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CONTROL OF OAK WILT AND THE GENETIC STRUCTURE
OF *CERATOCYSTIS FAGACEARUM*

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CONTROL OF OAK WILT AND THE GENETIC STRUCTURE OF *CERATOCYSTIS FAGACEARUM*

By

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ABSTRACT

CONTROL OF OAK WILT AND THE GENETIC STRUCTURE OF *CERATOCYSTIS FAGACEARUM*

By

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Oak wilt is a lethal disease of oak (*Quercus spp.*) caused by the fungus *Ceratocystis fagacearum.* Limited applicability of current control methods necessitates additional research into disease control. Three areas of research that could support future control strategies were considered. The first objective was to determine the length of time that the fungicide propiconazole (Alamo® 14.3% a.i, Syngenta Speciality Products) protects a tree from infection following fungicide injection. Results from a research plot showed that all control trees inoculated with *C. fagacearum* wilted in the same year as inoculation whereas only six of eleven trees inoculated 34 months following fungicide injection developed wilt symptoms by 2006, one year after inoculation. These trees had delayed symptom development when compared with the untreated controls, indicating that propiconazole may inhibit wilt up to 34 months following fungicide treatment. The second objective was to determine the efficacy of a laboratory-induced hypovirulent, *C. fagacearum* mutant, PM447, as a biocontrol agent. Results from oak seedling assays were varied, with some indication that, dependent on environmental factors, PM447 may protect seedlings when inoculated prior to inoculation with a wild-type *C. fagacearum* isolate. Results from field plot studies indicate that trees first inoculated with the hypovirulent

strain and then challenged with a wild-type strain exhibited slower disease progression; however, these trees ultimately succumbed to wilt. The final objective was to determine the level of genetic diversity within the *C. fagacearum* population, particularly within the Great Lakes region, and to identify genetic markers to assist in population studies using amplified fragment length polymorphisms (AFLPs). Twenty-five primer combinations were initially tested and three of these primer combinations were selected based on reproducibility and clarity of bands. Twenty-four isolates from Michigan (both the upper and lower peninsulas), Minnesota, and Wisconsin, and one isolate each from Texas and West Virginia were analyzed. Results show little genetic variation among all isolates tested indicating that this pathogen has only recently evolved or been introduced into the United States.

This is dedicated to my husband, Shawn, and our son, Zach, whose love and support enabled me to achieve my goals. Also to my parents, who stood by me through it all. And lastly to my sister for being the best friend I could ask for.

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Introduction and Literature Review

In the early twentieth century, several reports of an unknown, lethal disease of oak were recorded in various states including Wisconsin and Minnesota, and later Texas (Gibbs and French 1980, Appel 1995, Prey and Kuntz 1995). The disease was particularly prevalent in the Upper Mississippi Valley, and in 1942, a report in a Wisconsin Agriculture Experiment Station bulletin indicated that the causal agent was a previously unknown fungus (Anonymous 1942). In 1944, Henry described this fungus and proposed the name, *Chalara quercina*, based on the asexual stage observed. The common name "oak wilt" was prescribed to the disease as it seemed to best fit the symptoms of the disease and had been in use for several years (Henry et al. 1944). A decade later the sexual stage of the fungus was discovered when two isolates paired in culture produced perithecia, suggesting the heterothallic nature of the fungus (Bretz 1951). Based on the sexual stage, the fungus was placed within the Ceratostomella complex. In 1952, however, Bretz renamed the fungus *Endoconidiophora fagacearum* based on its conidial stage as it was considered a better indicator of the true taxonomic placement of the fungus. The species epithet was changed to reflect the fact that the fungus was found to be pathogenic to other genera in the Fagaceae. The pathogen is now known as *Ceratocystis fagacearum* based on the taxonomy of the Ceratocystis genus as described by Hunt (1956).

Since its discovery, oak wilt has often been considered the most important disease of oak in North America. The pathogen, *Ceratocystis fagacearum* (Bretz)Hunt, invades the xylem tissues of oaks triggering the production of

tyloses and gums by the host in an attempt to inhibit the spread of the pathogen (Beckman et al. 1953, Struckmeyer et al. 1953). These defensive reactions, however, in addition to fungal material, lead to blockage of the xylem vessels and ultimate wilt of the tree. Oaks are ring-porous trees, with large springwood vessels; in such trees, water may move mostly through the vessels of the current season. This feature makes oak especially vulnerable to wilt as the pathogen readily and predominantly invades the current season's vessels. The disease is particularly devastating to red oaks (subgenus *Quercus* section *Lobatae*), with mature trees often completely wilting within six to eight weeks after initial introduction of the pathogen into xylem tissues.

Ceratocystis fagacearum is a heterothallic ascomycete and produces perithecia containing ascospores when compatible mating types (designated A or B) are present. Either mating type can function as the female thallus, with conidia from the opposite mating type serving as spermatia (Hepting et al. 1952). Both mating types appear to occur in a 1:1 ratio in all areas throughout the range of the disease (Barnett and Staley 1953, Kaufman and MacDonald 1973, Appel et al. 1985). Perithecia are described as minute, black, and flask-shaped, with unicellular, hyaline ascospores extruded from the ostiole in a white, sticky mass (Bretz 1951). In its anamorphic state, *C. fagacearum* produces conidia typically borne in chains in the terminal cells of conidiophores. In culture, the fungus produces an olive to dark gray mycelium with lighter grayish aerial mycelium, becoming more tan and often producing sclerotia as it ages. Microscopically, the hyphae and conidiophores are subhyaline to brown and septate (Henry 1944). The fungus also produces a distinctive overripe, fruity odor.

Within a host tree, the fungus produces few hyphae or conidia until advanced stages of wilt develop. Following wilt, hyphae may grow out of the sapwood into the space between the cambium and bark to produce mycelial mats with centrally located pressure pads that function to force the bark open, thereby exposing the mat (Curl et al. 1952, Gibbs and French 1980). Conidia are abundantly produced on the mats and, in areas where both mating types occur, perithecia may also be present (Curl et al. 1953). Mats thus serve as a source of inoculum for overland spread of the pathogen. In general, mats form the spring following tree wilt, dependent on wood moisture and air temperature. Thus, the timing of tree death appears to influence mat development. Mats only form on trees belonging to the red oak group, signifying the importance of this subgenus in the disease cycle.

Overland spread of the pathogen is thought to primarily occur via the sap-feeding nitidulid beetles (Coleoptera:Nitidulidae), particularly in the north central states (Jewell 1956, Hayslett et al. 2007). Nitidulid beetles are attracted to the odor produced by both tree wounds (sap of the tree) and fungal mats, and have consistently been associated with sporulating mats produced on wilted trees and fresh sap wounds on healthy trees (True et al. 1952, Dorsey et al. 1953, Jewell 1956). Juzwik (2001) suggests that certain species of nitidulids may possess an ecological specialization as vectors in the oak wilt disease cycle. Several studies have also implicated oak bark beetles (*Pseudopityophthorus* spp.) as important vectors of *C. fagacearum*, predominantly in areas where mat production appears to be rare (Buchanan 1956, Rexrode 1976); however, there is still only circumstantial evidence regarding the role of these insects in overland spread of

the disease. Under experimental conditions, several other insects have been shown to be able to transmit the fungus to oak (Curl 1956, Himelick and Curl 1958, Merrill and French 1995); however, the nature of these potential vectors and the biology of the pathogen suggest these insects play a limited role, if any, in the overland spread of the disease.

Local spread of the pathogen is primarily through root grafts, which form when the roots of two trees of the same species grow into contact (Kuntz and Riker 1950). The incidence of root grafting is dependent on several factors, including soil type, the diameter of the trees involved, and the distance between these trees (Bruhn et al. 1991). Intraspecies root grafting may occur with a frequency of up to 35% in a site, while interspecies root grafts are rare (Beckman and Kuntz 1951, Jones and Partridge 1961, Rexrode 1978). Root-graft spread accounts for the majority of new cases of wilt and can result in large disease centers that radiate out from an initially infected tree. Aerial surveys are often used to identify these centers.

The most common and obvious symptom of the disease on red oak is bronzing of the leaves from the margin inward, with defoliation of leaves expressing all stages of wilt common; however, depending on the time of infection, leaves may turn brown and remain attached to the tree. Symptoms of late summer/early fall infection can be confused with normal fall color changes, so wilt may not be apparent until the following year when either the tree does not leaf out or, after leafing out, the crown exhibits symptoms and the tree wilts. Epicormic shoots sometimes develop from wilted trees, particularly the year often following wilt, but these will usually wilt and die within the year. Infected oaks

exhibit a characteristic dark streaking in the sapwood.

Disease progression in white oaks (subgenus *Quercus* section *Quercus*) is distinct from red oaks. White oaks may experience branch dieback over a period of several years with many ultimately recovering; thus, white oaks are considered fairly resistant to oak wilt. Several factors likely account for this apparent difference in susceptibility. First, tylosis formation is generally more common in white oaks than red oaks, both as a natural process and in response to pathogens, which may help prevent vertical movement of the pathogen through the tree (Jacobi and MacDonald 1980). In addition, white oaks lack the abundant lateral vessel interconnections that red oaks possess, which limits lateral growth of the pathogen (as hyphae move from vessels to parenchyma through pits) and prevents fungal movement around vertical blockages. White oaks also produce a distinct zone of dark, dense material that accumulates in the parenchyma surrounding infected cells (Sachs et al. 1970, Jacobi and MacDonald 1980); this discoloration is scattered/diffuse in the parenchyma of red oaks. The material consists of phenolic compounds that inhibit pathogen movement. Due to these aforementioned defense responses of white oaks to *C. fagacearum*, these oaks are often able to confine the pathogen, form new wood over the infected wood, and recover (Sachs et al. 1970).

Live oaks (*Quercus virginiana and Q. fusiformis*), although considered part of the white oak group, are fairly susceptible to wilt and have been particularly devastated by the disease in Texas. These oaks have a xylem morphology that is more similar to red oaks, and while up to 20% of infected individuals may recover, the clonal sprouting habit of these trees makes root graft spread

problematic (Appel 1995).

Through the 1950's, disease control mainly relied on deep-girdling infected trees or treatment with silvicides in an attempt to contain the pathogen and prevent formation of mycelial mats. Destruction of all oaks within 50 feet of an infected oak was also standard practice; however, this resulted in unacceptable levels of tree loss and incomplete containment of the pathogen (MacDonald and Hindal 1981). Biological control using *Hypoxylon punctulatum* (Davis 1966), endophytic bacteria such as *Pseudomonas* spp. (Gonzalez et al. 1995, Wilson and Lester 1992), and *Ophiostoma quercus* (Juzwik et al. 1998) has been ineffective.

Currently, oak wilt control relies on prevention of overland introduction of the pathogen or prevention of root graft spread once the pathogen has been introduced into a stand. Community-based control programs have become a principal means of preventing and minimizing disease spread and occurrence in localized areas (Billings 2008, Juzwik et al. 2004). Control methods include preventative sanitation measures, trenching, and fungicide treatment. Preventing wounds (or application of wound paint to wound sites) on healthy oaks during the spring and early summer, when vector activity is thought to be greatest, potentially minimizes pathogen introduction (Camilli et al. 2008). In addition, removing wilted trees and completely covering any firewood from diseased trees with plastic helps prevent mat formation and subsequent vectoring (O'Brien et al. 2000).

Once the fungus is introduced into an area, control measures focus on prevention of local spread through root grafts. Root grafts are typically disrupted

by making five-feet-deep trenches; the location of trenches is dependent on the site (soil type) and the diameters of and distance between infected and healthy trees (Bruhn et al. 1991). The means of trenching (either with a trencher, vibratory plow, or rock saw) often excludes this procedure in many areas because the equipment is large, often not readily available, and relatively expensive. In addition, some residential sites may have buried power, phone, or cable lines, septic, well, driveways, etc. that would prevent the necessary location of the trench line to create an effective barrier. Several experiments testing various antibiotics and chemicals to treat oak wilt have had little success (Phelps et al. 1966, Appel 1995). Chemical injection of propiconazole may help to protect individual trees; however, treatment is often only effective for white oaks with variable, but little, success in red oaks that are already infected (Eggers et al. 2005, Osterbauer and French 1992, Osterbauer et al. 1994). In addition, the fungus is able to move past a treated tree and into the root system of the next adjacent tree, limiting the value of chemical treatment further (Appel 1995).

Since its identification in the early 1940's, oak wilt has been considered one of the most important and devastating diseases of oak. To date, oak wilt has been verified in 22 states. Although the origin of *Ceratocystis fagacearum* is unknown, the identification of oak wilt in these states following the discovery of the causal pathogen is likely due to recognition of the disease rather than rapid spread (MacDonald 1995). Current research on the genetic structure of the *C. fagacearum* population suggests that is an introduced pathogen (Kurdyla et al. 1995, Juzwik et al. 2008, unpublished data). Although it is not currently known

outside of the U.S., there are concerns that it could be transported to Canada or Europe. MacDonald et al. (2001) determined that European oaks are similarly susceptible to the disease as the red oaks of North America. There is also the possibility of pathogen spread to western states, such as California and Colorado, which have susceptible oaks and populations of nitidulids that are known to vector other species of *Ceratocytis* (Juzwik 2001, Appel 1994). An additional concern is the possibility of importing insect vectors that could move the fungus more efficiently/effectively overland. For example, the European bark beetle, *Scolytus intricatus* (Coleoptera:Scolytidae), is an aggressive feeder that causes wounds often extending into the xylem tissues of trees (Juzwik 2001, MacDonald et al. 2001). The limited control measures currently available belie the importance of further research into additional means of control.

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Chapter 1

Effective longevity of propiconazole following injection into *Quercus rubra*

Abstract

Propiconazole was injected into *Quercus rubra* to determine duration of efficacy against *Ceratocystis fagacearum*, the fungus that induces oak wilt. Eleven and thirteen trees were treated preventatively in 2002 and 2003 respectively; these trees were subsequently inoculated with a conidial suspension of *C. fagacearum* at 0, 9.5, 14, 21.5, 23, 24, or 34 months following fungicide injection. Controls included trees either injected with fungicide, inoculated, or untreated and non-inoculated. Five to six-foot-deep trenches isolated treatment groups. Propiconazole-injected trees inoculated in May 2005, as late as 34 months following fungicide treatments, did not express wilt symptoms for at least three months; however all untreated, inoculated control trees developed symptoms within six weeks. As of August 2006, over one year after final inoculations, fourteen of the twenty-four treated and inoculated trees (including five of eleven trees inoculated at 34 months), remained symptomless. Of the ten symptomatic trees, nine had been inoculated twice and the remaining one had not taken up the full amount of fungicide administered. Results suggest that inhibition of *C. fagacearum* occurs at 24 months, and even up to 34 months, post-injection and that the duration of protection is dependent on the actual amount of propiconazole solution injected and disease pressure.

Introduction

Oak wilt is a lethal disease of oaks caused by the fungus *Ceratocystis fagacearum*. The pathogen invades the xylem inducing tylosis and gummosis in the host, which, in addition to fungal material (primarily hyphal fragments), blocks the movement of water through sap tissues. Overland spread of *C. fagacearum*

occurs via insect vectors, while local spread is primarily through root grafts that form between neighboring trees of the same species.

Intravascular injections with a wide variety of antibiotics and fungicides have had limited success in treating or preventing oak wilt (Phelps et al. 1966, Appel 1995). In 1992, however, Appel and Kurdyla determined that live oaks (*Quercus fusiformis* and *Q. virginiana*) injected with the triazole fungicide, propiconazole (an ergosterol inhibitor), had significantly lower disease levels compared to untreated trees, particularly when treated preventatively. Based on tests with live oaks, propiconazole injection was deemed to be an effective preventative treatment for oak wilt and was registered for use on live oaks in Texas (Appel 1995). Osterbauer et al. (1992 and 1994) subsequently studied injection treatments in other species of red oak (subgenus *Quercus* section *Lobatae*) and white oak (subgenus *Quercus* section *Quercus*) in Minnesota and found that propiconazole could protect a treated tree for up to two years against root graft spread. Additional research on propiconazole injection treatments has shown that white oaks typically respond well to fungicide injection and can often be treated therapeutically, whereas red oaks often succumb to wilt despite treatment if they already are infected (Eggers et al. 2005, Osterbauer and French 1992, Osterbauer et al. 1994). Therefore, red oak injections are usually limited to high value trees with little or no symptoms of disease. Due to the high cost of treating trees (a few hundred dollars per tree, including chemical and labor costs, is common) and variable success, additional knowledge regarding the activity of propiconazole within a tree is necessary for effective management strategies.

As the fungus can remain viable for several years in the roots of wilted trees,

and spread through root grafts may take several years to occur, it is difficult to predict where the pathogen is within the root system at any given time (Yount 1955, Rexrode 1978). In addition, disease progression in root-infected trees is often delayed in comparison to trees inoculated aboveground (Cobb et al. 1965). Therefore, it is difficult to determine whether symptomless trees within infected stands already have the pathogen within their roots. Previous research has focused on the efficacy of propiconazole injection against root graft transmission of *C. fagacearum* in stands where oak wilt was present. We investigated the longevity of propiconazole activity in an oak stand in Michigan where root graft transmission was prevented by trenching prior to experimentation.

Materials and Methods

Study Site. All oaks (*Quercus rubra*) used in the study were located at a single site on the Michigan State University campus and ranged from 16 to 51 cm in diameter at breast height (dbh), with the average dbh equal to 30 cm (standard error = ± 2.4 cm) (Figure 1). Trees at the site were arranged in six rows. The soil consisted of Colwood-Brookston loam (62%) and Capac loam (38%), which are both characterized by deep, poorly drained, fine loamy soil (Soil Survey Staff 2007, Web Soil Survey 2007). Five to six-foot-deep trench lines were established in June 2002 using a Davis Fleetline 70+4 trencher, which isolated treatment groups by disrupting potential root grafts (Figures 1.2 and 1.3). The resulting trenches were subsequently backfilled with soil.

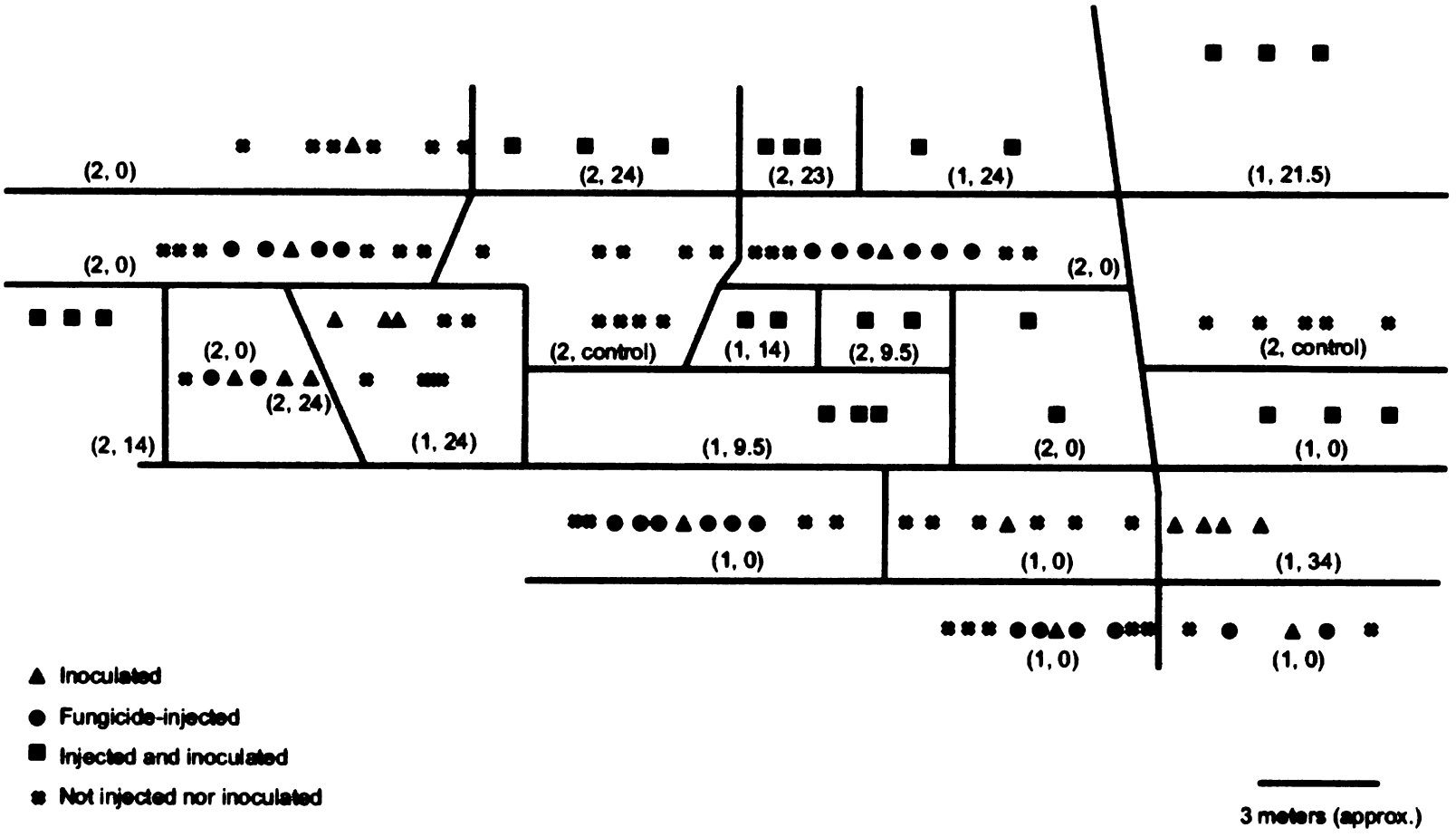

Figure 1. Map of Beaumont site. Black lines indicate location of trenches. Rep (1 or 2) and treatment are given in parentheses (rep, treatment) within each treatment group. Treatments indicate when trees were inoculated (in months following injection). Injections were performed in July 2002 (Rep 1) or June 2003 (Rep 2). Additional negative controls were considered until inoculated as positive controls.

Figure 1.2 Trench lines, dug with a Davis Fleetline 70+4 trencher, were used to isolate treatment groups.

Figure 1.3 Five to six-foot-deep (1.5 – 1.8 m) trenches break potential root grafts between neighboring trees. Trenches are approximately 4-6 in. (10-15 cm) wide.

Experimental Design. The experiment was replicated twice, first in July 2002 (Rep 1) and then in June 2003 (Rep 2). Eleven trees from Rep 1 and thirteen from Rep 2 were injected with propiconazole and then inoculated with a wild-type *C. fagacearum* isolate, with the time interval between chemical injection and fungicide inoculation ranging from 0 (inoculated immediately following fungicide injection) to 24 months (Table 1.1). Twenty-four negative control trees were injected with propiconazole without subsequent fungal inoculation. Seventeen untreated, positive control trees were inoculated with the wild-type strain throughout the course of the study. Twenty-one additional trees, both untreated and non-inoculated, were maintained throughout the site for comparison. However, thirteen of these twenty-one trees were incorporated into other studies in 2005 leaving eight untreated, non-inoculated controls in 2006.

Since none of the fungicide treated and pathogen-inoculated trees from Rep 1 developed symptoms by 2005, these eleven trees were inoculated a second time and combined into a 34-month treatment group. Therefore, at the conclusion of this study, all trees that were both injected and inoculated from Rep 1 had been inoculated twice: once at 0, 9.5, 14, 21.5, or 24 months after injection, and then again at 34 months post injection.

In Rep 2, trees from treatment groups 0, 9.5, and 14 months also remained symptomless in 2005 and so were incorporated into an additional 23-month treatment. Thus, the 23-month treatment groups in Rep 2 consisted of seven previously inoculated trees and three trees that were only inoculated once. Trees from the Rep 2, 24-month treatment were inoculated only once at 24 months following injection.

Table 1.1 Number of trees per treatment in A) Rep 1 and B) Rep 2. Treatments indicate the number of months following fungicide injection that a tree was inoculated.

A)

Treatment*	No. of trees
0*	3
9.5*	2
14*	2
21.5*	2
24*	2
0 and 34	3
9.5 and 34	2
14 and 34	2
21.5 and 34	2
24 and 34	2

B)

Treatment*	No. of trees
0*	2
9.5*	2
14*	3
23	3
24	3
0 and 23	2
9.5 and 23	2
14 and 23	3

*Trees from treatments followed by an asterisk were included in the treatments where trees received two inoculations.

Fungicide injection. Fungicide treatments using Alamo® (14.3% a.i., Syngenta Specialty products) were carried out via pressurized macro-injection into root flares with a 12V Flojet pump (ITT Corporation, White Plains, NY), according to the fungicide product label (Novartis Crop Protection, Greensboro, NC) in July 2002 (Rep 1) and June 2003 (Rep 2) (Figure 1.4). Injection pressure was maintained at 20 psi. Trees were treated with 20 ml of fungicide diluted in

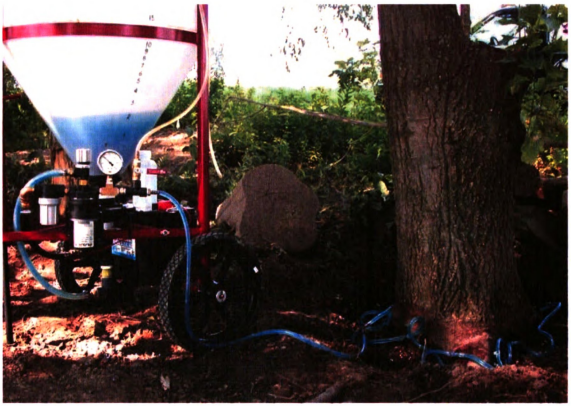

Figure 1.4 Injection apparatus used for propiconazole injection treatments. The fungicide solution was pumped out of the storage tank and into 2.5 cm deep holes drilled into the xylem of the root flares.

one liter of water per inch tree dbh (2.8 g a.i. or 0.09 oz per 2.5 cm dbh), which is the manufacturer's maximum recommended dosage for trees under high disease pressure. Injection wounds were painted with wound paint the day after injection before being covered again with soil.

In general, trees absorbed the fungicide solution within a few hours; however, several trees took much longer and six did not take up the full amount of product even when pressure was increased or the injection apparatus was left connected to the tree overnight. These six trees received less than two-thirds of the attempted injection amount and most had brownish, dry sapwood apparent when injection holes were drilled. Although these trees were retained in the study, their lack of full absorption was noted and any significant variations in results were considered.

Inoculations. Inoculations were performed with one wild-type strain, "Westcott", recovered from a diseased tree in Ogemaw County, Michigan in 2001. Conidia from Westcott cultures grown on plates containing potato-dextrose agar (PDA) were collected by placing 1-2 ml of distilled water onto the plate and gently rubbing the surface with a glass rod. The resulting suspension was strained through Miracloth™ and the concentration was adjusted to 10^5 conidia/ml with water and 20% glycerol. This suspension was divided into 1 ml aliquots and maintained at -80°C. The conidial suspension was thawed at room temperature for one hour prior to inoculation studies. Viability of spores was periodically assessed by serial dilution onto Petri dishes containing PDA; spore viability was consistently greater than 90%.

For tree inoculations, a 2.5 cm-deep hole was drilled into the north side of the trunk at 1.5 m aboveground with a 1/4 inch bit. One ml of the 10^5 conidia/ml suspension was then placed in the hole with a pipette. The suspension was generally absorbed within 5-10 minutes, and holes were subsequently covered with tape to prevent insects from entering the wound. For trees inoculated twice, the second inoculation hole was drilled 1/4 of the way around the trunk to the left of the first inoculation site.

Disease assessment. Trees were visually assessed monthly (May through October) for symptoms of oak wilt to determine the timing between inoculation and initial symptom development. Two symptomatic branches from each tree expressing disease symptoms were sampled for the presence of *C. fagacearum* by flame sterilizing samples after dipping in 90% ethanol, removing the outer bark, and then placing pieces of sap wood onto plates containing either PDA or glucose-phenylalanine agar. Final inspection of trees was done in early August 2006, fifteen months after final inoculations. For the purposes of this study, trees were rated as either diseased (1) or healthy (0).

Statistical analysis. The relationship between treatment parameters and disease development was analyzed by logistic regression using the LOGISTIC procedure in SAS version 9.1 software (SAS Institute Inc., Cary, NC). Syntax for exact conditional logistic regression was employed to account for small sample size (Derr 2000). Disease was modeled as a function of rep (1 or 2), month (when inoculation occurred), the number of times a tree was inoculated (once or twice), and whether trees received fungicide prior to inoculation. Significance of treatment variables to the model was determined according to a Score test, a

conditional exact test for the null hypothesis that the effect parameter is equal to zero (i.e. has no effect on disease). Exact parameter estimates (coefficients of the predictor variables in model) were also analyzed to determine the type of effect (positive or negative) each predictor variable had on disease occurrence. A p value ≤ 0.05 was used to determine statistical significance.

Results

All 17 positive control trees (inoculated but not treated with fungicide) from both reps developed wilt symptoms within six weeks following inoculation (Table 1.2). These trees completely wilted within the same year of inoculation, demonstrating the virulence of the Westcott strain and susceptibility of the trees within the site. In contrast, none of the untreated, non-inoculated trees throughout the site developed wilt symptoms, indicating that the trench lines initially established in 2002 remained effective and that insects were not vectoring inoculum.

Potential wilt symptoms on fungicide-treated trees within the plot developed during the fall of 2005. As this coincided with typical autumn leaf coloration changes, wilt was not confirmed until the following year. All symptomatic trees sampled in 2006 were positive for *C. fagacearum*. In August 2006, fifteen months after final inoculations, only six of the eleven propiconazole-treated trees from Rep 1 that had been inoculated 34 months post-injection showed disease symptoms. All six symptomatic trees had been inoculated twice: two at 14 and 34 months after fungicide injection, two at 21.5 and 34 months, and two at 24 and 34 months. One of the symptomatic trees inoculated at 14 and 34 months did not receive the full application of fungicide. None of the trees

inoculated at 0 and 34 months or 9.5 and 34 months developed symptoms.

Table 1.2 Proportion of wilting trees in August 2006 per rep. Treatments consisted of trees injected with propiconazole and then inoculated with *Ceratocystis fagacearum* at A) **0** (inoculated same day as injected), **9.5**, **14**, **21.5**, **23**, or **24** months later and B) once at 0, 9.5, 14, 21.5, or 24 months and a second time at **23** or **34** months. Positive, untreated control trees were inoculated at the respective time (in months) after experiments began. Rep 1 began in July 2002, while Rep 2 was initiated in June 2003. A '-' indicates treatment was not included in that rep.

A

	Treatment (months)							
							Control trees	
	0	***9.5***	***14***	***21.5***	***23***	***24***	***0***	***24***
Rep 1	0/3	0/2	0/2	0/2	-	0/2	4/4	3/3
Rep 2	0/2	0/2	0/3	-	1/3	0/3	4/4	2/2

B

	Treatment (months)			
			Control trees	
	23	***34***	***24***	***34***
Rep 1	-	6/11	3/3	4/4
Rep 2	3/7	-	2/2	-

Four of the thirteen treated and inoculated trees from Rep 2 displayed symptoms in 2006: one of the three trees that had been inoculated once only at 23 months and three of the seven trees that were inoculated twice (one at 0 and 23 months and two at 14 and 23 months). Two of the three trees inoculated only once at 23 months and all of the trees inoculated once at 24 months (Rep 2) remained symptomless over one year after inoculation. In contrast, the untreated, control trees inoculated at the same time developed wilt symptoms in 2005, the same year they were inoculated. All four of the trees from Rep 2 that expressed wilt symptoms in 2006 did not absorb the full amount of fungicide when injection was attempted. Only one tree in the study that did not take up the full amount of propiconazole (and was subsequently inoculated) did not develop wilt symptoms.

All fungicide-treated trees that developed wilt had delayed symptom development both initially and after symptoms appeared. Symptoms were not obvious for at least 3-13 months after inoculation and were confined to scattered branches where the disease progressed slowly. This is contrary to what was observed in the untreated control trees, which expressed wilt symptoms within six weeks following inoculation that progressed rapidly from the top of the crown, downward.

The number of times a tree was inoculated (once or twice) and whether or not a tree received fungicide prior to inoculation significantly contributed to the disease model (Table 1.3); however, month ($p = 0.43$) and rep ($p = 0.28$) were not significant explanatory variables and were excluded from the model. Based on parameter estimates, trees that did not receive a fungicide injection and those

trees inoculated twice had greater incidence of disease, while fungicide-treated trees had decreased disease incidence. The null hypothesis that one inoculation had no effect on disease cannot be rejected, thus indicating that fungicide-injected trees were significantly protected from disease after receiving one inoculation.

Table 1.3 Score test statistic (Score) with its associated exact p-value, and exact parameter estimates (Estimate) with associated p-value for treatment effects (Effect) for the disease predictor variables, injection and inoculation.

Effect	Score	Exact p-value	Estimate	p-value
Fungicide injection	25.66	<.0001	-2.95	<.0001
No fungicide injection	29.03	<.0001	1.94	<.0001
One inoculation	0.07	1.00	-0.18	1.00
Two inoculations	6.56	0.03	1.28	0.02

Discussion

Propiconazole injection was an effective preventative treatment for oak wilt for at least 23 months and, in some cases, up to 34 months following injection; however, disease pressure apparently affects the duration of efficacy, as intimated by disease occurrence in trees receiving two inoculations. Although the length of time between injection and inoculation (up to 34 months) was statistically insignificant in determining disease occurrence, the majority of trees inoculated over 23 months following injection were inoculated twice. Since the number of times a tree was inoculated was significant to the disease model, and no trees were inoculated only once at 34 months, the importance of inoculation timing beyond 24 months is unclear. Likewise, a single inoculation was not an important predictor of disease, perhaps because disease was better explained as a function of fungicide treatment.

It is unlikely that any of the observed symptoms in the treated trees were due to pathogen movement through root grafts for several reasons. First, all negative control trees located within the plot remained symptomless throughout the study. Secondly, a separate experiment conducted within the Beaumont plot indicated low root graft incidence (≤25%) at this site (unpublished data). Additionally, once roots have been severed (i.e. as a result of trenching), root grafts take several years to reform. Finally, pathogen spread through roots is typically slow and erratic and symptom development in root-inoculated trees is often delayed. Thus, it would take several years for root grafts to reform, the pathogen to move through them and then incite disease in a tree. The spatial pattern of disease development in this study also supports the non-involvement

of root graft movement.

Results indicate that some level of protection remains even at 34 months post-fungicide injection. As all treated trees from Rep 2 that developed symptoms did not take up the full amount of fungicide, the actual amount of propiconazole taken up by a tree may affect the duration of efficacy. Wilson and Lester (1992) found that lower rates of propiconazole are equally effective as higher rates; however, their study was performed with Texas live oaks (*Q. virginiana*) using multiple injections of two formulations (both different from Alamo®) of propiconazole. Therefore, the difference in our results (regarding those trees that did not absorb the full amount of propiconazole solution) could be due to using a more susceptible *Quercus spp.* (*Q. rubra*), the decreased amount of actual fungicide present within the tree, and/or the incomplete distribution of the fungicide within the tree due to a lower volume of solution injected. The health of the tree prior to injection may also influence efficacy of the treatment since tree health may have been the determining factor in injection failure.

All fungicide-treated trees that developed wilt did not express symptoms for at least 3 -13 months following inoculation. In contrast, all non-treated positive control trees developed symptoms within six weeks, indicating that the fungicide-injected trees inoculated at the same time were delayed in symptom development. Thus, even after 23 (and up to 34) months, the fungicide is acting in some capacity to inhibit disease development. Osterbauer and French (1992) were only able to detect propiconazole within sap tissues using a thin layer chromatography (TLC) assay up to 12 months following injection. At 16-18

months post-injection, TLC results were similar to those found when older supplies of propiconazole were analyzed, suggesting degradation of the product over time. At 20 months post-injection, no propiconazole was detected in any samples. Our results suggest that the amount of propiconazole injected may influence the length of efficacy of the product, as Osterbauer and French (1992) used much lower rates in their studies. Additionally, it is unclear how sensitive the TLC assay they used was since it tested for the presence of propiconazole based on activity against *Cladosporium caryigenum,* a fungal pathogen of pecan trees. Based on the EC_{50} and ED_{50} values reported for *C. fagacearum* (Appel and Kurdyla 1992) and *C. caryigenum* (Reynolds et al. 1997), respectively, *C. fagacearum* is sensitive to levels of propiconazole at concentrations 1000 times lower than *C. caryigenum.*

Propiconazole may only delay active colonization by *C. fagacearum* until the fungicide degrades to ineffective levels, because at lower concentrations of sterol biosynthesis inhibitors the inhibition of fungal growth is incomplete (Kuck and Scheinpflug 1986, Latteur and Jansen 2002, Nogueira et al. 2002, Wilson and Forse 2007). At sufficiently high levels, however, Wilson and Forse (1997) determined that propiconazole was fungicidal to *C. fagacearum.* Therefore, in trees inoculated soon after injection, the pathogen was potentially killed due to high initial levels of the fungicide. Our results support these conclusions: trees inoculated twice at 0 or 9.5 months and then 34 months did not develop symptoms, whereas trees inoculated twice at 14, 21.5, or 24 and again at 34 months all developed wilt. Similar results occurred in Rep 2; although one tree inoculated at 0 and 23 months developed wilt, this tree did not receive the full

dose of fungicide administered. Given these results, protection may actually begin to break down around 14 months, with increased disease pressure contributing to the eventual failure of product efficacy. However, under low disease pressure (i.e. a single inoculation), the fungicide provides protection for at least 23-24 months.

In addition to its fungistatic effects, triazoles, including propiconazole, are known to have plant growth regulating properties (Kuck and Scheinpflug 1986, Wetztein et al. 2002, Hanson et al. 2003). Phelps et al. (1966) reported that indole 3-acetic acid, a natural growth regulator, delayed symptom development in northern pin oak (*Q. ellipsoidalis*) up to 12 months or more when injected into the trunk. Although indole 3-acetic acid is an auxin and thus stimulates growth, whereas propiconazole has a growth retardation effect, by changing the balance of growth regulation in the plant (perhaps resulting in the enhancement of the tree's ability to cope with stress or by interfering with the production of tyloses in response to the pathogen) disease development is affected. Thus, it is possible that propiconazole works in two ways to inhibit disease development – first by interfering in ergosterol-biosynthesis and secondly by affecting growth regulation within the host.

Interestingly, the effects of propiconazole in this study are similar to those found with other compounds tested for the control of wilt. Phelps et al.'s (1966) research on northern pin oak shows that a few of the compounds tested for wilt suppression prolonged the incubation period of the disease up to 24 months. The pattern of symptom development on such treated trees differed from untreated trees in that wilt symptoms developed slowly, often branch by branch,

and sometimes over 1-2 years. Similar results of a temporary delay effect were found with trials using thiabendazole for oak wilt (Appel 1995). This effect was also observed on the propiconazole-treated trees in our plot that eventually developed symptoms. In addition, Phelps et al. (1966) reported that despite a prolonged incubation period, the fungus was isolated from 75% of symptomless trees 12 months after inoculation. This demonstrates the ability of the fungus to remain within a tree without inciting disease. Based on our results, however, there does appear to be a limit to how long the fungus can remain within a tree without provoking disease, since trees that were injected with propiconazole and inoculated with *C. fagacearum* the same day did not develop symptoms over two years later. It is possible that the fungicide treatment suppressed fungal colonization, and eventually the conidia (or hyphal fragments) are eventually 'flushed' out of the xylem (i.e. via transpiration). Alternatively, the conidia may have been killed as a result of the high initial concentration of fungicide as previously discussed. This scenario is likely different than what occurs with trees infected through root grafts since the pathogen can persist in root systems for several years (Rexrode 1978). Thus, *C. fagacearum* may endure until the fungicide degrades to low enough levels for the fungus to spread into the tree.

Natural infection with *C. fagacearum* is most likely to occur through branch wounds or root graft movement; therefore, the results from this study must be interpreted accordingly. Our experiment tested the effectiveness of propiconazole injection against non-root graft spread of the pathogen since trees were inoculated in the bole. The observed inhibitory effect may break down with natural overland infections, which probably occur in the crown, as it is believed

that the fungicide is not translocated or distributed evenly throughout the upper canopy (Osterbauer and French 1992). While bole inoculations have been found to have greater inoculation success than crown-inoculated trees (Jones 1964, Cobb et al. 1965), it is also probable that the greatest amount of fungicide was distributed within the trunk. Therefore, the observed delay in symptom development may or may not be related to the initial distribution of the fungicide in relation to the inoculation site, underscoring the need for further clarification of this relationship.

As the majority of research on oak wilt has focused on the host-pathogen interaction aboveground, there remain many unanswered questions regarding the movement and colonization of the pathogen in the root systems. Evidence of pathogen movement through root grafts may take one to three years (Rexrode 1978) and seemingly dormant disease centers may begin wilting again after several years, presumably due to root graft spread. Additionally, root-inoculated trees often display delayed wilt symptoms, up to one year from inoculation (Cobb et al. 1965). Thus, it is difficult to determine when and how the pathogen enters the root system and what happens once it is there. Defensive reactions in response to *C. fagacearum* are less extensive in the roots than in other parts of a tree (Struckmeyer et al. 1953). This suggests that the pathogen may be able to colonize parts of the root system, which has implications for disease development in fungicide-treated trees. There is evidence that propiconazole is distributed to the root system of injected trees (Blaedow et al. 2005, Tattar and Tattar 1999), but it is unclear to what extent and how this affects pathogen growth and movement within the root system. Therefore, the results of this study

cannot automatically be applied to trees at risk of root graft infection.

The possible dual inhibitory effects of propiconazole and advances in delivery via macro-injection have made propiconazole a promising fungicide treatment for oak wilt. However, since the early work by Osterbauer and French (1992) on propiconazole injections in red oaks, it has been apparent that red oak treatments are somewhat unpredictable. Our results imply that propiconazole, when fully administered at the highest recommended rate, may inhibit the development of wilt when trees are inoculated with *C. fagacearum* at least up to 24 months following injection; however, efficacy of the fungicide is probably dependent on several factors including disease pressure, timing between injection and inoculation, and the rate and/or amount of propiconazole injected. These results support extending the time between injections for non-root-grafted trees in at-risk areas and provide the impetus for additional studies to further clarify the factors that influence the effective longevity of propiconazole.

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Chapter 2

Efficacy of a hypovirulent mutant for biocontrol of oak wilt

Introduction

The term 'hypovirulence' was first used to describe strains of the chestnut blight pathogen, *Cryphonectria parasitica*, that were decreased in virulence (Grente 1965). These strains were recovered from non-lethal, healing cankers on chestnut trees and typically displayed abnormal growth and morphology in culture. The hypovirulent phenotype was attributed to the presence of cytoplasmic double-stranded RNA within the fungal mycelium (Day et al. 1977, Fulbright 1984); however, some hypovirulent strains of *C. parasitica* were identified that lacked dsRNA hypoviruses (Fulbright 1985). Mahanti et al. (1993) determined that these isolates had increased levels of alternative oxidase activity (the effect of a non-functional cytochrome-mediated respiratory pathway), which is indicative of mitochondrial dysfunction. Additionally, the hypovirulent phenotype was transmissible via hyphal anastomosis and maternally inherited in crosses, indicating that hypovirulence was mitochondrially based in these isolates. It is postulated that this phenotype persists in nature in part because mutated mtDNA is suppressive – a phenomenon whereby the mutant mitochondria eventually displace their wild-type counterparts due to selectively increased replication (Bertrand 2000). Additionally, the phenotype is conveyed to all progeny and is transferable via hyphal anastomosis, both of which provide a means of dissemination and propagation. Thus, the potential for an effective biologically-based control using a hypovirulent strain of a pathogen is apparent.

In an attempt to duplicate mitochondrial-based hypovirulence in *Ceratocystis fagacearum,* the fungal pathogen that causes oak wilt, Shaw (1999) exposed conidia from a wild-type strain, "Fenn", to ethidium bromide and UV

light. Once conidia germinated, the resultant, single-spore cultures were screened for slow growth, an indicator of possible mitochondrial dysfunction. The mitochondrial origin of the phenotype was later determined by testing for alternative respiration (cyanide resistance and salicylhydroxamic acid sensitivity) and maternal inheritance of the trait. One mutant, PM447, appeared to satisfy the above requirements and was subsequently used in seedling assays similar to those developed by Fenn et al. (1975). Using 28-day-old seedlings maintained in a growth chamber, Shaw (1999) found that seedlings first inoculated with the hypovirulent strain, PM447, and then challenged with the wild-type Fenn two weeks later, had significantly lower disease ratings than seedlings inoculated with Fenn only or seedlings challenged at 0 (co-inoculated with PM447 and Fenn) or 1 weeks.

In preliminary efforts to apply this technology to a wider application, we repeated these initial experiments, expanding the parameters to include challenges beyond two weeks, varying the strain of wild-type used in the challenge inoculation, varying the spore load of PM447, and including mature trees from three field plots in these studies. Seedling studies examined the effect of wild-type strain and spore concentration of PM447 used for inoculations, both separately and in conjunction with the timing between PM447 and wild-type challenge inoculations. The purpose of these studies was to identify any variation in virulence among the wild-type strains used, whether the dosage of PM447 had an effect on symptom development, and the optimal time between PM447 and challenge inoculations. Field plot studies were an extension of the seedling experiments and focused on the effects of various spore concentrations

of PM447 in addition to the effect of timing between PM447 and wild-type (challenge) inoculations.

An apparently hypovirulent strain (that exhibited reduced virulence and delayed or inhibited subsequent infection by a wild-type isolate) was recovered from the initial PM447 source maintained on PDA plates and was used for the experiments presented here. Results, however, indicate that the recovered strain may not be identical to the original PM447 isolate as developed and described by Shaw (1999).

Materials and Methods

Fungal cultures. Strain PM447 was re-isolated from cultures maintained on potato dextrose agar at 4-6°C. Attempts to re-isolate from soil stocks were unsuccessful. Cultures exhibited slower initial growth when compared to the wild-type, but did not possess sectors (alternating sections of slower and faster mycelial growth) as previously described by Shaw (1999). When inoculated into seedlings, conidia from these cultures did not incite any disease symptoms nor did they provide any level of protection against the wild-type strain upon challenge inoculation. In an attempt to recover the hypovirulent phenotype, spores from these cultures were screened for slow growth, and conidia from the slowest growing cultures were collected. Isolated conidia were then plated and again screened for slow growth following germination. Conidia from the four slowest-growing cultures were collected and then diluted to 10^5 conidia/ml with a 1:4 glycerol:water solution to make a spore stock solution. The resulting conidial suspension produced mild symptoms (similar to those reported by Shaw (1999))

when inoculated into seedlings and was therefore assumed hypovirulent; however, it was unknown whether the original, mitochondria-based, hypovirulent phenotype was recovered. The stock suspension was kept at -80°C and used for all subsequent PM447 inoculations.

Wild-type, virulent *C. fagacearum* isolates used in the studies were originally obtained from diseased trees in Michigan in 2001 (Westcott and Beal) or from culture kept in collections (Fenn). Single-spore cultures of all isolates were plated on potato-dextrose agar (PDA) and allowed to grow for 14 days at room temperature. Conidia were extracted by pipetting 2 ml of distilled water onto the plates and rubbing the top of the mycelia with a glass rod to suspend the conidia. The resulting suspension was strained through Miracloth™ and conidial density was determined using a hemacytometer. Samples were adjusted to 10^5 conidia per ml with distilled water and glycerol to make a 20% glycerol solution. Suspensions were divided into 1 ml aliquots and maintained at -80°C.

Quercus spp. Experiments performed in the greenhouse and growth chamber utilized 28 to 35-day-old red oak seedlings; *Quercus rubra* (red oak) seedlings were used in the greenhouse study, while *Quercus palustris* (pin oak) was used for the growth chamber study, due to a low crop of *Q. rubra* seed in the previous year. Seed that had been obtained from mulitiple trees located within a single (unknown) county was purchased from Sheffield's Seed Co., Inc. (Locke, NY). All field sites contained *Quercus rubra*; however, *Q. ellipsoidalis* was also present, and utilized, within the Jackson plot.

Field plots. Three field sites were utilized: East Farm, Beaumont (-1 and

-2), and Jackson. Trees (*Q. rubra*) at the East Farm site were of two size classes: saplings with a diameter at breast height (dbh) of 2-3 cm and those with a dbh equal to 5-6 cm. These trees had been purchased from a single Michigan nursery and planted 1.25 meters apart in two rows at the site. All other field sites contained mature trees with a dbh greater than 12 cm. Trees (*Q. rubra*) at the Beaumont site appear to have been planted several decades ago (although the exact year is unknown), whereas the Jackson site is a mixed stand of predominantly *Q. rubra* and *Q. ellipsoidalis* with other hardwoods. Soil at the Beaumont site consisted of Colwood-Brookston (62%) and Capac (38%) loams which are characteristically deep, poorly drained, and fine loamy soils (Web Soil Survey 2007, Soil Survey Staff 2007). The Jackson site was typified by Riddles sandy loam, which is well drained soil with a moderate available water capacity (Web Soil Survey 2008).

Experimental Design. Experiments were exploratory in nature; thus, the number of treatments was maximized for all experiments. Though this sacrificed sample size, the purpose of these studies was to identify potentially effective treatments that would provide the basis for more in-depth experiments focusing on these specific treatments. Additionally, clear results were expected due to the nature of the disease: oaks inoculated with a wild-type *C. fagacearum* develop symptoms rapidly and completely wilt within a few months of infection. For all field sites, Westcott was the wild-type isolate used for inoculations.

Greenhouse: Q. rubra seed that had been floated and cold stratified prior to shipment was directly planted into 16 ounce cups containing Baccto™ planting mix. Seedlings were watered 2-3 times per week with one watering

including modified Hoagland's mix fertilizer (Hoagland and Amon 1950). Twenty-eight to 35 days post-emergence, healthy seedlings (of approximately the same size and stage of development) were randomly arranged in metal trays (five seedlings per nine-well tray) and kept on a single bench in the greenhouse. The experiment was replicated twice in 2003: Rep 1 inoculations began on July 29 and Rep 2 was initiated on August 18. Treatments included seedlings inoculated with PM447 and then inoculated with a wild-type strain (Fenn, Westcott, or Beal) after 0, 1, 3, or 4 weeks (Table 2.1).

Table 2.1 Number of seedlings (n) used per rep in Greenhouse study treatments.

Treatment (seedlings inoculated with)	**Rep 1 (n=)**	**Rep 2 (n=)**
Water	4	3
PM447	4	3
One wild-type strain: Fenn	4	3
Westcott	4	3
Beal	4	3
Co-inoculation: PM447 and one wild-type strain	3 (per strain)	3 (per strain)
Challenge inoculations: PM447, then a wild-type strain: 1 week later	0	2 (per strain)
3 weeks later	3 (per strain)	3 (per strain)
4 weeks later	3 (per strain)	0

Growth chamber: Q. palustris seed was soaked for 24 hours and then cold stratified for 60 days (to induce germination) before planting into 16 ounce cups containing Baccto™ planting mix. Seedlings were watered twice per week with one watering including modified Hoagland's mix (Hoagland and Arnon 1950). Seedlings were randomly arranged in metal trays (five seedlings per nine cup tray) and sustained in a single growth chamber maintained at 26°C with a 16 hour per day light period. Seedlings were inoculated with various concentrations of PM447 either alone or followed by a challenge inoculation 2 weeks later with the wild-type isolate, Westcott (Table 2.2).

East Farm and Beaumont-1: Treatments from this study focused on the effect of timing between PM447 and wild-type challenge inoculations (Table 2.3). The East Farm site contained nine 2-3 cm dbh saplings and eleven 5-6 cm dbh saplings. Two saplings from each size class were used per treatment except for the water control, which included only one sapling from each size class, and the one week challenge treatment, which only included two 5-6 cm dbh saplings. Seventeen trees from the Beaumont-1 site were utilized for this study. These trees were arranged into two groups of trees (containing eight and nine trees) and were isolated from other trees within the plot by six-foot-deep trenches. Inoculations began July 16, 2004 and data was recorded in September 2005 and June 2006 for statistical analysis. Analysis of tree size in relation to treatment efficacy was explored to determine if there was an effect of tree size on treatment results.

Table 2.2 Treatments included in the Growth Chamber experiment. There were four seedlings per treatment except the water control, which included six seedlings.

Treatment (seedlings inoculated with 10ul of)
Water
PM447: 10^3 spores/ml
10^4 spores/ml
10^5 spores/ml
10^3 spores/ml + 10^3 spores/ml two weeks later
Westcott: 10^3 spores/ml
10^4 spores/ml
10^5 spores/ml
Challenge inoculations*:
10^3 spores/ml of PM447, then Westcott two weeks later
10^4 spores/ml of PM447, then Westcott two weeks later
10^5 spores/ml of PM447, then Westcott two weeks later
10^3 spores/ml of PM447 + 10^3 spores/ml of PM447 two weeks later, then Westcott two weeks later

*10ul of 10^5 spores/ml of Westcott were used for all challenge inoculations.

Table 2.3 Number of trees (n) per treatment at the East Farm and Beaumont-1 sites.

Treatment (trees inoculated with) (n=)	**East Farm[1] (n=)**	**Beaumont-1**
Water	2	3
PM447	4	3
Westcott	4	3
PM447, then Westcott: 1 week later	2	2
2 weeks later	4	4
3 weeks later	4	2

[1]Sample number includes saplings from both size classes.

Jackson: Twenty-eight red oaks at this site were identified and treatments were randomly assigned to trees; however, three pairs of trees (where one tree was located less than 2 meters from the other in the pair) received the same treatment in order to minimize the effect of potential root grafts between the two trees. Treatments focused on the effect of various inoculum concentrations of PM447 on disease development (Table 2.4). Initial inoculations were performed June 29, 2005 and final disease results were obtained in June 2006.

Beaumont-2: Thirty-eight trees at the Beaumont site were utilized in this study. To account for the possibility of root graft connections between adjacent trees, treatments were randomly assigned to certain groupings of trees

(consisting of 1-3 trees per 'group') rather than individual trees; therefore, neighboring trees received identical treatments. Six-foot-deep trenches and/or the presence of several untreated oaks isolated most treatment groups. Treatments began June 20, 2005 and consisted of inoculation with various concentrations of PM447 followed by a challenge inoculation with Westcott 2 weeks later (Table 2.5). Final data was collected in June 2006.

Table 2.4 Number of trees per treatment at the Jackson field site

Treatment (trees inoculated with)	Number of trees
Water (control)	4
Westcott (wild-type)	1
PM447: 10^1 spores	2
10^2 spores	2
10^3 spores	2
10^5 spores	2
10^1 spores + 10^1 spores two weeks later	3
10^2 spores + 10^2 spores two weeks later	3
10^3 spores + 10^3 spores two weeks later	3
10^1 spores + 10^1 spores + 10^1 spores at one week intervals	3
10^2 spores + 10^2 spores + 10^2 spores at one week intervals	3

Table 2.5 Number of trees per treatment in the Beaumont-2 study.

Treatment (trees inoculated with)	Number of trees
Water (control)	4
PM447: 10^1 spores	2
10^3 spores	2
10^5 spores	2
10^1 spores + 10^1 spores two weeks later	3
10^1 spores + 10^3 spores two weeks later	3
10^3 spores + 10^3 spores two weeks later	3
Westcott (wild-type strain)	4
Challenges:	
10^1 spores of PM447 + challenge	2
10^3 spores of PM447+ challenge	2
10^5 spores of PM447+ challenge	2
10^1 + 10^1 spores two weeks later + challenge	3
10^1 + 10^3 spores two weeks later + challenge	3
10^3 + 10^3 spores two weeks later + challenge	3

Inoculations. For seedling inoculations, a 10ul drop of conidial suspension was placed at the base of the stem, approximately 2 cm above the soil line. A 26-gauge needle was then inserted through the droplet into the stem at a 45° angle. Absorption of the droplet was observed, indicating successful uptake of the suspension into the xylem.

For field plot trees, inoculation wounds were made at 1.4 meters aboveground into the north side of the trunk by drilling a small hole into the xylem. For the smallest trees located at the East Farm site, a 5/64 inch drill bit was used, while a 7/64 bit was used for the 5-6 cm size class. A 1/4 inch drill bit was used for all other (> 12 cm dbh) trees. In cases where trees received more than one inoculation, subsequent inoculation sites were located 1/4 of the way around the trunk to the left of the last inoculation location. All inoculum (from the conidial suspensions as previously described) was pipetted into inoculation wounds. The 2-3 cm dbh saplings received two doses of 10 ul of inoculum, five minutes apart, for a total of 20 ul, while saplings that were 5-6 cm dbh received 50 ul inoculum in one dose. Larger trees received 1 ml of inoculum. Control trees were inoculated with water. All inoculation wounds were covered with tape to prevent insects from entering the wounds.

Disease measurements. The disease rating of seedlings was based on the degree of symptom expression at six weeks (greenhouse) or eight weeks (growth chamber) post-wild-type inoculation using a qualitative 0 to 5 rating (Table 2.6). Field plot trees were visually assessed weekly or biweekly following inoculations (May through October) to determine when initial onset of symptoms occurred. Trees were rated as either healthy (0), intermediate (1), or wilted (2).

Intermediate ratings were assigned to trees that developed wilt symptoms that did not progress beyond 60% crown wilt over a one to two year period. A disease rating of 2 was given to trees with advanced stages of wilt that did not recover by the following year. Final disease ratings were recorded at one and/or two years following experiment initiation for use in statistical analysis.

Table 2.6 Disease ratings for seedlings based on level of symptom expression.

Disease rating	Symptom expression
0	Symptomless
1	Leaves with mild bronzing of tips; less than 25% leaf area affected
2	Mild curling and drying of leaves with bronzing more apparent; up to 50% leaf area affected
3	Leaves curled and dry with bronzing of up to 75% of leaf area
4	Leaves severely curled and dry with bronzing nearly to petiole
5	Leaves entirely brown (though few scattered green flecks may remain); defoliation common; nearly 100% of leaf area affected

Statistical methods. Statistical analysis of the data to determine the significant factors in disease development was performed using the Genmod procedure in SAS v.9.1 software (SAS Institute, Inc., Cary, NC). The Pearson's Chi-Square (value/degrees of freedom) and/or log likelihood values were used to assess model fit for various distributions; the data from the best fit model is reported. Likelihood ratios for a type III analysis were used to determine the significance of main effects (dependent on experiment) to the disease model, and analyses of parameter estimates provided significance data for the individual levels/values of explanatory variables (Johnston 2007). Parameter estimates were based on a reference parameter whose value was set to zero and then used for comparison; comparisons between treatments were determined by changing the reference values accordingly. A p value ≤ 0.05 (based on the test statistic for the Chi-Square distribution) was used to determine statistical significance of the variable in question, where p is the probability that the null hypothesis (no relationship between predictor variable and disease) is valid.

Results

Greenhouse Inoculation Studies

Disease ratings were significantly different between reps ($p=0.02$), so the data from both experiments were analyzed separately (Figure 2.1). For each rep, disease was modeled as a function of the type of wild-type strain used for inoculation, the timing between PM447 and wild-type challenge inoculations, and the interaction between type and timing (type*timing). In Rep 1, only timing was significant to the model ($p<0.0001$). Since type was not significant to disease, data from all wild-type isolates were combined. Analysis of parameter estimates

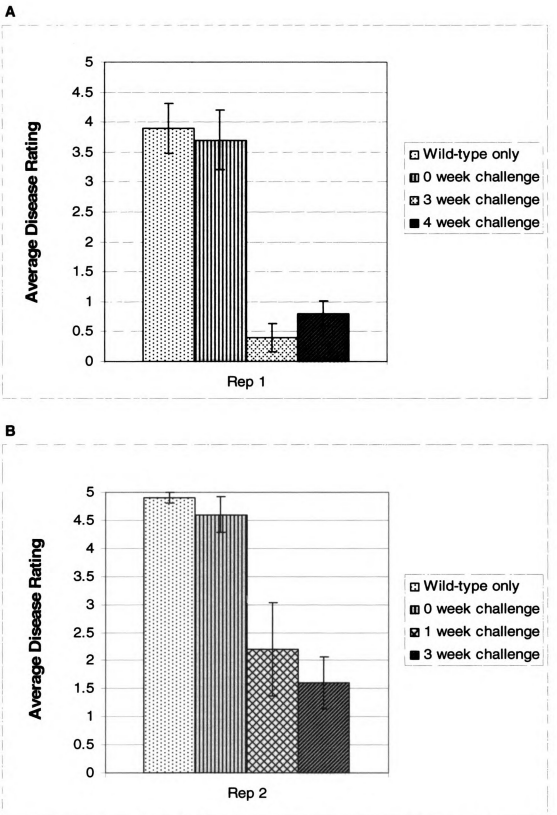

Figure 2.1 Average disease ratings (+/- SE) for *Quercus rubra* seedlings six weeks after inoculation with a wild-type strain in A) Rep 1 and B) Rep 2. Data from all wild-type strains is combined. Sample size ranged from 3-4 depending on rep.

indicates that the PM447 inoculations and the three- and four-week challenge treatments were not significantly different from the water inoculations (Table 2.7). Co-inoculation with PM447 and a wild-type strain (0-week challenge) was not significantly different from inoculation with a wild-type only; these treatments resulted in significantly more disease than the water control.

In Rep 2, timing ($p<0.0001$) and type*timing ($p=0.02$) were significant to the model. Statistically, PM447 inoculation treatments were not different from the water control ($p=0.49$). Overall, the one-week and three-week challenge treatments were significantly different from the water control, but not different from PM447 or each other; however, the Fenn 3-week challenge was significantly different from both the 0 (co-inoculated) and 1-week Fenn challenges (resulting in the significance of the crossed, type*timing effect). Co-inoculation with PM447 and a wild-type strain was not different from inoculation with a wild-type strain only.

Growth Chamber Inoculation Studies

There was a strong correlation between the amount of spores of the wild-type, Westcott strain used for inoculation and disease rating (Table 2.8). However, there was not a significant effect on symptom expression due to spore concentration of PM447 either when inoculated alone or when seedlings were subsequently challenged with Westcott (Figure 2.2, Table 2.9). Therefore, data from all spore concentrations was combined for both the PM447 only and the challenge treatments. Since all challenge treatments received 10 ul of 10^5 spores/ml of Westcott, challenges were compared to the Westcott-only disease

ratings at this spore concentration. Disease ratings for challenge treatments were significantly different from inoculations with PM447 only ($p<0.0001$). Inoculation with PM447 was not significantly different from water inoculations ($p=0.83$).

Table 2.7 Greenhouse treatment results in comparison to the water control (reference) six weeks after inoculation with a wild-type strain for A) Rep 1 and B) Rep 2.

A

Treatment[1]	Estimate	Chi-Square	p-value
Water (a)	0.00		
PM447 (a)	-0.00	0.00	1.00
Wild-type (b)	3.92	41.05	<0.0001
Co-inoculation (0 week) (b)	3.67	33.21	<0.0001
3 week challenge (a)	0.44	0.49	0.48
4 week challenge (a)	0.78	1.49	0.22

B

Treatment[1]	Estimate	Chi-Square	p-value
Water (a)	0.00		
PM447 (ab)	0.67	0.47	0.49
Wild-type (c)	4.89	38.25	<0.0001
Co-inoculation (0 week) (c)	4.56	33.21	<0.0001
1 week challenge (b)	2.17	6.68	0.01
3 week challenge (b)	1.56	3.87	0.05

[1]Treatments followed by the same letter (in parentheses) are not significantly different at $p \leq 0.05$.

Table 2.8 Disease ratings (DR) for seedlings eight weeks after inoculation with Westcott.

Treatment (inoculated with 10ul of)	n	Disease rating	Average DR
10^3 spores/ml	1	1	1
	2	1	
	3	1	
	4	1	
10^4 spores/ml	1	5	3
	2	5	
	3	1	
	4	1	
10^5 spores/ml	1	5	5
	2	5	
	3	5	
	4	5	

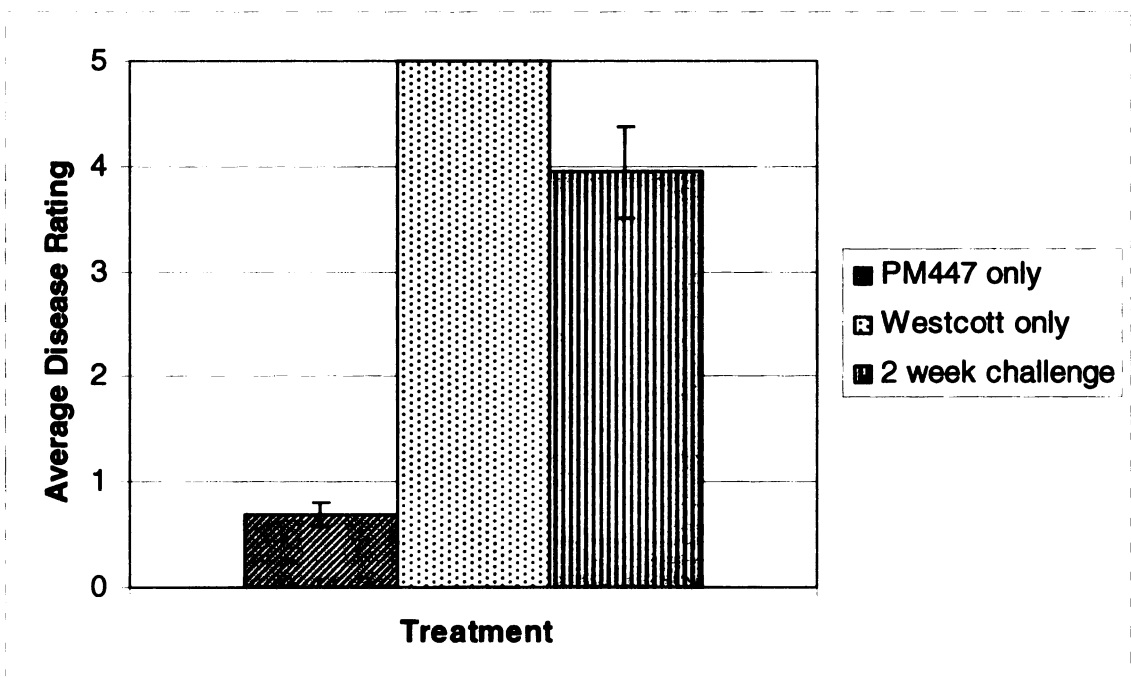

Figure 2.2 Average disease ratings (+/- SE) for *Quercus palustris* seedlings eight weeks after final inoculations. Concentration of PM447 inoculum was not a significant factor in disease, so data for all spore concentrations was combined for PM447 only (n=16) and 2-week challenges (n=16). Westcott data shown is for 10^5 spores/ml, which was the concentration used for all challenge inoculations (n=4).

Table 2.9 Likelihood ratio statistics for treatment effects in the Growth Chamber study. P-values indicate effects are not significant to the disease model.

Effect	Chi-Square	p-value
PM447 spore concentration	1.48	0.83
PM447 spore concentration re: challenges	3.13	0.37

East Farm and Beaumont-1 Field plots

Tree size was not a significant explanatory variable in the disease model ($p = 0.15$). Regardless of size, all water-inoculated control trees remained symptomless and all trees inoculated with Westcott only wilted the same year as inoculation. All 2-3 cm dbh trees inoculated with Westcott (whether alone or challenged) wilted within the first year and did not leaf out the following year; however, saplings inoculated with PM447 only had not developed symptoms by 2006.

Disease ratings for the 5-6 cm dbh trees varied by year, showing a trend similar to that observed with the larger trees located at the Beaumont site, where PM447-inoculated and 2- and 3-week challenged trees developed symptoms more slowly than other wild-type inoculated treatments (Figure 2.3, Table 2.10). After two years, the PM447-inoculated 5-6 cm saplings had new flushes of growth from epicormic shoots; however, the new growth eventually developed wilt symptoms. One of the 3-week challenged 5-6 cm saplings did not develop

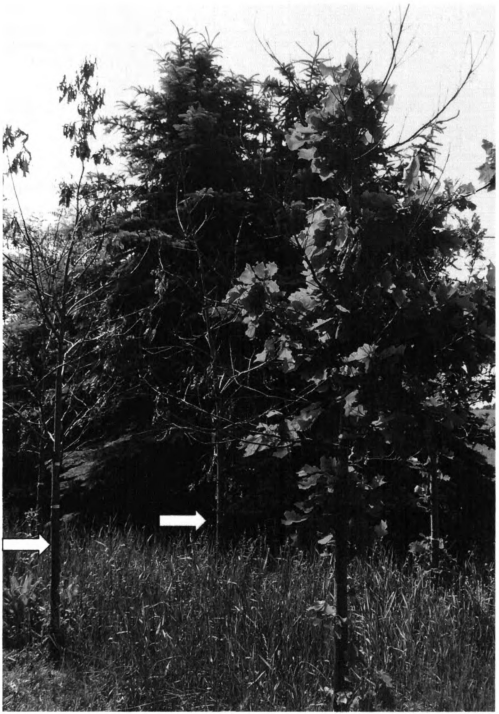

Figure 2.3 Saplings (5-6 cm dbh) at the East Farm site. The saplings on the left (indicated by the white arrows) were inoculated with the wild-type strain, Westcott, in 2004 (middle back) and 2005 (front left). The sapling in the foreground on the right was inoculated with PM447 in 2004. Note the new flush of growth off of the main stem; wilt symptoms were just appearing on the leaves at the time this photo was taken in June 2006.

Table 2.10 Average disease ratings (DR) of trees within the Beaumont-1 plot one year (2005) and two years (2006) after inoculation. Trees were assigned a DR of 0 if they had no symptoms, a 1 if they had mild to moderate symptoms (less than 60% of crown affected), or a 2 if they developed advanced symptoms or completely wilted.

Treatment	Average DR[1]: 2005	Average DR[1]: 2006
Control	0^{a}	0^{a}
PM447	1^{b}	2^{b}
Westcott	2^{c}	2^{b}
Challenge: 1 week	2^{c}	2^{b}
Challenge: 2 weeks	1^{b}	2^{b}
Challenge: 3 weeks	1^{b}	2^{b}

[1]Disease ratings for each treatment followed by the same letter are not significantly different at $p \leq 0.05$.

wilt, although based on other results (i.e. from the other 3-week challenged 5-6 cm and other sized trees) this is likely an 'escape' or caused by potential resistance in the tree rather than protection induced by PM447.

There was a significant effect of the timing of the challenge inoculation on disease rating with the larger Beaumont-1 trees ($p=0.00$) one year after inoculation. Although symptom development on 2- and 3-week challenged trees progressed much slower than on trees challenged at 1 week or inoculated with the wild-type only, all trees (including those inoculated with PM447 only) eventually succumbed to wilt by the second year following inoculation.

Jackson Field plot

The inoculum concentration of PM447 significantly affected disease ratings of trees ($p=0.00$). PM447 conidial concentrations of 10^1, 10^2, 10^3, and

10^1+10^1 did not produce symptoms that were significantly different from each other or the water controls (Table 2.11, Figure 2.4). Inoculation with 10^5, $10^1+10^1+10^1$, or $10^2+10^2+10^2$ spores produced symptoms that were not significantly different from each other or the Westcott (wild-type) control (Figure 2.5). The 10^2+10^2 and 10^3+10^3 inoculation treatments were not significantly different from each other or the $10^1+10^1+10^1$ and $10^2+10^2+10^2$ treatments; however, they were significantly different from both the water and Westcott controls.

Beaumont-2 Field plot

All water-inoculated (control) trees remained symptomless. All trees inoculated with Westcott only wilted in 2005, the same year as inoculation (Figure 2.6, Table 2.12). The treatment a tree received significantly affected disease development ($p<0.0001$). Based on parameter estimates (and corresponding p-values) using the water and Westcott control treatments as references, four treatments were identified that were not significantly different from the water control, but were significantly different than the Westcott control. Three of these four treatments involved inoculation with PM447 only ($10^1 + 10^1$, $10^1 + 10^3$, and $10^3 + 10^3$ inoculation treatments) and the remaining treatment consisted of an inoculation with $10^1 + 10^3$ spores of PM447 followed by a challenge inoculation with Westcott two weeks later. All other treatments were not significantly different from inoculation with Westcott only.

Table 2.11 Average disease ratings for treatments at the Jackson site. Trees were scored based on the level of symptom development one year following inoculation. A 0 was assigned to symptomless trees, a 1 to trees with mild to moderate symptoms (less than 60% of crown affected), and a 2 for those trees displaying advanced or complete wilt.

Treatment (trees inoculated with)	Average Disease Rating[1]
Water (control)	0^{a}
Westcott (wild-type strain)	2^{c}
PM447:	
10^{1} spores	0^{a}
10^{2} spores	0^{a}
10^{3} spores	0.5^{a}
10^{5} spores	2^{c}
$10^{1} + 10^{1}$ spores two weeks later	0.33^{a}
$10^{2} + 10^{2}$ spores two weeks later	1^{b}
$10^{3} + 10^{3}$ spores two weeks later	1^{b}
$10^{1} + 10^{1} + 10^{1}$ spores at one week intervals	1.67^{bc}
$10^{2} + 10^{2} + 10^{2}$ spores at one week intervals	1.33^{bc}

[1]Disease ratings for each treatment followed by the same letter are not significantly different at $p \leq 0.05$.

Figure 2.4 Two treated trees at the Jackson site. The tree on the left was inoculated with 10^2 spores of PM447 and the tree on the right with 10^1 spores. Both trees appear healthy in June 2006, 13 months after inoculation.

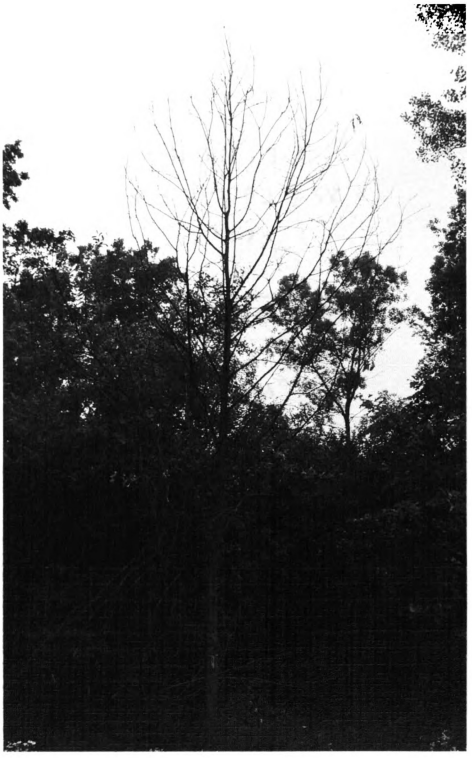

Figure 2.5 One of three trees in the Jackson study inoculated with 10^5 spores of PM447. Photo taken in June 2006, 13 months following inoculation.

Figure 2.6 Wilted oaks in the Beaumont-2 plot. These trees had been challenge inoculated with Westcott two weeks after inoculation with variable spore concentrations of PM447.

Table 2.12 Average disease ratings for treatments in the Beaumont-2 study. Disease ratings were recorded the year following inoculation and were assigned as follows: 0 = no apparent symptoms, 1 = mild to moderate symptoms (less than 60% of crown affected), and 2 = advanced or complete wilt.

Treatment (trees inoculated with)	Average Disease Rating[1]
Water (control)	0[a]
PM447:	
10^1 spores	1[b]
10^3 spores	2[b]
10^5 spores	2[b]
$10^1 + 10^1$ spores two weeks later	0[a]
$10^1 + 10^3$ spores two weeks later	0.67[a]
$10^3 + 10^3$ spores two weeks later	0.67[a]
Westcott (wild-type strain)	2[b]
Challenges[2]:	
10^1 spores of PM447	2[b]
10^3 spores of PM447	2[b]
10^5 spores of PM447	2[b]
$10^1 + 10^1$ spores two weeks later	1.67[b]
$10^1 + 10^3$ spores two weeks later	0.67[a]
$10^3 + 10^3$ spores two weeks later	1.67[b]

[1]Disease ratings followed by the same letter are not significantly different at $p \leq 0.05$.
[2]All challenge treatments were followed by inoculation with the wild-type, Westcott, two weeks after final PM447 inoculation.

Discussion

Results from the greenhouse experiment are similar to those reported by Shaw (1999). Unlike previous findings, however, all challenge inoculations beyond 0 weeks (co-inoculation) produced significantly less disease than resulted from co-inoculation or inoculation with a wild-type strain only, but were not different from inoculation with PM447 only. Growth chamber results demonstrate the hypovirulent nature of PM447, but subsequent protection against the virulent strain (when inoculated two weeks later) was not observed. Challenged seedlings in the growth chamber had significantly greater disease ratings than seedlings inoculated with PM447 only. The difference in results between the greenhouse and growth chamber should not be due to the *Quercus spp.* used since all red oaks have been found to have similar susceptibilities. In addition, although the disease ratings were recorded at different times, relative symptom expression at six or eight weeks post-inoculation in either study was essentially the same. The observed disparity could be due to environmental factors, which may have been more conducive to disease development in the growth chamber. This is a possibility given the difference in disease results between reps in the greenhouse, where the only difference between reps was the month of initiation.

Although there was no significant effect due to the spore load of PM447 when inoculated into seedlings, inoculum concentration did affect the timing and progression of disease development in seedlings inoculated with the wild-type, Westcott and in trees inoculated with PM447. However, though PM447 seemed to delay symptom development, most trees eventually wilted within 1-2 years.

PM447 inoculation with $10^1 + 10^3$ spores two weeks before wild-type inoculation was the most promising treatment for trees at the Beaumont-2 site. Although statistically this treatment could not be considered different from the water control, these trees still displayed symptoms, and, based on overall results, could still ultimately succumb to wilt.

The relatively small sample size per treatment and the overlapping range of disease ratings within and among treatments may have led to type I or type II error when evaluating treatment effects on disease. However, since the fundamental goal was to determine potential treatments for the prevention of wilt, the significance of these studies is in the observed delay in disease development in trees inoculated with PM447, which provides the basis for additional studies into understanding and enhancing the host defensive response.

Although complete protection against a virulent strain was not observed, results indicate that red oaks do respond to the presence of PM447 in some capacity that slows the progression of the disease. Perhaps the slow growth of PM447 in culture translates to slower growth and/or reduced fitness within a seedling or tree, thus enabling the host to respond to the presence of this pathogen. It is likely that this response is an induced defensive reaction that restricts the pathogen to some extent, although the pathogen is ultimately able to overcome any defenses produced by the host. Phelps et al. (1966) reported that several compounds were found to delay initial wilt development when applied to trees prior to inoculation, but were ultimately ineffective at controlling the disease. The difficulty in finding preventative treatments is probably due to the pathogen's ability to survive within a tree without actively inciting disease and red oaks'

inability to contain the pathogen on any level.

Another possible way PM447 delays symptom development, involves the fact that only one mating type (strain) of *C. fagacearum*, when two mating types are known to be present, can be isolated from a tree (Appel et al. 1985). This suggests some mechanism exists that restricts the establishment of a second strain within one tree. It is unlikely that this exclusion mechanism is host-based given the inability of trees to completely restrict the pathogen under other circumstances. As mitochondrial and nuclear DNA restrictions (Shaw 1999), and rep-PCR using $(GTG)_5$ (unpublished data) revealed no differences between the genomes of PM447 and wild-type strains, it currently cannot be determined whether PM447 is able to prohibit host colonization by a wild-type strain when inoculated beforehand. However, recent results with AFLPs (unpublished data) indicate there may be a particular genetic marker that would facilitate this discovery.

A final important consideration for disease results is that the hypovirulent PM447 used in these studies may be fundamentally different than the strain as used by Shaw (1999). *C. fagacearum* does not persist in culture longer than 8 months, in part due to its poor saprophytic nature. The cultures from which PM447 was re-isolated had been stored on PDA for over a year in a cold room. Also, although selection at the intracellular level appears to favor dysfunctional mitochondria (Bertrand 2000), it is possible that the described conditions favored mycelia that were the least deficient in respiration, resulting in cultures with zero or low levels of the mutant mitochondria apparently present in the initial strain. Additionally, degeneration of cultures after being maintained in culture for several

years is a common occurrence within the *Ceratocytis* and *Ophiostoma* genera (G. Adams, personal communication), and could lead to changes in virulence properties. Relatedly, the observed hypovirulence in our studies could be due to nuclear mutation(s), but rep-PCR and AFLP data (unpublished) show no differences between PM447 and the wild-type from which it was derived. Unfortunately, the only phenotype attributable to hypovirulence that can be observed in culture is slow growth. This phenotype is not specific to dysfunctional mitochondria, so is not a reliable indicator that the PM447 strain used in these studies is genetically identical to the PM447 strain first developed by Shaw (1999).

This study demonstrates the difficulty in establishing and maintaining a laboratory-induced hypovirulent mutant that has not undergone natural selective pressures and may not be stable under laboratory conditions. However, the importance of these results should not be overlooked. PM447 behaves in a reduced capacity and has some effect on disease progression even when a wild-type strain is subsequently introduced, thus suggesting that a better understanding of what is occurring in a tree in response to PM447, as opposed to a wild-type isolate, could provide insight into an inducible defense response against the pathogen. Based on this idea, a product that induces systemic acquired resistance may provide some protection against wilt.

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Chapter 3

Analysis of genotypic variation in *Ceratocystis fagacearum* using AFLPs

Abstract

Amplified fragment length polymorphisms (AFLPs) were used to characterize the genetic diversity of 24 *Ceratocystis fagacearum* isolates recovered from the Great Lakes Region. Two additional isolates from distinct geographic regions (one from Texas and one from West Virginia) were concurrently analyzed for comparison. Of the 82 bands initially scored, only one was polymorphic. Primers for this single polymorphic locus generated a 194 bp fragment in all strains except the Texas strain (which possessed a 162 bp fragment lacking in all other strains). These findings are consistent with previous results using other techniques that indicate very low diversity within the *C. fagacearum* population.

Introduction

Ceratocystis fagacearum is the causative agent of oak wilt, a lethal disease of oaks (*Quercus spp.*) found throughout the eastern half of the United States. *C. fagacearum* is spread to new locations via insect vectors, primarily the nitidulid beetles (Nitidulidae) (Jewell 1956). The fungus produces mycelial mats just below the surface of the bark on infected trees. These mats produce an aroma that attracts the nitidulids, where they pick up fungal spores that can then be carried to fresh wound sites on healthy trees. Once spores enter a wound site, the fungus invades the xylem, inducing wilt, and can move locally through intraspecific root grafts into nearby trees (Kuntz and Riker 1950). Although the disease has been known in North America since the early 20th century, some questions regarding the origin and spread of this fungal pathogen remain unanswered. For instance, it is unclear how this disease has become so

widespread despite the supposed inefficiency of the vector and the low frequency of fungal mat formation in many regions.

To better understand the movement and spread of *C. fagacearum*, a genetic marker system needs to be established that would allow isolates to be differentiated and the relationship among them evaluated. Concerns regarding spread of the pathogen via pruning tools and tree climbing equipment have recently arisen. To verify or disprove these modes of transmission, it is essential to be able to determine which strain has been introduced into a tree. In addition, the identification of individual isolates and the relationship between them would be useful for better understanding vector-mediated movement of the pathogen. Finally, strain differentiation is also necessary to conduct competition and movement studies within a single tree and between root-grafted trees.

Using restriction fragment length polymorphisms (RFLPs), Kurdyla et al. (1995) found that only eight percent (35/437) of the probe-enzyme combinations tested detected polymorphisms among nine isolates from three states, indicating a low level of genetic variation within the *C. fagacearum* population. In this study, the utility of AFLPs was explored, as an alternative to RFLPs, to identify nuclear markers within *C. fagacearum* strains.

The AFLP technique is a DNA fingerprinting procedure that takes advantage of the polymerase chain reaction (PCR) to amplify a limited set of genomic fragments from a specific DNA sample (Vos et al. 1995, Blears et al. 1998). AFLPs are highly reproducible and have been used successfully to differentiate individuals within a population (Jones et al. 1997, Robinson and Harris 1999, Majer et al. 1996). In some studies, AFLPs have proven more

successful than other methods at detecting polymorphisms (Restrepo et al. 1999, Majer et al. 1996). In addition to its high multiplex ratio (i.e. a large number of markers can be generated per reaction), it is also less labor intensive than RFLP and requires much less starting DNA (Powell et al. 1996, Ridout and Donini 1999). AFLPs have been used extensively in medical applications, including tracking individual strains of pathogens responsible for disease outbreaks, highlighting the usefulness of AFLPs for epidemiological studies (Thompson et al. 1999, Reche et al. 2002, Keto-Timonen et al. 2007). Based on the advantages of AFLPs, we applied this technique to *C. fagacearum* isolates from Michigan, Wisconsin, and Minnesota to determine the intrinsic genetic variation of the population within the Great Lakes region of the U.S. and to identify potential markers for strain differentiation.

Materials and Methods

Fungal cultures. Isolates were obtained from diseased trees in Michigan from 2001-2004 (Lower Peninsula) and from culture collections maintained in the Upper Peninsula of Michigan, Minnesota, and West Virginia. Thirteen isolates from Michigan, five from Wisconsin, four from Minnesota, and one isolate from both Texas and West Virginia were employed in the study (Table 3.1). One additional *Ceratocystis* species (*C. coerulescens*) was included for comparison in all analyses. All isolates were maintained on potato dextrose agar and glucose-phenylalinine agar.

DNA extraction. Single spore isolates were grown on plates containing Sabouraud's (1892) medium (20g glucose, 10g peptone, 3g yeast extract, and 15g agar per liter water) covered with cellophane. After two weeks, mycelium

Table 3.1 Isolate name, species, and state of origin (county given when available) for strains used in AFLP analysis.

Isolate	Species	State
Westcott	*C. fagacearum*	Michigan (Ogemaw)
Alden	*C. fagacearum*	MI (Alpena)
Beal	*C. fagacearum*	MI
Prudenville	*C. fagacearum*	MI (Roscommon)
Higgins Lake	*C. fagacearum*	MI (Roscommon)
Houghton Lake	*C. fagacearum*	MI (Roscommon)
MTU 2	*C. fagacearum*	MI (Iron)
MTU 3	*C. fagacearum*	MI (Iron)
MTU 4	*C. fagacearum*	MI (Menominee)
MTU 6	*C. fagacearum*	MI (Menominee)
MTU 8	*C. fagacearum*	MI (Iron)
MTU 10	*C. fagacearum*	MI (Iron)
DR 452	*C. fagacearum*	MI (Menominee)
Fenn	*C. fagacearum*	Wisconsin
PM447	*C. fagacearum*	(mutant derived from Fenn)
Wisc. 2	*C. fagacearum*	WI
Wisc. 5	*C. fagacearum*	WI
Tebeest	*C. fagacearum*	WI
Mississippi	*C. fagacearum*	Minnesota
Dakota	*C. fagacearum*	MN (Dakota)
Bloomsfield	*C. fagacearum*	MN (Fillmore)
Stacey	*C. fagacearum*	MN (Chisago)
Texas	*C. fagacearum*	Texas
W. VA.	*C. fagacearum*	West Virginia
MTU 5	*C. coerulescens*	MI (Lower Peninsula) – on maple

was removed from the cellophane and lyophilized. Lyophilized fungal mats were ground and DNA from 0.02 g of ground mycelium was extracted with DNAzol following the manufacturer's directions (Invitrogen™, Carlsbad, CA).

Generation of AFLPs. Genomic DNA was used in the AFLP protocol as described in the Applied Biosystems AFLP Microbial Fingerprinting Protocol Part # 402977 Rev.B with reagents available in the Applied Biosystems AFLP™ Microbial Fingerprinting Kit (Foster City, CA). Pre-selective amplification reactions were prepared using the MseI and EcoRI core primer sequences. For selective amplification, all 16 primer combinations with +2 3' nucleotide extensions and various combinations with 0, +1, and +2 nucleotides (for a total of 25 primer combinations) were assessed. Selective primers are labeled as either EcoRI- or MseI- with selective nucleotides (A, T, C, and/or G), dependent on which restriction site (generated by either EcoRI or MseI restriction endonucleases) the primer 'recognizes' and which additional (selective) nucleotides are located on the 3' end of the primer. EcoRI selective primers are 5' fluorescent dye-labeled. All PCR reactions were performed using an Applied Biosystems 2720 Thermal Cycler. The amplified DNA was then passed through a Sephadex 350 column and placed in an AB3130 sequencer, which uses capillary electrophoresis and measures fluorescence. Results were analyzed using the GeneScan Analysis v.3.7 software, which produced an electropherogram that enabled visualization and manual scoring of bands.

Data analysis. The *C. fagacearum* strains "Westcott" (Michigan) and "Fenn" (Wisconsin) were analyzed several times over a period of six months to

determine reproducibility of bands using the most promising primer combinations of the 25 tested. Only bands with peak intensity values greater than 50 and those that reliably exhibited clearly resolved profiles (i.e. appeared in each Westcott trial and were at least 1 bp different from other amplified fragments) were used for final analysis. I assumed that bands of the same molecular size in different isolates were identical. Bands were scored as present or absent; bands were scored as present if the fragment size was within 1 bp of the expected fragment. In addition to the *C. coerulescens* isolate, all runs included a negative water control for comparison.

Results

Of the 25 primer combinations tested for reproducibility and polymorphisms, three combinations (with 2 selective nucleotides on both primers) produced the greatest number of detectable bands and the most consistent results: EcoRI-AT/MseI-CA, EcoRI-AG/MseI-CC, and EcoRI-AC/MseI-CA (Table 3.2). A typical, successful AFLP reaction will generate approximately 50-100 restriction fragments (Vos et al. 1995). Although many bands were generated with the three primer combinations tested in this study, only a subset had intensities above the threshold value. Each of these three combinations yielded between 26 and 37 clearly defined bands. Other combinations with only 0 or 1 selective nucleotides with either EcoRI or MseI primers resulted in the amplification of too many bands with intensity levels well below threshold (< 50). Thus, no clear banding pattern was attainable using these combinations. Negative controls typically showed between 0 and 5 bands; however, none of these fragments were comparable to those amplified from the *Ceratocystis*

strains. The *C. coerulescens* (MTU5) isolate produced distinctive banding patterns from *C. fagacearum* with all primer combinations tested.

Table 3.2 Primer combinations tested for AFLP analysis of *Ceratocystis fagacearum.* A "+" indicates successful amplification, while a "-" denotes that few or no fragments were amplified. A "+/-" designation means that the combination produced inconsistent results.

Primer combination	amplification
EcoRI-A/MseI-A	-
-A/-C	+/-
-A/-CA	+
-A/-CC	+
-AA/-CA	-
-AA/-CC	+
-AA/-CG	-
-AA/-CT	+
-AC/-CA*	+
-AC/-CC	+
-AC/-CG	+
-AC/-CT	+
-AG/-0	-
-AG/-A	-
-AG/-C	-
-AG/-T	-
-AG/-CA	-
-AG/-CC*	+
-AG/-CG	+/-
-AG/-CT	-
-AT/-C	+
-AT/-CA*	+
-AT/-CC	+
-AT/-CT	-
-AT/-CG	+

*These combinations were chosen for final analysis.

Most strains that initially appeared to be missing particular fragments were all missing the same bands rather than showing a more random distribution of presence and absence of bands. These findings prompted further evaluation of the fragments at intensities below threshold. It was apparent that the intensities of bands varied between trials and isolates; thus, bands with peak amplitudes near 50 did not always 'appear' (i.e. sometimes their intensity value would be just below 50 and therefore they were not scored as present). For example, with EcoRI-AT/MseI-CA, a 103.31 bp fragment was not detected in strain MTU2 or Bloomsfield; however, a closer inspection revealed that the fragment was there in both strains but at intensities of 48 and 46, respectively. Since actual intensity values tend to fluctuate, the relative intensities of the bands (i.e. in comparison to the amplified fragments generated from the same strain) were considered (Hong and Chuah 2003). Thus, we assumed that the initial 'absence' of bands was likely due to random error inherent in the technique rather than true polymorphisms. Increasing the threshold amplitude value to 100 reduced the number of scorable bands, but showed a more reliable and accurate assessment of presence and absence of bands. All bands analyzed with the peak intensity threshold value set at 100 were monomorphic (Table 3.3).

Results with EcoRI-AT/MseI-CA

Data from five runs with Westcott were pooled and the average difference in homologous fragment lengths observed from run to run was determined to be 0.20 base pairs (standard error ± 0.05 bp). Of 32 well-defined bands, 31 were initially scored based on fragment size and reproducibility. However, raising the

Table 3.3 Results for the three primer combinations used in the AFLP analysis of *C. fagacearum* strains. Shown are the number of bands scored (with intensity threshold values equal to 50 or 100) and the percentage of bands that were polymorphic for each primer set.

Primer combination	Intensity threshold = 50		Intensity threshold = 100	
	scored	polymorphic	scored	polymorphic
AT-CA	31	3% (1 band)	13	0%
AG-CC	24	0%	11	0%
AC-CA	27	0%	10	0%

peak amplitude threshold to 100 resulted in only 13 scorable bands. Of the 31 fragments initially considered, one polymorphic locus was detected. All strains, except the isolate from Texas, showed a cluster of three bands at 194, 195, and 196 base pairs. The Texas isolate, however, was consistently missing a 194 bp fragment in each of three trials. Additionally, this isolate had a fragment of approximately 162 bp that reliably appeared in each run and was not observed in any other strain tested. MTU5 (*C. coerulescens*) was easily distinguishable from *C. fagacearum* with 23 out of 26 bands distinct from all *C. fagacearum* strains.

Results with EcoRI-AG/MseI-CC

Data from three trials with Westcott were analyzed and the average difference between homologous band sizes was found to be 0.30 base pairs (SE ± 0.08 bp). Twenty-seven clear bands were generated, but only 24 were originally used for analysis based on reproducibility and distinctive fragment

length. Increasing the band intensity threshold value to 100 decreased the number of scorable bands to 11. Bands were monomorphic for all *C. fagacearum* isolates. This primer set resulted in amplification of 19 fragments from MTU5 (*C. coerulescens*); 7 of these bands were of similar size to those of the *Ceratocystis fagacearum* strains (within 1 base pair) and 12 of the bands were distinctly different.

Results with EcoRI-AC/MseI-CA

Westcott was analyzed twice with this primer combination, with the average size difference between homologous fragments equal to 0.09 base pairs (SE ± 0.04 bp). A maximum of 37 bands was generated using this primer combination; however, only 27 of these bands were initially scored based on consistency. Increasing the peak amplitude threshold to 100 decreased the number of scorable bands to 10. All scored loci were monomorphic across all isolates.

Discussion

One polymorphic locus was identified from the 82 observed fragments. This polymorphism was only seen in the Texas strain, which lacked the 194 bp fragment that was present in all other isolates tested, but possessed a 162 bp fragment that was absent in all other isolates. The 162 bp fragment potentially resulted from a 32 bp deletion in the original 194 bp fragment (or alternatively a 32 bp insertion into the 162 bp fragment resulting in a 194 bp fragment).

My results are consistent with previous findings indicating little genetic diversity within the *C. fagacearum* population (Kurdyla et al. 1995, unpublished data). Several more polymorphic markers, however, were expected to be

generated based on earlier results using RFLPs (Kurdyla et al. 1995). It is possible that more polymorphisms may be observed using different restriction enzymes with different primer combinations as the restriction enzymes used and/or primers selected can greatly influence the quantity and quality of variation detected (Restrepo et al. 1999, Ridout and Donini 1999, Robinson and Harris 1999, von Korff et al. 2004, Kiros-Meles et al. 2005).

The origin of *Ceratocystis fagacearum* remains unclear; though, results with AFLPs indicate that *C. fagacearum* is not endemic to the Great Lakes region. As it is not found outside of the United States, it would appear to be an endemic pathogen or perhaps recently evolved from another endemic species. However, analysis of its rDNA sequence shows that it is distinct from all known *Ceratocystis* species (Zambino and Harrington 2005) and the lack of genetic diversity inherent in the population suggests it is not an endemic pathogen. If it is an introduced pathogen, it is reasonable to expect that it may have greater virulence on a non-native host; however, its necessary relationship with red oaks for sporulation and thus propagation, suggests it evolved on this host type. Therefore, perhaps the pathogen co-evolved with red oak species outside of North America, where the pathogen may be unknown either because it exists in nondescript locales and/or it does not incite acute disease symptoms.

Bengtsson (2003) estimates that recently evolved organisms with less than one sexual event per generation are most likely to show monomorphism. Even strictly asexual organisms are expected to be diverse over a long enough period of time because allelic changes would become fixed, leading to divergence. These ideas are consistent with *C. fagacearum* being a newly

established pathogen (either recently introduced or evolved around the beginning of the 20th century) and supports the idea that its primary means of reproduction is asexual in nature. An important consideration when analyzing the level of genetic diversity within the *C. fagacearum* population is that all isolates were obtained from infected, wilted oaks, so it is unknown what kind of diversity might exist outside of this niche. A heterogeneous population may exist, with one selectively successful individual being responsible for the observed clonal population structure.

C. fagacearum is heterothallic and therefore requires both mating types (A and B) to sexually reproduce. These mating types have been found in an approximate 1:1 ratio throughout the range of the disease, although apparently only one or the other mating type can colonize a single tree at a time (Barnett and Staley 1953, Kaufman and MacDonald 1973, Appel et al. 1985). Additionally, perithecia have regularly been observed on fungal mats that are produced just beneath the bark of wilted trees and serve as an inoculum source for vector transmission to new host trees. Based on these factors, one would expect that recombination would be apparent within the *C. fagacearum* population, but data from rep-PCR with $(GTG)_5$ (data not shown), RFLPs (Kurdyla et al. 1995), and the AFLPs we evaluated indicate that the population is for the most part clonal. This suggests that despite a sexual mechanism for reproduction, ascospores are largely unimportant in the disease cycle. Because ascospores have a greater survival potential and can theoretically disperse as well as conidia, the limited role they play in disease transmission could be due

either to lower production numbers or the timing of their formation in relation to conidia and vector emergence.

A situation comparable to that seen with *C. fagacearum* occurs with *Ceratocystis fimbriata f. sp. platani* (*Cfp*), which causes canker stain of plane trees, a pathogen and disease that share certain similarities with *C. fagacearum* and oak wilt. Recently, Engelbrecht et al. (2004) examined the genetic variation in geographically isolated populations of *Cfp*. Using the microsatellite $(CAT)_5$, they found only one genotype among the 27 isolates sampled in Europe where the pathogen was introduced in the early 1940's. In contrast, of 33 isolates sampled from the eastern U.S., where the fungus is believed to be endemic, 17 distinct genotypes were uncovered. Certain comparisons can be made between the recently introduced population of *Cfp* in Europe and the *C. fagacearum* population within North America. As with many *Ceratocystis spp.* capable of selfing, *Cfp* is capable of uni-directional mating-type switching (Harrington and McNew 1997). Thus, although it also has a sexual mechanism for reproduction, like *C. fagacearum* it shows no evidence of recombination within the population. Such monomorphism as is seen in the newly introduced *Cfp* population is typical of a pathogen that has recently undergone a genetic bottleneck. The case with *Cfp* supports the idea of a recent introduction or speciation event with *C. fagacearum*, and that, if it has been introduced, it is geographically isolated from the initial source population.

Another pathogen for comparison that has a similar history as *C. fagacearum* (is relatively new and geographically restricted) is *Fusarium oxysporum f. sp. albedinis* (*Foa*), which causes Bayoud disease of date palm.

The disease first appeared in Morocco in 1870 and has since spread into Algeria; however, the fungal pathogen is not known to occur in any other date palm cultivating region. Tantaoui et al. (1996) examined the diversity of 44 *Foa* isolates from Morocco using vegetative compatibility groupings, RFLP analysis of mtDNA, RAPD assays, and hybridization patterns of DNA with the DNA transposable element, Fot1. Their results revealed that all isolates belonged to a single vc group and the RFLP and RAPD analyses showed no polymorphisms; however, there were multiple hybridization patterns with Fot1. Fernandez et al. (1997) looked at Algerian isolates in comparison to several of the previously analyzed Moroccan isolates and had similar results; though, the use of additional primers in the RAPD assay revealed a few polymorphisms (three primers produced one polymorphic band each and one primer produced four polymorphic bands), again illustrating the importance of primer selection in identifying polymorphisms. The findings from both studies are consistent with the theory that a single, virulent clone was introduced to or arose in Morocco and subsequently spread. The results highlight the ability of certain pathogens' genomes to remain relatively stable over several decades and support the idea that conidia of *C. fagacearum* are the primary unit of dissemination as *Foa* spreads via asexual chlamydospores.

Harrington et al. (1998) also identified a correlation between reproductive mode and genetic diversity in three other *Ceratocystis* species: *C. eucalypti*, an obligately outcrossing species, *C. virescens*, which is capable of selfing, and *Chalara australis*, an asexual species with a single mating type. Using the nuclear markers $(CAT)_5$ and $(CAG)_5$ as probes, they found a high level of

diversity among *C. eucalypti* isolates, but very few polymorphisms were identified among the *Chalara australis* isolates. *C. virescens* isolates displayed an intermediate level of diversity. Restriction digestion of the mitochondrial DNA yielded similar results, with the only mitochondrial polymorphism among *Chalara australis* isolates associating with a plasmid. The results of little genetic diversity among *Chalara australis* isolates are similar to those found with *C. fagacearum* and again suggest that conidia of *C. fagacearum* are the primary, and perhaps the only source of, inoculum for disease spread.

A marker system in conjunction with spatial analysis of the oak wilt disease would facilitate epidemiological studies that may help answer questions regarding the origin and spread of *C. fagacearum*. Locating a region of increased diversity may signify where the pathogen was introduced or recently evolved. Analysis of the variation among geographic isolates in relation to their spatial pattern would help identify the processes influencing the spread of the pathogen, which would assist in more targeted management approaches. Additional population studies could be performed to better understand the movement of the pathogen. For example, researchers could compare the movement and relative distribution of two isolates within a single tree and/or stand under controlled conditions using the Texas strain and one other *C. fagacearum* strain that instead possesses the 194 bp fragment, but not the 161 bp fragment produced with the primer combination EcoRI-AT/MseI-CA as reported here. Although the results from this study show almost no diversity within the sampled *C. fagacearum* population, it is likely that an increased number of polymorphisms may be observed by using a different set of restriction

enzymes with various primer combinations and by including additional, geographically diverse strains in the analysis.

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Conclusion

Results from my research on propiconazole injection showed that treatments may inhibit oak wilt development for up to 34 months, but that multiple treatments of trees at risk of infection is probably necessary. *Ceratocystis fagacearum* appears to be able to persist for several months in an infected tree until the fungicide degrades to the point where the pathogen can actively colonize the host (assuming propiconazole is not present at fungicidal levels when the pathogen is introduced). The amount and/or rate of propiconazole injected seemingly affects the duration of efficacy of this product. Additional studies on the distribution of propiconazole within a treated tree in addition and in relation to the distribution of the pathogen may help clarify why this fungicide is generally less effective against root graft spread of the pathogen. More effective methods to determine the presence of both the fungicide and pathogen within the roots are essential for future research. Traditional fungal culturing methods from root tissue are difficult due to the presence of numerous other (contaminating) microorganisms within the soil; however, a PCR-based assay (with *C. fagacearum* specific primers) may provide a solution for detection of the pathogen within the root system of infected trees.

Results from inoculation studies with PM447 illustrate the less virulent nature of this strain, although it is unknown whether the observed hypovirulence can be attributed to mitochondrial dysfunction, as originally indicated. Both seedlings and trees generally exhibited less severe symptoms and slower wilt development when inoculated with PM447 at some point before inoculation with a wild-type, virulent *C. fagacearum* strain, in comparison to seedlings and trees

inoculated with a wild-type strain only. However, the majority of seedlings and trees challenged with a wild-type strain ultimately wilted regardless of whether or not they received an initial inoculation with PM447.

The latent period for symptom expression and subsequent disease development seen in seedlings and trees inoculated with PM447 or treated with propiconazole is typical of several other compounds previously tested for the control of wilt. This suggests that while a number of products may help delay the onset of disease, the pathogen is ultimately able to colonize a treated tree. In the case of PM447, it is likely that the presence of this strain triggers an induced defensive reaction within the host. PM447 may prime this defensive response so that when a virulent strain is introduced, the tree can more quickly restrict the pathogen to some degree. However, it is apparent that *C. fagacearum* is eventually able to overcome any defensive products or barriers produced by the host. Additional studies utilizing SAR (**s**ystemic **a**cquired **r**esistance) -inducing compounds could show more promising results than those employing PM447; however, similar results could also be observed since the defensive response may prove inadequate regardless of when it is initiated due to the physical properties of the host-pathogen interaction.

Results of AFLP analysis are consistent with previous findings of little genetic variation within the *C. fagacearum* population. Based on results using the AFLP technique with other fungal species and previous results with *C. fagacearum* using RFLPs, several polymorphic bands were expected to be resolved in these trials; however, this was not the case. Utilization of different restriction enzymes and primer combinations in addition to the inclusion of results

from several primer combinations for analysis would expectedly increase the number of scorable bands and, consequently, polymorphisms produced. Also, a more inclusive sample of isolates from all states where oak wilt is known to occur may reveal additional information about the origin of *C. fagacearum.*

One polymorphic locus was detected in the Texas isolate – no other strains contained a 162 bp fragment, while the Texas isolate did not have the 194 bp fragment present in all other strains. In relation to the work presented here, this Texas strain could be used in studies with the mutant strain, PM447, to identify the factors associated with colonization of one strain as opposed to the other and the interactions between these strains within a host.