol also has the benefit of taking only two to three days to obtain results, which is a significant improvement on the current 10 to 14 day waiting period for pathogen isolation.

The DNA extraction method used in this study employs a commercially available kit (QIAamp®) that has previously been used to successfully detect fungi directly from wood (Guglielmo et al. 2010). The decision to use a commercial kit offered the assurance the DNA extraction process was near identical for each sample being assayed. Diagnosticians are often familiar with the use of Qiagen® and other DNA extraction kits, which tend to minimize the handling of hazardous chemicals.

It is noteworthy to address the occurrence of both false positive and false negative results that may occur while using this protocol. Nested PCR protocols are often scrutinized because of the potential for contamination between PCR rounds (Neumaier et al. 1998). In order to ensure the accuracy of the assay, it is necessary to stress the importance of negative controls within each round of amplification.

All of the trees used in this study were known to have oak wilt, yet 100% detection was not obtained for any of the species. It is possible that some of the branch segments that tested negative for the fungus in this study may have been colonized by the pathogen, but resulting sequences were not of high enough quality to meet the defined standards (200bp, >98% Identity) and were thus rated “negative”. For research purposes, the use of DNA extraction methods that allow for greater recovery of fungal DNA from wood could optimize the extraction method. The amplification of DNA can be significantly limited by the presence of phenolic compounds, tannins, polysaccharides,

and other materials that inhibit PCR (Couch and Fritz 1990; Zeigenhagen et al. 1993). Furthermore the quality of DNA in dead or dying trees may be quite degraded, making the extraction method critical for pathogen detection (Lindhal 1993; Deguilloux 2002). Additional approaches to wood sample preparation and DNA extraction such as liquid nitrogen powdering or CTAB extraction may help to overcome some of the troublesome barriers posed by degraded DNA in wood samples but are not necessarily practical for use in diagnostic clinics (Hayden et al. 2004; Rachmayanti et al. 2006; Guglielmo et al. 2007).

The use of molecular techniques for the rapid detection of plant pathogens has gained attention and molecular assays have been developed for different types of plant material and pathosystems (Sikdar et al. 2014). Similar assays utilizing primers based on the ITS region have successfully been developed for the detection of fungi directly from woody material for diseases such as laurel wilt (*Raffaelea lauricola*) and *Verticillium* wilt (*Verticillium dahliae*) on smoke tree (*Cotinus coggygria*) (Jeyaprakash et al. 2013; Wang et al. 2013). The drilling method used in this study was adapted from the Banik et al. (2012) sapwood sample collection kit developed to detect fungi in declining red pine. The decision to modify the protocol by recommending that the sapwood drillings be obtained in the laboratory was made for two reasons. The first reason was to maintain the current oak wilt sampling guidelines without adding complicated steps for trained tree health professionals. The second reason was that sapwood samples could be obtained in a sterile environment with less risk of environmental contamination. Similar to the material presented here, the assay for *R. lauricola* also utilized a commercially available DNA

extraction kit. The value of these studies lies within their potential for simple routine use to detect pathogens and diagnose tree diseases.

The nested PCR protocol yielded a positive result from every branch sampled in this assay, regardless of tree species. These results support the current sampling guidelines set by Pokorny (1999), which recommend sampling from three symptomatic branches. Collecting drill shavings from multiple locations along the length of the branch (approximately one collection per 30 cm) will increase the possibility of detecting the fungus. However the progressive bulking of drill shavings will reduce the likelihood of pathogen detection. Dilutions of colonized red oak tissue with healthy red oak tissue had a detection limit at the 1:64 dilution in this study. The results of this dilution series suggest that drill shavings from actively wilting branches may be combined for processing, but should not be doubled in volume more than six times. For branches sampled the year following full wilt, it is not recommended to double the volume of drill shavings more than three times. If the cost were justified, it is recommended that multiple samples should be run for each symptomatic branch. The sensitivity testing was repeated with an adjusted lysis step before the DNA extraction using a tissue homogenizer (Fast Prep, MP Biomedicals, Santa Ana, CA) in place of the one-minute vortexing with the goal of increasing the detection threshold (data not shown). The use of the Fast Prep® did not improve detection; however it did greatly reduce the DNA extraction time.

This DNA-based detection method can lengthen the timeframe in which successful oak wilt diagnostics may occur by allowing for detection from samples that have been degraded, exposed to high heat, or have been stored improperly prior to

disease clinic submission. Bretz and Morison (1953) found that *C. fagacearum* is no longer viable in small diameter branches (≤ 1.9 cm) stored at 20°C for six days or more. Branch samples stored at 35°C had no viable fungus remaining after three days. It was also noted that as the time interval between sample collection and isolation increased, the development of secondary organisms from the wood chips also increased. Thus, the detection of *C. fagacearum* can be very limited by environmental and sample storage conditions. With nested PCR, the detection of *C. fagacearum* DNA would not be limited by the poor survival of the fungus or by the presence of contaminating microorganisms. Currently, successful fungal isolation from woodchips is unlikely after mid-September (University of Minnesota Plant Disease Clinic, *personal communication*). With the nested PCR protocol, the detection of oak wilt is no long restricted to periods of peak symptom development.

The detection of *C. fagacearum* in dead branch samples and main stems creates new possibilities for management of oak wilt in many scenarios. Detection of its presence is significant information, even in situations where only the DNA of non-viable fungus is detected. Regardless of whether or not the fungus is viable, *C. fagacearum* remains a virulent pathogen in northern oak species and timely management should be considered. The scattered nature of oak wilt symptoms in bur and white oak trees can limit the amount of material available for accurate pathogen testing using isolation methods. Branches that die as a result of the disease commonly exhibit black to brown discoloration in the outer sapwood. With the method tested here, dead white and bur oak branches (≤ 1 year dead) exhibiting sapwood discoloration caused by an oak wilt

infection can be successfully used to detect *C. fagacearum*. The ability to detect the fungus from this type of material allow for detection and treatment, if justified, on high value trees.

In red oak species, the protocol presented here could provide the opportunity for a post-mortem diagnosis of the disease from stem samples that have not produced or no longer possess fungal mats. The use of this detection method in wooded areas could help to quickly identify new infection centers and lead to the rapid removal of potential spore producing trees.

In conclusion, detection of *C. fagacearum* from sapwood drill shavings using nested PCR was compared to detection using traditional isolation methods. The PCR based assay tested here offers two significant improvements in detection of *C. fagacearum*. The first is an increase in detection reliability within specific sample types. The second improvement is the substantial decrease in the time it takes to detect the pathogen and, potentially, the time between diagnosis and treatment. While further refinements to the DNA extraction method may optimize the molecular assay, the DNA extraction and nested PCR protocol presented here is ready for use in plant disease diagnostic clinics. In addition to use in diagnostics, the protocol may also be used in future oak wilt research endeavors.

Table 2.1. Detection of *Ceratocystis fagacearum* in branch subsamples from actively wilting crowns of *Q. rubra/Q. ellipsoidalis* (red oak), *Q. macrocarpa* (bur oak), and *Q. alba* (white oak) study trees using traditional isolation techniques.

Species	Branch Segment Assayed		Branch Basis*		Tree Basis*	
	Total No. Assayed	No. Positive	Total No. Assayed	No. Positive	Total No. Assayed	No. Positive
Red	108	105	27	27	9	9
Bur	96	59	24	17	8	7
White	96	22	24	8	8	6

*Results of branch segment assays were composited on a branch or a tree basis

Table 2.2. Detection of *Ceratocystis fagacearum* in branch subsamples from actively wilting crowns of *Q. rubra/Q. ellipsoidalis* (red oak), *Q. macrocarpa* (bur oak), and *Q. alba* (white oak) study trees using nested PCR.

Species	Branch Segment Assayed		Branch Basis*		Tree Basis*	
	Total No. Assayed	No. Positive	Total No. Assayed	No. Positive	Total No. Assayed	No. Positive
Red	108	102	27	27	9	9
Bur	96	88	24	24	8	8
White	96	89	24	24	8	8

*Results of branch segment assays were composited on a branch or a tree basis

Table 2.3. Detection of *Ceratocystis fagacearum* from main stem samples on *Q. rubra/Q. ellipsoidalis* (red oak) trees one year after complete crown wilt using nested PCR.

Sapwood Sample Type*	Stem Sample		Tree†	
	Total No. Assayed	No. Positive	Total No. Assayed	No. Positive
Window	18	18	6	6
Mat Scar	21	19	7	7

*Window = sapwood sample removed from below cambium exhibiting vascular streaking or uniform brown in color; †Mat scar = sapwood underlying remnant of fungus sporulation mat.

Table 2.4. Model resulting from logistic-normal mixed model analysis of variables that affect the likelihood of detecting *Ceratocystis fagacearum* in actively wilting branch segments of *Q. rubra/Q. ellipsoidalis* (red oak), *Q. macrocarpa* (bur oak), and *Q. alba* (white oak) trees (Type II Tests of Fixed Effects).

Effect	Num DF	Den DF	P-Value
Species	2	31.9	0.0005
Diagnostic Method	1	594.0	<0.0001
Species*Diagnostic Method	1	594.0	<0.0001

Table 2.5. Models resulting from fixed effects logistic-normal regression of the interactions of actively wilting *Q. rubra/Q. ellipsoidalis* (red oak), *Q. macrocarpa* (bur oak), and *Q. alba* (white oak) trees and diagnostic method used for detection of *Ceratocystis fagacearum* in the sapwood.

Species	Diagnostic Method	Estimate	SE	P-Value	Estimated Probability*	Actual Probability†
Red	Isolation	4.1506	0.7803	<0.0001	0.98	0.97
	Nested PCR	2.9925	0.5202	<0.0001	0.95	0.96
Bur	Isolation	0.5573	0.3782	0.1552	0.63	0.64
	Nested PCR	2.9031	0.5097	<0.0001	0.95	0.92
White	Isolation	-1.4587	0.4041	0.0012	0.19	0.23
	Nested PCR	2.9055	0.5128	<0.0001	0.95	0.93

* Estimated probability based on logit transformation from model estimates.

† Actual probability based on calculated proportion of positive samples used in this study.

Table 2.6. Results of nested PCR band visibility from serially diluted *Ceratocystis fagacearum*-colonized *Q. rubra/Q. ellipsoidalis* (red oak) drill shavings serially diluted in healthy red oak drill shavings and ≥1 year dead red oak drill shavings.

Dilution	Nested PCR Band Visibility	
	Mixed in healthy sapwood	Mixed in decayed sapwood
Colonized wood only	+	+
Non-colonized wood only	-	-
1:2*	+	+
1:4	+	+
1:8	+	+
1:16	+	-
1:32	+	-
1:64	+	-
1:128	-	-
1:256	-	-
1:512	-	-
1:1024	-	-
1:2048	-	-

*Ratio is for colonized:non-colonized wood (w/w).

Figure 2.1. Proportion of *Ceratocystis fagacearum (C.f.)*-positive branch segments based on total number of segments assayed from wilting branches sampled from *Q. rubra/Q. ellipsoidalis* (red oak), *Q. macrocarpa* (bur oak), and *Q. alba* (white oak) trees .

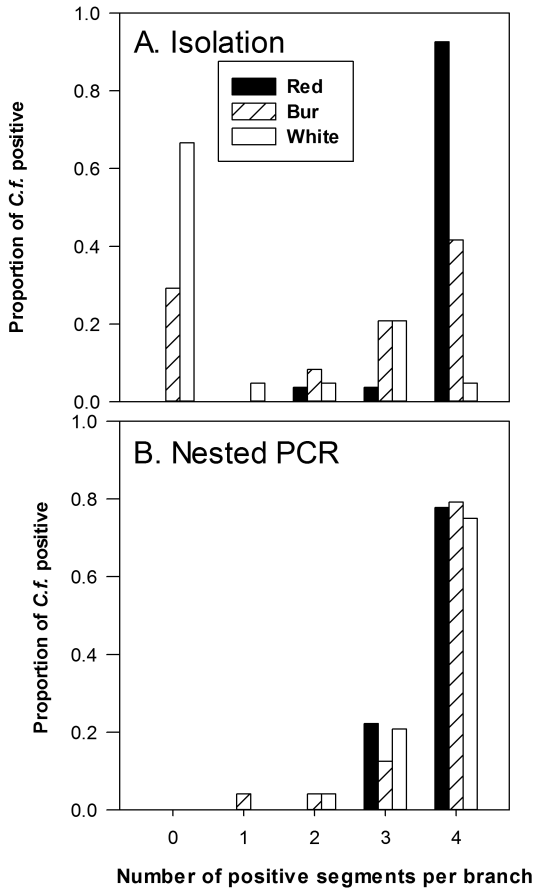


Figure 2.2. Proportion of *C. fagacearum (C.f.)*-positive branch segments of *Q. macrocarpa* (bur oak) and *Q. alba* (white oak) trees based on total number of segments assayed for ≥ one-year dead branches.

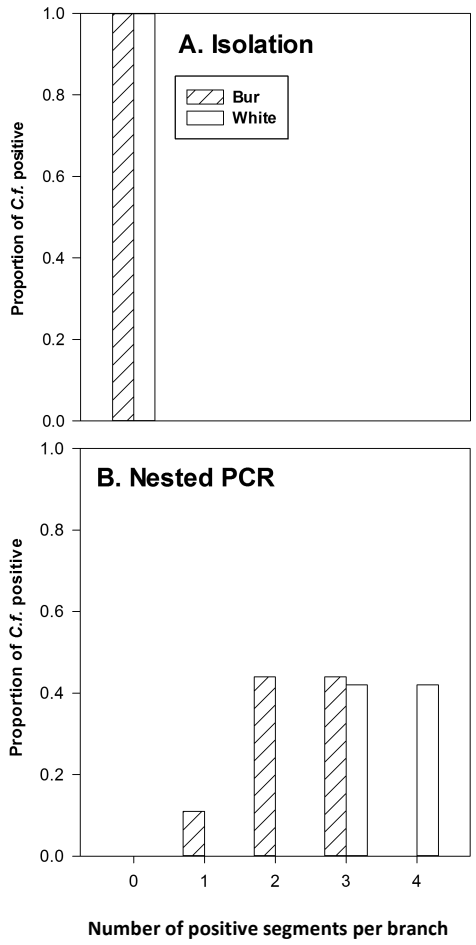
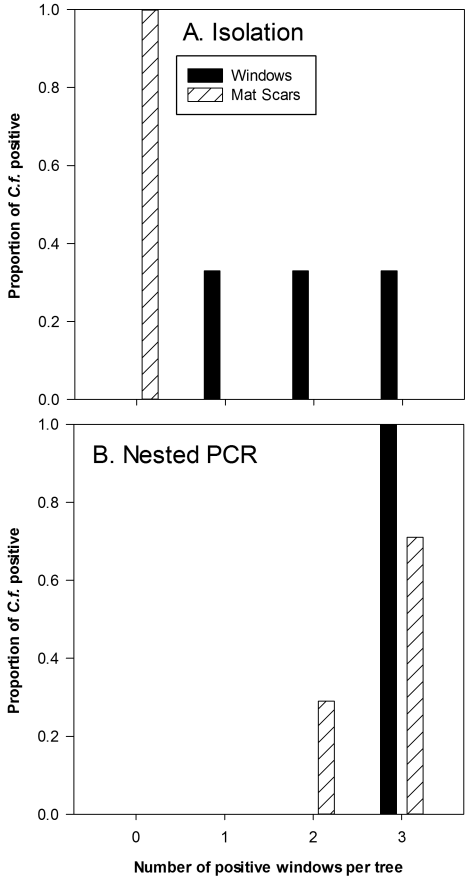


Figure 2.3. Proportion of sapwood windows and mat scars from one-year dead *Q. rubra/Q. ellipsoidalis* (red oak) that were positive for *Ceratocystis fagacearum (C.f.)* using isolation and nested PCR methods.



Chapter 3. Use of real-time PCR for the detection of the oak wilt pathogen, *Ceratocystis fagacearum*, from the sapwood of naturally infected *Quercus* species

Anna M. Yang

Introduction

Ceratocystis fagacearum, the causal agent of oak wilt, was discovered in the early 1940's and currently affects oak species (*Quercus* spp.) across the eastern United States and in Texas (Juzwik et al. 2011). While oak wilt has been well studied, the accurate diagnosis of the disease can be difficult. Species in the red oak group (section *Lobata*e) respond to the pathogen by exhibiting full wilt within months of infection (Juzwik 2011, Tainter and Baker 1996). In contrast, white oak species (section *Quercus*) express a scattered to slow, single branch dieback that can occur over several to many years. In addition to the differences in symptom development, other pathogens, insects, and abiotic agents may cause symptoms that mimic those of an oak wilt infection (Gould et al. 2005; Haack and Acciavatti 1992). Preventative and therapeutic treatments such as vibratory plowing and fungicide injections are very costly, thus placing a deserved emphasis on the need for accurate disease diagnosis.

The distinct and dramatic appearance of symptoms on red oak trees is generally recognizable by trained tree health professionals. However, some situations may require an official laboratory diagnosis of the fungus. Laboratory diagnosis is ordinarily recommended for bur and white oak trees with suspected infections. Field sampling and laboratory diagnostic protocols have been published (Pokorny 1999). The latter involves removal of the bark from branches and plating pieces of symptomatic sapwood on acidified potato dextrose agar (APDA). Following a 10-14 day incubation period, the macroscopic and microscopic characteristics of the resulting colony are used to identify *C. fagacearum*. Although this method has been successful in detecting *C. fagacearum*, it

has significant limitations related to sample quality that may lead to a false, negative result. Laboratory isolation is completely dependent on the viability of the fungus in the sapwood of the assayed sample. Sample desiccation and/or exposure to high heat prior to submission to the laboratory may kill the fungus within the sample. Furthermore, *C. fagacearum* is a poor saprophyte making it very difficult to culture from dead or moribund material that has been colonized by secondary fungi. Thus, there is a need for a rapid, practical assay that is not exclusively dependent on the ability to culture the fungus.

Molecular based methods for pathogen detection have emerged as useful tools for plant disease diagnosis due to the advantage of speed and sensitivity (McCartney et al. 2003; Schena et al. 2004). Molecular assays employing the use of polymerase chain reaction (PCR) are often less laborious than traditional detection methods and have the ability to provide faster and more accurate results. The most common methods are standard and real-time PCR, which utilize specific primers to amplify diagnostic DNA fragments. The ribosomal DNA (rDNA) internal transcribed spacer region (ITS) is commonly used in primer development for detection of and/or distinguishing among similar species using PCR (Schoch et al. 2011). Real-time PCR assays eliminate the need for high template DNA concentrations and post-processing steps, such as gel electrophoresis. Detection and quantification of target DNA in experimental samples is monitored using the threshold cycle (Ct), which occurs during the exponential stage of DNA amplification. The amplification curves of experimental samples are then compared to amplification curves of known standards. Numerous real-time PCR protocols utilizing

the rDNA ITS region have been developed for the rapid detection and quantification of plant pathogens in soil and plant material (Kurdyla et al. 2011; Samuelian et al. 2011; Sidkar et al. 2014; Wu et al. 2011). Specific primers and a probe with a fluorescent label have been developed for use with real-time PCR for the detection of *C. fagacearum* in spore suspensions from a pure culture, purified target DNA, and main stem samples from symptomatic live oak and Monterey oak trees in Texas (Kurdyla et al. 2011). The application of this real-time PCR protocol to detect *C. fagacearum* in diseased northern oak species could improve the speed and specificity of laboratory diagnosis in northern states.

The primary goal of this study was to test and evaluate a real-time PCR protocol for the detection of *C. fagacearum* in 1) the sapwood of actively wilting oak trees in Minnesota, and 2) the sapwood of trees one year after death in the same locations. This paper reports the modified real-time PCR protocol with *C. fagacearum*-specific primers and probe and the ability to detect the pathogen in red (*Q. rubra* and *Q. ellipsoidalis*), bur (*Q. macrocarpa*), and white (*Q. alba*) oak trees using this protocol.

Materials and Methods

Real-time PCR primer design and evaluation. *C. fagacearum*-specific primers and probe labeled with 6-carboxyfluorescein (FAM) were developed in a preliminary study by Kurdyla and Appel (2011) using Primer Express ® software (Applied Biosystems) (Table 3.1). The developed primer/probe set successfully detected the pathogen from spore suspensions made from a pure culture of the fungus and

purified, genomic DNA. The primer/probe set was also tested for specificity against closely related, non-target fungal species. All non-target species failed to amplify in the tests (Kurdyla and Appel 2011).

Real-time PCR standard curve development. A standard curve was constructed by using serial dilutions of gel-purified *C. fagacearum* PCR product (26.5 ng/ul) generated from nested PCR using ITS1F/4 and CF01/02 primers (see Chapter 2, this thesis). Each sample was amplified in triplicate. To obtain the slope of the real-time PCR standard curve, the log transformation of the DNA concentration was plotted against the average Ct value for each sample. Efficiency of the assay was calculated by using the formula $E = 10^{\left(\frac{1}{-slope}\right)} - 1 \times 100$ (Ginzinger 2002). Calculations were conducted using Microsoft Excel (Microsoft Office 2010).

Sampling sites and protocols. The study sites and trees were the same as those used for evaluation of a nested PCR protocol (see Chapter 2, this thesis) and the reader is directed to that chapter for more specific details.

Actively wilting red, bur, and white oaks were selected for sampling within the Minneapolis-St. Paul, MN, metropolitan area in 2012. In 2013, dead branches from the same oak wilt-positive bur and white oak trees with crown dieback were selected for assay. The bur and white oak branches sampled were estimated to have wilted in late summer/early fall of 2012. Lastly, main stem samples were taken from red oaks that had exhibited complete crown wilt in 2012. In late spring 2013, samples (= "windows") were cut from the sapwood beneath exposed cambium exhibiting either vascular staining or

were uniform brown in color and moist to the touch. During the same time period, vertical cracks associated with recently formed oak wilt mats were marked with paint on other 2012 wilted red oaks. In late August 2013, samples (= "mat scars") were cut from the sapwood underlying the highly deteriorated remnants or scars of the spring mats.

Processing using standard isolation protocol. All branch and stem samples were processed using published isolation protocols (Pokorny 1999). Small tissue pieces were aseptically removed from exposed sapwood exhibiting vascular staining and plated on acidified potato dextrose agar (APDA). Sapwood pieces were incubated and the resulting cultures identified as outlined in Chapter 2 of this thesis. The shavings were collected in a 2 mL microcentrifuge tube and stored at -20°C until processed. DNA was extracted from drill shavings using a QIAmp DNA Stool Kit (Qiagen) according to the manufacturer's instructions.

Processing using real-time PCR protocol. Drill shavings were obtained from each branch and stem sample using the modified techniques of Guglielmo et al. (2010) in the same manner described in the previous chapter. DNA was extracted from drill shavings using a QIAmp DNA Stool Kit (Qiagen) according to the manufacturer's instructions.

Real-time PCR was performed using a Smartcyler® (Cepheid, Sunnyvale, CA). Reactions were completed with the forward primer CfP2-01 (5'-TGGCAGGGACTTCTTTCTTCA-3'), reverse primer CfP2-02 (5'-

TTGTTAAATGCAACTCAGCAATGA-3'), and CfP2 (5'-/56-FAM/ATGTTTCTGCCAGTAGTATT/3BHQ1-3') developed by Kurdyla et al. (2011). Each 25 µL reaction contained 10 µL of 5Prime Real Mastermix (5 Prime, Gaithersburg, MD), 0.5 µL of each primer and probe (200 nM), 1 µL of extracted genomic DNA, and 12.5 µL of molecular grade water. Reactions were performed under the following conditions: 94˚C for two minutes, 40 cycles of 95˚C for 15 seconds and 60˚C for 1 minute to calculate the cycle threshold (Ct) for each sample. All reactions were performed in duplicate and included parallel reactions of three separate dilution series of pure standard DNA and a control free of any template DNA.

Statistical analyses. A logistic-normal mixed model analysis (Agresti 2002) was used to identify differences between real-time PCR and traditional isolation methods and to obtain predicted probabilities of detecting the fungus from actively wilting branch segments. In addition, the same mixed model was used to examine differences in fungal detection between real-time PCR and nested PCR (Chapter 2, this thesis). The logistic-normal mixed model has the form

$$\text{L1: } Y_{ijkl} \sim Bernoulli\ (P_{ijkl})$$

$$Logit\left[P\left(Y_{ijkl}=1\right)\right] = \mu + S_i + D_j + SxD_{ij} + \alpha_k + \beta_{l(k)}$$

$$\text{L2: } \alpha_k \sim N(0, \sigma^2_{Tree}),\ \beta_{l(k)} \sim N(0, \sigma^2_{Branch}),$$

where P is the probability of detecting the fungus, µ is the overall mean, S is the oak species variable, D is the diagnostic method variable, α is the error associated with tree, and β is the error associated with branch. The model was run as a hierarchical generalized

linear mixed model with PROC GLIMMIX of SAS (SAS/STAT® Institute, version 9.3 of the SAS system for Windows platform). A Tukey-Kramer means comparison test was used to detect differences among detection method within actively wilting branches. The probability of detecting the fungus from each species using either isolation methods or real-time PCR was calculated using the logit transformation:

$$f(X) = \frac{\exp(X)}{1 + \exp(X)}$$

An exact Wilcoxon rank sum test was use to test for differences in successful pathogen detection between bur and white oak branches. The rank sum test was performed for both real-time PCR and isolation methods. A paired Wilcoxon ranked sign test was then used to compare the probabilities of disease detection between diagnostic methods within branches of bur and white oak trees. Multiple p-value corrections were performed using the Bonferroni-Holm adjustment for the one year-dead bur and white oak samples and also for the red oak main stem samples. The same Wilcoxon rank sign test was used to examine differences in detection probability between the real-time PCR and nested PCR protocol.

McNemar's test (Agresti 2002) was used to determine whether or not the marginal probabilities of pathogen detection in red oak windows and mat scars were the same between real-time PCR and traditional isolation methods used. Calculations were also done to compare detection using real-time PCR compared to nested PCR. All calculations with McNemar's test were performed using R (version 3.0.2, R Core Team).

$$H_0: P_{isolation\ detection} = P_{nested\ PCR\ detection}$$

$$H_1: P_{isolation\ detection} \neq P_{nested\ PCR\ detection}$$

Detection threshold in spiked drill shavings. To examine the sensitivity of the DNA extraction and real-time PCR method for the molecular detection of *C. fagacearum* in diseased sapwood, the same serial dilutions of colonized sapwood were completed as described in the previous chapter of this thesis on nested PCR.

Results

Real-time PCR standard curve generation. The detection limit for *C. fagacearum* in the real-time PCR assays was 0.265 fg, with the lowest DNA concentration having a Ct value of 31.68 (Figure 3.1). A linear relationship was established between the log DNA concentration of detected *C. fagacearum* DNA and the Ct value of the PCR assay (R^2=0.95985). Based on the slope of the standard curve, the amplification efficiency of the real-time PCR reactions was calculated to be 96.72%.

***C. fagacearum* detection by traditional isolation.** In red oak trees with actively wilting crowns, 97% of branch segments were positive using standard isolation techniques (Table 3.2). For samples taken from bur oak trees, 64% of branch segments were positive for the fungus using standard isolation. Using the same technique, the pathogen was only detected in 23% of white oak branch segments. When summarized at the branch level, all wilting branches from red oak trees were positive for the fungus

using isolation techniques. For bur oak, 17 of the 24 branches were positive. Finally, in white oak trees, only 8 of the 24 branches were positive for the fungus. When isolation results were compiled at the tree level, the pathogen was detected in all red oak trees, seven of the eight bur oak trees, and in six of the nine white oak trees. The fungus was not detected in any of the healthy control trees.

C. fagacearum was not detected from bur and white oak branches that had completely wilted the previous year. The fungus also was not cultured from any of the one-year dead, non-oak wilt control trees.

The pathogen was detected in 11 of the 18 streaking or uniform brown-colored sapwood windows removed from the main stems of red oak trees approximately one year after full wilt occurred. *C. fagacearum* was not isolated from any of the subsamples taken from the sapwood underlying the remnants of deteriorated sporulation mats. The fungus was not cultured from any of the non-oak wilt affected control trees.

***C. fagacearum* detection by real-time PCR.** In actively wilting red oak trees, 75% of the segments from nine trees were positive for *C. fagacearum* using the real-time PCR protocol (Table 3.3). In wilting bur oak and white oak, the fungus was detected in at least 41% of the branch segments. In all of the species sampled, at least one segment from each branch tested positive for the presence of the pathogen. For red oak trees, all of the branches sampled were positive, while in bur and white oak, 21 out of the 24 branches were positive for *C. fagacearum*. When we collapsed the data to the tree level,

we found all actively wilting trees assayed were positive for *C. fagacearum* using the real-time PCR protocol.

In one-year dead white oak branches, *C. fagacearum* was detected in 9 of the 48 subsamples using real-time PCR. The fungus was not detected in any of the one-year dead bur oak trees using this method. In main stem samples of red oak taken approximately one year after full wilt, real-time PCR detected the pathogen in 12 of the 28 samples from trees with uniform brown-colored cambium and streaking cambium (Table 3.4). The fungus was detected in two or more subsamples of sapwood underlying sporulation mats, i.e. mat scars from five of the seven trees.

Comparison of detection by isolation and real-time PCR methods. In actively wilting red and bur oak trees, *C. fagacearum* was more frequently detected in branch segments using traditional isolation than in real-time PCR (Figure 3.2). In white oak, the fungus was detected more frequently by real time PCR versus the isolation protocol. However, when the data were examined at the branch level, more branches were positive for *C. fagacearum* using the real-time PCR protocol. When examined at the tree level, real-time PCR more frequently detection the fungus. The model resulting from the logistic-normal mixed model analysis found differences ($P \leq 0.0343$) for species, diagnostic method, and species x diagnostic method for actively wilting trees (Table 3.5).

The Tukey-Kramer means comparison test found differences in *C. fagacearum* detection between isolation and real-time PCR within actively wilting red and white oak

branch segments (Adj. P <0.0006) (Figure 3.2). There was no difference in detection methods for bur oak branch segments (Adj. P = 0.9641) (Figure 3.2).

Based on the logistic-normal mixed model, success of fungus detection using traditional isolation for red, bur, and white oak was predicted to be 97%, 64%, and 32%, respectively (Table 3.6). Predicted probabilities of pathogen detection for red, bur and white oak using real-time PCR were 75%, 41%, and 45%.

With the ≥1-year-old dead branches, the fungus was only detected in white oak branch segments by real-time PCR, while the pathogen was not detected in any of the bur oak branch segments (Figure 3.3). The exact Wilcoxon rank-sum test found no difference in the proportion of positive branches sampled from both species using isolation methods (W = 54, Adj. P = 1) or real-time PCR (W = 76.5, Adj. P = 0.0902). Similarly, the exact Wilcoxon rank-sign test found no differences between the two diagnostic tests in both bur (P = 1) and white oak branches (W = .0.15, Adj. P = 0.06). Bonferroni-Holm adjustments were used for all P-values reported for ≥1-year dead bur and white oak branches.

Sapwood windows removed from red oaks with a streaking cambium tested positive for the fungus with similar frequency using both methods; traditional methods detected nine out of twelve samples and real-time PCR detected eight positive (Figure 3.4). We failed to reject the hypothesis of McNemar's Chi-Square test significant for a difference in the probability of detecting the fungus using traditional isolation or real time PCR ($X^2 = 0$, Adj. P = 1) for the red oak windows. For mat scars from one-year dead red oak main stems, there was a difference between methods in the probability of *C.*

fagacearum detection (X^2 = 11.08, Adj. P = 0.0017). The adjusted P-values reported are based on McNemar's X^2 test for isolation, nested PCR, and real time PCR.

Comparison of detection by nested PCR and real-time PCR methods. Overall, the nested PCR method was able to detect *C. fagacearum* more often than the real-time PCR protocol (for full results from the nested PCR assay, see chapter 2). Based on the logistic-normal mixed model analysis, diagnostic method was the only significant main effect (Table 3.7).

Differences (Adj. P ≤ 0.0008) were found in detection rates for each species (Tukey-Kramer means comparison). When comparing the estimated and actual probabilities of detecting the fungus between the two molecular methods, we found that the nested PCR protocol had a higher probability of detecting the pathogen in the three oak species assayed (Table 3.8).

For the branches of the bur and white oak that had been dead for at least one year, the proportion of branches in which *C. fagacearum* was detected differed by species with nested PCR but not with real-time PCR (W = 96, Adj. P = 0.003 for nested PCR and W = 76.5, Adj. P = 0.090 for real-time, Exact Wilcoxon rank sum test). Within each species, the proportion of branches in which the pathogen was detected differed by the method used (W = 0, Adj. P = 0.0058 for bur and W = 0, Adj. P = 0.0015 for white, Exact Wilcoxon rank sign test). Similar probabilities of pathogen detection were found similar from sapwood windows of ≥ 10 months dead red oaks and from sapwood sampled

beneath mat scars using both molecular protocols (McNemar's $X^2 = 4.1667$, Adj. P = 0.0824 for sapwood windows and McNemar's $X^2 = 2.5$, Adj. P = 0.1138 for mat scars).

Real-time PCR detection sensitivity in drill shavings. The real-time PCR protocol detected the pathogen in mixtures of *C. fagacearum*-colonized sapwood shavings (from an actively wilting branch) in non-colonized sapwood shavings up to the 1:8 dilution (Table 3.9). When colonized sapwood drill shavings were diluted with ≥ 1-year dead sapwood shavings from *C. fagacearum*-free branches, DNA of the pathogen was not detected.

Discussion

In this study, a real-time PCR diagnostic method for detecting *C. fagacearum* from currently wilting *Q. rubra*, *Q. macrocarpa*, and *Q. alba* branches was tested. The same method was also tested on branches from *Q. macrocarpa* and *Q. alba* and main stem samples from *Q. rubra* trees that had wilted the previous year. In comparison to traditional isolation methods, there was no improvement in detection of the fungus in actively wilting red and bur oak samples, but the real-time PCR protocol did yield a higher predicted probability of detection in white oak samples. Additionally, there was no improvement of pathogen detection in branches sampled from one-year dead white and bur oak trees. Although there was no observed difference between real-time PCR and traditional isolation in fungal detection from red oak main stem windows with either a streaking or uniform-brown cambium, the real-time PCR protocol was able to confirm

the presence of *C. fagacearum* in samples taken from sapwood underlying the remnants of sporulation mats (mat scars). The ability to detect the fungus in mat scars using real-time PCR and nested PCR (chapter 2) will allow for diagnosis in situations where diagnosis was not previously possible.

The use of real-time PCR as a tool for the diagnosis of plant pathogens has gained attraction due to its speed, reproducibility, low contamination risk, and added ability of a melting curve analysis. The protocol tested here theoretically offers a significant improvement to the average turnaround time for disease diagnosis. Traditionally, oak wilt diagnostics require up to two weeks before an accurate fungal identification can be made, but the molecular method described here may be completed within a single day. Collecting drill shavings from symptomatic sapwood is a step that could be easily substituted into the current laboratory processing guidelines. Additionally, the DNA extraction method used is derived from a commercially available kit (QIAamp®) that diagnosticians may already be accustomed to using. Commercial kits are subjected to rigorous quality control, making them ideal for routine use in diagnostic clinics.

In situations where fungal DNA is being detected from the remnants of a sporulation mat, the fungus is no longer viable and therefore will not grow in culture. Fungal mats on red oak are a definitive sign of oak wilt, but are very temporary structures and persist for only a few weeks before deteriorating. After deteriorating, the remaining mat scars become nearly indistinguishable and can often be masked by sapwood discoloration associated with fungi such as *Armillaria mellea* (Wargo et al. 1983). Red oak species are highly susceptible to oak wilt and often transmit the disease via root

grafts, making detection critical for appropriate disease management. In addition to the obvious benefit of detecting the fungus in this type of problematic tissue, this protocol can also decrease diagnosis turnaround time for wilted red oak with a streaking or browned cambium and for currently wilting trees.

In this study, every actively wilting oak assayed was positive for the fungus using real-time PCR. However, not every branch on a tree was positive for the fungus leading to the conclusion that a minimum of three branches per tree should be sampled in order to ensure the accuracy of the testing. Furthermore, it is essential to collect drill shavings from multiple locations along the branch. Results from this study suggest that collecting drill shavings approximately every 20 to 30 cm is sufficient for accurate assay results. The results from the sensitivity testing show that combining drill shavings from different locations along the branch is not advised. The accuracy of this test for actively wilting bur and white oak branches appears to be similar to traditional isolation methods, making it most appropriate in situations where a rapid result is justified and real-time PCR is available. For actively wilting red oaks, traditional isolation methods would likely be preferred, unless diagnosis is required within a short timeframe. While the real-time PCR was able to occasionally detect *C. fagacearum* in one-year dead branches from white oak, this type of material is not recommended for use with this test. Kurdya and Appel (2011) noted similar limitations during the development of the primer/probe pair used here. Out of the nine symptomatic live oak assayed, only six of the trees tested positive with the real-time PCR assay.

There are a number of factors that influence the success of a real-time PCR assay that may have affected the outcome of the study presented here. The first factor is the method of nucleic acid extraction used and quality of extracted DNA. The decision to use a commercially available DNA extraction kit was made due to its familiarity in most diagnostic clinics and their minimal use of hazardous chemicals. It is known that DNA degrades in wood, making the extraction process critical for sufficient recovery (Degiolloux 2002; Lindhal 1993). Different methods of DNA extraction such as liquid nitrogen powdering or CTAB extraction have been used to obtain nucleic acids from woody samples but are not always practical for use in diagnostic clinics (Guglielmo et al. 2007; Hayden et al. 2004; Rachmayanti et al. 2006).

The second factor negatively influencing real-time PCR is the presence of inhibitory substances. Other methods of DNA extraction that remove PCR inhibitors present in wood (such as phenolic compounds and tannins) may optimize amplification (Couch and Fritz 1990; Zeigenhagen et al. 1993). Higher PCR reaction volumes often dilute inhibitory substances that are left over from the nucleic acid extraction.

Studies also have reported that the analytical sensitivity of real-time PCR assays can be proportional to the volume of template DNA used (Bastien et al. 2008). The Smartcyler® employed in this study operates with a maximum reaction volume of 25 uL, however it is unclear whether a higher reaction volume with additional template volume would have remedied some of the false-negative results that were observed. It is possible that there was a low concentration of *C. fagacearum* DNA relative to plant DNA in the DNA extracted from symptomatic oak tissues. The assay described here could potentially

be improved by adding an initial round of PCR, real-time or conventional, utilizing general fungal primers (ITS1F and ITS4) followed by the real-time PCR protocol. A nested real-time PCR protocol may result in a higher probability of *C. fagacearum* DNA amplification and detection. A nested real-time PCR protocol has been developed for the detection of *Verticillium dahliae* in symptomatic smoke trees (*Cotinus coggygria*) and in inoculated soil (Wang et al. 2013).

The bulk of published studies examining the specificity and sensitivity of real-time PCR have been within the realm of human disease detection (Bastien et al. 2008). Heirl et al. (2004) compared two real-time PCR protocols to a nested PCR protocol to detect *Toxoplasma gondii*, the parasitic protozoan causing toxoplasmosis. Specimens infected with *T. gondii* were subjected to a nucleic acid extraction followed by a nested PCR protocol to detect the pathogen. All specimens that tested positive using nested PCR were re-tested using two real-time PCR protocols. The results of the real-time PCR protocols were compared to the nested PCR protocol to determine accuracy of the real-time assays. The pathogen was detected in a higher number of infected samples using the nested PCR protocol when compared to the real-time PCR protocols. Heirl et al. (2004) attributed this discrepancy in detection to a number of factors including low copy number of the target sequence in the extracted DNA and large amounts of non-pathogen DNA that inhibited the amplification process.

Real-time PCR has the advantage of very rapid results and the high specificity of a fluorescent probe, but it does not appear to be robust for the detection of *C. fagacearum* from all sample types. Previously, the use of a nested PCR protocol for the detection of

C. fagacearum was examined and was compared to the real-time PCR protocol described here. The comparison of the two molecular assays found that the nested PCR protocol detected the pathogen more often in all species than the real-time protocol, aligning with the reports of Heirl et al. (2004). The underlying reasons for the differences in results from the two molecular methods are not entirely clear. It could be that the extracted DNA did not have a high enough quantity of target DNA for the real-time protocol to amplify sufficiently and that the two consecutive rounds of amplification with the nested PCR had more opportunity to amplify the target DNA. It could also be that the second round of nested PCR diluted non-target DNA and other inhibitory substances, which allowed for better amplification of *C. fagacearum.*

A number of studies exist that utilize specific primer pairs with a fluorescent probe or SYBR Green based protocol to detect xylem inhabiting fungal pathogens (Wang et al. 2013; Jeyaprakash et al. 2014). Wu et al. (2010) published a similar protocol, which used a SYBR Green real-time PCR reaction to amplify *C. fagacearum* DNA. The published SYBR Green method was attempted on a number of our samples, but amplification was very inconsistent and melting curve analyses were inconclusive (data not shown). It is possible that the PCR inhibitors present in natural wood interfered with amplification more than the artificially inoculated feeder strips used by Wu et al. (2010).

Further testing and adjustments to the real-time PCR protocol, such as the DNA extraction methods and PCR conditions, may increase the usefulness of this method in routine diagnosis of this disease. Oak wilt is a common disease that affects all oak species in Minnesota, yet current diagnostics are limited in their detection abilities. Therefore, it

is necessary to develop an accurate and fast method for *C. fagacearum* detection. In this study, a real-time PCR protocol that improved disease detection on red oak main stem samples was tested. This protocol, paired with the nested PCR protocol (chapter 2), can be used to quickly detect *C. fagacearum* in a range of sample types, some of which were not previously suitable for detection.

Table 3.1. CfP2 primer/probe sequences used for real-time PCR reactions* for the detection of *Ceratocystis fagacearum*.

Primer/Probe	Sequence
CfP2-01	5'-TGGCAGGGACTTCTTTCTTCA-3'
CfP2-02	5'-TGGTTAAATGCAACTCAGCAATGA-3'
CfP2 Probe	5'-/56-FAM/ATGTTTCTGCCAGTAGTATT/3BHQ_1/-3'

*As developed by Kurdyla and Appel (2011).

Table 3.2. Detection of *Ceratocystis fagacearum* in branch subsamples from actively wilting crowns of *Q. rubra/Q. ellipsoidalis* (red oak), *Q. macrocarpa* (bur oak), and *Q. alba* (white oak) study trees using traditional isolation techniques.

Species	Branch Segment Assayed		Branch Basis*		Tree Basis*	
	Total No. Assayed	No. Positive	Total No. Assayed	No. Positive	Total No. Assayed	No. Positive
Red	108	105	27	27	9	9
Bur	96	59	24	17	8	7
White	96	22	24	8	8	6

*Results of branch segment assays were composited on a branch or a tree basis

Table 3.3. Detection of *Ceratocystis fagacearum* in branch subsamples from actively wilting crowns of *Q. rubra/Q. ellipsoidalis* (red oak), *Q. macrocarpa* (bur oak), and *Q. alba* (white oak) study trees using real-time PCR.

Species	Branch Segment Assayed		Branch Basis*		Tree Basis*	
	Total No. Assayed	No. Positive	Total No. Assayed	No. Positive	Total No. Assayed	No. Positive
Red	108	81	27	27	9	9
Bur	96	39	24	21	8	8
White	96	43	24	21	8	8

*Results of branch segment assays were composited on a branch or a tree basis

Table 3.4. Detection of *Ceratocystis fagacearum* from main stem samples on *Q. rubra/Q. ellipsoidalis* (red oak) trees one year after complete crown wilt using real-time PCR.

Sapwood Sample Type*	Stem Sample		Tree Basis†	
	Total No. Assayed	No. Positive	Total No. Assayed	No. Positive
Window	18	12	6	6
Mat Scar	21	13	7	5

* Window = sapwood sample removed from below cambium exhibiting vascular streaking or uniform brown in color; mat scar = sapwood underlying remnant of fungus sporulation mat.

† Results of stem samples were composited on a tree basis

Table 3.5. Model resulting from logistic-normal mixed model analysis of variables that affect that likelihood of detecting *Ceratocystis fagacearum* in actively wilting *Q. rubra/Q. ellipsoidalis* (red oak), *Q. macrocarpa* (bur oak), and *Q. alba* (white oak) trees (Type II Tests of Fixed Effects).

Effect	Num DF	Den DF	P-Value
Species	2	26.6	0.0002
Diagnostic Method	1	4.5	0.0343
Species*Diagnostic Method	1	19.6	<0.0001

Table 3.6. Models resulting from fixed effects logistic-normal regression of the interactions of actively wilting *Q. rubra/Q. ellipsoidalis* (red oak), *Q. macrocarpa* (bur oak), and *Q. alba* (white oak) trees and diagnostic method used for detection of *Ceratocystis fagacearum* in the sapwood.

Species	Diagnostic Method	Estimate	SE	P-Value	Estimated Probability*	Actual Probability†
Red	Isolation	4.1452	0.7986	<0.0001	0.98	0.97
	Real-Time PCR	1.0248	0.4053	0.0180	0.73	0.75
Bur	Isolation	0.6228	0.4305	0.1602	0.65	0.64
	Real Time PCR	0.3539	0.4279	0.4160	0.58	0.41
White	Isolation	-1.4427	0.4489	0.0031	0.19	0.23
	Real Time PCR	0.2636	0.4230	0.5391	0.56	0.45

* Estimated probability based on logit transformation from model estimates.
† Actual probability based on calculated proportions from samples used in this study.

Table 3.7. Model resulting from logistic-normal mixed model analysis of variables that affect the likelihood of detecting *Ceratocystis fagacearum* in sapwood of actively wilting *Q. rubra/Q. ellipsoidalis* (red oak), *Q. macrocarpa* (bur oak), and *Q. alba* (white oak) trees (Type II Tests of Fixed Effects).

Effect	Num DF	Den DF	P-Value
Species	2	31.9	0.6853
Diagnostic Method	1	594.0	<0.0001
Species*Diagnostic Method	1	594.0	0.5944

Table 3.8. Models resulting from fixed effects logistic-normal regression of the interactions of actively wilting *Q. rubra/Q. ellipsoidalis* (red oak), *Q. macrocarpa* (bur oak), and *Q. alba* (white oak) trees and PCR method used for detection of *Ceratocystis fagacearum*.

Species	Diagnostic Method	Estimate	SE	P-Value	Estimated Probability*	Actual Probability†
Red	Nested PCR	2.9441	0.534	<0.0001	0.95	0.96
	Real-time PCR	1.0094	0.389	0.0163	0.73	0.75
Bur	Nested PCR	2.7538	0.524	<0.0001	0.94	0.92
	Real-time PCR	0.3283	0.409	0.4315	0.58	0.41
White	Nested PCR	2.8393	0.538	<0.0001	0.95	0.93
	Real-time PCR	0.2495	0.405	0.5506	0.56	0.45

* Estimated probability based on logit transformation from model estimates.
† Actual probability based on calculated proportions from samples used in this study.

Table 3.9. Results of real time PCR band visibility of *Ceratocystis fagacearum*-colonized red oak drill shavings serially diluted in healthy *Q. rubra/Q. ellipsoidalis* (red oak) drill shavings and in ≥1 year dead red oak drill shavings.

Dilution	Real Time PCR Result	
	Mixed in healthy sapwood	Mixed in ≥1 year dead sapwood
Colonized wood	+	+
Non-colonized wood	-	-
1:2*	+	-
1:4	+	-
1:8	+	-
1:16	-	-
1:32	-	-
1:64	-	-
1:128	-	-
1:256	-	-
1:512	-	-
1:1024	-	-
1:2048	-	-

*Ratio is for colonized:non-colonized wood (w/w).

Figure 3.1. Standard curve generated using genomic DNA from pure cultures and real-time polymerase chain reaction (PCR) for *Ceratocystis fagacearum*. A 10-fold serial dilution of DNA concentrations from 0.265 ng/µL to 0.265 fg/µL was used in generating the standard curve.

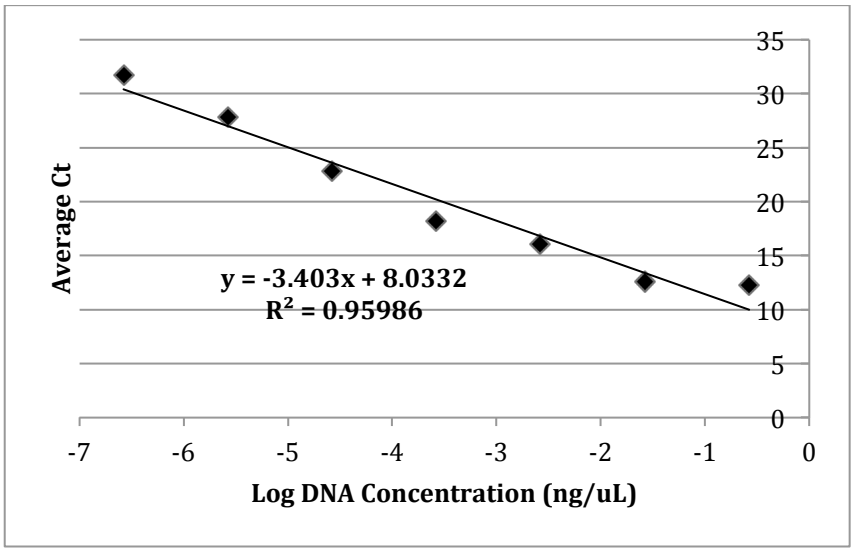


Figure 3.2. Proportion of *Ceratocystis fagacearum (C.f.)* - positive branch segments based on total number of segments assayed from wilting *Q. rubra/Q. ellipsoidalis* (red oak), *Q. macrocarpa* (bur oak), and *Q. alba* (white oak) trees.

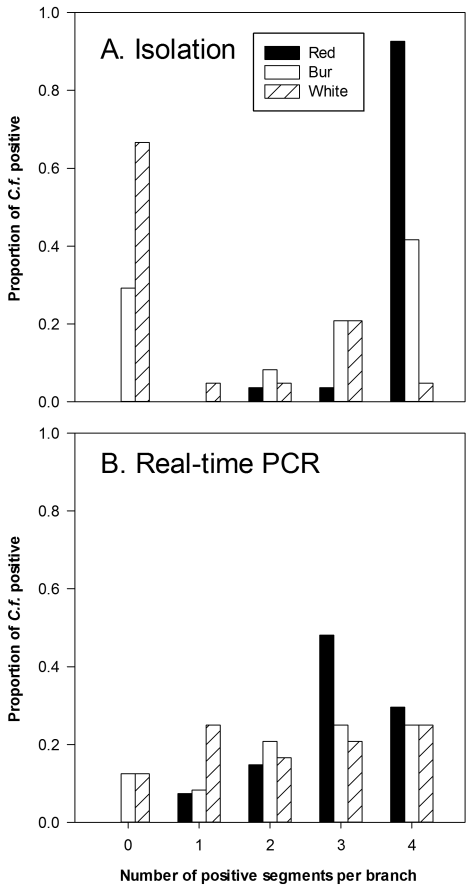


Figure 3.3. Proportion occurrence of branch segments per one-year dead branch of *Q. macrocarpa* (bur oak) and *Q. alba* (white oak) trees that were positive for *Ceratocystis fagacearum (C.f.)*.

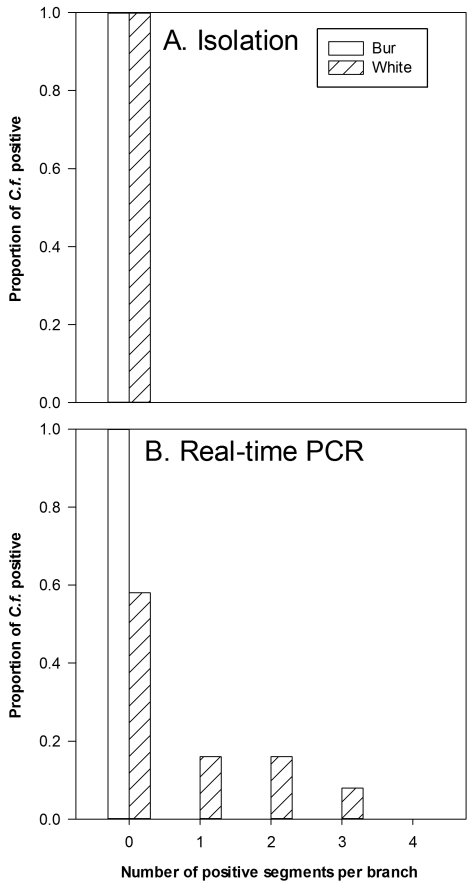
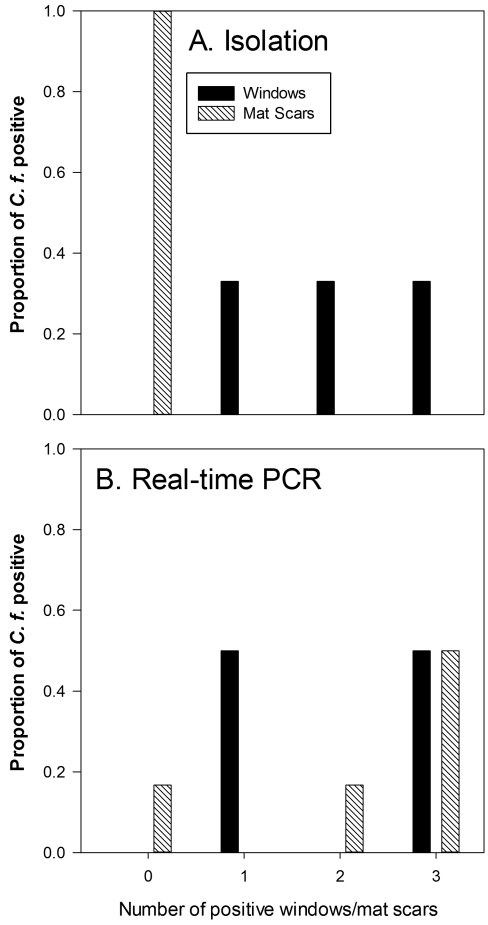


Figure 3.4. Proportion of sapwood windows from one-year dead *Q. alba/Q. ellipsoidalis* (red oak) trees that were positive for *Ceratocystis fagacearum (C.f.)* using traditional isolation and real-time PCR methods.



Chapter 4. Testing of nested PCR and real-time PCR protocols for the detection of *Ceratocystis fagacearum* in operational plant disease diagnostic clinics

Anna M. Yang

Introduction

Oak wilt is a major pathogen on oak species present in Minnesota as well as on other oak species present in the eastern United States and Texas. The causal agent of oak wilt, *Ceratocystis fagacearum*, is responsible for the death of numerous trees in Minnesota each year. Red oak species (section *Lobatae*) are highly susceptible to infection and the disease can commonly be identified by a dramatic full crown wilt that may occur in a single growing season (Juzwik et al. 2011; Tainter and Baker 1996). In contrast, species in the white oak group (section *Quercus*) have varied levels of susceptibility to disease development. Infected bur oak trees (*Q. macrocarpa*) have moderate levels of susceptibility, which are expressed by the appearance of scattered branch dieback that may persist for several years before leading to tree death. White oaks (*Q. alba*) with an oak wilt infection often wilt one branch or fork at a time over many years.

C. fagacearum infections in red oak species may be easily recognized in the field by trained tree health professionals; however, some circumstances may require an official confirmation via laboratory diagnosis. A diagnosis from a plant diagnostic clinic is generally required for symptomatic bur and white oak trees. Other pathogens, insects, or abiotic agents may confound a visual diagnosis of oak wilt in these species. Bacterial leaf scorch (*Xylella fastidiosa*) and the two-lined chestnut borer (*Agrilus bilineatus*) may result in similar visual symptoms in the crowns of red oak trees (Gould et al. 2005; Haack and Acciavatti 1992). In situations where the cost of management is justified, an accurate diagnosis is critical.

Plant disease diagnostic clinics currently follow oak wilt diagnostic protocols from published guidelines (Pokorny 1999). The guidelines involve plating pieces of symptomatic sapwood onto acidified potato dextrose agar (APDA) and providing a 6 to 14 day incubation period for the fungus to grow. Following incubation, the fungus is identified by 1) colony morphology and odor, and 2) presence of *C. fagacearum* endoconidia. The successful isolation of *C. fagacearum* from colonized sapwood is dependent on the quality of the material submitted to the diagnostic clinic. Branches that have been stored in a heated environment for prolonged periods of time lower the likelihood of culturing the fungus, thus leading to a false negative report (Bretz and Morison 1953). Dry branch material, either due to drought conditions or improper storage conditions, may also lead to a negative report.

Molecular techniques employing the use of DNA extraction and polymerase chain reaction (PCR) have become increasingly popular in plant disease diagnostics (McCartney et al. 2003; Schena et al. 2004). The use of molecular diagnostics can allow for faster, high-throughput testing that may increase productivity and decrease the turnaround time for disease diagnosis. Previously, pure cultures of fungal mycelium were necessary in order to use DNA-based identification techniques. However, methods of DNA extraction directly from xylem tissues of tree species have recently been published (Guglielmo et al. 2007, Guglielmo et al. 2010). The extraction of DNA directly from xylem tissues negates the previously required step of fungal isolation and dramatically reduces processing time.

Molecular protocols have been developed for the detection of *C. fagacearum* from wood (Kurdyla et al. 2011; Wu et al. 2011). The first protocol, developed by Wu et al. (2011), is a nested PCR design that utilizes *C. fagacearum*-specific primers. The nested PCR includes two rounds of amplification; the first round using universal fungal primers ITS1-F (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and the second round using *C. fagacearum* specific primers CF01 (5'-GGCGACTTCTTTCTT-3') and CF02 (5'-AAGGCTTGAGTGGTGAAA-3'). The developed primers were able to amplify DNA from the pathogen from artificially inoculated, sterile wood strips (3 x 1 x 0.2 cm) (Wu et al. 2011). The second protocol is a real-time PCR protocol, developed by Kurdyla and Appel (2011), which uses primers CfP2-01 (5'-TGGCAGGGACTTCTTTCTTCA-3'), CfP2-02 (5'-TGGTTAAATGCAACTCAGCAATGA-3'), and probe CfP2 (5'-/56-FAM/ATGTTTCTGCCAGTAGTATT/3BHQ_1/-3') developed specifically for *C. fagacearum* amplification and detection. The protocol was tested on *C. fagacearum* spore suspensions from pure cultures, purified genomic DNA, and stem samples from infected live oak and Montererey oak trees in Texas (Kurdyla and Appel 2011). The application of PCR protocols for oak wilt diagnostics in diseased northern species could improve on the speed and specificity of current diagnostics.

Chapters 2 and 3 of this thesis describe the application of the nested and real-time PCR protocol for the detection of *C. fagacearum* from the sapwood of naturally infected northern oak species and compares their accuracy to traditional isolation methods. Four types of tree material were tested, 1) red, bur, and white oak trees with actively wilting

branches, 2) bur and white oak branches ≥1 after branch death, 3) sapwood windows of red oak main stems with either vascular streaking or a uniform brown color, and 4) sapwood sampled beneath the remnants of deteriorated oak wilt fungal mats (= "mat scars"). The use of nested PCR resulted in a higher predicted probability of pathogen detection in actively wilting bur and white oak branch segments, ≥1-year dead bur and white oak branches, and red oak mat scars. There was no difference in predicted probability of *C. fagacearum* detection between nested PCR and isolation in actively wilting red oak branch segments or in main stem windows.

Use of the real-time PCR protocol did not result in a significant difference in the frequency of fungus detection in actively wilting red, bur, and white oak branch segments. The real-time PCR protocol resulted in less frequent detection of the fungus in actively wilting red and bur oak branch segments compared to the standard isolation method. However, when results for assayed branch segments were collapsed to the whole tree basis, there was no overall difference between detection methods. Neither of the methods was able to reliably detect the fungus from the ≥1-year dead bur and white oak branches. There was no observed difference in detection from bark windows taken from red oak main stems, but real-time PCR did yield a higher predicted probability of detecting the fungus from red oak mat scars compared to isolation methods. For full details on the methods and results, please refer to Chapters 2 (nested PCR) and 3 (real-time PCR) of this thesis.

The nested and real-time PCR methods were tested with the ultimate goal of application in plant disease diagnostic clinics. An inter-laboratory test was used to

examine the efficiency, accuracy, and cost efficiency of nested and real-time PCR for the detection of *Ceratocystis fagacearum* compared to traditional isolation methods. The trial reported here was completed in the summer and fall of 2014.

Materials and Methods

Sampling sites and protocols. In August of 2014, four locations with actively wilting (*Quercus rubra* or *Q. ellopsoidalis*), bur (*Q. macrocarpa*), and/or white oaks (*Q. alba*) were selected within the Minneapolis-St. Paul, MN metropolitan area. Sampling sites were located within the communities of Apple Valley, Oakdale, Rogers (Crow-Hassen Park Reserve), and Savage (Murphy-Hanrehan Park Reserve). Three branches, 5.0 to 7.6 cm in diameter, were collected from the crowns of four red, four bur, and three white oak trees with active wilt symptoms following the sampling protocols of Pokorny (1999). Three branches were also collected from the crowns of one apparently healthy tree with no wilt symptoms of each species to serve as a negative control. Up to ten 20.3 to 30.5 cm long segments were cut from each branch. Segments cut from each branch were separated into piles of three or four segments. The total number of branch segments was divided in half, sealed in plastic bags, and prepared for transported to the plant diagnostic laboratories at the University of Minnesota – St. Paul or University and the University of Wisconsin-Madison.

Processing using standard isolation protocol. One to three extra branch segments were processed in the St. Paul Annex, U.S. Forest Service Laboratory following previously published isolation protocols (Pokorny 1999) to document that

all branches were positive for the fungus. Branch segments were surface sterilized by spraying with 95% ethanol and flaming. The bark was peeled back with a sterile drawknife to expose the outer sapwood. Four 0.4 cm^2 pieces of stained sapwood were removed with a flame-sterilized wood gouge and forceps. The sapwood pieces were placed on acidified (5 mL of 20% lactic acid per 1 L) potato dextrose agar (APDA) in 100 mm deep Petri plates. The plates were incubated at room temperature and checked regularly for the presence of *C. fagacearum* for a maximum of 14 days. Wood chips that yielded mixed fungal isolates were sub-cultured until a pure culture of *C. fagacearum* was obtained. Colonies were identified by the presence of grey to olive-green mycelium, characteristic fruity odor, and presence of endoconidia.

Submission to plant disease clinics. Half of the sampled branch segments were placed on ice packs and delivered to the University of Minnesota Plant Disease Clinic. The second half of the branch segments were placed on ice packs and delivered to the University of Wisconsin-Madison Plant Disease Diagnostic Clinic. Each diagnostic clinic completed the same traditional isolation methods as previously outlined. Diagnosticians selected as many segments from each tree as determined necessary to optimize the possibility of obtaining the fungus and to also mimic their standard subsampling protocol for oak wilt testing. The disease clinics were asked to record which trees tested positive for the fungus and the average incubation time required to obtain the fungus from the sapwood (Table 4.1).

Each diagnostic clinic also tested one of the two molecular protocols for the detection of *C. fagacearum* from the same branch segments used for the isolation protocol. Both clinics followed the sapwood drilling and DNA extraction techniques outlined in Chapters 2 and 3 of this thesis. Following DNA extraction, the University of Minnesota Plant Disease Clinic tested the nested PCR protocol (see Chapter 2, this thesis, for protocol details) and the University of Wisconsin-Madison tested the real-time PCR protocol (see Chapter 3, this thesis, for protocol details). Clinics were asked to record which trees tested positive using the molecular diagnostic protocol and the average time to complete the DNA extraction and PCR (Table 4.1). Clinics were then asked to complete an evaluation and feedback form regarding the efficiency, accuracy, reliability and cost of the PCR protocol tested. Diagnosticians were asked to provide feedback on the following topics/questions regarding the molecular detection protocols:

1. General feedback on the sapwood drilling procedure (was it easy, difficult, tedious, etc.).
2. Were any problems encountered using the sapwood drilling procedure?
3. General feedback on use of the Qiagen ® DNA extraction kit.
4. Would the clinic consider using more complicated methods of DNA extraction that optimize DNA recovery from wood?
5. Were any problems encountered during the DNA extraction and PCR method? If so, what were they?

The diagnostic clinics were asked to provide feedback on the following topic/questions regarding typical oak wilt diagnostics and the potential of routinely using the molecular protocol(s):

1. What is your current fee for oak wilt testing?
2. What is your current fee for molecular diagnostics?
3. Do you currently sequence DNA for molecular diagnostic tests?
4. If the molecular protocol were in operational use, how long do you estimate that it would take from sample submission to obtaining results?

Results

St. Paul Annex, U.S. Forest Service isolation results. All symptomatic branch segments resulted in a positive culture of *C. fagacearum*. Branch segments sampled from healthy appearing control trees resulted in no *C. fagacearum* growth.

University of Minnesota Plant Disease Clinic assay results and feedback. The total time for sapwood plating and sapwood drilling was estimated to be the same (20 – 30 minutes) for both protocols. The DNA extraction was reported to take four hours to complete. The nested PCR protocol takes an estimated six hours to complete. *C. fagacearum* was detected from six of the same trees using the traditional isolation method and the nested PCR protocol (Table 4.2). There was one instance where isolation detected the fungus from a branch and nested PCR did not. There was also one instance where nested PCR detected the fungus when isolation did not. Three trees known to be positive

for the fungus tested negative by both tests. All branches sampled from healthy control trees tested negative for *C. fagacearum*. Overall, the comparison of traditional isolation and nested PCR by the University of Minnesota laboratory resulted in the same number of trees testing positive for the pathogen by both detection methods. Full responses for the DNA extraction and nested PCR protocol evaluation are contained in Appendix I.

University of Wisconsin-Madison Plant Disease Diagnostic Clinic assay results and feedback. Processing of branch samples using traditional isolation techniques was estimated to take a total of 30 minutes per sample, while the drilling protocol was estimated to take a total of 20 minutes. The DNA extraction was reported to take 45 minutes to complete and the PCR reaction took 1.5 hours to run. Isolation and real-time PCR had overlapping detection of *C. fagacaerum* for branches from three of the submitted trees (Table 4.3). Traditional isolation methods detected the fungus on three trees that tested negative with real-time PCR. In contrast, real-time PCR was able to detect the pathogen on a tree that tested negative using isolation methods. Four trees known to be positive for the fungus tested negative by both methods. All branches sampled from healthy control trees tested negative for *C. fagacearum*. The trial at the University of Wisconsin – Madison laboratory resulted in a higher rate of detection using isolation methods versus real-time PCR. This supports similar previous findings from real-time PCR assays to detect *C. fagacearum* (see Chapter 3, this thesis). Full responses for the DNA extraction real-time PCR protocol evaluation are contained within Appendix II.

Discussion

In this inter-laboratory test, two molecular methods for the detection of *C. fagacearum* from diseased sapwood were tested and evaluated in operational plant disease diagnostic laboratories. In summary, the real-time PCR protocol as tested is not recommended for use by plant diagnostic clinics. Further development and testing is needed before protocols are ready for operational use. However, the nested PCR protocol was successfully used to detect the pathogen from infected branch samples.

The duration of PCR is dependent on the type of PCR being performed. Nested PCR requires six hours while real-time PCR can be completed in as little as two hours (estimates do not include PCR preparation time). The estimated processing times suggest the opportunity for faster diagnostics; however, they may not necessarily reflect a realistic turnaround time for an oak wilt diagnosis.

Plant diagnostic clinics often manage hundreds of samples during a single growing season and time management must be carefully monitored in order to ensure the most efficient turnaround on all clinic submissions. The PCR protocols have the ability to provide a disease diagnosis within one to two days, but this rapid diagnosis requires dedicated laboratory time from the diagnostician that may not be warranted for all situations. Feedback obtained from the two laboratories suggested that the nested PCR protocol might, for the time being, be most useful as a tool for detection of *C. fagacearum* in material that will unlikely be able to result in a positive fungal culture through traditional isolation. Branches that have been subjected to high heat, dry branches, and dead branches are all examples of material that is unlikely to result in *C.*

fagacearum growth. A previous study (see Chapter 2, this thesis) found that the nested PCR protocol was able to detect DNA of the pathogen in dead bur and white oak branches that had died from a *C. fagacearum* infection and in bole samples from red oak trees the year following full crown wilt. Using the nested PCR protocol, diagnostic clinics should theoretically be able to detect the pathogen in one-year dead sapwood from which *C. fagacearum* isolation would not be possible. In the Minnesota clinic, oak wilt testing is limited to the growing season with fall cut-off dates occurring in September. Nested PCR could offer a much longer time period in which oak wilt diagnostics may occur, but obtaining a symptomatic sample during the leaf-off period would be problematic.

When traditional isolation methods were compared to real-time PCR (see Chapter 2, this thesis) on branch segments sampled from actively wilting oak crowns, there was either no difference in detection rate or a decrease in detection using real-time PCR. It is possible that the low level of pathogen detection may be attributed to low quality of extracted DNA or inhibitory substances in the PCR reaction.

Feedback from both diagnostic clinics suggests that the use of different DNA extraction methods may improve on the quality of DNA extracted and the reproducibility of testing results. However, methods resulting in higher quality nucleic acid isolation frequently utilize hazardous/toxic chemicals. Many diagnostic laboratories are open to the public and may not have fume hoods, thus making the use of these extraction protocols a larger safety concern.

Naturally, an increase in the number of drill samples and DNA extractions increases the likelihood of pathogen detection. In the previous chapters, as few as one branch segment out of the twelve total taken per tree may have been positive for the fungus, thus yielding a positive result for the entire tree. While combining drill shavings from multiple branches is cost effective, the progressive bulking of drill shavings has been shown to decrease the likelihood of pathogen detection (see Chapters 2 and 3, this thesis). Diagnostic clinics and customers must weigh the cost-benefits of such molecular protocols, especially when multiple samples may need to be run to ensure assay reliability. Currently, PCR-based diagnostic costs between $35 and $95 per sample, while culture-based oak wilt test costs $20 to $59 for the two laboratories participating in this study.

PCR testing for *C. fagacearum* presence has the potential to confirm a diagnosis of oak wilt with a faster turnaround than culturing methods. The PCR assay is also more sensitive than traditional diagnostic methods that are prone to false negative results from low quality samples. The University of Wisconsin-Madison Plant Disease Diagnostic Clinic plans to continue working on the real-time protocol with the anticipation of using it for routine oak wilt diagnostics. The University of Minnesota Plant Disease Clinic currently plans to use the nested PCR protocol on samples that are unlikely to result in a positive culture. These protocols could also prove useful as a research tool for oak wilt surveys in the upper Midwest states, with positive implications for oak wilt management.

Table 4.1. Template feedback table provided to each diagnostic clinic testing one of two PCR protocol for the detection of *C. fagacearum* from the sapwood of wilting *Q.* rubra/*Q. ellipsoidalis* (red oak), *Q. macrocarpa* (bur oak), and *Q. alba* (white oak) crowns.

Tree ID	Branches Numbers Used (1, 2, or 3)	Isolation Result (+/-)	Number of Days Incubated	PCR Result (+/-)	Extraction and PCR Time (min.)
R-1					
R-2					
R-3					
R-4					
R-5					
B-1					
B-2					
B-3					
B-4					
B-5					
W-1					
W-3					
W-4					
W-5					

Table 4.2. University of Minnesota Plant Disease Clinic results of isolation and nested PCR assays to detect *Ceratocystis fagacearum* from the sapwood *Q.* rubra/*Q. ellipsoidalis* (red oak), *Q. macrocarpa* (bur oak), and *Q. alba* (white oak) crowns.

Tree ID[a]	Isolation	Nested PCR
R-1	+	+
R-2*	-	-
R-3	+	+
R-4	+	+
R-5	+	+
B-1	+	-
B-2	-	+
B-3*	-	-
B-4	-	-
B-5	+	+
W-1	+	+
W-3	-	-
W-4	-	-
W-5*	-	-

[a] R = red oak; B = bur oak, W = white oak;
*Indicates apparently healthy oak trees (controls)

Table 4.3. University of Wisconsin-Madison Plant Disease Diagnostics Clinic results of isolation and real-time PCR assays to detect *Ceratocystis fagacearum* in the sapwood *Q.* rubra/*Q. ellipsoidalis* (red oak), *Q. macrocarpa* (bur oak), and *Q. alba* (white oak) crowns.

Tree ID†	Isolation	Real-Time PCR
R-1	-	-
R-2*	-	-
R-3	-	+
R-4	+	+
R-5	+	+
B-1	+	-
B-2	+	-
B-3*	-	-
B-4	-	-
B-5	+	+y
W-1	+	-
W-3	-	-
W-4	-	-
W-5*	-	-

†R = red oak; B = bur oak, W = white oak;
*Indicates apparently healthy oak trees (controls);
yCt of 37.83

Bibliography

Agresti, A. 2002. Categorical Data Analysis. John Wiley & Sons, Inc. Hoboken, New Jersey.

Ambourn, A. K., Juzwik, J., and Moon, R. D. 2005. Seasonal dispersal of the oak wilt fungus by *Colopterus truncatus* and *Carpophilus sayi* in Minnesota. Plant Dis. 89:1067-1076.

Appel, D. 1995. The oak wilt enigma: perspectives from the Texas Epidemic. Annu. Rev. Phytopathol. 33:103-118.

Banik, M.T., Lindner, D.L., Juzwik, J., and Glaeser, J.A. 2013. Use of DNA sequencing to detect pathogenic, saprotrophic, and stain fungi in sapwood of declining red pine (*Pinus resinosa*) in the Upper Midwest. In: Browning, John; Palacios, Patsy, comps. Proceedings of the Western International Forest Disease Work Conference; 2012 Oct. 8-12; Lake Tahoe, CA. Western International Forest Disease Work Conference: 101-110.

Barnett, H. L. 1953. Isolation and identification of the oak wilt fungus. W. Va. Agric. Exp. Stn. Bull. 359T. 15 p.

Bastien, P., Procop, G., and Reischl, U. 2008. Quantitative real-time PCR is not more sensitive than conventional PCR. J. Clin Microbiol. 6:1897-1900.

Blaedow, R. A., Juzwik, J., and Barber, B. 2010. Propiconazole distribution and effects on *Ceratocystis fagacearum* survival in roots of treated red oaks. Phytopathology 100:979-985.

Bretz, T. W. 1951. Oak wilt. J. Forest 49:169-171.

Bretz, T. W. 1952. The ascigerous stage of the oak wilt fungus. Phytopathology 42:435-437.

Bretz, T. W. and Morrison, D. W. 1953. Effect of time and temperature on isolation of the oak wilt fungus from infected twig samples. Plant Dis. Rep. 37:162-163.

Bruhn, J. N., Pickens, J. B., and Stanfield, D. B. 1991. Probit analysis of oak with transmission through root grafts in red oak stands. For. Sci. 37:28-44.

Cones, W.L. 1967. Oak wilt mats on white oak in West Virginia. Plant Dis. Rep. 51:430-431.

Couch, J. and Fritz, P. 1990. Isolaton of DNA from plants high in polyphenolics. Plant Mol. Biol. Rep. 8:8-12.

Deguilloux, M., Pemonge, M., and Petit, R. 2002. Novel perspectives in wood certification and forensics: dry wood as a source of DNA. Proc. R. Soc. Lond. B. Biol. Sci. 269:1039-46.

Eggers, J., Juzwik, J., Bernick, S., and Morduant, L. 2005. Evaluation of propiconizole operation treatments of oaks for oak wilt control. Research Note NC-390. USDA Forest Service, Northern Research Station, St. Paul, MN.

French, D. W. and Juzwik, J. 2002. Oak wilt in Minnesota. University of Minnesota Extension Service Publication. MI-3174-GO. Minnesota Extension Service, St. Paul, MN.

French, D. W. and Stienstra, W. C. 1975. Oak wilt. University of Minnesota, Agric. Exp. Stn., Extension folder 310.

Gibbs, J. N. and French, D. W. 1980. The transmission of oak wilt. Research Paper NC-185, St. Paul, MN: USDA Forest Service, North Central Research Station.

Ginzinger, D. G. 2002. Gene amplification using real-time quantitative PCR: an emerging technology hits the mainstream. Exp. Hematol. 30:503-512.

Gould, A.B. and Lashomb, J.H. 2007. Bacterial leaf scorch (BLS) of shade trees. The Plant Health Instructor. APSnet DOI: 10.1094/PHI-I-2007-0403-07.

Guglielmo, F., Bergemann, S., Gontheir, P., Nicolotti, G., and Garbelotto, M. 2007. A multiplex PCR assay for the detection and early identification of wood rotting fungi in standing trees. J. App. Microbiol. 103:1490-1507.

Guglielmo, F., Gonthier, P., Garbelotto, M., and Nicolotti, G. 2010. Optimization of sampling procedures for DNA-based diagnosis of wood decay fungi in standing trees. Lett. App. Microbiol. 51:90-97.

Haack, R. A. and Acciavatti, R. E. 1992. Twolined chestnut borer. USDA Forest Service. Forest Insect and Disease Leaflet 168, East Lansing, MI: USDA Forest Service, North Central Forest Experiment Station.

Hayden, K., Rizzo, D., Tse, J., and Garbelotto, M. 2004. Detection and quantification of *Phytophthora ramorum* from California forests using real-time polymerase chain reaction assay. Phytopathology 94:1075-1083.

Heirl, T., Reischl, U., Lang, P., Hebart, H., Stark, M., Kyme, P., and Autenrieth, I. B. 2004. Preliminary evaluation of one conventional nested and two real-time PCR assays for the detection of *Toxmoplasma gondii* in immunocomprimised patients. J. Med. Microbiol. 7:629-632.

Henry, B. W. 1944. *Chalara quercini* n.sp., the cause of oak wilt. Phytopathology 34:631-635.

Hunt, B. W. 1956. Taxonomoy of the genus *Ceratocystis*. Lloydia 19:1-58.

Jacobi, W. R. and MacDonald, W. L. 1980. Colonization of resistant and susceptible oaks by *Ceratocystis fagacearum*. Phytopathology 70: 618-623.

Jeyaprakash, A., Davison, D. A., and Schubert, T. S. 2013. Molecular detection of the laurel wilt fungus, *Raffaelea lauricola*. Plant Dis. 98:559-564.

Johnson, P. S., Shifley, S.R., and Rogers, R. 2002. The Ecology and Silviculture of Oaks. CABI Publishing, Oxford, England.

Juzwik, J., Appel, D. N., MacDonald, W. L., and Burks, S. 2011. Challenges and successes in managing oak wilt in the United States. Plant Dis. 95:8.

Juzwik, J., French, D. W., and Jeresek, J. 1985. Overland spread of the oak wilt fungus in Minnesota. J. Arbor. 11:323-327.

Juzwik, J. and Schmidt, T. L. 2000. Oak wilt and oak decline in the upper Midwest USA, pp. 139-145. *In* Oszako, T. and Delatour, C.: Proc recent advances on oak health in Europe. Forest Research Institute. Warsaw, PL.

Kowalski, T. T. and Winget, C. H. 1963. Patterns of water movement in forest trees. Bot. Gaz. 124:301-311.

Kurdyla, T. and Appel, D. 2011. The Detection of *Ceratocystis fagacearum* in Texas live oak using real-time polymerase chain reaction. Phytopathology 101:S95.

Lindahl, T. 1993. Instability and decay of the primary structure of DNA. Nature 362:709-715.

MacDonald, W. L. and Hindal, D. F. 1981. Life cycle and epidemiology of *Ceratocystis fagacearum* pp. 128-138. *In*: Fungal Wilt Diseases of Plants. Ed. Mace, M., Bell, A. A., Beckman, C. H. Academic Press, Inc. New York, New York.

McCartney, H., Foster, S., Fraaije, B., and Ward, E. 2003. Molecular diagnostics for fungal plant pathogens. Pest. Manag. Sci. 59:129-42.

Miles, P. D. and VanderSchaaf, C. 2014. Forests of Minnesota, 2013. Resource Update FS-9. Newton Square, PA: U.S. Department of Agriculture, Forest Service, Northern Research Station. 4 p.

Nair, V. M. G. and Kuntz, J. E. 1963. Mat formation in bur oaks infected with *Ceratocystis fagacearum*. Wis. Coll. Agric. For. Res. Note 95. 6 p.

Neumaier, M., Braun, A., and Wagener, C. 1998. Fundamentals of quality assessment of molecular amplification in clinical diagnostics. Clinic. Chem. 44:12-26.

O'Brien, J. G., Mielke, M. E., Starkey, D., and Juzwik, J. 2011. How to identify, prevent, and control oak wilt. USDA Northeastern Area State and Private Forestry, St. Paul, MN: NA-FR-01-11. 30 p.

Pokorny, J. 1999. How to collect field samples and identify the oak wilt fungus in the laboratory. USDA Forest Service, Northeastern Area State and Private Forestry. St. Paul, MN: NA-FR-01-99. 11 p.

Pokorny, J. D. and Harrington, T. C. 2011. Bur Oak Blight. USDA Forest Service Northeastern Area State and Private Forestry, St. Paul, MN: Pest Alert NA-PR-02-11. 2 p.

R Core Team. 2013. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org/.

Rachmayati, Y., Leinemann, L., Gailing, O., and Finkeldey, R. 2006. Extraction, amplification and characterization of wood DNA from *Dipterocarpaceae*. Plant. Mol. Biol. Rep. 24:45-55.

Samuelian, S.K., Greer, L.A., Savocchia, S., and Steel, C.C. 2011. Detection and monitoring of *Greeneria uvicola* and *Colletotrichum acutatum* development on grapevines by real-time PCR. Plant Dis. 95: 298-303.

Schena, L., Nigro, F., Ippolito, A., and Gallitelli, D. 2004. Real-time PCR: a new technology to detect and study phytopathogenic and antagonistic fungi. Euro. J. Plant Pathol. 110:893-908.

Schoch, C. L., Seifert, K. A., Huundorf, S., Robert, V., Spouge, J. L., Levesque, C. A., and Chen, W. 2012. Nuclear ribosomal internal transcribed spacer (ITS) region as universal DNA barcode marker for Fungi. Nat. Acad. Sci. Proc. 109:6241-6246.

Shifley, S. R. and Sullivan, N. H. 2002. The status of timber resources in the North Central United States. Gen Tech Rep NC-228, St. Paul, MN: USDA Forest Service, Northern Central Research Station.

Sikdar, P., Okibara, P., Mazzola, M., and Xiao, C. L. 2013. Development of PCR assays for diagnosis and detection of the pathogens *Phacidiopycnis washingtonensis* and *Sphaeropsis pyriputrescens* in apple fruit. Plant Dis. 98:241-246.

Tainter, F. H. and Baker, F. A. 1996. Oak wilt. Pp. 673-682. *In*: Principles of Forest Pathology. John Wiley and Sons, Inc. New York, New York.

Wang, Y., Wang, Y. and Tian, C. 2013. Quantitative detection of pathogen DNA of Verticillium wilt on smoke tree *Cotinus coggygria*. Plant Dis. 97:1645-1651.

Wargo, P. M. Houston, D. R., and LaMadeleine, L. A. 1983. Oak Decline. USDA Forest Service Northeastern Forest Experiment Station, Hamden, CT: Forest Insect and Disease Leaflet 165.

Wu, C. P., G. Y. Chen, B. Li, H. Su, Y. L. An, S. Z. Zhen, and J. R. Ye. 2011. Rapid and accurate detection of *Ceratocystis fagacearum* from stained wood and soil by nested and real-time PCR. For. Pathol. 41: 15–21.

Yang, A., Juzwik, J., and Mollov, D. 2014. Use of nested PCR to detect *Ceratocystis fagacearum* in sapwood of diseased northern oak species. Phytopathology 104-34-O.

Zeigenhagen, B., Guillemaut, P., and Scholz, F. 1993. A procedure for mini-preparations of genomic DNA from needles of silver fir (*Abies alba* Mill.). Plant. Mol. Biol. Rep. 11:117-121.

Appendix I: Feedback received from the University of Minnesota Plant Disease Clinic regarding the nested PCR protocol tested.

Respondents: Dr. Brett Arenz and Jennifer Flynn

1. **General feedback on the sapwood drilling procedure (was it easy, difficult, tedious, etc.):**

A sample with obvious symptoms probably took about 20 minutes to peel, collect wood cores for culture and collect drill shavings. On samples without clear symptoms, it probably took about 30 minutes due to extra time peeling bark to look for symptoms. The drilling was easy as long as I had all drill bits ready ahead of time. Once bark was peeled, it didn't take long to do the drilling – about a minute.

If just 1 sample is run, it takes approximately half of a working day. Adding more samples to that same run does not significantly increase the time.

2. **Were any problems encountered using the sapwood drilling procedure?**

When it came to weighing out the shavings on the analytical balance, they had static and this made the wood shavings unruly. This could be a step where cross-contamination could occur if one wasn't very careful with the unruly shavings. I found it better to not weigh them and just use the whole 1.5mL tube of shavings for the extraction.

3. **General feedback on use of the Qiagen® DNA extraction kit:**

It's easy, but there are a lot of steps.

4. **Would the clinic consider using more complicated methods of DNA extraction that optimize DNA recovery from wood?**

While no one step of the DNA testing is terribly difficult, it all adds up to a lot of time. Currently, we cannot spend so much time per Oak Wilt test when there are hundreds of samples to examine. I did find the DNA test useful and worthwhile for some samples from counties that hadn't had a lab-confirmed case of Oak Wilt. Three such samples arrived late in the growing season in 2014. The cultures were negative, but the cases

were suspicious for the oak wilt fungus. The DNA gave clear positive on 2 of the 3 samples.

5. **Were any problems encountered during the DNA extraction and PCR method? If so, what were they?**

It appeared that a couple of the samples that had positive cultures had negative DNA results. I suspect a more rigorous bead beating method might improve things.

Oak Wilt Diagnostics Feedback:

1. **What is your current fee for oak wilt testing?**

$59

2. **What is your current fee for molecular diagnostics?**

PCR: $95

3. **Do you currently sequence DNA for molecular diagnostic tests?**

Yes, though it is typically reserved for samples that cannot be confirmed through regular testing.

4. **If the molecular protocol were in operational use, how long do you estimate that it would take from sample submission to obtaining results?**

The problem is that it's neither time nor cost effective to run just 1 or 2 samples. Samples for oak wilt tests tend to arrive at the Clinic in small numbers from June to the end of September. By the time we "saved up" enough samples to economically justify the DNA test, it's possible that we could have the culture results done. In other words, the culture might be done before the DNA test got started.

It's hard to do multistep procedures during the growing season when there are a lot of interruptions as we have in a Plant Disease Clinic setting.

Appendix II: Feedback received from the University of Wisconsin-Madison Plant Disease Diagnostic Clinic regarding the real-time PCR protocol tested.

Respondents: Brian Huddelson, Ann Joy, Sean Toporek

1. **General feedback on the sapwood drilling procedure (was it easy, difficult, tedious, etc.):**

Average time to peel bark and plate sapwood chips: 30 minutes

Average time to complete sapwood drilling: 20 minutes

I found the drilling itself easy enough, but sterilizing the drill bits was tedious. Could they not have been autoclaved instead?

2. **Were any problems encountered using the sapwood drilling procedure?**

When branches were small one tended to drill right through and lose some sample, and combine that with branches of limited symptom area and it could be difficult to collect enough.

3. **General feedback on use of the Qiagen® DNA extraction kit:**

Average time to complete DNA extraction per tree: 45 min to 1 hour

4. **Would the clinic consider using more complicated methods of DNA extraction that optimize DNA recovery from wood?**

I think more complicated methods, although more time consuming and toxic, might be more reproducible. However, given that our lab is a facility open to the public, safety issues might prevent use of these extraction techniques.

5. **Were any problems encountered during the DNA extraction and PCR method? If so, what were they?**

Real time PCR yielded a) results that were difficult to reproduce and b) high ct values, often times no values. I worried that if there was DNA in the sample that maybe it was

too dilute/sparse after using the outlined extraction methods or it just did not succeed in extracting DNA to begin with.

Oak Wilt Diagnostics Feedback:

5. **What is your current fee for oak wilt testing?**

$20

6. **What is your current fee for molecular diagnostics?**

We price this based the particular test. Right now the most common molecular test that we run is one for phytoplasmas. The charge is $35. Because woody tissue is more difficult and time-consuming to work with, we might charge more (e.g., $50).

7. **Do you currently sequence DNA for molecular diagnostic tests?**

Yes, in situations where we do not have other techniques to confirm the identity of a pathogen, or in situations where we may be dealing with a new pathogen or pathogen/host combination.

8. **If the molecular protocol were in operational use, how long do you estimate that it would take from sample submission to obtaining results?**

Given the results that we obtained, I am not sure that I would use this technique. Culturing from branches seems more reliable and confirmed more positive samples.