Use of the Systemic Fungicide Propiconazole for Oak Wilt Management: An Assessment of Uncharacterized Host – Pathogen – Fungicide Interactions

A DISSERTATION
SUBMITTED TO THE FACUTLY OF THE GRADUATE SCHOOL
OF THE UNIVERSITY OF MINNESOTA
BY

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IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

Jennifer Juzwik, Adviser

April 2009

Acknowledgements

This dissertation is the culmination of an education that spans many years and was enabled by many educators, collaborators, mentors, professional acquaintances, friends, and family members. To all of them I extend my most sincere thanks; I will be forever grateful for the opportunities I have had, the people I have met, and the education I have received. I would like to thank the International Society of Arboriculture, the Minnesota Turf and Grounds Association, Rainbow Tree Care Scientific Advancements, the University of Minnesota, and the USDA Forest Service – North Central Research Station for their financial support. To Peter Ojiambo and Consuelo Arellano of North Carolina State University, thank you for your guidance and assistance in the statistical analyses required for this dissertation. To Brian Barber, who spent many long hours processing thousands of samples for me, and many more hours fixing the damage that resulted, I thank you for your patience, hard work, guidance, and friendship. I would like to acknowledge Gib Ahlstrand for his assistance and innovative approach in developing the microwave extraction technique that was critical for this investigation. I am grateful to all those who have assisted with my field work and all the injecting, digging, cutting, chopping, sawing, and grinding it entailed: Jordan Eggers, Brian Schwingle, Sara French, Maya Hayslett, Angie Ambourn, Ka Zang, and others. In particular I would like to thank Paul Castillo and Autumn McKnite for their hard work and friendship; our most difficult tasks are among my favorite memories. I would like to thank my friends and family who have made me who I am and have supported me in all of my endeavors. I would especially like to thank my parents Gary and Lynn, my grandparents Bob, Marion, Lois, and Art, and Uncle George and Aunt Marilyn. To my fiancé Karen, thank you for all of your support, assistance, love, and endless patience. This research brought us together and I will forever smile when I think of the effects propiconazole had on us! Finally I would like to thank the members of my Ph.D. committee: Robert Blanchette, William Chaney, Richard Zeyen, and especially Jennifer Juzwik for their guidance and patience. You have instilled in me the knowledge and passion needed to accomplish this work and to achieve my future goals. I extend my sincerest thanks to you for your willingness to mentor me during my time at the University of Minnesota and beyond.

Dedication

To my Family,

For everything you have given me.

Abstract

Propiconazole is a systemic fungicide widely used for the control of oak wilt, however, the long-term efficacy of this fungicide has not been well established and treatment effectiveness may be below levels that justify its use in many situations. To date, it is not known if propiconazole applications prevent root graft transmission of *Ceratocystis fagacearum* nor if they can completely eradicate this pathogen from the root system of an infected tree. Significant translocation of propiconazole into the roots from the point of injection has not been demonstrated and fungitoxic concentrations of the fungicide have not been determined *in vivo*. Furthermore, symptom development is not induced by pathogen colonization of the root system; oaks respond to infection only when *C. fagacearum* spreads above ground in the vascular system. Therefore, it is impossible to determine the extent of pathogen distribution in the root systems of trees near disease centers. Treatment failure may result from poorly informed management decisions that do not take into account pathogen distribution or the capabilities of the systemic fungicide being utilized. This dissertation presents the results of three investigations conducted to examine use of propiconazole to control root graft transmission of *C. fagacearum* in red oaks (Section *Lobatae*). The distribution of propiconazole in the root system of treated trees was examined using gas chromatography-mass spectrometry. While substantial movement of the fungicide into the root system did occur following injection, the inability of propiconazole to prevent infection or eradicate the pathogen from root-inoculated oaks was demonstrated. The spatial and temporal spread of *C. fagacearum* in the root system of oaks in or near disease centers, and the role of self- and inter-tree root grafts in pathogen spread was examined. Distribution of *C. fagacearum* in the root systems of wilted and wilting trees was sporadic, and the prevalence and importance of self-grafts was noted. Finally, the effects of propiconazole on the growth and anatomy of treated oaks was investigated. Plant growth regulating properties of the fungicide suggest that fungitoxicity alone may not be responsible for propiconazole-induced disease protection. The implications of these previously unknown aspects of the host-pathogen-fungicide interactions in propiconazole treated trees, and suggestions for improved treatment efficacy and oak wilt management are discussed.

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CHAPTER 1

Literature Review

1.0. Introduction

Oak wilt is considered to be the most important disease of oaks in the United States and few pathogens encountered in the field of forest pathology rival the virulence of the oak wilt fungus *Ceratocystis fagacearum* (T.W. Bretz) J. Hunt (Appel, 1995; Gibbs and French, 1980; Wilson and Lester, 2002). Yet, oak wilt management resulting in acceptable levels of oak mortality is possible if the control measures used to achieve that objective are understood and utilized in the most efficacious manner. The purpose of the research presented in this dissertation was to critically investigate the use of propiconazole for controlling below-ground transmission of *C. fagacearum*. Use of propiconazole as part of an integrated strategy for managing oak wilt has become increasingly popular since it was first used for this purpose in the late 1980's (Appel, 1990). The long-term effectiveness of vascular injections of propiconazole into oaks at high risk of infection has not been well established however, and there is growing evidence to suggest that treatment efficacy may not justify its use in some situations (Eggers et. al., 2005). To date, it is not known if propiconazole is fungicidal or fungistatic *in vivo*; nor if it is able to protect trees from infection or to completely eradicate the pathogen from an already infected tree. Furthermore, there is currently no proven mechanism by which propiconazole would prevent root graft transmission of *C. fagacearum*; all current methods of application rely upon translocation of the fungicide in the tree's transpiration stream for distribution in the crown. Significant translocation of propiconazole into roots from the point of injection has not been demonstrated definitively, nor has the longevity of the prophylactic effects of the treatment been well documented (Eggers et. al., 2005). The spatial and temporal distribution of the *C. fagacearum* within infected trees and its spread through expanding disease centers is also poorly characterized. Treatment failure may result from management decisions that fail to take into account the complex interactions between the host, pathogen, and fungicide.

Treatment failure is unacceptable because of the expense associated with fungicide injections, and more importantly, because a latent or masked infection represents an inoculum source that is not accounted for in management regimes.

1.1. Oak Wilt

1.1.1. History and Importance

Oak wilt is caused by the vascular pathogen *Ceratocystis fagacearum* (T.W. Bretz) J. Hunt. Since its discovery in Wisconsin in 1942, oak wilt has been found throughout much of the eastern United States where it is considered to be the most serious disease of oak (*Quercus spp.*). While *C. fagacearum* is known to be pathogenic on all 33 native oak species in the U.S., many species such as those belonging to the Section *Quercus* (collectively referred to as white oaks) exhibit various degrees of resistance and can survive infection for many years or even recover through successful compartmentalization of the fungus. However, in the Section *Lobatae* (red oaks) the pathogen has the capacity to kill its host very rapidly; within a few weeks or months. For this reason, there was great concern following its discovery that oak wilt posed a considerable threat to the nation's oak forests and associated resources; a concern that seemed all the more justified in the wake of the Dutch elm disease and chestnut blight epidemics earlier in the century. To date, devastation similar to that resulting from the introduction of *Ophiostoma ulmi* or *Cryphonectria parasitica* has not been realized for *C. fagacearum*. This is somewhat surprising because historical land use and forest management practices have altered forest stand composition significantly, resulting in more oak forests with greater proportions of highly susceptible red oak species.

The current distribution of oak wilt is limited to the eastern half of the United States (Figure 1.1). In most areas, epidemics remain relatively localized, or lack an intensity that would result in large annual losses of oak at the landscape level. The reason for this is unclear, but may be the result of limitations in the life history of the pathogen such as lack of a highly efficient vector, or co-evolution of host and pathogen resulting in a relatively stable population structure. Although the pathogen has not been discovered outside of the United States and overland spread is dependent upon well-characterized symbiotic relationships with native insect vectors, other evidence supports

the hypothesis that the origin of *C. fagacearum* lies outside of its current range (Juzwik et. al., 2008). Restriction Fragment Length Polymorphism (RFLP) analyses of mitochondrial and nuclear DNA indicate *C. fagacearum* has low genetic diversity, suggesting the pathogen was recently introduced (Kurdyla et al., 1995). Furthermore, in central Texas severe oak wilt epidemics appear to be more characteristic of an introduced pathogen. Although *C. fagacearum* may have been present as early as the 1930's, the devastating losses of live oaks (*Quercus fusiformis* and *Quercus virginiana*) that continue to this day were not observed until the 1970's. Cumulative losses during the 40 year epidemic have been conservatively estimated to be over one billion dollars in Texas alone (Wilson, 2001). Other epidemics such as those occurring in Minnesota and Wisconsin are most severe in areas adjacent to human development (Juzwik and Schmidt, 2000). They are particularly destructive because of losses incurred to high value trees in parks, wooded lots, and around homes and businesses.

1.1.2. Pathogen Biology

The fungus *C. fagacearum* belongs to the Pyrenomycetes of the Ascomycota. The species is heterothallic; consisting of two sexual compatability types (Types A and B) (Upadhyay, 1981; Wingfield, Sefiert, and Webber, 1993). The fungus produces two types of spores: asexually produced endoconidia, and sexually produced ascospores in long-necked perithecia when both mating types are present. Both types of spores can be produced on spore mats on many oak species, particularly on red and black oaks. Spore mats are produced beneath the bark of recently wilted trees, and consist of specialized mycelia and one or more pressure pads capable of rupturing the bark and exposing the mat when sufficient pressure has developed (Gibbs and French, 1980). The mat gives off a fruity odor attractive to a variety of insects and squirrels which can access the mat through the bark slit. Endoconidia act as spermatia, and are carried by insects from mats on recently wilted trees to other mats where sexual reproduction of the pathogen occurs (Hepting, Toole, and Boyce, 1952; True et. al., 1960).

C. fagacearum is spread by two mechanisms: above-ground where long distance transport of the fungus is mediated by insect vectors resulting in the initiation of new disease centers, and movement from tree to tree over short distances via root grafts.

Approximately 95% of new infections are the result of localized root graft transmission in existing disease centers, while only a small portion of new infections result in the formation of a new disease center following insect transmission over land (Juzwik, French, and Jeresek, 1985).

In Minnesota, the most important insect vectors are believed to be several species of sap feeding beetles of the family *Nitidulidae* (Ambourn, Juzwik, and Eggers, 2006; Ambourn, Juzwik, and Moon, 2005), while the small *Pseudopityophthorus minutissimus* is of minor importance. Bark beetles and sap feeding beetles may carry both spore types, and become contaminated upon emergence from infected trees or while feeding on sporulating mats, respectively (Gibbs and French, 1980). Spores must be introduced to healthy trees through fresh wounds. While bark beetles create small wounds that are suitable as infection courts during maturation feeding, sap feeding beetles must visit and deposit spores on fresh wounds created, for example, as a result of storm damage or human activity such as pruning and construction-associated damage. Insect vector transmission may occur over distances as great as several kilometers, but is generally limited to distances of less than 1 kilometer (Juzwik, French, and Jeresek, 1985). If transmission results in disease development and the pathogen is able to spread into the tree's root system, a disease center may result.

The movement of *C. fagacearum* through root grafts was confirmed early in the study of oak wilt transmission (Jones and Partridge, 1961). Although the evolutionary reason for the formation of root grafts is poorly understood, root grafts may aid in nutrient and water acquisition or may increase resistance to wind-throw (Graham and Bormann, 1966). In forests where management practices have promoted near homogenous stands of oak such as in Minnesota and Wisconsin, or in Texas where live oaks grow in shallow rocky soils and can propagate vegetatively via root sprouting, large interconnected networks of root grafts enable the pathogen to spread from tree to tree resulting in an expanding disease center which is characterized by a ring of infected or actively wilting trees surrounding a cluster of oak wilt-killed trees (Appel, 1995; Gibbs and French, 1980).

Wilt symptoms are accompanied by the formation of tyloses in large xylem vessels and gum production in smaller vessels and tracheids, which results in the

disruption of water flow in the vascular system (Nair, Kuntz, and Sachs, 1967; Struckmeyer et al., 1954). When the negative pressure produced by transpiration in the xylem of infected trees begins to fall, healthy trees root grafted to an infected tree may begin to draw endoconidia across the root graft resulting in the spread of the pathogen into uninfected hosts (Nair, 1995; Nair and Kuntz, 1975). Evidence to support this claim was provided by Shain, Fergus, and Stambaugh (1962). In their study of the absorption and translocation of azosulfamide solutions applied to cut stumps of diseased oak trees, the authors observed that both processes were increased when the stumps were root grafted to adjacent trees which were healthy and actively transpiring. The fungus is capable of survival in the root systems of deceased trees for one to five years (Skelly and Wood, 1974b). The frequency of endo-parasitic interactions between the pathogen and root cells was found to be relatively low, and the survival of the fungus in roots for long periods is not well understood (Struckmeyer, Kuntz, and Riker, 1958). Longevity of the fungus in the roots of dead trees may be aided by a supply of water and nutrients obtained through root grafts with healthy trees (Yount, 1955).

Following infection, the pathogen is able to spread rapidly throughout a tree via endoconidia that are carried upward in the transpiration stream, characteristic of a true vascular wilt (Kile, 1993). Interestingly, the pathogen's mycelia are generally not present in the xylem vessels until after permanent wilt is initiated (Struckmeyer, Kuntz, and Riker, 1958). Sparse vegetative growth of pathogens is common in wilt diseases, as the ratio of carbon to nitrogen in the xylem fluids is generally very low (Brown, 1936). Instead, pathogens are dependent upon organic nitrogen compounds as opposed to sugars for an energy source in xylem vessels prior to the onset of wilt symptoms (Beckman et al., 1953; Kessler, 1966). Sparse growth of *C. fagacearum* in oak prior to the onset of wilt is well documented. Vegetative growth of the pathogen in xylem vessels, tracheids, and fibers is relatively limited, and there is limited penetration of parenchyma cells adjacent to conductive tissues. Rather, the presence of the pathogen in vessels is usually limited to the easily distributed endoconidial form of the fungus prior to the development of wilt symptoms (Jacobi and MacDonald, 1980; Nair, 1964; Nair and Kuntz, 1960; Parmeter, Kuntz, and Riker, 1956; Sachs, Nair, and Kuntz, 1970; Struckmeyer, Beckman, Kuntz, and Riker, 1954; Struckmeyer, Esther, and Kuntz, 1954; Struckmeyer, Kuntz, and

Riker, 1958; Beckman, Kuntz, Riker, and Berbee, 1953; Wilson, 1961; Wilson and Montgomery, 1960).

1.1.3. Host-Pathogen Interactions

It is not until trees are severely wilted that extensive colonization by mycelia occurs. Vegetative growth begins to develop in xylem vessels from which surrounding parenchyma cells are invaded (Nair and Kuntz, 1975). Movement of the pathogen laterally occurs via penetration of the bordered pit pairs of the xylem vessels into adjacent vessels, ray cells, and parenchyma cells (Jacobi and MacDonald, 1980; Struckmeyer, Esther, and Kuntz, 1954; Struckmeyer, Kuntz, and Riker, 1958; Wilson, 1961). Partridge (1961) indicated that lateral movement of the pathogen was primarily through the wood rays, both intracellularly and intercellularly. *C. fagacearum* is also capable of forming appressoria and directly penetrating the thick cell walls of the xylem vessel, tracheid, and parenchyma cells (Sachs, Nair, and Kuntz, 1970). Degradation of lignin and pectin in the middle lamellae and secondary cell wall layers accompany penetration (Sachs, Nair, and Kuntz, 1970). No appressoria are formed when the fungus penetrates bordered pits (Kuntz, 1954; Nair, 1964). Penetration through pits is more common than direct penetration (Tainter, 1995). Most of the vegetative growth of *C. fagacearum* in a wilting tree occurs in parenchymatous tissue (Nair and Kuntz, 1975).

The direct cause of wilt symptoms is currently unknown. While tylose formation is most commonly implicated, pathogen structures (e.g. mycelia or spores) and pathogen metabolites may plug conductive elements to limit water movement. The disintegration of xylem elements such as vessel walls may increase the viscosity of tracheal fluids. Toxins produced by the pathogen may be directly responsible for inducing wilt symptoms. An α-mannan toxin has been shown to induce wilt symptoms in oak seedlings. It may be that a combination of some or all of these factors contribute to wilt induction (Beckman, Kuntz, Riker, and Berbee, 1953), however, there is extensive evidence suggesting that tylose formation, as part of a defense response to limit pathogen movement and to compartmentalize the fungus, is the greatest contributing factor to the development of wilt symptoms. Considering the sparse colonization of host tissues prior to wilt, it is likely that endoconidia alone are capable of inducing widespread tylose

formation in red oaks, suggesting that metabolites of high activity are at work. The difficulty in finding mycelia even in the early stages of wilt suggests the importance of endoconidia in the rapid internal spread of the pathogen throughout the tree. It also suggests that spore physiology may be of great importance in early host reactions; spores likely produce metabolites that stimulate processes such as gummosis and tylose development (Nair and Kuntz, 1960; Struckmeyer, Kuntz, and Riker, 1958).

The severity of wilt is directly correlated with the extent of tylose formation (Parmeter, Kuntz, and Riker, 1954; Struckmeyer, Beckman, Kuntz, and Riker, 1954, Tainter, 1995). Tyloses are outgrowths of parenchyma cells located adjacent to xylem vessels. During tylose formation, the pit membrane is enzymatically degraded while a protective layer laid over the pit membrane balloons out into vessel lumen. Tylose formation is only possible if the pit aperture is greater than ten microns wide (Esau, 1977). In general, tyloses form in infected trees three to five days prior to incipient wilt (Nair, Kuntz and Sachs, 1967; Parmeter, Kuntz, and Riker, 1954). Beckman et al. (1953) observed that the movement of radioactive rubidium in the transpiration streams of inoculated pin oaks was normal until four or five days before wilt symptoms were observed. Then movement in the transpiration stream dropped 90%. The authors concluded that plugging of the vascular system was directly responsible for decreased water movement and the resulting water deficit was the direct cause of wilt symptoms and tree death. In the same study, the authors were able to show that tylose formation was a response of the host to the presence of the pathogen and was the cause rather than the result of decreased water movement. This is an important distinction, as tylose formation occurs in response to the cessation of water movement, in response to injury, or as a normal developmental process during the life of a tree. These results have been confirmed in numerous studies (Parmeter, Kuntz, and Riker, 1954; Struckmeyer, Beckman, Kuntz, and Riker, 1954; Struckmeyer, Kuntz, and Riker, 1958). The vessel diameter in the twigs of the upper branches are generally larger than in the twigs of lower branches, and thus vessels of the upper twigs are at greater risk for the formation of embolisms (Tainter, 1995). This may be one of the reasons why wilt symptoms first appear in the upper crown of infected trees. However, the results of other studies suggest that mechanisms other than water stress are involved in the expression of oak wilt

symptoms. For instance, it was shown that the leaf abscission layer induced by oak wilt is similar in anatomical development to that described for natural leaf abscission, but dissimilar to the premature leaf loss induced by water stress and tylose formation (TeBeest, Durbin, and Kuntz, 1973). Furthermore, the dissolution of cell walls necessary for tylose formation occurs via a similar mechanism as that observed for leaf abscission. Wilson (1961) suggested that tree death results from the invasion and subsequent death of parenchyma cells and not from a lack of water due to vessel occlusion.

Tylose formation is extensive in the stems and branches of infected red oaks, but occurs only in narrow arcs or bands in the xylem tissue of white oaks (Beckman, Kuntz, Riker, and Berbee, 1953). Tylose formation is generally most common in the large springwood vessels in the current year's growth ring, while tylose formation is quite limited in the small vessels of summerwood. In the later stages of wilt, gummosis becomes prevalent in smaller vessels, parenchyma cells, and tracheids.

The number of vessels containing tyloses in the roots of infected trees is considerably less than in the stem, and tylose formation in the roots has been shown to be sporadic even though the same roots may contain both the hyphae and spores of the pathogen (Nair and Kuntz, 1975; Parmeter, Kuntz, and Riker, 1954; Struckmeyer, et al., 1954; Struckmeyer, Kuntz, and Riker, 1958).

Tyloses formation also occurs at a relatively low rate in the roots of wilted trees after death, and gummosis has not been reported in roots. Thus, it appears that pathogen movement can proceed uninhibited across root grafts (Nair and Kuntz, 1975; Struckmeyer et al., 1954; Yelenosky and Fergus, 1959). Inoculation studies have shown that although root inoculations may be less successful than trunk inoculations, the length of time between the appearance of the first foliar symptoms of wilt and root inoculation may be several years. In addition, Rexrode (1978) confirmed the presence of root grafts with the use of dyes injected into *C. fagacearum* infected trees, yet disease symptoms were not observed in grafted trees for up to three years after the wilting and death of the initially infected tree. This suggests that movement of the pathogen through roots is very slow, and that the pathogen is capable of surviving undetected in the roots of dead, wilting, and asymptomatic trees for many years (Skelly and Wood, 1974a; Yount, 1958).

It is believed that the ability of certain oak species to limit pathogen spread and recover from infection by *C. fagacearum* is due to anatomical features that allow them to compartmentalize the fungus while maintaining physiological integrity. In northern pin oak (*Q. ellipsoidalis*) and northern red oak (*Q. rubra*), the pathogen is rapidly distributed throughout the tree and the xylem vessels of the outer annual ring become plugged with tyloses and gums (Beckman et. al., 1953; Nair, Kuntz, and Sachs, 1961; Nair, Kuntz, and Sachs, 1967). In bur oaks however, fungus distribution is limited to vertical sectors of the trunk and to vascularly connected branches (Nair and Kuntz, 1960; Nair, Kuntz, and Sachs, 1967). Plugged vessels were also shown to be confined to areas extending directly upward and downward from the point of inoculation in white oaks (Parmeter, Kuntz, and Riker, 1956). Plugged vessels in the outer annual ring of these trees extended from the point of inoculation upward to twigs in the crown and downward to the root collar in an arc not much larger than the wound produced during inoculation. Plugging was found around the entire circumference of inoculated northern red oaks and northern pin oaks, effectively girdling the trees. Nair (1964) also observed that plugging was confined to a vertical sector of the xylem in inoculated bur oaks, but tyloses were produced for only a limited distance above the inoculation point. Oaks which are able to confine the pathogen and plugged vessels in such a manner will often produce a new annual ring the following year. If the new annual rings do not become invaded, the diseased tissues become effectively buried and the pathogen will die (Schoeneweiss, 1959).

Schoeneweiss (1959) and Kuntz (1954) both observed that often a new broad growth ring with open vessels is produced in resistant oak species such as white oak in the same year in which symptoms first appear. This new growth ring lacked tyloses and gums, suggesting that the pathogen was restricted to the plugged bands of springwood. The authors also noted that a sharp line of very small cells is laid down between the outermost infected ring and the adjacent healthy tissues in white oak, perhaps acting as a barrier which prevents the pathogen from spreading outward. In a separate study, a reaction zone made up of darkly stained parenchyma cells was noted in infected white oak (*Q. alba*) and chestnut oak (*Q. prinus*), but the zone was diffuse and did not prevent the movement of the fungus in northern red oak (Jacobi and MacDonald, 1976). In bur oaks, masses of dark, electron dense material developed in parenchyma cells adjacent to

infected vessels. Hyphae were rarely observed to penetrate parenchyma cells containing this material, which was thought to be composed of phenolic tannins (Sachs, Nair, and Kuntz, 1970).

Jacobi and MacDonald (1980) found that penetration of pits by *C. fagacearum* is rare in white oak, but lateral spread via pit penetration is rapid in northern red oaks. In the same study, the authors noted a sharply defined area of darkly stained parenchyma cells surrounding colonized and tylose-occluded vessels in white oak and chestnut oaks, a feature lacking in red oaks species. The authors concluded this darkly stained region was critical for restriction of the fungus to infected tissues, and may represent a mechanism for resistance. Tylose formation was much more extensive in white oaks than in red oaks. The authors also concluded that lateral and longitudinal restrictions such as tyloses, gums, anatomical restrictions and cellular responses in white oak limited growth of the pathogen much more effectively than in red oaks. *C. fagacearum* was unable to move into uncolonized vessels nor bypass blockages caused by gums, tyloses, and plugged perforation plates in white oak. Fungal growth was not restricted in northern red oaks. This may have been caused by a delay in fungal growth in red oaks as compared to white oak, and subsequently the red oaks induced fewer early responses to infection in comparison to white oaks. In infected northern red oaks, tylose formation is generally limited to the most recently formed growth ring, and there is normally no tylose formation in these areas in healthy trees. Tyloses are quite common in the outermost rings of healthy white oaks however, where they may impede the spread of vascular pathogens (Tainter, 1995). Parmeter, Kuntz, and Riker (1956) also concluded that differences in wilt development in bur and northern pin oaks was due to the more rapid spread and extensive distribution of the pathogen in northern pin oaks as compared to the slow spread and poor distribution of the fungus in bur oaks. The authors observed that as long as a portion of the vascular system remained unaffected, new annual rings were laid down each year. Often, the pathogen was able to invade this new growth ring and symptoms would reoccur. This cycle would continue until the fungus had spread throughout the entire crown and subsequently induced plugging of vessels around the entire circumference of the tree, or until the tree was able to produce a new growth ring that was not subsequently invaded.

Susceptibility of oaks to infection by *C. fagacearum* appears to be greatest during the time period of maximum physiological activity of the host (Nair, 1964). It has also been noted that symptom development is closely associated with the degree of leaf expansion and the formation of springwood in oaks. Generally, the larger springwood vessels are more conducive to rapid distribution of endoconidia. In addition, tylose formation is much more extensive in large springwood vessels as compared to smaller summerwood vessels (Drake, 1956; Nair, 1964). Larger vessels are also more susceptible to embolisms. Trees infected late in the summer or fall of the previous year often do not show wilt symptoms until the formation of springwood begins the following year. This has been noted in numerous cases where trees inoculated on various dates late in season, all show symptoms on nearly the same date the following year (Nair, 1964; Skelly and Wood, 1974a ; Skelly and Merrill, 1968). It may be that a trigger such as springwood formation or the production of growth regulators is needed to induce wilt symptoms (Skelly and Merrill, 1968).

It is known that *C. fagacearum* is able to stimulate the proliferation of ray cells as it moves outward towards the cambium. Later, these rapidly dividing ray cells collapse and disintegrate creating a cavity that contributes to the loosening of bark and provides a site for the fungal mat to form. Other cavities formed from such activity provide intercellular spaces which the pathogen colonizes and forms a parasitic relationship with adjacent parenchyma cells (Struckmeyer, Esther, and Kuntz, 1954; Struckmeyer, Kuntz, and Riker, 1958; Tainter, 1995).

1.1.4. Integrated Management

Successful management of oak wilt in the upper Midwest can be accomplished through an integrated management strategy that limits both overland and root graft transmission of *C. fagacearum* (Obrien et. al., 2000; Tainter and Baker, 1996).

Overland transmission of *C. fagacearum* is controlled by eliminating sources of inoculum, and minimizing the opportunity for introduction of pathogen propagules into the vascular system of healthy trees (Juzwik et. al., 2004). Recently wilted or wilting trees represent the only inoculum source from which insect vector contamination can originate. *C. fagacearum* is a relatively poor saprophyte and is rapidly displaced in wilt-

killed trees (Gibbs and French, 1980). However, before such displacement occurs, there is a burst of saprophytic growth following tree death during which time the inner bark tissue is invaded and sporulating mats are produced. Removal of trees prior to mat production eliminates the possibility for transmission by insects which feed or breed in sporulating mats (Juzwik et. al., 2004); and also prevents sexual reproduction in the pathogen population (Gibbs and French, 1980). However, it has been suggested (Nair, 1995; Nair and Kuntz, 1975) that removal of wilted or wilting trees may be unadvisable if such removal would establish a negative pressure gradient across grafted roots that would draw pathogen propagules from the root system of the diseased tree to healthy trees which are actively transpiring. While there is evidence to support this contention (Shain, Fergus, and Stambaugh, 1962), the increased risk of root-graft transmission by tree removal may be offset by the benefit associated with removal of inoculum contributing to long-distance spread, especially if care is taken to control root graft transmission as described below. Wood from diseased trees should be burned, chipped, or otherwise properly disposed of to prevent potential mat production or emergence of contaminated beetles from cut logs. If immediate disposal is not possible or long-term storage for firewood is desired, logs should be stored under a tarp or plastic sheet which is sealed to the ground for a period of one year to ensure the wood is no longer a potential inoculum source (Cummins-Carlson and Martin, 2005; Juzwik et. al., 2004).

Establishment of new disease centers via overland transmission of *C. fagacearum* is most effectively controlled by reduction of available infection courts suitable for pathogen introduction into the vascular system of healthy trees. Transmission by sap feeding beetles of the *Nitidulidae* family requires fresh wounds that expose the vascular system (Gibbs and French, 1980). Natural wounding through storm damage, branch to branch contact, and herbivory can provide for suitable infection courts. Human-induced wounding during pruning, construction damage, or logging operations is also a significant contributor to overland transmission (Cummins-Carlson and Martin, 2005; Juzwik et. al., 2004). Cessation of pruning and logging operations during periods of high insect vector activity, particularly when coinciding with periods of sporulating mat production, is the most effective way to minimize the risk of overland transmission and the creation of new disease centers. When wounding does occur or cannot be avoided,

immediate treatment of wounds with pruning paint can render the site unattractive to insects and unsuitable for infection (French and Juzwik, 1999).

Root graft transmission of *C. fagacearum* has historically been managed through mechanical disruption of root grafts between infected and asymptomatic trees, effectively containing the expansion of individual disease centers along root graft barrier lines (French and Juzwik, 1999; Juzwik et. al., 2004). Commonly, root grafts are severed with the use of a vibratory plow, trencher, or backhoe. The installation of trench inserts that prevent re-establishment of root grafts across root graft barrier lines have successfully been used to prolong the effectiveness of mechanical root graft disruption (Wilson and Lester, 2002). While expensive, the additional cost of trench inserts may be justified at sites where high-value landscape trees mandate enhanced protection and the additional costs and site disturbance associated with retrenching is undesirable.

Effective barrier placement must surround all infected trees, including those that are dead, wilting, or asymptomatic. Because mechanical root graft disruption is expensive and disruptive to the site, failure to contain all infected trees is unacceptable as it will subsequently require the establishment of new barrier lines. Yet, the strategy of barrier placement is to not only contain the pathogen, but to save as many healthy trees as possible. There is currently no way to determine the extent of pathogen distribution in the root systems of symptomatic or asymptomatic trees, therefore barrier placement is as much an art as a science. Conservative barrier line placement is more likely to succeed in pathogen containment, but will require the sacrifice of potentially healthy trees that may have been saved if the line was placed closer to the disease center. The use of primary and secondary root graft barrier lines was first proposed by French and Stienstra (1972) as a guideline for control of root graft transmission of *C. fagacearum*. Bruhn, Pickens, and Stanfield (1991) also suggested the placement of a primary and secondary barrier line, one conservatively placed to ensure pathogen containment and one placed closer to the disease center to save as many healthy trees as possible (French and Juzwik, 1999). This pattern of barrier placement has become a commonly accepted practice. Bruhn et. al. (1991) developed a disease spread model that can be used to predict the probability that *C. fagacearum* will spread to a healthy tree within one year of symptom development in a neighboring diseased tree through root grafts in Michigan. Because the incidence of

grafting is thought to be determined by a number of factors such as soil type, specific models need to be developed which reflect different spatial and temporal patterns of disease spread in different areas. Such models can be used to improve the reliability of barrier placement while minimizing the tendency to include healthy trees within the barrier line.

1.2. Control of Root Graft Transmission of *Ceratocystis fagacearum* with Propiconazole

1.2.1. History of Propiconazole

The search for chemicals that could be used against *C. fagacearum* has been ongoing since the initiation of studies at Iowa State University in 1947 with chemicals such as sodium dimethyldithiocarbamate (Bragonier, 1955). Success was quite limited until the introduction of the triazole compounds in the 1970's, such as propiconazole. The triazoles represented a new group of compounds that were highly systemic in plants and had exceptional fungicidal and plant growth regulating properties (Schwinn, 1983). Propiconazole has been used experimentally in the control of oak wilt since the mid-1980's (Appel, 2001). Successful use of the compound to reduce oak-wilt incidence in Texas live oaks and Dutch elm disease caused by *Ophiostoma novo-ulmi* prompted more widespread use of propiconazole in both preventative and therapeutic treatments of such diseases. Propiconazole's ability to be transported systemically in plants, high fungicidal activity against a wide range of fungal pathogens at very low concentrations, low phytotoxicity, ability to be mobilized in water as opposed to an organic solvent carrier, and uniform distribution in mature trees made it a far superior tool in the management of woody plant diseases when compared to previous compounds such as thiabendazole or benomyl (Appel, 2001). Today, injection of oaks with propiconazole is an important component of an integrated approach to prevent the spread of oak wilt (Appel, 2001; Juzwik et. al., 2004).

1.2.2. Current Utilization Procedures

Propiconazole, when introduced into the vascular system, has been used to protect healthy trees that are within root grafting distance of wilting trees from contracting oak

wilt. In addition to the use of propiconazole to prevent root graft transmission of *C. fagacearum*, the systemic fungicide is also used for curative or therapeutic treatments of oaks that are infected and exhibiting wilt symptoms. The therapeutic use of propiconazole is generally limited to more resistant oak species such as white oak (*Q. alba*) and bur oak (*Q. macrocarpa*) which can more effectively limit pathogen spread via tree defensive responses and compartmentalization, particularly in cases where symptoms are restricted to only a small proportion of the crown. However, those branches which display symptoms at the time of treatment usually do not recover and are pruned out after treatment (Eggers et. al., 2005). When prescribing therapeutic treatments, it is critical to treat only those trees in which the pathogen has not spread into the root system; therapeutic treatments are reserved for those cases where pathogen distribution is limited to the tree's crown.

The success of therapeutic treatments in more resistant oak species also means that treatment to prevent root graft transmission is generally unnecessary, i.e. the cost of treatment and injury to the tree incurred during the application of a preventative treatment is not offset by the reduced risk for infection. Conversely, therapeutic treatments in susceptible red oak species are relatively ineffective, especially during advanced stages of crown wilt (Ward et. al., 2005); therefore propiconazole application prior to infection is the most appropriate treatment in these species. Treatment with propiconazole to prevent overland transmission of the pathogen is not warranted because of the low risk of infection and associated costs.

To be effective, good distribution of the fungicide throughout the tree is required. Thus, propiconazole must be translocated systemically throughout the tree. Translocation from the point of injection throughout the tree's crown occurs via the transpiration stream. Therefore, propiconazole application methods must introduce the fungicide into those portions of the xylem that are currently transporting water. Application via soil injection near the drip line of the target tree has been successful in the past (Wilson and Lester, 2002), however, the most common method of application is through micro- or macro- injection techniques into the outermost xylem increments.

Microinjection introduces small volumes of concentrated fungicide from prepackaged containers into the tree. The applicator tips are inserted into the tree, either

in the root flares or on the trunk, through holes drilled into the xylem. The number of microinjectors per tree is based upon tree diameter, and the injectors are distributed evenly around the circumference of the tree. While many microinjectors are passive and rely only upon the transpiration stream to draw propiconazole into the xylem, other microinjectors rely upon applied pressure to assist in fungicide uptake (Haugen and Stennes, 1999; Kondo, 1978).

Macroinjection differs from microinjection in that it utilizes large volumes of propiconazole diluted in water (4-8 ml of active ingredient suspended in 1 L of water per cm diameter at breast height) rather than small volumes of concentrated fungicide. The large volume of diluted fungicide utilized in the macroinjection technique appears to result in more uniform distribution of the systemic fungicide in the crown of trees than can be achieved with microinjectors (Haugen and Stennes, 1999; Kondo, 1978). During macroinjection, the fungicide/water suspension is applied under pressure directly into the transpiration stream through a system of linked hoses with plastic tips (tees) inserted into holes drilled in root flares. Injection into the root flares appears to result in more uniform distribution of propiconazole in the tree's crown and provides a greater surface area to drill injection holes as compared to the number that could be applied on the trunk. The tees are spaced evenly in the root flares around the entire circumference of the tree at a rate of approximately 1 tee for every 4 cm of tree diameter at breast height.

Both methods of injection rely upon translocation of the fungicidal compound in the transpiration stream from the point of injection into the tree crown. Therefore, application of propiconazole is dependent upon a healthy, actively transpiring crown. The rate of uptake will vary with the size of the tree, crown condition, soil moisture, humidity, and temperature for example (Prosser, Zwack, and Johnson, not dated). The process of injection, by necessity, results in injury around the circumference of the tree in the form of drilled injection holes. Phytotoxicity induced necrosis of tissues around injection sites has been reported, however damage caused by propiconazole is relatively minor compared to that induced by alternative compounds such as thiabendazole (Haugen and Stennes, 1999).

At this time, the rate of fungicide degradation subsequent to injection in oaks has not been investigated. In elm, propiconazole used for control of Dutch elm disease could

not be detected in 4 of 6 trees treated at a rate of 10ml/DBH 7 months after injection, and the half-life of propiconazole at room temperature was estimated to be 67 and 101 days for the trans and cis isomers, respectively (Armstrong, 1999). Using a thin layer chromatography assay procedure, Osterbauer and French (1992) showed that propiconazole could be detected in the vascular tissue of northern red oaks up to 12 months after injection, but the fungicide could not be detected in samples taken 20 or 23 months after treatment. Currently, the protection provided by propiconazole is believed to last for no more than two years (Eggers et. al., 2005).

1.2.3. Efficacy of Propiconazole Treatments

Appel and Kurdyla (1992) reported the effective concentration of propiconazole to reduce the growth of *C. fagacearum in vitro* ranges from 2.0 ppb to 15.7 ppb depending on the fungal isolate used for analysis. Wilson and Forse (1997) later determined that the concentration of propiconazole necessary to kill 50% of the inoculum *in vitro* ranged between 10 and 100 ppb. Yet, the usefulness of this information is limited as it may not correspond to the concentrations required *in vivo* to achieve a desirable level of disease control. The authors suggested that the estimates are useful in efficacy testing for comparisons against actual concentrations of the fungicide measured in plant tissues via residue analyses following specific fungicide applications at different rates. To date, this has not been investigated. Wilson and Forse (1997) also stated that while it appears the effect of fungicide application, whether fungicidal (lethal to the fungus) or fungistatic (prevents fungal growth), may be concentration dependent, their results did not conclusively prove fungicidal activity because their methods did not allow direct determination of the fungicide concentration to which inocula were exposed after transfer and incubation.

The effectiveness of propiconazole for the control of oak wilt has been documented in several studies (Appel, 1990; 2001; Eggers et al., 2005; Johnson, 2001; Nair, 1995; Osterbauer and French, 1992; Osterbauer et al. 1994; Peacock and Fulbright, 2007). However, the widespread use of propiconazole for oak wilt control is primarily based on anecdotal evidence and the personal experience of arborists who utilize the fungicide in their operational treatments. In Texas, Appel (1990) reported that

propiconazole was a 97-100% effective preventative treatment for oak wilt in live oaks in the field, and it suppressed wilt development in 100% of containerized seedlings. In another study, Appel and Kurdyla (1992) reported that up to 41% crown loss in treated plots of live oak, whereas 60 to 100% crown loss was observed in untreated control plots. In preventatively treated northern red and northern pin oaks, the incidence of oak wilt was 4.5% in treated trees and 46.2% in control trees in the year of treatment, but that the number of trees wilting increased as time after injection increased (Osterbauer and French, 1992). Eggers et al. (2005) conducted an evaluation of oaks treated with propiconazole as part of operational treatments. In preventatively treated red oaks, the authors observed a 100% survival rate up to three years, and a 61% survival rate five years after treatment. In all studies, investigators concluded that disease protection provided by propiconazole decreases over time. There is currently a general consensus among arborists that re-treatment within two growing seasons of the first treatment is necessary to sustain disease suppression in high disease pressure situations.

1.2.4. Potential Reasons for Treatment Ineffectiveness

The long-term effectiveness of vascular injections of propiconazole into trees at risk of root graft infection has not been well established. There is currently no proven mechanism by which propiconazole would act to prevent root graft transmission of *C. fagacearum*, especially when one considers that all methods of application rely upon the transpiration stream for translocation of the fungicide. Significant translocation of propiconazole into the roots from the point of injection has not been demonstrated definitively. If propiconazole is not translocated into the root system following injection, then it is difficult to envision how root graft transmission could be prevented by such treatments.

In attempts to control the spread of oak wilt in Texas live oaks, Wilson and Lester (2002) concluded that injections of propiconazole were ineffective in preventing the transmission of *C. fagacearum* through root grafts. The authors suggested that inadequate translocation of the fungicide into the root system could permit continued transmission of the fungus through root grafts. Other studies also support the conclusion that injections do not prevent the spread of the pathogen from a propiconazole-treated

tree to an untreated tree through interconnected root systems (Appel, 1995). In early studies of the movement of injected dye in red oak roots, it was observed that distribution of vascular elements in roots is often discontinuous or interrupted, perhaps presenting an impediment to the movement of chemical solutions in roots (Yelenosky and Fergus, 1959). In general, triazole compounds such as propiconazole are thought to have little if any phloem mobility. Such a conclusion is supported by observations of the distribution patterns of numerous triazole growth regulators and fungicides.

While substantial acropetal transport of propiconazole in the upper portion of the tree seems to prevent oak wilt symptom development above ground, it does not appear to provide a barrier to movement of the pathogen in the roots. In trees within root grafting distance of actively wilting trees, this should be a primary concern, especially if measures to disrupt root grafts have not been taken. Even when root grafts are severed between potentially infected trees and healthy trees, there is currently no practical way of determining the extent of pathogen distribution into nearby trees. It is possible, if not likely, that *C. fagacearum* can move into and colonize the roots of trees treated with propiconazole, surviving in the host without causing crown symptoms. Furthermore, following the period of protection from propiconazole application, the pathogen may then be able to move upward in the tree resulting in the subsequent development of wilt and colonization of the tree by the pathogen. Not only would this constitute an ineffective preventative treatment, this type of masked infection may create a larger problem than if symptoms had not been suppressed. Pathogen-containing asymptomatic trees may act as avenues for further spread of the pathogen to additional trees not considered in management regimes, resulting in below-ground, undetected expansion of the infection center. It may be that asymptomatic trees near diseased trees are often treated with propiconazole with unrealistic expectations that the tree will survive, when in fact the pathogen may already be present in the roots and disease onset will only be delayed. Clearly the difference between the eradication of the pathogen and the prevention of symptoms should be considered when managing oak wilt with propiconazole.

There is growing evidence that the translocation of triazole compounds may not be as strictly confined to the xylem as once thought (Blaedow, 2003). Limited phloem mobility over short distances, or pressure driven movement of propiconazole into roots

during the injection process, could result in basipetal movement of propiconazole from the point of injection. Even limited translocation of propiconazole into roots could significantly influence the ability of *C. fagacearum* to induce disease and successfully colonize the tree. Significant concentrations of propiconazole in primary roots, root flares, and root crown could be lethal to pathogen propagules, preventing the invasion of the above ground portions of the tree that subsequently leads to symptom development. In addition, a chemical barrier could also prevent pathogen spread throughout the root system if root graft transmission occurs, thus confining the pathogen to the root(s) grafted to the diseased tree. Pathogen containment to one or a few roots could also prevent pathogen transmission to additional trees that may not have been considered in management regimes as described above. Given that direct pathogenic relationships with cells in the root system appear to be rare, and that vegetative growth of *C. fagacearum* in roots is sparse due to low nutrient availability in xylem fluids, the period of protection provided by propiconazole may outlast the pathogen's ability to survive in the root system. Yet, there is significant evidence to suggest that the pathogen can survive undetected in the roots of dead, wilting, and asymptomatic trees for many years (Skelly and Wood, 1974a, b; Yount, 1958). Use of propiconazole for prevention of root graft spread should therefore take into account the longevity of *C. fagacearum* survival in roots of treated trees. In addition, the degree of basipetal movement of propiconazole into roots must occur in concentrations that are lethal to the fungus and to a depth that prevents substantial colonization of the root system through networks of self-grafts.

Even if propiconazole is found to lack sufficient phloem mobility for downward movement, there may be other mechanisms by which this fungicide could move into the roots and reduce the transmission of *C. fagacearum* through root grafts. In a study of the movement of injected chemicals through the roots of oak, it was noted that although movement of poisons, dyes, and radioactive isotopes into roots was generally low, an exception was noted in roots grafted to other actively transpiring trees (Struckmeyer, Kuntz, and Riker, 1958). These trees may be equally capable of pulling chemicals, such as propiconazole, along the same path as viable endoconidia. Exploratory studies with the herbicide hydroxydimethylarsine (cacodylic acid) in the 1970's showed that sufficient quantities of this chemical could be forced into the root system of diseased oak trees and

through root grafts to prevent transmission of oak wilt. However, multiple applications were needed to eliminate the reservoir of inoculum formed before treatments were initiated (Rexrode, 1977). Even without physical movement of propiconazole into the root system of oak trees, plant growth regulating properties of propiconazole may be capable of altering host morphology in a way which imparts at least partial resistance to infection (Nair, 1995). Similar results have been obtained in studies using other growth regulating compounds (Geary and Kuntz, 1962; Nair, Wolter, and Kuntz, 1969). It is generally believed that the greater resistance of white oak to oak wilt is due to patterns of plant growth and development initiated in response to infection, responses which are nearly absent in red oak species, which result in compartmentalization of the pathogen (Schoeneweiss, 1959).

To date, it is not known if propiconazole is fungicidal or fungistatic *in vivo* (Wilson and Forse, 1997). If strictly fungistatic, then trees already infected (symptomatic or asymptomatic) may be treated with unrealistic expectations for survival. The success of therapeutic treatments in resistant white oak species likely results from their ability to successfully compartmentalize the fungus, particularly if growth of the pathogen is temporarily arrested due to the presence of propiconazole. Therapeutic treatments in highly susceptible red oak species may not be as successful because those species do not have the capacity to compartmentalize the fungus; or at least not to a degree which prevents reinvasion of uninfected tissue when the fungistatic effects of propiconazole deteriorate over time. If propiconazole is fungicidal *in vivo*, eradication of the pathogen is much more likely; particularly when disease symptoms have not yet developed. But limited symplastic movement of triazoles have been observed (Kuck and Scheinpflug, 1986), and therefore complete eradication of the pathogen after the invasion of parenchyma cells has begun may not be possible.

1.3. Biological Activities Associated with Propiconazole

1.3.1. Fungicidal Properties and Effects on Oak Wilt

The triazole derivatives, including propiconazole, are demethylation inhibitors (DMIs) which interfere with ergosterol biosynthesis by inhibiting the oxidative removal

of the 14α-methyl group from 24-methylenedihydrolanosterol in fungi (Sisler and Ragsdale, 1984), obtusifoliol in plants (Siegel, 1981; Van Cutsem et al., 1986) and other sterols methylated at C-14. The demethylation reaction is catalyzed by a cytochrome P450 dependent monooxygenase (Benveniste, 1986; Mercer, 1984), the inhibition of which causes accumulation of several sterol intermediates containing a methyl group at the C-14 position (Buchenauer, 1987; Ragsdale, 1975; Siegel, 1981; Weete, 1980).

Sterols are required to have a precise structural configuration to be effective in the plasma membrane, and it has been shown that intermediates methylated at C-14 do not fulfill this requirement. The additional methyl group or groups in 14-methyl and 14-dimethyl sterols respectively, protrude from the α-face of the sterol ring structure and prevent their proper placement in the plasma membrane (Bloch, 1983; Buchenauer, 1987; Weete, 1980). The lack of membrane sterols leads to phospholipid instability, resulting in membrane disorder and permeability. This has important physiological effects on the fungus, such as the loss of ability to obtain essential amino acids from the environment and cell death (Baldwin and Wiggins, 1984). However, the lack of structural sterols may be only one of a number of causes for the fungitoxicity of DMIs. There is evidence that triazole fungicide molecules may themselves be incorporated into the plasma membrane leading to further instability (Langcake et al., 1984). Triazoles may also effect membrane phospholipid desaturases (Buchenauer, 1987), inhibit the membrane enzyme responsible for chitin synthesis (Vanden Bossche et al., 1984), induce an irregular buildup of free fatty acids (Buchenauer, 1987), and disrupt the function of sterols as important signaling molecules leading to a wide variety of other adverse affects (Haughan et al., 1988).

1.3.2. Plant Growth Regulating Properties and Effects on Oak Wilt

In addition to interfering with sterol biosynthesis, triazoles interfere with the sysnthesis of other terpenoids such as gibberellins in a similar manner: by inhibiting cytochrome P450 dependent monooxygenases responsible for the hydroxylation reactions that transform isoprene-based precursors into biologically active molecules. Cytochrome P450 enzymes contain a ferric porphyrin prosthetic group that bind molecular oxygen (O_2). When oxygen binds to the porphyrin system, the complex is ‘activated’ and able to

accept an additional electron. During a hydroxylation reaction, the activated oxygen is transferred to various substrates (Koller, 1987a, b). In the sterol biosynthesis pathway of fungi, this substrate could be one of a variety of intermediates methylated at the C-14 position, such as 24-methylenedihydrolanosterol or lanosterol. Hydroxylation results in the removal of the methyl group. In gibberellin biosynthesis, the substrates include *ent*-kaurene, *ent*-kaurenol, and *ent*-kaurenal. The lone electron pair on the sp^2-hybridized nitrogen of triazoles (Koller, 1987b) allows them to bind to the ferric porphyrin prosthetic group of the cytochrome P450 monooxygenases, preventing the formation of the activated oxygen complex (Coolbaugh et al., 1978; Baldwin and Wiggins, 1984). Therefore, hydroxylation reactions of sterol and gibberellin biosynthetic pathways are inhibited, giving the compounds their respective fungicidal or plant growth regulating properties.

Since the introduction of the first triazole fungicide, triadimefon, in 1973 (Schwinn, 1983), a series of triazole compounds have been developed for use as either fungicides or as plant growth regulators (PGRs). Regardless of whether the compound is classified as a PGR or a fungicide, most triazole compounds exhibit both fungicidal and plant growth-regulating properties to some extent (Fletcher et al., 1986). During the development of the first triazole fungicides, it was observed that several compounds inhibited *ent*-kaurene oxidase in the gibberellin biosynthesis pathway (Hedden and Graebe, 1985; Rademacher et al., 1987; Sisler et al., 1984). After the fungicides triadimefon, and its successor triadimenol, were released in the 1970's, Buchenauer and Rohner (1981) observed that both chemicals inhibited plant growth. Most SBI fungicides induce some level of growth retardation (Kuck and Scheinpflug, 1986), and this side effect was in turn optimized to produce today's PGRs (Koller, 1987a, b). The opposite is also true. Fletcher et al. (1986) observed that paclobutrazol, a PGR widely used today, was more fungitoxic than the fungicide triadimefon. Other PGRs have also been shown to inhibit sterol biosynthesis at the 14α-demethylation step in fungi (Baldwin and Wiggins, 1984; Izumi et al., 1985; Takano et al., 1986; Wiggins and Baldwin, 1984) as well as in plants (Burden et al., 1987; Haughan et al., 1988; Koller, 1987a, b; Taton et al., 1988).

Compounds containing the 1,2,4-triazole ring system are effective fungicides because they inhibit ergosterol biosynthesis in basidiomycetes and ascomycetes (Buchenauer, 1979). Likewise, the many commercially successful fungicides have a triazole ring system incorporated into their chemical structure that allows them to inhibit the production of biologically active gibberellins. Furthermore, triazole compounds acting as fungicides or as PGRs possess a free electron pair on the sp^2-hybridized-nitrogen to inhibit cytochrome P450-dependent reactions (Koller, 1987a, b). Finally, both sterol and gibberellin biosynthesis pathways rely on cytochrome P450-dependent enzymes to catalyze essential transformations. Therefore, regardless of their designated use in agriculture, triazole compounds may possess both plant growth regulating and fungicidal properties because of their ability to inhibit cytochrome P450 dependent hydroxylations.

1.4. Objectives

1.4.1. Implications of the Distribution of Propiconazole in Red Oaks on the Management of Below Ground Spread of *Ceratocystis fagacearum*

To determine if propiconazole has the ability to protect red oaks from the spread of *C. fagacearum* through root grafts, I will characterize the distribution of propiconazole in the roots of injected red oaks over a two year period, and determine if *C. fagacearum* is capable of surviving in trees treated with propiconazole. Currently, it is not known if propiconazole is translocated into the roots of treated trees, nor through what mechanism propiconazole is capable of preventing root graft transmission of the pathogen if the fungicide is not present in biologically significant concentrations throughout the root system. It is possible that preventative propiconazole treatments do not prevent the transmission of *C. fagacearum* across root grafts, but do prevent the subsequent development of oak wilt. By examining the interaction between the host, pathogen, and fungicide, I will attempt to characterize the mechanism by which propiconazole induced suppression of oak wilt occurs, and the reliability of prophylactic propiconazole treatments.

1.4.2. Spatial and Temporal Distribution of *Ceratocystis fagacearum* and the Characterization of Root Grafting in Red Oak

The spatial and temporal distribution of *C. fagacearum* in the roots of red oaks in or near disease centers is poorly understood in spite of several attempts to describe the pattern of pathogen spread from diseased to neighboring healthy trees. Successful management of root graft transmission of *C. fagacearum* is dependent upon complete containment of the pathogen within root graft barriers and disease control with systemic fungicide treatments. Failure of these control measures may be due to placement of physical or chemical barriers in a way that does not completely contain the pathogen. While there has been some advancement in effective placement of root graft barriers so as to protect healthy trees but contain the pathogen in asymptomatic trees that may be infected; chemical treatments are often applied to trees with unrealistic expectations for survival. Treatment failure is unacceptable because of the high costs associated with fungicide injections, and more importantly, because a latent or masked infection represents an inoculum source that is not accounted for in management regimes. I will characterize the spatial and temporal distribution of *C. fagacearum* in the root systems of wilting and neighboring asymptomatic trees, and investigate the occurrence of root grafting and its role in *C. fagacearum* spread through red oaks on a sandy soil.

1.4.3. Investigation of Plant Growth Regulating Properties Associated with Propiconazole that may affect the Development of Oak Wilt

The triazole fungicide propiconazole injected into susceptible oak trees is being used as a means of preventing the spread of the oak wilt fungus. Although propiconazole is highly fungicidal to the oak wilt fungus *C. fagacearum in vitro*, it has been suggested that the compound may only be fungistatic to the pathogen *in vivo*. Furthermore, the ability of *C. fagacearum* to survive in propiconazole treated trees has serious consequences for the effectiveness of such a control measure. It does appear however, that propiconazole injections are effective, at least temporarily, in preventing the development of the disease. Plant growth regulating effects of propiconazole on oak anatomy and physiology may also play a role in arresting disease development. I will attempt to characterize the effects of propiconazole on anatomical features of northern

pin oak that when altered, may affect the ability of *C. fagacearum* to infect, colonize and incite disease in its host; and describe how the observed changes in northern pin oak anatomy following propiconazole application could potentially affect the host-parasite interaction and disease development.

1.5. Structure of Dissertation

In this dissertation are the results of three field and/or laboratory investigations conducted to examine use of propiconazole to control root graft transmission of the oak wilt pathogen *C. fagacearum* in red oak. These investigations were conducted in an attempt to better understand the mechanism by which the observed effectiveness of oak wilt suppression through intravascular injection of propiconazole occurs, and how the efficacy of those treatments may be enhanced.

In chapter 2 are the results of a two year investigation at the Blaine Airport (Anoka County, Minnesota) in which the distribution of propiconazole in the root system of treated red oaks was characterized using microwave assisted extraction and a modified gas chromatography – mass spectrometry based assay. In addition, inoculation of tree roots with the pathogen prior or following propiconazole injection allowed determination of the ability of *C. fagacearum* to survive in treated trees.

In chapter 3 are the results of an attempt to characterize the spatial and temporal distribution of *C. fagacearum* in root systems of diseased trees and the pattern of root graft transmission to neighboring trees. During this study, the incidence of root grafts (both self-grafts and inter-tree grafts) was examined.

In chapter 4 are the results of a preliminary investigation to determine if the predicted growth regulating properties of propiconazole affect growth and development of treated red oaks, and if so, what changes influence the host-parasite interaction. In this study, several anatomical properties of wood samples taken from trees treated with propiconazole in 2002, 2003, and 2004 were examined.

In discussing the results of these experiments, an attempt will be made in each chapter to characterize the host-pathogen-fungicide interaction that occurs in propiconazole treated trees. Overall conclusions and recommendations for the use of propiconazole in oak wilt management are provided in Chapter 5.

1.6. Figures

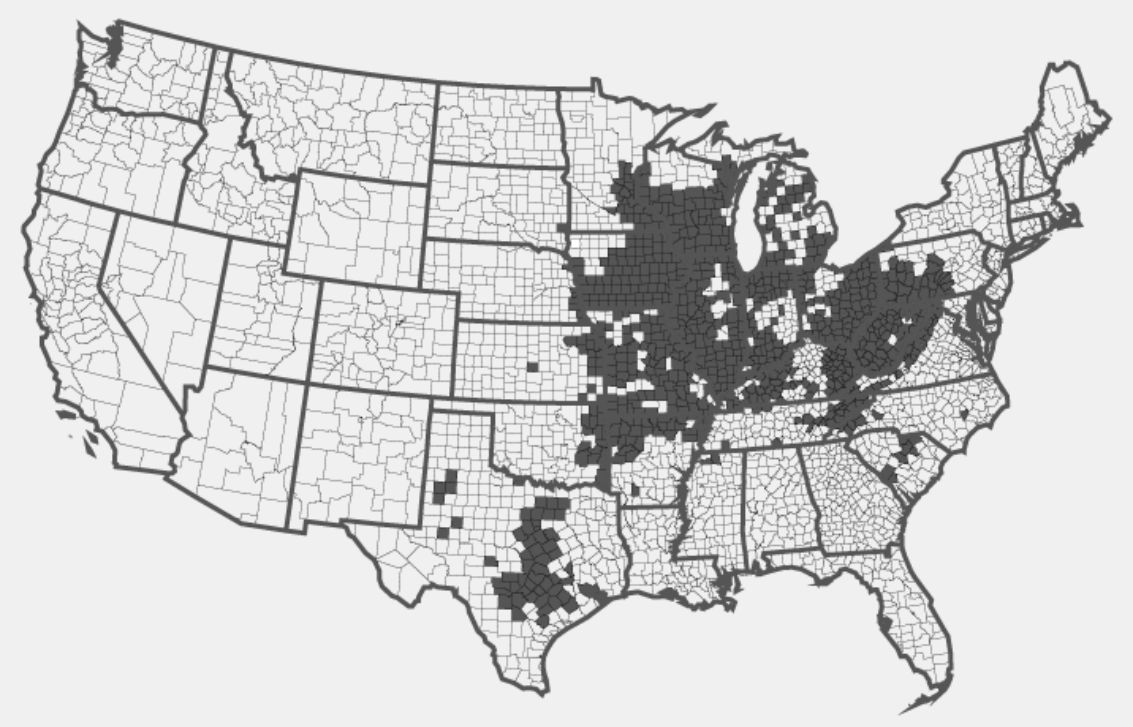

Figure 1.1. Distribution of oak wilt in the United States by county in 2005. Map available online: http://www.na.fs.fed.us/fhp/ow/maps/ow_distribution05.gif.

CHAPTER 2

Implications of the Distribution of Propiconazole in Red Oaks on the Management of Below Ground Spread of *Ceratocystis fagacearum*

2.0. Introduction

Oak wilt, caused by the vascular pathogen *Ceratocystis fagacearum*, is considered to be the most important disease of oaks (*Quercus spp.*) in the eastern United States. Its ability to kill red oaks (Section *Lobatae*) within a few weeks after the onset of incipient wilt makes *C. fagacearum* one of the most destructive forest pathogens known (Gibbs and French, 1980; Young, 1949). All known species of oak are susceptible to *C. fagacearum*, however, white oaks (Section *Quercus*) exhibit varying degrees of resistance and may survive for many years or fully recover from infection by compartmentalization of the fungus. The disease is particularly devastating for highly susceptible red oak species including northern red oak (*Quercus rubra*), black oak (*Quercus velutina*) and northern pin oak (*Quercus ellipsoidalis*) in the Lake States; and for live oaks (*Quercus fusiformis* and *Quercus virginiana*) which are a critical constituent of the oak savannah ecosystem in central Texas.

Overland transmission of the oak wilt pathogen via insect vectors such as oak bark beetles (*Pseudopityophthorus spp.*) and sap feeding beetles of the *Nitidulidae* family results in long distance spread of *C. fagacearum* across the landscape. More commonly, however, *C. fagacearum* spreads through root grafts between proximal oaks resulting in the formation of expanding areas of wilting and dead trees known as disease centers. Oak wilt management practices include strategies to reduce inoculum and the number of infection courts available for overland transmission. Infection via root graft transmission poses a more immediate threat to trees within root grafting distance of diseased trees. Below-ground spread of the pathogen is managed through mechanical root graft disruption (e.g. using a vibratory plow) or by systemic fungicide protection in nearby healthy oaks.

Propiconazole (1-[(2-(2,4-dichlorophenyl)-4-propyl-1,3-dioxolan-2-2-yl) methyl}-1*H*-1,2,4-triazole), a systemic fungicide applied via intra-vascular injection at the root flare or lower stem, was first used in the 1980's to prevent losses of live oaks at risk of infection via root graft transmission of *C. fagacearum* (Appel, 1990). The use of propiconazole to protect high-value trees and at sites where mechanical root graft disruption is not possible has since become a widespread and accepted practice for highly susceptible oak species. There is documented observational evidence to suggest that a single treatment with propiconazole is highly effective in preventing oak wilt, but for no more than two (Appel and Kurdyla, 1992; Eggers et al., 2005; Osterbauer and French, 1992; Wilson and Lester, 1995) or three (Peacock and Fulbright, 2007) years. Thus, some arborists recommend retreatment of high-value red oaks at 12 to 24 month intervals.

The underlying cause of increasing treatment failures in oaks two years after injection is putatively linked to degradation of propiconazole over time. Osterbauer and French (1992) were able to detect propiconazole 1.4 m above the point of injection using a thin-layer chromatography assay in treated red oaks 12 months, but not 20 months after injection. Armstrong (1999) detected the fungicide in the crown of American elms (*Ulmus americana*), treated with propiconazole for Dutch elm disease control, 7 months after treatment, but not after 12 months using gas chromatography-mass spectrometry. In the same study, Armstrong determined that propiconazole degradation, primarily the result of hydrolysis and enzymatic degradation, is temperature dependent and that the half-life of propiconazole at 25ºC was between 67 - 101 days.

The absence of detectable levels of propiconazole in the stem or branches after one year suggests that trees are vulnerable to infection after that time. *C. fagacearum* is capable of surviving in roots of wilted oaks for 5 years or more (Skelly and Wood, 1974; Yount, 1958), highlighting the importance of root graft barrier installation or retreatment with systemic fungicides at regular intervals until disease pressure has subsided.

Early observations of treatment efficacy led Appel (1991) to suggest that propiconazole injections do not prevent the spread of *C. fagacearum* through the root system or across root grafts. While substantial acropetal translocation of propiconazole from the point of injection into the stem and crown of the tree is believed to prevent

disease expression above ground, this does not appear to provide a barrier to the spread of *C. fagacearum* in roots. Propiconazole is a demethylation-inhibiting fungicide that requires direct contact with the pathogen for fungicidal action. Few studies have been conducted which would suggest that propiconazole is capable of symplastic translocation and movement through the phloem into the roots, but triazole compounds in general have little if any phloem mobility (Nair, 1995). Currently, propiconazole has not been shown to move into the root system of red oaks following intra-vascular injection; however the fungicide has been detected in the primary roots of live oak (Appel, pers. comm.) and peach (*Prunus* sp.) (Amiri et al., 2008) less than 0.5 m from the point of injection.

Intra-vascular treatments with propiconazole may be incapable of completely eradicating *C. fagacearum* from oaks once infected. Latent infections of the root system lasting from several months to a year or more are known to occur, and as discussed above, there is currently little evidence to support basipetal translocation of propiconazole into the root system necessary for pathogen eradication. Spread of *C. fagacearum* from the root system into the crown of the tree is followed by colonization of xylem parenchyma and ray cells after the onset of wilt symptoms (Struckmeyer, Kuntz, and Riker, 1958); therefore a systemic fungicide with significant mobility in the symplast would be better suited for pathogen eradication once living cells have been invaded. When tested against several Texas strains of *C. fagacearum*, propiconazole was fungistatic at 10-100 ppb and fungicidal at 400-600 ppb *in vitro* (Wilson and Forse, 1997). Currently, there is no evidence to suggest that propiconazole is translocated in quantities that would eradicate the fungus from roots and other colonized tissues in an infected tree.

Should oaks become infected prior to or after treatment with propiconazole, survival of *C. fagacearum* in treated trees could subsequently permit disease development after the fungicide degrades over time. Therefore, it is possible that observed treatment failures after 24 months are a result of propiconazole's inability to prevent pathogen transmission across root grafts and/or to completely eradicate the fungus from infected trees. A more complete understanding of interactions between host, pathogen, and fungicide is necessary to characterize the effect of and most efficacious use for propiconazole applications in oak wilt management regimes. The objectives of this study

were to determine if propiconazole movement into primary roots occurs following macroinjection at the root flare; and to determine if *C. fagacearum* can infect and/or survive in the root system of treated trees. Characterization of the host-pathogen-fungicide interaction over time could improve our understanding of observed prophylactic effects of propiconazole application and the reasons for treatment failure after two years.

2.2. Materials and Methods

2.2.1. Experimental Design

Four spatially separated experimental plots, consisting of six randomly selected mature northern red oaks, were established in a fragmented red oak forest in Anoka County, Minnesota. Oak wilt was not observed in any of the selected forest fragments. Each of the six trees within a plot received the same treatment combination of fungicide and/or pathogen, to prevent possible transmission of the pathogen or translocation of fungicide across root grafts from trees assigned to different treatment combinations. Treatment combinations include:

1) Prophylactic treatment: a single macro-infusion application of propiconazole applied two weeks prior to root inoculation with *C. fagacearum*
2) Therapeutic treatment: a single macro-infusion application of propiconazole applied two weeks after root inoculation with *C. fagacearum*
3) Fungicide control: a single macro-infusion application of propiconazole, simulated root inoculation with sterilized distilled water
4) Disease control: single macro-infusion application of water, root inoculation with *C. fagacearum*

Propiconazole (Syngenta Crop Protection Inc., Greensboro, NC), formulated as the suspensconcentrate Alamo® (14.3% active ingredient), was applied to northern red oaks in July 2004 using a standard macro-infusion technique consisting of 20.0 ml of the fungicide suspended in 1.0 L of water per 2.5 cm dbh (diameter of stem 1.4 m above the root collar), applied under pressure (138 – 276 kPa). Injection tees, connected to a pressurized chemical tank via a network of plastic tubing, were placed into the root flares just below the root collar. Tees were inserted into the outermost ring of xylem through a

3/8 inch hole created with a high-helix drill bit. Tees were spaced so as to utilize approximately 1 tee per 1.7 cm dbh around the circumference of the tree.

Trees were inoculated with *C. fagacearum* through three primary roots per tree, 1.0 m from the root collar to simulate root graft transmission of the pathogen. An air excavation tool (Airspade®, Guardair Corp., Chicopee, MA) was used to displace mineral soil and expose roots to be inoculated. Each of the three inoculations were performed by drilling a ¼" diameter hole with a high-helix drill bit 2.0 cm deep into the root, then applying 1.0 ml of a conidial suspension (10^6 conidia ml^{-1} in sterilized distilled water) through a 5.0 ml pipette tip held in place in the hole using moldable epoxy putty. Following complete uptake of the conidial suspension, the holes were sealed with putty, wrapped with laboratory film, and exposed roots were covered with sand to the original soil grade.

Following propiconazole application (2, 12, and 24 months), the root systems of two trees from each treatment/plot were re-excavated. For each of the three inoculated primary roots from each tree, the segment of root between the propiconazole injection site and inoculation site was removed and placed in cold storage (4ºC). Each collected root was sub-sampled (8.0 cm long root segments) at 33 cm intervals. In addition, three wood samples were taken from the stem of each tree 33 cm above the injection point, from the same vascular columns as each sampled root. Root sub-samples and stem wedges were split radially; one-half of the subsample was assayed for the presence of *C. fagacearum* and the other assayed for propiconazole.

2.2.2. Pathogen Assay

Root and stem samples were assayed for *C. fagacearum* using a standard isolation protocol (Pokorny, 1999). Following a period of 2-3 weeks of refrigerated storage that allowed for extensive pathogen colonization of root segments, samples were surface sterilized with 95% ethanol and briefly flamed. Twelve small wood chips (~ 0.5 cm long) were removed from outer rings of xylem along the length of the root segment with a sterilized wood gouge. Wood chips were plated onto 10% lactic acid-amended potato dextrose agar (Difeo Laboratories, Becton, Dickinson, and Co., Sparks, MD) and incubated in the dark at 25ºC for 30 days. Presence of *C. fagacearum* was monitored

weekly on plates; a second attempt to isolate the pathogen was made from any root or stem samples which did not yield the pathogen from the first attempt.

2.2.3. Fungicide Assay

The concentration of propiconazole in root and stem samples was determined using a microwave-assisted extraction procedure and gas chromatography – mass spectrometry (GCMS). The microwave extraction procedure was adapted from a protocol developed at Virginia Polytechnic Institute (Armstrong, 1999). The concentration in each root or stem sample was individually determined twice to demonstrate repeatability. Samples were rinsed to remove mineral soil, chopped into small cubes (approximately 1.0 cm^3), frozen, coarsely ground using a Model 4 Wiley® Mill (Thomas Scientific, Swedesboro, New Jersey) to a size of less than 2.0 mm, and then dried at room temperature. For extraction, 5.0 g dried wood were placed in a 85 mm polystyrene Petri dish, saturated with 30.0 ml of methanol (99.9% purity), and covered. Extractions were conducted in a Pelco 3441 Laboratory Microwave System (Ted Pella Inc., Redding, California) fitted with a Pelco 3430 Microwave Power Controller, a Pelco ColdSpot water-cooled stage cooled by an external chiller unit (Forma Scientific Bath and Circulator Model 2945, Forma Scientific Inc., Marietta, Ohio), and a thermocouple temperature probe. Up to nine samples could be processed per extraction; operating conditions were 100 W for 15 minutes with the water-cooled stage set to 14ºC. The water-cooled stage was used to keep sample temperature below the boiling point of methanol, and to provide a water load for microwave absorption which maintains an evenly distributed microwave flux up to 8.0 cm above the stage area for uniform processing.

Following extraction, samples were filtered through a fiberglass filter in a polypropylene funnel, and the solvent collected in 15 mm borosilicate glass vials. The filtrate was evaporated to dryness using nitrogen blowdown, re-diluted in a 5:1:1 hexane/acetone/methanol mixture, and the supernatant removed and evaporated again using nitrogen blowdown. Samples were re-suspended in 1.5 ml of methanol, centrifuged at high speed for 10 minutes, and 1.0 ml of the supernatant was transferred to gas chromatography vials for analysis.

Analysis was performed with a HP6890N Gas Chromatography System with MSD (mass selective detector) (Agilent Technologies, Wilmington, DE) operating in SIM (select ion monitoring) mode. Operating conditions were as follows: the initial oven temp was 180°C with a temperature ramp of 15° C/min to 300°C and held for 10 min. A 2.0 µL injection in splitless mode with an approximate flow rate of 54 ml/min onto a HP-5MS 5% Phenyl Methyl Siloxane 30 m capillary column (Agilent Technologies, Wilmington, DE) was used for chemical separation. Ions monitored in SIM were 173, 175, 191, 259 m/z.

Peak area values of propiconazole concentrations were obtained from the chromatogram for each sample. Propiconazole concentrations (µg/ml) in analyzed extracts was calculated by the external calibration method. Calibration curves were constructed by triplicate analysis of at least three standards made by serial dilution of a stock solution of propiconazole (Tilt® 97.2% purity, Chemical Services Inc., West Chester, Pennsylvania) dissolved in methanol (99.9% purity); correlation coefficients for calibration curves were $r > 0.99$. Calibration curves were constructed for every 10 to 15 tissue samples analyzed.

Propiconazole concentration (µg/g) in root and stem samples were then calculated by dividing propiconazole concentration in analyzed extracts by the average percent recovery, which was calculated from analysis of wood samples spiked with propiconazole. Spiked samples consisted of five grams of milled and dried root tissue from non-treated northern red oak. Samples were spiked with 1.0 ml aliquots of 150, 100, 50, 25, 10, 5, and 1 ppm dilutions of propiconazole prepared from a stock solution of analytical grade propiconazole (Tilt® 97.2% purity, Chemical Service Inc., West Chester, Pennsylvania) dissolved in methanol (99.9% purity). For each concentration, three samples were spiked and the test replicated six times. Standard solutions were added slowly to cover the surface of the wood matrix. The solvent was allowed to evaporate, and the spiked wood matrix was allowed to equilibrate for 36 hours in darkness. Spiked samples were analyzed by GCMS following microwave extraction as described above, and percent recovery calculated.

2.2.4. Data Analysis

Data from assays for propiconazole and *C. fagacearum* were analyzed in PROC MIXED of SAS Version 9.1.3 (SAS Institute, Cary, North Carolina) to accommodate covariance parameters. Incidence of *C. fagacearum* was calculated from isolation (binary) data for each tree at each distance from the injection point (percent of samples yielding the fungus), and pathogen incidences were arcsine transformed for the analysis. The concentration of propiconazole in each wood sample was calculated as the mean of each of the two individual determinations because consistent repeatability of the propiconazole assay was confirmed. Propiconazole concentration data were normally distributed, and concentrations (ppm) were not transformed for analysis. Distance from the injection point and time since injection were considered fixed effects, while tree and root effects were considered random. The dependency of measures at all four distances within a root was recognized through the REPEATED statement, and covariance among distance measures was identified as Toeplitz. When interactions between main effects were significant, the SLICE option of PROC MIXED was used to further examine individual effects. Significant differences among means were determined by pairwise comparisons of least-squares means using the LS MEANS statement with the DIFF option (α=0.05).

Two roots, one from Treatment 1 excavated 12 months after treatment and one from Treatment 2 excavated 2 months after treatment, were dead and severely decayed at the time of sampling. Assays for propiconazole and *C. fagacearum* were not successful in these roots, and the samples were therefore removed from statistical analyses.

2.3 Results

2.3.1. Propiconazole Distribution in Treated Trees

Propiconazole was detected at all sampled locations in the primary roots and lower stems of trees treated prophylactically (treatment 1), therapeutically (treatment 2), or as part of the fungicide control treatment (treatment 3) 2, 12, and 24 months after injection. Fungicide distribution was similar for all treatments, i.e. inoculation with *C. fagacearum* did not affect propiconazole uptake and distribution in trees after injection (P=0.7729). Although the concentration of propiconazole in the injected solution

(approximately 3050 ppm) was the same for all treated trees, when the concentration of the fungicide in wood samples taken at the same time and distance from the injection point where compared, a high degree of variability in propiconazole concentration was observed among trees and even among roots of the same tree (Table 2.1). The highest concentration of propiconazole detected, 815.02 ppm, was in a wood sample taken from the stem (0.33 m above the injection point) two months after treatment. The concentration of propiconazole in an adjacent stem sample from the same tree was only 458.46 ppm In only five root samples was propiconazole not detected via the GCMS assay, all of which were sampled 24 months after treatment. Tree diameter and the corresponding number of injection tees did not affect the concentration of propiconazole in individual samples (P=0.4542).

The concentration of propiconazole decreased with increasing distance from the injection point ($P < 0.0001$) (Figure 2.1), and was consistently higher in stem samples than in the adjacent root samples 0.33 m below the injection point. Two months after treatment, the average concentration of propiconazole was 64% lower in roots 0.33 m from the injection point than in the stem at the same distance, and 91% lower 1.0 m below the injection point in the roots. Similar patterns of distribution were observed during other sampling periods. On average, propiconazole concentration decreased by 54% for each successive root sample, beginning in the stem 0.33 m above the injection point, and proceeding basipetally into the roots 1.0 m from the injection point. Significant differences in concentration with increasing distance from the injection point were observed during all sampling periods at all distances sampled.

Although propiconazole concentration could not be determined in the same individual roots during each of the three sampling periods because of destructive sampling techniques, the average concentration of the fungicide did decrease significantly over time in treated trees ($P<0.0001$) (Figure 2.2). Between 2 and 24 months post-treatment, the average concentration of propiconazole in samples decreased by 72%. The decrease was greatest in stem samples (79%) and in the roots 1.0 m from the injection point (81%) over the 22 month period. However, significant decreases in propiconazole concentration over time were only observed in those samples adjacent to the point of injection (0.33 m above and below the injection point) over all three

sampling periods. In samples taken 0.66 m and 1.0 m below the injection point, no significant decreases occurred after 12 months. Propiconazole was detected in 92% (n=72) of wood samples after 24 months. Of the five samples that did not contain propiconazole, four came from the same tree, and three of those four from the same root (i.e. propiconazole was not found in that root below the injection point).

2.3.2. Pathogen Distribution in Disease Control Trees

Oak wilt symptoms were first observed in disease control trees (treatment 4) approximately four weeks after inoculation. Symptoms appeared in all six disease control trees within a six day period, and all trees were completely wilted within eight weeks of inoculation (four weeks after incipient wilt development) with *C. fagacearum*. The incidence of the pathogen decreased with time elapsed since inoculation (P= 0.0072) (Figure 2.3). Two months after inoculation, *C. fagacearum* was isolated from 96% (n=24) of samples from disease control trees. At this time, we were unable to isolate the pathogen from only one root or stem sample (4.1%, n=24), which was taken 0.33 m below the injection point. Twelve months after injection, the pathogen was isolated from 100% (n=18) of root samples but only 50% (n=6) of stem samples because the pathogen was no longer found in the stem of one of the two trees sampled. After 24 months, the pathogen was isolated from only 17% (n=24) of samples, and was found only in the roots. It was observed that the outermost xylem increment of trees sampled 24 months after inoculation (approximately 20 months after tree death) was too dry to support *C. fagacearum*. The highest pathogen incidence observed 24 months after inoculation was 0.33 m below the injection point (50%, n=6), followed by 0.66 m below (17%, n=6). At all sampled distances, pathogen incidence decreased by an average of 82% during the two year study.

2.3.3. Pathogen Distribution in Treated Trees

Pathogen incidence in propiconazole treated trees (treatments 1 and 2 only) was correlated with distance from the injection point ($P < 0.0001$) (Figure 2.6), and the spatial and temporal distribution of the pathogen was different than that observed in disease control trees ($P<0.0001$) (Figure 2.3). At 2 and 12 months after injection, *C. fagacearum*

was found only in the roots of propiconazole treated trees 0.66 m and 1.0 m below the injection point. During the first 12 months of the study *C. fagacearum* was not successfully isolated from root and stem samples adjacent to the injection point. However, 24 months post-injection, the pattern of pathogen distribution in propiconazole treated trees changed. While the pathogen was again not isolated from roots 0.33 m below the injection point, pathogen incidence in stem samples increased to 50% (n=12) and pathogen incidence at or below 0.66 m decreased to 17% (n=24). Pathogen incidence in stem samples was significantly greater after 24 months compared to stem samples taken 2 and 12 months after treatment. Conversely, pathogen incidence decreased in root samples 0.66 m and 1.0 m below the injection point between 12 and 24 months post treatment, though the difference at 0.66 m was not statistically significant. *C. fagacearum* was never isolated from root samples 0.33 m below the injection point in propiconazole treated trees during this study.

Although average pathogen incidence was significantly lower in samples from propiconazole-treated trees than from disease control trees 2 and 12 months after treatment ($P < 0.0001$), at no sampling period was a treated tree (therapeutically or preventatively treated with propiconazole) found to be completely free of the pathogen. After 24 months, there was no significant difference in average pathogen incidence among disease control trees and treated trees from either treatment ($P = .6623$) (Figure 2.4). At no time after treatment or distance from the injection point did the incidence of *C. fagacearum* differ between therapeutically and prophylactically treated trees (Figure 2.4 and 2.5). Thus data were combined for analysis as shown in Figure 2.6. Over all three sampling periods, 53% of inoculated roots yielded the pathogen from at least one of the three sampled distances below the injection point.

Because the pathogen was isolated so infrequently in treated trees, particularly in samples adjacent to the injection point where fungicide concentrations were highest, there was not enough data to make reliable conclusions as to how fungicide concentration affected the presence of *C. fagacearum* at specific distances from the injection point at certain time intervals post-treatment. Pathogen incidence in wood samples from trees treated with propiconazole and inoculated with *C. fagacearum* was much lower in samples taken 2 and 12 months post-treatment application ($P = 0.0016$ and 0.009,

respectively) (Figure 2.7). Pathogen incidence was not correlated with fungicide concentration at 24 months (P = 0.3005) (Figure 2.8). *C. fagacearum* was not isolated from wood samples with concentrations of propiconazole higher than 36 ppm within 12 months of treatment, but the pathogen was isolated from samples with propiconazole concentrations as high as 254 ppm 24 months after treatment. The average concentration of propiconazole in samples yielding the pathogen was 5.25 ppm 2 and 12 months after treatment, and 64.51 ppm after 24 months. *C. fagacearum* was never isolated from propiconazole control trees (Treatment 3) during this study.

Wilt symptoms were observed in five of six therapeutically treated trees eight weeks after inoculation, just prior to the initial set of root and stem sample collections. Wilt symptoms affected 25-50% of the crown of affected trees just prior to the onset of fall coloration, which made subsequent observations of symptom development difficult that year. Non-treated / non-inoculated trees in the same forest stand did not exhibit similar wilt symptoms. Presence of *C. fagacearum* in the crowns of symptomatic trees was not confirmed by pathogen isolation at this time. However, both of the therapeutically treated trees sampled two months post-injection displayed these symptoms, and the pathogen was isolated from the root system of each of these trees. All symptomatic, therapeutically-treated trees leafed out normally the following spring (no crown dieback), and wilt symptoms never reappeared during our 36 month observation period. In June 2007, 35 months after treatment, two prophylactically treated trees developed oak wilt, and were completely wilted by July 2007. One of these trees was sampled 12 months after propiconazole treatment, the other 24 months after treatment. In the latter tree, *C. fagacearum* was detected in all 3 stem samples taken 0.33 m above the root collar. The pathogen was also detected in the former, but only in root samples 0.66 and 1.0 m below the root collar. Therefore, propiconazole prevented wilt in 100% (n=12) of treated trees for the first 24 months after treatment regardless of application timing in relation in to inoculation and the incidence of *C. fagacearum* in root or stem tissue, but treatment failures did begin to occur approximately 36 months after treatment.

2.4. Discussion

2.4.1. Propiconazole distribution in treated trees

Significant movement of propiconazole into primary roots following macro-infusion treatment was confirmed using gas chromatography – mass spectrometry. This is the first confirmed report of propiconazole movement into the root systems of northern red oaks, a species highly susceptible to *C. fagacearum* and commonly treated to prevent root graft-transmission in active disease centers. The presence of propiconazole in the root system was not expected because it had previously been thought that translocation in the phloem would be required for downward movement into the root system, and propiconazole is not thought to be a phloem-mobile systemic fungicide. We did not assay specific tissues for propiconazole; the concentration of the fungicide was determined for the entire cross section of the sample being analyzed. Therefore, it was not determined if propiconazole was translocated in the phloem, or if another mechanism was responsible for movement into the root system. Visual inspections of sample cross sections revealed blue streaking in the xylem of both stem and root samples, indicating that at least a portion of the injected solution (which is blue in color) was mobilized in the xylem. It is likely that the injected solution was translocated both acropetally into the stem and crown, and forced downward into the root system by the pressure generated from the injection system.

The concentration of propiconazole decreased with increasing distance from the injection point; however, propiconazole was detected at the most distal point sampled in the root systems of all trees during the two year-study (Table 2.1). Ideally, the total extent of propiconazole translocation in the root system would have been measured; however, the depth of the root system in sandy soils prevented sampling beyond 1.0 m from the root collar. In addition, only those roots inoculated with *C. fagacearum* (treatments 1 and 2) or water (treatment 3) were assayed for the fungicide. Therefore, the total extent of propiconazole movement into the root system (e.g. in roots that had not been directly penetrated by injection tees) and the distance it travels in the root system is unknown. Based on the observed differences in concentration between samples taken 0.33 m below and 1.0 m below the injection site (Figure 2.1), it is improbable that significant concentrations of propiconazole would be observed more than a few meters

from the root collar. It is therefore unlikely that propiconazole could prevent root graft transmission of *C. fagacearum* throughout the entire root system of a mature oak. However, levels of propiconazole in root tissue that are likely to be fungistatic, if not fungicidal, *in vivo* could play an important role in disease control. For instance, although high concentrations of the fungicide within a few meters of the root collar may not prevent root graft transmission, they may limit pathogen movement into and subsequent colonization of above-ground tissues leading to disease development. Also, propiconazole may prevent extensive colonization of the root system should a single grafted root become infected. Movement from root to root would require the pathogen to pass though the root collar zone, or through self-grafts which tend to occur more frequently near the root collar where the concentration of large roots is the highest (Chapter 3).

Although the roots were destructively sampled, and therefore the concentration of propiconazole could not be determined in the same root more than once, our data indicate that average fungicide concentration decreased significantly in the roots and stem over time (Figure 2.3). A decrease in propiconazole concentration was observed at all sampled distances between two and twelve months after treatment, but not at 0.66 m and 1.0 m from the injection site after twelve months. The estimated degradation rate of propiconazole in sampled roots over the two year study was 69% (41% annually), permitting detectable levels of residual fungicide in the roots after 24 months. This is in contrast to observations of residual propiconazole levels above ground. For instance, propiconazole could not be detected in the crown of American elm (Armstrong, 1999) or red oaks (Bernick, pers. comm.) 12 months after being treated with Alamo® at the 20 ml per 2.5 cm DBH rate. Propiconazole degradation is proportional to temperature (Armstrong, 1999), and the absence of propiconazole in the tree crown may be the result of exposure to elevated temperatures during the growing season. Propiconazole concentrations as high as 93.1 ppm and 58.89 ppm could still be detected in the lower stem and primary roots, respectively, 24 months after treatment (Table 2.1). High concentrations of propiconazole near the root collar and in the root system after several years may be due to binding of the active ingredient to the walls of the xylem near the injection point (Lever, 1986) and lower rates of degradation in roots which are insulated

by the soil, respectively. The documented absence of propiconazole in the stem or branches after one year does suggest that continued disease protection in subsequent years is not dependent upon the presence of detectable levels of the fungicide above ground, but that the efficacy of propiconazole applications after one year is the result of residual active ingredient in the lower stem and root crown of treated trees.

2.4.2. Effect of propiconazole on *Ceratocystis fagacearum in vivo*

Samples with a relatively high propiconazole concentration were very unlikely to yield *C. fagacearum* two and twelve months after treatment, yet the pathogen was isolated from samples with fungicide concentrations in the parts per million range during this time (Figure 2.7). The effective concentrations (fungicidal or fungistatic) of propiconazole on this pathogen *in vitro* may have limited applicability *in vivo*. Based on our observations, *C. fagacearum* can survive in tissues with concentrations of propiconazole above the parts per billion range, though the actual concentration to which the pathogen was directly exposed in these tissues was not determined. The effect of fungicide concentration on the survival of *C. fagacearum* diminished after the first year post-treatment, and the pathogen was isolated from samples with propiconazole in the tens to hundreds of parts per million range at 24 months (Figure 2.8). Pathogen survival in treated trees was greatest in root samples taken furthest from the root collar within a year after treatment, but the trend was reversed after two years (Figure 2.6). Propiconazole has not been shown to be mobilized into new growth from year to year, and therefore the fungicide may have been compartmentalized in older growth rings of the stem allowing the pathogen to spread into the newer growth rings. This hypothesis may be supported by the noticeable absence of *C. fagacearum* 0.33 m below the injection site at all three sampling periods (Figure 2.6). The vascular system at the root/stem interface is more interconnected, and xylem elements in the root system are not annually occluded by gums and tyloses as they are in the stem and branches, potentially allowing sufficient translocation of residual active ingredient into the new vascular elements of the root system. Long term efficacy of propiconazole may be limited by compartmentalization in certain tissues, but residual levels of propiconazole at the root crown could extend the period of protection provided by systemic fungicide injections.

However, the pathogen was detected in the stems of several asymptomatic trees 24 months after treatment, and disease development was observed in two treated trees at 35 months, suggesting that pathogen propagules may be able to pass through the root crown zone and begin colonization of the stem regardless of residual propiconazole levels.

2.4.3. Treatment Efficacy

The rapid onset of wilt symptoms and subsequent death of disease control trees confirms the effectiveness of the root inoculation method. The continued presence of *C. fagacearum* in the root system two growing seasons after mortality (Figure 2.3) highlights the need to consider root systems as potential inoculum sources long after tree death. Propiconazole applications were unable to prevent successful infection or to eradicate *C. fagacearum* from any prophylactically or therapeutically treated trees, respectively (Figure 2.7). Therefore, treated but infected trees should also be considered potential inoculum sources. Because there was no significant difference in the incidence of *C. fagacearum* among prophylactically or therapeutically treated trees at any time during this study (Figure 2.8), it may be justified to make the decision to treat a tree based on the presence of symptoms alone. Latent infections pose a problem for the placement of root graft barrier lines because managers need to balance the need to contain the pathogen in active disease centers while saving as many healthy trees as possible. The inability of propiconazole to prevent infection in this study and the lack of treatment failures in every infected tree for two years suggests that treatment efficacy is the result of disease suppression rather than by preventing infection. If symptom development has not begun, it may be justifiable to treat high-value trees even when infection is suspected. The long term survival of *C. fagacearum* should be investigated in trees repeatedly treated with propiconazole on an annual or bi-annual basis. Repeated treatments could continue to suppress disease development, but propiconazole's capacity to successfully eradicate the pathogen with repeated injections is unknown.

2.5. Conclusions

Translocation of propiconazole into the root system of trees injected by the macro-infusion application method has been demonstrated, but the concentration of

active ingredient in roots rapidly decreases with increasing distance from the root flare injection site. Intravascular injections with propiconazole do not prevent root infections, nor are they capable of eradicating *C. fagacearum* from the root system of infected trees. The efficacy of propiconazole applications is likely the result of disease suppression, the duration of which is extended by residual levels of propiconazole found in the root crown and lower stem. Survival of *C. fagacearum* in the root system of propiconazole treated trees for more than two years and subsequent movement out of the root system into the stem indicates that retreatment every two years would be necessary for continued disease suppression. Several propiconazole formulations are available and application methods vary considerably. Choice of formulation and application method based on maximum movement into and longevity in the root system may result in greater treatment efficacy in asymptomatic trees than those options which achieve greatest and most uniform distribution in the crown. Recognition of the capabilities and limitations of propiconazole applications will be conducive to the continued use of this control option for vascular diseases such as oak wilt, Dutch elm disease, and most recently, laurel wilt in the southeastern United States (Mayfield et. al., 2008).

2.6. Figures and Tables

Months Post-Treatment	Distance From Injection Point (m)	Propiconazole Concentration (PPM) in Samples				
		n	Mean[z]	Std Dev	Minimum	Maximum
2	+ 0.33	17	463.94 [a]	215.45	132.87	815.02
	- 0.33	17	167.50 [b]	132.53	30.57	507.89
	- 0.66	17	67.87 [cd]	65.02	1.58	242.85
	- 1.00	17	41.04 [e]	57.40	1.21	189.05
12	+ 0.33	17	180.80 [b]	96.51	55.18	468.31
	- 0.33	17	99.67 [c]	61.31	4.95	239.35
	- 0.66	17	16.10 [f]	24.96	1.06	83.84
	- 1.00	17	9.76 [g]	18.93	0.69	73.74
24	+ 0.33	18	93.10 [c]	61.11	7.47	254.87
	- 0.33	18	58.89 [de]	47.68	0.00	162.63
	- 0.66	18	25.16 [f]	21.63	0.00	70.51
	- 1.00	18	7.66 [g]	11.77	0.00	42.24
	Total	208	101.54	149.25	0.00	815.02

Table 2.1. Propiconazole concentration (PPM) over time in wood tissues sampled at various distances above (+) and below (-) chemical injection sites in root flares[y].

[y] Wood samples were taken from northern red oaks treated with Alamo® (20.0 ml per 2.5 cm DBH) in prophylactic, therapeutic, and chemical control treatments.
[z] Significant differences among means are indicated by different letters as determined by pairwise comparisons of least-squares means (α=0.05).

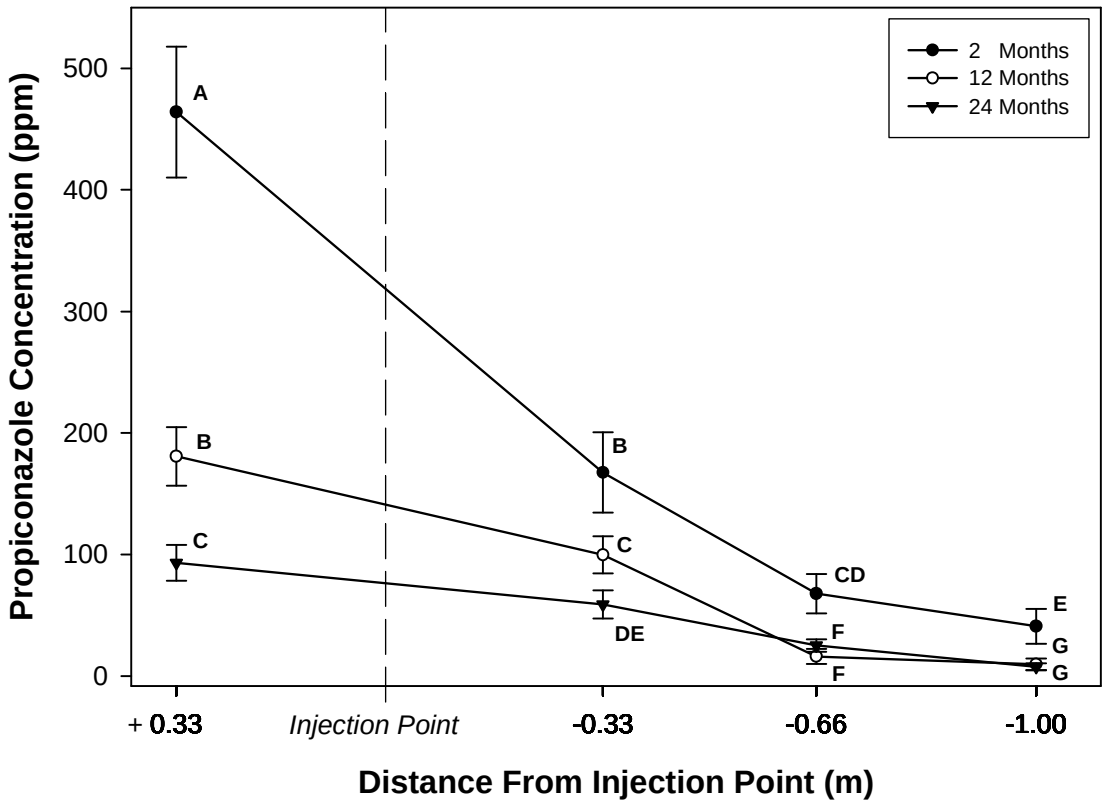

Figure 2.1. Average concentration of propiconazole (PPM) in wood samples taken at different distances above (+) and below (-) the root flare injection site in trees treated with Alamo (20.0 ml per 2.5 cm DBH) prophylactically (treatment 1), therapeutically (treatment 2), and in trees not inoculated with *C. fagacearum* (treatment 3) by months after injection. Significant differences among means are indicated by different letters as determined by pairwise comparisons of least-squares means (α=0.05). Error bars indicate +/- standard error of the mean; n=17 for months 2 and 12, n=18 for month 24.

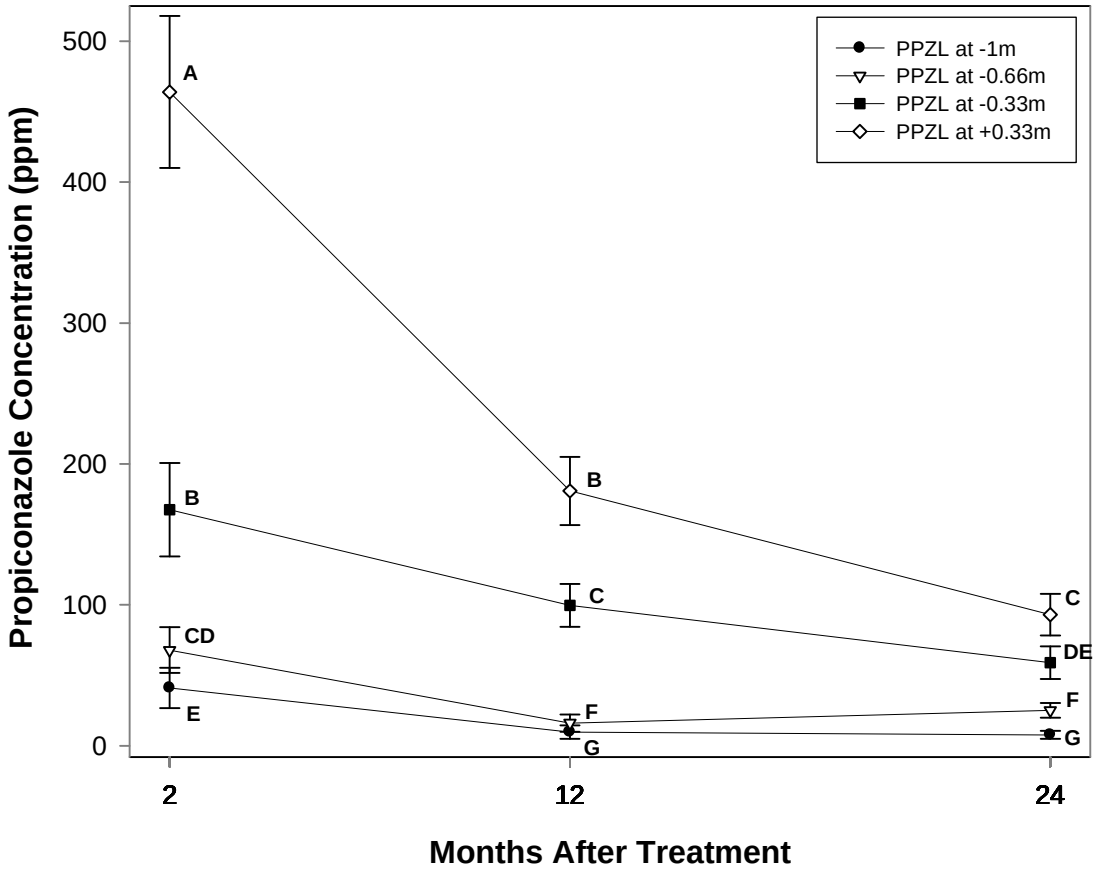

Figure 2.2. Average concentration of propiconazole over time (PPM) in wood samples taken above (+) and below (-) the root flare injection site in trees treated with Alamo (20.0 ml per 2.5 cm DBH) prophylactically (treatment 1), therapeutically (treatment 2), and in trees not inoculated with *C. fagacearum* (treatment 3) by distance from the injection point. Significant differences among means are indicated by different letters as determined by pairwise comparisons of least-squares means (α=0.05). Error bars indicate +/- standard error of the mean; n=17 for months 2 and 12, n=18 for month 24.

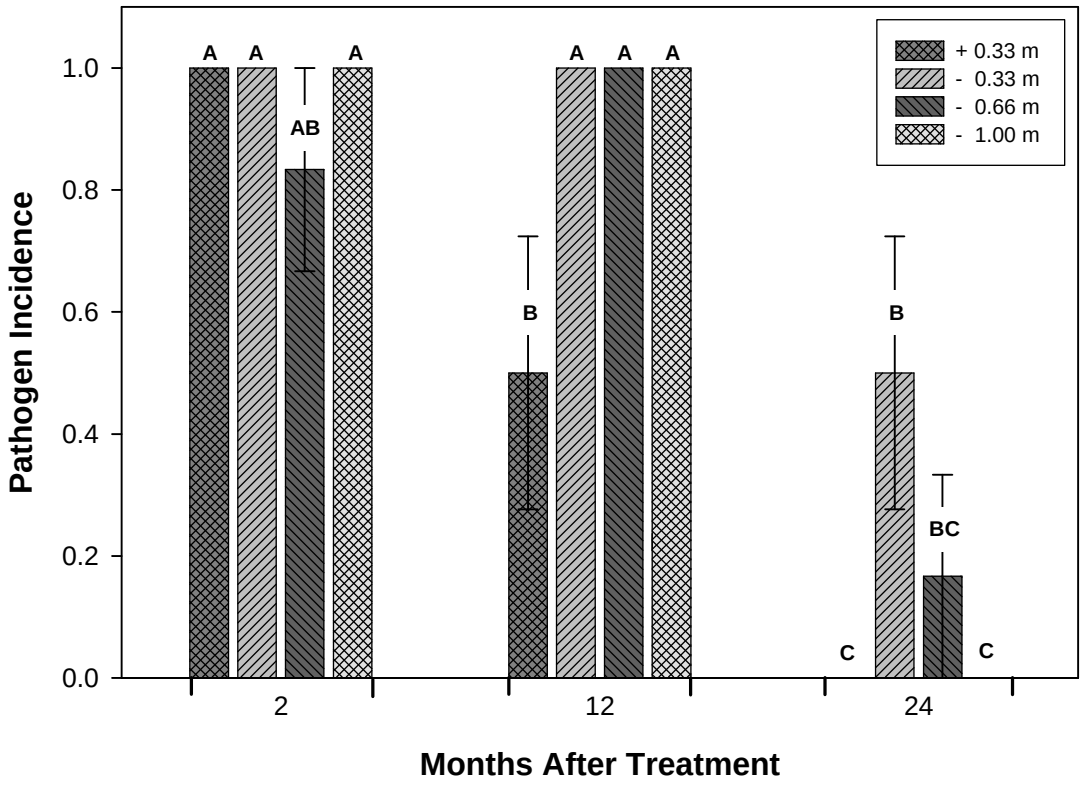

Figure 2.3. Pathogen incidence in disease control trees (treatment 4) by months after inoculation expressed as the proportion of wood samples taken at different distances above (+) and below (-) the root flare injection site yielding *C. fagacearum*. Three roots on each tree were inoculated with a 1.0 ml spore suspension (1.0×10^6 spores/ml) of *C. fagacearum* 1.0 m from the root flare injection site. Significant differences among means are indicated by different letters as determined by pairwise comparisons of least-squares means (α=0.05). Error bars indicate +/- standard error of the mean; n=6.

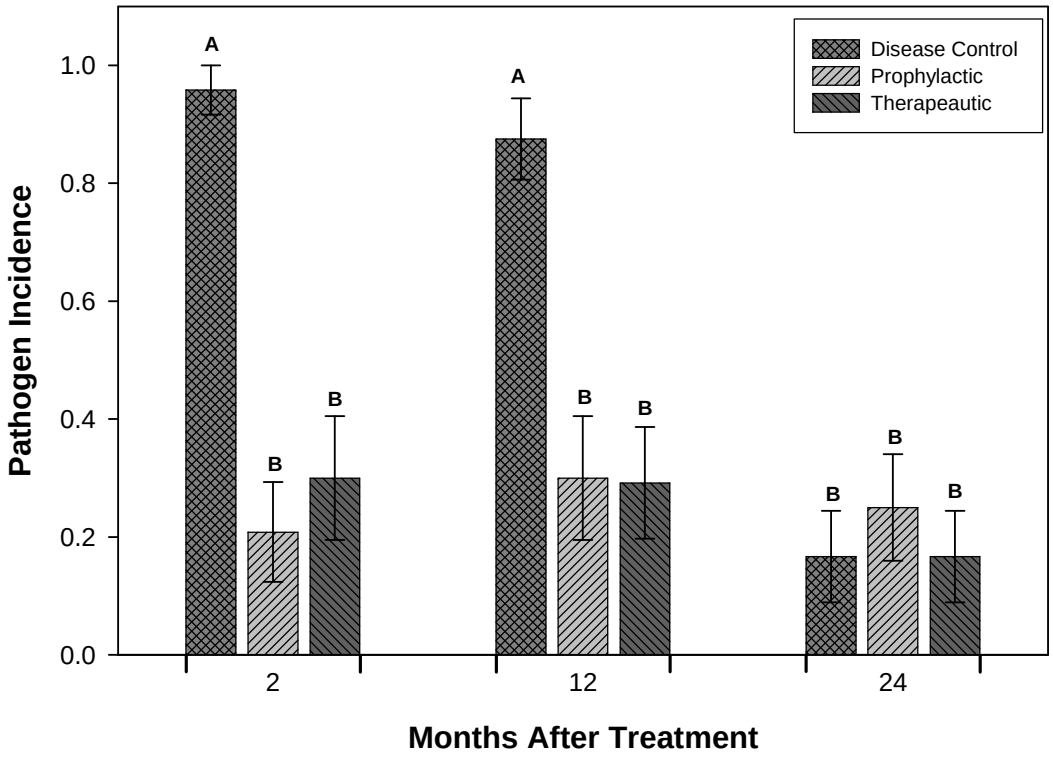

Figure 2.4. Pathogen incidence in trees treated with Alamo (20.0 ml per 2.5 cm DBH) prophylactically (treatment 1), therapeutically (treatment 2) or in disease control trees (treatment 4) by months after inoculation expressed as the proportion of wood samples yielding *C. fagacearum* for all distances from the injection site. Three roots on each tree were inoculated with a 1.0 ml spore suspension (1.0×10^6 spores/ml) of *C. fagacearum* 1.0 m from the root flare injection site. Significant differences among means are indicated by different letters as determined by pairwise comparisons of least-squares means (α=0.05). Error bars indicate +/- standard error of the mean; n=21 for month 12 prophylactic and month 2 therapeutic, n=6 otherwise.

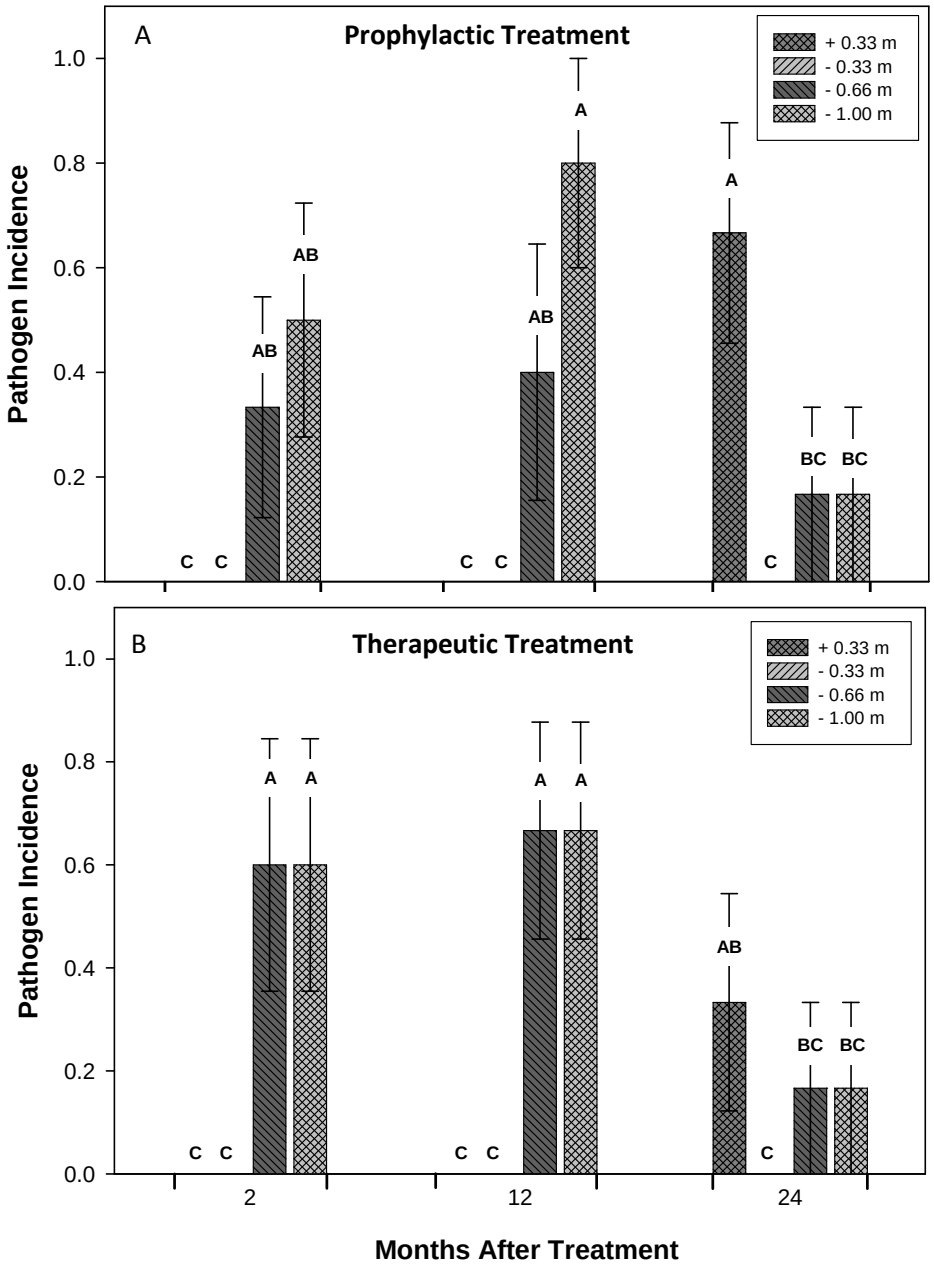

Figure 2.5. Pathogen incidence in trees treated with Alamo (20.0 ml per 2.5 cm DBH) prophylactically (treatment 1, A) and therapeutically (treatment 2, B) by months after inoculation expressed as the proportion of wood samples taken at different distances above (+) and below (-) the root flare injection site yielding *C. fagacearum.* Three roots on each tree were inoculated with a 1.0 ml spore suspension (1.0 x 10^6 spores/ml) of *C. fagacearum* 1.0 m from the root flare injection site. Significant differences among means are indicated by different letters as determined by pairwise comparisons of least-squares means (α=0.05). Error bars indicate +/- standard error of the mean; n=5 for month 12 Figure 7A and month 2 Figure 7B, n=6 otherwise.

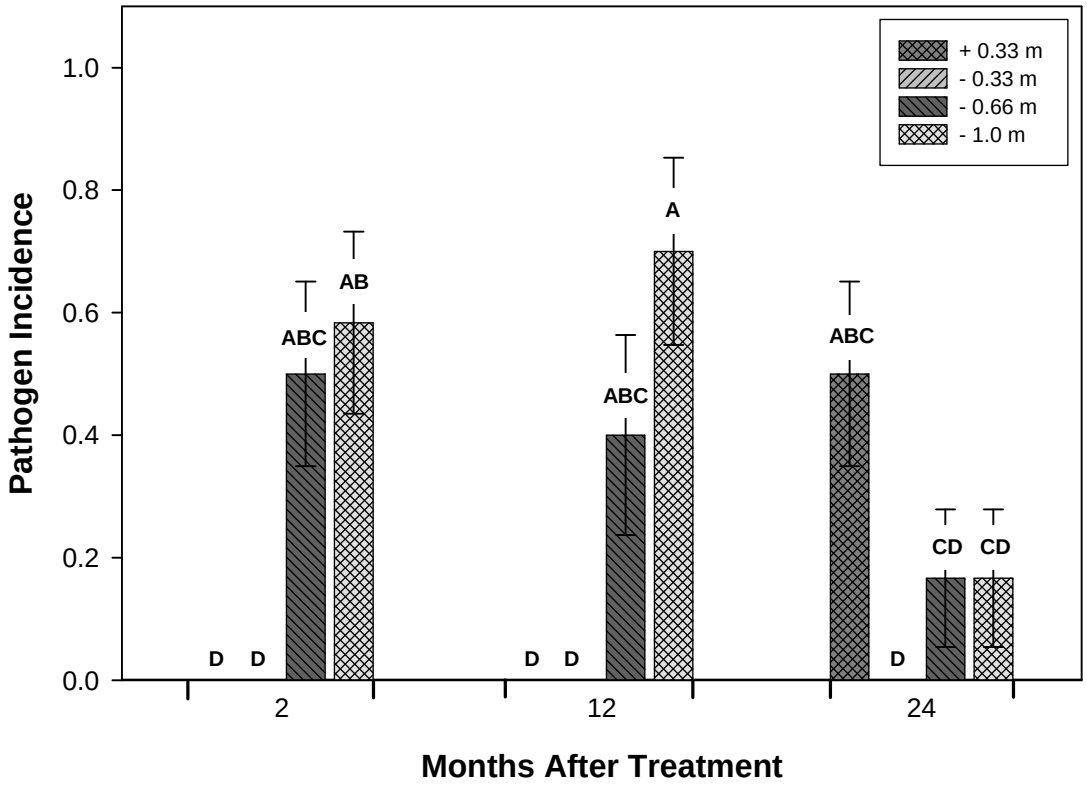

Figure 2.6. Pathogen incidence in trees treated with Alamo (20.0 ml per 2.5 cm DBH) prophylactically (treatment 1), and therapeutically (treatment 2) by months after inoculation expressed as the proportion of wood samples taken at different distances above (+) and below (-) the root flare injection site yielding *C. fagacearum*. Three roots on each tree were inoculated with a 1.0 ml spore suspension (1.0×10^6 spores/ml) of *C. fagacearum* 1.0 m from the root flare injection site. Significant differences among means are indicated by different letters as determined by pairwise comparisons of least-squares means (α=0.05). Error bars indicate +/- standard error of the mean; n=11 for months 2 and 12, n=12 for month 24.

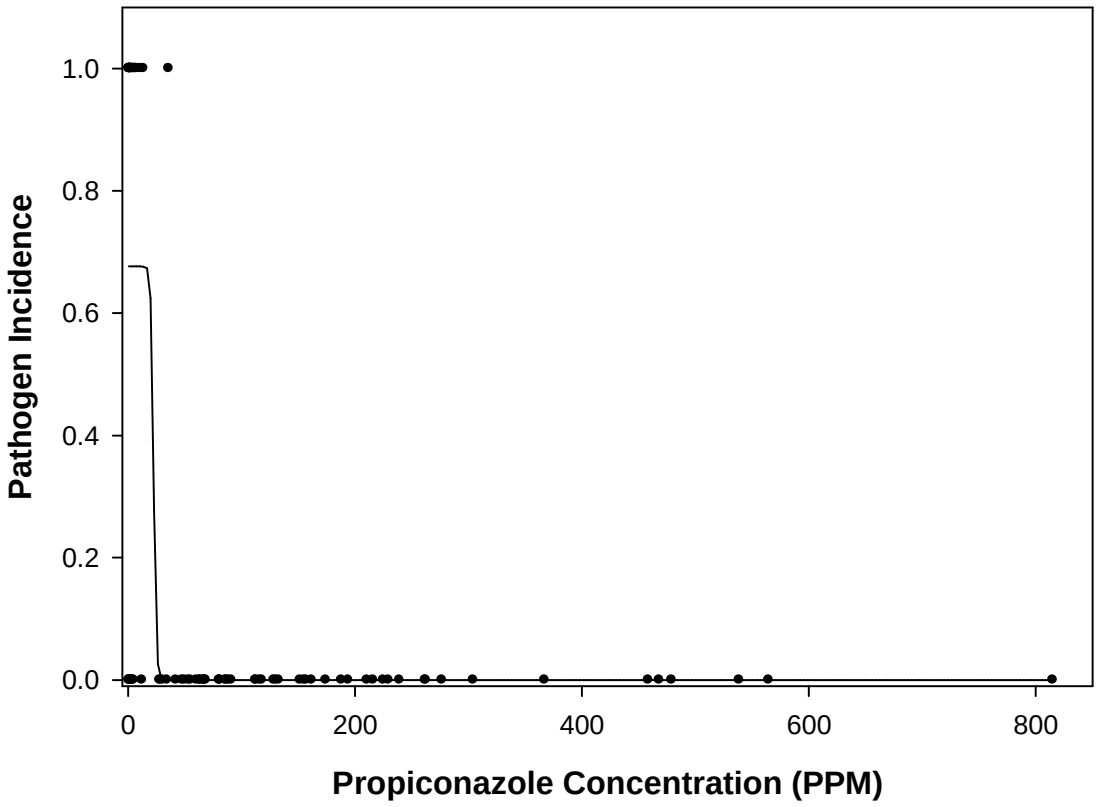

Figure 2.7. Pathogen incidence 2 and 12 months after treatment application by concentration of propiconazole (ppm) in wood samples taken from the stem (0.33 m above the injection site) and roots (0.33m to 1.0m below the injection site) of trees treated with Alamo (20.0 ml per 2.5 cm DBH) prophylactically (treatment 1) and therapeutically (treatment 2). Three roots on each tree were inoculated with a 1.0 ml spore suspension (1.0×10^6 spores/ml) of *C. fagacearum* 1.0 m from the root flare injection site. Pathogen incidence is expressed on a scale from 0 (sample did not yield *C. fagacearum*) to 1 (sample yielded *C. fagacearum*). Data were fit with a sigmoid trend line ($y = .6765 / (1+ \exp(-(x-22.5419)/-1.1165))$; $R^2=0.5264$) for reference; n=136.

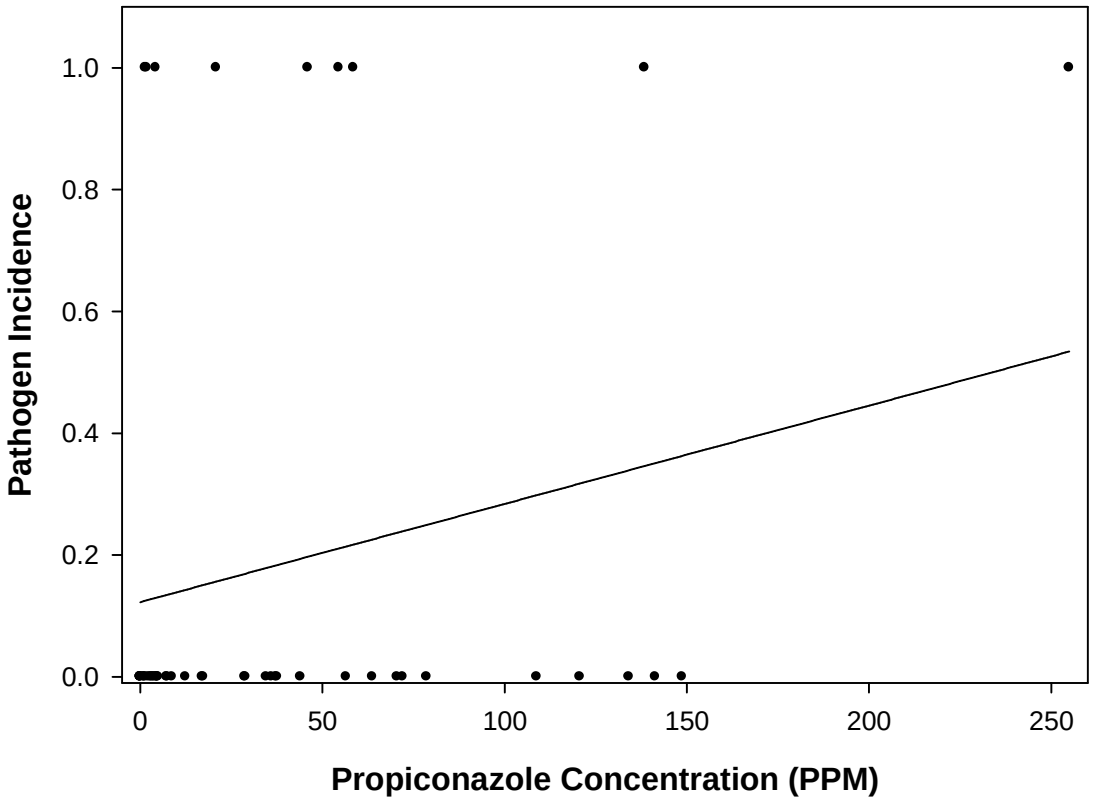

Figure 2.8. Pathogen incidence 24 months after treatment application by concentration of propiconazole (ppm) in wood samples taken from the stem (0.33 m above the injection site) and roots (0.33m to 1.0m below the injection site) of trees treated with Alamo (20.0 ml per 2.5 cm DBH) prophylactically (treatment 1) and therapeutically (treatment 2). Three roots on each tree were inoculated with a 1.0 ml spore suspension (1.0×10^6 spores/ml) of *C. fagacearum* 1.0 m from the root flare injection site. Pathogen incidence is expressed on a scale from 0 (sample did not yield *C. fagacearum*) to 1 (sample yielded *C. fagacearum*). Data were fit with a linear trend line ($y = 0.1126 + 0.0016x$; $R^2=0.2196$) for reference; n=72.

CHAPTER 3

Spatial and Temporal Distribution of *Ceratocystis fagacearum* and the Characterization of Root Grafting in Red Oak

3.0. Introduction

Oak wilt, caused by the vascular pathogen *Ceratocystis fagacearum*, is considered to be the most important disease of oaks (*Quercus spp.*) in the eastern United States (Tainter and Baker, 1996). In Minnesota, both white oaks (Section *Quercus*) and red oaks (Section *Lobatae*) are susceptible to *C. fagacearum*, but the disease is particularly devastating for red oak species including northern red oak (*Quercus rubra*) and northern pin oak (*Quercus ellipsoidalis*) which wilt and die within weeks to months of incipient wilt development.

The oak wilt fungus spreads through root grafts between neighboring oaks, resulting in the formation of expanding areas of wilting and dead trees known as disease centers (Gibbs and French, 1980). New disease centers are established via overland transmission of the pathogen to healthy trees by insect vectors such as sap feeding beetles of the *Nitidulidae* family. While overland transmission is responsible for long distance spread of *C. fagacearum* in the landscape, the majority of new infections occur as a result of root graft transmission of the pathogen in expanding disease centers (Juzwik, French, and Jeresek, 1985).

Tylose production and gummosis in response to infection, while prolific in the above-ground water-conducting vessel elements, appears to occur at a relatively low rate in the roots of infected trees even after tree death. Thus the spread of the fungus proceeds relatively uninhibited through roots and root grafts (Nair and Kuntz 1975; Struckmeyer et al. 1954; Yelenosky and Fergus 1959). Inoculation studies have shown the length of time between root inoculations and incipient wilt development ranges from several weeks to several years (Rexrode, 1978; Skelly and Wood, 1974a; Yount, 1955; 1958). This suggests that the rate of movement of *C. fagacearum* through roots is variable, and that

the pathogen is capable of surviving undetected in the roots of apparently healthy trees for many years (Skelly and Wood 1974; Yount 1958). This hypothesis was supported when dyes injected into *C. fagacearum* infected trees helped to definitively confirm the presence of root grafts, and yet disease symptoms were not observed in grafted trees for up to three years after the death of the initially infected tree (Rexrode 1978). The frequency of parasitic interactions between the pathogen and parenchymatous root cells was found to be relatively low, and the survival of the fungus in roots for long periods is not well understood (Struckmeyer, Kuntz, and Riker 1958). Longevity of the fungus in the root systems of trees that succumbed to oak wilt may be aided by water and nutrients supplied through root grafts to healthy trees (Yount 1955).

The frequency of root graft transmission is influenced by soil, host, and stand characteristics and may vary widely between regions or on more local scales (Bruhn, Pickens, and Stanfield 1991; Gibbs and French, 1980). Attempts to characterize oak wilt spread in disease centers have been made in Michigan's Upper Peninsula (Bruhn, Pickens, and Stanfield 1991), southern Wisconsin (Menges and Kuntz 1985), North Carolina (Boyce 1960), Texas (Appel et al. 1989), and Missouri (Jones and Partridge 1961). Management guidelines developed as a result of these investigations have increased the efficacy of integrated management strategies for oak wilt in those regions. However, the application of guidelines outside regions where the disease spread was modeled can lead to reduced or inconsistent control.

Root graft transmission in Minnesota is managed by mechanically severing grafted root systems, most commonly with a vibratory plow, thus establishing a "root graft barrier" (French and Juzwik, 1999). Effective root graft barrier placement is difficult because the pathogen must be contained within the disease center while sacrificing as few healthy oaks as possible within the barrier line. Pathogen distribution in the root system of trees in or near disease centers cannot be determined reliably based on symptom development above ground, so "primary" lines are installed to contain the pathogen effectively and "secondary" lines are placed within the primary line in an attempt to save healthy oaks in close proximity to wilting ones (French and Juzwik, 1999).

Systemic fungicides such as propiconazole have been used to protect oaks in situations where, for example, a vibratory plow cannot be used to mechanically disrupt root grafts (Appel, 2001). Propiconazole applications also provide additional protection for high value asymptomatic trees within root graft barrier lines (Juzwik et. al., 2004). However, propiconazole has not been shown to prevent root graft transmission; rather it is believed to prevent disease development through an unknown mechanism.

Management of oak wilt via control of root graft transmission of *C. fagacearum* has the potential to be more efficacious should managers achieve a more thorough understanding of pathogen movement into and colonization of the root system, because treatments are directed at preventing infection of healthy trees that generally do not show symptoms immediately following root infection. These latent infections are a substantial obstacle to oak wilt management. They can lead to costly treatment failures, for example, in situations where the pathogen has already spread beyond root graft barrier lines at the time of treatment or when disease development occurs in chemically-treated oaks following fungicide degradation, and can put additional trees at risk that are normally not considered in management regimes.

The spatial distribution of *C. fagacearum* in the near-surface roots of wilted or wilting northern red oaks and proximal asymptomatic northern red oaks was investigated in east-central Minnesota. Field studies involving pairs of oak wilt symptomatic and asymptomatic trees were conducted in 2005 and 2006 to determine: 1) the presence of the pathogen in primary roots (roots originating directly from the root collar) in wilting trees, 2) the frequency of inter-tree grafts and self grafts (grafts between two roots of the same tree) within 1.0 m (3.3 ft) of the soil surface, and 3) the presence of the pathogen in roots joined in functional root grafts.

3.2. Materials and Methods

3.2.1. Experimental Design

The field study was conducted at 12 sites, each with an active disease center, within the Carlos Avery Wildlife Refuge in Chisago County in east-central Minnesota. All sites were located on Zimmerman loamy sand or Sartell fine sand, and northern red oak was the predominant species. Wilt development of individual northern red oaks in

disease centers was monitored between 2004 and 2006. A pair of trees consisting of one diseased tree and the nearest asymptomatic tree were selected from each disease center during this time period. The diseased trees were suspected to have been infected via root graft transmission of the pathogen because of their close proximity (< 5.0 m; 16.4 ft) to actively wilting or recently wilted trees. The extent of pathogen distribution in the root system of wilting trees and the incidence of root graft transmission to the neighboring asymptomatic tree was expected to increase with increasing symptom progression. Therefore a minimum of two diseased trees from each of the following stages of crown wilt (percent of crown displaying symptomatic foliage) were selected for this study: 1) 20 – 40 % crown wilt, 2) 41 – 85 % crown wilt, 3) 86 – 99 % crown wilt, and 4) 100 % crown wilt for 12-14 months (dead). The diameter at breast height (dbh) of selected trees ranged from 12.7 – 63.5 cm (5.0 - 25.0 in); average dbh was 31.2 cm (12.3 in) (Table 3.1). Distance between trees within a pair ranged from 0.4 – 4.6 m (1.3 – 15.1 ft); average distance was 2.6 m (8.5 ft).

3.2.2. Root Excavation and Sampling

Partial root system excavation of each of the 24 selected trees (12 pairs of diseased and proximal asymptomatic trees) was conducted with an air excavation tool (Airspade®, Guardair Corp, Chicopee, MA). After removing approximately the upper 15 cm (6.0 in) of soil containing fine roots and understory plants, the underlying mineral soil was removed while leaving the root system of study trees intact and undamaged. At the selected field sites, the roots of the red oaks had frequently grown at a high angle of descent, beyond one meter below grade. However, the maximum excavation depth that could be efficiently achieved in the sandy soil with the excavation tool was approximately one meter.

One primary root in each quadrant of the four cardinal directions was sampled to investigate the spatial distribution of the pathogen in each tree. Root segments approximately 10 cm (3.9 in) in length were removed from each primary root at 0.3, 0.6, 0.9, and 1.2 m (1.0, 2.0, 3.0, and 3.9 ft) from the root collar. The incidence of root grafting between pairs of trees was also examined at each site. All soil was removed from an initial search area 3.0 m (9.8 ft) wide that spanned the distance between the

wilting and asymptomatic tree to a depth of 0.6 m (2.0 ft). Each root exposed in the initial search area was examined beginning at the root collar of the originating tree until one of the following conditions were met: 1) the root grafted to the root of a neighboring tree, 2) the root descended to a depth greater than 1.0 m (3.3 ft) below grade, 3) the root diameter tapered to less than 1.0 cm (0.4 in), or 4) the root significantly deviated from a course that would have led into the canopy drip line of the proximal tree. If an inter-tree graft between the two paired trees was discovered, root segments approximately 10 cm (3.9 in) in length were removed from both roots involved in the graft union at 0.3 m (1.0 ft) intervals beginning at the root graft to the root collar. Within the total excavated area between the two trees, samples were also taken from roots involved in self-grafts when present. Inter-tree and self-grafts were also sampled from the excavated primary roots described above. For each excavated root system, the length of all exposed roots that could be successfully traced back to the root collar, the number of inter-tree and self grafts discovered, and the linear distance of grafts along the root from the root flare were recorded. Inter-tree grafting was confirmed by removal of bark and outer layers of vascular tissue to confirm continuity of the vascular system across the root graft union between the two roots involved.

All root samples were sealed in plastic bags and placed into cold-storage shortly after removal from the soil. Root samples were then assaued for the presence of *C. fagacearum*. Following a period of 2-3 weeks of refrigerated storage that allowed for extensive pathogen colonization, root samples were surface sterilized with 95% ethanol and briefly flamed. Small wood chips approximately 0.5 cm (0.2 in) in length were removed from outer rings of xylem along the length of the root segment. Chips were plated onto 10% lactic acid-amended potato dextrose agar and incubated in the dark at 25ºC for 30 days. Plates were examined weekly for the presence of *C. fagacearum.* A second attempt to isolate the pathogen was made from any root segments which did not yield the pathogen from the first attempt.

3.2.3. Data Analysis

The effects of tree wilt category and distance of root segments from the root collar on the incidence of *C. fagacearum* were analyzed by Analysis of Variance (ANOVA)

using the general linear model procedure (PROC GLM) in SAS (SAS Institute, Inc. Cary, NC). Differences between means were determined using Tukey's *w* procedure (α=0.05).

3.3. Results

3.3.1. Pathogen Presence in Primary Roots

C. fagacearum was isolated from 18.7% (n=96) of primary roots excavated and assayed (Table 3.1). Overall, the pathogen was isolated from 27.5% (n=192) of root segments from symptomatic (wilting or dead) trees and from 1% (n=192) root segments of asymptomatic trees (Table 3.2). In the latter category, *C. fagacearum* was isolated from one asymptomatic red oak (24.1 cm; 9.5 in dbh) that was 4.6 m (15.1 ft) west of a red oak (30.7 cm; 12.1 in dbh) that exhibited 100% crown wilt at the time of excavation; a tree that began wilting only two months prior in mid-June. In the nearby wilted tree, the pathogen was isolated from all segments of the root nearest the asymptomatic tree; however, no grafts were found in the excavated zone between the two trees. In the asymptomatic tree, the pathogen was sporadically distributed; being found in only one segment of each of the northern and southern originating roots.

The number of primary roots from each tree yielding the pathogen was similar in all categories of wilting or wilted trees (P = 0.2874) (Table 3.1). In all but one case, *C. fagacearum* was isolated from no more than two of the primary roots extending in each cardinal direction from the tree stem. The exception was an oak with 60% crown wilt which yielded the pathogen in primary roots from all four cardinal directions. This tree was 4.0 m (13.1 ft) from two trees (35.6 cm and 40.6 cm; 14.0 in and 16.0 in dbh) that had completely wilted the previous year.

Within each crown wilt stage for actively wilting trees, the proportions of root segments yielding *C. fagacearum* were not correlated with distance from the root collar (0.3 to 1.2 m, 1.0 to 2.0 ft) ($P \geq 0.3351$), however, pairwise comparisons of those proportions revealed several significant differences (α=0.05) (Table 3.2). The frequency of segments yielding the pathogen increased as distance from the root collar increased in dead trees (P = 0.0422) (Table 3.2). During all stages of crown wilt, in the majority of roots from which the pathogen was successfully isolated (77.7%, n=18), at least one-half

of the four root subsamples yielded the fungus, but *C. fagacearum* was only isolated from all four subsamples of pathogen-yielding roots in 44.4% of cases.

3.3.2. Root Graft Occurrence

Eleven inter-tree root grafts were found along 514 m (1,686 ft) of roots excavated between pairs of 12 wilting/wilted oaks and their closest asymptomatic neighbors. The grafts occurred between 3 of the 12 tree pairs and most grafts involved either primary or secondary roots (Table 3.3). Two additional grafts were discovered during excavations of a single symptomatic/asymptomatic pair of trees, but the grafts were traced from the symptomatic tree to another oak (also symptomatic) that was not originally included in the study. Over 30% (n=21) of grafted roots were involved in more than one inter-tree graft. Of the seven roots involved in more than one root graft, three roots were involved in two grafts, two roots were involved in three grafts, and one root was grafted at four different locations to other roots. Thus, the number of inter-tree grafted roots (n=21) is less than the expected number (n=26) for 13 grafts, because 7 of the grafted roots were involved in more than inter-tree root graft.

All of the inter-tree grafts occurred within 2.5 m (5.2 ft) of the root collar of both trees in the pair, but all grafts occurred within 1.0 m (3.3 ft) of the root collar of at least one tree in the pair (Figure 3.1). The average distance between grafted pairs of trees was 1.1 m (3.6 ft), while the maximum distance was 1.9 m. Many “false grafts” were found, i.e. superficial grafts where continuity of the vascular system across the union was absent.

Self grafts were observed in all 24 root systems; a total of 162 self grafts were found along the same length of roots examined for inter-tree grafts. They were prevalent in close proximity to the root collar where the density of roots was the highest (Figure 3.2A); however, self-grafts were found among all root orders and up to five meters from the stem. The frequency of self grafts was positively correlated with tree diameter (Figure 3.2B). No “false grafts” were found among the observed self grafts.

3.3.3. Pathogen Presence in Root Grafts

Tissue samples taken in close proximity to inter-tree graft unions yielded the pathogen in 2 of the 13 inter-tree grafts. However, both grafts testing positive for *C.*

fagacearum were the two grafts found inadvertently between a symptomatic study tree and a symptomatic non-study tree (1.9 m; 6.2 ft apart) as described above. The study tree (25.4 cm; 10.0 in dbh) had begun to wilt in early July 2005 and was 100% wilted at the time of excavation in August 2005, while the non-study tree (12.7 cm; 5.0 in dbh) was 20% wilted. In both grafts, the pathogen was found in both roots involved in the graft union.

C. fagacearum was isolated from 13 of 62 self-grafts assessed for presence of the pathogen. Eleven of those self grafts yielded *C. fagacearum* in both roots of the graft union, while the pathogen was found in only one root in the other two cases.

3.4. Discussion

Absence of *C. fagacearum* from the root system of asymptomatic trees in close proximity (0.5 – 4.6 m; 1.6 – 15.1 ft) to wilting or wilted trees, and from the majority of sampled primary roots of dead or diseased trees in the most advanced stages of disease progression, suggests that pathogen distribution in the root system may not be widespread. Alternatively, extensive pathogen colonization of the root system may require months or years following tree death, a hypothesis that seems to be supported, at least in certain cases, by latent spread of the fungus over the course of many years between wilted and healthy trees (Skelly and Wood 1974a, b).

Slow movement of the pathogen into the root system during wilting or following tree death could be explained by the necessity for spread via vegetative growth. In other cases where the pathogen is found in roots more rapidly than could be accounted for by vegetative growth (even at the earliest stages of incipient wilt), it has been suggested that the negative water potential generated by healthy, actively transpiring trees grafted to the wilting tree may draw pathogen propagules from the diseased tree into the root system and across root grafts (Nair 1995; Nair and Kuntz 1975). Rapid movement into a few grafted roots as opposed to colonization of the entire root system via vegetative growth could explain the sporadic distribution of the pathogen in primary roots of diseased or dead oaks. We did not attempt isolations from all primary roots, so it is possible that pathogen distribution in the root system is more widespread than our results indicate.

The distribution of *C. fagacearum* observed in this investigation suggests colonization of the root system in wilting or wilted trees is limited however.

In dead trees, absence of the pathogen near the root collar may be due to displacement of *C. fagacearum* by more efficient saprophytes. Presence of the pathogen did increase with increasing distance from the root collar in the dead trees, likely because moisture availability and temperature are more suitable for pathogen survival with increasing distance from the soil surface (Tainter 1995).

While we did not attempt to examine the entire root system of the study trees, the frequency of inter-tree grafting, particularly between trees in close proximity to one another (< 5.0 m; 16.4 ft) in sandy soils, was surprisingly low. Large portions of the root systems of most excavated trees appeared to be distributed well below 1.0 m (3.3 ft) the soil surface (the maximum depth examined in this study). Inter-tree grafting in the upper one meter (3.3 ft) of soil was a relatively infrequent phenomenon when compared to the incidence of self grafting. However, inter-tree graft occurrence is likely frequent enough to account for the rate of expansion of disease centers observed in sandy soils. In North Carolina, Boyce (1960) was only able to locate 3 inter-tree root grafts between 42 wilting oaks and proximal wilt-killed trees, and only one of these grafts were shown to be the pathway for infection. Yet, the author noted that 96% of wilting oaks in the state were within 15 m (50 ft) of wilt-killed trees, suggesting that root graft transmission was the primary mode of infection. In Missouri, a slightly higher percentage of black oaks (16%) and white oaks (20%) were found to be grafted to other trees (Jones and Partridge, 1961). Parmeter et al. (1956) reported that over 70% of northern pin oaks in stands on deep sand soils in Central Wisconsin were grafted. Therefore, it is likely that inter-tree grafts on sites such as those used in this study may exist below the range of our excavation equipment, as well as that of vibratory plows and trenchers commonly used for the installation of root graft barrier lines.

Self-grafts, while rarely discussed in the literature, may play an important role in pathogen transmission in the root systems of oaks. In this investigation, the results of isolations from self-grafts demonstrate that the pathogen is able to readily move from root to root through self-grafts. Therefore, lateral spread of *C. fagacearum* from one root to another may not require pathogen movement into the root collar zone, but could occur

through networks of self-grafts. Such a mechanism of pathogen movement could allow the fungus to spread throughout large portions of the root system while bypassing the root collar zone where systemic chemical treatments are applied. It was previously believed that translocation of propiconazole into roots following injection is limited and does not prevent infection via root grafts (Appel 2001). Recently however, high concentrations of propiconazole in primary roots following macro-infusion treatment have been observed (Blaedow and Juzwik, 2008). Substantial acropetal transport of propiconazole from the point of injection into the upper portion of the tree seems to prevent wilt development in infected trees. If systemic fungicides such as propiconazole move downward into the roots (even short distances) following injection, it could provide the added benefit of limiting latent root system colonization in infected trees because of the prevalence of self-grafts within 1.0 m (3.3 ft) of the root collar.

3.5. Conclusions

The results of this study reveal the sporadic and unpredictable distribution of *C. fagacearum* in the root systems of wilted and wilting trees. Based on these results, distribution of the oak wilt pathogen in the root systems of diseased or neighboring asymptomatic trees cannot reliably be determined by assessment of above ground symptoms. Current control approaches that assume the pathogen is present in all roots at the time of symptom appearance, and call for conservative placement of root graft barriers and preventative chemical treatments that account for latent infections are warranted. The frequent inability to isolate *C. fagacearum* from the root system of diseased trees, even from trees that had died the previous growing season, suggests pathogen movement into and subsequent colonization of the root system may take months or years following tree death in most cases.

Even when efforts are made to prevent root graft transmission, and all root grafts between diseased and asymptomatic trees are severed, it may be possible that latent infections in asymptomatic trees result in pathogen movement beyond control barriers and disease development outside barrier lines. Chemical treatments such as propiconazole which do not prevent root graft transmission but prevent disease development (Chapter 2), may allow latent colonization of root systems and even

transmission to neighboring trees. Following the period of protection provided by chemical treatments, generally less than two years, disease development may be initiated as a result of masked infections. However, propiconazole translocation into roots could limit pathogen colonization of large portions of the root system and spread to uninfected trees if distribution of the fungicidal compound is sufficient to prevent pathogen spread out of inter-tree grafted roots containing the invading pathogen into self grafts and the root collar.

3.6. Figures and Tables

Crown wilt stage (% Wilt)	Number of Trees Sampled	Number of Primary Roots [z]		Number of Primary Roots Per Tree Yielding *C. fagacearum*	
		Assayed	Yielding fungus	avg.	max.
0	12	48	2	0.2	2
20 - 40	2	8	4	2.0	2
41 - 85	4	16	6	1.5	4
86-99	4	16	4	1.0	2
Dead	2	8	2	1.0	2

Table 3.1. The number of primary roots (average and maximum number of roots) per tree that yielded *C. fagacearum* by crown wilt stage.

[z] Four primary roots were sampled from each northern red oak, and the incidence of *C. fagacearum* was determined in four 10 cm long sub-samples taken at 0.3 m (1 ft) intervals from the root collar.

Crown wilt stage	Number Assessed		Percent of Root Segments Yielding *Ceratocystis fagacearum* by Distance from Root Collar (m) [z]				
(% Wilt)	Trees	Root segments [y]	0.3	0.6	0.9	1.2	All segments
0	12	192	2.1 [e]	2.1 [e]	0.0 [e]	0.0 [e]	1.1 [D]
20 - 40	2	32	50.0 [a]	50.0 [a]	50.0 [a]	25.0 [bc]	43.8 [A]
41 - 85	4	64	31.3 [b]	25.0 [bc]	18.8 [cd]	25.0 [bc]	25.0 [B]
86 - 99	4	64	25.0 [bc]	25.0 [bc]	18.8 [cd]	18.8 [cd]	21.5 [B]
Dead	2	32	0.0 [e]	12.5 [d]	25.0 [bc]	25.0 [bc]	15.5 [C]

Table 3.2. Proportions of primary root segments that yielded *C. fagacearum* by sampling distance from the root collar and by crown wilt stage.

[y] Four roots were sampled from each northern red oak, and the incidence of *C. fagacearum* was determined in four 10 cm long sub-samples taken at 0.3 m (1 ft) intervals from the root collar.
[z] Different letters indicate significant differences between means (α=0.05) for percent of root segments yielding *C. fagacearum* by distance (a-e) and total segments (A-E).

Root Order	Number of Roots Examined	Length of Roots Examined (m)	Number of Roots Involved in Inter-Tree Grafts
Primary	174	306	12
Secondary	259	144	7
Tertiary	85	64	2
Total	518	514	21

Table 3.3. Number, order, and total length of roots excavated and the number of roots per tree involved in inter-tree root grafting based on the occurrence of 13 inter-tree grafts found between four pairs of northern red oaks within 1.0 m (3.3 ft) of the soil surface. In several instances, individual roots were involved in more than one inter-tree graft.

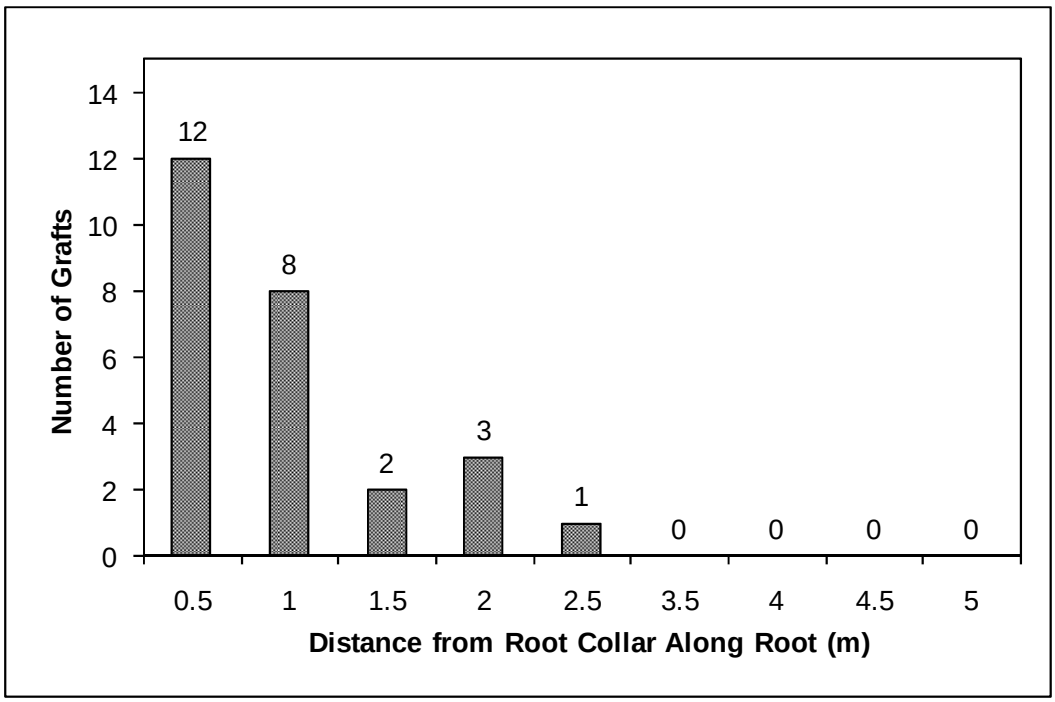

Figure 3.1. Frequencies of inter-tree graft distance from the root collar, measured along the length of each root involved in the graft union, based on the occurrence of 13 inter-tree grafts found between four pairs of northern red oaks within 1.0 m (3.3 ft) of the soil surface. Each inter-tree graft is represented by two measurements, one for each root involved in the graft union.

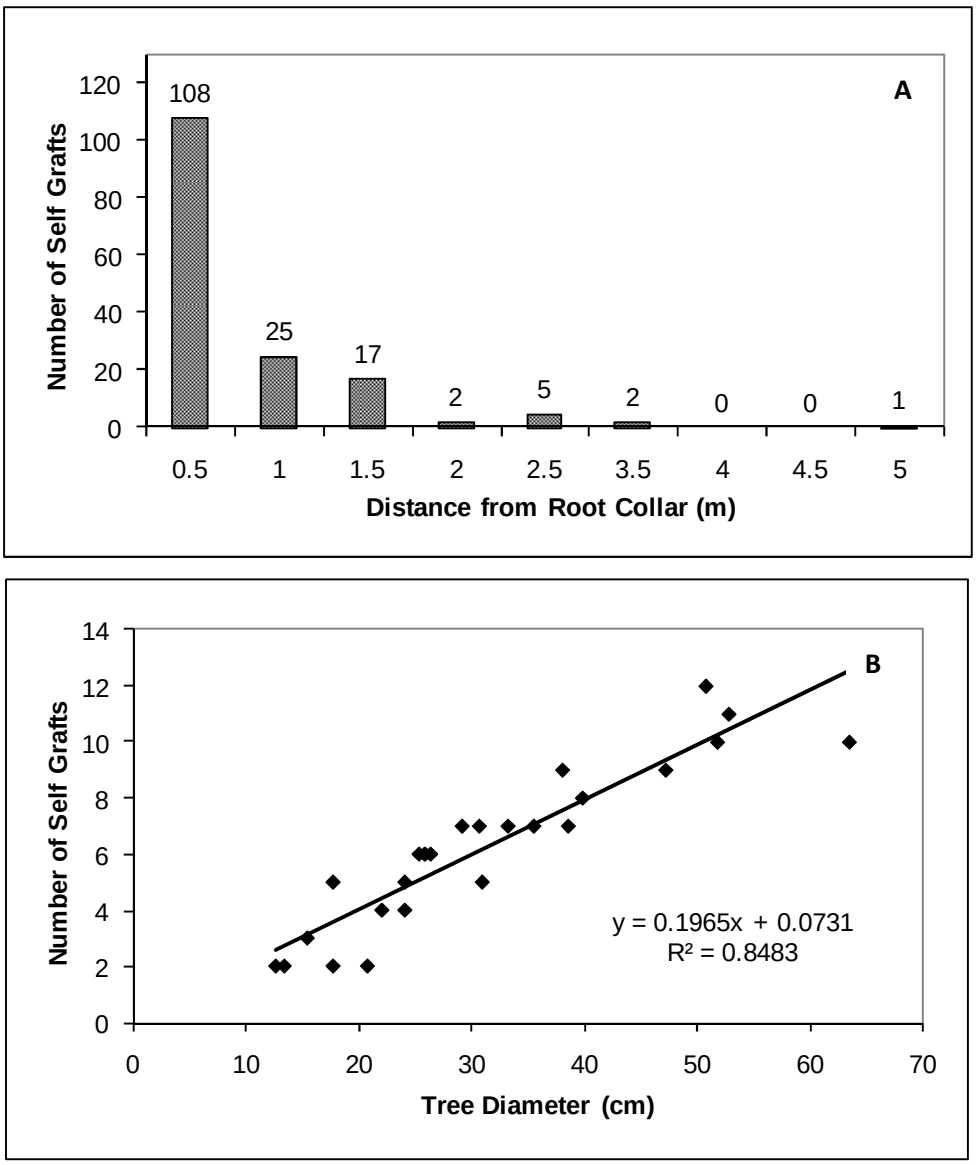

Figure 3.2. (A) Frequency of self-graft distance from the root collar, measured along the length of one root involved in the graft union, based on the occurrence of 162 self grafts in 24 northern red oaks. Each graft is represented by one measurement corresponding to the shortest distance to the graft along the two roots involved in the graft union. (B) Number of self grafts per northern red oak by stem diameter (cm) at breast height.

CHAPTER 4

Investigation of Plant Growth Regulating Properties associated with Propiconazole that may affect the Development of Oak Wilt

4.0. Introduction

Oak wilt, caused by the fungus *Ceratocystis fagacearum,* is a vascular disease of oak (*Quercus* spp.) in the eastern United States. The pathogen is carried by insect vectors such as sap feeding beetles of the *Nitidulidae* (Ambourn, Juzwik, and Moon, 2005) and is introduced into the vascular system of susceptible hosts during visitation to fresh wounds. In response to infection, oaks attempt to limit pathogen spread via prolific production of gums and tyloses in xylem elements. While *C. fagacearum* is known to be pathogenic on all 33 native oak species in the U.S. (Tainter and Baker, 1996), many species such as those belonging to the Section *Quercus* (collectively referred to as white oaks) exhibit various degrees of resistance and can survive infection for many years or even recover after successful compartmentalization of the fungus. However, in the Section *Lobatae* (red oaks) the pathogen has the capacity to kill its host very rapidly; widespread disruption of water conduction in the xylem results in mortality in a few weeks to several months. Once the fungus has entered the root system, *C. fagacearum* can spread across root grafts to proximal oaks, resulting in an expanding oak wilt disease center.

The systemic fungicide propiconazole has become an important tool for oak wilt management, and is primarily used to protect high-value red oaks near disease centers (Appel 2001). Widespread use of propiconazole began in the mid-1980's when it was shown to reduce the incidence of oak wilt in Texas live oaks (*Quercus fusiformis* and *Quercus virginiana*) (Appel, 1990) and Dutch elm disease caused by *Ophiostoma novo-ulmi* (Haugen and Stennes, 1999). Since that time, the effectiveness of propiconazole for the control of oak wilt has been well documented in several studies (Appel, 1990; 2001; Appel and Kurdyla 1992; Eggers et al., 2005; Johnson, 2001; Nair, 1995; Osterbauer and French, 1992; Osterbauer et al. 1994).

Propiconazole belongs to a class of demethylation inhibiting (DMI) compounds known as the triazoles. Since the introduction of the first triazole triadimefon in 1973 (Schwinn, 1983), a series of triazole compounds have been developed for use as either fungicides or as synthetic plant growth regulators (PGRs). The biological activity of these compounds arises from their inhibition of cytochrome P450-dependant monooxygenases that catalyze the hydroxylation reactions that transform isoprene-based precursors into biologically active molecules. In fungi, triazoles interfere with ergosterol biosynthesis via inhibition of the oxidative removal of the 14α-methyl group from 24-methylenedihydrolanosterol (Sisler and Ragsdale, 1984). This results in phospholipid instability, membrane disorder and permeability, and cell death. In plants, triazole inhibition of cytochrome P450 monoxygenases interferes with the synthesis of the natural plant growth regulator gibberellin, and can alter the metabolism of others including auxins, cytokinins, and absisic acid (Coolbaugh et al., 1978; Koller, 1987ab; Wiggins and Baldwin, 1984). The specificity of triazole molecules for monoxygenases of the sterol and gibberellin biosynthetic pathways varies considerably and ultimately determines if a compound will be utilized as a fungicide or PGR (Burden et al., 1987; Lurrsen, 1988; Sugavanam, 1984). Regardless of a compound's classification however, because they possess a common mode of action, most triazole compounds exhibit both fungicidal and plant growth-regulating properties to some degree (Baldwin and Wiggins, 1984; Fletcher et al., 1986; Izumi et al., 1985; Kuck and Scheinpflug, 1986; Takano et al., 1986; Wiggins and Baldwin, 1984).

While it is probable that fungitoxic concentrations of propiconazole exist in treated trees and direct inhibition of fungal growth is at least partially responsible for the chemical's proven efficacy, another possible mode of action for oak wilt control is through plant growth regulation. Resistance in some oak species is linked with natural PGR-regulated responses to infection, and fungal colonization and symptom development is highly dependent on tree growth, anatomy, and the development of structures including springwood vessels and tyloses (Jacobi and McDonald, 1980; Kuntz, 1964; Nair 1964; Nair and Kuntz, 1960; Nair, Kuntz, and Sachs, 1967; Parmeter, Kuntz, and Riker, 1956; Schoeneweiss, 1959; Sachs, Nair, and Kuntz, 1970; Tainter, 1995). *C. fagacearum* also

produces a variety of growth regulating substances that have a number of roles in the disease cycle (Fenn, Durbin, and Kuntz, 1978; Fergus and Wharton, 1957; Geary and Kuntz, 1962). Therefore, it is not surprising that there have been several reports of growth regulators being used to alter host physiology and anatomy in a way that reduces or prevents disease development (Kuntz, Nair, and Venn 1968). Northern pin oaks (*Q. ellipsoidalis*) treated with 2,3,6-trichlorophenyl acetic acid formed wood without xylem vessels. These trees were able to conduct water for normal physiological activity via transpiration through xylem parenchyma, fibers, and tracheids. After trees were inoculated, the pathogen and vascular plugging was confined to vessels formed prior to treatment and disease development was suppressed (Venn, Nair, and Kuntz, 1968). In another study, tylose formation was inhibited in oaks treated with cytokinins. Although fungal assays revealed that the pathogen was distributed throughout these trees, wilt symptoms were suppressed (Nair, Wolter, and Kuntz, 1969). Inhibition of earlywood formation and disease incidence was observed following treatments with the growth regulator 2,3,5-tri-iodobenzoic acid in pin oaks inoculated with *C. fagacearum* (Geary and Kuntz, 1962). Interestingly, an extra band of xylem similar to those produced in resistant oak species was produced artificially in pin oaks treated with the antimicrobial agent Vancide 51 (2-mercaptobenzothiazole) (Schoeneweiss, 1959), a broad spectrum monooxygenase inhibitor. (McCarty 1999; Weever, Van Den Neste, Verachtert, 1997). The compound 2-mercaptobenzothiazole was also effective in prolonging the latent period of *C. fagacearum* in inoculated pin oaks (Phelps, Kuntz, and Ross, 1966).

Propiconazole applications may alter tree growth, development, and the normal balance of plant growth regulating compounds in a manner that is antagonistic to infection, colonization, and disease development by *C. fagacearum.* A better understanding of a propiconazole's effects on both the pathogen *C. fagacearum* and oak species is needed to optimize the use of this chemical for the management of oak wilt. Therefore, the purpose of this preliminary investigation is to characterize the effects of propiconazole on anatomical features of northern pin oak that when altered, may affect the ability of *C. fagacearum* to infect, colonize and incite disease in its host.

4.1. Materials and Methods

4.1.1. Study Sites and Trees

The effect of propiconazole injections on the growth and anatomy of oak was investigated at three study sites in southeastern Minnesota: the Anoka County Airport in Blaine, Long Lake Regional Park in New Brighton, and the Carlos Avery Wildlife Refuge in Anoka County. The sites consisted of oak-dominated hardwood stands on loamy sand to sandy soil. At each site, eight co-dominant northern red oaks were selected for the study; four trees were treated with propiconazole and the remaining four trees were used as untreated controls. The average diameter of selected trees was 26 cm, and ranged from 17 cm to 56 cm; all trees were visually inspected, showed no symptoms of major diseases or other disorders, and had no major growth defects or injury. Fungicide treatments consisted of a single intravascular application of propiconazole, formulated as Alamo® (14.3% active ingredient), applied at the label rate of 20 ml of product diluted in 1.0 L of water per 2.5 cm of trunk diameter. The diluted product was injected into the base of treated trees on or near the root flares using a standard macroinfusion technique (Prosser, Zwack, and Johnson, not dated). Propiconazole applications were conducted in early July 2002 at Carlos Avery, early September 2003 at Long Lake, and mid-July 2004 at the Anoka County Airport.

4.1.2. Sample Collection and Processing

In October 2005, four branches (one branch from each quadrant of the four cardinal directions) were removed from the upper crown of each treated and control tree at all three sites. Harvested branches were 7 to 13 years old, a minimum of 3 years old at the time of fungicide application, and originated directly from the main stem or a main leader if a single dominant stem was not present. A 25 cm long segment was cut from the basal end of each harvested branch and placed into cold storage (4.5ºC).

A 10.0 cm long sub-sample was taken from each branch sample at a point where the branch exhibited the most straight and uniform growth. Each sub-sample was split radially and boiled in water for 10 minutes. Tangential and transverse sections 100-200 µm thick were cut from softened subsamples with a miter trimmer (Rockler Woodworking and Hardware, Medina, MN), submersed in a 10% bleach solution for 10

minutes, and dried at room temperature for 24 hours under compression between two glass slides. Prepared sections were photographed digitally under magnification with a Nikon DXM1200F Digital Camera mounted to a Nikon Optiphot compound microscope or a Nikon SMZ-10 stereoscope (Nikon Instruments Inc., Melville, NY).

4.1.3. Measurements and Data Collection

Growth changes in propiconazole-treated trees were assessed by comparing anatomical features in the growth ring formed during the year prior to treatment (Year -1) with the same anatomical features in growth rings formed in the year of treatment (Year 0) or in subsequent years: Year 1 (all sites), Year 2 (Long Lake and Carlos Avery), and Year 3 (Carlos Avery). Significant changes from Year -1 were then compared to changes observed in the growth rings of control trees during the corresponding time period. Nine anatomical features of the vascular system that were hypothesized to affect the oak wilt disease cycle and would be indicative of growth regulating effects were selected for assessment in each sampled branch: width of annual growth increment (µm), width of earlywood (µm), width of latewood (µm), percent area in transverse-section of earlywood occupied by vessel elements (%), average area of vessels in transverse-section (μm^2), largest and smallest vessel diameter (µm), concentration of vessels in earlywood (# vessels/cm^2), and the diameter of pits in the wall of xylem vessels (µm). Measurements were made using ImageJ (U.S. National Institute of Health, Bethesda, Maryland), a Java image processing program. Growth increment measurements were made at a minimum of three locations on each transverse section, vessel measurements were made on all vessels in each transverse section, and pit measurements were made on all pits within a single field of view under 400X magnification from minimum of three springwood vessels in each growth increment.

4.1.4. Data Summarization and Analysis

There was a high degree of variability in the average size of anatomical features among branches (Table 4.1). Therefore, measurements from different branches were not pooled for data analysis because we were only interested in changes over time within a branch rather than the inherent differences in feature size between different branches. To

assess for changes in growth over time in each branch, the average size of anatomical features in the growth ring formed during the year prior to treatment (Year -1) were compared with average size of features formed in each subsequent growth increment (Year 0 through Year 3) and expressed as a percent change. The average percent change in feature size was then calculated by growth increment for all branches combined and used for statistical analysis.

Data were analyzed in PROC MIXED of SAS Version 9.1.3 (SAS Institute, Cary, North Carolina) to accommodate covariance parameters. There was no significant difference in growth changes between sites or corresponding date of propiconazole application ($P=0.5454$), therefore data from all three sites were pooled for analysis. Treatment (treated or control) and year relative to treatment (Year -1 to Year 3) were treated as fixed effects; branch, tree, and site variables were treated as random effects. The dependency of measures from the same branch and tree was recognized through the REPEATED statement. Significant differences between means were determined using Tukey's multiple comparison test ($\alpha=0.05$).

4.2. Results

All trees sampled displayed no oak wilt symptoms prior to sample collection in 2005. Limited leaf cupping or curling indicative of minor phytotoxicity or growth regulation was noted in treated trees at all sites in the year after treatment. Transverse sections from each branch were examined for any abnormal growth patterns, however, no obvious macroscopic changes in growth were observed.

The widths (µm) of annual growth increments (Figure 4.1), earlywood (Figure 4.2A), and latewood (Figure 4.2B) decreased slightly but non-significantly ($P=0.2001$) in branches of control trees during the years after treatment, however observed changes did not significantly differ from zero ($\alpha=0.05$). No significant growth changes were observed in the year of treatment (Year 0) in propiconazole treated trees. Significant decreases in the widths of annual growth increments, latewood, and earlywood were observed in treated trees in Years 1, 2, and 3 after treatment compared to Year -1 ($\alpha=0.05$). These reductions were significantly different from those observed in control trees only in the year immediately following treatment (Year 1). This indicates that while significant

growth reductions were induced by propiconazole one year after treatment, the reductions observed after Year 1 may be due to some other factor(s) common to both control and treated trees. On average, the widths of growth increments in treated trees one year after treatment were 98% smaller than increments in Year -1. The reductions in the widths of earlywood and latewood one year after treatment were 26% and 406%, respectively. On average, latewood made up 62% of the total width of the annual growth increment in treated and control oaks in Year -1, therefore the overall reduction in the total width of the annual growth increment in treated trees was due mainly to the reduction of latewood production.

The size of vessels (μm^2) (Figure 4.3) and largest and smallest diameters (μm) of vessels (Figure 4.4) were significantly reduced in propiconazole treated trees one and two years after treatment compared to the Year -1 and control branches (α=0.05). There was a slight but non-significant increase in vessel size (P=0.6699) and large and small diameters (P=0.4360 and 0.4468, respectively) over time in control trees. In contrast, during the first two years after treatment (Year 1 and 2) vessel size was reduced by 20% (P=0.0008) and vessel diameters were reduced by 10% (P<0.0006) on average in treated trees. A slight reduction of vessel size (8%) and vessel diameters (4%) was observed in the third year after treatment, but this reduction was not significantly different from zero (α=0.05). The diameter of pits in the wall of xylem vessels (Figure 4.5) did not significantly change over time post-treatment (P=0.7646) and did not differ from the diameter from pits in control trees (α=0.05).

The concentration of vessels in earlywood (no. vessels/cm^2) (Figure 4.6A) was also significantly reduced subsequent to treatment in trees treated with the fungicide (P<0.0001); the 22.3% and 21.7% reduction in vessel concentration observed one and two years after treatment respectively, significantly differed from changes observed in control trees (2.4% and 2.0%, respectively) (α=0.05). A 20% reduction in vessel concentration was also observed in Year 3, but this difference was not significantly different from a small 1% reduction observed in control trees during the same time period (α=0.05). The percent of earlywood occupied by vessels in cross-section (Figure 4.6B) was also reduced in treated trees after propiconazole application (P<0.0001), and the reductions observed one year (22.2%) and two years (21.5%) after treatment significantly

differed from the statistically non-significant changes observed in the controls during the same time period (2.3% and 2.1 %, respectively) (α=0.05).

4.3. Discussion

Significant growth reduction occurred in the vascular system of propiconazole treated trees in the years following treatment at all study sites (Figure 4.7). Regardless of the year when treatments were applied, reductions in growth were highest in the first one to two years subsequent to treatment depending on the anatomical feature being assessed. The width of annual growth rings, both earlywood and latewood, were reduced significantly in the year after propiconazole injections, but the effects were not statistically significant thereafter. Effects were longer lasting on the size and number of vessels produced in the xylem with significant reductions observed for two years. Of the nine features assessed, only the diameter of the pits in walls of xylem vessels were unaffected by propiconazole applications. This is the first report that propiconazole possesses plant growth regulating properties in oak, and that use of this fungicide can affect the anatomy of the host in addition to inhibiting growth of the pathogen when used for oak wilt control.

Plant growth regulation by propiconazole may play a role in preventing infection or disease development in treated trees. Natural resistance to *C. fagacearum* in some oak species is frequently attributed to anatomical features and growth changes in response to infection that assist with pathogen compartmentalization. For instance, resistance in infected white and chestnut oaks has been linked to the development of an extra band of xylem cells (Kuntz, 1964; Nair 1964; Schoeneweiss, 1959), production of specialized cell layers that assist in compartmentalization (Kuntz, 1964), formation of dark masses of electron dense material around large xylem vessels (Jacobi and McDonald, 1980; Sachs, Nair, and Kuntz, 1970), and temporally expedient but spatially limited tylose development (Jacobi and MacDonald, 1980; Nair, 1964; Nair and Kuntz, 1960; Nair, Kuntz, and Sachs, 1967; Parmeter, Kuntz, and Riker, 1956; Tainter, 1995). Susceptibility of oaks to infection by *C. fagacearum* appears to be greatest during maximum physiological activity of the host, and symptom development is closely associated with the formation of the earlywood vessels (Nair, 1964) that are conducive to rapid

distribution of conidia and prone to extensive tylose production and embolisms (Drake, 1956; Parmeter, 1955; Nair, 1964). In several studies, symptoms were reported to develop around the same date regardless of the date of inoculation, and it has been suggested that natural plant growth regulators or their effects on growth and development act as a trigger for symptom induction (Nair, 1964; Skelly and Wood, 1974; Skelly and Merrill, 1968). *C. fagacearum* produces growth regulating enzymes and substances such as indole acetic acid (IAA) (Fenn, Durbin, and Kuntz, 1978; Fergus and Wharton, 1957; Geary and Kuntz, 1962) that increase the plasticity of cell walls, weaken the cytoskeleton, assist the hydrolysis of pectic materials in the cell wall, and cause rapid proliferation of ray parenchyma (Struckmeyer, Esther, and Kuntz, 1954; Struckmeyer, Kuntz, and Riker, 1958; Tainter, 1995). Clearly, natural growth regulating substances play an important role in the oak wilt disease cycle.

Although the effects of propiconazole-induced growth changes on the oak wilt disease cycle (e.g. infection, colonization, or symptom development) were not directly assessed, some of the observed changes in oak anatomy could affect infection, colonization, and disease development by *C. fagacearum*. For instance, fewer and narrower vessels may be less prone to embolisms (Tainter, 1995), could inhibit movement of pathogen propagules in the vascular system, reduce the frequency of invasion of parenchyma cells associated with vessels, or alter host-pathogen interactions that may trigger symptom development. Smaller annual growth rings occupied by fewer vessels may be denser and more capable of compartmentalizing the pathogen, however in this study, dense latewood production was reduced to a much greater degree than was earlywood production. There is extensive evidence suggesting that tylose formation is the greatest contributing factor to the development of wilt symptoms and the severity of wilt is directly related to the extent of tylose formation (Parmeter, Kuntz, and Riker, 1954; Struckmeyer, Beckman, Kuntz, and Riker, 1954, Tainter, 1995), but tylose formation is only possible if the pit aperture is greater than 10 µm wide (Esau, 1977). While diameter of pits was not reduced in this study, it may be possible that pit diameter reduction could be induced with different fungicide rates or application methods. However, the greatest potential for plant growth regulator-induced control of oak wilt may lie with changes in anatomical features not assessed in this study. This preliminary

investigation shows that oak anatomy is affected by propiconazole in a number of ways, but changes in cell wall thickness, the distribution of xylem parenchyma, and patterns of tylose formation for example, could play a substantial role in disease suppression if they are affected by propiconazole. Direct investigations of the effect of artificially-induced changes in oak anatomy on the oak wilt disease cycle are therefore needed.

Although propiconazole is commonly utilized to protect high value oaks near oak wilt disease centers, a number of questions and concerns regarding the extent of fungicide translocation in treated trees and propiconazole's effects on the pathogen remain unanswered. For instance, propiconazole is thought to have little if any phloem mobility and extensive translocation into the root system following injection is unlikely (Nair 1995). It is now believed that propiconazole does not prevent infection via root graft transmission of *C. fagacearum* nor does it eradicate the pathogen from the root system. Rather, propiconazole is thought only to prevent or delay symptom development in infected trees (Chapter 2; Appel, 1995; Wilson and Lester, 2002). *C. fagacearum* can survive for more than five years in the root system of trees that have succumbed to oak wilt (Skelly and Wood, 1974a; Yount, 1958), and latent infections lasting two or more years in trees treated with propiconazole have now been confirmed (Chapter 2). However, the fungicide is not detectable in the crowns of trees after 12 months (Armstrong, 1999; Osterbauer and French, 1992) because of thermal and enzymatic degradation of the molecule as found in American elm (*Ulmus Americana*) (Armstrong, 1999). Furthermore, the effective fungicidal and fungistatic concentrations of propiconazole have not been determined *in vivo*, the fungicide has not been shown to move into new growth each year, and translocation in the symplast has not been demonstrated. Therefore, the eradicative and prophylactic capabilities of propiconazole based on fungitoxicity alone are in question. Recently, it was shown that substantial concentrations of propiconazole remain in the root crown and lower stem for at least two years in treated trees (Chapter 2). Propiconazole-induced changes in the balance of natural plant growth regulators produced in oak and the subsequent changes in growth and development induced by treatment, could be an additional mechanism for longer-lasting protection throughout treated trees because direct contact of the propiconazole molecule with the fungus would not be required.

4.4. Conclusions

While the fungicidal or fungistatic activity of propiconazole may contribute to treatment efficacy, the compound's longevity and distribution in injected oaks is limited. Considering these limitations, it is not currently understood how propiconazole can provide disease protection throughout the entire tree for two or more years (Eggers et al., 2005) based on fungitoxicity alone. Propiconazole's efficacy may be enhanced by the compound's effects on the production and metabolism of plant growth regulators such as gibberellins, auxins, abscisic acid, and cytokinins which are translocated throughout the entire tree and can affect many physiological processes and anatomical features that could interfere with the oak wilt disease cycle. Our data reveal that propiconazole applications do affect growth and anatomy in the xylem, though the effects on infection and diseases progression are unknown. A more thorough understanding of the host-pathogen-fungicide interaction is needed to adapt fungicide formulations, rates, and application methods to increase treatment efficacy should propiconazole-induced growth regulation be clearly shown to play a role in protection from oak wilt in treated trees.

4.5. Figures and Tables

Measurement			Control					Propiconazole				
Feature [z]	Units	Statistic	Year -1 n=48	Year 0 n=48	Year 1 n=48	Year 2 n=32	Year 3 n=16	Year -1 n=48	Year 0 n=48	Year 1 n=48	Year 2 n=32	Year 3 n=16
WGR	µm	*Mean*	1083	1098	954	937	998	970	836	541	546	526
		StDev	593	586	621	612	686	601	525	375	477	537
WEW	µm	*Mean*	414	444	427	443	511	358	342	291	266	251
		StDev	142	177	203	227	254	154	147	141	124	96
WLW	µm	*Mean*	669	654	526	494	486	624	504	254	297	292
		StDev	515	493	477	436	474	494	421	271	419	461
VDL	µm	*Mean*	131	132	133	142	157	116	116	109	105	110
		StDev	31	27	29	24	23	17	16	27	17	12
VDS	µm	*Mean*	100	101	101	110	123	88	89	82	80	83
		StDev	25	22	25	21	19	14	14	13	13	8
VAR	μm^2	*Mean*	11966	12056	12218	14044	17313	9095	9185	9110	7648	8179
		StDev	5649	4962	5610	4900	4841	2751	2641	10059	2643	1635
VCN	vessels/cm^2	*Mean*	73170	77041	75517	79190	89465	58593	62997	48756	46454	49975
		StDev	25273	25702	24191	22941	25000	13490	15682	11198	11680	14619
VPA	%	*Mean*	15.9	17.2	17.5	17.8	19.7	15.8	14.9	16.1	16.8	17.2
		StDev	3.7	10.6	10.9	3.9	4.1	4.4	3.4	3.9	4	3.2
PDI	µm	*Mean*	17	18	18	19	21	16	16	14	14	14
		StDev	4	3	4	3	3	2	2	3	2	1

Table 4.1. Average measurement and standard deviation of anatomical features in the xylem of branches from red oaks treated with Alamo® (20.0 ml per 2.5 cm DBH) or untreated (control) trees at three sites in Minnesota by year relative to treatment application date.

[z] WGR=width of annual growth increment; WEW=width of earlywood in annual growth increment; WLW=width of latewood in annual growth increment; VDL=vessel diameter along the longest axis; VDS=vessel diameter along smallest axis; VAR=area of vessel in transverse-section; VCN=vessel concentration in annual growth increment; VPA=percent area occupied by vessels in transverse section in annual growth increment; PDI=diameter of pits in vessel walls.

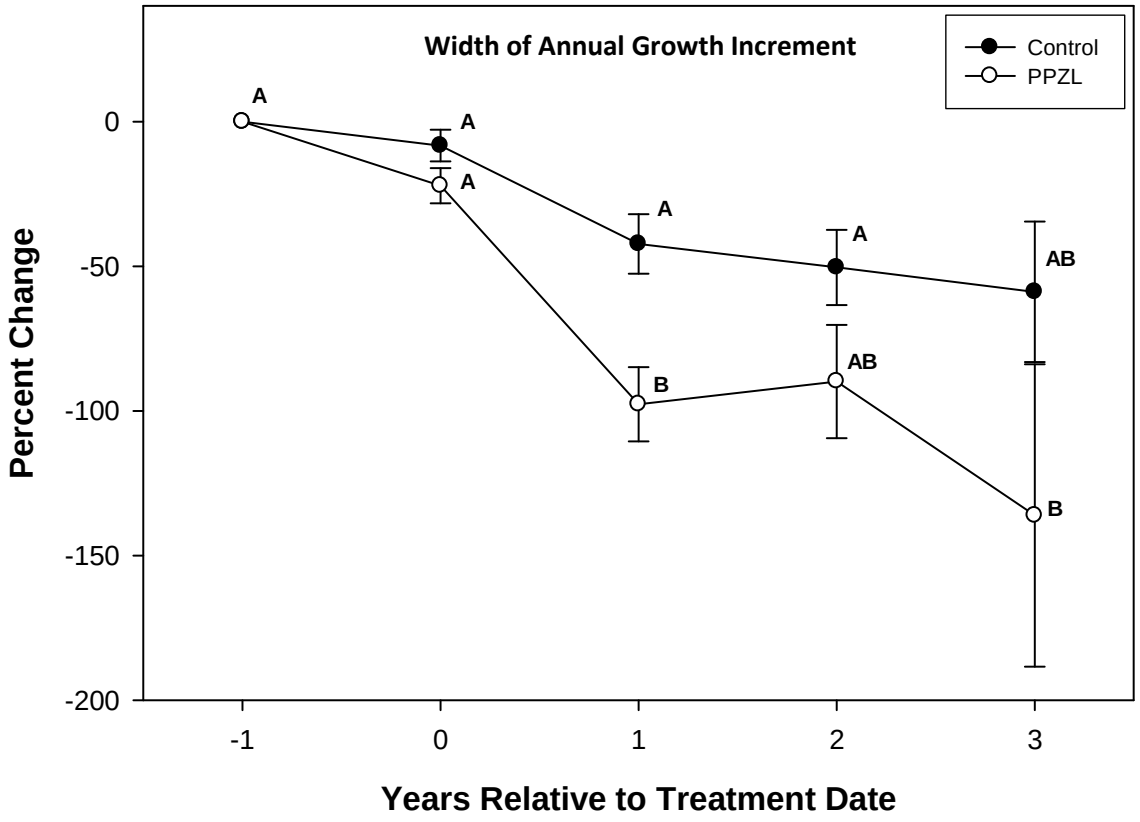

Figure 4.1. Percent change (relative to Year -1) in the width of annual growth increments in the year of treatment (Year 0) and subsequent years, in the branches of trees treated with propiconazole (PPZL; 20.0 ml Alamo® per 2.5 cm dbh) or untreated control trees. Significant differences among means are indicated by different letters as determined by Tukey's multiple comparison test (α=0.05). Error bars indicate +/- standard error of the mean.

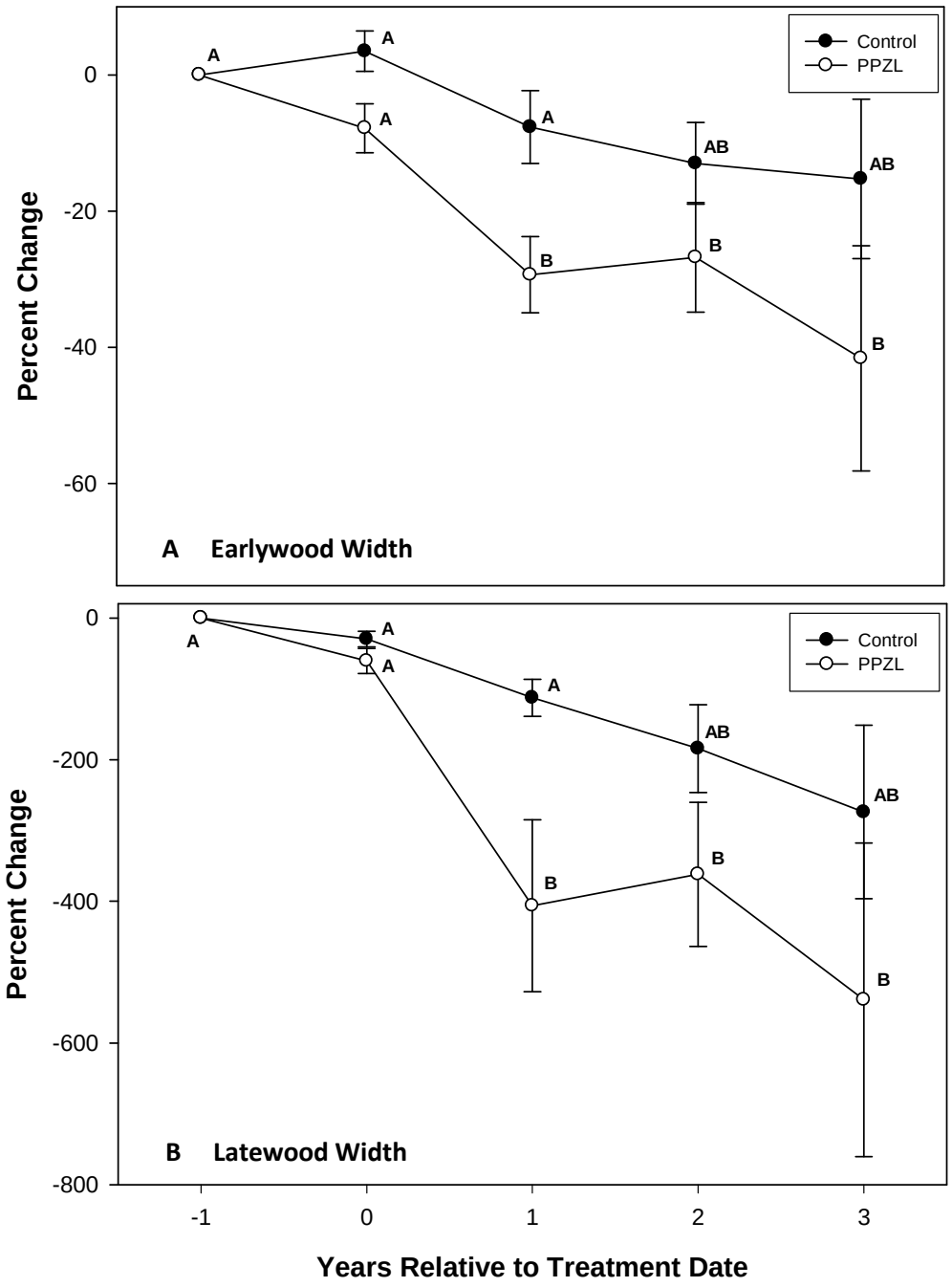

Figure 4.2. Percent change (relative to Year -1) in the width of earlywood (A) and latewood (B) in the year of treatment (Year 0) and subsequent years, in the branches of trees treated with propiconazole (PPZL; 20.0 ml Alamo® per 2.5 cm dbh) or untreated control trees. Significant differences among means are indicated by different letters as determined by Tukey's multiple comparison test (α=0.05). Error bars indicate +/- standard error of the mean.

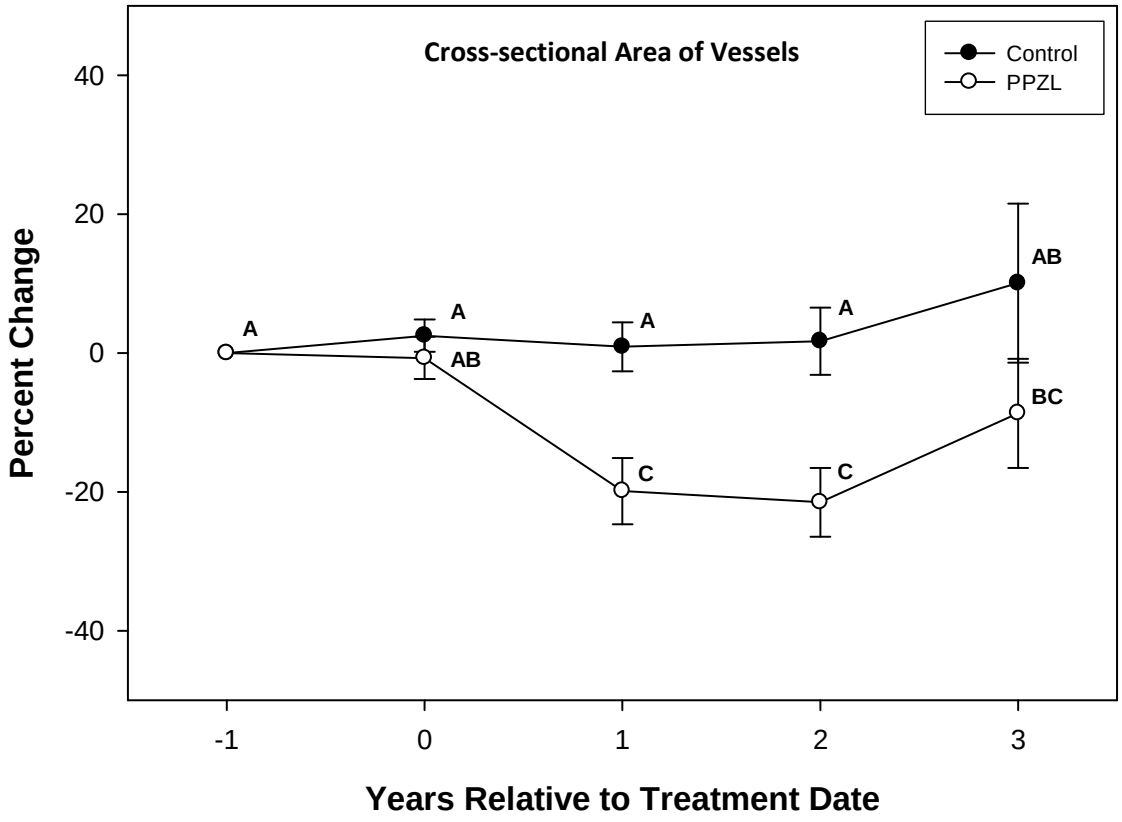

Figure 4.3. Percent change (relative to Year -1) in the size (area) of vessels in transverse sections in the year of treatment (Year 0) and subsequent years, in the branches of trees treated with propiconazole (PPZL; 20.0 ml Alamo® per 2.5 cm dbh) or untreated control trees. Significant differences among means are indicated by different letters as determined by Tukey's multiple comparison test (α=0.05). Error bars indicate +/- standard error of the mean.

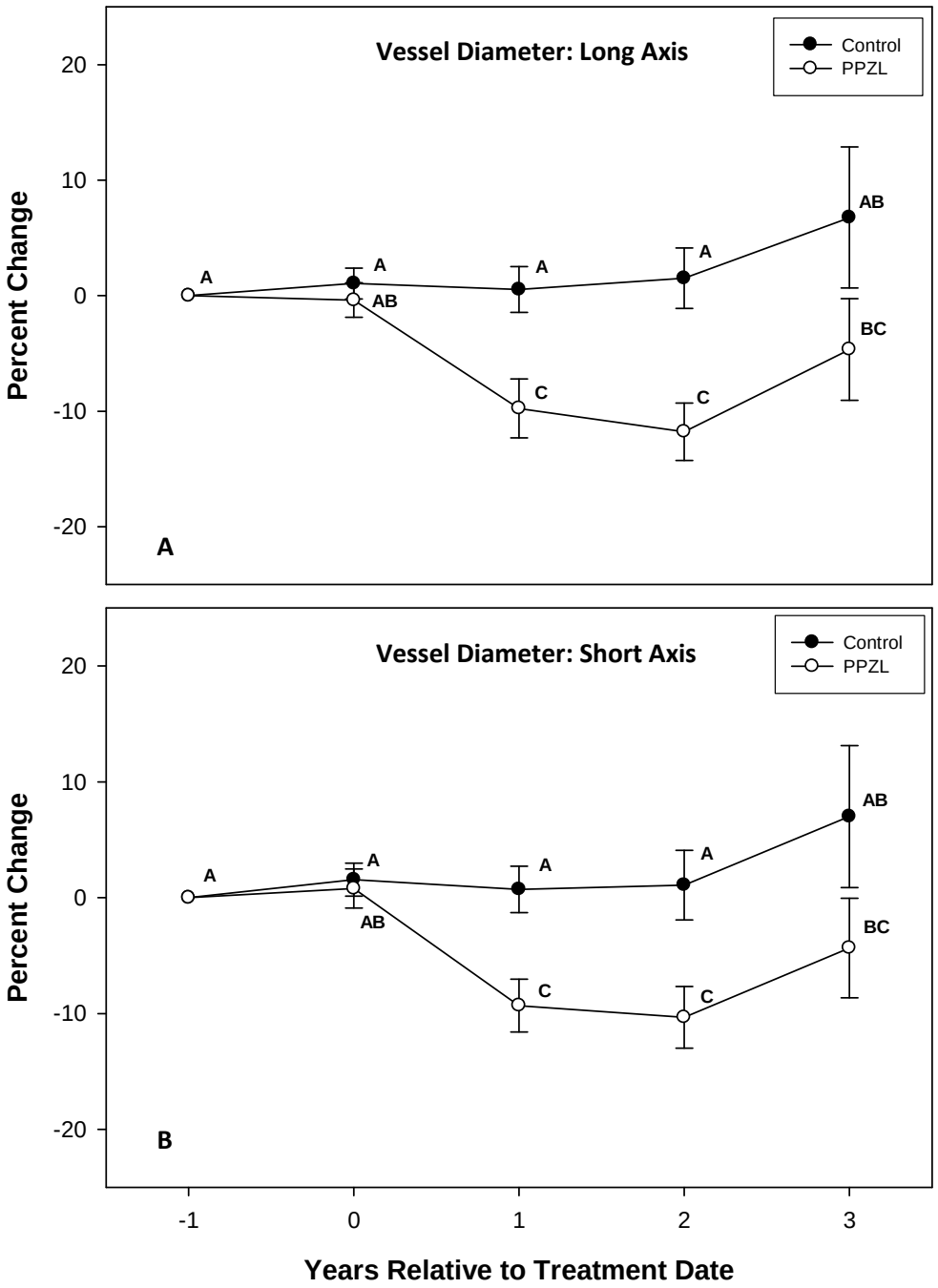

Figure 4.4. Percent change (relative to Year -1) in the largest (A) and smallest(B) diameter of vessels in the year of treatment (Year 0) and subsequent years, in the branches of trees treated with propiconazole (PPZL; 20.0 ml Alamo® per 2.5 cm dbh) or untreated control trees. Significant differences among means are indicated by different letters as determined by Tukey's multiple comparison test (α=0.05). Error bars indicate +/- standard error of the mean.

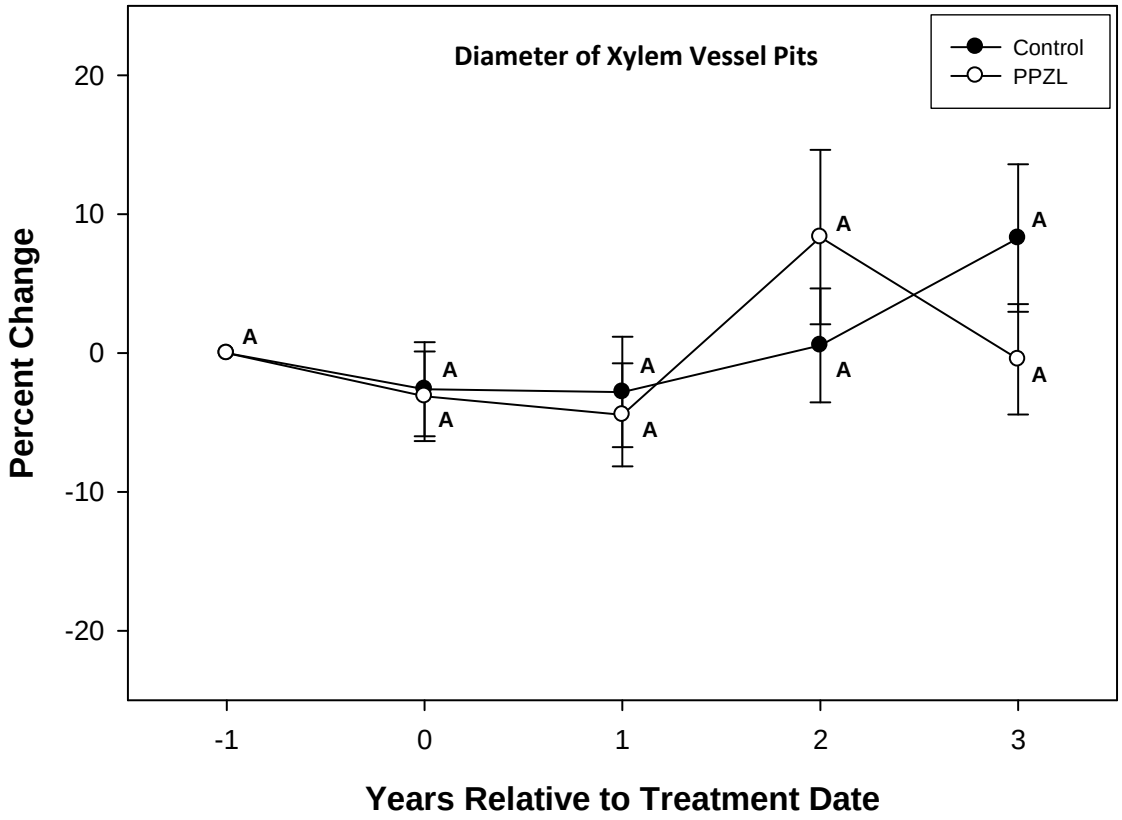

Figure 4.5. Percent change (relative to Year -1) in the diameter of pits in the xylem vessel walls in the year of treatment (Year 0) and subsequent years, in the branches of trees treated with propiconazole (PPZL; 20.0 ml Alamo® per 2.5 cm dbh) or untreated control trees. Significant differences among means are indicated by different letters as determined by Tukey's multiple comparison test (α=0.05). Error bars indicate +/- standard error of the mean.

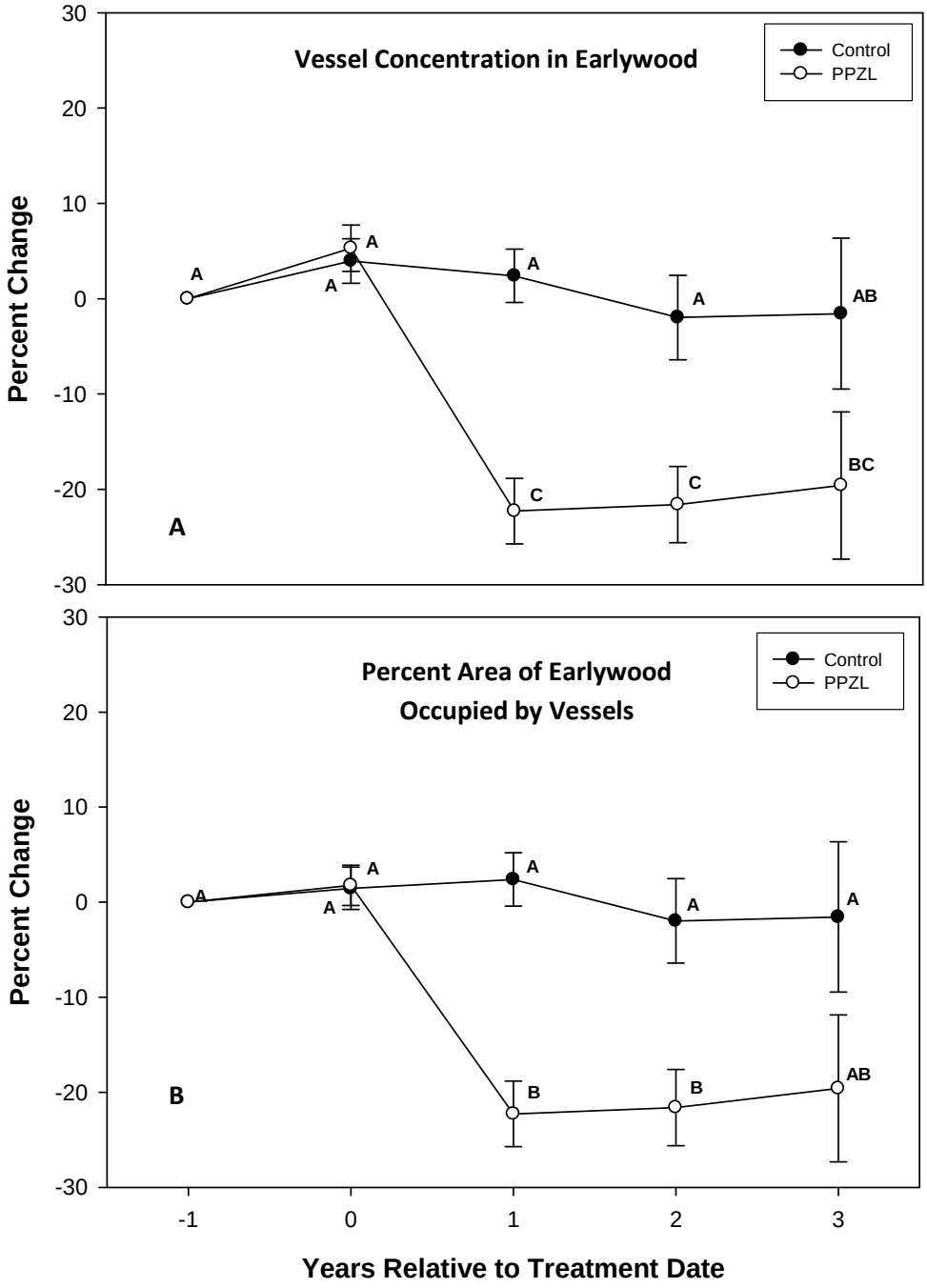

Figure 4.6. Percent change (relative to Year -1) in the concentration of vessels in earlywood (A) and the percent of earlywood occupied by vessels in transverse sections (B) in the year of treatment (Year 0) and subsequent years, in the branches of trees treated with propiconazole (PPZL; 20.0 ml Alamo® per 2.5 cm dbh) or untreated control trees. Significant differences among means are indicated by different letters as determined by Tukey's multiple comparison test (α=0.05). Error bars indicate +/- standard error of the mean.

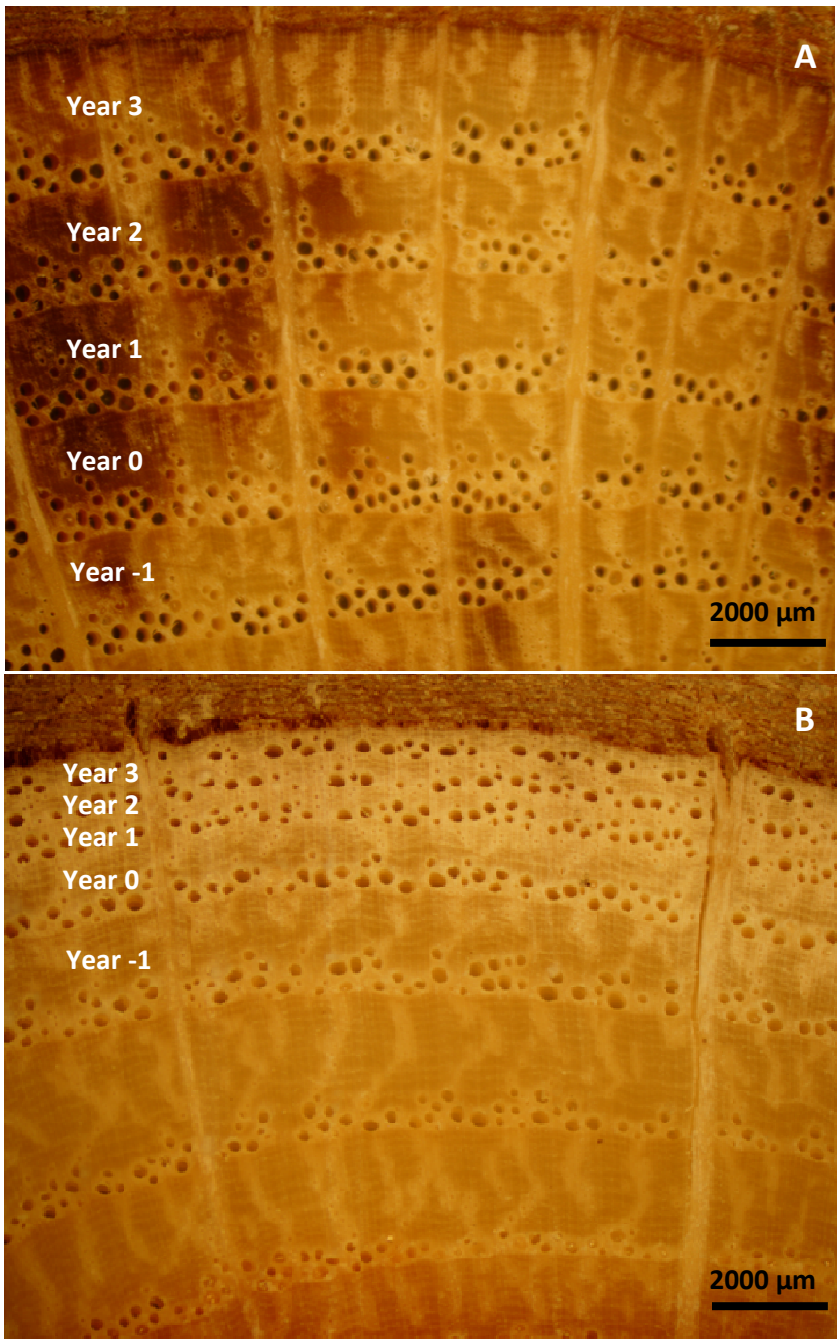

Figure 4.7. Representative transverse sections of branches from an untreated control tree (A) and a tree treated with propiconazole (20.0 ml Alamo® per 2.5 cm dbh) (B) at Carlos Avery Wildlife Refuge, Anoka County, MN. Trees were treated in June 2002 (Year 0).

CHAPTER 5

Summary and Implications for Oak Wilt Management

5.0. Introduction

Oak wilt, caused by the vascular pathogen *Ceratocystis fagacearum* (T.W. Bretz) J. Hunt, is considered to be the most important disease of oaks (*Quercus* spp.) in the United States. While all native species of oaks (*Quercus* spp.) are susceptible, resistance mechanisms in the white oaks (Section Quercus) allow species such as white oak (*Q. alba*) and bur oak (*Q. macrocarpa*) to survive with the disease for many years, or fully recover after successful compartmentalization of the fungus. The red oaks (Section *Lobatae*) and live oaks (*Q. fusiformis* and *Q. virginiana*) are highly susceptible however, and mortality rapdily follows infection within a few weeks to a few months. Insect vectors are responsible for overland transmission of the *C. fagacearum* and the establishment of new disease centers, but the pathogen is transmitted primarily through inter-tree root grafts between proximal trees.

A successful integrated management program for oak wilt limits both overland and root graft transmission of *C. fagacearum.* Overland transmission is managed by removal of inoculum-producing trees, and cultural controls such as avoidance of pruning oaks during periods of high beetle activity and inoculum production. Root graft transmission is managed more directly because the spatial distribution of the fungus is limited. Mechanical disruption of root grafts using vibratory plows or trenching equipment severs conduits for infection, and when constructed correctly, the effect of these “root graft barriers” generally last for a sufficient period of time to prevent further expansion of a disease center. In many instances, the installation of root graft barriers may not be feasible because of site conditions and the lack of appropriate equipment. Systemic fungicides have been used for nearly 30 years as a viable alternative to root graft disruption, and as an additional level of protection for high-value trees.

Propiconazole is a systemic fungicide introduced in the early 1980's and is widely used for the control of oak wilt today. The fungicide can be introduced into the vascular system of trees using a variety of application methods, but intravascular injections of highly diluted active ingredient are preferred because they result in thorough distribution and low phytotoxicity. Propiconazole is used prophylactically to protect healthy trees that are within root grafting distance of wilting trees from contracting oak wilt, and therapeutically to eradicate the pathogen from wilting trees. Generally, therapeutic use of propiconazole is limited to more resistant oak species that can compartmentalize the pathogen after treatment application, and only in cases where symptoms are restricted to a relatively small proportion of the crown. The success of therapeutic treatments in more resistant oak species also means that treatment to prevent root graft transmission in those species is generally unnecessary, i.e. the cost of treatment and injury to the tree incurred during the application of a preventative treatment is not offset by the reduced risk for infection. Conversely, therapeutic treatments in susceptible red oak species are relatively ineffective, especially during advanced stages of crown wilt; therefore propiconazole application prior to infection is the most appropriate treatment in these species.

Although propiconazole has become increasingly popular in recent years, little is known about the distribution and longevity of the fungicide in the vascular system, its effects on the pathogen and host, and its long term efficacy. Recent studies indicate that propiconazole injections may be effective for no more than two years (Eggers et al., 2005), therefore arborists have begun to retreat oaks on a biannual basis. Arborists and researchers have reported high mortality rates in propiconazole-treated trees when follow-up treatments were not conducted. Treatment failure is unacceptable due to the expense associated with fungicide injections, and more importantly, because a latent or masked infection represents an inoculum source that is not accounted for in management regimes. Oak wilt development in treated trees may result from poorly informed management decisions that do not take into account pathogen distribution or the capabilities of the systemic fungicide being utilized.

In this dissertation are the results of three investigations conducted to examine use of propiconazole to control root graft transmission of *C. fagacearum* in red oaks (Section *Lobatae*). It was hypothesized that oak wilt development in propiconazole-

treated trees is an indirect result of poorly informed decisions to inject trees without a complete understanding of the host-pathogen-fungicide interactions at work. Many of these interactions are uncharacterized to date. Therefore, these investigations were designed to characterize five aspects of the host-pathogen-fungicide interactions that could improve our understanding and use of propiconazole for oak wilt control:

1) The extent of propiconazole distribution and longevity of the fungitoxic molecule in the root system of intravascularly injected trees.
2) The prophylactic and eradicative capabilities of propiconazole on *C. fagacearum* in the root system of intravascularly injected trees
3) The spatial and temporal distribution and pattern of spread of *C. fagacearum* in roots of trees in oak wilt disease centers
4) The frequency of root graft occurrence and the presence of *C. fagacearum* in roots involved in root grafts
5) The effect of putative plant growth regulating properties of propiconazole on the growth and anatomy of oak and the oak wilt disease cycle.

The remainder of this chapter is a discussion of the results of these investigations in the broader context of oak wilt management and the use of propiconazole for the control of vascular diseases. The reader is encouraged to refer to Chapter 1 for relevant supporting literature and justification / rationale for the approach taken.

5.1. Propiconazole Distribution and Longevity in the Root System

Intravascular propiconazole injections introduce the active ingredient into the xylem, and translocation in the transpiration stream is needed to distribute the compound in the tree's stem and crown. Application methods have been optimized to achieve maximum distribution in the canopy, however little consideration was given to distribution of the fungicide in the root system. This is surprising considering propiconazole is used in highly susceptible oak species to prevent root graft transmission rather than infection via insect vectors. If prophylactic treatments are truly intended to prevent infection via root grafts, then thorough distribution of the fungicide in the root

system is required. It has previously been suggested that propiconazole does not prevent root graft transmission of *C. fagacearum* (Appel, 1995; Wilson and Lester 2002), yet this has not dissuaded use of propiconazole for this purpose because treatment efficacy is very good within two years of treatment.

An alternative explanation for treatment efficacy is that propiconazole, rather than preventing infection, suppresses disease expression. *C. fagacearum* induces prolific tylose development and gum production in the water conducting elements of the stem and branches, resulting in the disruption of the transpiration stream, wilting, and death. However, the root system does not respond to infection in a similar manner. The incidence of tylose formation in the roots is low, and gum production has not been observed (Nair and Kuntz, 1975; Parmeter, Kuntz, and Riker, 1954; Struckmeyer, et al., 1954; Struckmeyer, Kuntz, and Riker, 1958, Yelenosky and Fergus, 1959). Therefore, *C. fagacearum* can survive in the root system of oaks without causing disease symptoms or death. If a tree is treated with propiconazole and is/becomes infected, pathogen propagules carried upward in the transpiration stream may be unable to colonize or cause symptoms in above-ground tree tissues containing fungitoxic concentrations of the fungicide. Overtime however, propiconazole is degraded and above ground tissues are no longer protected; subsequently, symptoms develop and treatment failure is observed. Propiconazole has not been detected in the stem or branches of treated trees after 12 months (Osterbauer and French, 1992), and the half-life of the active ingredient is between 60 to 100 days at room temperature (Armstrong, 1999). This hypothesis could explain increasing treatment failure rates two years after treatment.

This investigation revealed that propiconazole movement into the root system does occur following macroinjection. Although the total extent of propiconazole distribution in the root system could not be determined, propiconazole concentrations were commonly in the tens to hundreds of parts per million within one meter of the root flare injection site. The concentration of propiconazole decreased with increasing distance from the injection point however, and although not directly measured, it is unlikely that propiconazole would be found more than a few meters from the root flare following application. Therefore, it is unlikely that propiconazole could prevent root graft transmission throughout the entire root system, because root grafts are known to

commonly occur within 20 m of the stem (Juzwik et. al. 2004; Nair and Kuntz, 1975). High concentrations of propiconazole in the root crown zone could prevent the spread of *C. fagacearum* out of an inter-tree grafted root, should it become infected, into other roots or the stem because the pathogen would need to pass into the root crown zone to do so. Networks of self-grafts (Section 5.5) were common in the root system of oaks and could serve as a conduit for colonization of other roots while avoiding the root crown zone. However, the incidence of self-grafts is highest within a few meters of the stem where biologically significant concentrations of propiconazole may be present.

Because degradation of propiconazole is temperature dependent (Armstrong 1999), the highest rates of degradation would be expected to occur in the stem and branches of trees because they are not insulated by soil. Several studies have noted the absence of propiconazole in the stem and branches of treated trees after 12 months (Armstrong, 1999; Osterbauer and French, 1992) and sometimes as soon as seven months after treatment. Rapid degradation of the active ingredient above-ground could result in a lack of disease suppression much sooner than two years after treatment. During this thesis investigation, propiconazole was detected in the roots and lower stem 24 months after treatment. While degradation occurred in these tissues over the two year study, residual but potentially biologically significant levels of the fungicide in the roots and lower stem may be the underlying cause for treatment efficacy lasting two or more years. One possibility is that pathogen propagules passing through the root crown region where propiconazole concentrations are high may be killed or rendered inviable prior to spreading to above-ground tissues where propiconazole may be absent.

Propiconazole is not thought to be phloem mobile; therefore, it is unlikely that downward movement into the root system following injection at the root flares is the result of basipetal translocation in the symplast. Rather, the positive pressure generated by the injection equipment may force the fungicide both upward into the stem and downward for a limited distance into the roots. Propiconazole also does not move into new growth, rather the fungicide appears to be relatively confined to the growth ring(s) actively transporting water at the time of injection (Armstrong, 1999). Because of the vertically discontinuous but more interconnected nature of vascular elements in the root crown and root/stem union (Yelenosky and Fergus, 1959), and lack of annual tylose

production in the roots that would occlude older xylem elements (such as the annual occlusion that occurs in the stem and branches of most oak species), it is possible that propiconazole forced into the root crown during injection may be capable of limited translocation into new vascular elements, providing multi-year protection while residual active ingredient remains (see section 5.3). The discontinuous nature of the vascular elements in the root crown zone would also slow the spread of pathogen propagules from the roots to the stem, allowing greater exposure to the fungicidal molecule.

Based on these results and previous research, it appears that propiconazole cannot completely prevent root graft transmission of *C. fagacearum* in a treated tree because of limited distribution in the root system. Rather, the presence of propiconazole in above-ground tissues, and especially in the root crown, may prevent colonization and disease development following root graft transmission. Application methods, treatment timing, and fungicide formulations and rates that maximize distribution and retention in these tissues could improve the longevity and efficacy of propiconazole injections.

5.2. Prophylactic and Eradicative Capabilities of Propiconazole

Appel and Kurdyla (1992) reported the effective concentration of propiconazole to reduce growth of the *C. fagacearum in vitro* ranges from 2.0 ppb to 15.7 ppb depending on the isolate used for analysis. Wilson and Forse (1997) later determined that the concentration of propiconazole necessary to kill 50% of the inoculum *in vitro* ranged between 10 and 100 ppb. Yet the usefulness of this information is limited as concentrations may not correspond to those required *in vivo* to achieve a desirable level of disease control. To date, no studies have been conducted to determine fungicidal or fungistatic concentrations of propiconazole *in vivo*.

As discussed in section 5.2, propiconazole applications likely do not completely prevent root graft transmission of *C. fagacearum* because fungicide distribution in the root system is limited. However, the data from this study revealed that root-inoculations, intended to mimic root-graft transmission of the pathogen (one meter from the root flare) were successful in infecting trees injected with propiconazole two weeks prior to inoculation. This is very surprising because the concentration of propiconazole in the roots at the inoculation point were in the parts per million range two months after

treatment. It was not expected that the pathogen would survive in tissues where propiconazole was present at the time of inoculation. Never the less, two years after treatment, isolations from root samples continued to yield the pathogen. Thus, even when present in biologically significant concentrations, propiconazole may not prevent root graft transmission. Prophylactic treatments in this study did not prevent infection, only disease development for an extended period of time.

Therapeutic treatments were equally unsuccessful in preventing infection or eradicating the pathogen from the root system. Although the pathogen had a two week "head start" prior to propiconazole injections, disease symptoms were suppressed for the entire two year study, and pathogen incidence in the root system did not significantly differ from prophylactically treated trees. Yet, after two years the pathogen was still present in the root system, even in samples containing high concentrations of propiconazole.

The concentration of propiconazole was not determined in individual growth rings or xylem elements, and therefore it was not possible to determine the levels of the fungicide to which the pathogen was directly exposed. Yet, it appears that fungicidal concentrations of propiconazole are much lower *in vivo* than *in vitro.* Two and twelve months after treatment, *C. fagacearum* was not isolated from samples with propiconazole concentrations greater than 40 ppm. After 24 months however, fungicide concentration did not correlate with *C. fagacearum* viability. The abrupt change in propiconazole efficacy after 24 months could be explained by compartmentalization of the fungicide in the previous year's growth ring. If the fungicide is confined to older growth increments as the pathogen spreasd into and through new annual increments, direct contact of pathogen with the fungicide would be avoided and disease development could occur. Such a trend was observed in prophylactically and therapeutically treated trees. The pathogen was conspicuously absent from the stem and root crown two and twelve months after treatment, but pathogen incidence increased substantially in the stem after 24 months. Wilt was observed in two of these trees 36 months after treatment.

C. fagacearum was conspicuously absent from root samples taken adjacent (0.33 m) to the point of injection during all three sampling periods, even 24 months after injection when the pathogen was isolated from the stem in many trees. Propiconazole

concentrations were relatively high in these samples compared to root samples more distant from the root flare, but not as high on average as concentrations in the stem. The absence of *C. fagacearum* in the root crown two years after treatment, even in trees where the pathogen was isolated from the stem, could be the result of propiconazole movement from one annual increment to another in the root crown zone (see section 5.2). More continuous, less interconnected vascular elements in the roots and stem could permit fungicide compartmentalization, while lateral translocation of the fungicide in the root crown could inhibit the fungus. Regardless, *C. fagacearum* passed from the infected root system, through the root crown zone, and into the stem after 24 months, regardless of fungicide concentration in these regions, suggesting that a chemical barrier to pathogen propagules at the root collar may not be complete.

Results from this study indicate the efficacy of propiconazole is due to disease suppression rather than prevention of infection or of eradication of the pathogen from the root system of an infected tree. Further investigations of the long-term effects of repeated propiconazole applications on the survival of *C. fagacearum* in the root system are needed. The incidence of the pathogen did decrease in the root system of treated trees between 12 and 24 months, suggesting there may be a limit to the length of latent infections. The frequency of direct parasitism of xylem parenchyma cells prior to the onset of wilt is low. Rather, the pathogen is dependent upon organic nitrogen compounds in the sap as opposed to sugars for an energy source in xylem vessels prior to the onset of wilt symptoms (Beckman et al., 1953; Kessler, 1966). The ability to incite disease and/or the survival of *C. fagacearum* may be limited by repeated applications and an increased latent period.

An additional implication of propiconazole's inability to prevent root graft transmission or to eradicate the pathogen from the root system is the possibility that *C. fagacearum* may be able to infect proximal trees during a latent infection (Appel, 2001). If disease is suppressed in an infected tree by a propiconazole application, the pathogen may be able to spread through the root system of the infected tree, potentially through networks of self grafts avoiding the root crown zone where fungicide concentrations are greatest, and into additional roots and even into other trees across inter-tree root grafts. If biannual retreatment with propiconazole *ad infinitum* is utilized without the installation

of root graft barriers, there may be adequate time for the pathogen to spread through the interconnected root systems of treated trees into untreated trees beyond control areas. Root graft barriers therefore, should remain an important component of integrated oak wilt management strategies. Root graft barriers should be the primary option for control around disease centers; their effects are longer lasting than chemical controls (Juzwik et. al., 2008), they prevent infection rather than suppress disease symptoms, and there is no risk of fungicide-induced latent infections that could threaten other trees. Propiconazole, when used in coordination with barrier lines, provides an extra level of protection for asymptomatic but potentially infected trees inside/along line perimeters.

5.3. Distribution of *C. fagacearum* in Root of Trees in Disease Centers

Root graft transmission of *C. fagacearum* has historically been managed through mechanical disruption of root grafts between infected and asymptomatic trees, effectively containing the expansion of individual disease centers along root graft barrier lines. Effective barrier placement must surround all infected trees, including those that are dead, wilting, or asymptomatic while saving as many healthy trees as possible. There is currently no way to determine the extent of pathogen distribution in the root systems of symptomatic or asymptomatic trees, therefore barrier placement is as much an art as a science. French and Stienstra (1972) were the first to suggest the placement of a primary and secondary barrier line: the first conservatively placed to ensure pathogen containment and the second placed closer to the disease center to save as many healthy trees as possible. Bruhn, Pickens, and Stanfield (1991) developed guidelines for the placement of primary and secondary lines, and strongly recommended the dual-line approach to disease center containment. This pattern of barrier placement has become a commonly accepted practice. Numerous attempts to characterize pathogen spread in disease centers have been made (Appel et al. 1989; Boyce 1960; Bruhn, Pickens, and Stanfield 1991; Jones and Partridge 1961; Menges and Kuntz 1985), but management guidelines derived from these studies are specific to geographic region.

As noted in section 5.3, root graft barriers should be the primary mode of disease protection around disease centers. In situations where barrier lines cannot be installed, it is equally important to consider the placement of “fungicide lines”; those trees that will

be treated and those trees that are not at risk. As for root graft barriers, fungicide applications need to protect those trees at risk of infection, but need to be applied conservatively for cost considerations. Fungicide applications need to be applied prior to the onset of symptoms in red oaks; therapeutic treatments are rarely successful in these species.

Our attempt to characterize the spread of *C. fagacearum* from wilting trees to healthy trees based on above ground symptom development (% crown wilt) was unsuccessful because no correlation between the spacial distribution of the fungus and above ground symptoms could be elucidated. Pathogen incidence in the roots of diseased trees was sporadic and unpredictable, even in trees that had died the previous growing season. This suggests that widespread colonization of the root system need not occur prior to symptom development, nor will it necessarily occur soon after the tree dies.

C. fagacearum has the ability to spread from tree to tree through root grafts efficiently. Parmeter et al. (1956) reported that over 70% of northern pin oaks in stands on deep sand soils in Central Wisconsin were grafted, and it is somewhat uncommon to find a healthy tree amidst many dead trees in a disease center in such sites. By some mechanism, *C. fagacearum* is able to spread through grafted roots to healthy trees, without necessarily colonizing the entire root system, as is suggested by the results of this investigation. One explanation is that when the negative pressure produced by the transpiration stream in the xylem of infected trees begins to fall, healthy trees which are root grafted to the infected tree may begin to draw water and endoconidia through root grafts resulting in the spread of the pathogen into uninfected hosts (Nair, 1995; Nair and Kuntz, 1975). This mechanism would not require widespread colonization of the root system for root graft transmission; rather it would provide a means for the pathogen to move more rapidly and efficiently from tree to tree.

Propiconazole-induced disease suppression in an infected tree would prevent rapid transmission of *C. fagacearum* across root grafts via negative pressure, helping to contain the pathogen in the disease center. Further investigations are needed to determine if *C. fagacearum*, when given adequate time, can spread via vegetative growth or some other mechanism through a propiconazole treated tree into proximal trees. For now, management strategies that assume widespread colonization of the root system and that

consider latently infected trees as potential inoculum sources are encouraged. The extent of pathogen spread into asymptomatic trees cannot currently be determined based on symptom development alone. Disease managers and arborists need to be aware that while propiconazole is capable of protecting trees from oak wilt for up to two years, these treatments can mask infection. *C. fagacearum* can survive in the root systems of treated trees, may be able to spread to proximal trees, and disease may develop if retreatments are not conducted on a biannual basis.

5.4. The Importance of Root Grafts

The movement of *C. fagacearum* through root grafts was confirmed early in the study of oak wilt transmission (Jones and Partridge, 1961). In forests where management practices have promoted near homogenous stands of oak such as in Minnesota and Wisconsin, or in Texas where live oaks grow in shallow rocky soils and can propagate vegetatively via root sprouting, large interconnected networks of root grafts enable the pathogen to spread from tree to tree. During this study, it was difficult to locate inter-tree root grafts within the upper-most meter of soil, even after thorough excavation of the root system between proximal oaks; only 25% of excavated tree pairs were found to be grafted. It is highly unlikely that such a low rate of inter-tree grafting could account for the observed expansion of disease centers, therefore it is likely that many root grafts were not discovered because they occurred at distances greater from the stem than were excavated, or they occurred below one meter. If the latter is true, root graft disruption with vibratory plows or trenchers may not completely sever all inter-tree grafts if they occur below the reach of the equipment. This may be particularly true in sandy soils where root systems are not confined to the upper most soil horizons.

It should also be noted that although few inter-tree root grafts were found during this investigation, many false grafts were observed between trees. The incidence of false grafts was high compared to true inter-tree grafts, suggesting that graft formation may be under the control of a compatibility mechanism or some manner of genetic control rather than by physical contact alone. It may also be possible that *C. fagacearum* is transmitted across false grafts via vegetative growth even though the vascular system is not continuous across the graft, but to date this has not been investigated.

Self-grafts were very prevalent in the root systems of excavated oaks during this study, though there is little scientific literature regarding their occurrence or importance in oaks. As noted in previous sections, self-grafts may serve as an important conduit for pathogen spread through and colonization of the root system. If propiconazole only prevents disease development and is not able to eradicate *C. fagacearum* from the root system as was demonstrated during this study, self-grafts would allow spread of the pathogen from infected roots into other roots without passing through the root collar zone where fungicide concentrations are highest. Application methods and fungicide formulations that maximize fungicide distribution in the root system could inhibit pathogen movement through these connections because self-graft incidence is highest within a few meters of the stem.

5.5. Plant Growth Regulation by Propiconazole

Perhaps one of the most intriguing results from these investigations was the discovery that propiconazole may have plant growth regulating properties that affect growth and the anatomy of the vascular system in oaks. Propiconazole, a demethylation-inhibiting triazole fungicide, was shown to possess plant growth regulating properties that likely arise from inhibition of cytochrome P-450 dependent hydroxylation reactions that are responsible for the synthesis of gibberellins and metabolism of other natural plant growth regulating compounds (Coolbaugh et al., 1978; Koller, 1987ab; Wiggins and Baldwin, 1984). This was anticipated however, as most triazole posses both fungicidal and plant growth regulating properties to some degree (Baldwin and Wiggins, 1984; Fletcher et al., 1986; Izumi et al., 1985; Kuck and Scheinpflug, 1986; Takano et al., 1986; Wiggins and Baldwin, 1984).

Annual growth increments, latewood production, earlywood production, vessel size, the concentration of vessels, and proportion of earlywood occupied by vessels were all reduced in propiconazole-treated trees within the first two growing seasons after treatment. While the documented changes in growth are unlikely to completely prevent disease development, they may play a role in restricting pathogen movement in the vascular system or in aiding in compartmentalization of the pathogen. However, because the processes of infection, colonization, and disease development are linked with host

anatomy and related physiological processes, small growth regulator-induced changes could dramatically affect the oak wilt disease cycle.

The fungitoxic activity of propiconazole is likely the main contributor to treatment efficacy, however, the compound's longevity and distribution in injected oaks is limited (section 5.2). The observed efficacy of propiconazole may be enhanced by the compound's effects on the production and metabolism of plant growth regulators such as gibberellins, auxins, abscisic acid, and cytokinins which are translocated throughout the entire tree and can affect a wide variety of physiological processes and anatomical features that could interfere with the oak wilt disease cycle, though a more thorough understanding of host-pathogen-fungicide interactions would be needed before fungicide formulations, rates, and application methods were optimized that could improve treatment efficacy through growth regulation.

5.6. Conclusions

Propiconazole has been and will continue to be an important management tool for the control of oak wilt. Propiconazole's ability to be transported systemically in plants, high fungitoxic activity against a wide range of fungal pathogens at very low concentrations, low phytotoxicity, ability to be mobilized in water as opposed to an organic solvent carrier, and uniform distribution in trees make it an extremely useful tool in the management of woody plant diseases. Other vascular diseases such as Dutch elm disease are also managed successfully with propiconazole. More recently, the compound has been utilized successfully for the control of laurel wilt in red bay (*Persia borbonia*) (Mayfield et al., 2008), for blue stain fungi introduced by bark beetles in pine (Dubois, Byrne, and Clark, 2000), and for Armillaria root rot in peach (Amiri et al, 2008). Longevity and adequate distribution in targeted plant tissues is important however, and should be considered when using propiconazole for disease control. The lack of significant eradicative or prophylactic capabilities when used for oak wilt control highlight the importance of using propiconazole as part of an integrated management program. Root graft barriers and management of overland transmission will be needed to contain *C. fagacearum* in disease centers. Use of propiconazole for the control of other vascular diseases will also necessitate consideration of the compound's limitations.

Although the compound has been utilized for nearly 30 years for the control of woody plant diseases, much work remains to characterize the unknown host-pathogen-fungicide interactions and to optimize fungicide formulations, application methods, and rates so as to maximize treatment efficacy and to make informed decisions regarding utilization of propiconazole for disease control.

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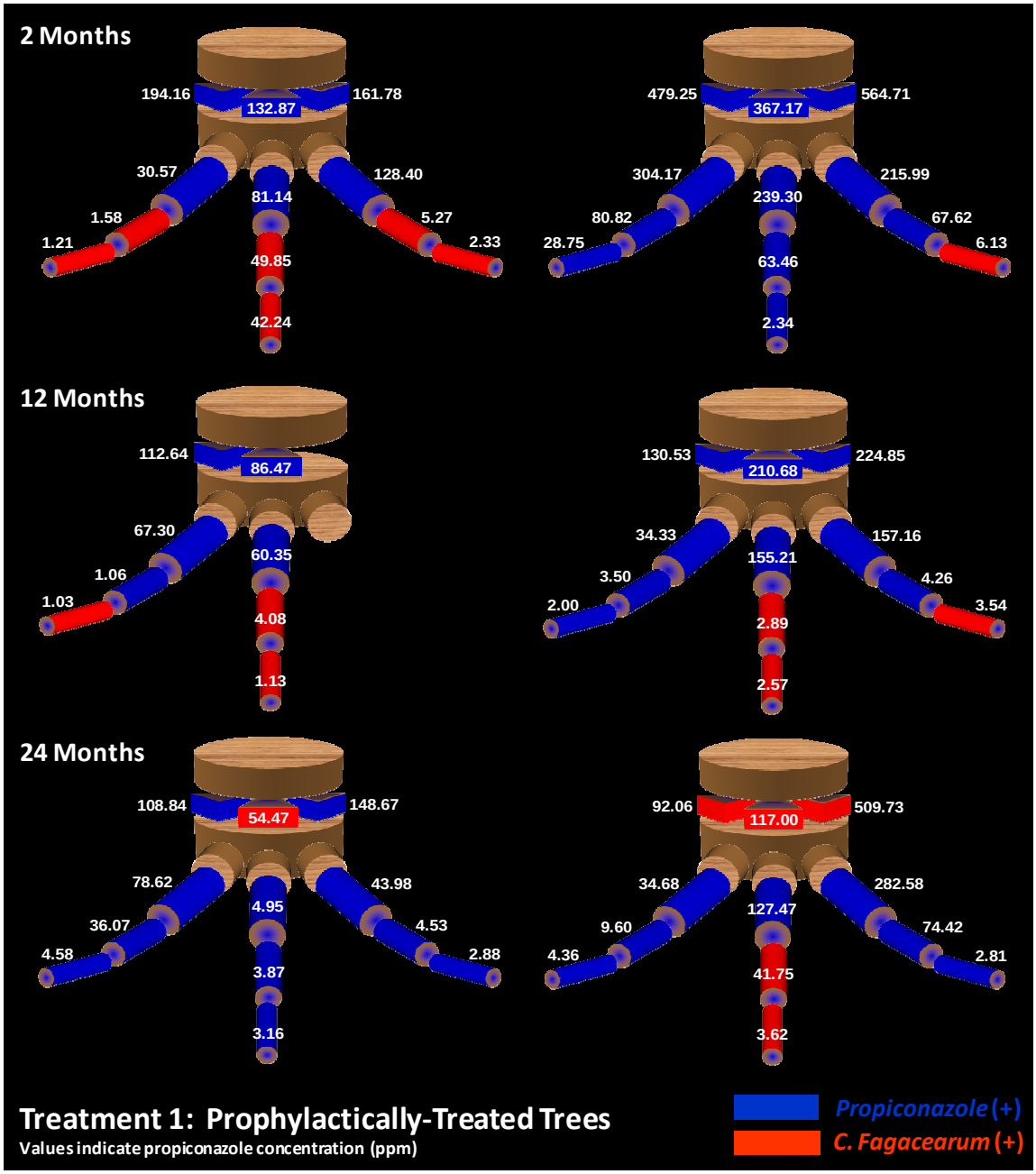
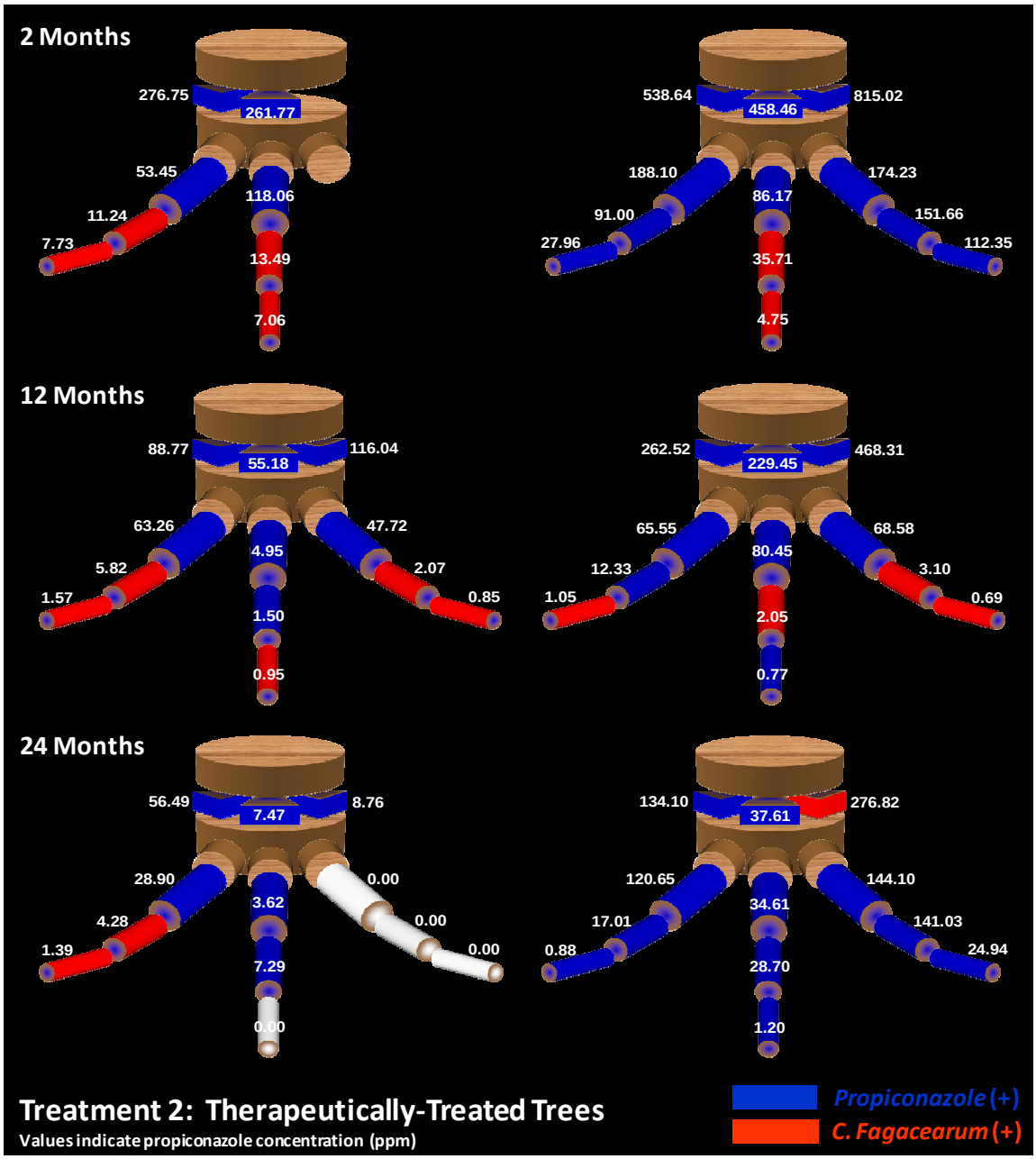
Appendix A –Tree Root Diagrams

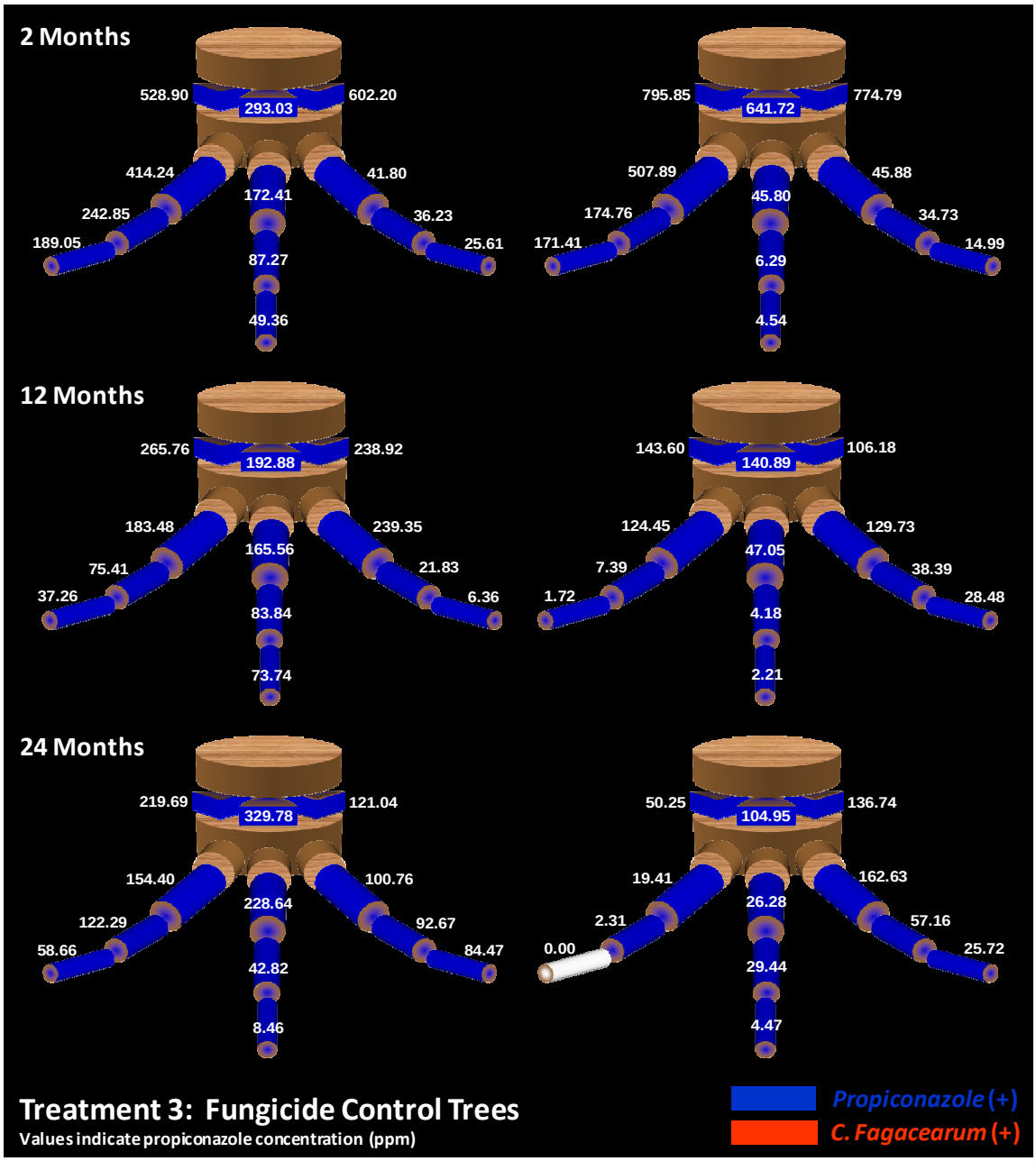
The Distribution of C. fagacearum and Propiconazole in Treatment 1: Prophylactically-Treated Trees



The Distribution of C. fagacearum and Propiconazole in Treatment 2: Therapeutically-Treated Trees



The Distribution of C. fagacearum and Propiconazole in Treatment 3: Fungicide Control Trees



The Distribution of C. fagacearum and Propiconazole in Treatment 4: Disease Control Trees