Etiology of crown decline and dieback in bitternut hickory

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

Ji-Hyun Park

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

Jennifer Juzwik, Adviser

September, 2011

Acknowledgements

I wish to express my deepest appreciation to my advisor, Dr. Jennifer Juzwik, for the opportunity to continue my graduate education in Forest Pathology at the University of Minnesota. Her insight, guidance, encouragement and support throughout the years were essential for me to complete this program. I gratefully acknowledge the advice, valuable discussion, and dissertation review offered by my committee members, Drs. Robert Blanchette, Jeannine Cavender-Bares, and Dean Malvick.

The technical assistance of the following individuals in the collection and examination of samples, DNA sequencing, inoculations, and sap flow monitoring studies is gratefully acknowledged: Andrew Klein, Bill Stroobants, Bobbi Zenner, Carmen Collazo, Clara Shaw, Greg Reynolds, Kari Koehler, Kat Sweeney, Linda Haugen, Melanie Moore, Mike Boll, Paul Castillo, and Stacey Olszewski. Ned Tisserat and Tom Harrington are gratefully acknowledged for providing isolates of *Ceratocystis caryae, C. smalleyi*, and *Fusarium solani*.

I am grateful to Jeannine Cavender-Bares and her lab for providing sap flow monitoring system, to Kevin Smith at the Forest Service for providing the sliding microtome and various supplies for anatomical studies, and to Robert Seavey in the department of Bioproducts and Biosystems Engineering for providing a band saw. Without the help of Alex Wiedenhoeft at the Forest Products Lab, Emily Peters, Jeannine Cavender-Bares and Joseph Shannon, it would not be possible to acquire the deeper knowledge of theory and practice of microtomy and sap flow monitoring system. I am also grateful to Dr. Robert Blanchette for his valuable advice on the plan and interpretation of the histological study.

Linda and Dennis Haugen, the Chippewa County Forest, and the Minnesota Department of Natural Resources are acknowledged for providing access to lands and trees for this work.

Special thanks are extended to Paul Castillo for numerous hours spent in the field and his friendship and Melanie Moore for her helps with my life in the lab and office. I especially thank my mentor, friends and family for their supports in all of my endeavors. This is the fruit of God's grace expressed in my life. All glory to you, Lord!

This work was supported by USDA Forest Service, Forest Health Evaluation Monitoring Grant (FHEM-NC-EM-07-01). Additional funding was provided by Department of Plant Pathology, University of Minnesota.

Table of Contents

List of Tables

List of Figures

Chapter 1. Introduction

Hickories are an important associate of oak-hickory forest type that covers 56 million hectares (35.9%) of the Eastern hardwood forests in the USA (Smith et al. 2009). During 1985 – 1995, greater than normal mortality rates of hickories were noted throughout the Eastern United States by the Forest Health Monitoring (FHM) program of USDA Forest Service (Steinman 2004). Furthermore, estimates of tree crown condition obtained from Forest Inventory and Analysis (FIA) plots indicated that hickories exhibited unhealthy crowns in Maryland, Missouri, New York, Pennsylvania, West Virginia, and Wisconsin for the past two decades (Steinman 2004). According to the FHM report (2004), unhealthy crowns are defined as trees having at least 25% dieback, at least 30% foliage transparency, or less than 35% crown density. More recently, severe decline and mortality of hickory were noticed from throughout Wisconsin (Wisconsin Department of Natural Resources 2005) and Southern Ontario, Canada (Tucker et al. 2006).

Widespread mortality and decline of certain tree species has been periodically reported over large geographical regions in the USA during the past century. In some cases, a biotic disease involving a pathogen or closely related pathogens with high virulence was responsible for the regional tree health problem. Chestnut blight, Dutch elm disease, and oak wilt are such examples. However, many other incidences of the widespread mortality were categorized as “decline disease” because there were likely multiple interacting factors or obscure causal factors involved. For hardwood forests in the eastern United States, Millers et al (1989) documented historical events of hardwood declines that had occurred over the last century. Although the report was limited to mortality occurrences covering an area larger than 10 ha, many major species of hardwood forest such as oak, maple, birch, and ash appeared to have experienced extensive mortality and decline throughout the 20th century (Millers et al. 1989). The adverse effects of the decline diseases appeared as important as other types of forest

diseases causing losses. For instance, oak decline together with oak wilt are two major regional problems that have affected oak trees (Millers et al. 1989). Outbreaks of oak decline have occurred on thousands of acres with mortality up to 100% in some forest stands of eastern and central USA since 1950 (Millers et al. 1989; Sinclair and Lyon 2005).

Decline disease is a unique category of disease in forest pathology even though the concept of declines as a distinct category of disease is not totally accepted by all forest pathologists (Manion 1991; Ostry et al. 2011). In contrast, diseases of annual plants are generally categorized as either biotic (infectious) and abiotic (noninfectious) (Agrios 2005). Manion (1991) defined decline diseases as diseases caused by the interaction of a number of interchangeable, abiotic and biotic factors to produce a gradual, general deterioration, often ending in the death of trees. However, the term decline is also a common word that refers to a progressive loss of vigor and health regardless of causes such as disease, injury, and age. To prevent the confusion in the term, Manion (1991) and Sinclair and Lyon (2005) limited decline disease to premature, unnatural, or abnormal decline of complex abiotic and biotic origin.

It is considered that the complex etiology of decline diseases cannot be demonstrated by Koch's postulates (Manion 1991). Thus, there have been efforts by some forest pathologists to explain decline diseases using various conceptual models. Houston (1992) suggested the host/stress/saprogen concept. In this model, decline diseases are caused by the successive action of stress factors followed by actions of secondary biotic agents. Sinclair (1965) proposed the predisposing/inciting/contributing factors concept to explain declines of white ash, oaks and sugar maple in the Northeastern forests. In this model, **predisposing factors** weaken a tree's ability to respond to stress factors, **inciting factors** cause the initial decline symptoms for a short term, and then **contributing factors** ultimately lead to the tree death. Based on Sinclair's model, Manion (1991) developed the disease spiral concept by adding age, genetic potential, and viruses to the predisposing factors. Mueller-Dombois et al (1992), in their cohort senescence model, considered declines a part of forest dynamics and succession rather than disease. These conceptual models were all trying to understand complex

etiologies of decline diseases as a whole by illustrating the roles of multiple interacting factors (Ostry et al. 2011).

The disease triangle is the classic conceptual framework of plant disease that visualizes the interactions of three components of disease: susceptible host, virulent pathogen, and conducive environment (Agrios 2005). Each side of the triangle represents one of the components and plant diseases develop when all components favor disease (Agrios 2005). Ostry et al (2011) contended that the disease triangle model can adequately describe the expression of decline symptoms and, thus, other conceptual models are not necessary. They proposed the modified disease triangle that incorporates Sinclair's (1965) predisposing, inciting, and contributing factors into susceptible host, conducive environment and damaging agents, respectively (Ostry et al. 2011). It could also incorporate Houston (1992)'s host, stress, and saprogen factors in the same way. A key difference between the modified and original disease triangle models is that the damaging agent can include two or more biotic agents, including insects, or abiotic agents (Ostry et al. 2011). This model could be particularly useful in facilitating communications among forest pathologists as well as between forest and plant pathologists by using widely accepted terms and concepts.

However, the greatest hindrance to the understanding of some decline diseases is the lack of close examination and of agreement on the cause(s) and importance of the specific factors implicated in declines (Ciesla and Donaubauer 1994). A recent rush to attribute tree health problems to decline in too many instances (Ostry et al. 2011) has increased the number of reported cases of tree decline and mortality for reasons unknown or difficult to determine. Thus, etiologies of many decline situations remain elusive providing no practical insights for understanding and managing the tree health problems. For this reason, Ostry et al (2011) stressed the need for systematic investigations of decline diseases in order to elucidate major determinants and to provide more accurate description of the disease.

As a matter of fact, there are cases where what was initially regarded as a decline disease of complex or unknown causes turned out to be a simple disease, a complex of multiple diseases, or overlapping effects of different local problems. As the most recent

example, black walnut (*Juglans nigra*) decline had been noted throughout western states since the early 1990's (U.S. Department of Agriculture 2011). Previous reports attributed the widespread mortality of black walnut to environmental stress, especially a drought condition for black walnut trees planted outside its natural range with walnut twig beetle (*Pityophthorus juglandis*) as a secondary pest (Murray 2008; Tisserat et al. 2009; U.S. Department of Agriculture 2011). Through investigations of biotic organisms involved, Tisserat et al (2009) concluded recent episodes of black walnut decline are a result of geographic expansion of the walnut twig beetle, "host jump" of the beetle from native walnuts to black walnut in southwest USA, its aggressive behavior on black walnut, and numerous cankers caused by its associated fungus *Geosmithia morbida*. Following elucidation of these key causal agents, the disease was renamed as "thousand cankers disease."

Sudden oak death is another example of a complex disease problem that required careful investigation to determine the primary causal agent. Large numbers of dead and dying coast live oak (*Quercus agrifolia*), tanoak (*Lithocarpus densiflorus*) and black oak (*Q. kelloggii*) trees were first noticed in 1995 in California (McPherson et al. 2000). This syndrome was soon referred to as sudden oak death by the popular press (Rizzo et al. 2002). Sudden oak death was never considered a "decline disease", but was first called "oak mortality syndrome" (McPherson et al. 2000). Various causes whether anthropogenic (air and soil pollution, acid deposition, poor forestry practices) or climatic (unusual rainfall patterns attributed to the El Nino) were suggested for the mortality until the causal organism was successfully isolated through selective media and its biology was understood (McPherson et al. 2001). There also were many biotic organisms that were likely to have influenced the syndrome such as bark beetles, ambrosia beetles, *Phytophthora* spp., and *Hypoxylon thouarsianum* (McPherson et al. 2000). Rizzo et al (2002) finally demonstrated that *Phytophthora ramorum* is the major determinant of the problem and suggested "Phytophthora canker" as the disease name. Current understanding is that *P. ramorum* can cause three distinct diseases (i.e. stem or bole canker, ramorum leaf blight, and ramorum shoot dieback) depending on the host species and affected tissues (Cave et al. 2008; Hansen et al. 2002; Kliejunas 2010). Yet, rapid

crown wilt of oaks and tanoak followed by tree death is characterized by cankers (Hansen et al. 2002) and sapwood colonization underneath the canker (Collins et al. 2009; Hansen 2008; Parke et al. 2007).

While a simple disease is responsible for much of tree decline and mortality across the affected region in the above cases, a complex of multiple local health problems was involved in some other cases of decline diseases. Dieback and decline of white ash (*Fraxinus americana*) which was called ash dieback was first observed in northeastern USA and adjacent Canadian provinces in the 1930s and became a serious concern in the 1950s (Ciesla and Donaubauer 1994). One of several biotic and abiotic factors was recognized as a main determinant of ash dieback from place to place. Involvement of a phytoplasma causing ash yellows was determined as the main cause of incidences in New York State (Ciesla and Donaubauer 1994; Manion 1991; Sinclair and Lyon 2005). The association of ash yellows with declining ashes was also suggested in Iowa, Indiana, and Pennsylvania (Millers et al. 1989). Canker diseases caused by *Cytospora pruinosa* and *Fusicoccum* sp. contributed to the syndrome in other locations (Ciesla and Donaubauer 1994; Millers et al. 1989; Sinclair and Lyon 2005). Episodes of ash dieback in Quebec in the 1920's were attributed to root freezing leading to an abiotic disease (Sinclair and Lyon 2005). Thus, dieback of white ash was determined to be a collection of several distinct diseases.

Maple decline is a similar case where multiple diseases brought about overlapping effects across a wide geographic range. Decline of sugar maple has been commonly noted in the Northeast and Midwest United States since 1912 (Millers et al. 1989). Since most of early reports did not distinguish landscape settings (e.g. forest stands, commercial plantations, roadside, and urban setting), reports of decline derived from different causes were viewed as a whole. However, maple decline was later distinguished into five different health problems: 1) salt damage of trees along roadsides in New England and the Lake States, 2) dry growing season and general decline in the Northeast USA, 3) damage from maple webworm in Wisconsin, 4) sap streak disease in North Carolina and the Lake States, and 5) multiple diseases of sugar bush maple.

Recent incidences of widespread mortality and decline of hickory in northeastern and north central USA were also described as a decline disease using terms of hickory decline and hickory dieback (U.S. Department of Agriculture 1994; Wisconsin Department of Natural Resources 2005). However, it is one of the decline diseases for which etiology has yet to be thoroughly investigated. Historically, episodes of hickory mortality have occurred periodically since the early 20th century. Forest entomologists have attributed the widespread decline and mortality of hickory to population outbreaks of the hickory bark beetle (*Scolytus quadrispinosus*), partly following several years of drought (U.S. Department of Agriculture 1985). Individual tree death was considered to result from girdling of stems by numerous beetle galleries (Hopkins 1912). Episodes of hickory mortality in New York State (Blackman et al. 1924; Felt 1914), in Western North Carolina (St. George 1929; U.S. Department of Agriculture 1956), and in Quebec (Tucker et al. 2006) were attributed to heavy infestation by hickory bark beetle.

At least two problems arise when one attempts to link the historical causation to recent incidences of widespread decline/mortality of hickory. First, hickory bark beetle outbreak may not be the single cause for all cases of hickory mortality. Evidence for the proposition that hickory trees were killed by hickory bark beetles was mostly acquired from observations of early epidemics in New York State. Gypsy moth defoliation was found to have contributed to hickory decline in Pennsylvania (Quimby 1986) and New Jersey (U.S. Department of Agriculture 1972). Yet, there have been no adequate diagnostic efforts on other biotic organisms associated with declining hickory than hickory bark beetle. In fact, no cause was determined for incidences in eastern Iowa (Sweets 1986), northwestern Indiana (U.S. Department of Agriculture 1980), and nine southeastern states (Starkey and Brown 1986). Secondly, many of previous reports of hickory decline did not identify the host species. Except for the incidence in New York where pignut hickory (*Carya glabra*) was affected (Blackman et al. 1924), hickories seem to have been mentioned as a group. This issue becomes more important when the historical reports are compared to recent episodes of hickory decline because among hickories in the northern natural range, bitternut hickory was found to have been the

species predominantly affected in recent events (Tucker et al. 2006; U.S. Department of Agriculture 1994; Wisconsin Department of Natural Resources 2005).

Recent findings from a regional survey also demand rethinking of the etiology of hickory decline. Based on the results of tree mortality and unhealthy crowns during the past two decade (Steinman 2004), FHEM (Forest Health Evaluation Monitoring) of USDA Forest Service awarded funds for a regional survey to sort out causes of the observed declines in hickory (U.S. Department of Agriculture 2007, 2008, 2009). The survey conducted in six states (IA, IN, MN, NY, OH, and WI) in 2007 and 2008 found that individual tree symptom and associated stem/branch damage was variable in declining hickories (Juzwik et al. 2008a). Unhealthy crown conditions were categorized into three types: 1) rapid crown decline characterized by thinning crowns with small, chlorotic leaves followed by tree death in one to two years; 2) top dieback with normal sized and normal green leaves below; 3) slow crown decline likely due to heavy gall formation on stems (Juzwik et al. 2008a). The declining hickories exhibited several types of stem damage including from most to least common: insect damage by hickory bark beetle, ambrosia beetles (e.g. *Xyleborus celsus*), and flathead woodborers (e.g. *Agrilus* sp.); cankers (diffuse or sunken, annual); stem and branch galls; sapsucker and woodpecker damage; wood decay; mechanical and fire damage; and broken tops (Juzwik et al. 2008b). For the insect damage, based on insect emergence from stem sections of declining hickory trees, hickory bark beetle and hickory timber beetle (*X. celsus*) were the predominant insect pests (Juzwik et al. 2009). The frequent presence of cankers and globose galls offered clues to possible involvement of fungal agents. A preliminary study on fungi associated with stem bark cankers and xylem lesions on declining hickory in three Midwestern States suggested three putative fungal pathogens (e.g. *Ceratocystis smalleyi*, *Fusarium solani*, and *Phomopsis* sp.) based on their prevalence on affected stem tissues.

While hickory decline is an “old” problem, the attention to possible association of fungi with declining hickory or hickory bark beetle is quite new. The possibility of a fungus contributing to hickory mortality was first suggested in 1994 when a fungus in the genus *Ceratocystis* was first isolated from sunken cankers and associated discolored

sapwood on shagbark hickory infested with hickory bark beetle (U.S. Department of Agriculture 1994). The fungus was later described as *C. smalleyi* and its unique association with hickory bark beetle was also suggested (Johnson et al. 2005). The suggestion was based on the frequent isolation of the fungus from beetle infested trees and morphological characteristics of the fungus such as the absence of narrow endoconidial state and aromatic volatiles when compared to its sister species, *C. caryae* (Johnson et al. 2005). *C. caryae* which was also newly described was found to commonly colonize wounds of bitternut and shagbark hickory (Johnson et al. 2005). Furthermore, the two *Ceratocystis* species were shown to be pathogenic to 2-year old seedlings of *Carya* spp. as well as *Juglans* spp. (Johnson et al. 2005). Based on these new findings, it appeared highly probable that the two fungi play a role in the recent widespread mortality of hickory, but the hypothesis needed further study.

Other fungi that were frequently found in the preliminary study included *Fusarium solani*. This fungus is known to cause cankers or twig dieback on many hardwood species including black walnut (Sinclair and Lyon 2005) which is a close relative of hickory. However, its presence has not been reported on hickory (Farr et al. 1989). *Phomopsis* species were also of interest because they were believed to cause stem and branch galls on various woody plants in eastern and central USA (Sinclair and Lyon 2005). Specifically, globose galls on bitternut hickory are widely considered to be caused by *Phomopsis* sp. and have been commonly called Phomopsis galls. However, the early reports of *Phomopsis* sp. from hickory in the 1930's (Brown 1938) have never been corroborated. Although very little is known about the association of these fungal agents with hickory, it seems likely that the outbreaks of hickory bark beetle and drought are only part of the story of the observed crown decline and dieback in hickory. To comprehend the etiology of the health issue of bitternut hickory more completely, it is obviously necessary to identify major determinants of the problem and to clarify their relationships with the affected tree.

Determinants of disease can be elucidated in several ways. First, symptoms sometimes suggest the identity of a pathogen when they are well-defined, specific, and consistent in occurrence (Wallace 1978). For instance, mycoplasma and foliar pathogens

are relatively easily recognized by symptoms. However, studying symptoms, in general, is not sufficient because symptoms are nonspecific, diverse, or cryptic in many diseases, especially when several causal factors are involved (Wallace 1978). Second, important determinants can be detected by manipulating proposed determinants such as by applying nematicides and controlling mycoplasmas (Wallace 1978). Like studying symptoms, this method only works for a limited number of simple diseases. Third, the etiology of disease can be understood by analyzing consistent or non-consistent association of a determinant with a disease (Wallace 1978). This is a useful approach where the symptoms are less well-defined, several organisms are present, or information is lacking (Wallace 1978). In this approach, it is crucial to obtain representative samples to be investigated by time and space. Lastly, understanding of complex etiology can be achieved by multiple regression analysis with a selection of variables (Wallace 1978). This is a synoptic approach to simultaneously assess contributions of multiple factors and their interactions to disease. Yet, because of the time-consuming and labor intensive nature of such investigation, this approach is recommended for the most refractory and economically important problems (Wallace 1978). Thus, the analysis of consistent association of a major determinant with various symptom and damage types might be the most appropriate approach to elucidate the etiology of hickory decline.

With all this knowledge on various symptoms, stem damage types, and fungal agents associated with declining hickory and on the applicable approach to assess multiple factors of disease etiology, this dissertation research revisited the “old” problem, hickory decline. Studies in this dissertation focused on fungal agents associated with three major crown symptoms observed in a regional survey. To analyze the consistent association between crown symptoms, stem damage types and potential fungal pathogens, stem sections from individual trees representing three major crown and stem symptoms were sampled from 27 sites in six states (IA, IN, MN, NY, OH, and WI). Major fungal agents were identified and their association with symptoms in crowns and stems was analyzed. Subsequently, the ability of major determinants to induce the crown and stem symptoms was determined. Since rapid crown decline followed by tree mortality was the predominant symptom observed in affected sites, this health problem

was examined in depth. To address the symptom development, the effects of *C. smalleyi* infection on the physiological functioning of bitternut hickory were examined at the whole tree level using a sap flow monitoring system. Furthermore, host responses to the pathogen infection were investigated at the tissue level using anatomical and histological approaches. Ultimately, this thesis tries to provide a more accurate description of the hickory decline problem using the disease triangle concept.

Effective management of diseases depends on the comprehensive information of the etiology of disease. Elucidating fungal agents that are likely involved in the hickory decline problem and their relationships with declining trees could assist in understanding the etiology of this elusive problem. The knowledge gained should also provide a scientific basis for the development of management strategies and offer insights into the elucidation and interpretation of other decline diseases which have not been thoroughly examined.

Chapter 2. Fungi associated with stem damage on declining hickory

2.1. Introduction

Severe decline and mortality of hickory has become a serious forest health issue in recent years in parts of the North Central and Northeastern Regions of the United States and in southern Ontario, Canada (Steinman 2004; Tucker et al. 2006; Wisconsin Department of Natural Resources 2005). This problem has been found primarily on bitternut hickory and to a lesser extent on shagbark hickory. Historically, episodes of hickory mortality have occurred periodically since the early part of the 20th century. For about 15 years (circa 1900), serious hickory mortality affected hickory trees in much of its natural range from Wisconsin to Vermont and south to central Georgia (Hopkins 1912). During the same period, thousands of hickory trees died within a decade in central New York (New York State Museum 1910).

A series of early entomological surveys of the affected stands found that outbreaks of hickory bark beetle (*Scolytus quadrispinosus)* were associated with declining hickory. Evidence for bark beetle involvement that was obtained during these surveys included: 1) presence of millions of larvae, 2) presence of excavated tunnels caused by the larvae on declining trees, and 3) observations of fan-shaped galleries that girdled the trees. Based on these findings, researchers concluded that the hickory bark beetle was the primary or direct cause of the death of the affected hickories (Hopkins 1912; New York State Museum 1910, 1915). Because of the seriousness of the situation, i.e. mortality of large numbers of hickories in numerous and widely distributed forest stands, *S. quadrispinosus* was listed as one of the most destructive insect pests of North American forests (Hopkins 1910). The hickory bark beetle was the only beetle attacking hardwoods on the list while the others were well-known destructive coniferous bark beetles such as the southern pine beetle, eastern spruce beetle and mountain pine beetle (Hopkins 1910). A considerable amount of mortality of hickories has occurred at various times and places since then. Such occurrences in western North Carolina in the mid-1950's during drought periods and in southern Ontario, Canada were also attributed to the

beetle attacks (Tucker et al. 2006; U.S. Department of Agriculture 1956). However, the actual cause was not elucidated for other cases in eight southern states, Pennsylvania, Indiana, and Iowa in the 1970's and 80's, with the exception of reported gypsy moth defoliation in New Jersey and Pennsylvania (Pennsylvania Department of Environmental Resources 1983; Quimby 1986; Starkey and Brown 1986; Sweets 1986; U.S. Department of Agriculture 1972, 1980).

Other than the hickory bark beetle, no other serious insect pests or diseases have been reported on hickories. Wood boring and ambrosia beetles including *Agrilus* sp. and *Xyleborus* sp. and fungal organisms such as *Armillaria* sp. and *Poria* sp. were observed on dying hickories (Campbell and Davidson 1942; Pennsylvania Department of Environmental Resources 1983; Sweets 1986). However, these biotic agents normally occur as secondary invaders on trees with declining vigor following drought, hickory bark beetle outbreaks or fire damage (Blackman et al. 1924; Campbell and Davidson 1942; Millers et al. 1989). Globose galls (= Phomopsis galls) on branches and main stems of hickory have been commonly observed (Sinclair and Lyon 2005). Mineral streaks and sapsucker-induced streaks are common problems in hickories but their impacts on the overall tree health are unknown (Burns and Honkala 1990). Bitternut hickory (*Carya cordiformis*) is known to be susceptible to fire damage at all ages due to its shallow bark (Burns and Honkala 1990).

The association of a putative pathogenic fungus (*Ceratocystis* sp.) with hickory bark beetle attacks was first reported in 1994 (U.S. Department of Agriculture 1994). Bark beetle – pathogen associations are well known and commonly occur in coniferous hosts. Fewer cases are known for hardwood species, but important examples do exist (e.g. *Scolytus* species and *Ophiostoma ulmi* and *O. novo-ulmi*). Furthermore, fungi in the genus *Ceratocystis* are commonly associated with various insects – a number of which serve as vectors of pathogenic *Ceratocystis* species (Paine et al. 1997). Eleven years after the initial report of a *Ceratocystis* sp. on declining hickory, two new species *C. smalleyi* and *C. caryae* were described (Johnson et al. 2005). These discoveries plus recent episodes of widespread crown decline and tree mortality of hickory prompted a closer examination of the problem.

In 2007 and 2008, a USDA Forest Service-led survey was conducted to elucidate the major determinants of the problem (Juzwik et al. 2009; Juzwik et al. 2008b). The most widespread and predominant insect pest associated with declining bitternut and pignut (*Ca. glabra*) hickory in 27 stands surveyed in the six state (IA, IN, MN, NY, OH, and WI) was the hickory bark beetle (Juzwik et al. 2009). The hickory timber beetle, *Xyleborus celsus*, was the second most common insect pest documented. Of 1,334 insects that emerged from log sections taken from 33 felled declining hickory in 2008, 91% were *S. quadrispinosus* and 8%, *X. celsus* (Juzwik et al. 2009).

Entry or exit holes of different diameters were the most common type of stem damage observed during point plot surveys in 21 stands with actively declining bitternut hickory (Juzwik, pers. comm.). Cankers and globose galls were the second most common type of stem damage (12 and 11%, respectively).

Fungi were suspected to be the cause of the cankers and galls observed based on related reports in the literature (Sinclair and Lyon 2005; U.S. Department of Agriculture 1994). Two different types of stem cankers were observed. Diffuse cankers are characterized by lack of callus tissue, slightly sunken appearance, oval shape, and occasional bleeding spots in late spring (Fig. 2-1A). When the bark is removed, elongate to oval, reddish-brown discoloration of the outer sapwood is found associated with the bark canker (Fig. 2-1B). Entry or exit holes, egg galleries and sometimes limited larval galleries typical of *S. quadrispinosus* are commonly found associated with diffuse cankers. The crowns of trees with these stem symptoms were characterized by rapid decline with small, chlorotic leaves and thin crowns. Annual cankers are oval in shape, sunken and bounded by heavy callus tissue that appears to prevent further canker development (Fig. 2-1C). The bark is often sloughed off the canker face exposing the wood. A small insect entry or exit hole such as that caused by ambrosia beetles (e.g. *X. celsus*) was commonly associated with annual cankers. Trees with multiple annual cankers on the upper main stem resulted in top dieback with normal sized green leaves below. Globose galls or swellings (single or several clustered) were found on main stems of several dying trees in the surveyed stands. Globose branch galls were usually observed

in the crowns of such trees. Numerous globose galls on branches or main stems resulted in top dieback above the heavily infected area.

The goal of this study was to determine whether putatively pathogenic fungi are associated with canker and gall samples obtained from trees exhibiting crown decline or top dieback in stands visited during the 2007-2008 Forest Service survey. This chapter examines 1) the identities of fungi associated with these cankers and galls, and 2) frequencies of fungi found by geographical locations and types of stem damage, i.e. diffuse or annual cankers or globose stem galls.

Figure 2-1. Two different types of stem cankers that occur on hickory. A. A diffuse canker without surrounding callus. **B.** Reddish-brown discoloration of sapwood underneath a diffuse canker. **C.** Callus-surrounded annual cankers.

2.2. Materials and Methods

2.2.1. Study sites

Twenty seven stands in six states were visited between 2007 and 2008 during the US Forest Service survey (Fig. 2-2). Site selection was based on known locations of decline-affected stands reported by state cooperators. Most stands visited were classified as oak-hickory-maple northern hardwood with bitternut hickory accounting for the majority of the *Carya* species present. The remainder was shagbark hickory (*Carya ovata*) and pignut hickory (*C. glabra*).

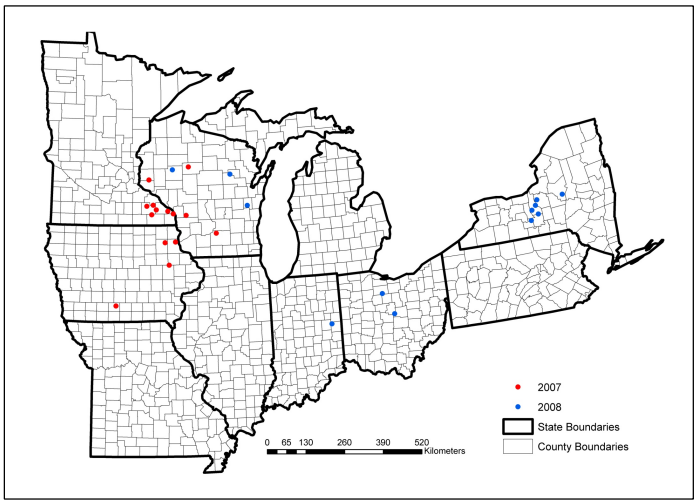

Figure 2-2. Geographical distribution of forest stands surveyed during 2007-08. Total number of stands visited for each year is as follows: In 2007, 4 stands in Iowa, 6 stands in Minnesota, and 4 stands in Wisconsin. In 2008, 1 stand in Indiana, 6 stands in New York, 2 stands in Ohio, and 4 stands in Wisconsin. Source: P. Castillo, US Forest Service.

2.2.2. Sample collection and subsampling

Three trees exhibiting the most common types of stem damage observed in the stands were felled. Two stem sections (0.6 m in length) per tree that included affected areas were cut and transported to the laboratory for stem pathogen assay. In the lab, each section was visually examined for any symptoms and signs of diffuse canker, annual canker, globose gall, xylem discoloration, and insect activities. The presence of any visual damage observed, the size and shape of insect boring holes, and the pattern and relative size of insect galleries were recorded. The presence of any insects and their life stages were also recorded. Using a chainsaw, two 5 cm thick wood discs were cut from where the symptoms/signs were obvious in each log section. From the two wood discs, three to four subsamples of diseased bark and/or sapwood were obtained using a small sledge hammer and a chisel. Each subsample was placed in a separate polyethylene bag, labeled, and subsequently stored at 4°C until fungal isolation was attempted.

Subsamples were categorized according to the type of stem damage on the log yielding the subsample. The damage categories included 1) hickory bark beetle (hereafter referred to as hbb) damage only, 2) diffuse canker, 3) annual canker, and 4) globose stem gall. When a sample had both canker and insect damage, it was placed in the category of

either diffuse canker or annual canker according to the symptoms observed. Subsamples taken from a tree that had isolated bleeding spots were included in the category of diffuse canker because such bleeding was found to be associated with diffuse cankers during the field survey.

2.2.3. Fungal isolation

Each subsample was divided into two portions, one for isolating *Ceratocystis*-like species and the other for isolating nonspecific fungal species. For the *Ceratocystis*-like fungi, small wood cubes of symptomatic sapwood associated with observed stem damage were kept in small moist chambers to stimulate formation of fruiting structures of fungi. The cubes in each moist chamber were examined under a stereomicroscope 10 days later for the presence of any fungal fruiting bodies. Ascospore masses on tips of perithecia sporulating on the wood cubes were transferred to 2% malt yeast extract agar amended with 100 ppm streptomycin sulfate (MYEA+SS). In cases where no perithecia were produced or were not yet fully developed, wood cubes were kept in the same moist chamber for 10 more days and then re-examined. Spore masses from other fruiting structures (e.g. synemmata, sporodochia) were also transferred to agar media. To isolate nonspecific fungal taxa, the other portion of each subsample was surface-sterilized by dipping in 70% alcohol solution (EtOH) and quickly burning off the EtOH. Wood chips were taken from the advancing margin of discolored sapwood or bark tissues of the surface-sterilized subsamples and plated onto media. Culture media used in this type of isolation included lactic acid-amended potato dextrose agar (APDA), Nash-Snyder medium (Nelson et al. 1983), and MYEA+SS. After incubation at room temperature for one week, cultures were examined and sorted into groups based on colony morphology.

2.2.4. Fungal identification

Pure cultures were obtained for the isolated fungi. The most prevalent isolate types were identified to genus and stored on agar slants at 4°C.

To identify *Ceratocystis* isolates to species, cultural and morphological characteristics were compared to reference cultures of *C. smalleyi* and *C. caryae* obtained

from T.C. Harrington, Iowa State University. Cultural features of interest included color and scent of culture, location and pattern of perithecia formation, and color of ascospore mass. For morphological characteristics, endoconidia, endoconidiophores, and aleurioconidia were observed in terms of their presence, size, and shape. To confirm identification, the internal transcribed spacer (ITS) region and the translation elongation factor (tef) 1-α gene were sequenced. DNA for both regions was extracted from mycelia of 7 day old cultures grown on MYEA. The sequences obtained were compared to those in GenBank using a BLAST search.

Single spore cultures were derived from all *Fusarium* isolates. Species identification of the isolates was determined by cultural and morphological features on carnation leaf agar (CLA) and homemade PDA (Booth 1971; Leslie and Summerell 2006; Nelson et al. 1983). Cultural characteristics included growth rate, color of culture and sporodochia, and proliferation of aerial mycelia. For morphological characteristics, presence, shape, and size of macroconidia, microconidia, conidiophores, and chlamydospores were evaluated. The DNA sequences of tef 1-α gene is commonly used to assist with identification of *Fusarium* species. Thus, to confirm the identification, DNA was extracted from 10 day old cultures and the tef 1-α gene was sequenced. Sequences obtained were compared to those in GenBank and in FUSARIUM-ID v. 1.0 database (http://fusarium.cbio.psu.edu) at Pennsylvania State University using a BLAST search.

Identification of *Phomopsis* isolates was made based on cultural and morphological features including color of culture, pattern of conidiomata formation, shape and size of macroconidia and microconidia. DNA sequencing of the ITS region was also completed per protocol of Murali and others (2006).

Pure cultures of other fungal taxa obtained were maintained in stock culture after tentative identification was made. These isolates were not processed any further.

2.2.5. Data analysis

DNA sequences of each taxon were aligned and manually adjusted to minimize insertion/deletion events using BioEdit software (http://www.mbio.ncsu.edu/BioEdit/

bioedit.html). Maxium parimony analysis is commonly performed to differentiate phylogenetically distinct species in the genera *Ceratocystis* (Johnson et al. 2005) and *Fusarium* (O'Donnell 2000; Zhang et al. 2006). Thus, phylogenetic trees for representative isolates of *C. smalleyi* and *F. solani* obtained from different geographic locations were constructed using a maximum parsimony heuristic search with stepwise addition using PAUP 4.0b10 (Swofford, 2002). Phylogenetic accuracy was tested by bootstrapping 1000 replicates of heuristic searches. Representative isolates sequenced in this study and reference isolates used for phylogenetic analyses were listed in Table 2-1 and Table 2-2.

The association between frequencies of fungal taxa and stem damage types were assessed by the Pearson's chi-square test using SAS 9.1 (SAS Institute, Inc. Cary, NC). A significant level of 0.05 was used for the analysis.

Table 2-1. Isolates of *Ceratocystis* used for phylogenetic analysis

Species	Isolate no.[a]	GenBank Accession no. (ITS region, tef 1-α gene)	Host	Geographic origin
Ceratocystis smalleyi	CS0709	GU190738, GU201530	*Carya cordiformis*	Minnesota
	CS0716	GU190737, GU201537	*C. cordiformis*	Minnesota
	CS0720	GU201531	*C. cordiformis*	Minnesota
	CS0726	GU190736, GU201529	*C. cordiformis*	Minnesota
	CS0729	GU190739, GU201539	*Carya ovata*	Minnesota
	CS0823	GU190735, GU201532	*C. cordiformis*	New York
	CS0825	GU190734, GU201533	*C. cordiformis*	New York
	CS0827	GU190740, GU201538	*C. cordiformis*	New York
	CS0830	GU190741	*C. cordiformis*	New York
	CS0832	GU190742, GU201534	*C. cordiformis*	Ohio
	CS0833	GU190743, GU201535	*C. cordiformis*	Ohio
	CS0834	GU190744	*C. cordiformis*	Ohio
	CS0835	GU190745, GU201536	*C. cordiformis*	Indiana
	C684	AY907030	*C. cordiformis*	Wisconsin
	C1410	AY907031, HM569621	*C. cordiformis*	Iowa
	C1828	AY907032	*C. cordiformis*	Iowa
	CMW14800	EF070420, EF070408	*C. cordiformis*	Wisconsin
	CMW26383	EU426553, EU426556	*C. cordiformis*	Wisconsin
Ceratocystis caryae	C1412	AY907033, HM569620	*C. cordiformis*	Iowa
	C1827	AY907034	*C. ovata*	Iowa
	C1829	AY907035	*C. cordiformis*	Iowa
	CMW14793	EF070424, EF070412	*C. cordiformis*	Iowa
	CMW14808	EF070423, EF070411	*C. ovata*	Iowa

[a] Isolate numbers preceded by CS were sequenced as part of this study. Other sequences for phylogenetic comparison were retrieved from GenBank and isolate numbers of each in GenBank is given.

Table 2-2. Isolates of *Fusarium* used for phylogenetic analysis

Species	Isolate no.[a]	GenBank Accession no. (tef 1-α gene)	Host	Geographic origin
Fusarium solani	FS06C1		*Carya cordiformis*	Iowa
	FS06C2		*C. cordiformis*	Wisconsin
	FS06C3		*C. cordiformis*	Wisconsin
	FS06C4	HQ647292	*Carya ovata*	Wisconsin
	FS06C5		*C. cordiformis*	Minnesota
	FS06C7		*C. cordiformis*	Minnesota
	FS06C9		*C. cordiformis*	Minnesota
	FS06C10	HQ647289	*C. cordiformis*	Minnesota
	FS06J1	HQ647285	*C. cordiformis*	Minnesota
	FS06J2		*C. cordiformis*	Iowa
	FS0705		*C. cordiformis*	Wisconsin
	FS0715		*C. cordiformis*	Minnesota
	FS0720		*C. cordiformis*	Minnesota
	FS0723	HQ647286	*C. cordiformis*	Minnesota
	FS0726	HQ647290	*C. cordiformis*	Minnesota
	FS0731		*C. cordiformis*	Minnesota
	FS0735		*C. cordiformis*	Wisconsin
	FS0740		*C. ovata*	Minnesota
	FS0742		*C. cordiformis*	Iowa
	FS0745		*C. cordiformis*	Iowa
	FS0747		*C. cordiformis*	Iowa
	FS0750		*C. cordiformis*	Wisconsin
	FS0757		*C. cordiformis*	Iowa
	FS0801	HQ647291	*C. cordiformis*	Wisconsin
	FS0802	HQ647287	*C. cordiformis*	Wisconsin
	FS0803	HQ647288	*C. cordiformis*	Wisconsin
	FS0806		*C. cordiformis*	New York
	FS0810		*C. cordiformis*	New York
	FS0817		*C. cordiformis*	New York
	FS0818		*C. cordiformis*	New York

Table 2-2. Continued.

Species	Isolate no.[a]	GenBank Accession no. (tef 1-α gene)	Host	Geographic origin
Fusarium solani	FS0819		*Carya cordiformis*	New York
	FS0822		*C. cordiformis*	Ohio
	FS0823		*Carya ovata*	Ohio
	FS0825		*C. cordiformis*	Indiana
	FS0828		*C. cordiformis*	New York
	FS0830		*C. cordiformis*	New York
	FS0832		*C. cordiformis*	Ohio
	FS0834		*C. cordiformis*	Indiana
	FS917		*Juglans nigra*	Colorado
	FS1179		*J. nigra*	Colorado
	FS1241		*J. nigra*	Colorado
	FRC S1	DQ247282	*Betula lenta*	Pennsylvania
	FRC S1124	DQ247436	*J. nigra*	Kansas
Fusarium sporotrichioides	NRRL25479	HM347118	*Pinus nigra*	Germany

[a] Isolate numbers preceded by FS were sequenced as part of this study. Other sequences for phylogenetic comparison were retrieved from GenBank and isolate numbers of each in GenBank is given.

2.3. Results

2.3.1. Subsamples obtained from damaged stems

The 2007-08 survey of hickory decline resulted in 299 subsamples from 87 trees on 27 field sites (Table 2-3). A large number of subsamples (88%) were obtained from trees with either diffuse (50%) or annual cankers (38%) regardless of insect damage. Since hbb damage frequently co-occurred with diffuse cankers, the category of "hbb damage only" made up 4% of the total subsamples. Insect holes (associated with ambrosia beetles and hickory bark beetles) were also observed in and around annual cankers. Subsamples from the cankered area associated with insect damage accounted for 16% of the total. Twenty six subsamples (9%) were obtained from stem galls.

Table 2-3. Characteristics of stem subsamples obtained for fungal isolation

State	No. stands sampled	No. trees sampled	No. subsamples with					
			Hbb damage only	Diffuse cankers		Annual cankers		Globose stem galls
				Canker only	Canker and hbb damage	Canker only	Canker and insect damage[a]	
Indiana	1	3	1	0	4	1	8	0
Iowa[b]	4	13	-	-	-	10	3	0
Minnesota	6	18	0	11	30	12	4	12
New York	6	17	6	4	12[c]	31	17	7
Ohio	2	7	4	1	5	8	7	1
Wisconsin	8	29	0	16	66	4	8	6
Total	27	87	11	32	117	66	47	26

[a] Insect damage includes entry/exit holes typical of ambrosia beetles and hickory bark beetles.
[b] Subsamples for hbb damage and diffuse cankers obtained from Iowa sites were excluded because dead trees or dry wood were sampled which may have affected ability to isolate *C. smalleyi*.
[c] Three samples were obtained from a tree with bleeding spots.

2.3.2. Most commonly found putative pathogens

2.3.2.1. Overall

Each subsample yielded one fungal taxon except for 20 subsamples (7%) which yielded two fungal species. *Ceratocystis smalleyi*, *Fusarium solani*, and *Phomopsis* sp. were commonly isolated from the field samples (Fig. 2-3). Among 299 subsamples in total, 132 subsamples (44%) yielded one or two of the three species. The most commonly found fungal taxon was *C. smalleyi*, being obtained from 84 subsamples (28%). Second most common was *F. solani* isolated from 61 samples (20%). Compared to the two species, *Phomopsis* sp. was rarely obtained (2%). *Ophiostoma quercina* and *Penicillium* sp. were found very infrequently on the wood cubes or as contaminants on agar plates.

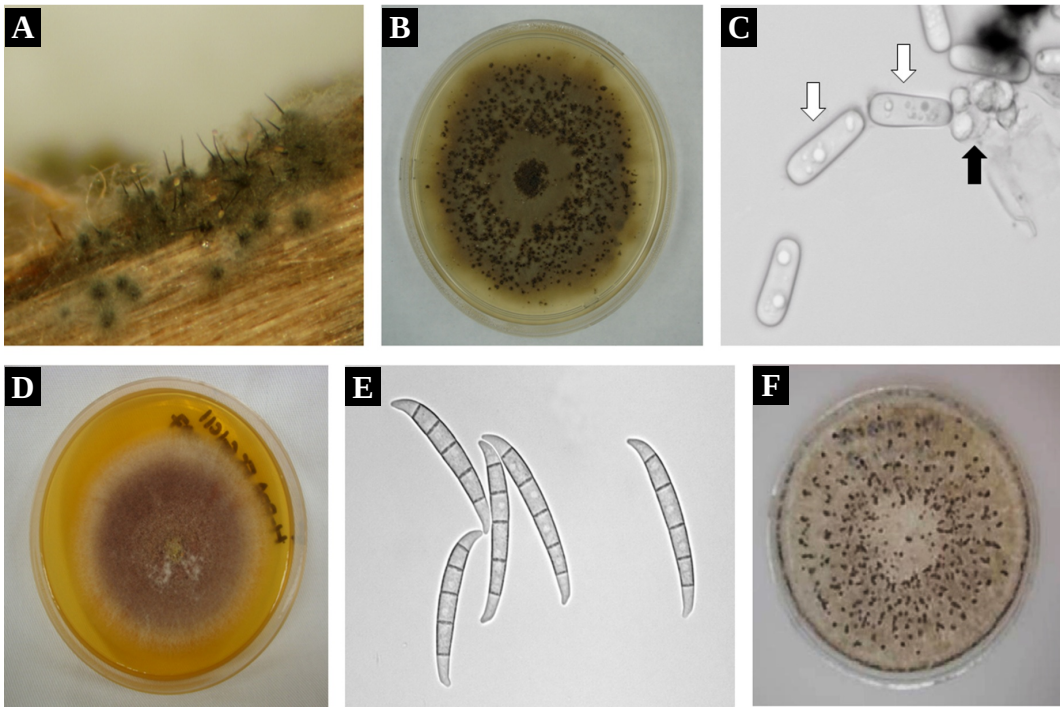

Figure 2-3. Fungal taxa commonly isolated from field survey samples. A-C. *Ceratocystis smalleyi*. A. Perithecia forming on a wood cube in a moist chamber. Some mature perithecia have sticky ascospore masses extruded at their tips. B. Culture on malt yeast extract agar (MYEA). This homothallic species abundantly produces its fruiting body, perithecia, when cultured on MYEA. C. Doliiform endoconidia (white arrows) and ascospores (black arrow). **D-E. *Fusarium solani*.** D. Culture on homemade potato dextrose agar. E. Macroconidia obtained from sporodochia forming on carnation leaf agar (CLA). **F. *Phomopsis* sp.** Black conidiomata were abundantly produced when cultured on PDA.

2.3.2.2. *Ceratocystis* sp.

Ceratocystis isolates were all obtained from wood cubes in the moist chambers. All isolates appeared to be *C. smalleyi* based on cultural and morphological features. When cultured on MYEA+SS, they produced perithecia, ascospores, endoconidiophores, and conidia typical of *C. smalleyi* as previously described (Johnson et al. 2005). Three isolates (CS0832, CS0833, and CS0835) showed two morphological variations such as the production of flask-shaped endoconidiophores and chlamydospores which resembled morphological characteristics of *C. caryae*. However, none of them had pink ascospore masses, banana oil odor, and aleurioconidia which are other distinguishing features of *C. caryae*.

The species identification was confirmed by DNA sequences produced from the ITS region and tef 1-α gene of 13 representative isolates. Maximum identities to *C. smalleyi* ranged from 98 to 100 % for the ITS sequences over 579 bp (GenBank Accession Nos. AY907030 - 907032). The ITS region of *C. smalleyi* was found to have a long stretch of either A or T that is generally attributed to secondary structure presence. Thus, the sequences from the ITS region are often unsatisfactory for some isolates. Therefore, the tef 1-α gene was also sequenced to corroborate the validity of the identification based on the ITS sequences. DNA sequences of the tef 1-α gene were informative enough to distinguish *C. smalleyi* and *C. caryae*. Nucleotide identities for the tef 1-α gene were 99 to 100% over 708 bp (GenBank Accession No. EF070408).

Phylogenetic trees indicated that the representative isolates were indistinguishable from other reference *C. smalleyi* isolates (Fig. 2-4; 2-5). Reference isolates of *C. caryae* were clustered together into a distinct clade with high bootstrap values (89% for the ITS region and 98% for the tef 1-α gene). The three *C. smalleyi* isolates with greater morphological variation fell within known *C. smalleyi* isolates but were grouped into a separate linage within the clade.

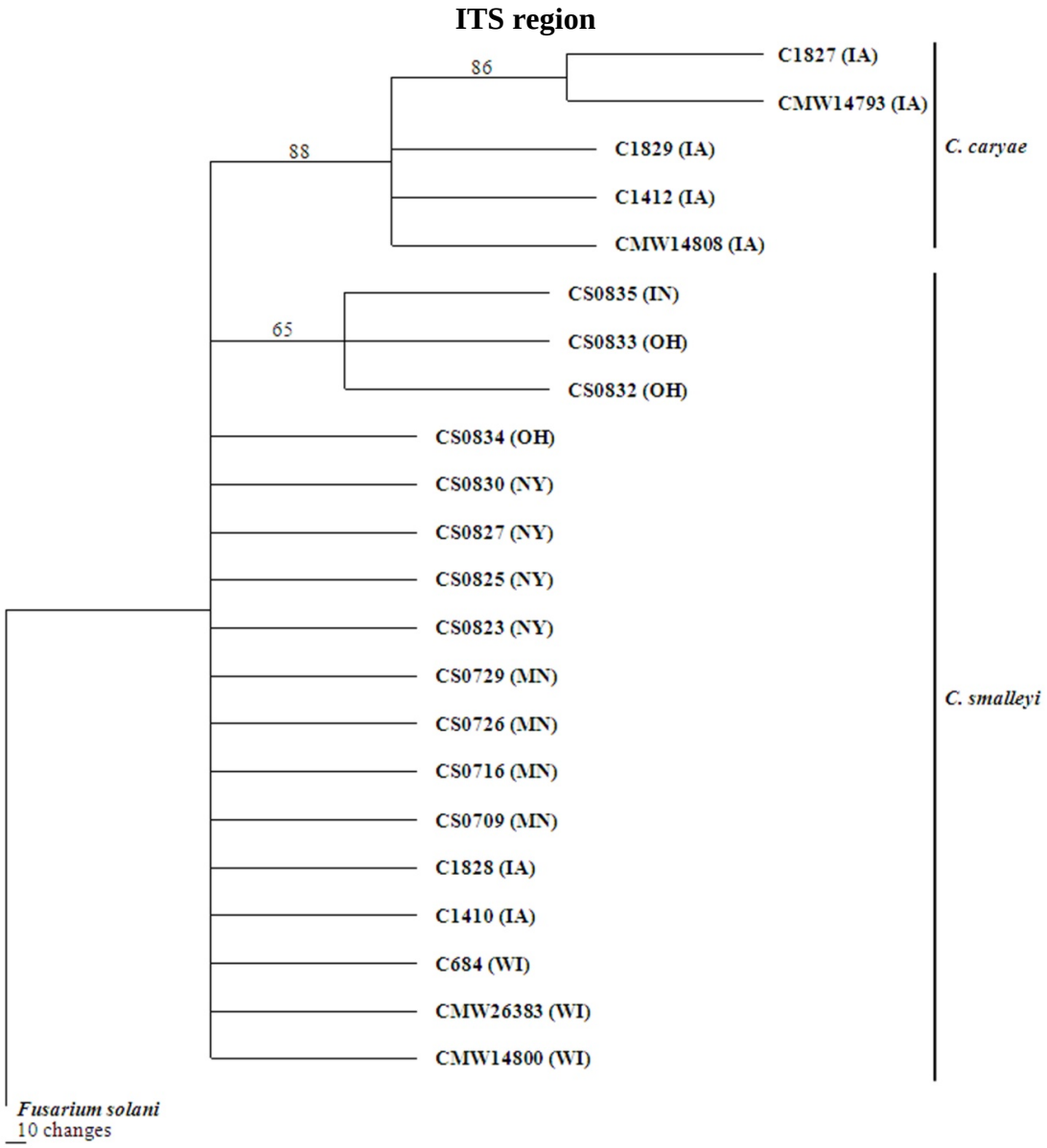

Figure 2-4. Most parsimonious phylogenetic tree based on the ITS region of *Ceratocystis smalleyi* and *C. caryae* isolates. Consistency index (CI) = 0.9756, rescaled consistency index (RC) = 0.8268, retention index (RI) = 0.8475. Bootstrap values greater than 50% are indicated above the branches. *Fusarium solani* represents the out-group taxon.

tef 1-α gene region

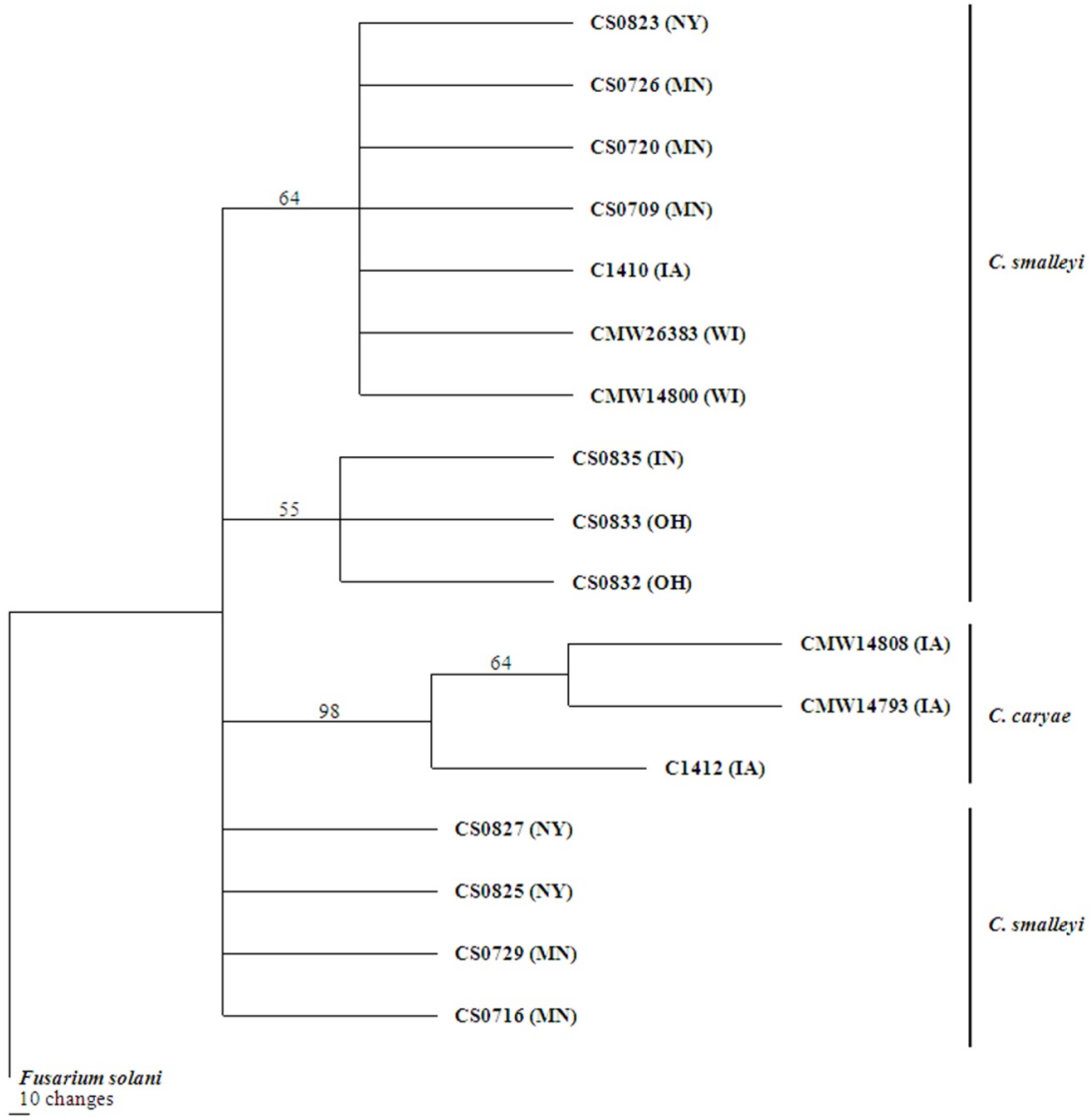

Figure 2-5. Most parsimonious phylogenetic tree based on the tef 1-α gene region of *Ceratocystis smalleyi* and *C. caryae* isolates. Consistency index (CI) = 0.8947, rescaled consistency index (RC) = 0.9285, retention index (RI) = 0.9333. Bootstrap values greater than 50% are indicated above the branches. *Fusarium solani* represents the out-group taxon.

2.3.2.3. *Fusarium* sp.

Most *Fusarium* isolates were obtained through wood chip plating on APDA or Nash-Snyder medium while a few were obtained from sporodochia produced on wood cubes in moist chambers. All of the *Fusarium* isolates were identified as *F. solani* based on cultural and morphological characteristics. For 42 representative isolates, a BLAST search of their tef 1-α sequences against GenBank and Fusarium ID databases resulted in 99 to 100% match to the accessions of *F. solani*.

All but one of the *F. solani* isolates obtained in this study were distinguished into two isolate types (designated BB and BC) with regard to their sequence matches. BC isolates had a sequence that best matched *F. solani* strain FRC S1124 in GenBank database (GenBank accession DQ247436; 99% similarity) which was obtained from a black walnut canker. BB isolates had 100% nucleotide homology with several strains of *F. solani* in GenBank database (GenBank accession DQ247282, DQ247025, and DQ246841), which were related to various sources including birch (*Betula lenta*), human skin, and human blood. A BLAST search of Fusarium-ID database indicated sequences of BB and BC isolates were, respectively, 98% and 99% similar to *F. solani* isolate FD01041 obtained from black locust. Sequence alignment and nucleotide comparison indicated that there were 10 nucleotide differences between sequences of the two isolate types distinguishing each other. The phylogenetic tree based on the tef 1-α sequences supported the separate groupings of the two isolate types (Fig. 2-6). Sequences within each group were homogeneous. In regard to phenotypes, most BC isolates, but not all, produced a slow-growing colony with brown to reddish brown or dark violet pigmentation when cultured on homemade PDA. In contrast, BB isolates formed rapid-growing colonies cream to violet on the upper surface and colorless on the undersurface.

tef 1-α gene region

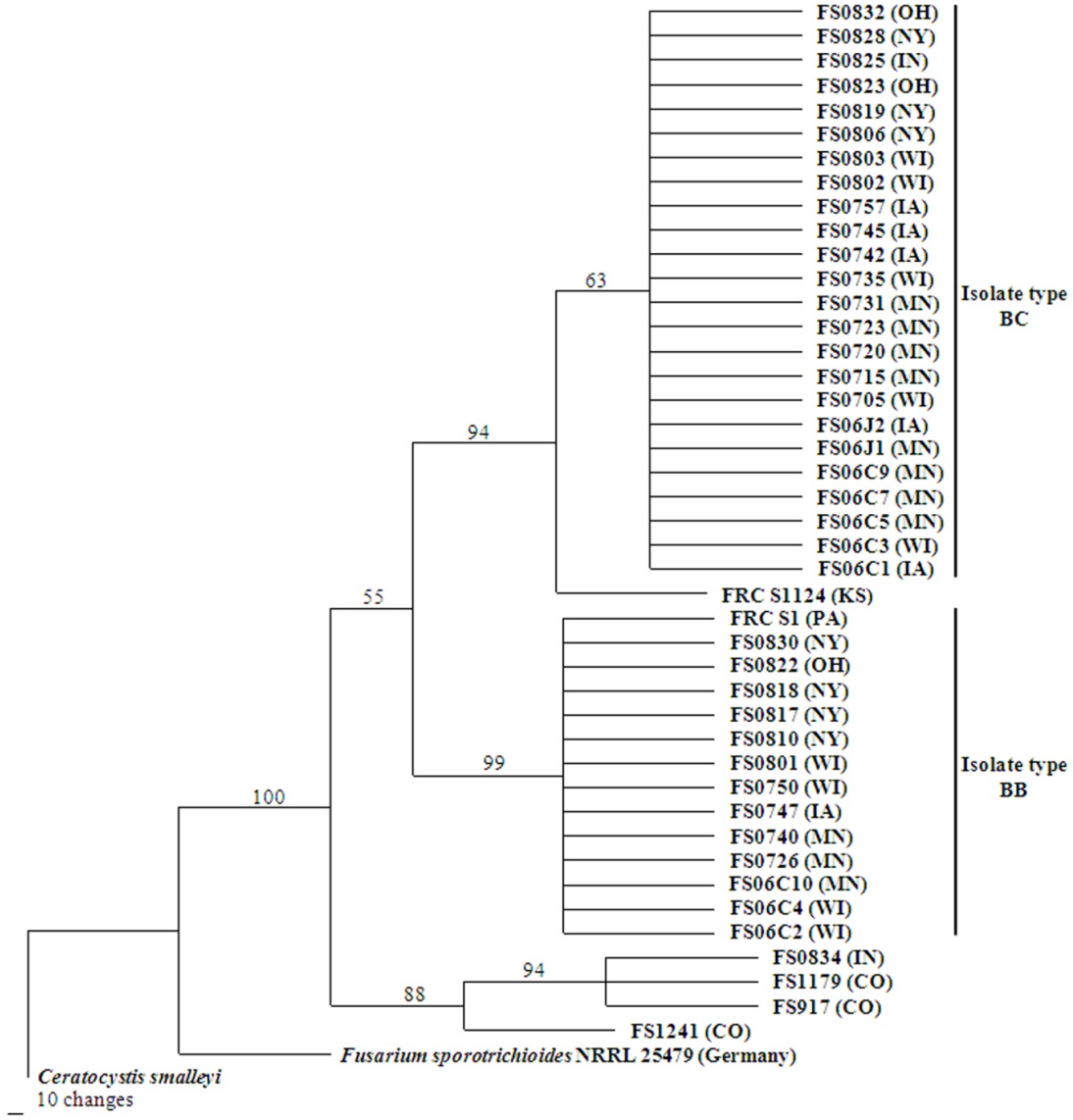

Figure 2-6. Most parsimonious phylogenetic tree based on the tef 1-α gene sequences of *Fusarium* isolates. Consistency index (CI) = 0.9827, rescaled consistency index (RC) = 0.9442, retention index (RI) = 0.9609. Bootstrap values greater than 50% are indicated above the branches. *Ceratocystis smalleyi* represents the out-group taxon. BC = Black walnut canker type and BB = Birch type.

2.3.2.4. *Phomopsis* sp.

Phomopsis isolates (n=7) were all obtained via wood chip plating on APDA. They could only be identified as *Phomopsis* species because morphological and cultural characters separating species and DNA sequence database for the genus in GenBank were insufficient for identifying to species level. Therefore, it is unknown whether the seven isolates of *Phomopsis* sp. are the same species.

2.3.3. Frequency distribution of fungi

2.3.3.1. By geographical location

Frequencies of *C. smalleyi*, *F. solani*, and *Phomopsis* sp. isolates obtained from field samples are summarized by state in Table 2-4. *C. smalleyi* was obtained from 22 (96%) of 23 field sites. Counties (and respective State) where *C. smalleyi* presence was reported for the first time include: Randolph (IN); Filmore, Houston, Olmsted, Wabasha and Winona (MN); Cayuga, Oneida and Schuyler (NY); and Morrow and Seneca (OH). *F. solani* was also frequently isolated occurring at 24 (89%) of 27 sites surveyed. *Phomopsis* sp. was found to exist in five sites sampled.

Table 2-4. Isolation frequency of *Ceratocystis smalleyi*, *Fusarium solani*, and *Phomopsis* sp. in each state

State	No. stands sampled	No. fungus-positive stands[a]			No. trees sampled	No. fungus-positive trees		
		CS	FS	PH		CS	FS	PH
Indiana	1	1	1	0	3	1	3	0
Iowa[b]	4	-	4	1	13	-	7	1
Minnesota	6	6	6	2	18	10	10	2
New York	6	5	6	1	17	9	9	3
Ohio	2	2	2	0	7	3	3	0
Wisconsin	8	8	5	1	29	20	11	1
Total	27	22	24	5	87	43	43	7

[a] CS = *Ceratocystis smalleyi*, FS = *Fusarium solani*, and PH = *Phomopsis* sp.
[b] Subsamples obtained from Iowa sites for *C. smalleyi* isolation were excluded because dead trees or dry wood were sampled which may have affected ability to isolate *C. smalleyi*.

2.3.3.2. By stem damage type

Of 299 subsamples, 11 were associated with hbb damage only, i.e. without any symptoms of cankers or stem galls. *C. smalleyi* was isolated from 5 of the 11 (Fig. 2-7A).

For subsamples taken from 33 trees exhibiting declining crowns, 149 subsamples were obtained from diffuse cankers. *C. smalleyi* was most frequently isolated from these subsamples (42%) followed by *F. solani* (19%) (Fig. 2-7B). On the other hand, *F. solani* was more frequently obtained (28%) from subsamples associated with annual cankers than *C. smalleyi* (12%) (Fig. 2-7C). The subsamples of annual cankers were obtained from 23 trees with top dieback. The isolation frequencies of the two fungal taxa, *C. smalleyi* and *F. solani*, were significantly associated with different stem canker types (by Pearson's chi-square test, $P < 0.0001$), i.e. *C. smalleyi* with diffuse cankers and *F. solani* with annual cankers. A relatively small number of samples (n=26) were obtained from seven trees with stem galls compared to diffuse and annual cankers. Although not identified to the species level, *Phomopsis* sp. was obtained at a higher frequency (23%) than other fungal taxa (Fig. 2-7D).

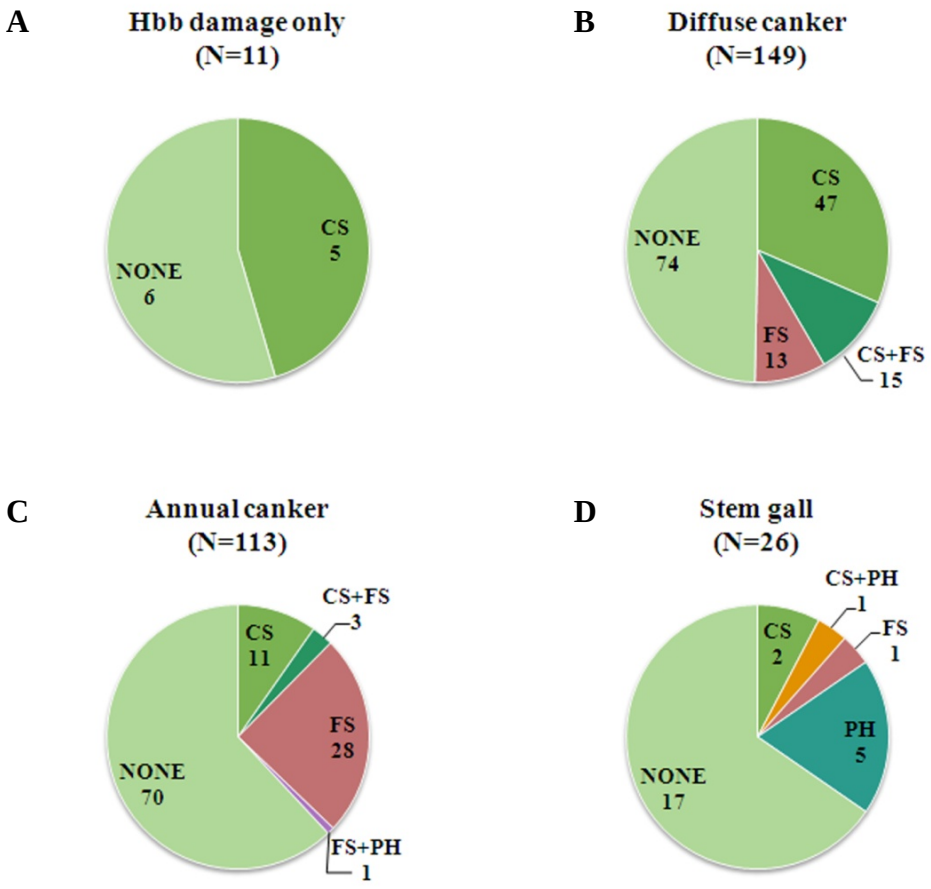

Figure 2-7. Frequency of fungal taxa obtained from field samples associated with different stem damage types. B and C are with and without associated insect damage. CS = *Ceratocystis smalleyi*, FS = *Fusarium solani*, and PH = *Phomopsis* sp.

2.4. Discussion

Three putative fungal pathogens were commonly obtained from damaged stems of hickories that were sampled in 2007 and 2008 survey.

All *Ceratocystis* isolates were found to be *C. smalleyi* based on morphological and cultural characteristics and DNA sequences (the ITS region and tef 1-α gene). Bitternut hickory was the major source from which *C. smalleyi* isolates were obtained while four trees of shagbark hickory infested with hickory bark beetles or wounded (e.g. by sapsuckers) in Minnesota and Wisconsin yielded the fungus. Since several trees sampled had wounds caused by sapsuckers or physical injuries, some wound colonizing

isolates of *C. carya* (Johnson et al. 2005) were anticipated. However, none of them yielded *C. carya*. Rather, *C. smalleyi* appeared to be associated with sapsucker damage in sapwood of two trees at two sites. It was an unexpected finding because *C. smalleyi* is believed to be newly diverged having developed a unique association with hickory bark beetle (Johnson et al. 2005).

Variations in fungal morphology were found in three isolates of *C. smalleyi* obtained from Indiana and Ohio sites. These isolates formed a separate lineage in the clade of *C. smalleyi* in both phylogenetic trees based on the aligned ITS and tef 1-α gene sequences. Given the sexual compatibility between *C. smalleyi* and *C. caryae* that was proven by interfertility tests by Johnson et al. (2005), it may not be surprising that some intermediate phenotypes exist between the two species. This finding also suggests that some degree of genetic variation is present in natural populations of *C. smalleyi*.

Despite the relatively short history of the fungal taxon since first discovered in 1994, *C. smalleyi* appeared widely distributed in the North Central and Northeastern regions. Its wide existence plus other findings such as the presence of a close sibling species (*C. caryae*), some indications of variation in genetic and phenotypic characteristics, and presence of relatively resistant or tolerant trees observed within affected sites, suggest that *C. smalleyi* is likely to be a native organism in the United States. However, this speculation will require further investigations on genetic variability of the fungus and the level of resistance and susceptibility of host plants. The isolates of *C. smalleyi* from several states obtained during the survey constitute the first report of the taxon's presence on hickory in Indiana, Minnesota, New York and Ohio (Park et al. 2010). Natural ranges of bitternut hickory and hickory bark beetle are coincident throughout the eastern United States from Southern Quebec, west to Minnesota, south to Texas, and east to Georgia (Burns and Honkala 1990; U.S. Department of Agriculture 1985). However, it is unknown whether *C. smalleyi* occurs in the southern part of bitternut hickory range where hickory bark beetle is commonly found.

All *Fusarium* isolates were identified as *F. solani* based on morphology, cultural features, and the tef 1-α gene sequences. It is well known that many members of *F. solani* species complex are plant pathogens responsible for serious plant diseases. Examples of

such diseases, to name a few, include root and fruit rot of *Cucurbita* spp., sudden death syndrome of *Glycine max*, and root rot of *Phaseolus vulgaris* (O'Donnell 2000). On the other hand, *F. solani* received minimal attention in forest pathology in the past because it was considered a common saprophyte or a contaminant. Its pathogenicity with regard to hardwood species has been noted from the 1960's, primarily as a cause of canker diseases. More recently, increasing numbers of tree diseases caused by *F. solani* have been reported from a variety of host species such as English walnut, London plane, and red oak (Chen and Swart 2000; Pilotti et al. 2002; Vujanovic et al. 1999). Among them, Fusarium stem cankers reported from two other species in the genus *Juglans* (Chen and Swart 2000; Tisserat 1987) were intriguing because it is a close relative of *Carya* sp. and in the same family Juglandaceae. The similarity of the reported disease symptoms to those observed on bitternut hickory was also highly suggestive of the pathogenicity of *F. solani* to bitternut hickory.

Based on sequence comparisons, *F. solani* isolates were split into two isolate types, type BC that best matched the *F. solani* strain obtained from black walnut canker and type BB that were identical to several strains obtained from a birch tree and human tissues in their sequences. *F. solani* species complex consists of over 45 phylogenetically distinct species whose ecological functions vary from soil saprophytes, decomposers, plant pathogens to human and animal pathogens (Zhang et al. 2006). Therefore, it is logical to speculate that the two isolate types might act differently in their associations with bitternut hickory. Specifically, it will be interesting to determine whether BC isolates show higher virulence to bitternut hickory than BB isolates because they have a closer DNA sequence to that of canker causing strains (e.g. isolate FRC S1124 from black walnut, isolate FD01041 from black locust). In addition, further study will be required to determine the potential host speciation of BC isolates. Two formae speciales of *F. solani* have been reported from woody plants. *F. solani* f. sp. *robiniae* causes twig blight on black locust (*Robinia pseudomonas*) and f. sp. *mori* causes stem blight on mulberry (*Morus* sp.) (Matuo and Sakurai 1965; Sakurai and Matuo 1957, 1959). Such host specificity which is commonly found in *F. solani* species complex can be

determined using cross inoculation tests among various formae speciales of *F. solani* obtained from different hosts.

F. solani was found widely distributed throughout the North Central and Northeastern regions. Its presence was confirmed in 24 out of 27 sites surveyed across six states. There have been no previous reports of *F. solani* as a pathogen or saprophyte on *Carya* species (Farr et al. 1989). Therefore, once its pathogenicity is verified (chapter 3), it will constitute the first report of *F. solani* and associated stem canker disease on *Carya* sp.

Phomopsis sp. was more commonly isolated from samples of stem galls on bitternut hickory compared to other fungal taxa. However, the isolation rate of fungi from such samples was relatively low and it is uncertain if all the *Phomopsis* isolates were the same species because the identification to the species level was not successful. According to the world list of *Phomopsis* species by Uecker (1988), no *Phomopsis* species has been described from *Carya* species. Among its close relatives, English walnut (*Juglans regia*) has been reported to host two *Phomopsis* species, *P. juglandina* and *P. lixivia*. In spite of Uecker's list, gobose gall (= Phomopsis gall) on bitternut hickory is widely known and considered to be caused by *Phomopsis* sp. in USA.

Phomopsis sp. is known to be a cause or a putative cause of gall and tumor diseases of various woody plants including *Acer*, *Carya*, and *Quercus* species in the United States (Sinclair and Lyon 2005). On *Carya* species, its presence was first observed as mycelium in sections of young growing galls by Brown (1938). As *Phomopsis* sp. was isolated from those tissues and a pathogenicity study was conducted by the same author, the disease has been called Phomopsis galls. However, there are several reasons that this disease should be viewed with caution regarding the cause: (1) the *Phomopsis* sp. appeared to be a weak pathogen to hickory forming a small gall at best in the pathogenicity study conducted by Brown (1938); (2) in the same study, the success rate to induce galls by the inoculation was very low at about 9%; (3) when another pathogenicity test was conducted in 2007 using *Phomopsis* isolates obtained from stem galls of bitternut hickory, no evidence of resulting gall formation was found within 1 year (J.-H. Park, data not shown); (4) as discussed above, the frequency at which *Phomopsis*

species was isolated from samples of stem galls was not high (23%). These inconclusive results, therefore, possibly suggest that galls found on bitternut hickory may be induced by another organism, but further study is needed.

C. smalleyi and *F. solani* were consistently obtained from samples of stem cankers of hickories. Specifically, *C. smalleyi* was more commonly associated with diffuse cankers of trees exhibiting rapid crown decline than annual cankers. On the other hand, *F. solani* was more frequently obtained from annual cankers of trees showing top dieback in the tree crown. Both species were not found from stem galls as commonly as from cankers. The finding of multiple stem damage types on declining hickories and their associations with different putative pathogens suggest that recently reported hickory decline is not just attributable to outbreaks of hickory bark beetle alone.

Because earlier studies of hickory mortality were dominated by investigations of insect species, particularly the hickory bark beetle, it is important to consider the relationship between known insect pests and other stem damage types as well as fungal species commonly found in this study. It turned out that a large number of samples had hickory bark beetle damage accompanying diffuse cankers. However, in spite of the apparently close association between the two damage types, there were occasions when each of them individually occurred. Evidence of hickory bark beetle activities was observed in some instances of annual cankers as well. This observation agrees with the previous report of hickory mortality where sunken bark cankers were associated with beetle attacks (U.S. Department of Agriculture 1994). This may be a genetic resistance response by certain bitternut hickory or may indicate the high vigor of the tree and its ability to prevent expansion of cankers more than one or two years. Another noticeable aspect was insect activity of pinhole borers (e.g. *Xyleborus celsus*) that more commonly occurred inside or at the edge of the annual cankers than hickory bark beetles.

The hickory bark beetle was suspected to be a vector of *C. smalleyi* because the fungus was frequently obtained from trees infested by the beetle and several morphological features of the fungus were likely derived while presumably coevolving with the beetle (Johnson et al. 2005). High frequency of *C. smalleyi* isolation from samples associated with hickory bark beetle damage regardless of canker presence

supported the close association between the two organisms. *C. smalleyi* was obtained even from small patches of bark lesions surrounding the freshly attacked area by the beetle. However, *C. smalleyi* was not always associated with hickory bark beetle damage as it is shown that not every beetle attack site yielded the fungus. The fungus, conversely, was obtained from at least 11% of 149 subsamples of diffuse cankers in the absence of the beetle damage. These results raise questions about the consistency of association and strength of the relationship between the two organisms. Based on the observation of the extensive discolored sapwood beneath diffuse cankers, it is probable that there are other means of transmission of *C. smalleyi* such as vascular movement within an infected tree as well as by hickory bark beetle.

The frequent observations of ambrosia beetle activities associated with annual cankers on bitternut hickory were interesting because *F. solani*-ambrosia beetle complexes have been studied in relation to their associations with canker diseases of other woody plants. *F. solani* was found to be a canker causing pathogen to tulip poplar, black walnut and flowering dogwood. On these host plants, the fungus was frequently obtained from galleries of ambrosia beetles including *Xyleborus sayi*, *Xylosandrus germanus*, and *Xylosandrus compactus* found in the cankered area (Anderson and Hoffard 1978; Ngoan et al. 1976; Weber and McPherson 1984). According to Weber and McPherson (1984), *F. solani* was not an ambrosia fungus carried in mycangia but the presence of *F. solani* was consistently associated with beetle damage. Therefore, if the ability of *F. solani* to cause annual cankers on bitternut hickory is confirmed, the role of ambrosia beetles as vectors will need to be investigated. *Xyleborus celsus* is the ambrosia beetle of interest since it was the second most common beetle that emerged from hickory log sections taken from declining trees during the 2008 USDA FS hickory survey (Juzwik et al. 2009).

Recently reported hickory decline and mortality appeared to involve several different causal agents, not solely the hickory bark beetle. *C. smalleyi* among them seems particularly important with regard to its role involved in tree death. It is because *C. smalleyi* was most closely associated with the most prevalent symptom observed in affected sites: rapidly declining crown associated with hickory bark beetle attacks and

diffuse cankers. In addition, its associated cankers affected much larger areas of bark and sapwood than other damage types.

In summary, this study investigated the fungi associated with three different types of stem damage associated with dying and dead hickory: diffuse cankers, annual cankers, and globose stem galls. The putative pathogens obtained from these affected trees were *C. smalleyi*, *F. solani*, and *Phomopsis* sp. *C. smalleyi* and *F. solani* were found to be widely distributed across the Northeast and North Central regions. *C. smalleyi* appeared to be more commonly associated with diffuse cankers and *F. solani* with annual cankers. Because *Phomopsis* sp. was infrequently obtained from globose galls and its pathogenicity was low or did not occur on bitternut hickory, further examination of microorganisms associated with this disease is warranted. Ability of *C. smalleyi* and *F. solani* to cause cankers on mature bitternut hickory (chapter 3), their associations with insects, and their roles in causing the recently wide-spread hickory decline and mortality need to be determined.

Chapter 3. Pathogenicity of fungi associated with cankers on bitternut hickory

3.1. Introduction

Bitternut hickory (*Carya cordiformis*) which belongs to the Walnut family (Juglandaceae) is considered the most abundant and uniformly distributed hickory in North America. The native range of bitternut hickory lies throughout the eastern United States. Although there have been a few reports of declining hickory which were attributed to outbreaks of hickory bark beetle (*Scolytus quadrispinosus*) and drought at times from 1900, bitternut hickory has been considered to have no severe diseases. Other common problems on hickories were fire damage due to its shallow bark and sapsucker-induced streaks (Burns and Honkala 1990).

Since 2000, higher rates of hickory decline or mortality than expected has been noticed from multiple states in the range of bitternut hickory including Iowa, Maryland, Missouri, New York, Pennsylvania, West Virginia, and Wisconsin (Johnson et al. 2005; Steinman 2004; Tucker et al. 2006; Wisconsin Department of Natural Resources 2005). The loss of a high proportion of this tree species on these sites occurred over a very short period of time (3 to 5 years) and has resulted in a significant adverse impact on wildlife, timber value, and biodiversity. This recent sudden decline of hickories in North Central and Northeastern forests prompted surveys of affected forest stands focused on site conditions, tree health, and biotic and abiotic problems observed. Based on results of the 2-year survey (2007 to 2008), a high percentage of declining hickory trees were found with several types of fungal-associated stem and branch damage (chapter 2). These findings lead to an examination of the putative pathogens associated with the disease complex in order to clarify which fungi played a major role in decline development and tree death.

One fungus associated with declining hickories was recently described as a new species (*Ceratocystis smalleyi*) and the authors suggested that it might play a significant role in hickory mortality (Johnson et al. 2005). A *Ceratocystis* species was first isolated

from sapwood beneath bark cankers associated with hickory bark beetle attacks in 1994 (U.S. Department of Agriculture 1994). Later, greenhouse inoculation tests with *C. smalleyi* showed its ability to cause bark necrosis and xylem discoloration on 2-yr-old *Carya* spp. (Johnson et al. 2005). The fungus, subsequently, has been isolated at a high frequency from cankered tissues of trees exhibiting rapid crown decline (chapter 2).

Many members of the genus *Ceratocystis* are well known as causes of a number of tree diseases including cankers, root diseases, wood stains, and vascular wilts. Their hosts range from agronomic crops to hardwood and conifer trees. Well-known tree pathogens are *C. fagacearum* causing oak wilt in North America, *C. fimbriata* f. sp. *platani* causing canker stain of plane trees in Europe, and *C. polonica* causing blue stain on Norway spruce in Europe. As a canker pathogen of hardwood, *C. fimbriata* f. sp. *platani* has caused serious losses of urban plantings of plane trees in European cities (Panconesi 1999). *C. cacaofunesta, C. fimbriata* sensu lato, *C. manginecans*, *C. colombiana* and *C. papillata* have been reported to cause canker diseases of economically important tree species such as cacao, stone fruit, mango, coffee and citrus (Al Adawi et al. 2006; Engelbrecht et al. 2007; Teviotdale and Harper 1996; Van Wyk et al. 2007; Van Wyk et al. 2010). These pathogenic members of the genus *Ceratocystis* are also, in general, associated with or vectored by insects. Thus, the likelihood of *C. smalleyi* being a pathogen on hickory seemed high because it is commonly associated with stem cankers and with insect damage on affected trees.

Another fungus commonly found on declining hickory in the 2007-08 survey was *Fusarium solani*. *F. solani* has been dealt with as a species complex rather than a species because of its high host specificity and phylogenetic variability (Zhang et al. 2006). Due to its importance as a plant pathogen, its intraspecific category as a forma specialis has been well-established for crop diseases. However, this is not the case for tree pathogens in spite of *F. solani*'s wide geographical distribution and frequent association with tree cankers or dieback. Rather, *F. solani* tends to be considered as a saprophyte or a contaminant in isolation because of its ubiquitous nature as a saprophyte (Boyer 1961). *F. solani* has been noted from the 1960s as Fusarium cankers on hardwoods were reported from multiple states in the United States and Canada and its pathogenicity was tested on a

list of tree species. Such cases are canker diseases of eastern cottonwood (*Populus deltoides*) in Quebec, black walnut (*Juglans nigra*) in Kansas, yellow poplar (*Liriodendron tulipifera*) in Ohio, sugar maple (*Acer Saccharum*) in Pennsylvania, and sweet orange (*Citrus sinensis*) cultivars in Florida (Boyer 1961; Dochinger and Seliskar 1962; Nemec 1987; Skelly and Wood 1966; Tisserat 1987). Reports of cankers or canker related dieback caused by *F. solani* on new hardwood species have continued to appear worldwide as follows: severe dieback on box elder (*Acer negundo*) in Turkey, stem canker of English walnut (*Juglans regia*) in South Africa, stem disorders of London plane cultivars (*Platanus* × *acerifolia*) in Italy, canker and wilt of red oak (*Quercus rubra*) in Canada, and bark canker disease of a leguminous tree (*Cedrelinga cateniformis*) in Equador (Chen and Swart 2000; Demirci and Maden 2006; Lombard et al. 2008; Pilotti et al. 2002; Vujanovic et al. 1999).

Fusarium solani has not been reported as a pathogen or saprophyte on *Carya* species (Farr et al. 1989). However, based on results of the 2007-08 survey (chapter 2), the species was suspected to be a pathogen for several reasons. First, it is consistently associated with annual, sunken cankers of bitternut hickory. Secondly, *F. solani* is known to cause annual cankers on other hardwoods, and specifically on the other species in *Juglans* (Juglandaceae). Lastly, the proximity of DNA sequences of BC isolates of *F. solani* to the one obtained from black walnut canker during the survey provided the high probability of its pathogenicity to bitternut hickory.

The goal of this study was to evaluate the pathogenicity of *C. smalleyi* and *F. solani* on maturing bitternut hickory. Information on the pathogenicity of these fungi will contribute to determination of the role of putative pathogens isolated from declining bitternut hickory in causing crown decline and dieback in the species.

3.2. Materials and Methods

3.2.1. Isolates

C. smalleyi isolates selected for the pathogenicity trials were C1952 and C1928 (obtained from T. C. Harrington, Iowa State University) for the Iowa trial; isolates

CS0709 and CS0729 for Minnesota and isolates CS0731 and CS0734 for Wisconsin (Table 3-1). Isolates of two *F. solani* isolate types (BB = birch bark source; BC = black walnut canker source) were selected for two pathogenicity trials. Two BB and two BC type *F. solani* isolates were used in each of the two field trials (Table 3-2).

Table 3-1. Sources and GenBank accession numbers for ITS region DNA sequences of *C. smalleyi* isolates

Isolate number	GenBank accession no.	Collector	Source	Collection location
C1952	-	Johnson*	*Carya cordiformis* with *Scolytus* beetle gallery	Linn Co., IA
C1828	AY907032	Johnson	*C. cordiformis* with *Scolytus*	Linn Co., IA
CS0709	GU190738	Park and Juzwik	*C. cordiformis* with cankers	Wabasha Co., MN
CS0729	GU190739	Park and Juzwik	*C. ovata* with cankers	Wabasha Co., MN
CS0731	-	Park and Juzwik	*C. cordiformis* with cankers and *Scolytus* beetle holes and galleries	Monroe Co., WI
CS0734	-	Park and Juzwik	*C. cordiformis* with cankers and *Scolytus* beetle galleries	Monroe Co., WI

* (Johnson et al. 2005)

Table 3-2. Sources and GenBank accession numbers for tef 1-α gene DNA sequences of *F. solani* isolates

Isolate type	Isolate number	GenBank accession no.	Collector	Source	Collection location
BC	FS06J1	HQ647285	Park and Juzwik	*C. cordiformis* with dieback and cankers	Winona Co., MN
BC	FS0723	HQ647286	Park and Juzwik	Declining *C. cordiformis*	Olmstead Co., MN
BB	FS06C10	HQ647289	Park and Juzwik	*C. cordiformis* with dieback and cankers	Wabasha Co., MN
BB	FS0726	HQ647290	Park and Juzwik	*C. cordiformis* with cankers	Olmstead Co., MN
BC	FS0802	HQ647287	Park and Juzwik	*C. cordiformis* with cankers and insect damage	Chippewa Co., WI
BC	FS0803	HQ647288	Park and Juzwik	*C. cordiformis* with cankers	Chippewa Co., WI
BB	FS0801	HQ647291	Park and Juzwik	*C. cordiformis* with cankers and insect damage	Chippewa Co., WI
BB	FS06C4	HQ647292	Park and Juzwik	*C. ovata* with dieback, cankers, and *Scolytus* beetle galleries	Marathon Co., WI

3.2.2. Pathogenicity study

3.2.2.1. Inoculum preparation

For the Iowa trial, *C. smalleyi* isolates were cultured for 3 weeks on 2% malt yeast extract agar (MYEA) and colonized agar plugs (0.6 cm diameter) were used for inoculum. For Minnesota and Wisconsin trials, ascospores of *C. smalleyi* were collected from extruded masses on tips of perithecia of 1 to 2 week old cultures growing on MYEA and suspended in 1.0 ml sterile distilled water. Due to the sticky nature of ascospore masses, the suspension was homogenized with a tip sonicator. The spore suspension was adjusted to a concentration of 1×10^4 ascospores/ml. For *F. solani*, single spore cultures were transferred onto carnation leaf agar (CLA) to stimulate the fungus to produce macroconidia. After 10 days, these spores were scraped from sporodochia on carnation leaves and suspended in 1 ml sterile distilled water. The suspension was vortexed and adjusted to a concentration of 1×10^4 macroconidia/ml.

3.2.2.2. Study sites and trees

Three sites were selected in three states as follows: a mixed hardwood stand undergoing timber stand improvement in Allamakee Co., IA; mixed hardwood stands in Wabasha Co., MN, and Chippewa Co., WI. Pole-size bitternut hickory trees (13 to 28 cm in diameter) with healthy crowns (< 15% dieback) and stems without visible defect or damage were selected for *C. smalleyi* pathogenicity trials in the three sites. They were considered intermediate to dominant in the canopy. Healthy bitternut hickory trees of smaller size (12 to 21 cm in diameter) were selected for *F. solani* pathogenicity studies in Minnesota and Wisconsin sites. They were intermediate to co-dominant in their crown class and had healthy crowns and stems without visible defect or damage.

3.2.2.3. Inoculations

In May 2007, one fungus colonized MYEA disk or sterile agar was placed in each of two holes (0.6 cm diameter) drilled into the cambium on stems of 10 trees in Allamakee Co., IA. Moist cotton and laboratory film held the disks in place. In June 2008, 0.1 ml spore suspension (1×10^4 ascospores/ml) of *C. smalleyi* or sterile distilled water was pipetted into four drilled-holes (to the outer sapwood) of four trees in Chippewa Co., WI. Holes were sealed with moist cotton and masking tape. In a similar

trial, a spore suspension or sterile water was placed into four drilled holes covered with moist cotton and moldable epoxy putty on each of six trees in Wabasha Co., MN. In May 2009, 0.1 ml of spore suspension (1×10^4 macroconidia/ml) of *F. solani* isolate of each BC type and BB type or sterile distilled water was pipetted into one of three holes (0.6 cm in diameter) drilled into the cambium on stems of the study trees. Holes were sealed with moist cotton and moldable putty. In June 2009, a duplicate pathogenicity trial was carried out in Chippewa Co., WI, but with the WI source isolates.

3.2.3. Evaluation and data analysis

Symptom development following the fungal inoculation was evaluated by presence and extent of necrotic bark area and the volume of discolored sapwood. In the Iowa site, both the area of inner bark necrosis and the volume of discolored sapwood were measured 12 months after inoculation. For the latter two trials of *C. smalleyi* inoculation, only inner bark necrosis was measured after two months in the Minnesota trial and fourteen months in Wisconsin. Re-isolation of *C. smalleyi* was attempted from every inoculation point using the moist chamber process previously described (chapter 2). For *F. solani* pathogenicity study, the extent of inner bark necrosis was assessed 13 months after inoculation in both sites. *F. solani* was recovered by plating small wood chips from advancing margin of inner bark lesion onto APDA. Re-isolated *F. solani* isolates were identified by cultural characteristics and DNA sequences of the tef 1-α gene to differentiate the isolate types. Effects of each treatment on symptom development were analyzed by one-way Analysis of Variance (ANOVA) in SAS 9.1 (SAS Institute, Inc. Cary, NC). Differences of means were determined using Tukey's HSD at the significance level of $\alpha=0.05$.

3.3. Results

3.3.1. Canker development by *C. smalleyi*

In Allamakee Co., IA, diffuse cankers were found for all but one fungus inoculation site; no cankers occurred with control inoculations after 12 months of inoculation. Reddish-brown inner bark necrosis (mean area 22.4 ± 3.20 cm^2) and

sapwood discoloration (mean volume 38.1 ± 9.21 cm^3) were associated with the cankers (Fig. 3-1; 3-2B-D). There was no difference between effects of two isolates in canker development (Fig. 3-1). *C. smalleyi* was recovered from five of nine cankers, but not from the control wounds. Two months after inoculation in Chippewa Co., WI, diffuse cankers with reddish inner bark (mean 49.7 cm^2) surrounded 16 inoculation points; no cankers or inner bark necrosis was observed for the control points (Fig. 3-2A). In Wabasha Co., MN, either diffuse or sunken cankers with reddish inner bark necrosis (mean 22.3 cm^2) were observed surrounding all inoculated points while all control points were callus-closed fourteen months later (Fig. 3-1; 3-2A). For the latter two trials long, narrow zones of discoloration (reddish brown) were found in the sapwood associated with each canker; no sapwood discoloration was observed for the control wounds (Fig. 3-2A). There was no significant difference between the effects of the two isolates within each location in canker disease development in both trials (Fig. 3-1). In addition, *C. smalleyi* was re-isolated from all cankered stems in WI and from 21 of 24 cankers in MN, but not from the controls.

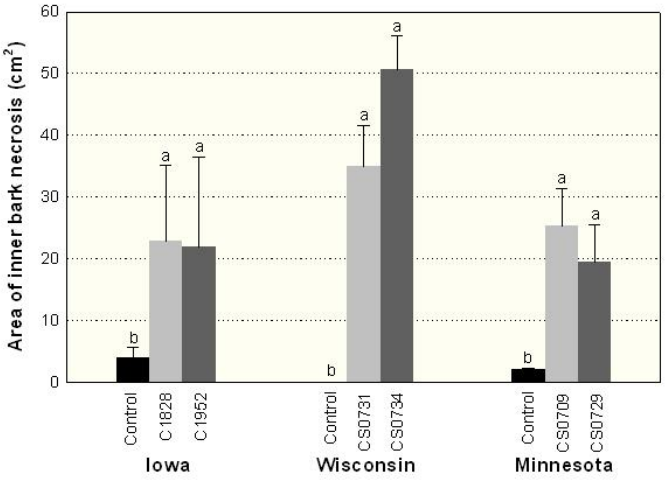

Figure 3-1. Mean area of inner bark necrosis by *C. smalleyi* inoculation compared to water control in three study sites. Error bars represent standard error for inner bark necrosis. Different letters above the bars indicate treatments that are statistically different based on Tukey's HSD test at a 95% confidence limit.

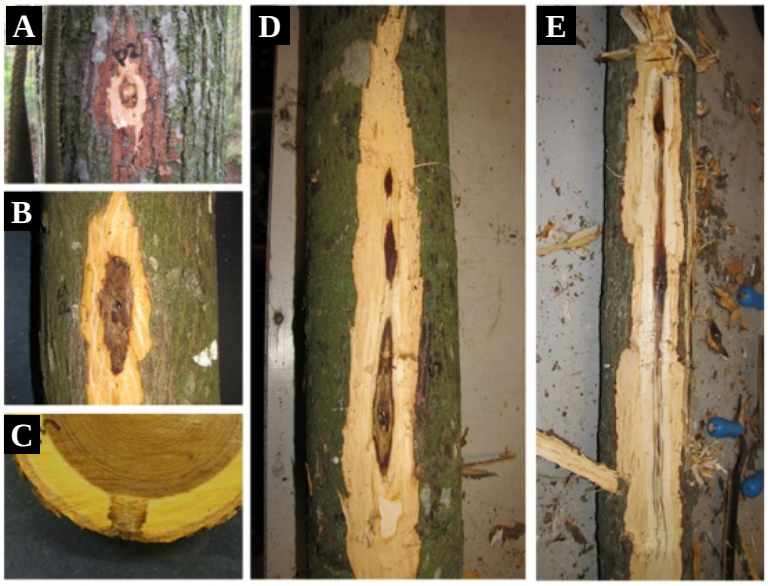

Figure 3-2. Canker development by *C. smalleyi* inoculation in Wabasha Co., MN. A. Water inoculated with wound closed by newly formed callus tissues. **B.** Necrotic inner bark caused by *C. smalleyi*. **C.** Brownish sapwood discoloration induced by *C. smalleyi* inoculation. It extended all the way to the borderline between heartwood and sapwood. **D.** Canker resurgence along the length of the stem on a fungus-inoculated tree. **E.** Reddish brown, long, narrow discoloration of sapwood induced by *C. smalleyi* inoculation.

3.3.2. Canker development by *F. solani*

In Wabasha Co., MN, sunken or open cankers were induced by BC type inoculation (Fig. 3-3C) while smaller bark lesions or callus-closed holes were found around BB type inoculation points (Fig. 3-3B). All control inoculation points were callused over at the point of evaluation (Fig. 3-3A). Brownish inner bark necrosis (mean length 29.7 ± 0.27 mm) resulting from BC type inoculation was significantly longer than that produced by BB type isolates or control ($P < 0.0001$). There was no significant difference between effects of BB type isolates and control on canker development (Fig. 3-4). The same isolate type of *F. solani* was recovered from all lesions as inoculated and it was confirmed by tef 1-α sequence comparison and cultural characteristics. In Chippewa Co., WI, sunken cankers with brownish inner bark necrosis (mean 37.5 ± 3.1 mm) surrounded all BC type inoculation points but one. Relatively smaller lesions (mean 33.5 ± 2.4 mm) were induced by BB type inoculation and no canker formed for control. Necrotic inner bark produced by BC and BB type isolates were significantly longer than the wounds made for control treatment ($P = 0.0014$). *F. solani* was recovered from all bark lesions induced by fungal inoculation and its cultural characteristics and DNA sequences were identical to those observed for the inoculum. Overall, the length of inner bark lesions or wounds for each treatment was larger on examined trees in Wisconsin than in Minnesota ($P < 0.0001$). However, effects of different treatments showed the same pattern in both locations.

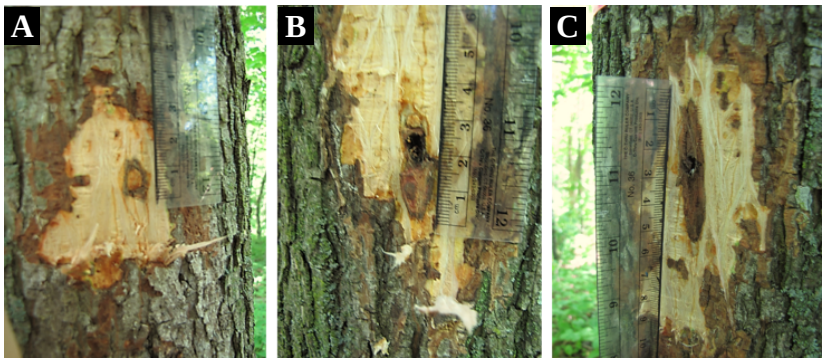

Figure 3-3. Canker development by *F. solani* inoculation in Wabasha Co., MN. A. Control inoculation point and newly formed callus tissue closed the wound. **B.** Necrotic bark lesion formed by BB type *F. solani* inoculation. **C.** Inner bark necrosis induced by BC type *F. solani* inoculation.

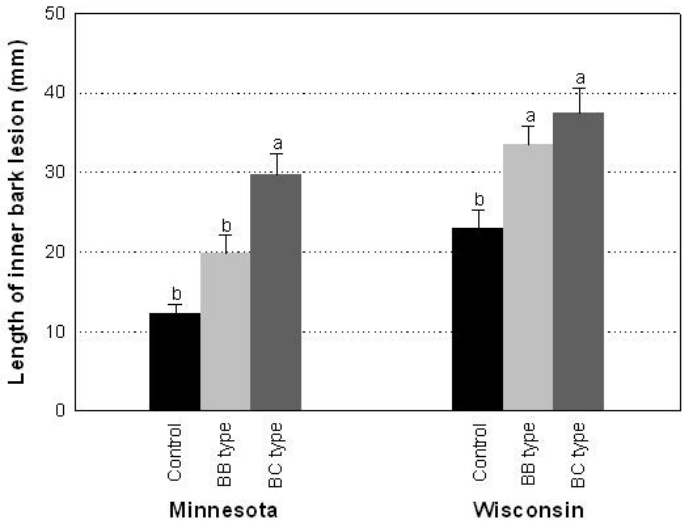

Figure 3-4. Mean length of inner bark lesions formed by two isolate types of *F. solani* inoculation compared to control in two study sites. Error bars represent standard error for inner bark necrosis. Different letters above the bars indicate treatments that are statistically different based on Tukey's HSD test at a 95% confidence limit.

3.4. Discussion

This study verified the ability of *C. smalleyi* to cause a newly described canker disease on pole-timber sized hickories. This is the first report of Ceratocystis canker of bitternut hickory caused by *C. smalleyi* in the North-central and Northeastern United States. USDA Forest Service (1994) and Johnson and others (2005) found *C. smalleyi* can be pathogenic to bitternut hickory (*Carya cordiformis*), shagbark hickory (*Ca. ovata*), pecan (*Ca. illinoensis*), black walnut (*Juglans nigra*) and butternut (*J. cinerea*) based on greenhouse studies conducted in Wisconsin and Iowa. However, Koch's postulates were not fully performed or described in those previous studies and seedling inoculations do not provide a satisfactory description of the disease which mainly occurs on pole-timber and saw-timber sized trees in the field. By fulfilling Koch's postulates with maturing trees in three study sites, *C. smalleyi* was found to be not only pathogenic but highly virulent to maturing bitternut hickory resulting in extensive bark necrosis and sapwood discoloration.

Any external symptoms of declining crown did not appear on infected trees during the examination period until 14 months after inoculation. However, artificial

inoculations with *C. smalleyi* produced typical symptoms of Ceratocystis canker diseases of hardwoods such as extensive bark lesion and associated sapwood discoloration. The extent of canker formation by *Ceratocystis* sp. on hardwood species does vary by pathogen, host species, tree age, and the duration of canker development. In this study, *C. smalleyi* induced larger cankers of mean 21.8 cm (7.8 – 37.2 cm) in length 2 months after inoculation on bitternut hickory compared to cankers on coffee trees caused by *C. fimbriata* (2.6 – 13.6 cm 6.5 months after inoculation) (Marin et al. 2003). The linear extent of Ceratocystis canker of bitternut hickory was similar to what was shown on mango trees by *C. manginacans* (formally known as *C. fimbriata*) which averaged 29.4 cm (19.8 – 46.1 cm) after 42 days of inoculation (Al Adawi et al. 2006). The fact that both pathogens *C. fimbriata* and *C. manginacans* have been recognized as a primary determinant of sudden declines of coffee in Colombia (Marin et al. 2003) and mango in Oman (Al Adawi et al. 2006) leads to the hypothesis that *C. smalleyi* might also be a major contributor to the historically known "hickory decline" problem.

Reddish brown discoloration of sapwood was consistently found under the cankers produced by *C. smalleyi* inoculation. The association of discolored sapwood with diffuse cankers has been frequently observed in hickory bark beetle attacked, declining hickories. Many members of the genus *Ceratocystis* are known to change the color of sapwood where they invade. Related to canker diseases of hardwood, *C. manginacans, C. fimbriata,* and *C. fimbrata* f. sp. *platani* are such examples (Marin et al. 2003; Panconesi 1999; Van Wyk et al. 2007). The color pattern in affected xylem of Ceratocystis canker of bitternut hickory most resembles what was described for stain canker of plane trees (Walter et al. 1952). Two-year-old hickory seedlings also developed the xylem discoloration in response to *C. smalleyi* inoculation in greenhouse studies (Johnson et al. 2005) but the extent was found much longer on maturing trees examined in the present study.

Besides the extensive symptom development in phloem and xylem, canker resurgence was commonly observed on fungus-inoculated bitternut hickory trees. Canker resurgence refers to the reappearance of necrotic bark at a considerable distance from the original infection site. In common with the color pattern of xylem discoloration, canker

resurgence was known to appear in canker stain of plane trees (Panconesi 1999). Since *C. fimbriata* f. sp. *platani* is able to invade both phloem and xylem tissues, canker resurgence is considered to occur as the pathogen spreads through conducting vessels and moves outward to the cambium through ray parenchyma cells (Panconesi 1999). Therefore, the frequent occurrences of canker resurgence on infected bitternut hickory provide a strong indication that *C. smalleyi* has the ability to invade and colonize sapwood beyond the cambium and phloem.

C. smalleyi was found to remain viable for 14 months at the margin of canker or discolored sapwood. Upon attempting to recover the fungus 14 months after inoculation, the recovery was not successful in cases where secondary organisms colonized the necrotic tissues. Such failure of re-isolation was also noted for *C. fimbriata* f. sp. *platani* where ability to isolate it is rapidly lost as other fungi such as *Pestalotiopsis* sp., *Fusarium* sp., *Dendrochium* sp., *Sphaeropsis* sp., and *Asterosporium* sp. colonize the affected tissues (Panconesi 1999). Because of the viability of *C. smalleyi* in the advancing area of cankers and the possibility of following secondary infection, it is likely important to take a considerable number of samples for successful recovery of the fungus.

BC type *F. solani* was found to be pathogenic to bitternut hickory causing annual cankers in both study sites. Canker disease caused by *F. solani* has not been reported from any related hickories in the United States (Farr et al. 1989). *F. solani*, and *Fusarium* sp. have not been found to be associated with stem diseases of any *Carya* sp. in the United States (Farr et al. 1989). Canker diseases have been reported from black walnut and English walnut in the genus *Juglans* which is a close relative of *Carya* sp., and *F. solani* was suggested as the main factor of the problem based on the isolation frequency and pathogenicity study (Carlson et al. 1993; Chen and Swart 2000; Tisserat 1987). Interestingly, the pathogenic isolates (BC isolates) had the best proximity of tef 1-α DNA sequence to the one obtained from black walnut canker and showed high homogeneity among isolates (chapter 2). Based on those close relations between host groups, bitternut hickory and black walnut, as well as between the fungus isolates obtained from each host group, the high host specificity of a *F. solani* complex in crop diseases likely apply to tree diseases as well.

BB isolates of *F. solani* were found to be non-pathogenic to weakly pathogenic to bitternut hickory in different sites. The presence of multiple *F. solani* isolates which differ in their virulence from non-pathogenic to pathogenic has been found in other host species such as cottonwood (Boyer 1961) and *Platanus* ×*acerifolia* (Pilotti et al. 2002). Since this isolate type showed inconsistent results in two field experiments, its pathogenicity may need to be re-examined in more controlled settings. It is probable that BB isolates depend on the tree vigor and environmental conditions to cause cankers and they may usually exist as a facultative saprophyte.

Artificial inoculation of the stem of bitternut hickory with *F. solani* induced sunken cankers which were often accompanied by callus formation. This result is in agreement with the survey results that *F. solani* was most frequently obtained from annual cankers of bitternut hickory exhibiting crown dieback (chapter 2). In pathogenicity trials on other hosts, *F. solani* was found to cause small (< 50 mm long), annual cankers on species such as *Juglans regia*, *Cedrelinga cateniformis*, and *Platanus* ×*acerifolia* (Chen and Swart 2000; Lombard et al. 2008; Pilotti et al. 2002). However, large Fusarium cankers can occur on yellow poplar, sugar maple, and black walnut; although the cankers were occasionally limited by subsequent callus formation in natural or artificial infection (Dochinger and Seliskar 1962; Skelly and Wood 1966; Tisserat 1987). Therefore, Fusarium canker of bitternut hickory is similar to cankers caused by *F. solani* on other hardwood species. The extent of canker development on a species may depend on time of inoculation and tree vigor because of the limited virulence of the pathogen. In many of the above cited cases, canker occurrence was related to unfavorable environmental conditions. These conditions included 1) water excess or deficit associated with flooding, saturated soils, or drought, and 2) poor site or growing conditions attributed to overstocking, close spacing, suppression by other species, and soil compaction (Boyer 1961; Dochinger and Seliskar 1962; Nemec 1987; Pilotti et al. 2002). In addition, wind damage and bark freeze-thaw events and insect attacks resulted in bark wounds that allowed for pathogen entry (Weidensaul and Wood 1973).

Pathogenicity studies of two predominant fungi *C. smalleyi* and *F. solani* associated with stem damage on declining bitternut hickory showed both are pathogenic

to maturing bitternut hickory resulting in stem cankers. However, based on the extent of canker development and tree wound closure response, *C. smalleyi* was found to be more virulent than *F. solani* to bitternut hickory under the given experimental conditions. *C. smalleyi* was most frequently obtained from hickory beetle-attacked trees during the 2 year survey in the North-central and Northeastern United States (chapter 2). In a separate study, up to 500 cankers were found on trees with approximately 1,500 beetle attack points (Juzwik et al. 2009). Bark and sapwood lesions extend beyond the beetle damage regardless of whether the attack was aborted after entry or a full gallery system was formed (Juzwik et al. 2010). It is likely, but not yet proven, that the hickory bark beetle is a major vector of the pathogen (Johnson et al. 2005; U.S. Department of Agriculture 1994).

In conclusion, these results and observations support the conclusion that *C. smalleyi* is more important than *F. solani* in causing crown decline and death of bitternut hickory in North Central and Northeastern States. Furthermore, these results also challenge the long-held view that stem girdling resulting from coalescing of hickory bark beetle galleries in the inner bark and cambium causes crown decline and death (U.S. Department of Agriculture 1985). Rather, the coalescing of hundreds of *C. smalleyi* cankers and/or fungus-caused dysfunction of the vascular system might be the main cause(s). Additional research is needed to test this hypothesis.

Chapter 4. Consequences of multiple *Ceratocystis smalleyi* infections for stem water transport in maturing bitternut hickory

4.1. Introduction

Severe decline and mortality of hickory have been reported to occur periodically over the past 100 years in the North Central and Northeastern United States. Recent occurrences of the hickory decline events have been noticed in parts of the same regions since 2000. The recent reports appeared like a repeat of previous occurrences which were considered to be caused by outbreaks of hickory bark beetle that often followed drought periods. The long-held conjecture that the hickory bark beetle is the sole cause of hickory decline, however, was challenged by new discoveries of two fungal pathogens of hickory (*Ceratocystis caryae* and *C. smalleyi*) and other biotic organisms observed during local surveys.

A recent two year survey of hickory decline led by USDA Forest Service found the predominant health problem in affected forest stands was rapidly declining crowns of bitternut hickory. Affected trees readily succumb to death within two years. Upon close examination of declining trees, numerous cankers and evidence of hickory bark beetle colonization were found. Diffuse cankers caused by *C. smalleyi* and accompanying sapwood discoloration were commonly associated with the hickory bark beetle attacks (chapter 2).

Progressive, declining crowns of affected bitternut hickory may be caused by coalescing of hickory bark beetle galleries, by numerous Ceratocystis cankers girdling the stem, and/or local disruption of water flow in discolored sapwood associated with the beetle attacks and the cankers. However, the exact mechanism by which the rapid crown decline and mortality occur is not known. When the bark was stripped from three actively declining trees (40 to 80% crown decline) and stems were examined for insect activity, the gallery systems observed were small and not coalescing (Juzwik, personal observation). This observation was not consistent with the reported complete girdling of tree stems by insect galleries in most previous reports (Hopkins 1912; New York State Museum 1910, 1915; Solomon and Payne 1986). Furthermore, numerous Ceratocystis

cankers affected much larger areas of bark and sapwood of affected trees than any other stem damage observed.

Locally discolored sapwood where xylem occlusion and an interruption of water transport occur has been suggested as an underlying cause of Japanese oak wilt and Sudden oak death neither of which are systemic vascular wilt diseases. Oak trees exhibiting Japanese oak wilt show a large amount of insect damage by a wood boring beetle (*Platypus quercivorus*) and associated fungal infection of *Raffaelea quercivora*. As *P. quercivorus* constructs gallery systems in the sapwood, it simultaneously inoculates the walls of the insect tunnels with its symbiotic fungus, *R. quercivora*. The fungus subsequently colonizes sapwood tissues which are recognized by its dark brown color from adjacent healthy tissues as similarly demonstrated on bitternut hickory by *C. smalleyi* infection (Kuroda 2001; Murata et al. 2007; Park et al. 2010). Xylem vessels in the discolored area were found to lose their functionality, thus reducing the amount and rate of water transport in infected oaks (Kuroda and Yamada 1996; Murata et al. 2005; Murata et al. 2007).

Sudden oak death of tanoak has been considered a shoot blight disease which causes localized tissue death. Interestingly, this disease was recently found to exhibit characteristics of vascular wilt. The causal agent, *Phytophthora ramorum* appeared to colonize not only phloem tissues but also xylem vessels by hyphal growth and chlamydospore reproduction (Parke et al. 2007). As the pathogen colonization extends into sapwood, sapwood discoloration was found to concurrently develop underneath bark necrosis (Parke et al. 2007). As was shown in the case of Japanese oak wilt, fewer vessels were found functioning in the discolored sapwood of tanoak than in the non-discolored sapwood when hydraulic conductivity was measured (Collins et al. 2009; Parke et al. 2007).

With such disease examples causing partial or limited wilt of trees, one of the key questions that arose from study of the hickory decline problem was whether multiple infections by *C. smalleyi* were responsible for the rapid wilting of bitternut hickory. The number of inoculations to be made per tree might be an important factor in designing an experimental setting for inducing hickory crown decline because this disease was not

considered to be a systemic disease. The importance of multiple infections by a pathogen to demonstrate severe crown symptoms have been also stressed in previous studies on Japanese oak wilt (Ito et al. 1998; Kinuura and Kobayashi 2006; Murata et al. 2005). In fact, it turned out that a small number of fungal inoculations (one and four holes per pole-size tree of 13 - 28 cm DBH) did not lead to any visible symptoms in the crown such as wilting, dieback, and defoliation in bitternut hickory (chapter 3).

Sapwood colonization of *C. smalleyi* and associated xylem lesion are highly likely to be associated with local disruption of water relations of bitternut hickory as the same phenomenon occurred not only in Japanese oak wilt but in other diseases such as Sudden oak death and trees infected with blue stain fungi (Collins et al. 2009; Kirisits and Offenthaler 2002; Murata et al. 2005; Parke et al. 2007). Sapwood discoloration induced by *C. smalleyi* infection extends in vertical and transverse directions, but evidence of systemic spread throughout the tree is not evident. However, multiple areas of the discolored sapwood often cover a large portion of a transverse area of wood as it commonly occurs in case of Japanese oak wilt. This observation of extensive discolored sapwood associated with *C. smalleyi* infection suggests that similarities in symptom development may exist between the hickory problem and the diseases mentioned above. Specifically, the possibility that xylem dysfunction occurs in discolored sapwood of *C. smalleyi* infected hickory required investigation.

Xylem water conductance is a key physiological function with implications for tree crown condition. This hydraulic function in relation to tree diseases has been measured in different ways. A dye injection method has been used to distinguish conductive versus nonconductive xylem areas and paths of water conduction (Kuroda and Yamada 1996; Kuroda et al. 1988; Murata et al. 2007). Xylem pressure potential has been measured using a pressure chamber to determine the effects of Japanese oak wilt and of pine wilt disease on tree physiology (Fukuda et al. 1992; Murata et al. 2005). Measurement of hydraulic conductivity is commonly used to determine the extent of xylem blockage as shown in studies on Japanese oak wilt (Yamato et al. 2003) and sudden oak death (Collins et al. 2009; Parke et al. 2007). Another useful tool for examining hydraulic conditions of trees is a sap flow monitoring system such as the heat

pulse velocity (HPV) instrument and the Granier's thermal dissipation probe (TDP) (Granier 1987; Lu et al. 2004; Smith and Allen 1996). Using these techniques, reduced sap flow velocity has been observed with sudden oak death in tanoak (Parke et al. 2007) and blue stain in pines and Norway spruce (Kirisits and Offenthaler 2002; Rice and Langor 2008; Yamaoka et al. 1990).

The use of a sap flow method seemed to be the most appropriate choice in studies of physiological responses of bitternut hickory to multiple infections by *C. smalleyi* for several reasons. First, the dye injection method using an acid fuchsin solution (0.1%) that has been commonly used in studies on Japanese oak wilt and pine wilt disease was tested for its ability to detect non-conductive areas of sapwood in naturally infected hickory trees. However, the dye solution was found to easily diffuse laterally and radially when trees were felled and before any measurement could be made (Park, unpublished data). Furthermore, the solution absorption rate was highly dependent on the time of the day and weather conditions and, practically speaking, many trees could not be tested simultaneously with this technique. Secondly, measuring hydraulic conductivity and xylem pressure deficit require immediate measurement following sample collection. Such a procedure is not easy to apply to mature trees growing in a field setting. Compared to these other techniques, the sap flow monitoring system holds important advantages. It is a non-destructive, easily automated method that allows obtaining long-term, continuous data of plant water use with high time resolution (Smith and Allen 1996). It can also be used anywhere with very little disturbance to the site (Smith and Allen 1996).

Understanding the mechanism of symptom development is crucial to predict impacts of the disease and to find approaches to managing it. Based on similarities in disease characteristics between hickory crown decline and other pathosystems such as Japanese oak wilt and Sudden oak death, it is hypothesized that *C. smalleyi* causes limited vascular occlusion, thus contributing to the rapid wilting symptom of bitternut hickory. The sap flow monitoring system was likely to be a useful technique to investigate effects of multiple stem infections of *C. smalleyi* on xylem functioning in bitternut hickory.

The objectives of this study were to 1) evaluate differences in sap flow velocity of cankered trees versus canker-free trees, and 2) determine the relationship between sap flow velocity and a) disease severity as a proportion of the total area of inner bark necrosis associated with the cankered stem and b) selected xylem properties related to water transport within hickory trees.

4.2. Materials and Methods

4.2.1. Study sites and trees

Two study sites were selected in mixed hardwood stands in Wabasha Co., MN, and Chippewa Co., WI. The Wisconsin stand had undergone thinning in winter of 2007 - 2008. Twelve pole-size bitternut hickory trees (13 to 28 cm in diameter at 1.4 m) with healthy crowns (<15% visible dieback) and stems without visible defect or damage were selected for multiple inoculations with *C. smalleyi* in each site. The trees were intermediate to dominant in the canopy.

4.2.2. Fungus inoculation

To mimic numerous canker occurrences on single stems, multiple sites on stems of the study tree were inoculated with *C. smalleyi*. In July 2008, fifty holes (0.6 cm diameter) were made by aseptically drilling into the outer sapwood on the main stem of nine trees between 1.8 m and 3.7 m stem height. The fifty holes consisted of twelve to thirteen holes in each of four longitudinal rows along the stem. Aliquots (0.1 ml) of spore suspensions (1.0×10^4 ascospores/ml) of two local isolates of the fungus and sterile distilled water (control) were pipetted into the 50 drilled holes on each of three trees, respectively. Fungal inoculum was prepared as previous described (chapter 3). Holes were sealed with moist cotton and moldable epoxy putty. Three trees remained non-inoculated and served as negative controls. A duplicate trial, but with WI isolates, was initiated in Chippewa Co., WI in July 2009.

4.2.3. Sap flow velocity measurements

The Granier-type thermal dissipation probe (TDP) system was used to monitor sap flow velocity (J_s, sap flow rate per unit conducting sapwood area) of the inoculated trees and non-inoculated, healthy trees 12 to 14 months after the multiple inoculations. In September 2009, four fungus-inoculated trees and three control trees (one water-inoculated and two non-inoculated) were selected in the Minnesota site. Three 3 cm long thermal dissipation probes (Dynamax Inc., Houston, TX), each of which consists of two sensors (heated and unheated serving as a reference) were radially inserted into sapwood approximately 30 cm above the uppermost inoculation heights around the stem of each tree. To prevent thermal interference, the heated sensor was installed 4 cm above the unheated sensor as recommended by the manufacturer. Two probes were placed along two selected inoculation rows and one probe was placed between two inoculation rows. To provide waterproofing insulation, the sapwood-air interface of each probe location was sealed with silicone and covered with a plastic cup. The stem area where three probes were placed was covered with reflective bubble wrap for thermal insulation. Signals from the sensors were monitored every 15 seconds and 30-minute means were recorded by a data logger (CR 10X, Campbell Scientific Inc., Logan, UT) for18 days (September 18 – October 5). The signal recorded was the temperature difference between the heated and unheated sensors that is dependent on the rate of sap flow around the probes. As sap flow rates increase, heat is dissipated more rapidly and the temperature difference decreases (Smith and Allen 1996).

J_s in g H_2O m^{-2} s^{-1} was calculated following Granier's equation (1987):

$$J_s = 119\left(\frac{\Delta T_M - \Delta T}{\Delta T}\right)^{1.231}$$

where ΔT (°C) is the mean temperature difference between sensors during each half-hour measurement interval and ΔT_M is the maximum ΔT when there is no sap flow. Since sapwood depth (average 2 cm) was shorter than the length of 3 cm long probe, the equation was calibrated for data obtained from the 3 cm long probes following Lu et al (2004). The software package BaseLiner (version 2.4.1, Hydro-Ecology Group, Duke University, Durham, NC) was used to calculate ΔT_M and J_s. ΔT_M was calculated as the

maximum temperature difference measured over each 24 h cycle. As this method can lead to an underestimate of norturnal J_s, ΔT_M was calculated only when there were no rain events within 24 hours and the nighttime vapor pressure deficit remained below 0.2 kPa.

In July 2010, sap flow of five fungus-inoculated trees, three water control trees, and three non-inoculated trees were monitored in the Wisconsin site in the same way, but with two 2 cm long hand-made probes and one 3 cm long probe (Dynamax Inc., Houston, TX) for each tree. Sensors of a 2 cm long probe were placed 10 cm apart according to Granier(1987)'s method. Probes were installed approximately 30 cm above the uppermost inoculation heights along three vertical inoculation rows on the east, west, and north sides. Signals from the sensors were monitored every 15 seconds and 30-minute means were recorded by two data loggers (CR 23X, Campbell Scientific Inc., Logan, UT) for 24 days (July 30 – August 22).

4.2.4. Evaluation

4.2.4.1. Visual symptoms and pathogen assay

External symptoms of crown decline such as yellowing, wilting, and defoliation were evaluated one year after inoculation prior to sap flow monitoring. After sap flow monitoring was completed and TDP sensors were removed, the size of inner bark necrosis (length and width) was measured for each inoculation point. For Minnesota trees, the size of sapwood discoloration (length, width, and radial depth) beneath each canker was also measured after trees were felled. Wood blocks that contained the edges of four cankers or control wounds were taken from each tree in order to confirm the presence/absence of the pathogen. *C. smalleyi* was re-isolated by maintaining small wood cubes cut from the sampled wood blocks in small moist chambers in order to stimulate formation of fruiting structures of the fungus. The presence of any fungal fruiting bodies was determined 10 days and 20 days later. Ascospore masses on tips of perithecia sporulating on the wood cubes were transferred to 2% malt yeast extract agar amended with 100 ppm streptomycin sulfate to obtain fungal isolates.

4.2.4.2. Selected xylem properties

Water transport within a plant largely depends on gradients in water potential along the soil-plant-atmosphere continuum and xylem structures that control hydraulic properties. Size of vessels is one of the important anatomical features of the xylem. According to the Hagen-Poiseuille law for volume flow through ideal capillary tubes, volume flow rate through a vessel increases with the fourth power of the vessel radius (Pallardy 2008). Thus, hydraulic efficiency is governed by the proportion of large vessels that conduct more water. To determine if there were any intrinsic differences in vessel sizes that could have affected water conduction in study trees, mean vessel diameter, mean hydraulic vessel diameter which gives more weight to large vessels, and the size distribution of vessels were measured. Two sapwood cubes (1.5 cm × 1.5 cm × 2.0 cm) were taken from above and below each probe location. Cross sections (20 – 25 µm) were made from each wood cube using a sliding microtome (Model 860, American Optical, Southbridge, MA), stained in toluidine blue O (0.5% aq.) and mounted in glycerol (10%). A total of 500 vessels were analyzed for each probe location (250 vessels for each wood cube). To estimate vessel diameter (d), the maximum and minimum width of vessels were measured at 100× magnification using imaging software NIS-Elements (Nikon, Japan) and averaged. Mean vessel diameter of trees in each treatment group was calculated by averaging vessel diameters estimated in every probe location. Mean hydraulic vessel diameter was also calculated for each treatment as $\sum d^5/\sum d^4$. For size distribution of vessels, 500 vessels measured for each probe location were classified into 30 µm diameter classes and the numbers of each class from fungal inoculated trees versus control trees (water control and non-inoculated trees) were summed for comparison.

Tyloses are overgrowths of the protoplast from adjacent living parenchyma cells, which protrudes into xylem vessels through pits (Agrios 2005). Vessel occlusion by tyloses has been observed in trees with vascular wilt diseases such as oak wilt and Dutch elm disease (Elgersma 1973; Pallardy 2008). Since extensive vascular plugging by tyloses has been closely linked to xylem dysfunction in infected trees, the frequency of tyloses formed in response to each treatment was evaluated (Collins et al. 2009; Kuroda 2001; Parke et al. 2007). Tylose formation was observed at 100× magnification with the

same cross sections used for vessel diameter measurements and the number of vessels with and without tyloses in each annual ring from the latest to the 9 or 10 year old annual ring was counted (n = 500 per probe location). Tylose frequencies in the two outermost annual rings which might have been affected by the inoculation and in the outer 9 - 10 annual rings were separately estimated.

4.2.5. Data analyses

Effects of each inoculum type on symptom development (the average size of inner bark lesions) were analyzed by one-way Analysis of Variance (ANOVA). Differences of means were determined using Tukey's HSD at the significance level of $\alpha = 0.05$. Average of peak sap flow velocity (J_s) values measured on multiple days was estimated for each tree by repeated measures ANOVA. Mean peak J_s, mean vessel diameter, mean hydraulic vessel diameter, and tylose abundance of fungal inoculated trees were compared to those of control trees (water control and non-inoculated trees) using two sample t-test for means at 95% confidence level. Effects of different sites on any parameters were evaluated by factorial ANOVA at the significance level of $\alpha = 0.05$. Pearson's chi-square statistics at $\alpha = 0.05$ were used to determine the presence of correlation among J_s, disease severity (inner bark lesion proportion), and xylem features. Regression analysis was conducted to investigate the strength of relationships of sap flow velocity to disease severity and selected xylem features. All statistical analyses were conducted using SAS 9.1 (SAS Institute, Inc. Cary, NC).

4.3. Results

4.3.1. Visual disease symptoms

Numerous cankers were found on the *C. smalleyi* inoculated stems 12 to 14 months after treatment (Fig. 4-2A). Fourteen months after inoculation, no obvious external symptoms in the crown region such as wilting leaves or crown dieback were seen in any inoculated trees in Minnesota. Whereas, one of six fungal inoculated trees

exhibited about 60% declining crown 12 months after inoculation in Wisconsin. Other trees had healthy looking crowns at the time of evaluation.

Twelve to fourteen months after the inoculation, *C. smalleyi* inoculated trees exhibited significantly larger cankers characterized by bark necrosis and sapwood discoloration versus no cankers on the water inoculated trees (by ANOVA, Tukey's HSD at $\alpha = 0.05$) (Fig. 4-1). The cankers were mostly long, narrow, diffuse cankers with reddish inner bark as described in the pathogenicity study (Fig. 4-2D and E) (chapter 3) while sunken cankers of a limited size were also found at some inoculation points. One of the fungal inoculated trees in Minnesota actively produced callus tissues around relatively small cankers suggesting high tree vigor or resistance responses (Fig. 4-2F). Discontinuous cankers, as previously described as "canker resurgence" (chapter 3), were commonly observed in fungal inoculated trees (Fig. 4-2H). Larger cankers (mean 64.3 ± 2.2 cm^2) developed in Wisconsin trees compared to that of Minnesota trees (mean 15.3 ± 0.6 cm^2) in spite of being evaluated two months earlier (by two sample t-test for means, $P < 0.0001$). In Wisconsin, many of the cankers were longer than the distance between the above and below inoculation points, thus they coalesced (Fig. 4-2D). In such cases, lesion length was measured up to the middle point of the two inoculation points. This may have resulted in underestimation of canker area. For water controls, bark necrosis was restricted to the wound site itself and many inoculation points were callused over at the time of evaluation (Fig. 4-2B and C). Lesion proportion was calculated for trees used for sap flow measurement by dividing the sum of the 50 inner bark lesion areas by the stem surface area where the 50 inoculations were made (Table 4-1).

Sapwood discoloration extended further beyond its corresponding bark lesion along the vertical direction of stem when inoculated with the fungus (Fig. 4-2H). Occurrences of two to three discontinuous bark lesions corresponding to a continuous, discolored sapwood area were not unusual in fungus inoculated trees (Fig. 4-2H). Since many of fungus-caused cankers were coalesced, it was hard to delineate discolored sapwood corresponding to an individual inoculation. Thus, the length or volume of discolored sapwood was not measured. In the radial direction, sapwood was discolored up to 3.3 cm from the cambium in response to the fungal infection, reaching the outer

boundary of heartwood in many cases (Fig. 4-2G). Control wounds had little, if any, discolored sapwood that was confined to a very small area around the inoculation points (Fig. 4-2G).

Sapwood samples were taken from four cankers or control wounds for each inoculated tree or water control tree. *C. smalleyi* was re-isolated from all canker samples, but not from the controls.

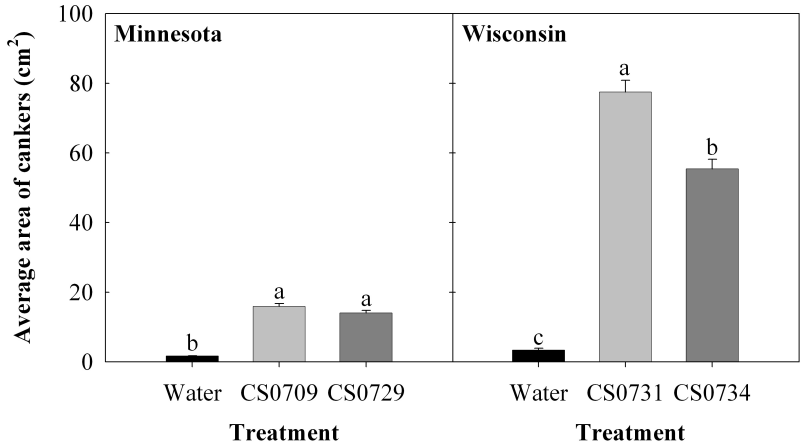

Figure 4-1. Mean area of inner bark necrosis associated with *C. smalleyi* inoculation (CS0709, CS0729, CS0731, CS0734) compared to water controls in two study sites. Canker development was evaluated 14 months after inoculation in Minnesota and 12 months after inoculation in Wisconsin. Measurements from 150 cankers/wounds of three replicate trees were averaged for each inoculum type except for CS0731 (n = 100). Error bars represent standard error for inner bark necrosis. Different letters above the bars indicate treatments that are statistically different within a site based on Tukey's HSD at a 95% confidence limit.

Figure 4-2. Bark lesions and sapwood discoloration resulted from multiple inoculations with *C. smalleyi* and water (control). A. Numerous cankers observed on a naturally infected tree after the bark was removed. **B.** Inoculation wounds on a water inoculated tree 12 months after inoculation. **C.** Water-inoculated wound healed over by newly formed callus tissues on a water inoculated tree. **D.** Coalescing cankers caused by *C. smalleyi* in two rows on a fungus-inoculated tree. **E.** Diffuse cankers exhibiting long, reddish brown inner bark lesions on a *C. smalleyi*-inoculated tree. **F.** Actively developing callus tissue (arrow) that restricted canker development on a resistant or vigorous tree to *C. smalleyi* infection. **G.** Extensive sapwood (SW) discoloration beneath *C. smalleyi* cankers (on the left side and in the center) compared to restricted effects of water control on sapwood (on the right side). Sapwood discoloration often reaches to heartwood (HW) in fungus-inoculated trees. **F.** Underlying sapwood discoloration (delineated by red colored solid line) corresponding to three discontinuous bark lesions (black arrows) in longitudinal radial face of a fungus inoculated tree. White arrow indicates the inoculation point.

Table 4-1. Characteristics of bitternut hickory trees used for sap flow monitoring study and extent of inner bark lesion area evaluated 14 months after inoculation in MN and 12 months after inoculation in WI.

Tree no. by location and treatment	Stem DBH[1] (cm)	Total inner bark lesion area[2] (cm^2)	Stem surface area (cm^2)	Inner bark lesion proportion[3] (%)
Minnesota				
Fungus-inoculated trees				
413	17.2	1231	10715	11.5
414	19.6	421	11216	3.8
416	16.7	1159	10381	11.2
Mean (SE)	19.5 (0.90)			
Water inoculated tree				
409	12.6	66	7129	0.9
Non-inoculated trees				
1	27.7	-[4]	-	0.0
2	18.2	-	-	0.0
Mean (SE)[5]	17.8 (4.41)			
Wisconsin				
Fungus inoculated trees				
25	20.2	1723	11228	15.3
43	19.2	1958	12055	16.2
46	17.0	4514	10920	41.3
47	22.5	5050	16704	30.2
48	16.0	2693	9582	28.1
Mean (SE)	19.0 (1.16) a			
Water inoculated trees				
21	27.3	311	14212	2.2
34	21.6	109	10863	1.0
40	20.5	68	10514	0.6
Mean (SE)	23.1 (2.10) ab			
Non-inoculated trees				
29	25.5	-	-	0.0
45	24.0	-	-	0.0
49	26.0	-	-	0.0
Mean (SE)	25.2 (0.60) b			

[1] Stem diameter measured at 1.4 m height on the stem. Mean stem diameters did not significantly differ between control (water inoculated and non-inoculated trees) and fungus-inoculated trees in the Minnesota site (by two sample t-test for means, $P = 0.7297$). In the Wisconsin site, statistical difference is significant (Tukey's HSD test, $P < 0.05$) between groups labeled with different letters (a and b).
[2] Total inner bark lesion areas are based on 50 inoculation points per tree.
[3] Inner bark lesion proportion was calculated by dividing the total inner bark lesion area by the stem surface area for each inoculated tree.
[4] Not applicable
[5] Data were averaged for one water inoculated and two non-inoculated, healthy trees for control group.

4.3.2. Diurnal patterns of sap flow velocity

All trees exhibited similar diurnal trends of sap flow, i.e. sap flow began to increase around 6 am, continued to increase during the morning, peaked just after 12 noon, declined sharply after sunset, and remained low at night (Fig. 4-3). The values of sap flow velocity did not differ between water inoculated and non-inoculated, healthy trees (Fig. 4-3B). Compared to the control groups (water inoculated and non-inoculated trees combined), inoculated trees had consistently lower flux of water during the midday hours in both sites (Fig. 4-3A and B).

To compare the differences in daily sap flow velocity between control (pooled data of water inoculated and non-inoculated trees) and inoculated groups, maximum values of sap flow velocity on selected days were averaged by repeated measures ANOVA. Selected days were chosen when representative trends of sap flow were detected for the full set of trees. For control trees, maximum sap flow velocity ranged 26.3 – 30.0 g m^{-2} s^{-1} in Minnesota and 34.2 – 49.3 g m^{-2} s^{-1} in Wisconsin. Inoculated trees only achieved the maximum sap flow velocity of 10.7 – 18.1 g m^{-2} s^{-1} in Minnesota and 5.7 – 27.2 g m^{-2} s^{-1} in Wisconsin. In Minnesota, the average maximum sap flow velocity of all infected trees (14.0 ± 2.1 g m^{-2} s^{-1}) were significantly reduced by 51% compared to control trees (28.6 ± 1.1 g m^{-2} s^{-1}) (by two sample t-test for means, P = 0.009). In Wisconsin, inoculated trees experienced a 64% decrease in the maximum sap flow velocity (15.3 ± 3.8 g m^{-2} s^{-1}) compared to control trees (41.9 ± 2.2 g m^{-2} s^{-1}) (by two sample t-test for means, P = 0.0001). Sap flow velocity was consistently lower in Minnesota where it was monitored in the later part of the year (September 18 to October 5, 2009) than in Wisconsin (monitored during the late summer, July 30 to August 22, 2010) (Fig. 4-3).

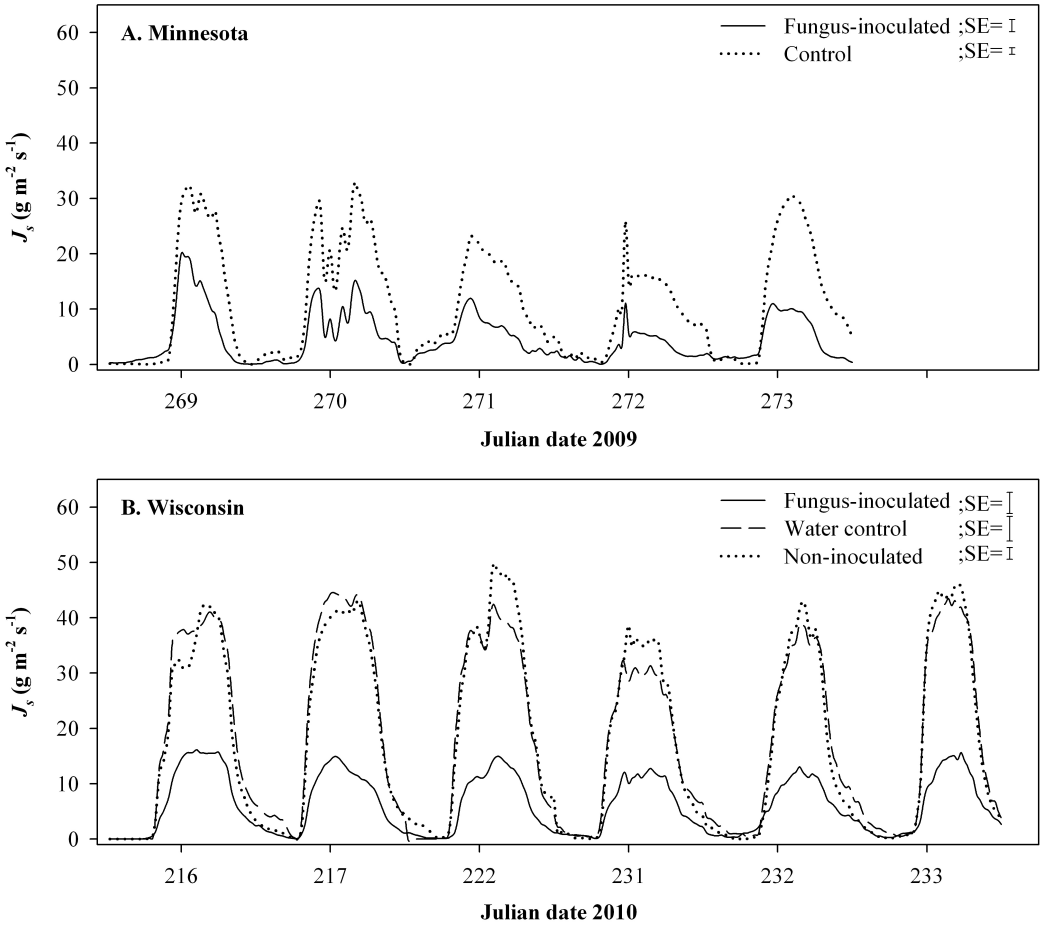

Figure 4-3. Diurnal changes in sap flow velocity (J_s) in *C. smalleyi* inoculated trees versus water inoculated and non-inoculated controls on selected days during the study period. A. In Minnesota, data were averaged for three fungus-inoculated trees and for one water inoculated and two non-inoculated, healthy trees for control group. **B.** In Wisconsin, data were averaged for five fungus-inoculated trees, three water inoculated trees, and three non-inoculated healthy trees for each treatment. Ticks in x-axis indicate noon on each day. Bars indicate standard errors of the mean peak sap flow velocity values for each treatment.

4.3.3. Xylem properties of stems

4.3.3.1. Vessel characteristics

Vessel characteristics were analyzed to determine if there were any inherent differences in tree anatomy that could have affected the hydraulic function of study trees. Since sites did not affect the characteristics, data from both sites were pooled and presented in Table 4-2 (by factorial ANOVA, P = 0.17 for vessel diameter and 0.07 for hydraulic vessel diameter). Mean vessel diameters and mean hydraulic vessel diameters did not significantly differ between control and fungus-inoculated trees in both sites (by

two sample t-test for means, P = 0.54 and 0.19, respectively). One of five fungus-inoculated trees had relatively small mean vessel diameter (84.7 µm) and mean hydraulic vessel diameter (191.7 µm) in Wisconsin, thereby reducing the average values of the treatment group. Yet, differences in mean vessel diameters and mean hydraulic vessel diameters between inoculated versus control trees remained insignificant. Vessel diameter distributions of control and inoculated trees were also homogeneous (by chi-square test, P = 0.23) (Fig. 4-4).

Table 4-2. Vessel characteristics of control and inoculated trees

Vessel characteristics by study location[a]	**Control**[b]			**Inoculated**			**P**
	n	Mean	SE	n	Mean	SE	
Mean vessel diameter (µm)							
Minnesota	3	105.0	4.2	3	105.6	5.0	0.93
Wisconsin	6	102.4	1.6	5	98.7	3.3	0.32
Pooled	9	103.3	8.9	8	101.3	2.8	0.54
Mean hydraulic vessel diameter[c] (µm)							
Minnesota	3	238.2	6.4	3	237.4	4.5	0.92
Wisconsin	6	233.9	2.3	5	224.7	4.9	0.11
Pooled	9	235.3	2.6	8	229.5	3.7	0.19

[a] 1500 vessels were observed per tree.
[b] Data were pooled from water inoculated trees and non-inoculated trees.
[c] Hydraulic vessel diameter was calculated as $\sum d^5/\sum d^4$ for each tree.

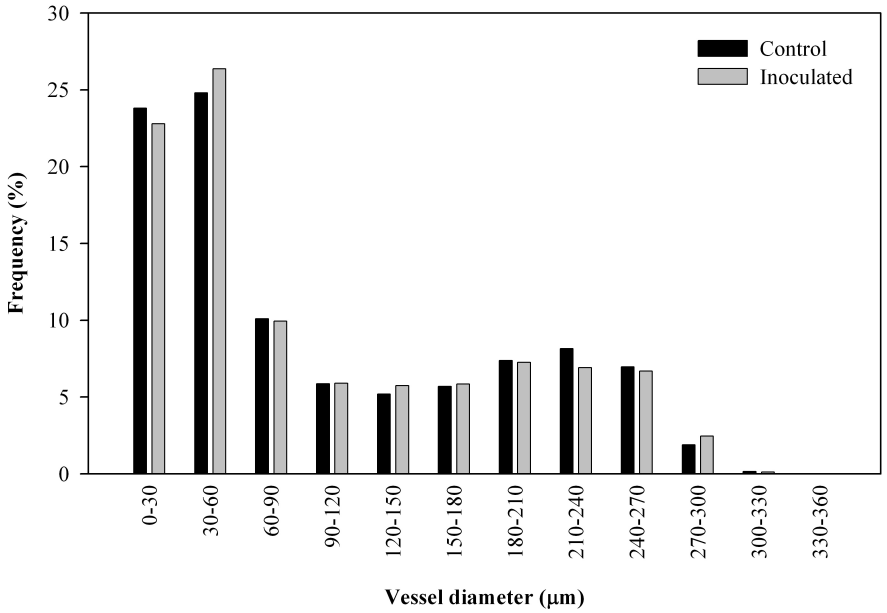

Figure 4-4. Distribution of vessel diameters for control and fungal-inoculated trees. Data shown were pooled results of the Minnesota site (n=1800 vessels per group) and Wisconsin site (n=3000 vessels per group).

4.3.3.2. Tylose abundance

Tyloses within vessels were observed in both control and fungus-inoculated trees, but more frequently in fungus-inoculated trees (Table 4-3). In cross section, multiple tyloses were found to be occasionally produced from surrounding parenchyma cells into a vessel, thus completely occluding the vessel. Differences in tylose abundance in the outer 9-10 annual rings and the two outermost annual rings were all significant in both sites. The difference was more pronounced in the two outermost annual rings where tyloses were formed at low frequency (9%) in the absence of fungus inoculation (by two sample t-test for means, $P = 0.0012$ in Minnesota and < 0.0001 in Wisconsin). In control trees, the presence of tyloses was more frequently observed in vessels of the inner part of the sapwood because tylose production is a natural process that occurs as sapwood transitions to heartwood. Yet, fungus-inoculated trees had more vessels with tyloses in the outer 9-10 annual rings compared to control trees (by two sample t-test for means, $P = 0.0059$ in Minnesota and < 0.0001 in Wisconsin).

Table 4-3. Tylose abundance in response to different treatments

Tylose abundance by study location[a]	Control[b]			Inoculated			P
	n	Mean	SE	n	Mean	SE	
In the two outermost annual rings (%)							
Minnesota	3	8.9	2.6	3	30.4	4.8	0.0012
Wisconsin	6	8.8	1.5	5	56.0	5.4	<0.0001
In the outer 9-10 annual rings (%)							
Minnesota	3	24.5	2.2	3	36.5	3.1	0.0059
Wisconsin	6	42.4	2.0	5	58.9	2.5	<0.0001

[a] 1500 vessels were observed per tree.
[b] Data were pooled from water inoculated trees and non-inoculated trees.

4.3.4. Relationships between sap flow velocity and disease severity or selected xylem properties

In Minnesota, correlation analyses by Pearson's Coefficient indicated that significant interactions are present between mean peak sap flow velocity and inner bark lesion proportion ($P = 0.0042$), mean peak sap flow velocity and tylose abundance in the two outermost annual rings ($P = 0.0084$), and between inner bark lesion proportion and tylose abundance in the two outermost annual rings ($P = 0.0045$). In Wisconsin, mean peak sap flow velocity was strongly associated with inner bark lesion proportion ($P < 0.0001$), tylose abundance in the two outermost annual rings ($P < 0.0001$), as well as tylose abundance in the outer 9-10 annual rings ($P = 0.0004$). Inner bark lesion proportion was also significantly associated with tylose abundance both in the outer two annual rings and in the outer 9-10 annual rings ($P < 0.0001$ and $P = 0.0005$, respectively).

Based on the correlation results, regression analyses for mean peak sap flow velocity were conducted against the three significantly associated parameters: inner bark lesion proportion, tylose abundance in the two outermost annual rings, and tylose abundance in the outer 9 – 10 annual rings (Fig. 4-5). Regression analyses indicated that mean peak sap flow velocity could be explained by inner bark lesion proportion and tylose abundance in the outer two annual rings in both sites. Mean peak sap flow velocity exhibited negative linear responses to increasing inner bark lesion proportion ($R^2 = 0.90$,

P = 0.0042 in MN, R^2 = 0.90, P < 0.0001 in WI). The similar inverse linear relationship was also found between mean peak sap flow velocity and tylose abundance in the outer two annual rings (R^2 = 0.85, P = 0.0084 in MN, R^2 = 0.88, P < 0.0001 in WI). In Wisconsin, a decrease in mean peak sap flow velocity was also significantly associated with the increase in tylose abundance in the outer 9 – 10 annual rings (R^2 = 0.77, P = 0.0004).

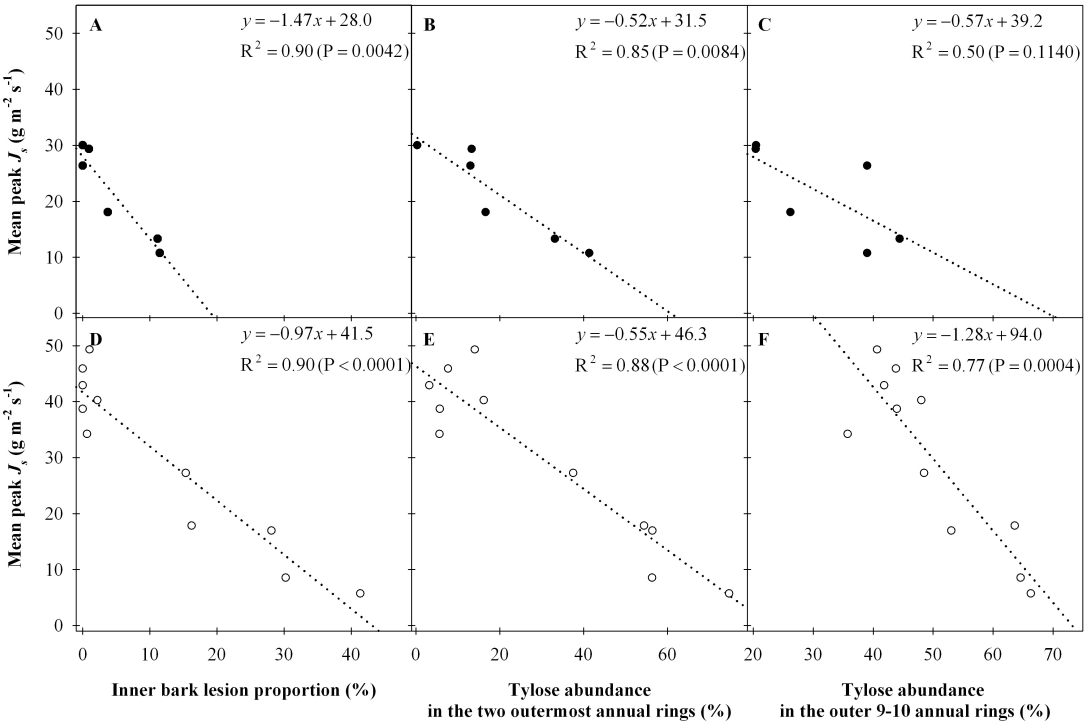

Figure 4-5. Linear regression results for mean peak sap flow velocity (J_s) versus inner bark lesion proportion and tylose abundance with different sites shown separately (A-C: Minnesota, D-F: Wisconsin)

4.4. Discussion

No crown symptoms developed on all but one tree within 12 to 14 months of treatment. The symptomatic tree exhibited over 60% declining crown and, among all inoculated trees, had the highest disease severity indicated by the inner bark lesion proportion that covered 41% of stem surface area. Although a symptom of crown decline or wilt was only observed in a single fungus-inoculated tree and severity of canker response varied by tree, for the trees monitored for sap flow a general trend was observed

of reduced sap flow velocity in diseased compared to control trees. It suggests that physiological disruption occurs before visible, external symptoms appear and that multiple stem infections of *C. smalleyi* impair water transport in visually healthy bitternut hickory.

Sap flow measurements of water-inoculated trees were similar to or even higher than that of non-inoculated, healthy trees suggesting that mechanical wounds which were subsequently closed over did not affect the water conductance of trees. Therefore, mechanical damage by attacking bark beetles alone, for instance failed or aborted attacks without fungus introduction, may not sufficiently explain crown symptoms observed in declining hickories. However, one should note that even the aborted attack of hickory bark beetle is frequently associated with *C. smalleyi* infection in naturally affected trees. On the other hand, the reduced hydraulic functioning observed in fungus-inoculated trees suggests the impediment to water transport by fungal infection and resulting water depletion in the above area of infected trees likely explain the rapid wilting and declining symptoms. Such reduced sap flow velocities in relation to sapwood colonization of fungal pathogens have also been observed in wilting trees with Japanese oak wilt and sudden oak death (Kuroda and Yamada 1996; Parke et al. 2007).

Using an artificial inoculation system with only 50 inoculation points per tree, extensive crown decline was not expected to occur within one year. There were practical limitations in mimicking the natural occurrence of multiple infections in the experimental study conducted. The number of inoculations per tree (50 points) was far below the number of lesions observed in naturally affected trees and of inoculations made in other studies. When three actively declining trees of bitternut hickory (40, 55 and 80% crown decline) were closely examined in a different study, 24 to 555 lesions were found on the main stems (Juzwik et al. 2010). In studies of other similar pathogens which cause local sapwood lesions or stains, visible symptoms such as crown decline or tree mortality only occurred when trees were inoculated with relatively high inoculation densities, e. g. 100 points/m^2 for *C. polonica* on Norway spruce, 200 points/m^2 for *C. rufipenni* on Sitka spruce, 400 points/m^2 for *Leptographium wingfieldii* on Scots pine (Christiansen 1985; Croisé et al. 1998; Solheim and Safranyik 1997). Secondly, inoculations made along four

separated rows in this study also did not represent the spatial distribution of hundreds of canker occurrences in nature. While many of the cankers coalesce around the tree circumference in naturally infected trees, cankers induced by the inoculation tended to be narrower and longer than that observed in nature, thus only coalescing in a vertical direction when inoculated in a row. Consequently, discolored sapwood associated with these cankers, which is possibly implicated in the process of tree wilting, did not cover as much cross-sectional area of wood as observed in naturally infected trees. Thirdly, height of inoculation was another limiting factor of the simulation. Inoculations were limited up to 3.7 m for practical reasons despite the fact that cankers frequently occur higher up in naturally affected trees, often up to 20 m above the ground (Juzwik et al. 2010). As the upper part of the tree stem has a smaller diameter, neighboring cankers are more likely to coalesce. Given these limitations that were not met in this study, it is highly probable that a closer inoculation setting to the naturally occurring situation could have accelerated the symptom expression of crown decline more rapidly than observed.

Multiple inoculations of *C. smalleyi* did not affect wood anatomy evaluated by vessel diameter and vessel diameter distribution in inoculated trees. The lack of significant differences in vessel characteristics provides evidence that reduced sap flow velocity in inoculated trees is not a result of tree's inherent xylem properties. However, *C. smalleyi* inoculations were correlated with significantly more vessels plugged by tyloses compared to that in water-inoculated and non-inoculated control trees. The higher incidence of tyloses in infected trees versus control trees is not a surprising result because tylosis formation is a well-known host response to xylem pathogen colonization (Beckman 1987). Tyloses are known to form in response to xylem cavitation which can be induced by pathogens either by physically blocking the xylem vessel or by secreting substances that alter the physical properties of the water within the vessel (Kuroda 1991; Tyree and Sperry 1989). Tylose development was also found to occur by increased ethylene production in grapevine stems which can be stimulated by wounding or pathogen colonization (Sun et al. 2007). Vessel occlusion by such tyloses has been commonly observed in trees with vascular wilt diseases such as oak wilt and Dutch elm disease (Beckman 1987; Elgersma 1973; Pallardy 2008). *P. ramorum* causing sudden oak

death was also found to induce tylosis formation in tanoak sapwood (Collins et al. 2009; Parke et al. 2007).

Of selected xylem properties, only the frequency of tyloses in the outer annual rings was inversely and significantly correlated with sap flow velocity in both sites. Tyloses increase resistance to flow of water by reducing the radius of vessels and can completely occlude the vessel when large or abundant (Dimond 1970). Limited distribution of tyloses may have little effect on total water transport because, when an individual vessel ceases to function, water can flow around the affected vessel through pit openings into adjacent functioning vessels and continue to move to distal tissues (Pallardy 2008). However, extensive occlusion of vessels by tyloses could affect sustained hydraulic transport of trees by dehydration (Pallardy 2008). Furthermore, such extensive blockage of xylem vessels increases tension in the remaining vessels to maintain water flow to tree crowns resulting in more embolism and more reduced hydraulic conductivity (Tyree and Sperry 1989). For instance, reduced hydraulic function of xylem in relation to tylose abundance has been observed in tanoak trees naturally infected and inoculated with *P. ramorum* by measuring sap flow and hydraulic conductivity (Collins et al. 2009; Parke et al. 2007). Therefore, tylose formation associated with *C. smallyi* infections, specifically in outer annual rings where the most volume of water is transported in ring-porous trees like bitternut hickory, is likely responsible for much of this water transport disruption.

The xylem dysfunction that resulted from fungal inoculation provides evidence to speculate that *C. smalleyi* might be a vascular pathogen as well as a bark canker pathogen. Frequent isolation of *C. smalleyi* from discolored sapwood and secondary canker occurrences associated with continuous xylem discoloration observed in pathogenicity studies further support this speculation (chapters 2 and 3). Confirming the ability of *P. ramorum* to interfere with water transport and to colonize xylem, Parke et al (2007) suggested that *P. ramorum* may be a vascular wilt pathogen on tanoak rather than a canker-causing pathogen. This argument is based on the distinction between canker diseases and vascular wilts. Canker-causing pathogens are adapted to colonize living cells of the phloem, cambium, and infrequently outer xylem cells, thus interrupting downward

translocation of photosynthates (Agrios 2005; Manion 1991). *Ophiognomonia clavigignenti-juglandacearum* (previously *Sirococcus clavigignenti-juglandacearum*) causing butternut canker, *Geosmithia morbida* causing thousand cankers disease, and many members of the genus *Nectria* are examples of canker-causing pathogens. In contrast, vascular wilt pathogens invade and systemically spread throughout the xylem vessels of roots and stems, thus interfering with translocation of water and minerals to the crown of the plant (Agrios 2005; Manion 1991). Dutch elm disease and oak wilt are classic wilt diseases and specifically considered to be "true vascular wilts". However, there are examples of other canker-causing pathogens that can also invade xylem tissues such as *P. ramorum*. *Ceratocystis fimbriata* f. sp. *platani* causing stain canker of plane trees colonizes sapwood as hyphae or chlamydospores (Panconesi et al. 2003). *Cryptosphaeria populina* causes cankers as well as internal sapwood stain by invading healthy sapwood of aspen (Sinclair and Lyon 2005). Although not classified as canker pathogens, blue stain fungi in the genera *Ceratocystis* and *Ophiostoma* associated with bark beetles are well known to infect both phloem and xylem tissues (Sinclair and Lyon 2005). *C. smalleyi* is likely to be a phloem and xylem infecting pathogen as in the last examples, but its physical presence in xylem tissues need to be further investigated.

Although pathogen-induced wilting is involved in disease development of hickory crown decline, this pathosystem has a major difference from true vascular wilts such as Dutch elm disease and oak wilt. The fungal invasion in xylem tissues and the spread of sapwood discoloration are slow and locally restricted. Because of the limited nature of xylem colonization by *C. smalleyi*, wilting in bitternut hickory seems to require multiple infections of *C. smalleyi*. This is in contrast to single infection being sufficient to cause tree wilt or mortality in true vascular wilt diseases. One of possible reasons for the local limiting phenomenon might be fungal structures that colonize the vascular system in that rapid advance of vascular pathogens is achieved by rapid reproduction of spores rather than hyphal growth (Beckman 1987). Those fungal spores are carried quickly and for long distances along the water flow in vascular wilt diseases (Beckman 1987). Vessel length of host trees, paths of water movement within the sapwood, and failure/success of

host resistance responses in localizing the initial infection might also account for whether this disease can develop systemically or not.

The preference of *C. smalleyi* for xylem rather than phloem tissues for its growth and reproduction is another question that needs to be answered before it was called a vascular pathogen. True vascular pathogens such as *Ophiostoma ulmi* and *C. fagacearum* preferentially spread in xylem vessels producing different types of conidia (Agrios 2005). Short-coming of this study was that the inoculation was made by drilling through the phloem into the outer sapwood and then pipetting a spore suspension into the hole, thus inoculating both phloem and sapwood. This question, therefore, may simply be answered by inoculating the fungus one or the other stem tissues. Based on all these similarities and differences between hickory crown decline and true vascular wilts and further considerations discussed above, it might be proper to consider hickory crown decline as a limited vascular wilt disease.

One could still consider the possibility that stem girdling caused by either coalescing of hickory bark beetle galleries or coalescing of hundreds of bark cankers might cause tree decline or death. Tree girdling that removes or kills phloem and/or cambium tissues interferes with the downward movement of carbohydrates. The interrupted translocation of organic substances is believed to disrupt major metabolic systems resulting in auxin imbalance, increased respiration, reduced leaf area/numbers, and C/N ration imbalance as well as to limit root survival (Noel 1970). Girdling of a stem does not necessarily interrupt the water movement. For that reason, many girdled trees, even in the absence of girdle healing, can survive for a considerable period after girdling depending on the initial carbohydrate availability below the girdle and the maintenance of transpiration (Noel 1970). For instance, thousand cankers disease of black walnut which is caused by *Geosmithia morbida* in association with walnut twig beetle (*Pityophthorus juglans*) slowly progresses in an individual tree compared to true vascular wilts as well as hickory crown decline. On black walnut trees exhibiting early symptoms such as yellowing and twig dieback, numerous small patches of dead bark which might have initiated unnoticed years ago are often found to already have coalesced, thus girdling branches and twigs. Girdling effect itself, therefore, may not be sufficient to explain the

rapid symptom progress from thinning crowns to complete tree death observed on bitternut hickory trees.

Nevertheless, damaged phloem and cambium by bark cankers and hickory bark beetle attacks cannot be neglected due to their indirect influences on the general water regime of the tree and tree vigor. Furthermore, what is normally seen on declining bitternut hickory is a close association among the three damage types, hickory bark beetle attacks, bark canker development, and extension of discolored sapwood. Overall, field observations and findings from this study suggest that a synergistic interaction of *C. smalleyi* and hickory bark beetles leads to the rapid tree decline and mortality. In the interaction, bark beetles are likely involved in initiation of cankers and multiple stem infections by *C. smalleyi* disrupt physiological functioning of phloem and xylem. The resulting loss of tree vigor might attract more beetles to affected trees, thus accelerating the disease progress.

In conclusion, this study showed that multiple infections of *C. smalleyi* affected physiological function involving water transport in mature bitternut hickory. Disease severity was associated with the reduced level of sap flow velocity of diseased trees. More specifically, tylose formation in current year vessels associated with *C. smalleyi* infections is likely responsible for much of the water transport disruption. This mechanism of tree wilting suggests that rapid crown decline of bitternut hickory is a result of limited vascular wilt caused by multiple infections of *C. smalleyi*.

Chapter 5. Host colonization pattern of xylem and tree response to infection by *Ceratocystis smalleyi*

5.1. Introduction

Extensive discoloration in sapwood (or young xylem) is a recognized feature of bitternut hickory infected with *Ceratocystis smalleyi* (chapter 3). *C. smalleyi*-induced discoloration spreads radially and vertically, thus covering most of tree's sapwood cross section when a tree has numerous infections. This type of response has been considered a diagnostic trait of xylem pathogens (Sinclair and Lyon 2005). As classic examples, true vascular pathogens such as *Ophiostoma ulmi*, *C. fagacearum,* and *Verticillium dahliae* cause vascular streaking in the outer sapwood of host trees (Sinclair and Lyon 2005). Patterns of xylem discoloration similar to that of *C. smalleyi*-induced discoloration (e.g. wedge-shaped, local lesion) have been documented for canker stain of plane trees, sudden oak death of tan oak, Japanese oak wilt, and Phytophthora stem diseases of hardwood trees (Brown and Brasier 2007; Kuroda and Yamada 1996; Panconesi 1999; Parke et al. 2007). Although none of these diseases are systemic wilts, the pathogen of each of the diseases was found or suggested to be able to establish itself in xylem tissues based on microscopic observations or fungal isolations. Thus, it is likely that the xylem discoloration of diseased bitternut hickory is a result of the sapwood colonization by *C. smalleyi*. High frequency of isolation of *C. smalleyi* from the discolored sapwood supports this speculation (chapter 2).

Another, more interesting feature that suggests *C. smalleyi* is a xylem as well as phloem pathogen is the occurrence of bark lesions that arise from discolored sapwood and speculated vertical spread of the pathogen through xylem elements (chapter 3). Phloem lesions associated with the vertically expanding strips of discolored xylem have been described by different terms in different studies, such as secondary lesion, canker resurgence, necrotic reappearance, and island lesion (Brown and Brasier 2007; Panconesi 1999). *C. fimbriata* f. sp. *platani* on *Platanus acerifolia, P. ramorum* or *P. kernoviae* on *Fagus sylvatica* and *Quercus* spp., and *Chalara fraxinea* on *Fraxinus excelsior* induced

the same symptom upon infection (Brown and Brasier 2007; Panconesi 1999; Schumacher et al. 2010). There is a probability of secondary infection from an outside source such as natural infections initiated by pathogen propagules disseminated via wind, water, insect vectors or other means. However, secondary phloem lesions demonstrated by inoculation tests provide evidence for the ability of the pathogen to spread through vessels and break out from the xylem into unaffected phloem tissues, thus initiating a new phloem lesion.

In addition, xylem functions of bitternut hickory appear to be impaired as a result of numerous infections by *C. smalleyi* (chapter 4). Young xylem or sapwood plays a vital role in tree physiology by transporting water and minerals through vessels from roots to crowns and storing food in living parenchyma cells (Pallardy 2008). Due to the crucial role of the xylem in conducting water, hydraulic disruption caused by xylem infection of a pathogen has been examined with regard to not only true vascular wilts but also some of stem canker and stain diseases (Beckman 1987; Collins et al. 2009; Kirisits and Offenthaler 2002; Kuroda and Yamada 1996; Murata et al. 2005; Parke et al. 2007; Yamaoka et al. 1990). In most cases, xylem vessels were found to lose their functionality when colonized with propagules of a pathogen or when embolized or occluded as a result of host defense responses. Such dysfunction of water conduction was observed in bitternut hickory infected with *C. smalleyi* where reduced sap flow was closely associated with disease severity as measured by proportion of stem affected by bark cankers (chapter 4). On this basis, it was speculated that the potential xylem infection of *C. smalleyi* and accompanying tree defense responses in the xylem are a key factor in inducing development of rapid crown decline in affected trees.

As a xylem pathogen infects living sapwood, the host tree responds with a range of defense mechanisms. The induced host reactions include cell wall alterations such as lignification and suberization of cell walls of parenchyma cells, production of constitutive and induced antimicrobial compounds, and occlusion of xylem elements with tyloses or gels (Beckman 1987; Pearce 1996; Yamada 2001). These host reactions have been observed with varying degrees in trees with true vascular wilt diseases as well as local

lesion diseases such as Japanese oak wilt and stain canker of plane trees (Dimond 1970; Jacobi and MacDonald 1980; Panconesi 1999; Yamada et al. 2003).

For instance, tylose formation, one of the defense strategies, appeared to frequently occur in the xylem of bitternut hickory infected with *C. smalleyi* (chapter 4). Furthermore, the extent of vessel occlusion with tyloses was closely associated with disease severity measured by the proportion of bark lesions to stem surface area as well as the loss of water conduction in *C. smalleyi*-infected trees (chapter 4). Tyloses are formed as a part of the natural process of xylem senescence from sapwood to heartwood. In young xylem vessels of many hardwoods, however, tyloses commonly form as a result of wounding, or infection from fungi or bacteria (Kuroda 1991; Sun et al. 2007; Tyree and Sperry 1989). The tylose proliferation observed in *C. smalleyi*-infected bitternut hickory apparently occurred as a part of sapwood defense strategies against the xylem infection because wounding of the control trees did not induce a comparable abundance of tylose formation (chapter 4). It is possible that other defense responses might have occurred in the sapwood upon the *C. smalleyi* infection in the sap flow study (chapter 4).

Host defense responses actively occur in the internal interface between the living host and the pathogen at the xylem lesion margin or reaction zone (Pearce 1991). The success of the pathogen in establishing itself in the host depends on the rapidity and intensity of the host defense responses to contain the pathogen in the initial infection site (Dimond 1970). If the host fails to block the advancing pathogen in time and space or if the pathogen has the ability to overcome defense barriers by breaking them down, the disease will continue to progress. It is well known that true vascular pathogens such as *C. fagacearum* and *O. ulmi* produce small conidia that can be transported rapidly throughout the xylem vessels. In addition, susceptible host trees of *C. fagacearum* were found to have slow initial responses that would otherwise limit systemic spread of the pathogen (Jacobi and MacDonald 1980). *O. ulmi* produces cell wall degrading enzyme, toxins, and growth substances that could help overcome defense responses. Since the xylem lesion induced by *C. smalleyi* does not develop throughout an infected tree, it is speculated that *C. smalleyi* is more restricted in its ability to invade and grow in the vascular system than *C. fagacearum* and *O. ulmi*. However, it needs to be further investigated and the reaction

zone would be the most appropriate area for the examination of the host-pathogen interaction.

Overall, this research is focused on the xylem colonization of bitternut hickory by *C. smalleyi* and host defense responses to the xylem infection. This investigation on the host-pathogen interaction in the sapwood addresses the following questions: 1) how extensively and over what time period does *C. smalleyi* invade the xylem of bitternut hickory? 2) does the host react to the xylem infection with various defense responses, particularly vascular occlusion by tyloses which is considered to contribute to tree wilting? and 3) why does *C. smalleyi* only cause a limited wilt disease rather than a systemic wilt?

The objectives of this study were 1) to determine the ability and extent of *C. smalleyi* to colonize the sapwood tissue of bitternut hickory spatially and temporally with microscopic investigation and 2) to identify host defense responses against the pathogen using anatomical and histological approaches.

5.2. Materials and Methods

5.2.1. Study sites and trees

The study site was located in a mixed hardwood stand in Chippewa Co., WI. The stand was thinned during the winter of 2007 - 2008. Eighteen pole-size bitternut hickory trees (13 to 28 cm in diameter at 1.4 m) with healthy crowns (<15% visible dieback) and stems without visible defect or damage were selected for inoculations. The trees were intermediate to dominant in the canopy.

5.2.2. Fungus inoculation

Two isolates of *Ceratocystis smalleyi* (CS0731 and CS0734) were selected for inoculation. Both isolates were obtained from bitternut hickory with diffuse cankers and hickory bark beetle galleries in Monroe Co., WI. Ascospores of *C. smalleyi* were collected from extruded masses on tips of perithecia of 1 to 2 week old cultures growing on 2% malt yeast extract agar amended with 100 ppm streptomycin sulfate per liter

(MYEA+SS) and suspended in 1.0 ml sterile distilled water. Due to the sticky nature of ascospore masses, the suspension was homogenized with a tip sonicator. The spore suspension was adjusted to a concentration of 1×10^4 ascospores/ml. In June 2008, four holes (0.6 cm diameter) were made by aseptically drilling into the outer sapwood on the main stem of eighteen trees at 1.2 m stem height. The four holes were evenly distributed along the tree's circumference. Aliquots (0.1 ml) of spore suspensions (1.0×10^4 ascospores/ml) of two fungal isolates and sterile distilled water (control) were pipetted into the four drilled holes on each of four trees, respectively. Holes were sealed with moist cotton and masking tape.

5.2.3. Temporal sampling

All trees were monitored after 2 and 12 months for any crown symptoms such as yellowing, crown thinning, and branch dieback. Two trees of each treatment were harvested 2 months after inoculation. Only one tree inoculated with each of two *C. smalleyi* isolates was harvested 12 months after inoculation because the remainder of the study trees were inadvertently removed during the thinning operation in winter of 2007 – 2008. Stem section (1.0 m in length) of each tree that included four inoculated and cankered areas was cut and transported to the laboratory.

5.2.4. Sample processing

5.2.4.1. Canker measurement

Each section was visually examined for any symptoms of diffuse bark cankers and xylem lesions or discoloration. The outer bark of each stem section was removed to reveal inner bark lesions. Canker development for each inoculation point was evaluated as the width and length of necrotic inner bark and of discolored sapwood.

5.2.4.2. Subsampling

Each stem section was longitudinally split into four quarters to separate each canker or control wound using a band saw. For fungus recovery, 5 cm thick wood slices were taken from the top and bottom of each canker and from each inoculation wound in

case of water inoculation (Fig. 5-1). For histological study, three 2 cm thick wood slices per inoculation were taken from the stem quarter at three points (Fig. 5-1). Points 1, 2, and 3 were, respectively, 2 cm, 12 cm, and 22 cm above each inoculation point. When the wood slice contained discolored sapwood, two samples (wood cubes of 1.5 cm × 1.5 cm × 2 cm) were obtained from the outer sapwood at both marginal side edges of discoloration so that each sample consisted of half discolored tissues and half healthy tissues (i.e. the supposed reaction zone). The samples were fixed in FAA solution (formaldehyde: acetic acid: 50% ethanol = 5: 5: 90 v/v) prior to sectioning.

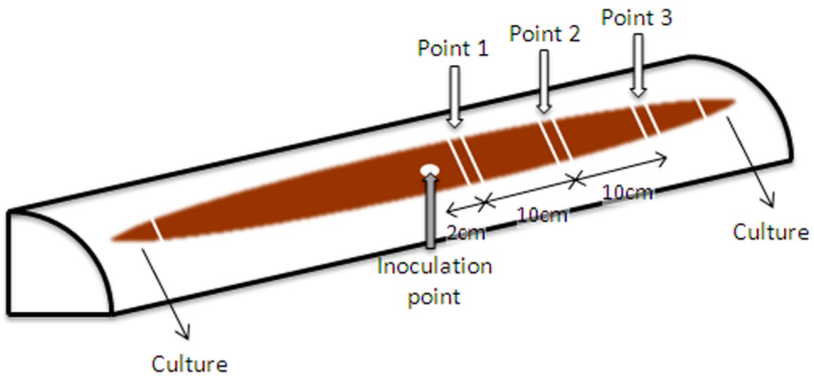

Figure 5-1. Sampling from a quarter log of *Ceratocystis smalleyi*-inoculated bitternut hickory. Samples for fungus recovery were taken from both ends of a canker. Samples for histology were taken at point 1, 2, and 3.

5.2.4.3. Fungus recovery

For re-isolating *Ceratocystis smalleyi*, stem slices were cut into small wood cubes of sapwood using a miter cutter and were kept in small moist chambers to stimulate formation of fruiting structures of fungi. The cubes in each moist chamber were examined under a stereomicroscope 10 days later for the presence of any fungal fruiting bodies. Ascospore masses on tips of perithecia sporulating on the wood cubes were transferred to 2% MYEA+SS. In cases where no perithecia were produced or were not fully developed, wood cubes were kept in the same moist chamber for 10 more days and then re-examined.

5.2.4.4. Sectioning and staining

Fixed samples for histology were thoroughly washed in running tap water. Of two samples obtained at a sampling point, one sample was sectioned transversely and the other longitudinally to a 20 - 25 µm thickness using a sliding microtome (Model 860, American Optical, Southbridge, MA). The sections were stained with 0.1% toluidine blue O as a general stain (Clark 1981), 0.1% Nile blue to detect lipid (Chayen and Bitensky 1991), 0.005% ruthenium red for pectin detection (Jensen 1962), 0.05% 4-nitrosophenol in concentrated H_2SO_4 for phenolic compounds (Gersbach et al. 2001), and phloroglucinol (1% aq.) plus HCl for lignin (Redman et al. 1999). Stained sections were subsequently mounted in 10% glycerol on glass slides and viewed with a microscope.

5.2.5. Evaluation of qualitative and quantitative features

Presence of fungal structures, tyloses and gels in vessels was observed in transverse sections stained with toluidine blue O. On each section, 250 vessels were examined under a light microscope to count the number of: 1) occluded vessels with tyloses or gels, 2) non-occluded, fungus-colonized vessels, and 3) healthy vessels. Healthy vessels refer to vessels without fungal hyphae, gels, and tyloses. Their occurrences were recorded as the proportion of vessels (n = 250) with each feature. Chemical reactions of the host to fungus colonization or wounding were determined by evaluation of the presence of lipids, pectins, phenolics, and increased lignification with stained sections. All observations were made with a light microscope at 200× or 400× power.

5.2.6. Data analyses

Effects of each inoculum type on symptom development (the size of inner bark lesions and sapwood discoloration) were analyzed by one-way Analysis of Variance (ANOVA). Since there was no difference in pathogenicity of two fungal inoculum sources (CS0731 and CS0734) (chapter 3), data from the two inoculum types were pooled. Differences of means were determined using Tukey's HSD at the significance level of $\alpha = 0.05$. Results of each feature types (mean proportions of occluded, fungus-

colonized, and apparently healthy vessels) were compared to each other using two sample t-test for means at 95% confidence level. All statistical analyses were conducted using SAS 9.1 (SAS Institute, Inc. Cary, NC).

5.3. Results

5.3.1. Canker development by time and fungus recovery

Cankers formed around each fungus-inoculation point while no cankers occurred with control inoculation points 2 months after inoculation (Fig. 5-3B and C). The mean length of inner bark lesion associated with the cankers was 21.8 ± 2.6 cm and significantly increased to 64.2 ± 3.2 cm 10 months later (by ANOVA, Tukey's HSD at $\alpha = 0.05$)(Fig. 5-2). Cankers developed slowly in a lateral direction compared to their vertical expansion. Yet, the mean width of the inner bark lesion still implied a statistically significant increase over time from 2.6 ± 0.08 cm 2 months after inoculation to 3.0 ± 0.09 cm 12 months after inoculation (by ANOVA, Tukey's HSD at $\alpha = 0.05$)(Fig. 5-2).

Long, narrow, reddish brown discoloration of sapwood (xylem lesion) was evident for all fungus-inoculated points (Fig. 5-3E). In cross section, xylem lesion was wedge-shaped (Fig. 5-3D). In the absence of *C. smalleyi*, all control wounds were closed over with callus tissue and no discoloration was observed in the sapwood (Fig. 5-3A). Xylem lesion extended with the inner bark lesion and its length was, in general, larger than the corresponding bark necrosis (Fig. 5-2). Xylem lesion also significantly extended by time (Fig. 5-2). A continuous xylem lesion was associated with one to four distinct bark cankers that resurged (Fig. 5-3B and C); commonly with one (13 of 24 inoculations) or two (7 of 24 inoculations) and seldom with three (1 of 24 inoculations) or four (3 of 24 inoculations).

C. smalleyi was re-isolated from all cankers, but not from the control wounds. No differences were found in spatial range of vertical distance above versus below from inoculation point that *C. smalleyi* was recovered. Two months after inoculation, *C. smalleyi* was recovered from xylem tissues 3.0 – 27.5 cm above and 4.0 – 23.5 cm below

inoculation point. 12 months after inoculation, the fungus was recovered from xylem tissues 26.0 – 40.0 cm above and 26.0 – 39.0 cm below inoculation point.

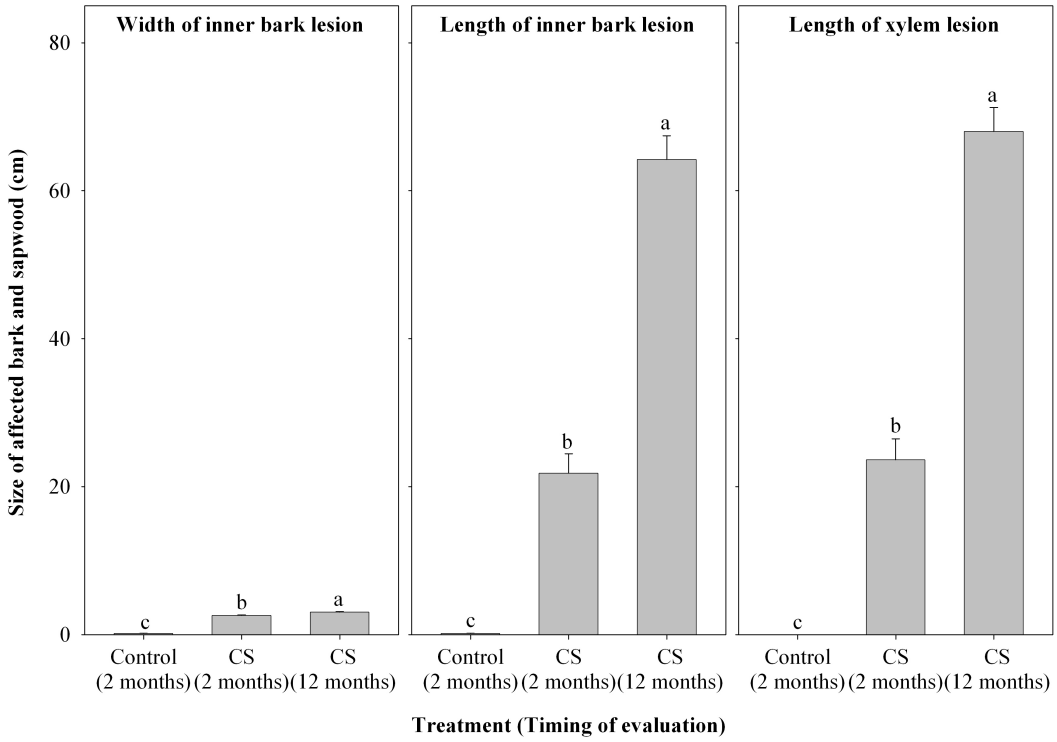

Figure 5-2. Average size of inner bark lesion and xylem lesion caused by *Ceratocystis smalleyi* (CS) inoculation compared to water control by time. Error bars represent standard error for each measurement. Different letters above the bars indicate treatments that are statistically different based on Tukey's HSD test at a 95% confidence limit. Data for CS were pooled from inoculated trees with CS0731 and CS0734. Total numbers of inoculation points and of trees that were evaluated for each treatment are as follows: 8 points (2 trees) for control, 16 points (4 trees) for CS 2 months, and 8 points (2 trees) for CS 12 months.

Figure 5-3. Canker development by *Ceratocystis smalleyi* inoculation. A. Water inoculated wound 2 months after inoculation. **B.** Necrotic inner bark caused by *C. smalleyi* 2 months after inoculation. **C.** Four separate inner bark lesions formed on a fungus-inoculation point 2 months after inoculation. **D.** Wedge-shaped, reddish brown sapwood discoloration on a fungus-inoculation point 2 months after inoculation. **E.** Long, narrow discoloration of sapwood induced by *C. smalleyi* inoculation 12 months later.

5.3.2. Visual presence of *C. smalleyi*

Many fungal hyphae were observed in the discolored sapwood area and advancing hyphae at the margin of the discolored xylem. In all sections examined, no sporulation of *C. smalleyi* was observed. Hyphae were commonly observed in all types of xylem tissues such as axial parenchyma, ray parenchyma, fibers, and vessels (Fig. 5-4) and were particularly abundant in the axial and ray parenchyma tissues. Hyphae growing along large vessels were frequently branched in the vessel lumen (Fig. 5-4A). The primary passage of hyphae from cell to cell was through bordered pits, probably the result of penetration by mechanical pressure as seen by hyphae becoming thin in the pit regions (Fig. 5-4D and E). Hyphal projections were commonly observed protruding from ray parenchyma cells to vessel lumens (Fig. 5-4F).

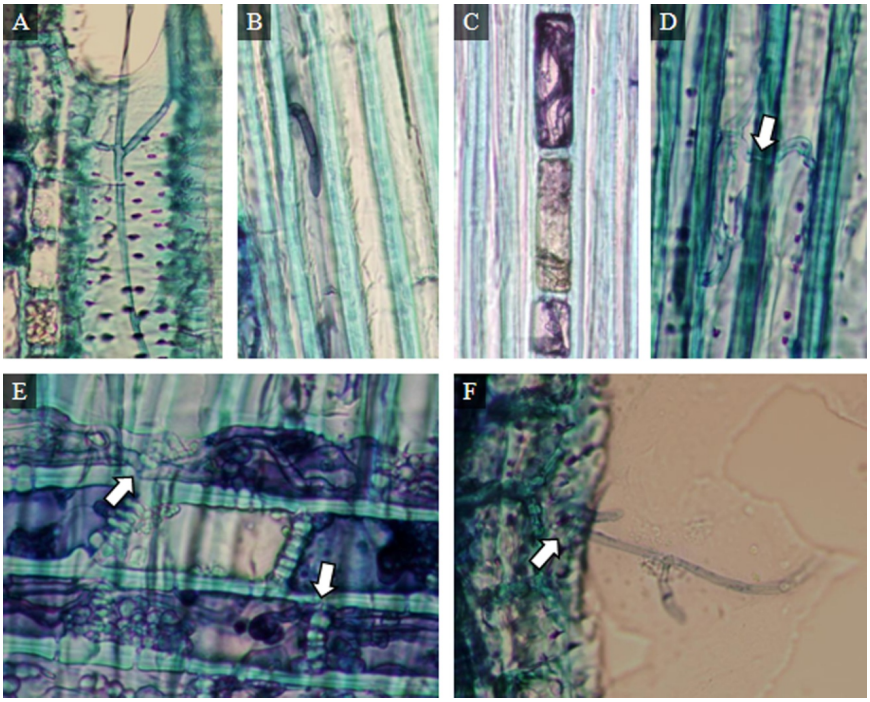

Figure 5-4. The appearance of *Ceratocystis smalleyi* in colonized sapwood tissues of bitternut hickory. Sections were stained with toluidine blue O. **A.** Branching of hyphae in a large vessel. **B.** Advancing hyphal tip in a wood fiber. **C.** Hyphae in axial parenchyma cells. **D.** Hyphal penetration (arrow) of a bordered pit between wood fibers. **E.** Hyphal penetration (arrows) of bordered pits between radial parenchyma cells. **F.** Hyphae (arrow) projecting from radial parenchyma cell to a vessel lumen.

5.3.3. Vessel occlusion by tyloses and gels

Tyloses formed abundantly in earlywood vessels of all fungus-inoculated trees while gels partially or completely occluded latewood vessels (Fig. 5-5A-C). Most tyloses were thin-walled. Vessel lumens of earlywood vessels were normally closed by multiple, contiguous tyloses (Fig. 5-5D). When latewood vessels were occluded with gels, the same gel substances were observed abundantly in surrounding parenchyma cells (Fig. 5-5E).

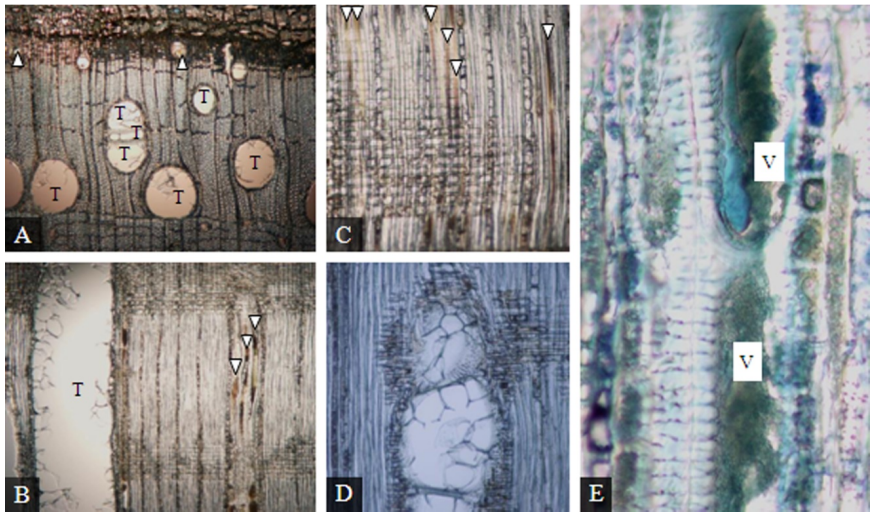

Figure 5-5. Vessel occlusion by tyloses or gels in reaction zone. A. Tyloses (T) in earlywood vessels and gels (arrow heads) in latewood vessels in transverse view. **B.** Tyloses (T) in an earlywood vessel element and gels (arrow heads) in latewood vessels in radial view. **C.** Latewood vessels occluded with gels in radial view (arrow heads). **D.** Earlywood vessel elements showing contiguous tyloses in radial view. **E.** Gels in late-wood vessel elements and surrounding parenchyma cells in radial view. **A, D.** Sections stained with Nile blue. **B, C.** Sections without staining. **E.** Section stained with toluidine blue O.

5.3.4. Frequencies of vessel occlusion by tyloses and gels and vessel colonization by fungal hyphae

Vessel occlusion occurred in response to both water-inoculation and fungus-inoculation but it was significantly more abundant in fungus-inoculated trees (by ANOVA, Tukey's HSD test at $\alpha = 0.05$). After 2 months, 36 – 70% (mean 54 ± 2.4%) of vessels were occluded by tyloses or gels at the margin of the infected area. After 12 months, the amount of vessel plugging (34 – 78%, mean 57.3 ± 2.5%) was slightly more exceeded that occurring after 2 months. The proportion of occluded vessels was consistent or increased by distance from the inoculation point (Fig. 5-6). Wound-induced tylosis formation occurred in 16 – 32% of vessels in water-inoculation points with a single exception in the nearest point (point 1) of one inoculation site where 48% of vessels were plugged. Gel accumulation in a vessel did not occur in water-inoculated trees. The frequency of wound-induced tyloses decreased by distance from the inoculation point in water inoculated trees (Fig. 5-6).

In the rest of vessels which were non-occluded, fungal hyphae were observed at frequencies of 0.4 – 12.4% of vessels after 2 months and 0.4 – 34.7% of vessels after 12 months in fungus-inoculated trees. The mean proportion of fungus-colonized vessels was significantly higher after 12 months (8.1 ± 2.1%) than after 2 months (2.1 ± 0.7%) (Two sample t-test for means, $P = 0.01$). Colonized vessels were more abundant near the point of inoculation (Fig. 5-6). No fungal hyphae were observed in water inoculated trees.

Consequently, the proportion of healthy vessels at the margin of discolored sapwood was significantly less in fungus-inoculated trees (44 ± 2.6% after 2 months and 35 ± 2.8% after 12 months) compared to that of water-inoculated trees (74 ± 2.7%) (by ANOVA, Tukey's HSD at $\alpha = 0.05$).

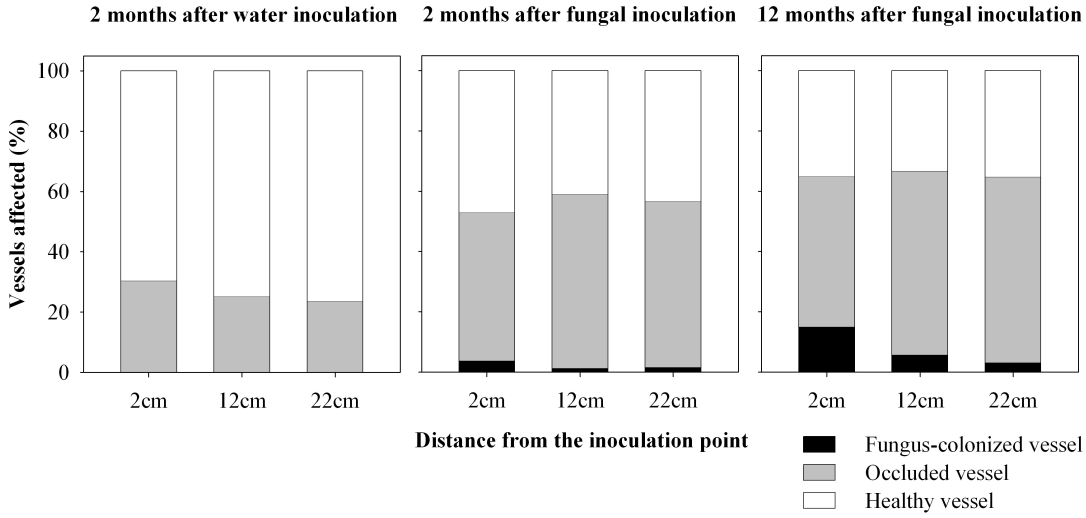

Figure 5-6. Frequencies of healthy, occluded by tyloses or gels, and fungus-colonized vessels at the margin of discolored sapwood 2 months and 12 months after inoculation with *Ceratocystis smalleyi* compared to those inoculated with sterile wate (2 months only). The results shown are means obtained from 8 replicates of fungus-inoculation and 4 replicates of water control.

5.3.5. Chemical responses of host to infection

In discolored sapwood, parenchyma cells appeared necrotic based on the lack of any cell contents (Fig. 5-7A). In contrast, the reserve starch was abundant in healthy parenchyma cells in the sapwood of water-inoculated trees and in the unaffected sapwood of fungus-inoculated trees (Fig. 5-7B).

At the margin of discolored sapwood, lipid was detected (i.e. blue by Nile blue staining) to accumulate in axial and ray parenchyma cells (Fig. 5-7A and C). In parenchyma cells adjacent to a vessel, the nearer to the discolored area they occurred, the more lipid content appeared to have leaked into the vessel through bordered pits (Fig. 5-7D). In discolored cells, all cell contents of such surrounding parenchyma cells were depleted and vessels in the center were partially or completely occluded by gels (Fig. 5-7E).

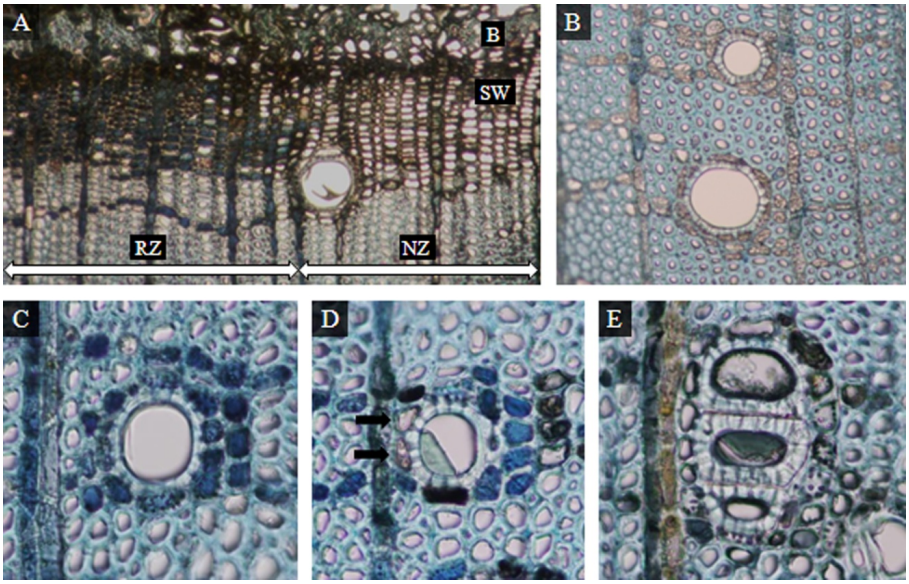

Figure 5-7. Lipid accumulation in reaction zone. A. Radially aligned lipid-rich parenchyma cells in reaction zone (RZ) at the margin of discolored sapwood. Parenchyma cells in necrotic zone (NZ) depleted their cell contents. B: bark, SW: sapwood. **B.** Healthy parenchyma cells in non-discolored tissue containing abundant starch. **C.** Lipid accumulation in parenchyma cells in reaction zone of a fungus-inoculated tree. **D.** Lipid depletion in parenchyma cells (arrows) and an adjacent vessel partially occluded with gel in discolored sapwood. **E.** Necrotic parenchyma cells showing cell contents depleted and vessels occluded with gels in discolored sapwood. **A-E.** Transverse sections stained with Nile blue.

Brownish darkening of cells commonly occurred in colonized tissues of phloem, cambium and xylem of fungus-inoculated trees (Fig. 5-8A). In the xylem, it was more distinct in the ray parenchyma. In the controls, dark-brown discoloration was observed in some ray parenchyma but it was limited to the area near the inoculation wound.

Phenolic compounds were seldom detected by 4-nitrosophenol in fungus-inoculated trees. When observed, they were mostly present in axial parenchyma cells (Fig. 5-8C) and in a few latewood vessels that were occluded with gels in the advance of the reaction zone (Fig. 5-8B). No phenolic compounds were observed in healthy sapwood tissues of water-inoculated trees nor in unaffected sapwood of fungus-inoculated trees.

Pectin materials were initially detected in axial parenchyma cells surrounding vessels in the reaction zone of fungus-inoculated trees (Fig. 5-8D). As sapwood tissues became more extensively colonized, pectin in the parenchyma cells was mobilized into and accumulated in the adjacent vessels (Fig. 5-8E). Some wood fibers also contained pectin materials (Fig. 5-8E). In controls and unaffected sapwood tissues, there was no accumulation of pectin.

No distinctive lignification of xylem cell walls was observed in reaction zone.

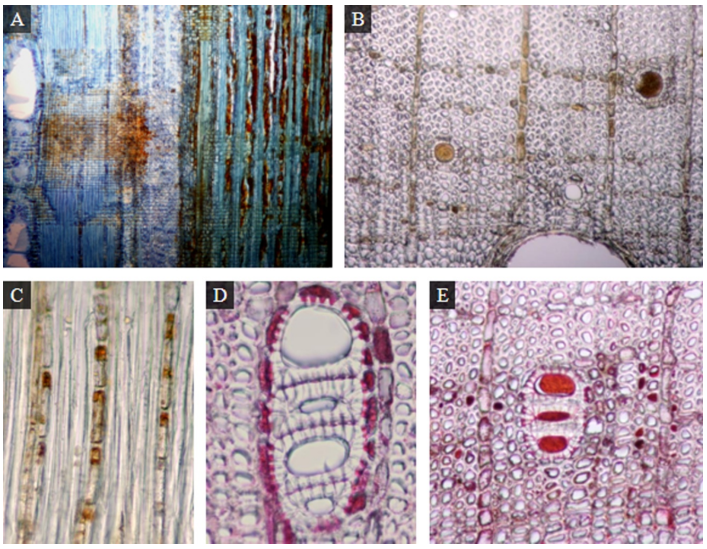

Figure 5-8. Browning of cells and accumulation of phenolic and pectic compounds in reaction zone. A. Brown discoloration of cells in colonized phloem, cambium, and ray parenchyma of xylem in radial view. **B.** Phenolic compounds detected in latewood vessels. **C.** Phenolic compounds concentrated near end-walls of axial parenchyma cells in radial view. **D.** Pectin materials in surrounding parenchyma cells of latewood vessels. **E.** Pectin materials mobilized and accumulated in latewood vessels and wood fibers in discolored wood. **A.** Section stained with toluidine blue O. **B, C.** Sections stained with 4-nitrosophenol. **D, E.** Sections stained with ruthenium red.

5.4. Discussion

Extensive xylem colonization of *C. smalleyi* was clearly visible in principal components of the axial structure of xylem including vessels, fibers, and parenchyma cells in the reaction zone. *C. smalleyi* was also abundantly present in radial parenchyma cells. This physical presence of *C. smalleyi*, particularly of advancing hyphae, in the reaction zone and the high recovery rate of the fungus from xylem lesions indicate that *C. smalleyi* has the ability to invade, colonize, and remain viable in the xylem tissues. Hyphal projections from radial parenchyma cells to vessel lumens and hyphae growing along vessels were commonly observed. These features may be related to the radial and vertical spread of the fungus in the xylem that could likely lead to the expansion of xylem lesions in both directions. Hyphal growth from cell to cell occurred exclusively through bordered pits. Hyphal constriction seemed to be the way of fungal growth between cells in that the hyphae became thin in the pits when they crossed from one cell to another. This fungal penetration by mechanical pressure was also observed in the infection process of *Chalara fraxinea* on *Fraxinus excelsior* (Schumacher et al. 2010). Neither intercellular growth in the middle lamella nor direct penetration through cell walls was found.

Hyphal growth appeared to be the main means of spread of *C. smalleyi* in the xylem. This is contrary to true wilt pathogens such as *Ophiostoma ulmi* and *C. fagacearum* which produce two types of fungal structures (e.g. spores and hyphae) in the colonization of the sapwood. Spore reproduction is considered to be crucial for the establishment of the true wilt pathogens in the xylem because spores can be transported more rapidly than hyphae throughout the vascular system. No such sporulation was observed in the reaction zone of bitternut hickory infected with *C. smalleyi*. It is, however, consistent to the case of Japanese oak wilt where only hyphae of *Raffaelea quercivora* have been observed in infected oaks (Takahashi et al. 2010). This is an interesting similarity in that both diseases exhibit numerous, local xylem lesions that could lead to tree wilting. Other pathogens such as *C. fimbriata* f. sp. *platani* and *Phytophthora ramorum* that cause the same type of xylem lesions were found to produce chlamydospores in xylem vessels (Panconesi 1999; Parke et al. 2007). However, the

contribution of the spore type to fungal spread in the xylem is unknown. In general, chlamydospores are fungal structures important for fungus survival through unfavorable environmental conditions, but are not important for dissemination unless aided (e.g. human mediated). The fact that chlamydospores of *P. ramorum* were only found in the area where the infection was advanced in *Rhododendron* twigs with Ramorum blight (Pogoda and Werres 2004) may indicate that the primary means of the fungal spread is mycelium rather than chlamydospores. The dependency of *C. smalleyi*, *R. quercivora*, and *P. ramorum* on hyphal growth for advancing in the xylem may be one of the factors accounting for why the stem diseases caused by these fungi do not develop systemically throughout the plants.

A set of general defense responses to xylem infection common to many microorganisms were detected in bitternut hickory infected with *C. smalleyi*. Vessel occlusion by tyloses or gel deposits is a general defense mechanism of angiosperms associated with sapwood infections. Vascular occlusion is considered to act in preventing pathogen progression within vessels as well as water loss from the area around damaged tissue (Pearce 1996; Rioux et al. 1998; Sun et al. 2008). It has been reported that a plant species predominantly induces either tyloses or gels (Pearce 1990; Sun et al. 2008). *Ulmus hollandica* and *Prunus pensylvania* are examples of a tylose-producing and gel-forming species, respectively (Yamada 2001). However, bitternut hickory exhibited both types of vascular occlusions, tylosis formation and gel accumulation in response to *C. smalleyi* infection. Such cases in which both tyloses and gels are produced in a plant species were also found in *Vitis vinifera* and *Platanus* × *acerifolia* (Clerivet et al. 2000; Sun et al. 2008). Sun et al. (2008), through a study of wound-induced vascular occlusions, pointed out that the type of vessel occlusion may depend on the season when wounding occurs. Specifically, tyloses primarily formed in summer and gels in winter in *V. vinifera* in their study. This seasonal dependence is somewhat consistent with the case of bitternut hickory infected with *C. smalleyi* where tyloses exclusively formed in earlywood vessels and gels were deposited in latewood vessels.

The basic component of vascular gels is known to be pectin-rich substances which are derived from paratracheal parenchyma cells surrounding a vessel (Rioux et al. 1998;

Sun et al. 2008). Thus, it was not unusual that pectic substances were abundantly produced in parenchyma cells, particularly in the vicinity of vessels, in the reaction zone of infected bitternut hickory. The pectin materials appeared to be subsequently secreted into the adjoining vessel through pits, thus partly or completely clogging the vessel. This process of gel formation is consistent with observations in previous studies (Rioux et al. 1998; Sun et al. 2008). Phenolic compounds were detected in a few of occluded vessels of infected bitternut hickory in this study as they were shown to partly compose gel depositions in cricket bat willow and royal palm trees (Weiner and Liese 1995; Wong and Preece 1978).

Parenchyma cells that were lipid-rich in the reaction zone were distinctive from either healthy, unaffected cells which contained abundant starch grains or necrotic, colonized cells of which cell contents were all depleted. Such increase in lipid in parenchyma cells in association with tree diseases has been observed in susceptible pine seedlings inoculated with pine wood nematode (*Bursaphelenchus xylophilus*) and oaks naturally infected or artificially inoculated with *R. quercivora* (Hara et al. 2006; Yamada et al. 2003). Using successive sampling over time and by distance from inoculation point, Hara et al. (2006) found that lipid in the parenchyma cells diffuses into surrounding trachieds as nematode infection proceeds in susceptible pine trees. This transitioning of parenchyma cells in terms of cell composition agrees with the findings reported in this chapter for bitternut hickory and *C. smalleyi* interaction.

The lipid response has been reported as a consequence of cell damage or senescence or an active defense response in host-microbial interaction (Newcombe and Robb 1989). In the latter case, the lipid contributed to the formation of an impermeable barrier by being incorporated into coating materials (Bishop and Cooper 1983; Newcombe and Robb 1989). Such coating material in parenchyma cells adjacent to a colonized vessel and eventually in vessel lumina were found to impede the lateral penetration of *Verticillium albo-altrum* in alfalfa (Newcombe and Robb 1988). Therefore, this process of lipid response, where lipid is synthesized in living parenchyma cells and incorporated into coating, likely plays an important role in limiting the further penetration of *C. smalleyi* in the xylem unless the fungus produces abundant lipase.

Phenols were rarely detected in a few axial parenchyma cells in the advance of the reaction zone. Phenolic compounds are known to act as constitutive components of sapwood or as phytoalexins which are produced in response to biotic or abiotic stresses (Pearce 1996). In either case, however, phenols create inhibitory environment to fungal growth. Phytoalexins such as mansonones of *Ulmus glabra*, -7- hydroxycalamenene of *Tilia europaea*, and α-, β-, and γ- pyrufurans of *Pyrus communis* were found to accumulate in the reaction zones at the margin between healthy and invaded tissues in these hosts (Pearce 1987), just as it was shown in bitternut hickory. However, further investigation is needed to determine whether the phenols detected in bitternut hickory are functioning as phytoalexins. Even though this may be the case, their fungistatic effects are uncertain because the distribution of detected phenols was sparse.

The lesion development in the phloem and xylem seemed to reflect the growth pattern of *C. smalleyi* and accompanying host defense responses. Xylem lesions more readily extended in a vertical and radial direction than in a lateral direction. Although there is a possibility that fungal spores inoculated into the outer xylem were forcibly drawn up via the transpiration stream in xylem vessels, no spores have been found in xylem tissues in this study. Thus, it may suggest that *C. smalleyi* hyphae more rapidly progresses through vertical and radial routes than a lateral route even though its spread is possible in all directions. In terms of wood structure, both pathways (axially along vessels and radially in the rays) are known to be the easiest routes of fungal spread compared to the lateral movement from vessel to vessel (Pearce 1989). In contrast, *C. fagacearum* which is a true vascular pathogen was found to successfully spread laterally and longitudinally into many vessels in susceptible red oak (Jacobi and MacDonald 1980). Therefore, the limited lateral progression of *C. smalleyi* in bitternut hickory may account for the resulting local lesions that do not extend circumferentially to the entire tree rings.

Xylem lesions tended to extend further vertically than their corresponding bark lesions in general and a long, continuous xylem lesion corresponding to several bark lesions was commonly observed. The separated bark lesions which have also been observed in other hardwood diseases (e.g. *Fraxinus excelsior* infected by *Chalara*

fraxinea (Schumacher et al. 2010), *Platanus acerifolia* infected by *C. fimbriata* f. sp. *platani* (Panconesi 1999), *Quercus* spp., and *Fagus sylvatica* by *P. ramorum* and *P. kernoviae* (Brown and Brasier 2007)) may indicate *C. smalleyi* has the ability to progress outward from xylem to cambium and phloem, thus initiating the development of a new bark canker. Therefore, these features of *C. smalleyi*, together with its abundant hyphal presence and ability to remain viable for over one year in the xylem, may support the speculation that xylem tissues are the preferred substrate of *C. smalleyi* over phloem tissues. However, this hypothesis needs to be investigated.

The extent of the pathogen development in the xylem depends on the effectiveness of resistance mechanisms to limit the pathogen spread. Among the defense responses, Beckman (1966) emphasized the important role of vascular occlusion with gels and tyloses in sealing off the infected area and screening out migrating fungal structures. Such occluded vessels were extensively found in bitternut hickory in response to *C. smalleyi* infection. However, since *C. smalleyi* hyphae were found to colonize up to 35% of otherwise unaffected vessels in the reaction zone, it seems vessel occlusions did not successfully restrict the progressing fungal colonization. This overcoming of host defense responses, which eventually allows the further colonization of the entire tree, commonly occurs in susceptible hosts with true vascular wilts such as oak wilt, Dutch elm disease, and pine wilt disease (Dimond 1970; Hara et al. 2006; Jacobi and MacDonald 1980).

It should also be noted that bitternut hickory failed to contain the pathogen in the initial infection site as the xylem lesion significantly expanded in infected trees compared to that of wounded trees. Without the initial containment, the lesions significantly enlarged over time in *C. smalleyi*-infected trees. The initial response around the inoculation site is critical to restricting the pathogen from invading tissues any further. Through the investigation of the symptom development of pine wilt disease, Hara et al (2006) concluded that the failure of initial defense responses and excessive responses following the subsequent pathogen invasion contribute to the mortality of pines infected with pine wood nematode. Although vessel occlusion was found to be very effective in restricting further *C. fagacearum* spread following initial infection in white oaks, 5-year

old seedlings of the same species exhibited severe wilt symptoms when 50 - 60% of vessels were occluded (Jacobi and MacDonald 1980). Thus, the hydraulic dysfunction observed in *C. smalleyi* infected trees (chapter 4) could be the consequence of this excessive defense response of bitternut hickory, particularly the substantial amount of vessel occlusion.

The larger question of how and why *C. smalleyi* is restricted in the host compared to true vascular wilt pathogens in spite of the limited host defense responses remains uncertain. Jacobi and McDonald (1980) pointed out three factors that likely contribute to the successful colonization of *C. fagacearum*: 1) the production of small conidia that can be transported rapidly throughout the functioning xylem, 2) the common occurrence of natural vessel anastomosing, and 3) the apparent lack of restriction to lateral growth of *C. fagacearum*. Among these factors, the first and third are different from observations in the *C. smalleyi* - bitternut hickory interactions. (The second factor is not discussed because of inadequate information about the wood structure of bitternut hickory.) No sporulation was observed at least in the reaction zone of bitternut hickory and the principal means of *C. smalleyi* for progression was found to be hyphal growth. Of the three dimensions observed, lesion development was restricted laterally in bitternut hickory suggesting the lateral colonization of *C. smalleyi* is limited. It is probable that the lipid-rich parenchyma cells that are radially aligned along the reaction zone form an effective barrier to the lateral invasion of *C. smalleyi*. Suberized walls of parenchyma cells which also have lipid incorporated are known to constitute a structural barrier to the lateral and radial invasion of other pathogens in reaction zones (Newcombe and Robb 1989; Pearce 1996; Yamada 2001; Yamada et al. 2003). Another factor that should be considered is the involvement of cell degrading enzymes. Wilt pathogens such as *V. dahlia, O. ulmi, C. fagacearum, F. oxysporum*, as well as *Pseudomonas solanacearum* are known to secrete extracellular pectolytic and cellulolytic enzymes (Dimond 1970). These degrading enzymes are particularly beneficial for directly penetrating host cells as well as for dissolving the materials making up the defense barrier. If *C. smalleyi* lacks or is deficient in such extracellular enzymes, this may account for the pathogen's limited

ability to overcome host defense responses and spread throughout the xylem like true wilt pathogens. Additional investigation of this point is warranted.

In conclusion, this study showed *C. smalleyi* has the ability to invade and colonize the xylem based on evidence of its physical presence in all xylem tissues. This indicates *C. smalleyi* is not only a phloem pathogen causing bark lesions, but also a xylem pathogen causing xylem lesions that can impair the physiological functioning of the vascular system (chapter 4). Bitternut hickory does activate general defense responses against xylem infection by *C. smalleyi*, such as vessel occlusion with gels and tyloses, lipid accumulation, and production of phenolic compounds. Pectic substances, lipids, and, to a rare extent, phenolics were detected in vascular gels and these were likely derived from paratracheal parenchyma cells. *C. smalleyi* colonization was found to progress gradually, particularly, in a vertical direction in spite of the activated host defense responses. The lack of sporulation and unsuccessful lateral growth of *C. smalleyi* likely limit disease development in the local xylem tissues in contrast to pathogen spread throughout the host vascular system with true vascular wilt organisms.

Chapter 6. Conclusions

Severe decline and mortality of hickory has recently been noted in Maryland, Missouri, New York, Pennsylvania and West Virginia by Forest Health Monitoring (Steinman 2004) and Wisconsin (Wisconsin Department of Natural Resources 2005). Episodes of hickory mortality have periodically occurred in the Eastern United States since the early part of the 20th century. Various terms have been used to document these events, such as "the dying hickory trees" (Hopkins 1912), outbreaks of "hickory bark borer" or "hickory bark beetle" (New York State Museum 1910, 1912, 1915; St. George 1929; Tucker et al. 2006), and "hickory mortality" (U.S. Department of Agriculture 1994). For recent incidences, it has been described as an example of a "decline disease" for which major determinants have not been identified (Juzwik et al. 2008a; Millers et al. 1989; Wisconsin Department of Natural Resources 2005).

Historically, widespread mortality of hickory has been attributed to outbreaks of the hickory bark beetle (*Scolytus quadrispinosus*) during drought periods (Felt 1914; St. George 1929; U.S. Department of Agriculture 1985). Drought alone was found unlikely to lead to tree death because trees not attacked by hickory bark beetles survived and even regained their normal vigor and growth after the drought had ended (St. George 1929). Therefore, tree death was believed to result from stem girdling by numerous galleries formed by the beetle between the inner bark and surface of the wood (Hopkins 1912; U.S. Department of Agriculture 1985). Trees severely infested by the beetles appeared to rapidly develop crown symptoms such as chlorosis and crown thinning followed by tree death within 1 to 2 years (Blackman et al. 1924; St. George 1929).

Systematic assessment of recent epidemics of hickory decline was conducted by a 2 year survey led by the U.S. Forest Service, studies in this dissertation, and other related studies. In contrast to previous reports, the recent assessment concluded that there are at least three scenarios accounting for widespread hickory decline in the North Central and Northeastern regions. First, rapidly declining crown associated with numerous Ceratocystis cankers (Park et al. 2010) and hickory bark beetle colonization (Juzwik et al.

2010) on the main stem was the major tree health problem of hickory observed in recently affected sites. Smooth bark hickories, primarily bitternut hickory (*Carya cordiformis*), were found to be most affected (Juzwik et al. 2010). Trees exhibiting rapid crown wilt readily succumb to death within two years. These symptomatic features of the first scenario are similar to that which many previous reports described. The second scenario is top dieback in the tree crown. Multiple annual cankers caused by *Fusarium solani* on the upper stem of bitternut hickory may account for this crown symptom. Ambrosia beetles (e.g. *Xyleborus celsus*) may be involved in this scenario as a vector of *F. solani*, a creator of infection courts, or as a secondary pest. Hickory bark beetles also carry propagules of *F. solani* and likely serve as a vector as well. This syndrome may not necessarily lead to tree death because affected trees have normal size green leaves below the dead top sufficient to sustain tree life. In a few cases, crown top dieback can also be caused by heavy gall formation on branches and upper main stem. The third scenario is slow crown decline for which symptoms of branch death progressing to death of an entire tree may occur slowly over the course of many years. It could be attributed to many factors such as stem galls, root diseases, and high stand density. However, this scenario accounts for a very small portion of declining trees compared to the other two scenarios. Based on these results, widespread hickory decline is likely similar to ash dieback and maple decline where multiple health problems were not initially differentiated; rather, they were viewed as a single syndrome.

Since the rapid crown decline was the predominant health problem of hickory, the first scenario was further investigated by elucidating major determinants and the mechanism of symptom development in studies of this dissertation. Trees exhibiting rapidly declining crowns are characterized by numerous diffuse cankers and xylem lesions caused by *C. smalleyi*, often in association with hickory bark beetle attack and colonization. According to pathogenicity studies, Ceratocystis cankers can develop up to 80 cm long on bitternut hickory in one year but they are hard to detect unless the outer bark is removed. It is likely that the recognition of this canker disease and the causal agent has been delayed until recently because of this cryptic nature of the symptom and the lack of attempts to isolate microorganisms. A xylem lesion which appears reddish

brown underneath the bark canker is a unique feature of stem infection by *C. smalleyi*. The hickory bark beetle is suspected to be a vector of *C. smalleyi* because the fungus was frequently obtained from trees infested by the beetle, its sticky spores are a good adaptation for acquisition by the beetles, and it was detected on a high percentage of attacking beetles in another study (Juzwik and Banik 2011). However, *C. smalleyi* was not always associated with hickory bark beetle activity as it is shown that not every beetle attack site yielded the fungus and, conversely, *C. smalleyi* was obtained from some stem samples in the absence of the beetle damage. It is possible that there are other means of transmission of *C. smalleyi* such as vascular movement and other wound-causing agents as well as by hickory bark beetle.

Symptoms of rapid decline in tree crowns may be caused by coalescing of hickory bark beetle galleries, by numerous Ceratocystis cankers girdling the stem, and/or local disruption of water flow in xylem lesions associated with the cankers and beetle attacks. This study supports the third hypothesis for several reasons. First, when the bark was stripped from actively declining trees to examine insect activity, the gallery systems observed were small and not coalescing (Juzwik et al. 2010). Second, girdling itself may not be sufficient to explain the rapid progress of symptoms to the mortality of an entire tree because girdling effects, in general, take a considerable period of time until tree death depending on the initial carbohydrate availability below the girdle and the maintenance of transpiration (Noel 1970). In contrast, research results in this dissertation (chapter 4) showed that a significant reduction in sap flow velocity occurred on bitternut hickory one year after they were artificially inoculated with *C. smalleyi* at multiple points on the stem compared to control trees.

Results of this dissertation research (chapters 4 and 5) lead to the hypothesis that the impediment in water conduction is likely the consequence of xylem infection by the fungus and resulting host responses. The reduced level of sap flow velocity was associated with disease severity and the frequency of occluded vessels by tyloses in the current two years' annual rings. This is consistent with the fact that extensive vessel occlusion by tyloses affects water transport in a tree by dehydration (Pallardy 2008) and increases tension in the remaining vessels to maintain water flow to tree crowns resulting

in more embolism and more reduced hydraulic conductivity (Tyree and Sperry 1989). The negative effects of tylose formation in outer annual rings can be much worse in ring porous trees like bitternut hickory because most water is transported through vessels formed within the last few years. Tylose formation was apparently one of host defense responses against to xylem colonization by *C. smalleyi*. Vessel occlusion by tyloses in earlywood vessels and gel deposits in latewood vessels commonly occurred in the internal interface between the living host cell and the pathogen. However, vessel occlusion was not found to be very effective in the complete containment of the pathogen. This failure of initial defense responses may result in subsequent pathogen invasion and excessive host responses, which lead to xylem dysfunction in infected xylem tissues.

Xylem water conductance is a key physiological function with implications for tree crown condition and disruption of such physiological functioning occurs before visible, external symptoms appear. Sap flow monitoring system using the Granier's thermal dissipation probe (TDP) was found to be a useful tool for measuring the hydraulic function in relation to the declining symptom. In bitternut hickory, results from sap flow monitoring (chapter 4) clearly showed that multiple stem infections of *C. smalleyi* impaired water transport in visually healthy trees. The relationship between the reduced sap flow velocity and the degree of external symptom development was not the subject of this study. Yet, an infected tree of which the average maximum sap flow velocity was reduced by 86% compared to control trees exhibited over 60% declining crown one year after inoculation. A sap flow monitoring system is widely used to measure whole-tree water use in physiological studies. This technique has also been used to monitor effects of fungal infection on xylem functioning in pathology studies such as for sudden oak death in tanoak (Parke et al. 2007) and for blue stain of pines and Norway spruce (Kirisits and Offenthaler 2002; Rice and Langor 2008; Yamaoka et al. 1990). This study is the first to demonstrate the utility of the sap flow monitoring system for elucidating the role of a pathogen in a complex disease, previously referred to as a decline disease. Except for foliar diseases, crown symptoms such as crown decline, dieback, foliage wilt, and cholorisis, in general, appear as the end result of physiological

problems in other parts of a tree (e.g. stem and roots), thus making it hard to evaluate symptom progress in a short period of a one-year-experiment and with objectivity. In this regard, the usage of sap flow monitoring system is highly recommended to evaluate the degree of individual tree decline as well as effects of potential determinants on symptom development of decline diseases.

Although pathogen-induced wilting is involved in disease development of hickory crown decline, this disease is different from true vascular wilts such as oak wilt and Dutch elm disease where systemic infection occurs. *C. smalleyi* does not systemically spread throughout the tree. The lack of sporulation in the xylem and unsuccessful lateral growth may account for the limited vascular infection of *C. smalleyi*. Radially aligned lipid-rich parenchyma cells may contribute to limiting the lateral expansion of the fungus in the sapwood. From this aspect, mass attack of hickory bark beetles should be very important because they can create numerous infection courts and/or vector the fungus while attacking a tree. Fan-shaped gallery formation of the beetles may also compensate for the limited ability of the fungus to grow laterally in sapwood. Crown decline and tree death may rapidly occur when much of tree's sapwood cross section is covered by numerous xylem lesions and resulting limited wilt occurs in an extensive xylem area. This nature of a limited vascular wilt is most like that of Japanese oak wilt (Table 6-1). Oak trees exhibiting Japanese oak wilt show a large amount of insect damage by an ambrosia beetle species (*Platypus quercivorus*) and associated fungal infection by *Raffaelea quercivora* (Kubono and Ito 2002; Kuroda 2001). The fungus causes local discoloration in sapwood where xylem occlusion and an interruption of water transport occur (Kuroda 2001; Kuroda and Yamada 1996; Murata et al. 2005; Murata et al. 2007). Although *P. quercivorus* acts differently from *S. quadrispinosus* in terms of constructing gallery systems into the sapwood and having a mutually symbiotic relationship with the fungus, the underlying cause of tree wilt and death is the same.

Table 6-1. Comparisons of hickory wilt to other limited wilt diseases and thousand cankers disease

Disease	Hickory wilt (Potentially)	Japanese oak wilt[a]	Sudden oak death on tanoak[b]	Thousand cankers disease[c]
Host	*Carya cordiformis*	*Quercus crispula, Qercus serrata*	*Lithocarpus densiflorus*	*Juglans nigra*
Pathogen	*Ceratocystis smalleyi*	*Raffaelea quercivora*	*Phytophthora ramorum*	*Geosmithia morbida*
Stem colonization				
Xylem	Yes	Yes	Yes	No
Phloem	Yes	Not reported	Yes	Yes
Main means of spread in xylem	Hyphae	Hyphae	Hyphae, chlamydospores	Not applied
Insect associates	Hickory bark beetle (*Scolytus quadrispinosus*)	Ambrosia beetle (*Platypus quercivorus*)	bark beetles, ambrosia beetles	Walnut twig beetle (*Pityophthorus juglandis*)
Pathogen-insect relationship	Possible vector	Symbiotic	Secondary insect pests	Symbiotic
Infection incidence per tree	Numerous	Numerous	Not reported	Numerous
Mechanism of tree wilt and death	Multiple, limited vascular wilts	Multiple, limited vascular wilts	Canker and likely limited vascular wilt	Stem girdling by numerous bark cankers

[a] (Kuroda 2001; Kuroda and Yamada 1996; Murata et al. 2005; Murata et al. 2007; Takahashi et al. 2010)
[b] (Hansen 2008; McPherson et al. 2000; Pallardy 2008; Parke et al. 2007)
[c] (Tisserat et al. 2009)

Characteristics of such limited vascular wilt were recently observed in tanoak (*Lithocarpus densiflorus*) infected by *Phytophthora ramorum* (Collins et al. 2009; Hansen 2008; Parke et al. 2007). *P. ramorum* was found to colonize not only phloem tissues but also xylem vessels by hyphae and chlamydospore reproduction, thus inducing xylem dysfunction in colonized xylem areas (Collins et al. 2009; Parke et al. 2007). However, sudden oak death is different from hickory crown decline and Japanese oak wilt in that insect involvement is considered secondary to the fungal infection (Table 6-1). Thousand cankers disease, which is an emerging disease of black walnut, involves numerous attacks of the walnut twig beetle (*Pityophthorus juglans*) and following infections of its symbiotic fungus (*Geosmithia morbida*) in twigs and main stem (Tisserat et al. 2009). Yet, the fungus does not invade the xylem and tree death results from stem girdling by numerous bark cankers (Tisserat et al. 2009). Therefore, tree decline and

mortality of affected trees progress more slowly compared to the other limited wilt diseases mentioned above (Table 6-1).

Given this new knowledge on the mechanism involved in symptom development of hickory crown decline and the comparisons with other patho-systems (Table 6-1), the new name “hickory wilt” is recommended. This revised name better describes the rapid crown decline syndrome observed in bitternut hickory. Furthermore, the revised name is consistent with the use of Japanese oak wilt, a disease with many similarities to the hickory disease. According to the modified disease triangle concept (Ostry et al. 2011), the primary host species of hickory wilt is bitternut hickory and damaging agents are *C. smalleyi* and hickory bark beetle. Hickory bark beetles may be responsible for dissemination and inoculation of *C. smalleyi* into tree stems although further study is needed on this topic. The synergistic interaction of the hickory bark beetle and *C. smalleyi* results in numerous bark cankers and xylem lesions on stems of bitternut hickory. The deleterious effects of multiple cankers and xylem dysfunction caused by *C. smalleyi* may lead to rapid crown decline and tree death. Drought is still considered an important environment factor for build-up of hickory bark beetle populations.

C. smalleyi is speculated to be a native organism in the United States for several reasons. First, *C. smalleyi* appeared widely distributed in the North Central and Northeastern regions despite the relatively short history of the fungal taxon since first discovered in 1994. Second, close examination of fungal morphology and phylogenetic analyses indicated that some degree of genetic and phenotypic variations exist in its natural populations. Third, the presence of its close sibling species (*C. caryae*) was recently discovered (Johnson et al. 2005). Last, among naturally infected and artificially inoculated trees, some trees were found to be relatively resistant or tolerant to *C. smalleyi* infection. However, this latter speculation will require further investigations on genetic variability of the fungus and the level of resistance and susceptibility of host plants. In addition, the presence of *C. smalleyi* has yet to be detected in the southern part of bitternut hickory range where hickory bark beetle is commonly found (i.e. Southern USA).

Annual cankers caused by *F. solani* and insect activity of ambrosia beetles were closely associated with the second scenario (crown top dieback). Although *F. solani* appeared widely distributed throughout the North Central and Northeastern regions, substantial variation in genetic and cultural characteristics was not seen. Among *F. solani* isolates, two isolate types (BB and BC) were predominant and they were distinguished from each other by 10 base pair differences in sequence comparisons. BC type *F. solani* was pathogenic to maturing bitternut hickory causing sunken cankers up to 5.1 cm long 13 months after inoculation. This canker development by *F. solani* infection was much slower compared to trees inoculated with *C. smalleyi*. BB type *F. solani* was found to be nonpathogenic to weakly pathogenic on bitternut hickory by site. Thus, it is probable that BB isolates depend on the tree vigor and environmental conditions to cause cankers and they may exist as a facultative saprophyte. The pathogenic BC isolates have tef 1-α sequences that best matched the *F. solani* strain obtained from a canker on black walnut and showed high homogeneity among isolates. These features suggest the potential host speciation of BC isolates as seen in *F. solani* f. sp. *robiniae* causing twig blight on black locust (*Robinia pseudomonas*) (Matuo and Sakurai 1965) and *F. solani* f. sp. *mori* causing stem blight on mulberry (*Morus* sp.) (Sakurai and Matuo 1957, 1959). Modes of transmission of *F. solani* are unknown, but ambrosia beetles could serve as a vector for the fungus because pinholes typical of insects such as ambrosia beetle commonly occurred inside or at the edge of the annual cankers. *F. solani*-ambrosia beetle complexes associated with canker diseases have been observed on tulip poplar, black walnut, and flowering dogwood (Anderson and Hoffard 1978; Ngoan et al. 1976; Weber and McPherson 1984). *Xyleborus celsus* is of particular interest because it was the second most common beetle that emerged from hickory log sections taken from declining trees during the 2008 FS-led survey (Juzwik et al. 2009). Hickory bark beetles also were found to carry viable propagules of *F. solani* as well (Juzwik, unpublished data).

Globose galls on bitternut hickory that are often referred to as Phomopsis gall are widely known in USA and considered to be caused by *Phomopsis* sp. *Phomopsis* sp. was more commonly obtained from samples of stem galls on bitternut hickory compared to other fungal taxa. However, the overall isolation rates of fungi from those samples were

relatively low and it is uncertain if all isolates were the same species because the identification to the species level was not successful. Furthermore, the pathogenicity of *Phomopsis* isolates was low (Brown 1938) or did not occur on bitternut hickory (Park, unpublished data). These inconclusive results, therefore, possibly suggest that galls found on bitternut hickory may be induced by another organism, but further study is needed. Phomopsis galls are commonly found on maples, hickories, oaks, and elms throughout central and eastern USA (Sinclair and Lyon 2005), but the identity of the pathogen has not been thoroughly studied. To clarify the causal organism of this common disease, taxonomic study of the gall-inducing *Phomopsis* is warranted.

In conclusion, widespread decline and mortality of hickory, historically, have been attributed to drought and subsequent hickory bark beetle outbreaks (U.S. Department of Agriculture 1985) and previously described as a decline disease of the species (Juzwik et al. 2008a; Wisconsin Department of Natural Resources 2005). Results from studies in this dissertation and related studies document this health problem as being a combination of synchronous effects from at least three scenarios (rapid crown wilt, crown top dieback and slow crown decline). This dissertation demonstrates that the predominant health problem of bitternut hickory (rapid crown wilt) is due to hickory wilt caused by *C. smalleyi* in association with hickory bark beetle. For managing this disease, control of the hickory bark beetle may be the key. Reducing density of bitternut hickory in a stand may improve individual tree health by increasing access to water, carbon, and nutrient sources as well as reduce the attraction of hickory bark beetles to the stand (Solomon and Payne 1986; U.S. Department of Agriculture 1985). Sanitation is also recommended in order to prevent hickory bark beetle from readily building-up its population (Solomon and Payne 1986), but it is difficult for landowners to accomplish. Effects of systemic fungicide injection in restricting xylem colonization by *C. smalleyi* could be examined for its applicability to high-value trees.

Bibliography

Agrios, G.N. 2005. Plant Pathology. Elsevier Academic Press, Boston, MA. 922 p.

Al Adawi, A.O., M.L. Deadman, A.K. Al Rawahi, Y.M. Al Maqbali, A.A. Al Jahwari, B.A. Al Saadi, I.S. Al Amri, and M.J. Wingfield. 2006. Aetiology and causal agents of mango sudden decline disease in the Sultanate of Oman. European Journal of Plant Pathology 116(4):247-254.

Anderson, R.L., and W.H. Hoffard. 1978. Fusarium canker-ambrosia beetle complex on tulip poplar in Ohio. Plant Disease Reporter 62(8):751-751.

Beckman, C.H. 1966. Cell irritability and localization of vascular infections in plants. Phytopathology 56:821-824.

Beckman, C.H. 1987. The Nature of Wilt Diseases of Plants. APS Press, St. Paul, MN. 175 p.

Bishop, C.D., and R.M. Cooper. 1983. An ultrastructural study of vascular colonization in three vascular wilt diseases. 1. Colonization of susceptible cultivars. Physiological Plant Pathology 23(3):323-343.

Blackman, M.W., H.H. Stage, M.H. Hatch, F.G. Mundinger, and H. Notman. 1924. On the Succession of Insects Living in the Bark and Wood of Dying, Dead and Decaying Hickory. Syracuse University, Syracuse, NY. 336 p.

Booth, C. 1971. The Genus *Fusarium*. Commonwealth Mycological Institute, Kew, England. 237 p.

Boyer, M.G. 1961. A Fusarium canker disease of *Populus deltoides* marsh. Canadian Journal of Botany 39:1195-1204.

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

Brown, N.A. 1938. The tumor disease of oak and hickory trees. Phytopathology 28:401-411.

Burns, R.M., and B.H. Honkala. 1990. Silvics of North America: Volume 2. Hardwoods. United States Department of Agriculture, Forest Service, Agriculture handbook 654.

Campbell, W.A., and R.W. Davidson. 1942. A species of *Poria* causing rot and cankers of hickory and oak. Mycologia 34(1):17-26.

Carlson, J.C., M.E. Mielke, J.E. Appleby, R. Hatcher, E.M. Hayes, C.J. Luley, J.G. O'Brien, and D.J. Rugg. 1993. Survey of black walnut canker in plantations in five Central States. Northern Journal of Applied Forestry 10:10-13.

Cave, G.L., B. Randall-Schadel, and S.C. Redlin. 2008. Risk Analysis for *Phytophthora ramorum* Werres, de Cock & Man in't Veld, Causal Agent of Sudden Oak Death, Ramorum Leaf Blight, and Ramorum Dieback. U. S. Department of Agriculture, Animal and Plant Health Inspection Service, Raleigh, NC. 88 p.

Chayen, J., and L. Bitensky. 1991. Practical Histochemistry. Wiley, Chichester, NY. 321 p.

Chen, W., and W.J. Swart. 2000. First report of stem canker of English walnut caused by *Fusarium solani* in South Africa. Plant Disease 84(5):592.

Christiansen, E. 1985. *Ips*/*Ceratocystis*-infection of Norway spruce: what is a deadly dosage? Zeitschrift für Angewandte Entomologie 99:6-11.

Ciesla, W.M., and E. Donaubauer. 1994. Decline and Dieback of Trees and Forests : A Global Overview. Food and Agriculture Organization of the United Nations, Rome. 90 p.

Clark, G.B. 1981. Staining Procedures. Williams and Wilkins, Baltimore, MD. 512 p.

Clerivet, A., V. Deon, I. Alami, F. Lopez, J.P. Geiger, and M. Nicole. 2000. Tyloses and gels associated with cellulose accumulation in vessels are responses of plane tree seedlings (*Platanus* x *acerifolia*) to the vascular fungus *Ceratocystis fimbriata* f. sp *platani*. Trees 15(1):25-31.

Collins, B.R., J.L. Parke, B. Lachenbruch, and E.M. Hansen. 2009. The effects of *Phytophthora ramorum* infection on hydraulic conductivity and tylosis formation in tanoak sapwood. Canadian Journal of Forest Research 39(9):1766-1776.

Croisé, L., F. Lieutier, and E. Dreyer. 1998. Scots pine responses to number and density of inoculation points with *Leptographium wingfieldii* Morelet, a bark beetle-associated fungus. Annals of Forest Science 55(4):497-506.

Demirci, F., and S. Maden. 2006. A severe dieback of box elder (*Acer negundo*) caused by *Fusarium solani* (Mart.) Sacc. in Turkey. Australasian Plant Disease Notes 1(1):13-15.

Dimond, A.E. 1970. Biophysics and biochemistry of the vascular wilt syndrome. Annual Review of Phytopathology 8:301-322.

Dochinger, L.S., and C.E. Seliskar. 1962. Fusarium canker found on yellow poplar. Journal of Forestry 60:331-333.

Elgersma, D.M. 1973. Tylose formation in elms after inoculation with *Ceratocystis ulmi*, a possible resistance mechanism. European Journal of Plant Pathology 79(5):218-220.

Engelbrecht, C.J., T.C. Harrington, and A. Alfenas. 2007. Ceratocystis wilt of cacao - a disease of increasing importance. Phytopathology 97(12):1648-1649.

Farr, D.F., G.F. Bills, G.P. Chamuris, and A.Y. Rossman. 1989. Fungi on Plants and Plant Products in the United States. APS Press, American Phytopathological Society, St. Paul, MN. 1252 p.

Felt, E.P. 1914. Notes on forest insects. Journal of Economic Entomology 7:373-375.

Fukuda, K., T. Hogetsu, and K. Suzuki. 1992. Photosynthesis and water status in pine wood nematode-infected pine seedlings. Journal of the Japanese Forestry Society 74:289-299.

Gersbach, P.V., S.G. Wyllie, and V. Sarafis. 2001. A new histochemical method for localization of the site of monoterpene phenol accumulation in plant secretory structures. Annals of Botany 88(4):521-525.

Granier, A. 1987. Evaluation of transpiration in a Douglas fir stand by means of sap flow measurements. Tree Physiology 3(4):309-320.

Hansen, E.A. 2008. Rethinking *Phytophthora* - research opportunities and management. P. 5-14 *in* Proceedings of the Sudden Oak Death Third Science Symposium. Gen. Tech. Rep. PSW-GTR-214. Santa Rosa, CA, Frankel, S.J., J.T. Kliejunas, and K.M. Palmieri (eds.). USDA Forest Service, Pacific Southwest Research Station.

Hansen, E.M., J. Parke, R. Linderman, and W. Sutton. 2002. *Phytophthora ramorum* and Oregon forest trees – one pathogen, three diseases. P. 78 *in* Proceedings of Sudden oak death science symposium. Monterey, CA. U.S. Department of Agriculture, Forest Service, Pacific Southwest Research Station.

Hara, N., Y. Takeuchi, and K. Futai. 2006. Cytological changes in ray parenchyma cells of seedlings of three pine species infected with the pine wilt disease. Japanese Journal of Nematology 36(1):23-32.

Hopkins, A.D. 1910. Insects which kill forest trees: character and extent of their depredations and methods of control. Goverment printing office, Washington, DC. 9 p.

Hopkins, A.D. 1912. The dying hickory trees; cause and remedy. Goverment printing office, Washington, DC. 5 p.

Houston, D.R. 1992. A host-stress-saprogen model for forest dieback-decline diseases. P. 3-25 in Forest Decline Concepts, Manion, P.D., and D. Lachance (eds.). APS Press, St. Paul, MN.

Ito, S., T. Kubono, N. Sahashi, and T. Yamada. 1998. Associated fungi with the mass mortality of oak trees. Journal of Japanese Forest Society 80(3):170-175.

Jacobi, W.R., and W.L. MacDonald. 1980. Colonization of resistant and susceptible oaks by *Ceratocystis fagacearum*. Phytopathology 70(7):618-623.

Jensen, W.A. 1962. Botanical Histochemistry: Principles and Practice. W.H. Freeman and Company, San Francisco, CA. 408 p.

Johnson, J.A., T.C. Harrington, and C.J.B. Engelbrecht. 2005. Phylogeny and taxonomy of the North American clade of the *Ceratocystis fimbriata* complex. Mycologia 97(5):1067-1092.

Juzwik, J., and M. Banik. 2011. Nature of *Ceratocystis smalleyi* - *Scolytus quadrispinosus* interactions on stems of bitternut hickory with declining crowns. Phytopathology 101:S86.

Juzwik, J., P. Castillo, J.-H. Park, and L.M. Haugen. 2009. Assessment and etiology of hickory decline (NC-EM-07-01): Second year results. *in* Forest health monitoring working group meeting, Savannah, GA. Available online at http://www.fhm.fs.fed.us/posters/posters09/assessment_etiology_hickory_decline.pdf

Juzwik, J., L. Haugen, J.-H. Park, and M. Moore. 2008a. Fungi associated with stem cankers and coincidental scolytid beetles on declining hickory in the Upper Midwest. P. 476-482 *in* Proceedings of 16th Central Hardwood Forest Conference, New Town Square, PA, Gen. Tech. Rep. NRS-P-24, Jacobs, D.F., and C.H. Michler (eds.). USDA Forest Service, Northern Research Station.

Juzwik, J., S. Olszewski, J.-H. Park, and G. Reynolds. 2008b. Assessment and etiology of hickory decline (NC-EM-07-01): Preliminary results. *in* Forest health monitoring working group meeting, San Antonio, TX. Available online at http://www.fhm.fs.fed.us/posters/posters08/hickory_decline.pdf

Juzwik, J., J.-H. Park, P. Castillo, and L.M. Haugen. 2010. Assessment and etiology of hickory decline (NC-EM-07-01): Third year results. *in* Forest health monitoring working group meeting, Albuquerque, NM. Available online at http://www.fs.fed.us/foresthealth/fhm/posters/posters10/assessment_etiology_hickory_decline.pdf

Kinuura, H., and M. Kobayashi. 2006. Death of *Quercus crispula* by inoculation with adult *Platypus quercivorus* (Coleoptera : Platypodidae). Applied Entomology and Zoology 41(1):123-128.

Kirisits, T., and I. Offenthaler. 2002. Xylem sap flow of Norway spruce after inoculation with the blue-stain fungus *Ceratocystis polonica*. Plant Pathology 51(3):359-364.

Kliejunas, J.T. 2010. Sudden Oak Death and *Phytophthora ramorum*: A Summary of the Literature. 2010 Edition. Gen. Tech. Rep. PSW-GTR-234. USDA Forest Service, Pacific Southwest Research Station, Albany, CA. 181 p.

Kubono, T., and S. Ito. 2002. *Raffaelea quercivora* sp. nov. associated with mass mortality of Japanese oak, and the ambrosia beetle (*Platypus quercivorus*). Mycoscience 43:255-260.

Kuroda, K. 1991. Mechanism of cavitation development in the pine wilt disease. European Journal of Forest Pathology 21(2):82-89.

Kuroda, K. 2001. Responses of *Quercus* sapwood to infection with the pathogenic fungus of a new wilt disease vectored by the ambrosia beetle *Platypus quercivorus*. Journal of Wood Science 47(6):425-429.

Kuroda, K., and T. Yamada. 1996. Discoloration of sapwood and blockage of xylem sap ascent in the trunks of wilting *Quercus* spp. following attack by *Platypus quercivorus*. Journal of Japanese Forest Society 78:84-88. (in Japanese with English summary)

Kuroda, K., T. Yamada, K. Mineo, and H. Tamura. 1988. Effects of cavitation on the development of pine wilt disease caused by *Bursaphelenchus xylophilus*. Annals of the Phytopathological Society of Japan 54:606-615.

Leslie, J.F., and B.A. Summerell. 2006. The *Fusarium* Laboratory Manual. Blackwell Publishing, Ames, IA. 388 p.

Lombard, L., M. Bogale, F. Montenegro, B.D. Wingfield, and M.J. Wingfield. 2008. A new bark canker disease of the tropical hardwood tree *Cedrelinga cateniformis* in Ecuador. Fungal Diversity 31:73-81.

Lu, P., L. Urban, and Z. Ping. 2004. Granier's thermal dissipation probe (TDP) method for measuring sap flow in trees : theory and practice. Acta Botanica Sinica 46(6):631-646.

Manion, P.D. 1991. Tree Disease Concepts. Prentice Hall, Englewood Cliffs, NJ. 402 p.

Marin, M., B. Castro, A. Gaitan, O. Preisig, B.D. Wingfield, and M.J. Wingfield. 2003. Relationships of *Ceratocystis fimbriata* isolates from Colombian coffee-growing

regions based on molecular data and pathogenicity. Journal of Phytopathology 151(7-8):395-405.

Matuo, T., and Y. Sakurai. 1965. *Fusarium solani* f. *robiniae* n. f., one of the causal fusaria of the twig blight of *Robinia pseudoacacia*. Japanese Journal of Phytopathology 30(1):31-36.

McPherson, B.A., D.L. Wood, A.J. Storer, N. Maggi Kelly, and R.B. Standiford. 2001. Sudden oak death, a new forest disease in California. Integrated Pest Management Reviews 6(3):243-246.

McPherson, B.A., D.L. Wood, A.J. Storer, P. Svihra, D.M. Rizzo, N.M. Kelly, and R.B. Standiford. 2000. Oak mortality syndrome: Sudden death of oaks and tanoaks. California Department of Forestry and Fire Protection Tree Notes No. 26. California Department of Forestry and Fire Protection. 6 p.

Millers, I., D.S. Shriner, and D. Rizzo. 1989. History of Hardwood Decline in the Eastern United States. Gen. Tech. Rep. NE-126. Northeastern Forest Experiment Station, Broomall, PA. 75 p.

Mueller-Dombois, D. 1992. A natural dieback theory, cohort senescence as an alternative to the decline disease theory. P. 26-37 in Forest Disease Concepts, Manion, P.D., and D. Lachance (eds.). APS Press, St. Paul, MN.

Murali, T.S., T.S. Suryanarayanan, and R. Geeta. 2006. Endophytic *Phomopsis* species: host range and implications for diversity estimates. Canadian Journal of Microbiology 52(7):673-680.

Murata, M., T. Yamada, and S. Ito. 2005. Changes in water status in seedlings of six species in the Fagaceae after inoculation with *Raffaelea quercivora* Kubono et Shin-Ito. Journal of Forest Research 10(3):251-255.

Murata, M., T. Yamada, Y. Matsuda, and S. Ito. 2007. Discoloured and non-conductive sapwood among six Fagaceae species inoculated with *Raffaelea quercivora*. Forest Pathology 37(2):73-79.

Murray, M. 2008. New threat to walnuts: Walnut twig beetle and thousand cankers disease. Utah Pests News 2:1-2.

Nelson, P.E., T.A. Toussoun, and W.F.O. Marasas. 1983. *Fusarium* species : An Illustrated Manual for Identification. The Pennsylvania State University Press, University Park, PA. 193 p.

Nemec, S. 1987. *Fusarium solani* association with branch and trunk cankers on citrus weakened by cold weather in Florida. Mycopathologia 97(3):143-150.

New York State Museum. 1910. 25th report of the state entomologist on injurious and other insects of the State of New York 1909. P. 103-104 in Education Department Bulletin No. 475, Museum Bulletin 141. University of State of New York, Albany, NY.

New York State Museum. 1912. 27th report of the state entomologist on injurious and other insects of the State of New York 1911. P. 110-113 in Education Department Bulletin No. 510, Museum Bulletin 155 University of the State of New York, Albany, NY.

New York State Museum. 1915. 29th report of the state entomologist on injurious and other insects of the State of New York 1913. P. 33-36 in University of the State of New York Bulletin No. 589, Museum Bulletin 175. University of the State of New York, Albany, NY.

Newcombe, G., and J. Robb. 1988. The function and relative importance of the vascular coating response in highly resistant, moderately resistant and susceptible alfalfa infected by *Verticillium albo-atrum*. Physiological and Molecular Plant Pathology 33(1):47-58.

Newcombe, G., and J. Robb. 1989. The chronological development of a lipid-to-suberin response at *Verticillium* trapping sites in alfalfa. Physiological and Molecular Plant Pathology 34(1):55-73.

Ngoan, N.D., R.C. Wilkinson, D.E. Short, C.S. Moses, and J.R. Mangold. 1976. Biology of an introduced ambrosia beetle, *Xylosandrus compactus*, (Coleoptera-Scolytidae) in Florida. Annals of the Entomological Society of America 69(5):872-876.

Noel, A.R.A. 1970. The girdled tree. Botanical Review 36(2):162-195.

O'Donnell, K. 2000. Molecular phylogeny of the *Nectria haematococca-Fusarium solani* species complex. Mycologia 92(5):919-938.

Ostry, M.E., R.C. Venette, and J. Juzwik. 2011. Decline as a disease category: Is it helpful? Phytopathology 101(4):404-409.

Paine, T.D., K.F. Raffa, and T.C. Harrington. 1997. Interactions among *scolytid* bark beetles, their associated fungi, and live host conifers. Annual Review of Entomology 42:179-206.

Pallardy, S.G. 2008. Physiology of Woody Plants. Elsevier, Burlington, MA. 454 p.

Panconesi, A. 1999. Canker stain of plane trees: a serious danger to urban plantings in Europe. Journal of Plant Pathology 81(1):3-15.

Panconesi, A., S. Moricca, I. Dellavalle, and G. Torraca. 2003. The epidemiology of canker stain of plane tree and its spread from urban plantings to spontaneous groves and natural forests. P. 84-91 in Mitteilungen aus der Biologischen Bundesanstalt für Land- und Forstwirtschaft, Heft 394. Biologische Bundesanstalt für Land- und Forstwirtschaft, Berlin.

Park, J.-H., J. Juzwik, and L.M. Haugen. 2010. Ceratocystis canker of bitternut hickory caused by *Ceratocystis smalleyi* in the North-Central and Northeastern United States. Plant Disease 94(2):277.

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

Pearce, R.B. 1989. Cell wall alterations and antimicrobial defense. P. 346-360 in Plant Cell Wall Polymers - Biogenesis and Biodegradation. American Chemical Society, Washington, DC.

Pearce, R.B. 1990. Occurrence of decay-associated xylem suberization in a range of woody species. European Journal of Forest Pathology 20(5):275-289.

Pearce, R.B. 1991. Reaction zone relics and the dynamics of fungal spread in the xylem of woody angiosperms. Physiological and Molecular Plant Pathology 39(1):41-55.

Pearce, R.B. 1996. Antimicrobial defences in the wood of living trees. New Phytologist 132(2):203-233.

Pennsylvania Department of Environmental Resources. 1983. Forest pest management news, 1983 September. Department of Environmental Resources, Bureau of Forestry, Devision of Forest Pest Management, Middletown, PA.

Pilotti, M., V. Ponzio, and E. Motta. 2002. Disorders of *Platanus* × *acerifolia* in Italy associated with *Fusarium solani*. Forest Pathology 32:249-264.

Pogoda, F., and S. Werres. 2004. Histological studies of *Phytophthora ramorum* in *Rhododendron* twigs. Canadian Journal of Botany 82(10):1481-1489.

Quimby, J.W. 1986. Tree mortality in Pennsylvania forests defoliated by gypsy moth - a 1984 update. Department of Environmental Resources, Bureau of Forestry, Division of Forest Pest Management, Middletown, PA. 11 p.

Redman, R.S., S. Freeman, D.R. Clifton, J. Morrel, G. Brown, and R.J. Rodriguez. 1999. Biochemical analysis of plant protection afforded by a nonpathogenic endophytic mutant of *Colletotrichum magna*. Plant Physiology 119(2):795-804.

Rice, A.V., and D.W. Langor. 2008. A comparison of heat pulse velocity and lesion lengths for assessing the relative virulence of mountain pine beetle-associated fungi on jack pine. Forest Pathology 38(4):257-262.

Rioux, D., M. Nicole, M. Simard, and G.B. Ouellette. 1998. Immunocytochemical evidence that secretion of pectin occurs during gel (gum) and tylosis formation in trees. Phytopathology 88(6):494-505.

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

Sakurai, Y., and T. Matuo. 1957. On a Fusarium-disease of mulberry twigs caused by *Hypomyces solani* (Rke. et Berth.) Snyd. et Hans. Research Reports of Textile and Sericulture, Shinshu University 7:18-24. (in Japanese with English summary)

Sakurai, Y., and T. Matuo. 1959. On the form name and race of *Hypomyces solani* (Rke. et Berth.) Snyd. et Hans. which is pathogenic to the mulberry trees. Japanese Journal of Phytopathology 24(4):219-223. (in Japanese with English summary).

Schumacher, J., R. Kehr, and S. Leonhard. 2010. Mycological and histological investigations of *Fraxinus excelsior* nursery saplings naturally infected by *Chalara fraxinea*. Forest Pathology 40(5):419-429.

Sinclair, W.A. 1965. Comparisons of recent declines of white ash, oaks, and sugar maple in northeastern woodlands. Cornell Plantations 20:62-27.

Sinclair, W.A., and H.H. Lyon. 2005. Diseases of Trees and Shrubs. Comstock Pub. Associates, Ithaca, NY. 660 p.

Skelly, J.M., and F.A. Wood. 1966. The occurrence and etiology of an annual canker of sugar maple in Pennsylvania. Canadian Journal of Botany 44:1401-1411.

Smith, D.M., and S.J. Allen. 1996. Measurement of sap flow in plant stems. Journal of Experimental Botany 47:1833-1844.

Smith, W.B., tech. coord., P.D. Miles, data coord., C.H. Perry, map coord., and S.A. Pugh, Data CD coord. 2009. Forest Resources of the United States, 2007. Gen. Tech. Rep. WO-78. U.S. Department of Agriculture, Forest Service, Washington Office, Washington, DC. 336 p.

Solheim, H., and L. Safranyik. 1997. Pathogenicity to Sitka spruce of *Ceratocystis rufipenni* and *Leptographium abietinum*, blue-stain fungi associated with the spruce beetle. Canadian Journal of Forest Research 27(9):1336-1341.

Solomon, J.D., and J.A. Payne. 1986. A guide to the insect borers, pruners, and girdlers of pecan and hickory. USDA Forest Service Southern Forest Experiment Station, New Orleans, LA. 31 p.

St. George, R.A. 1929. Weather, a factor in outbreaks of the hickory bark beetle. Journal of Economic Entomology 22:573-580.

Starkey, D.A., and H.D. Brown. 1986. Oak decline and mortality in the Southeast - an assessment. P. 103-114 *in* Proceedings of 14th annual hardwood symposium of the Hardwood Research Council, Cashiers, NC. Hardwood Research Council, Cashiers, NC.

Steinman, J. 2004. Forest health monitoring in the northeastern United States: Disturbances and conditions 1993-2002. USDA Forest Service, Newtown Square, PA. 46 p.

Sun, Q., T.L. Rost, and M.A. Matthews. 2008. Wound-induced vascular occlusions in *Vitis vinifera* (Vitaceae): Tyloses in summer and gels in winter. American Journal of Botany 95(12):1498-1505.

Sun, Q., T.L. Rost, M.S. Reid, and M.A. Matthews. 2007. Ethylene and not embolism is required for wound-induced tylose development in stems of grapevines. Plant Physiology 145(4):1629-1636.

Sweets, L. 1986. Iowa's major forest problems in 1986. Iowa State University, Ames, IA.

Takahashi, Y., N. Matsushita, and T. Hogetsu. 2010. Spatial distribution of *Raffaelea quercivora* in xylem of naturally infested and inoculated oak trees. Phytopathology 100(8):747-755.

Teviotdale, B., and D. Harper. 1996. Almond pruning wounds, bark abrasions susceptible to *Ceratocystis*. California Agriculture 50(3):29-33.

Tisserat, N. 1987. Stem canker of black walnut caused by *Fusarium solani* in Kansas. Plant Disease 71(6):557.

Tisserat, N., W. Cranshaw, D. Leatherman, C. Utley, and K. Alexander. 2009. Black walnut mortality in Colorado caused by the walnut twig beetle and thousand cankers disease. doi:10.1094/PHP-2009-0811-01-RS. Plant Health Progress. Available Online at http://www.plantmanagementnetwork.net/php.

Tucker, L., E. Czerwinski, and T. Scarr. 2006. Forest health alert: Hickory bark beetle, *Scolytus quadrispinosus* (Say). Ontario Ministry of Natural Resources, Sault Ste. Marie, ON, Canada. 2 p.

Tyree, M.T., and J.S. Sperry. 1989. Vulnerability of xylem to cavitation and embolism. Annual Review of Plant Physiology and Plant Molecular Biology 40:19-38.

U.S. Department of Agriculture, Animal and Plant Health Inspection Service. 2011. Pathway assessment: *Geosmithia* sp. and *Pityophthorus juglandis* Blackman movement from the western into the eastern United States. USDA Animal and Plant Health Inspection Service, Raleigh, NC. 50 p.

U.S. Department of Agriculture, Forest Service. 1956. Forest insect conditions in 1955 : a status report. USDA Forest Service, Washington, DC. 16 p.

U.S. Department of Agriculture, Forest Service. 1972. Forest insect and disease conditions in the United States in 1971. USDA Forest Service, Washington, DC. 65 p.

U.S. Department of Agriculture, Forest Service. 1980. Forest insect and disease conditions in the United States in 1978. Gen. Tech. Rep. WO-19. USDA Forest Service, Washington, DC. 83 p.

U.S. Department of Agriculture, Forest Service. 1985. Insects of Eastern Forests. USDA Forest Service, Washington, DC. 608 p.

U.S. Department of Agriculture, Forest Service Northern Area State and Private Forestry and Region 8. 1994. Pest alert: Hickory mortality. NA-PR-02-94. USDA Forest Service, Northern Area State and Private Forestry, Radnor, PA. 2 p.

U.S. Department of Agriculture, Forest Service, National Forest Health Monitoring. 2007. FY 2007 Base Evaluation Monitoring Proposals. Available online at http://www.fs.fed.us/foresthealth/fhm/em/funded/07/proposals_base.shtml.

U.S. Department of Agriculture, Forest Service, National Forest Health Monitoring. 2008. FY 2008 Base Evaluation Monitoring Proposals. Available online at http://www.fs.fed.us/foresthealth/fhm/em/funded/08/proposals_base.shtml.

U.S. Department of Agriculture, Forest Service, National Forest Health Monitoring. 2009. FY 2009 Base Evaluation Monitoring Proposals. Available online at http://www.fs.fed.us/foresthealth/fhm/em/funded/09/proposals_base.shtml.

Uecker, F.A. 1988. A World List of *Phomopsis* Names with Notes on Nomenclature, Morphology, and Biology. J. Cramer, Berlin. 231 p.

Van Wyk, M., A.O. Al Adawi, Q.A. Khan, M.L. Deadman, A.A. Al Jahwari, B.D. Wingfield, R. Ploetz, and M.J. Wingfield. 2007. *Ceratocystis manginecans* sp nov., causal agent of a destructive mango wilt disease in Oman and Pakistan. Fungal Diversity 27(1):213-230.

Van Wyk, M., B. Wingfield, M. Marin, and M. Wingfield. 2010. New *Ceratocystis* species infecting coffee, cacao, citrus and native trees in Colombia. Fungal Diversity 40(1):103-117.

Vujanovic, V., A. Cogliastro, M. St-Arnaud, P. Neumann, and D. Gagnon. 1999. First report of *Fusarium solani* canker and wilt symptoms on red oak (*Quercus rubra*) in Quebec, Canada. Plant Disease 83(1):78.

Wallace, H.R. 1978. The diagnosis of plant diseases of complex etiology. Annual Review of Phytopathology 16:379-402.

Walter, J.M., E.G. Rex, and R. Schreiber. 1952. The rate of progress and destructiveness of canker stain of plane trees. Phytopathology 42:236-239.

Weber, B.C., and J.E. McPherson. 1984. The ambrosia fungus of *Xylosandrus germanus* (Coleoptera, Scolytidae). Canadian Entomologist 116(2):281-283.

Weidensaul, T.C., and F.A. Wood. 1973. Sources of species of *Fusarium* in Northern hardwood forests. Phytopathology 63:367-371.

Weiner, G., and W. Liese. 1995. Wound response in the stem of the royal palm. International Association of Wood Anatomists Journal 16:422-433.

Wisconsin Department of Natural Resources. 2005. Wisconsin forest health highlights, December 2005. Wisconsin Department of Natural Resources, Division of Forestry. 10 p.

Wong, W.C., and T.F. Preece. 1978. *Erwinia salicis* in cricket bat willows - phenolic constituents in healthy and diseased wood. Physiological Plant Pathology 12(3):349-357.

Yamada, T. 2001. Defense mechanisms in the sapwood of living trees against microbial infection. Journal of Forest Research 6:127-137.

Yamada, T., Y. Ichihara, and K. Hori. 2003. Defense responses of oak trees against the fungus *Raffaelea quercivora* vectored by the ambrosia beetle *Platypus quercivorus*. P. 132-135 *in* IUFRO "Forest Insect Population Dynamics and Host Influences", Kanazawa, Japan.

Yamaoka, Y., R.H. Swanson, and Y. Hiratsuka. 1990. Inoculation of lodgepole pine with four blue-stain fungi associated with mountain pine-beetle, monitored by a heat pulse velocity (Hpv) instrument. Canadian Journal of Forest Research 20(1):31-36.

Yamato, M., T. Yamada, and K. Suzuki. 2003. Water relations of *Quercus mongolica* var. *grosseserrata* seedlings inoculated with *Raffaelea quercivora*: Ambrosia

fungi related with mass mortality of oaks in Japan. P. 128-131 *in* Proceedings of IUFRO Kanazawa 2003 "Forest Insect Population Dynamics and Host Influences".

Zhang, N., K. O'Donnell, D.A. Sutton, F.A. Nalim, R.C. Summerbell, A.A. Padhye, and D.M. Geiser. 2006. Members of the *Fusarium solani* species complex that cause infections in both humans and plants are common in the environment. Journal of Clinical Microbiology 44(6):2186-2190.