Investigations on *Cryptodiaporthe corni*: a Pathogen of Pagoda Dogwood

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

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

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April 2013

Acknowledgements

There are many people I would like to acknowledge. To Dr. Stan Hokanson, thank you for urging me to pursue higher education. Your guidance and friendship is appreciated more than you know. I have learned more teaching with you than I ever could have hoped for. To Dr. Robert Blanchette, thank you for teaching me about the wonderful world of fungi. I also thank you for always having your door open when questions arose and for not giving me a straight answer, I am better for it. To the other members on my advisory committee, Dr. Jeffrey Gillman and Dr. Jennifer Juzwik, I thank you both for being a part of the planning process, I could not have done it without you. To Benjamin Held, thank you for the constant help in the lab and listening to my tales of woe. A finer lab mate would be difficult to find. To my family, I owe a great debt, you have always had faith in me and for that I am truly grateful. Mom and Dad, you taught me the meaning of hard work and without that I would have quit a long time ago. To Kara Brehm, thank you for helping me become a better person and always listening, I couldn't have done it without you. Also, I would like to thank the Minnesota Department of Natural Resources for allowing me to work in the various state parks. Thank you to all of the people who sent me disease samples, part of my thesis could not have been done without your help.

Abstract

Pagoda dogwood (*Cornus alternifolia* L.) is a small understory tree native to the eastern half of North America. The plant is found both in the managed landscape and growing native in rich woodlands and swampy thickets. A major problem for the tree in both the managed and natural landscape is a devastating canker disease called golden canker or *Cryptodiaporthe* canker of pagoda dogwood, which is caused by the Ascomycete fungus, *Cryptodiaporthe corni* Wehm. The devastating effects of this fungus have caused concern among gardeners and arborists. This thesis reports results on a variety of different aspects of the host/pathogen interaction and include whether *C. corni* is capable of living as an endophyte in pagoda dogwood, more information about the etiology and epidemiology of golden canker, description of an effective artificial inoculation protocol resulting in disease, and the proper taxonomic placement of *C. corni* based on phylogenetic analysis.

Table of Contents

	Page
Acknowledgements	i
Abstract	ii
Table of Contents	iii
List of Tables	iv
List of Figures	vi
Chapter 1. Introduction	
Pagoda Dogwood (*Cornus alternifolia* L.)	1
Golden Canker of Pagoda Dogwood	1
Cryptodiaporthe corni Wehm.	3
Objectives	4
Chapter 2. *Cryptodiaporthe corni* Wehm., a Latent Pathogen in Asymptomatic Stems of Pagoda Dogwood (*Cornus alternifolia* L.)	
Introduction	5
Materials and Methods	8
Results	11
Discussion	13
Tables and Figures	18
Chapter 3. Inoculation of *Cornus alternifolia* L. with *Cryptodiaporthe corni* Wehm. and Etiology of the Disease	
Introduction	24
Materials and Methods	26
Results	32
Discussion	36
Tables and Figures	44
Chapter 4. Optimal Temperature for Growth and Toxin Production of *Cryptodiaporthe corni* Wehm.	
Introduction	51
Materials and Methods	52
Results	56
Discussion	58
Tables and Figures	62
Chapter 5. Genetic Diversity of *Cryptodiaporthe corni* Wehm. and Placement Within the Cryphonectriaceae Family	
Introduction	66
Materials and Methods	68
Results	72
Discussion	74
Tables and Figures	76
Bibliography	85

List of Tables

Page

Table 2.1. Fungicide applications on the large nursery pagoda dogwoods for the 6 months prior to collection of the stems. Stems were later sampled for presence of *C. corni*. 18

Table 3.1. Description and geographical origin of isolates of *C. corni* collected from pagoda dogwood used in this study. 44

Table 3.2. Greenhouse Inoculation Experiment 45
Observations on pagoda dogwood stems 7 months post inoculation for four different treatments. Observations are given as a proportion of stems with a (+) scoring. Inoculated plants were inoculated with *C. corni* growing on malt extract agar, while mock-inoculated plants were inoculated with sterile grain seed. The main stem was severed at approximately 46 cm and inoculum was placed on the wound.

Table 3.3. Greenhouse Inoculation Experiment 46
Effects of 5 different *C. corni* isolates on variables observed on pagoda dogwood stems 3 and 7 months post inoculation (MPI) (wilt treatment and extended drought treatment were combined for analysis). Observations are given as a proportion of stems with a (+) scoring. The main stem was severed at approximately 46 cm and inoculum was placed on the wound.

Table 3.4. Greenhouse Inoculation Experiment 47
Observations on pagoda dogwood stems 7 months post inoculation for four different treatments. Measurements are giving as a mean±SD and observations are given as a proportion of stems with a (+) scoring. Inoculated plants were inoculated with *C. corni* growing on malt extract agar, while mock-inoculated plants were inoculated with sterile grain seed. The main stem was wounded 15 cm above the soil line with a 5 mm cork borer and inoculum was placed on the wound.

Table 3.5. Greenhouse Inoculation Experiment 48
Effects of 5 different *C. corni* isolates on variables observed on pagoda dogwood stems 3 and 7 months post inoculation (MPI) (wilt treatment and extended drought treatment were combined for analysis). Measurements are given as a mean±SD and observations are given as a proportion of stems with a (+) scoring. The main stem was wounded 15 cm above the soil line with a 5 mm cork borer and inoculum was placed on the wound.

Table 3.6. Field Inoculation Experiment 49
Measurements and observations on pagoda dogwood branches 3 months post inoculation (MPI) for branches severed either above or below a node to make a wound. Two isolates of *C. corni* were used as inoculum (MNS1005 and MNS1007) for the inoculated branches, and sterilized grain seeds were used for the mock-inoculated. Measurements are given as a mean percentage and observations are given as a proportion of branches with a (+) scoring.

Table 3.7. Field Inoculation Experiment 50
Measurements and observations on pagoda dogwood branches 3 months post inoculation (MPI) for branches wounded with a 5 mm cork borer. Two isolates of *C. corni* were used as inoculum (MNS1005 and MNS1007) for the inoculated branches, and sterilized grain seeds were used for the mock-inoculated branches. Measurements are given as a mean±SD and observations are given as a proportion of branches with a (+) scoring.

Table 4.1. Proportions of pagoda dogwood stems with wilted leaves (+) after being immersed in four different liquid media treatments. Inoculated media contained fungal exudates of *C. corni*. Observations were taken at 6 different time points. Stems with any leaves wilted were scored as a (+). 65

Table 5.1. Description and geographical origin of isolates of *C. corni* collected from pagoda dogwood used in this study. 76

Table 5.2. Statistical summary for phylogenetic analysis of the four different gene regions. 77

List of Figures

	Page
Figure 2.1. Illustration of stem processing of pagoda dogwood from initial cutting to final plating of stem tissue onto acidified malt extract agar for *C. corni* isolation.	19
Figure 2.2. Appearance of *C. corni* on malt extract agar before (left) and after (right) 3% KOH staining. This reagent provides a diagnostic test for fungi in the Cryphonectriaceae family, which causes orange stromatic tissue to turn purple.	20
Figure 2.3. Illustration showing presence or absence of *C. corni* throughout asymptomatic stems of pagoda dogwood collected in April 2011 (Collection 1) from 5 sites across Minnesota.	21
Figure 2.4. Illustration showing presence or absence of *C. corni* throughout asymptomatic stems of pagoda dogwood collected in July 2011 (Collection 2) from 5 sites across Minnesota.	22
Figure 2.5. Increase in odds ratio (odds of finding presence of *C. corni*) based on an increase in segment diameter (mm) within asymptomatic stems of pagoda dogwood collected from five sites across Minnesota. Dotted lines represent 95% confidence intervals.	23
Figure 4.1. Boxplots of growth of *C. corni* (isolates MNS1005 and MNS1006) at eight different temperature regimes (5 – 40 °C) for 3, 6, 9, and 11 days after being placed on malt extract agar.	62
Figure 4.2. Number of stems of pagoda dogwood with wilted leaves (+) after being placed in one of the four different treatments at 6 given time points after immersion in the liquid media. Inoculated media contained fungal exudates of *C. corni*. Stems containing a single leaf that was wilted were scored as (+).	63
Figure 4.3. Comparison of non-wilted (-) (left) to wilted (+) (right) leaves of pagoda dogwood after 52 hours in non-autoclaved mock-inoculated liquid media (left) and non-autoclaved inoculated liquid media (right). Inoculated media contained fungal exudates of *C. corni*.	64

Figure 5.1. Neighbor-joining tree created from Beta tubulin 1 gene sequences to compare isolates of *Cryptodiaporthe corni*. Confidence levels above 50% based on 1000 bootstrap replications are listed for maximum parsimony (top) and neighbor-joining (bottom). The species for sequences obtained from Genbank are followed by the strain. Two isolates of *Diaporthe ambigua* were used as an outgroup. 78

Figure 5.2. Neighbor-joining tree created from Beta tubulin 2 gene sequences to compare isolates of *Cryptodiaporthe corni*. Confidence levels above 50% based on 1000 bootstrap replications are listed for maximum parsimony (top) and neighbor-joining (bottom). The species for sequences obtained from Genbank are followed by the strain. Two isolates of *Diaporthe ambigua* were used as an outgroup. 79

Figure 5.3. Neighbor-joining tree created from ITS gene sequences to compare isolates of *Cryptodiaporthe corni*. Confidence levels above 50% based on 1000 bootstrap replications are listed for maximum parsimony (top) and neighbor-joining (bottom). The species for sequences obtained from Genbank are followed by the strain. Two isolates of *Diaporthe ambigua* were used as an outgroup. 80

Figure 5.4. Neighbor-joining tree created from Beta tubulin 1 gene sequences to compare isolates in the Cryphonectriaceae family. Confidence levels 70% and above based on 1000 bootstrap replications are listed for maximum parsimony (top) and neighbor-joining (bottom). The species for sequences obtained from Genbank are followed by the strain. Two isolates of *Diaporthe ambigua* were used as an outgroup. 81

Figure 5.5. Neighbor-joining tree created from Beta tubulin 2 gene sequences to compare isolates in the Cryphonectriaceae family. Confidence levels 70% and above based on 1000 bootstrap replications are listed for maximum parsimony (top) and neighbor-joining (bottom). The species for sequences obtained from Genbank are followed by the strain. Two isolates of *Diaporthe ambigua* were used as an outgroup. 82

Figure 5.6. Neighbor-joining tree created from ITS gene sequences to compare isolates in the Cryphonectriaceae family. Confidence levels 70% and above based on 1000 bootstrap replications are listed for maximum parsimony (top) and neighbor-joining (bottom). The species for sequences obtained from Genbank are followed by the strain. Two isolates of *Diaporthe ambigua* were used as an outgroup. 83

Figure 5.7. Neighbor-joining tree created from LSU gene sequences to compare isolates in the Diaporthales. Confidence levels 70% and above based on 1000 bootstrap replications are listed for maximum parsimony (top) and neighbor-joining (bottom). Isolates of *Cryptodiaporthe corni* are highlighted in light gray and other species of *Cryptodiaporthe* are highlighted in dark gray. The genus species for sequences obtained from Genbank are preceded by the accession number. Two isolates of *Gaeumannomyces graminis* were used as an outgroup. 84

Chapter 1. Introduction

Pagoda Dogwood (*Cornus alternifolia* L.)

Pagoda Dogwood (*Cornus alternifolia* L.) is a member of the Cornaceae family. It is native to the eastern half of North America, ranging from Newfoundland to Manitoba in the north, to northern Florida and Arkansas in the south (USDA, 2013). Found most frequently in rich woodlands and swampy thickets, the plant grows as a small understory tree usually ranging in height from 3 to 7.5 meters tall (Cullina, 2002). The pagoda dogwood, with its unique layered horizontal branches, creamy white flowers, which appear from May to June and its showy clusters of dark blue fruit in late summer, is a desirable specimen plant for the landscape (Dirr, 1998).

As residential lots are becoming smaller, due to an increase in human population, a need has developed for shade tolerant, small statured trees. Pagoda dogwoods have become more prevalent in the city landscape in recent years because of their unique architecture, small size, and preference for shady conditions. They are often used as a specimen plant or as a transitional plant between natural and managed landscapes.

Golden Canker of Pagoda Dogwood

The major problem associated with pagoda dogwood in the landscape is the occurrence of a devastating disease in the stems and branches, which is called golden canker or *Cryptodiaporthe* canker of pagoda dogwood, caused by the Ascomycete fungus, *Cryptodiaporthe corni* Wehm. This disease is readily

identifiable by the yellow to orange color of cankered stems as well as bright orange pycnidia bursting through lenticels (Redlin & Rossman, 1991). Stems of all sizes can succumb to the disease, which can devastate the plant. Often starting at the tips of branches, the disease continues to move down the stem until it reaches a branch attachment or node. Commonly the advancing lesion can progress past branch nodes, move into the main stem, and kill the entire plant.

There is little known about the etiology and epidemiology of golden canker of pagoda dogwood. Without an understanding of this basic biology and ecology, it is difficult to make management suggestions. A previous publication has suggested that golden canker is likely a stress related disease (Redlin and Rossman, 1991). There have been general management practices suggested by University extension websites, which include general control measures that are used for other cankers of hardwoods (Hudelson, 2004; Stanosz, 1998; Pataky, 2010; Ball, 2010; Grabowski, 2009), but no published studies have been made on the most appropriate control measures for golden canker. To gain more information, investigations were done on the impact of water stress and pruning on disease development. We also wanted to learn more about the fungus through studies on the effects of temperature on fungal growth and toxin production by the fungus.

Golden canker can be found throughout Minnesota. Due to the abundance of the disease, there have been questions regarding whether or not the fungus can be found in asymptomatic plant tissue. Also, in a previous study (unpublished data), asymptomatic nursery stock kept in the greenhouse developed the disease

months after being placed in the greenhouse. If the fungus is capable of living as an endophyte, a greater emphasis may need to be placed on cultural practices to reduce outbreaks of golden canker. This led us to investigate whether *Cryptodiaporthe corni* is capable of living in pagoda dogwood as an endophyte.

Since the disease is so widespread, some are concerned with using pagoda dogwood in the landscape. There are currently no cultivars marketed which are tolerant/resistant to golden canker. There have been no publications on effective methods of artificial inoculation of *Cornus alternifolia* with *Cryptodiaporthe corni*. Without a reliable, effective method of inoculation, developing resistant or tolerant cultivars to golden canker will be extremely difficult. In order to set up trials to evaluate responses to inoculation with the fungus of different genotypes of *Cornus alternifolia*, an effective protocol for inoculation was needed.

***Cryptodiaporthe corni* Wehm.**

The causal agent of golden canker of pagoda dogwood is the Ascomycete fungus, *Cryptodiaporthe corni*. This pathogen can be found throughout the range of its host and is thought to only infect pagoda dogwood (Redlin and Rossman, 1991). The genus *Cryptodiaporthe* is found in the order Diaporthales, which consists of nine different families (Rossman et al., 2007). A common trait of the Diaporthales is having dark perithecia imbedded in stroma or plant tissue (Barr, 1978).

Cryptodiaporthe is in the Gnomoniaceae family, however, placement of *Cryptodiaporthe corni* within the Gnomoniaceae family has been previously

questioned. After performing phylogenetic analysis, *C. corni* was found to be more appropriately placed in the Cryphonectriaceae family (Castlebury et al., 2002; Gryzenhout et al., 2006). The fungus also shares the uniting trait of the Cryphonectriaceae family, in which the orange stromatic tissue turns purple in the presence of 3% KOH (Redlin and Rossman, 1991). Although the fungus was placed within the Cryphonectriaceae family, phylogenetically it did not group within any of the other genera in the family. Both Gryzenhout et al. (2006) and Castlebury et al. (2002) believed that *C. corni* should be in a separate, yet to be defined genus, but believed more isolates for phylogenetic analysis and further morphological observations were needed.

Objectives

In order to gain a better understanding of *Cryptodiaporthe corni* and its interaction with *Cornus alternifolia*, we developed experiments to address the following four objectives:

1. Determine if *Cryptodiaporthe corni* is capable of living as an endophyte in *Cornus alternifolia*.
2. Learn more about the etiology and epidemiology of golden canker.
3. Develop an effective artificial inoculation protocol for pagoda dogwood that results in golden canker development.
4. Determine the proper taxonomic placement of *Cryptodiaporthe corni* within the Cryphonectriaceae family based on phylogenetic analysis.

Chapter 2. *Cryptodiaporthe corni* Wehm., a Latent Pathogen in Asymptomatic Stems of Pagoda Dogwood (*Cornus alternifolia* L.)

Introduction

Pagoda dogwood, *Cornus alternifolia* L, is a plant native to the Eastern half of North America (USDA, 2013). Found most frequently in rich woodlands and swampy thickets, the plant grows as a small understory tree. This plant is considered a small tree or large shrub ranging in height from 3 to 7.5 meters tall (Cullina, 2002). One special attribute of the pagoda dogwood is its unique architecture. It has layered tiers of branches, which resemble a pagoda, giving rise to its name. An increasing amount of shaded areas in the urban landscape due to increases in building height, building density, and maturation of boulevard trees has created a need for shade tolerant plants. Also, in many cities, lots are becoming smaller and smaller, limiting the use of large trees. Pagoda dogwoods have found increased use in the urban setting due to their preference for shady conditions, small stature, and their unique architecture.

One limitation to the use of pagoda dogwood in the landscape is the occurrence of a devastating disease in the stems and branches called golden canker or *Cryptodiaporthe* canker of pagoda dogwood, which is caused by the Ascomycete fungus, *Cryptodiaporthe corni* Wehm. The cankered wood appears light orange and pycnidia appear as small bright orange raised bumps on the surface of the dead bark making the disease easily recognizable (Redlin & Rossman, 1991). Cankers can devastate the plant by girdling stems of all sizes. The disease typically continues to move down the stem until it reaches a branch

attachment or node; however, it can progress past branch nodes, move into the main stem and kill the entire plant. The stumps of the plant often persist for years, locked in a cycle of regrowth and dieback.

Infections have been found in 21 states where the pagoda dogwood is native (Redlin & Rossman, 1991). States where the disease has not been described are all located in the South and South Central United States, and include South Carolina, Georgia, Alabama, Mississippi, Florida, Arkansas, and Missouri. A potential reason for the lack of description of the disease in these states is that the native populations are more isolated than in the northern states (USGS, 2012).

Pagoda dogwood is now planted extensively in many states where it is not native and the disease has been found in many of these locations. The incidence of golden canker on pagoda dogwood in ornamental plantings outside of the plant's native range raises questions of how the disease disperses over great distances. The disease is prevalent and isolates have been collected from eastern South Dakota, nearly 200 miles from native stands of pagoda dogwoods located in central Minnesota and Iowa. Also, landscape plantings in Fargo, North Dakota, which is outside the native range of pagoda dogwood, have been found to be infected with *C. corni* (Redlin & Stack, 2001). Spores from the fungus may be dispersed by wind over these large distances, but a more likely explanation is that this pathogen is present on or in plant material as it is transported out of the nursery as small cankers with pycnidia and spores, as spores on the surface of the bark, or as an endophyte.

In addition to the disease being found well outside the native range of pagoda dogwood, other factors led us to believe that the fungus may be an endophyte. A common association between the fungus and the plant led Sinclair and Lyon (2005) to hypothesize that *C. corni* was likely an endophyte of pagoda dogwood. We have observed asymptomatic nursery stock develop the disease months after being placed in the greenhouse (unpublished data).

There are a wide variety of definitions that exist for the term endophyte (Hyde & Soytong, 2008). Endophytes are often considered fungi living in plants that do not cause symptoms. However, whether any microorganism living asymptomatically for a time in a plant before becoming pathogenic is an endophyte is in question (Mostert et al., 2000, Reviewed by Hyde & Soytong, 2008; Wilson, 1995; Wennstrom, 1994). For this thesis, the definition used is: "All organisms inhabiting plant organs that at some time in their life, can colonize internal plant tissues without causing apparent harm to the host" (Petrini, 1991). An important aspect of this thesis is to determine the endophytic status of this fungus in pagoda dogwood.

Arborists and the general public are concerned about using pagoda dogwood in the landscape due to its susceptibility to golden canker. Currently, there are no cultivars available that are known to be tolerant/resistant to golden canker. This disease is extremely widespread in Minnesota and the fungus can be found on dead material as well as on live stems. General management practices suggested by University extension websites, include control measures that are used for other cankers of hardwoods, but no published studies have been made on

the most appropriate control measures for golden canker (Hudelson, 2004; Stanosz, 1998; Pataky, 2010; Ball, 2010; Grabowski, 2009).

There were multiple objectives for this study including: 1) Obtain more information on the etiology of the disease, 2) Determine if the fungus exists as an endophyte in pagoda dogwood, 3) Determine whether there was a difference in the presence of the fungus between natural and managed plants in Minnesota, and 4) Determine whether the fungus was present in both mature (large) and young (small) nursery material.

Materials and Methods

Plant Materials

Established Field Material

Plant samples were collected from 5 sites across Minnesota. Three sites contained natural stands of *Cornus alternifolia*: Itasca State Park (+47° 11' 48.36", -95° 10' 15.56"), Great River Bluffs State Park (+43° 56' 16.48", -91° 24' 56.64"), and William O' Brien State Park (+45° 13' 25.43", -92° 45' 37.85"). The other two sites were managed landscapes: University of Minnesota, St. Paul Campus (+44° 59' 13.78", -93° 11' 1.81") and University of Minnesota Landscape Arboretum (+44° 51' 37.99", -93° 37' 5.30"). At all sites trees were categorized as known to be planted or naturally occurring. Stems were collected at two different times for each collection site, the first collection occurred in April 2011 and the second occurred in July 2011. At all sites for each collection, four plants with at least one stem showing no symptoms of the disease were randomly selected. The asymptomatic stem from each plant was cut and in the event that a plant had more

than one asymptomatic stem, one was randomly selected. Due to the abundance of the disease, plants with both symptomatic and asymptomatic stems had to be used. Only 3 of the 40 trees sampled had no signs or symptoms of the disease on any of the multiple stems.

Large Nursery Material

In October 2011, 10 pagoda dogwoods ranging in height from 1.1 to 1.8 m were cut at ground level from a production nursery field. These plants had been repeatedly treated with fungicides during the six months prior to being collected (Table 2.1). Four of the ten plants had small broken branches, which were necrotic. None of the trees showed signs or symptoms of golden canker.

Small Nursery Material

Small pagoda dogwood liners, ranging in size from 30 – 46 cm, were purchased from a commercial nursery. Separate plants were sampled for the fungus both before potting and 6 months after potting. Two weeks after arrival, six 30 cm tall and two 46 cm tall bareroot plants were randomly selected from bundles of bareroot plants and sampled for the fungus. The remaining plants were grown in size 3 containers in Sun Gro's Metro-Mix 950 (Sun Gro, Agawam, MA) growing media. Plants were grown in a greenhouse at the University of Minnesota, St. Paul. Six months after the bareroot stock was planted, six new 46 cm tall plants were randomly selected for sampling for the fungus.

Sample Processing, Sterilization, and Culturing

For both the outdoor and container grown plants, stems were cut off at the ground and all leaves and branches were removed from the main stem (Figure

2.1). Stems were taken back to the lab and stored in a cold room at approximately 5 °C. Within 24 hours, the plant was measured from the base of the stem to the apical meristem. The bottom 8 cm of the stem was discarded and the remaining stem was cut into 15 cm long segments. The first segment was saved and labeled as segment 1, the next 15 cm segment was discarded, the third was labeled segment 2 etc. until reaching the apical meristem. If the last segment was less than 15 cm it was discarded. For the small nursery material, stems were cut into 20 cm segments instead of 15 cm segments. Stem segments were stored in individual plastic bags at approximately 5 °C for no more than 72 hours.

Each stem segment was individually surface sterilized. Segments went through a sequence of three sterilization treatments; 70% EtOH for 1 minute, 20% bleach solution (5.25% available chlorine) for 3 minutes, and 70% EtOH for 30 seconds. The stem segments were then allowed to air dry in a laminar flow hood.

After drying, the stem segments were cut through the bark around the circumference down to the xylem at 5 and 10 cm for the 15 cm segments and 5 and 15 cm for the 20 cm segments with a scalpel blade to make a ring approximately 3 mm wide. Each ring was removed and cut into five smaller pieces and plated onto a 100 x 15 mm Petri dish containing acidified malt extract agar (2 mL 85% lactic acid, 15 g malt extract, 15 g agar, and 1 L deionized water) (Fig. 2.1).

Samples were monitored for fungal growth for one month. If a Petri dish contained a fungus, which had mycelium resembling *Cryptodiaporthe corni*, a small portion of the mycelium was subcultured and plated onto a 100 x 15 mm

Petri dish with media consisting of malt extract agar (15 g malt extract, 15 g agar, and 1 L of deionized water). Once the fungus was isolated in pure culture it was identified by morphological characteristics, such as orange stromatic tissue and lack of aerial mycelium, and by the use of KOH that specifically stains stromatic tissue of fungi in the Cryphonectriaceae family (Gryzenhout et al., 2006). Orange stromatic tissue of *Cryptodiaporthe corni* turns purple in the presence of 3% KOH solution (Redlin & Rossman, 1991) (Fig 2.2).

Analysis

A chi-squared test was used to analyze the difference between the presence of fungi on the top half of the plant versus the bottom half of the plant. Analysis using a Fisher's exact test was used to assess 2 x 2 contingency tables for categorical data. A linear mixed model (by REML) was used to examine relationships between stem diameter and the increased probability of finding the fungus.

Results

Established Field Material

Plants at the sites were categorized as either native (naturally occurring) or landscaped (planted). A higher percentage of the plants that were native (68%) were found to have *C. corni* compared with the landscape plants (44.5%), but the difference was not found to be statistically significant (p=0.26) (Fig. 2.3 and 2.4). The height of the plants in the study ranged from 0.46 m to 2.79 m. Although, it was not found to be statistically significant, there was a difference found between the proportions of asymptomatic stems with *C. corni* present when stems of

different heights were compared. When stems were shorter than 68 cm, *C. corni* was only isolated in 40% of the stems, while in stems taller than 68 cm it was isolated in 70% of the stems ($p=0.13$). *C. corni* was isolated from asymptomatic stems from both collection dates in Minnesota. The mean number of plants with *C. corni* was almost the same between collection 1 (April 2011) and collection 2 (July 2011). Collection 1 had a slightly higher average at 65% compared with 60% for collection 2 ($p=1.0$). There was considerable variation in the number of plants with the fungus present at the different sites, but when collection 1 and collection 2 were combined the difference between locations was not found to be statistically significant ($p=0.13$) (Fig. 2.3 and 2.4). In collection 1 from the University of Minnesota it was found that none of the plants contained *C. corni*, while all of the plants from Itasca and the Great River Bluffs State Park examined in collection 1 contained *C. corni* ($p<0.05$) (Fig. 2.3). In collection 2 all of the locations had at least one plant with the fungus present and at least one plant without the fungus present ($p>0.05$) (Fig. 2.4). When collections 1 and 2 for all the sites were combined, 62.5 % of the stems examined had *C. corni* present. The percentage of segments within a plant with the fungus present varied widely from 0 to 100% with a mean of 33%.

When looking at individual stems within each location a pattern was found where a majority of the plants had a higher number of segments from the bottom half of the stem with the fungus present as compared with the top. For stems where the fungus was found to be present, it was found that 52.5% of the plants had more *C. corni* found on the top half of the plant and only 5% had more *C.*

corni found on the bottom half of the plant ($p<0.001$). The remaining plants with the fungus present had equal amounts of infection in the bottom and top halves.

Diameters for the stems ranged from 2.7 mm to 21.0 mm. Statistical analysis using a linear mixed model (by REML) was used to determine the potential relationship between stem diameter and presence or absence of the fungus. It was found that within a tree, odds for the presence of the fungus increases 1.15 times for each unit increase (mm) in stem diameter (Fig. 2.5.).

Large and Small Nursery Material

C. corni was obtained from 2 (20%) of the trees from the large asymptomatic nursery plants, while none of the isolation attempts in the small, asymptomatic nursery plants yielded *C. corni* ($p=0.16$). Of the 86 isolation attempts on the large asymptomatic tissue, only 3 had the fungus of interest. In the two plants where the fungus was found, the fungus was located in the lowest and second lowest 15 cm segment for one plant and in the lowest 15 cm segment for the other. One of the plants that the fungus was isolated from had necrotic regions at old branch stubs where branches had been removed, but did not show signs of the disease, while the other plant did not have any necrotic regions.

Discussion

Cryptodiaporthe corni was found in a large number of asymptomatic stems throughout the state of Minnesota. It was found not only in plants from native stands, but also those in the managed landscape. This is the first report of *C. corni* being an endophyte in *Cornus alternifolia*. Unfortunately, due to the scope of this experiment, we were unable to show that the endophytic form of the

fungus is the cause of the golden canker in natural settings. However, in a greenhouse experiment, isolates collected from this study did cause the disease on cut stems (Chapter 3). So it appears under appropriate conditions this fungus, isolated while living endophytically, is capable of causing golden canker.

C. corni was more commonly found in large asymptomatic stems compared with smaller asymptomatic stems (Fig. 2.3 and 2.4). However, field observations (unpublished data) indicate small plants can be attacked and it appears that juvenile resistance is likely not the explanation for the smaller percentage of small plants with *C. corni*. A study by Flowers et al. (2001), of *Diplodia pinea* (Desm.) J.J. Kickx (syn. *Sphaeropsis sapinea*) in pines found this fungus was also a latent pathogen and was more commonly found in older pines compared with younger plant material. One reason for this may be the longer time of exposure to inoculum the tree experiences, as they get larger.

The fungus was more common in the bottom half of asymptomatic stems compared with the top half, 52.5% vs. 5% respectively. A study by Johnson and Whitney (1989) of balsam fir also found that endophytes were more frequently isolated from lower parts of the plant compared with the top. There are a few potential explanations for this finding. New growth put on during the growing season has been exposed to the fungus for a shorter period of time than the more mature part of the stem at the base. Also, spores disseminated from symptomatic upper branches are likely brought downward due to gravity or in raindrops and can become lodged in the thicker bark near the base of the plant.

C. corni was present in asymptomatic stems of landscape plants at a relatively high incidence (44.5%) and all 9 of the landscape plants studied had golden canker present on at least one of the other multiple stems. Since only a small portion of the nursery material studied had the fungus present in asymptomatic tissue, 20% of large plants and 0% of small plants, it appears that uninfected plant material is infected from spores after it has been placed in the landscape either from infected plants growing in the area or from spores already on the nursery plant. However, if a less stringent fungicide regimen had been used on the large nursery material, the percentage of plants infected with the fungus may have been higher. The fact that the disease has been detected on pagoda dogwood trees in remote locations, hundreds of miles from native stands of pagoda dogwood suggests that although the fungus was found at low rates on asymptomatic nursery material, infected nursery stock could account for the presence of the disease in these locations. Alternatively, long distance dispersal of spores could be the cause.

Due to the increased use of pagoda dogwood in managed landscapes, and the relatively high rate of *C. corni* living endophytically in asymptomatic stems, further studies are needed to determine what is causing the fungus to switch from an endophytic to a parasitic phase. Previous publications have suggested that the development of golden canker is incited by stress (Redlin and Rossman, 1991; Steiner, 2011, Grabowski, 2009, Pataky, 2010). For other pathosystems where endophytes considered to be latent pathogens have become pathogenic, the main factor suggested to be responsible is drought stress (Bachi and Peterson, 1985;

Blodgett et al., 1997; Appel and Stipes, 1984; Bagga and Smalley, 1974a; Swart et al., 1987). Plant moisture stress caused by drought can also be a factor in allowing an endophyte to more quickly colonize a tree (Capretti and Battisti, 2007; Chapela and Boddy, 1988). Another factor that can cause an increase in the development or occurrence of disease is wounding. One of the common contributing factors to the outbreaks of the disease caused by the latent pathogen, *Diplodia pinea* (syn. *Sphaeropsis sapinea*), is hail damage (Kotze, 1935; Laughton, 1937; Luckhoff, 1964, Reviewed by Wingfield and Knox-Davis, 1980). Determining exactly what factors play a role in causing *C. corni* to switch to a parasitic phase will help to develop recommendations for controlling and minimizing this disease.

From this study, there are some recommendations for management and control of golden canker of pagoda dogwood. Although a plant showing signs or symptoms of the disease is likely to have the fungus in asymptomatic tissue, branches showing signs or symptoms of the disease should be removed to help prevent the spread to potentially uninfected plants nearby by reducing inoculation sources. Before recommendations for nursery purchases can be made, studies involving more nurseries with larger sample sizes should be conducted. However, based on information gathered from the study, where none of the small plants had the fungus present, there may be merit in recommending purchases of smaller nursery plants for areas outside of the pagoda dogwood's native range. Since few of the nursery plants that were regularly treated with fungicide had *C. corni* present, future research opportunities exist in investigating whether

preventative application of fungicides or surface sterilization of the plant material reduces colonization by *C. corni* in asymptomatic nursery material.

Table 2.1. Fungicide applications on the large nursery pagoda dogwoods for the 6 months prior to collection of the stems. Stems were later sampled for presence of *C. corni*.

Date of Application	Product Name	EPA Regulation #	Active Ingredient
3/30/2011	Compass® (OHP, Inc., Mainland, PA)	432-1371-59807	Trifloxystrobin
4/14/2011	Terraguard® SC (OHP, Inc., Mainland, PA)	400-521	Triflumizole
4/25/2011	Spectro (Cleary Chemicals LLC, Dayton, NJ)	1001-72	Chlorothalonil & Thiophanate-methyl
5/12/2011	Compass® (OHP, Inc., Mainland, PA)	432-1371-59807	Trifloxystrobin
6/1/2011	Terraguard® SC (OHP, Inc., Mainland, PA)	400-521	Triflumizole
6/22/2011	Spectro (Cleary Chemicals LLC, Dayton, NJ)	1001-72	Chlorothalonil & Thiophanate-methyl
7/20/2011	Compass® (OHP, Inc., Mainland, PA)	432-1371-59807	Trifloxystrobin
8/11/2011	Terraguard® SC (OHP, Inc., Mainland, PA)	400-521	Triflumizole
8/29/2011	Mainsail™ (Prokoz, Inc., Alpharetta, GA)	72112-5	Chlorothalonil

Figure 2.1. Illustration of stem processing of pagoda dogwood from initial cutting to final plating of stem tissue onto acidified malt extract agar for *C. corni* isolation.

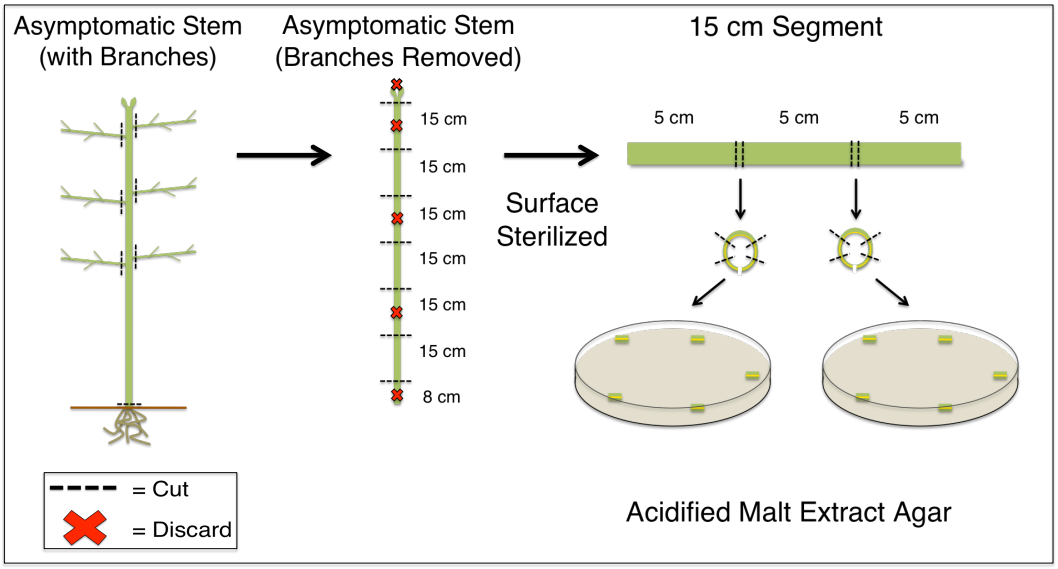


Figure 2.2. Appearance of *C. corni* on malt extract agar before (left) and after (right) 3% KOH staining. This reagent provides a diagnostic test for fungi in the Cryphonectriaceae family, which causes orange stromatic tissue to turn purple.

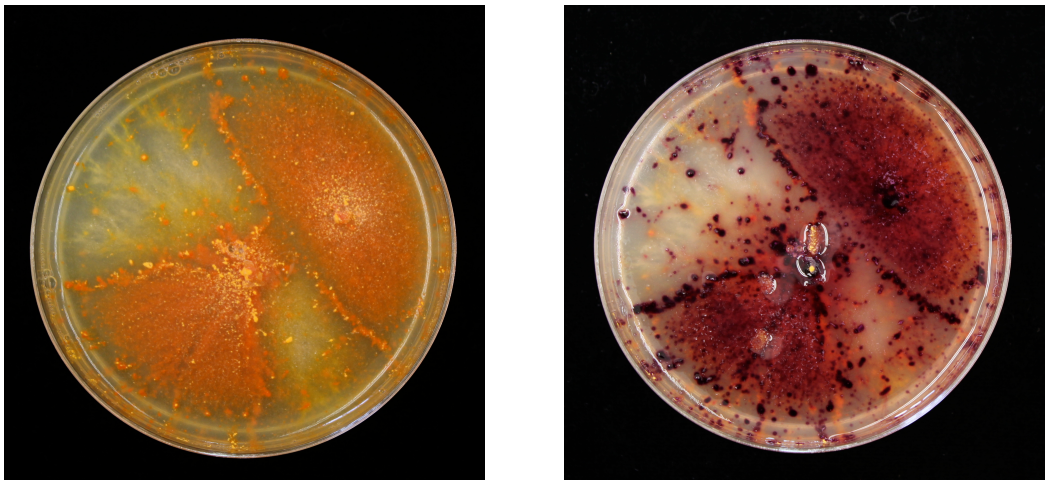
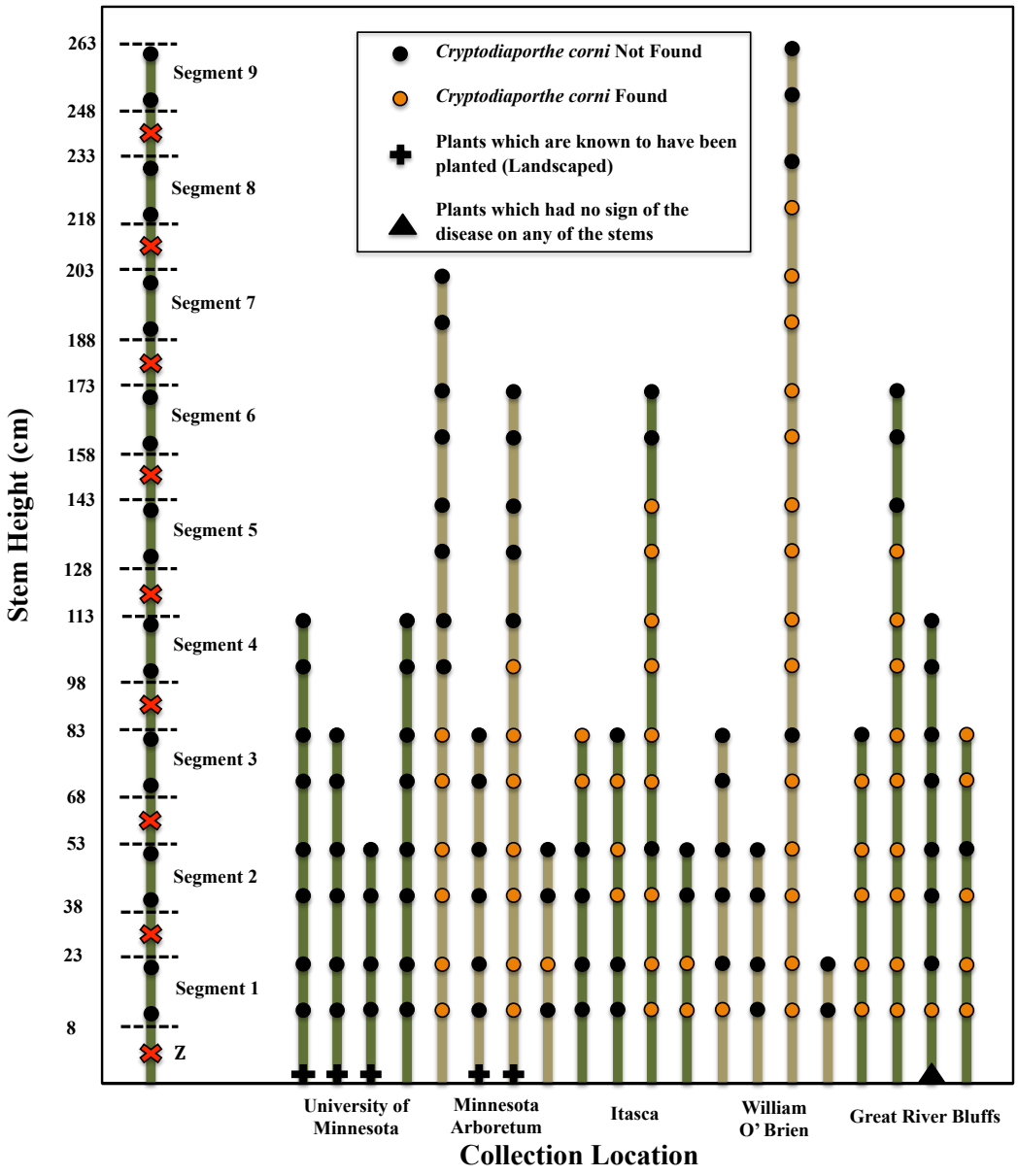
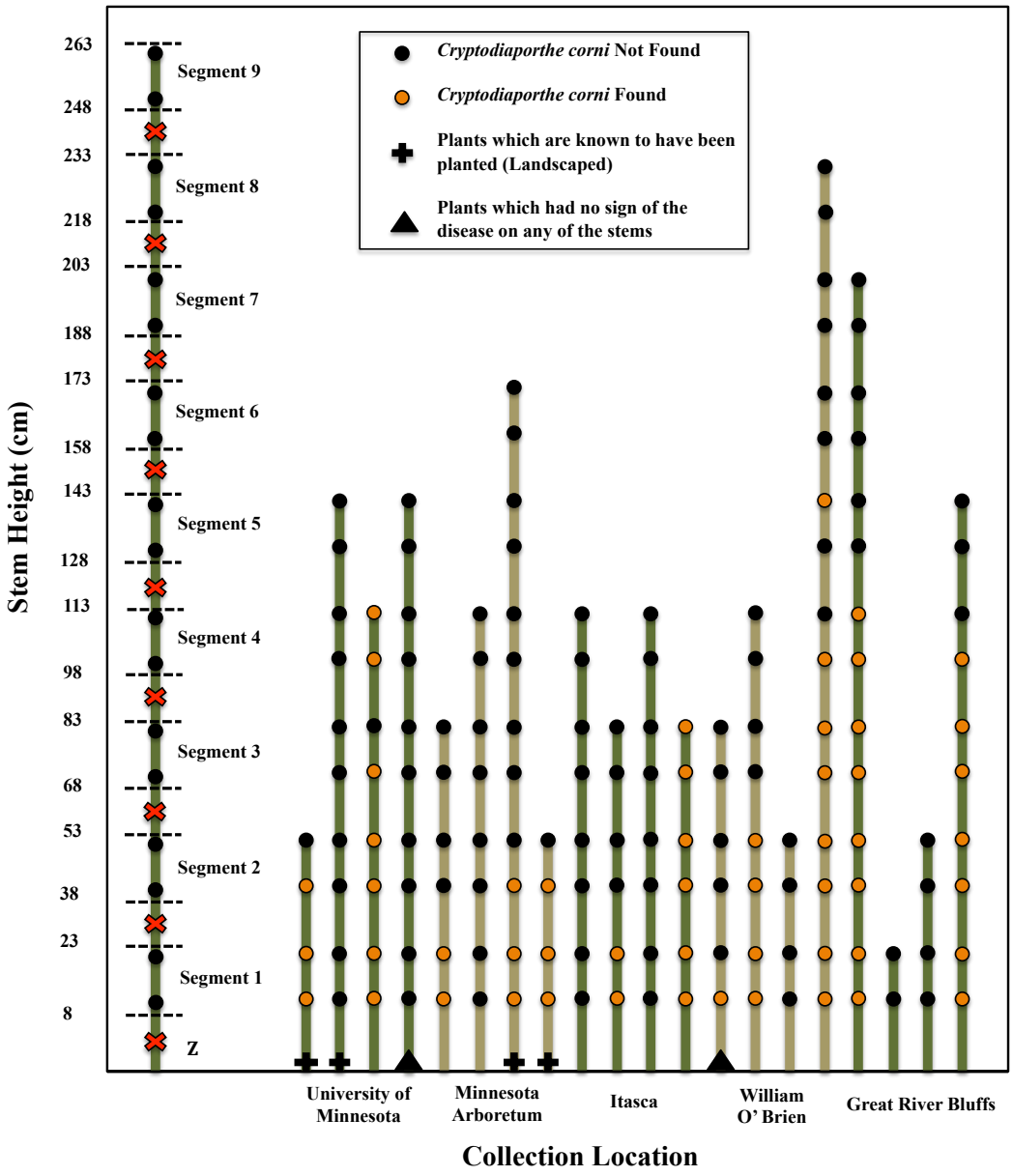


Figure 2.3. Illustration showing presence or absence of *C. corni* throughout asymptomatic stems of pagoda dogwood collected in April 2011 (Collection 1) from 5 sites across Minnesota.



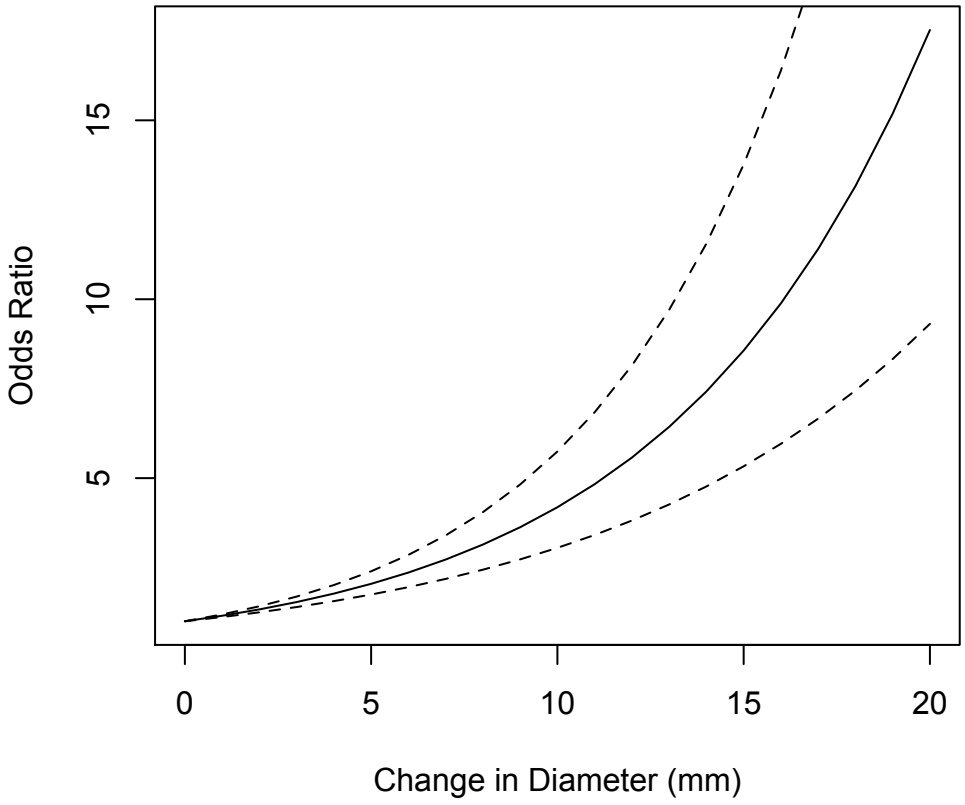
[Z] Segments with a red X were discarded.

Figure 2.4. Illustration showing presence or absence of *C. corni* throughout asymptomatic stems of pagoda dogwood collected in July 2011 (Collection 2) from 5 sites across Minnesota.



[Z] Segments with a red X were discarded.

Figure 2.5. Increase in odds ratio (odds of finding presence of *C. corni*) based on an increase in segment diameter (mm) within asymptomatic stems of pagoda dogwood collected from five sites across Minnesota. Dotted lines represent 95% confidence intervals.



Chapter 3. Inoculation of *Cornus alternifolia* L. with *Cryptodiaporthe corni* Wehm. and Etiology of the Disease

Introduction

Currently, there are no genotypes of pagoda dogwood that are known to be resistant to golden canker. Since serious losses occur throughout North America from this disease it would be advantageous for the horticultural industry to have resistant cultivars available. In order to evaluate and screen plants, an effective inoculation protocol for the disease must be established.

Inoculations for woody canker diseases in the Cryphonectriaceae family are most commonly done by wounding the main stem of the host with a cork borer and placing the fungus, which is growing on grain or some other substrate, on the wound (Hoegger et al., 2002; Seixas et al., 2004; Chen et al., 2011; Begoude et al., 2010; Gryzenhout et al., 2010; Vermeulen, et al., 2011). This method, as well as cutting off the apical portion of stems and applying inoculum to the cut stem, was evaluated as potential inoculation methodologies.

Disease management is an important part of preventing and/or controlling disease spread. There have been numerous extension publications discussing golden canker of pagoda dogwood. Some of the recommendations for managing the disease include pruning out infected material and keeping the plant stress free. All of the recommendations for controlling the disease appear to be based on general recommendations for controlling other hardwood canker diseases (Hudelson, 2004; Stanosz, 1998; Pataky, 2010; Ball, 2010; Grabowski, 2009). For canker diseases, it is recommended that infected branches are

removed by cutting at least 10 cm below the advancing front of the lesion (Wegulo and Gleason, 2001). There have been no specific studies on disease management of golden canker so we wanted to investigate the importance of proper pruning to help minimize the spread of the disease.

There are a variety of environmental factors that can have impacts on disease development such as water stress, freezing stress, defoliation stress, and physical damage. One of the most frequently discussed is the influence of drought stress on disease development (Desprez-Loustau et al., 2006; Schoeneweiss, 1981; Schoeneweiss, 1975). An extension article from the University of Illinois indicated canker diseases are more commonly found on drought stressed pagoda dogwoods, though they did not cite specific evidence (Pataky, 2010). However, there have been studies on two species of fungi in the Cryphonectriaceae family, *Endothia gyrosa* (Schwein.:Fr) and *Chrysoporthe cubensis* (Bruner) Gryzenhout & M.J. Wingf., in which drought stress did not result in significant increases in lesion length of inoculated Eucalyptus (Swart et al., 1992; Old et al., 1990). In contrast, an earlier study of *Endothia gyrosa* on pin oak, did result in increased lesion lengths due to drought stress (Appel and Stipes, 1984). Thus, there is conflicting evidence regarding the influence of drought in disease development caused by fungi in the Cryphonectriaceae family and no specific evidence for influence of drought on golden canker.

The objectives for this study were to 1) Identify an effective method to artificially inoculate pagoda dogwood to promote development of golden canker, 2) Develop information regarding the influence of drought on host susceptibility

and disease progression, and 3) Evaluate the effect of pruning methods on disease development.

Materials and Methods

Greenhouse Inoculation Experiment

Plant Material

One hundred and twenty 30 cm to 45 cm tall bareroot pagoda dogwood liners were grown in size 3 containers using Sun Gro's Metro-Mix 950 (Sun Gro, Agawam, MA, USA) growing media. Plants were grown for 9 months in a greenhouse at the University of Minnesota, St. Paul. The average temperature of the greenhouse was 18 °C with a 14-hour photoperiod. The plants were subsequently moved to a cooler maintained at a constant temperature of 4.5 °C with no light for a dormancy period of 4 months.

Inoculum

Five different isolates and a mock-inoculated control treatment were used in this study (Table 3.1). Two of the isolates were collected from asymptomatic pagoda dogwood stems (Chapter 2). Three of the isolates were collected from pagoda dogwood stems exhibiting signs of golden canker. Grain seed inoculum was prepared by measuring forty-three grams of wheat seed and placing it in a 100 mL beaker. The container was filled with deionized water and allowed to soak overnight. Excess water was poured off so that the water was just covering the seed. Growth media, 1.25 g of malt extract and 0.125 g of yeast extract, was added to the seed and mixed. The container was covered and autoclaved for 90 minutes. After allowing the container to cool, eight small pieces (approximately

0.5 x 0.5 cm) of the fungus grown on malt extract agar (15 g malt extract, 15 g agar, and 1 L deionized water) were added to the grain seed/malt/yeast mix in a laminar flow hood. The mixture was then stirred and the container was sealed with Parafilm. The fungus was allowed to grow at room temperature under natural day length on the grain media for 18 days before being used for inoculation. Controls were made using the same methods; except plugs of uncolonized malt extract media were added to the grain.

Treatments

There were two different methods of inoculation tested in the experiment. One method consisted of inoculating the main stem. Inoculations were made 15 cm above the soil line on the main stem. The area to be inoculated was wiped with a paper towel soaked in 95% EtOH in order to help eliminate surface contaminants. A #2 five-mm cork borer (flame sterilized) was used to make a circular cut down to the xylem. A sterile scalpel blade was used to remove the resulting circle of bark, leaving the xylem intact. Immediately after wounding, three pieces of grain with or without *C. corni* depending on the treatment was placed on the exposed xylem. A piece of Parafilm was wrapped around the seed and wound to hold it in place, to prevent desiccation, and to prevent possible contamination from other microbes. All tools were surface sterilized between uses by dipping in 95% EtOH and flaming.

The second method consisted of cutting the tip off the main stem approximately 46 cm above the soil line. Before cutting, the area to be inoculated was wiped with a paper towel soaked in 95% EtOH in order to help eliminate

surface contaminants. A hand pruner was used to cut just below the node that was closest to 46 cm resulting in a 'stub' of internodal stem. Three pieces of grain with or without *C. corni* were placed onto the end of the cut stem and held in place with Parafilm. Each of the two methods described was replicated 10 times for each of the 5 different isolate sources and the 1 mock-inoculated control resulting in a total of 120 treated plants. The experiment was only performed once.

After inoculation, the plants were placed into a cooler maintained at 10 °C with no light. After one month the plants were taken out of the cooler and placed in the greenhouse. The average temperature for the greenhouse over the course of the experiment was 23 °C, with a maximum of 38 °C and a minimum of 9 °C. Only natural light was used in the greenhouse, so day lengths varied. Until the different watering treatments began, the plants were watered when a majority of the plants showed slight wilting symptoms in their leaves. Three months after inoculation, half of the plants from each treatment were placed into a group that was watered after a majority of the plants showed wilt symptoms in any of their leaves (Wilt Treatment). The remaining half of the plants was subjected to a longer drought stress (Extended Drought Treatment). For trees in this treatment, water was withheld for 3 additional days longer than the Wilt Treatment. There was not a well-watered treatment.

Measurements

Three months post inoculation (MPI), data was collected for lesion length (measured vertically and included the wound if it had not callused), lesion minus

the wound length (measured vertically after the wound (5 mm) had been subtracted), presence of callus, amount of callus, and whether pycnidia were visibly present for plants wounded with the cork borer. For the cut stem wounded ('stubbed') plants measurements and observations were taken for percent necrosis (percentage of internode necrosis measured vertically from the wound to the first node down from the wound), whether the area between the wound and the node was fully necrotic or partially necrotic, whether pycnidia were visibly present, and whether the lesion progressed beyond the node immediately below the wound.

A second set of observations and measurements (as described above) were made 7 MPI (4 months after being placed in the wilt or extended drought treatments) for both wounding methods. In addition, 4 plants wounded with the cork borer from the wilt and extended drought treatments were randomly selected for each isolate source and the mock-inoculated (24 plants total were selected for both the wilt and extended drought treatments) in order to determine if the fungus was present. A 15 cm segment centered on the wound was cut and those segments were later sterilized with 70% EtOH for 1 minutes, 20% bleach (5.25% available Cl) solution for 3 minutes, and 70% EtOH for 0.5 minutes. The segments were allowed to air dry in a laminar flow hood. A 3 mm wide ring of bark down to the xylem was made at the wound, and 1.25 cm above and below the lesion/wound. Each ring was cut into five smaller segments and plated onto a 100 x 15 mm Petri dish containing an acidified malt extract agar media (2 mL 85% lactic acid, 15 g malt extract, 15 g yeast extract, and 1 L of deionized water). Petri dishes were monitored for fungal growth for one month. Specimens of

Cryptodiaporthe corni were identified using morphological characteristics, such as the lack of aerial mycelium and light white color, and were confirmed by observing their reaction to a 3% KOH solution. Members of the Cryphonectriaceae family have orange stromatic tissue that stains purple in the presence of KOH (Gryzenhout et al., 2006).

All of the cut stem wound 'stubbed' treatment plants were sampled to determine if the fungus was present. Cuts were made 15 cm below the initial cut and the segments were surface sterilized as described above. Bark rings (as described above) were made 1.25 cm above and below the advancing front of the lesion. If the lesion was less than 2.5 cm, the tissue sample from above the advancing front was collected from the middle of the lesion. Also, if no lesion was present samples were taken 1.25 cm from the initial cut and 1.25 cm below the next node down from the wound. Bark ring samples were plated and monitored as described above for the cork borer wounded plants.

Field Inoculation Experiment

Materials

Twenty pagoda dogwood plants with at least six asymptomatic branches were randomly selected at the Great River Bluffs State Park in Minnesota. The branches selected were between 1.5 and 2.5 meters from the ground.

Inoculum

C. corni cultures used for field inoculations were isolated from two different plants exhibiting cankers; one from the Great River Bluffs State Park in Minnesota (MNS1005) and the other from the University of Minnesota, St. Paul

campus (MNS1007) (Table 3.1). Grain seed inoculum was prepared as described above.

Inoculation

All trees were inoculated with the control grain (mock-inoculated treatment) and either the MNS1005 or the MNS1007 inoculated grain (inoculated treatment). The twenty trees were numbered and odd numbered trees were inoculated with MNS1005 and the even numbered trees were inoculated with MNS1007. Three types of wounding were created for each tree for both the inoculated and mock-inoculated treatments (6 wounds total per tree). Six branches in an asymptomatic area of the plant were randomly selected for treatments. Two of the branches were selected for each wound treatment with one being treated with inoculated seeds (inoculated) and the other with control seeds (mock-inoculated). Wounding treatment one consisted of cutting the branch off directly below a node (within 1 cm) creating a 'stub' of internodal stem. Wounding treatment two consisted of cutting the branch off directly above a node (within 1 cm). Wounding treatment three consisted of boring a hole in the branch through the bark down to the xylem in the internode using a #2 five mm cork borer. Branches and tools were surface sterilized before wounding using a 70% EtOH solution. Three of the inoculated grain seeds or sterilized grain seeds were placed on each of the wounds. The wound was wrapped with Parafilm to retain moisture and reduce the likelihood of contamination.

Data Collection

Branches were collected 3 MPI and data was collected the following day. Lesion length and branch diameter at the site of the wound were taken using a digital caliper. The number of nodes the lesion had grown past was determined. Necrosis and visible presence of pycnidia were also noted. Pycnidia were confirmed to be *C. corni* by using the KOH staining method previously described.

Analysis

All categorical data were analyzed using a 2 x 2 contingency table. Graph Pad Software (Graph Pad Software, Inc., La Jolla, CA) was used to perform Fisher's exact test on all contingency tables. Continuous data was analyzed using a Student's T-Test in R version 2.9.2© (R Development Core Team, Vienna, Austria).

Results

Greenhouse Inoculation Experiment

Cut Stem Wound 'Stubbed'

There was little difference in the variables observed and measured when the inoculated plants in the wilt and extended drought treatments were compared. None of the differences between them were found to be statistically significant (Table 3.2)

Differences were found between the mock-inoculated and inoculated groups in the wilt treatment (Table 3.2). Only 20% of the stems in the mock-inoculated group were completely dead from the wound to the first node down, while 96% of the inoculated group were dead to the node ($p<0.05$). Also, the

inoculated group had a higher proportion of lesions, which had advanced past the first node, compared with the mock-inoculated group, 16% versus 0%, respectively, but the difference was not found to be significant ($p>0.05$). None of the mock-inoculated plants had any pycnidia present or the fungus present (determined by isolation and identification), while in the inoculated group 80% had pycnidia present and 60% had the *C. corni* present ($p<0.05$).

Comparisons of the mock-inoculated and inoculated groups in the extended drought treatment were very similar to those found in the wilt treatment (Table 3.2). However, unlike in the wilt treatment, the mock-inoculated and inoculated groups both had 100% of the plants that had complete necrosis from the wound to the first node down. The inoculated group had a high proportion of plants with pycnidia present (80%) compared with the mock-inoculated (0%) ($p<0.05$). Also, *C. corni* was isolated from 60% of the inoculated plants, while it was not isolated from any of the mock-inoculated plants ($p<0.05$).

There was some variability in variables observed and measured amongst the 5 isolates (Table 3.1) used in the cut stem wound 'stubbed' inoculation experiment (Table 3.3). At 3 MPI the proportion of plants that were fully necrotic from the site of inoculation to the next node ranged from 40% for MNA1003 to 90% for MNS1007 ($p>0.05$). By 7 MPI, all inoculation sources resulted in ≥ 90% of the plants that were dead to the node ($p=1.0$). The greatest variation at both measurements (3 and 7 MPI) was for presence/absence of pycnidia on the plant tissue. At 3 MPI, MNA1003 (asymptomatic isolate source) resulted in 10% of the plants with pycnidia, while the four other isolate sources resulted in ≥ 70% of the

plants displaying pycnidia (p=0.02). At 7 MPI, 20% of the plants inoculated with MNA1003 had pycnidia, but the other four isolates resulted in 90% to 100% of the plants displaying pycnidia (p=0.006). Seven MPI the fungus was isolated from above the advancing front of the lesion in 60% of the plants treated with MNA1003 (asymptomatic isolate source), which was equal to the average of the other four isolates.

Cork Borer Wound

For plants wounded with the cork borer, the mock-inoculated plants had greater average lesion size than the inoculated plants at 7 MPI in both the wilt treatment and extended drought treatment, although the difference was not found to be statistically significant (Table 3.4). In both the wilted and extended drought treatments the inoculated plants had a higher proportion of plants that were at least partially callused compared with the mock-inoculated plants at 7 MPI, but the differences were not considered statistically significant (p>0.05) (Table 3.4). None of the control or inoculated plants had any pycnidia forming around the wound. The fungus was isolated from 47% of the inoculated plants sampled, while it was not isolated from any of the mock-inoculated plants sampled. When the fungus was isolated, it was found at the site of the wound 43% of the time or below the wound 7% of the time; the fungus was never isolated from above the wound.

The wilt and extended drought treatments, had a small effect on lesion length and callus development, but it was not found to be statistically significant (Table 3.4). Lesions amongst the inoculated plants group were 1.3 times larger in

the extended drought treatment compared with the wilt treatment (p=0.4). The extended drought group of inoculated plants had fewer plants that formed callus tissue compared to the controls, 68% vs. 88% respectively (p=0.2). None of the differences in lesion development were considered statistically significant when comparing the 5 different inoculation sources used in the cork borer inoculation treatment (Table 3.5).

Field Inoculation Experiment

Cut Branch Wound

Differences in disease progression were noted in comparisons between samples cut below a node 'stubbed' and those cut above a node 'not stubbed' (Table 3.6). However, the only statistically significant difference between the two treatments was presence of pycnidia. Eighty-nine percent of the branches cut below the node 'stubbed' had pycnidia, while 26% of those cut above the node 'not stubbed' had pycnidia ($p<0.001$). When the cut was made below the node, the percent of internode necrosis (percentage of necrosis measured vertically from the wound to the first node down from the wound) was 10% higher than when the cut was made above the node (p=0.2). Although not statistically significant, a higher proportion of the lesions, which developed in branches cut below the node 'stubbed' progressed beyond the next node compared with those cut above the node 'not stubbed', 36% and 20% respectively (p=0.2).

There were no statistically significant differences in any disease progression measurements or observations between branches cut above 'not stubbed' or below the node 'stubbed' inoculated with the two different isolates,

MNS1005 and MNS1007 (Table 3.6). Also, there were no statistically significant differences between the mock-inoculated and inoculated treatments for any of the observations or measurements for either the branches cut above 'not stubbed' or below the node 'stubbed' (Table 3.6).

Cork Borer Wound

There were no statistically significant differences in any measures or observations of disease progression between cork borer wounded branches inoculated with two different isolates (MNS1005 and MNS1007) and mock-inoculated controls (Table 3.7). The average lesion size for inoculated branches was 4.3 mm, which was only 0.1 mm larger than the mock-inoculated branches ($p>0.05$). Mock-inoculated branches did have a larger proportion of wounds that had began to callus, 83% compared with inoculated branches at 67%, but it was not found to be statistically significant ($p>0.05$). None of the branches in the mock-inoculated or inoculated treatments had pycnidia.

Discussion

Greenhouse Inoculation Experiment

Cut stem 'stubbed' inoculations resulted in more lesion progression than cork borer inoculation treatments. It is unclear why the fungus was so much better at causing disease in the cut stems compared with the cork borer wounded stems. Also, it is uncertain why the disease occasionally progressed past a node (16% in the inoculated wilt treatment and 8% in the inoculated extended drought treatment) and advanced down the stem in the cut stem plants. There is little research available on why some fungi are unable to cause disease when inoculated

in the main part of the stem compared with a cut terminal end of the stem. However, research on latent pathogens of fruit may provide some general insights into potential reasons why the fungus is differentially able to colonize various tissues. A review by Verhoeff (1974) discussed three potential reasons for latency of fungi in fruit: differences in compounds toxic to fungi found in unripe vs. ripe fruit, differences in nutrients needed by the fungus in unripe vs. ripe fruit, and differences in the enzyme production potential of the fungus in unripe vs. ripe fruit.

There are likely differences between the tissue of the cut stem and the cork borer wounded stem. By severing the stem from the apical meristem, hormonal production is highly disrupted, which likely has many effects on the plant. One process regulated by hormones in plants is abscission layer production (Addicott, 1982). It has been noted in other plant species that when an internode is damaged, an abscission layer forms at the base of the internode just above the lower node (Lloyd, 1914; Lloyd, 1916, reviewed by Addicott, 1982). With abscission potentially disrupting defenses in the internode such as cell wall modification and production of antimicrobial chemicals such as phenols, it is not surprising that the tissue is more susceptible to colonization. Also, the senescing tissue may be more susceptible to toxins from the fungus (Chapter 4) due to a lack of enzymes to degrade the toxin or extra protection in membranes or cell walls. There have been numerous reported toxins produced by other tree fungal pathogens (Bennett and Hindal, 1989; Stermer et al., 1984; Takai and Richards, 1978; Takai et al., 1983; White, 1955). Results of the toxin experiment in

Chapter 4 of this thesis suggests that *C. corni* is capable of producing a secondary metabolite or protein that is able to kill plant tissue. It is unclear what levels of the toxin, if any, the fungus is producing when living endophytically. However, when the fungus has aggressively colonized stem tissue, the amount of toxin would be expected to be large simply due to the large amount of fungus present. The toxin(s) would likely have an effect on healthy tissue.

The fungus was isolated from only 60% of the inoculated plants in the cut stem wound 'stubbed' treatment. The correlation between fungal presence and pycnidia presence was less than 0.001, when only inoculated plants were assessed. One potential explanation of why the fungus was not isolated from all of the inoculated plant material is that the environmental conditions in the greenhouse may have had an effect on the survival of the fungus. During certain times in the summer, temperatures in the greenhouse were as high as 38 °C for an undetermined amount of time. Ramsfield et al. (2010) found multiple fungal species growing in wood blocks that had reduced survival when treated with temperatures as low as 41 °C for 2 hours or less.

Plant stress induced by drought is often implicated with increased disease severity, including golden canker (Pataky, 2010). In this study, wilt versus extended drought did not have a statistically significant impact on disease progression (lesion expansion) measured in the inoculated treatments. Also, only 16% of the plants in the wilt treatment and 8% of the plants in the extended drought treatment had lesions, which advanced past the first node below the wound. This is similar to the findings of Swart et al. (1992) and Old et al. (1990),

where drought did not have a statistically significant impact on lesion lengths for other fungi in the Cryphonectriaceae family. A number of plants in the extended drought treatment had tip dieback away from the wound due to drought: 6 plants in the cut stem wound 'stubbed' treatment and 9 plants in the cork wound treatment, while none of the plants in the wilt treatment had tip dieback. None of these necrotic areas had any pycnidia present. For the cut stem wound 'stubbed' treatment nearly all of the inoculated plants in both the wilt and extended drought treatments were dead to the next node, 96% and 100% respectively. A small percentage of plants inoculated in the cut wound 'stubbed' treatment had lesions advance past the node for both the wilt and extended drought sub treatment, 16% and 8% respectively, but the difference between them was not statistically significant ($p=0.7$).

The cork borer inoculation method proved ineffective as a means of artificially promoting lesion development. Mock-inoculated control plants had larger average lesion sizes than inoculated plants. One possible explanation is that other contaminants were able to colonize the control wounds, while the inoculated wounds were colonized by *C. corni*. The average lesion size, which included the wound if it was not callused, 7 MPI for the inoculated plants in the extended drought treatment and the wilt treatment was 4.8 mm and 3.7 mm respectively, which was actually smaller than the original wound of 5 mm ($p>0.05$).

Field Inoculation Experiment

Ninety-four percent of the mock-inoculated branches for the cut below the node 'stubbed' treatment had pycnidia present, which were confirmed to be *C. corni* by staining with 3% KOH. Since the fungus is often found in asymptomatic plant material as an endophyte, this finding is not unexpected (Chapter 2). Two of the branch samples died completely due to golden canker so they were not included in analysis because the difference between treatments could not be assessed. Also, one of the cut above the node control samples broke off before measurements could be taken so it was not included in analysis.

The greatest insight gained from this study resulted from the comparison between the branches cut above 'not stubbed' and below the nodes 'stubbed'. Cutting above the node resulted in significantly fewer samples with pycnidia present ($p<0.05$). In addition, although the difference was not found to be statistically significant, cutting above the node resulted in fewer cases where the fungus advanced past the node, 20% compared with 36% ($p=0.2$). These results highlight the importance of proper pruning for disease management in the landscape. Cutting directly above a node so there is no 'stub' serves to reduce the spread of disease by limiting pycnidia formation. This observation falls in line with current general pruning guidelines, which suggest pruning cuts be made 0.6 cm above the bud (Fazio, 2011). However, it is important when pruning the tree to not damage the node. In a preliminary study, 4 out of 5 plants had disease which progressed past the node when the leaf and bud at the node, where the disease had previously ceased, were removed. By scraping the surface of the bark

at the node, we were able to make disease in the fifth plant advance past the node. Further studies with larger sample sizes should be done to determine if damage to nodes is resulting in the advancement of disease into and past the node, which were previously restricted above the node.

Stark differences were noted when comparing the cork borer and branch cut wounding methods for inoculation. Inoculation by wounding the branch with a cork borer was found to be very ineffective, which is surprising given all of the reported successes using this inoculation method with other fungi in the Cryphonectriaceae family (Hoegger et al., 2002; Seixas et al., 2004; Chen et al., 2011; Begoude et al., 2010; Gryzenhout et al., 2010; Vermeulen, et al., 2011). Interestingly, the average lesion size, which included the wound if it had not callused, three months after cork borer wounding for both the mock-inoculated and inoculated branches were smaller than the initial wound of 5 mm. The plants had begun to callus over the wound in well over half of all of the cork borer inoculations at that time.

Within the cut branch treatments, the cut below the node method (called "stubbing") was most effective at promoting pycnidia development. It seems possible that a disease resistance screening protocol could be developed that would involve cutting branch tips of established trees in the natural setting with a shears or hedge trimmer, making sure to create some 'stubs' and scoring disease development. The abundance of natural inoculum as well as endophytes in the plant tissue may eliminate the need for initial artificial inoculation. Once

'resistant' trees are identified, artificial inoculations would help confirm resistance.

There is another method of screening that could be developed in a nursery or greenhouse setting. Small seedlings would have their tops cut with a shears or hedge trimmer, which would result in 'stubs'. The plants would then be sprayed with a spore suspension containing *C. corni* conidia or ascospores. The studies described in this chapter used actively growing mycelium as an inoculation source, so if a spore suspension were to be used, further studies would need to be conducted to determine if the cut stems would prohibit spore germination. A screening method such as this could be done to screen a very large number of seedlings in a relatively small area.

Whether the isolate was from the Great River Bluffs State Park (MNS1005) or the University of Minnesota, St. Paul campus (MNS1007) had little effect on the variables measured and none of the differences were considered statistically significant. Even though the plants inoculated were located at the Great River Bluffs State Park, there was no discernable interaction between the trees and a specific isolate.

Unanswered Questions

There are still many questions about the disease etiology that remain unanswered. It is still unclear how the fungus gains entrance into the plant. A majority of the cankers found in both field and greenhouse plants were confined to branch tips, and the disease is often stopped just above a branch node. We often observe branch tips that appear to have been frozen in the winter. It is

uncertain if infection arises from fungi living in the tree endophytically or from conidia or ascospores which have germinated on the dead or dying tissue. Pukacki and Przybyl (2005) performed a study on ash trees (*Fraxinus excelsior* L.) and found that frost injury was likely the initial factor contributing to dieback of branch tips, which was followed by subsequent colonization of multiple fungi.

Importantly, we still do not know what causes the shift from an endophytic to pathogenic state. For the fungus *Sphaeropsis sapinea* (Fr.) Dyko & B. Sutton, which is often found as an endophyte that later becomes pathogenic in pine stems, stress agents such as drought play an important role (Swart et al., 1987; Flowers et al., 2001). Although the study did not have a well watered control for comparison, the inoculated plants in the wilt treatment and extended drought treatment had only a small proportion of lesions (16% and 8%, respectively) that advanced past the first node, which is critical in disease progression. As discussed earlier, the progression of disease is probably determined by a complex interaction between plant defenses and toxins produced by the fungus. What factors affect host defenses are still unclear.

Table 3.1. Description and geographical origin of isolates of *C. corni* collected from pagoda dogwood used in this study.

Isolate Number[Z]	Location	Collection Date
MNS1005	Great River Bluffs State Park, MN	2/3/12
MNA1003	William O' Brien State Park, MN	7/17/11
MNS1007	University of Minnesota, Twin Cities, MN	2/3/12
MNS1008	Pillsbury State Forest, MN	2/3/12
MNA1009	Minnesota Landscape Arboretum, MN	7/18/11

[Z] The first two letters in the isolate number are abbreviations for the state collected from and the third letter represents whether the host plant was symptomatic or asymptomatic (S = symptomatic, A = asymptomatic).

Table 3.2. Greenhouse Inoculation Experiment

Observations on pagoda dogwood stems 7 months post inoculation for four different treatments. Observations are given as a proportion of stems with a (+) scoring. Inoculated plants were inoculated with *C. corni* growing on grain seed, while mock-inoculated plants were inoculated with sterile grain seed. The main stem was severed at approximately 46 cm and inoculum was placed on the wound.

	Stem Cut Wound 'Stubbed'					
	Wilt[P]		Extended Drought[Q]		Inoculated[R]	
Observation	**Mock-Inoculated**	**Inoculated**	**Mock-Inoculated**	**Inoculated**	**Wilt**	**Extended Drought**
Dead to Node (+/-)[S]	0.20a[T]	0.96b	1.00a	1.00a	0.96a	1.00a
Internode Partial Necrosis (+/-)[U]	0.60a	0.00b	0.00a	0.00a	0.00a	0.00a
Necrosis Past First Node (+/-)[V]	0.00a	0.16a	0.00a	0.08a	0.16a	0.08a
Pycnidia Present (+/-)[W]	0.00a	0.80b	0.00a	0.80b	0.80a	0.80a
Fungal Presence (+/-)[X]	0.00a	0.60b	0.00a	0.60b	0.60a	0.60a
Fungal Presence Above Node (+/-)[Y]	0.00a	0.60b	0.00a	0.60b	0.60a	0.60a
Fungal Presence Below Node (+/-)[Z]	0.00a	0.00a	0.00a	0.00a	0.00a	0.00a

[P] Plants in the wilt treatment were watered when a majority of the plants began showing wilt symptoms

[Q] Plants in the extended drought treatment were watered 3 days after the wilt treatment cycle

[R] Plants in the inoculated treatment were inoculated with *C. corni*

[S] Whether the area from the wound to the first node down was fully necrotic

[T] Differences in proportions in rows within paired columns (columns adjacent to columns of the same color) with the same letter are not considered statistically significant ($p>0.05$) using Fisher's exact test

[U] Whether the area from the wound to the first node down was partially necrotic

[V] Whether the lesion advanced past the first node down from the wound

[W] Whether pycnidia were visibly present on the surface of the stem

[X] Whether the fungus could be isolated from either above or below the advancing lesion front

[Y] Whether the fungus was isolated above the advancing lesion front

[Z] Whether the fungus was isolated below the advancing lesion front

Table 3.3. Greenhouse Inoculation Experiment

Effects of 5 different *C. corni* isolates on variables observed on pagoda dogwood stems 3 and 7 months post inoculation (MPI) (wilt treatment and extended drought treatment were combined for analysis). Observations are given as a proportion of stems with a (+) scoring. The main stem was severed at approximately 46 cm and inoculum was placed on the wound.

	Cut Stem Wound 'Stubbed'									
	MNS1005		MNA1003		MNS1007		MNS1008		MNA1009	
Observations	**3 MPI**	**7 MPI**	**3 MPI**	**7 MPI**	**3 MPI**	**7 MPI**	**3 MPI**	**7 MPI**	**3 MPI**	**7 MPI**
Dead to Node (+/-)[T]	0.70a[U]	1.00a	0.40a	0.90a	0.90a	1.00a	0.60a	1.00a	0.60a	1.00a
Internode Partial Necrosis (+/-)[V]	0.20a	0.00a	0.30a	0.00a	0.00a	0.00a	0.40a	0.00a	0.40a	0.00a
Necrosis Past First Node (+/-)[W]	0.10a	0.20a	0.00a	0.20a	0.00a	0.00a	0.20a	0.20a	0.00a	0.00a
Pycnidia Present (+/-)[X]	0.80a	0.90a	0.10b	0.20b	0.90a	1.00a	0.70a	1.00a	0.70a	0.90a
Fungal Presence (+/-)[Y]	⊘[Z]	0.60a	⊘	0.60a	⊘	0.60a	⊘	0.50a	⊘	0.70a

[T] Whether the area from the wound to the first node down was fully necrotic

[U] Differences in proportions in rows with the same letter are not considered statistically significant ($p>0.05$) using Fisher's exact test

[V] Whether the area from the wound to the first node down was partially necrotic

[W] Whether the lesion advanced past the first node down from the wound

[X] Whether pycnidia were visibly present on the surface of the stem

[Y] Whether the fungus could be isolated from either above or below the advancing lesion front

[Z] Isolations were not made 3 MPI to determine if the *C. corni* was present

Table 3.4. Greenhouse Inoculation Experiment

Observations on pagoda dogwood stems 7 months post inoculation for four different treatments. Measurements are giving as a mean±SD and observations are given as a proportion of stems with a (+) scoring. Inoculated plants were inoculated with *C. corni* growing on grain seed, while mock-inoculated plants were inoculated with sterile grain seed. The main stem was wounded 15 cm above the soil line with a 5 mm cork borer and inoculum was placed on the wound.

	Cork Borer Wound					
	Wilt[P]		Extended Drought[Q]		Inoculated[R]	
Measurements/Observations	**Mock-Inoculated**	**Inoculated**	**Mock-Inoculated**	**Inoculated**	**Wilt**	**Extended Drought**
Measurements						
Lesion (mm)[S]	7.1±6.0a[T]	3.7±4.4a	8.3±5.1a	4.8±5.6a	3.7±4.4a	4.8±5.6a
Lesion Minus Original Wound (mm)[U]	3.1±4.8a	1.2±2.7a	4.3±3.1a	2.3±3.5a	1.2±2.7a	2.3±3.5a
Observations						
Callus (+/-)[V]	0.60a	0.88a	0.20a	0.68a	0.88a	0.68a
Callused Fully (+/-)[W]	0.20a	0.48a	0.20a	0.48a	0.48a	0.48a
Callused Over 50% (+/-)[X]	0.20a	0.12a	0.00a	0.04a	0.12a	0.04a
Callused Under 50% (+/-)[Y]	0.20a	0.28a	0.00a	0.16a	0.28a	0.16a
Pycnidia Present (+/-)[Z]	0.00a	0.00a	0.00a	0.00a	0.00a	0.00a

[P] Plants in the wilt treatment were watered when a majority of the plants began showing wilt symptoms

[Q] Plants in the extended drought treatment were watered 3 days after the wilt treatment cycle

[R] Plants in the inoculated treatment were inoculated with *C. corni*

[S] Lesions (the necrotic region) were measured vertically

[T] Differences in proportions or means in rows within paired columns (columns adjacent to columns of the same color) with the same letter are not considered statistically significant ($p>0.05$) using Fisher's exact test (Observations) or Student's T-Test (Measurements)

[U] The vertical measurement of the lesion minus the orginial wound of 5 mm (lesion minus wound≥0)

[V] Whether the wound had any callus formation

[W] Whether the wound was fully callused

[X] Whether the wound was callused over 50%

[Y] Whether the wound was callused under 50%

[Z] Whether pycnidia were visibly present on the surface of the stem

Table 3.5. Greenhouse Inoculation Experiment

Effects of 5 different *C. corni* isolates on variables observed on pagoda dogwood stems 3 and 7 months post inoculation (MPI) (wilt treatment and extended drought treatment were combined for analysis). Measurements are given as a mean±SD and observations are given as a proportion of stems with a (+) scoring. The main stem was wounded 15 cm above the soil line with a 5 mm cork borer and inoculum was placed on the wound.

	Cork Borer Wound									
	MNS1005		MNA1003		MNS1007		MNS1008		MNA1009	
Measurements/Observations	**3 MPI**	**7 MPI**	**3 MPI**	**7 MPI**	**3 MPI**	**7 MPI**	**3 MPI**	**7 MPI**	**3 MPI**	**7 MPI**
Measurements										
Lesion (mm)[S]	5.2±4.7a[T]	5.0±5.3a	1.7±2.7a	3.0±6.1a	4.2±3.5a	4.8±4.6a	4.2±4.2a	4.5±4.8a	3.4±3.1a	3.9±4.8a
Lesion Minus Original Wound (mm)[U]	1.9±3.1a	2.1±3.5a	0.2±0.3a	1.5±4.5a	1.0±1.7a	1.8±2.5a	1.2±2.5a	1.8±2.8a	0.6±0.9a	1.6±2.6a
Observations										
Callus (+/-)[V]	0.70a	0.70a	0.90a	0.90a	0.70a	0.70a	0.80a	0.80a	0.90a	0.80a
Callused Fully (+/-)[W]	0.30a	0.40a	0.70a	0.70a	0.30a	0.40a	0.30a	0.40a	0.40a	0.50a
Callused Over 50% (+/-)[X]	0.20a	0.10a	0.00a	0.00a	0.20a	0.10a	0.20a	0.10a	0.20a	0.10a
Callused Under 50% (+/-)[Y]	0.20a	0.20a	0.20a	0.20a	0.20a	0.20a	0.30a	0.30a	0.30a	0.20a
Pycnidia Present (+/-)[Z]	0.00a	0.00a	0.00a	0.00a	0.00a	0.00a	0.00a	0.00a	0.00a	0.00a

[S] Lesions (the necrotic region) were measured vertically

[T] Differences in means and proportions in rows with the same letter are not considered statistically significant ($p>0.05$) using Fisher's exact test (Observations) or Student's T-Test (Measurements)

[U] The vertical measurement of the lesion minus the orginial wound of 5 mm (lesion minus wound≥0)

[V] Whether the wound had any callus formation

[W] Whether the wound was fully callused

[X] Whether the wound was callused over 50%

[Y] Whether the wound was callused under 50%

[Z] Whether pycnidia were visibly present on the surface of the stem

Table 3.6. Field Inoculation Experiment

Measurements and observations on pagoda dogwood branches 3 months post inoculation (MPI) for branches cut either above or below a node to make a wound. Two isolates of *C. corni* were used as inoculum (MNS1005 and MNS1007) for the inoculated branches, and sterilized grain seeds were used for the mock-inoculated branches. Measurements are given as a mean percentage and observations are given as a proportion of branches with a (+) scoring.

	Branches Cut Below Node 'Stubbed'				Branches Cut Above Node 'Not Stubbed'				Mock-Inoculated + Inoculated[T]	
	Mock-Inoculated	Inoculated[U]	MNS1005	MNS1007	Mock-Inoculated	Inoculated	MNS1005	MNS1007	Below Node	Above Node
Measurements										
Percent Internode Necrosis[V]	0.97±0.24a[W]	0.83±0.38a	0.80±0.42a	0.88±0.35a	0.71±0.47a	0.89±0.32a	0.80±0.42a	1.00±0.0a	0.90±0.29a	0.80±0.41a
Observations										
Internode Necrosis (+/-)[X]	1.00a	0.83a	0.80a	0.88a	0.71a	0.89a	0.80a	1.00a	0.92a	0.80a
Necrosis Past First Node (+/-)[Y]	0.39a	0.33a	0.30a	0.38a	0.24a	0.17a	0.10a	0.25a	0.36a	0.20a
Pycnidia Present (+/-)[Z]	0.94a	0.83a	0.80a	0.88a	0.29a	0.22a	0.20a	0.25a	0.89a	0.26b

[T] Mock-inoculated and inoculated branches were combined for analysis

[U] MNS1005 and MNS1007 were combined for analysis

[V] Percent of the branch from the wound to the first node down showing necrosis (measured vertically)

[W] Differences in proportions or mean percentages in rows within paired columns (columns adjacent to columns of the same color) with the same letter are not considered statistically significant ($p>0.05$) using Fisher's exact test (Observations) or Student's T-Test (Measurements)

[X] Whether the area from the wound to the first node down showed any necrosis

[Y] Whether the lesion advanced past the first node down from the wound

[Z] Whether pycnidia were visibly present on the surface of the stem

Table 3.7. Field Inoculation Experiment

Measurements and observations on pagoda dogwood branches 3 months post inoculation (MPI) for branches wounded with a 5 mm cork borer. Two isolates of *C. corni* were used as inoculum (MNS1005 and MNS1007) for the inoculated branches, and sterilized grain seeds were used for the mock-inoculated branches. Measurements are given as a mean±SD and observations are given as a proportion of branches with a (+) scoring.

		Wound with a Cork Borer		
Measurements/Observations	**Mock-Inoculated**	**Inoculated[R]**	**MNS1005**	**MNS1007**
Measurements				
Lesion (mm)[S]	4.20±3.0a[T]	4.30±3.0a	5.20±3.1a	3.10±2.7a
Lesion Minus Wound (mm)[U]	0.78±1.3a	0.81±1.2a	1.2±1.5a	0.25±0.39a
Observations				
Callus (+/-)[V]	0.83a	0.67a	0.70a	0.63a
Callused Fully (+/-)[W]	0.28a	0.28a	0.20a	0.38a
Callused Over 50% (+/-)[X]	0.22a	0.06a	0.00a	0.13a
Callused Under 50% (+/-)[Y]	0.33a	0.33a	0.50a	0.13a
Pycnidia Present (+/-)[Z]	0.00a	0.00a	0.00a	0.00a

[R] Isolates MNS1005 and MNS1007 were combined for analysis

[S] Lesions (the necrotic region) were measured vertically

[T] Differences in proportions or means in rows within paired columns (columns adjacent to columns of the same color) with the same letter are not considered statistically significant ($p>0.05$) using Fisher's exact test (Observations) or Student's T-Test (Measurements)

[U] The vertical measurement of the lesion minus the orginial wound of 5 mm (lesion minus wound≥0)

[V] Whether the wound had any callus formation

[W] Whether the wound was fully callused

[X] Whether the wound was callused over 50%

[Y] Whether the wound was callused under 50%

[Z] Whether pycnidia were visibly present on the surface of the stem

Chapter 4. Optimal Temperature for Growth and Toxin Production of *Cryptodiaporthe corni* Wehm.

Introduction

Much remains unknown about the biology of *Cryptodiaporthe corni* Wehm., the causal agent of golden canker disease on pagoda dogwood. Redlin and Rossman (1991) described the taxonomic characteristics of the fungus, but did not discuss the biology of the fungus, the etiology of the canker disease, or pathogen dissemination. Cankers caused by the fungus have been noted to expand both during the dormant season (Sinclair and Lyon, 2005) and during the growing season (Chapter 3). There have been no published studies on the effect of temperature on fungal growth. As an initial step in the understanding of disease development, we wanted to determine the optimal temperature for fungal growth on artificial media in the laboratory. Studies on a different tree canker disease, Hypoxylon canker of aspen, showed that the optimal temperature for growth on artificial media coincided with the optimal temperature for lesion expansion (Bagga and Smalley, 1974a; Bagga and Smalley, 1974b). Determining the temperature at which the fungus grows most actively will help in the development of disease management strategies.

Many fungal tree pathogens are capable of producing toxins that damage their host and enable more efficient colonization. There have been numerous studies to identify some of these toxins involved in different tree diseases such as chestnut blight, Hypoxylon canker, Dutch elm disease, and oak wilt (Bennett and Hindal, 1989; Stermer, et al., 1984; Takai and Richards, 1978; Takai et al., 1983;

White, 1955). In field observations, *C. corni* has been found to infect and progress in two general modes. In some cases it appears to advance directly down the stem, while at other times it infects and kills just the stem tip and appears to be arrested at the branch node. We wanted to determine if the fungus is capable of producing toxins, which can kill living tissue of pagoda dogwood. In some pathosystems there is a correlation between resistance of plants to a specific pathogen and its specific culture filtrates. Screening procedures have been developed for multiple pathosystems that involve treating plants with culture filtrates to screen for disease resistance (Kuo et al., 1970; Behnke, 1980; Borras et al., 2001). Pijut et al. (1990) developed a procedure to screen elm cuttings for resistance to Dutch elm disease by placing cuttings in fungal exudates of *Ophiostoma ulmi* (Buisman) C. Moreau and observing wilt symptoms. If a toxin is found in the *C. corni* exudates, the culture filtrates may potentially be used in a screening procedure for golden canker resistance in pagoda dogwoods.

Materials and Methods

Temperature Optimum for Fungal Growth:

Isolates

Fungal isolates were obtained from two different pagoda dogwoods exhibiting cankers at the Great River Bluffs State Park in Minnesota. One isolate was collected on February 3, 2012 (MNS1005), while a second was collected on June 22, 2012 (MNS1006). Bark segments were taken from visible cankers and plated onto Petri dishes containing acidified malt extract agar (2 mL 85% lactic acid, 15 g malt extract, 15 g agar, and 1 L deionized water). Pure cultures

identified by morphological traits and staining with 3% KOH were placed onto Petri dishes of malt extract agar (15 g malt extract, 15 g agar, and 1 L deionized water). The pure cultures were allowed to grow for one week, at which point mycelium was removed with a #2 cork borer (5 mm) and plugs were used as a source of inoculum for the experiments. On July 17, 2012 the 5 mm circular inoculum plugs were placed at the center of 100 x 15 mm Petri dishes containing malt extract agar. The Petri dishes were then sealed with Parafilm and randomly assigned to treatments.

Treatments

Ten replicates per isolate (MNS1005 and MNS1006) for each temperature treatment were randomly placed into temperature-controlled incubators. There were eight different temperature treatments (10 replications per isolate) ranging from 5 to 40 °C at 5-degree intervals. Petri dishes were stored in the dark to eliminate any variation in light source.

Measurements

Mycelial growth was measured using a digital caliper. Diameter was determined by measuring the single widest point of the developing mycelium. Measurements were taken at 3, 6, 9, and 11 days after inoculation. The maximum diameter the fungus could reach in the Petri dish when the original size of the mycelium plug was subtracted was 81.5 mm.

Toxin Experiment:

Inoculation Source

The fungal isolate (MNS1007) used in this experiment was collected on February 3, 2012 from a symptomatic plant growing on the University of Minnesota, St. Paul campus. The fungal isolation and confirmation procedures were the same as that used in chapter 3. Isolates were transferred to Petri dishes containing malt extract agar to grow for 5 days before being used for the experiment.

Liquid Media

The liquid media used was malt extract broth (15 g malt extract broth and 1 L deionized water), which was autoclaved for 20 minutes. One hundred mL of the media was poured into 125 mL Pyrex Erlenmeyer flasks and autoclaved again for 20 minutes. After the media was allowed to cool, three 0.5 x 0.5 cm pieces of inoculum (MNS1007) from the malt extract agar, which had been colonized by *C. corni* for 5 days, were added. For the mock-inoculated (controls), un-colonized pieces of malt extract agar were added. Inoculated and mock-inoculated flasks were allowed to incubate for 24 days at room temperature under natural day length.

Filtering and Sterilization

Once the media had incubated for 24 days, it was filtered first through a Whatman's 1 filter paper and next through a Corning® 115 mL 0.22 um filtration system (Corning, Corning, NY, USA). Some small unidentified particulates were found in the filtrated liquid so half of the filtered liquid media for both the mock-

inoculated control and inoculated media were then autoclaved for 20 minutes to kill any parts of the fungus, which may have survived the filtration process. Twenty mL aliquots of the liquid media for all treatments were poured into Pyrex 16 x 100 mm disposable culture tubes.

Treatments

Four different treatments were initiated for this experiment with each treatment containing 10 replicates. Treatment 1 and 2 used mock-inoculated media with and without autoclaving respectively. Treatment 3 and 4 used inoculated media with and without autoclaving respectively. The pH for a randomly selected culture tube for each treatment was measured after the liquid media had been incubated for 24 days.

Plant Material

Asymptomatic terminal ends of branches were collected from 2 infected trees growing on the St. Paul campus of the University of Minnesota. Branches were not sampled to determine if *C. corni* was present in the asymptomatic tissue. The terminal tips of each branch were trimmed to leave 8 cm of stem below the lowest leaf. The number of leaves on the stems ranged from 6-8. The stems were randomly assigned to the different treatments and the distal basal ends of each were inserted into the liquid media in the culture tubes. The opening around the top of the tube was sealed with Parafilm to prevent evaporation and contamination. The culture tubes were randomized within a growth chamber maintained at 27 °C with constant light.

Observations

The stems were evaluated at 16, 23, 29, 40, 46, and 52 hours after being placed in the growth chamber for wilted (+) or non-wilted leaves (-). If any of the leaves on the stem showed wilting, the stem was scored as (+).

Analysis:

All categorical data was analyzed using a 2 x 2 contingency table. Graph Pad Software (Graph Pad Software, Inc., La Jolla, CA, USA) was used to perform Fisher's exact test on all contingency tables. Continuous data was analyzed using a Student's T-Test in R version 2.9.2© (R Development Core Team, Vienna, Austria).

Results

Temperature Optimum for Fungal Growth:

The optimal temperature for fungal growth for both MNS1005 and MNS1006 was 25 °C. Within 6 days at 25 °C, some replicates of MNS1005 had grown to the edge of the Petri dish (81.5 mm of new growth) and by 9 days all of the replicates for both MNS1005 and MNS1006 had grown to the edge of the Petri dish (Fig. 4.1). The fungal growth response to temperature assumed a bell curve distribution that peaked at 25 °C, with minimal growth at 5 °C and no growth when incubated at 40 °C for either of the isolates. At 35 °C the maximum growth for either isolate after 11 days was 6.5 mm. At the lowest temperature (5 °C) at 11 days, mean growth for MNS1005 was 4.6 mm and 2.9 mm for

MNS1006, but the difference between the means was not found to be statistically significant ($p=0.12$). The only temperatures where the differences in growth between the two isolates at 11 days were statistically significant were 15 °C and 35 °C ($p=0.04$ and 0.001, respectively).

Toxin Experiment:

Since small particulates were seen in the filtrated media, half of the samples for both the mock-inoculated treatment and the inoculated treatment were autoclaved before the stems were placed in the media to ensure that any parts of the fungus that may have passed through the filters were killed. Whether or not the mock-inoculated media was autoclaved had almost no effect on wilting (Table 4.1, Fig 4.2). The only difference was found at hour 29, when one of the stems in the autoclaved mock-inoculated treatment had wilted leaves, while no stems had wilted leaves in the non-autoclaved mock-inoculated treatment. However, considerable differences were seen when the inoculated media was autoclaved. At both hour 23 and hour 29, the autoclaved inoculated treatment had a significantly higher number of stems with wilted leaves (6) compared with the non-autoclaved inoculated treatment (0) ($p=0.01$). After hour 29 none of the differences between the autoclaved inoculated treatment and the non-autoclaved inoculated treatment were statistically significant ($p>0.05$) (Table 4.1).

When the stems in the non-autoclaved mock-inoculated media and the non-autoclaved inoculated media were compared, there was no statistically significant difference in the number of stems with wilted leaves at any time point. However, in the last three observations (hours 40, 46, and 52) the inoculated

media did have more stems with wilted leavers (Table 4.1). An example of wilted versus non-wilted leaves is shown in Fig. 4.3. When comparing the difference between the mock-inoculated treatment and the inoculated treatment that were autoclaved, the inoculated treatment always had a higher percentage of stems with wilted leaves. The greatest differences between the autoclaved mock-inoculated and autoclaved inoculated treatments occurred at hour 23, when the mock-inoculated had 0 and the inoculated treatment had 6 stems with wilted leaves, and at hour 46 when the mock-inoculated had 1 and the inoculated treatment had 7. At both time points the differences were found to be statistically significant ($p<0.05$).

There were differences found in the pH between the mock-inoculated control media and the inoculated media. The non-autoclaved mock-inoculated media had a pH of 4.59 and a pH of 4.56 after autoclaving. Both the non-autoclaved and autoclaved inoculated media had a pH of 3.79.

Discussion

Temperature for Optimal Fungal Growth Experiment:

The optimal temperature for fungal growth on malt extract media was 25 °C. One of the only canker causing fungi that have been studied for both optimal temperature for growth in culture and optimal temperature for canker expansion in the field is *Hypoxylon pruinatum* (Klot.) Cke. A study on Hypoxylon canker of aspen by Bagga and Smalley (1974b), showed the optimal growth on artificial media for *Hypoxylon pruinatum* ranged from 24 – 28 °C depending on the media used. They also found the optimal temperature for lesion development on living

hosts to be 24 – 28 °C depending on the isolate (Bagga and Smalley, 1974a). It is possible that pagoda dogwood would also have an optimal temperature for lesion development similar to optimal temperature for fungal development on media. Sinclair and Lyon (2005) suggest that most of the dieback on the tree occurs during the dormant season. This conclusion was based on the observation that no dead leaves were seen on infected branches. If canker development had occurred during the growing season, shriveled dead leaves should have been seen adhering to the stems. During the course of this study, I observed numerous stems, which had died due to golden canker during the growing season and leaves were almost always attached to the stems. Based on the fact that the fungus exhibited only minimal growth at temperatures of 5 – 10 °C and that most of the dead stems I have observed have shriveled leaves, it seems unlikely that the fungus is causing extensive dieback during the dormant season.

Although the optimal growing temperature for the fungus in-vitro is 25 °C the fungus may encounter elevated defenses from the plant at this temperature compared with those expressed at lower temperatures during its dormancy. A study by Jacobi (1984) found the optimal temperature for fungal growth for *Thyronectria austroamericana* (Speg.) Seeler on media occurred at 25-30 °C. However, a later study by Jacobi (1989) found that the greatest lesion expansion on honeylocust trees inoculated in the fall occurred during the dormant months of September and November, which are considerably colder than the in-vitro optimal growth temperatures for the fungus. He attributed this to lower defense reactions in the plant during the dormant season (Jacobi, 1989). Although it is evident that

the fungus is capable of colonizing tissue during the non-dormant part of the season (Chapter 3), it is unclear if the disease lesions expand in the dormant season. Additional studies should be done to determine the optimal temperature for lesion development. Using the cut inoculation method (Chapter 3) and growing plants at different temperatures, would likely resolve this unanswered question.

Toxin Experiment:

Fungal exudates in the autoclaved inoculated media did have a statistically significant effect on the number of stems with wilted leaves at hours 23 and 46 compared with either the autoclaved or non-autoclaved, mock-inoculated treatments. It is unclear why there was such a difference between autoclaved and non-autoclaved inoculated treatments at hours 23 and 29 ($p=0.01$). A potential explanation is that the compounds in the autoclaved media may have been altered during autoclaving making them more efficient at causing wilt. There was an obvious difference between the pH of the inoculated media (pH = 3.8) and the mock-inoculated media (pH = 4.6). Although it is unclear what the substance(s) are that are causing the plant to wilt, it is evident that some kind of compound that lowered the pH is being produced by the fungus. Whether that compound is a toxin or is capable of forming a toxic compound is not known. Bennett and Hindal (1989) found that *Cryphonectria parasitica* (Murrill) M.E. Barr, a canker fungus in the Cryphonectriaceae family, produced oxalic acid in liquid media, but they did not examine if it was associated with lesion development. A study by Godoy et al. (1990) of *Sclerotinia sclerotiorum* (Lib.) de Bary on beans showed

that mutant forms of the fungus, which could not produce oxalic acid were nonpathogenic, while non-mutants and restored mutants producing oxalic acid were pathogenic. Further studies are needed in order to identify the toxin(s) produced by *C. corni* that are causing the leaves to wilt. A water-soluble and a non-water soluble fraction could be made by passing the fungal exudates through a specialized resin (Tawaraya et al. (1998). Cuttings could be placed in the fractions to determine which fraction caused wilting. Once the fraction containing the toxin is identified, high-performance liquid chromatography could be used to identify the compound of interest.

Although insight was gained from this study about the interaction of *C. corni* and pagoda dogwood, a lot of information regarding toxin production is still unknown. We do not know when the toxin is produced by the fungus in the plant or how much is produced. Further studies to investigate the production of the toxin while the fungus is actively growing at an infection front and in asymptomatic plant tissue would need to be performed before a greater understanding can be achieved. Also, before culture filtrates are used in disease screening, further studies must be performed to determine if there is a correlation between the response of the plant to the pathogen and to the pathogen's culture filtrates.

Figure 4.1. Boxplots of growth of *C. corni* (isolates MNS1005 and MNS1006) at eight different temperature regimes (5 – 40 °C) for 3, 6, 9, and 11 days after being placed on malt extract agar.

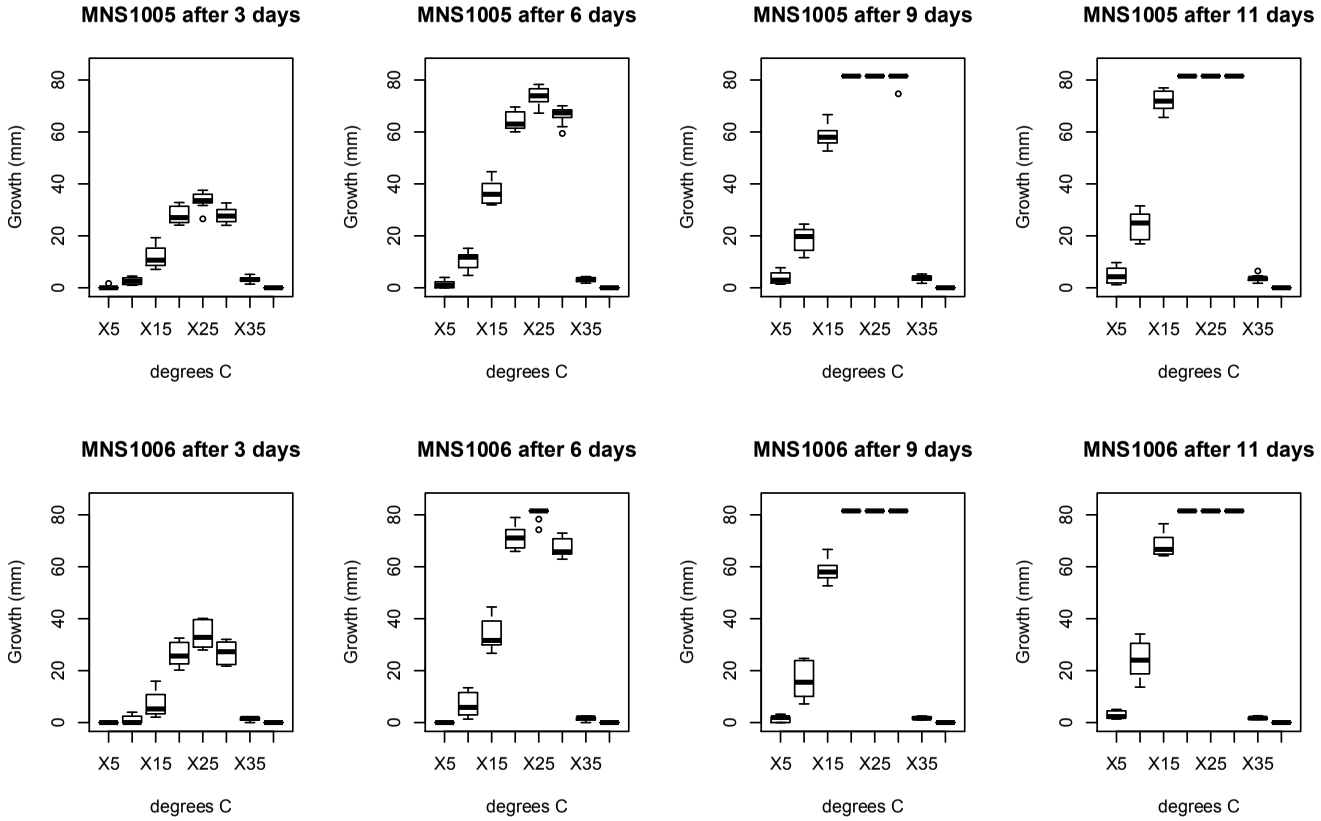


Figure 4.2. Number of stems of pagoda dogwood with wilted leaves (+) after being placed in one of the four different treatments at 6 given time points after immersion in the liquid media. Inoculated media contained fungal exudates of *C. corni*. Stems containing a single leaf that was wilted were scored as (+).

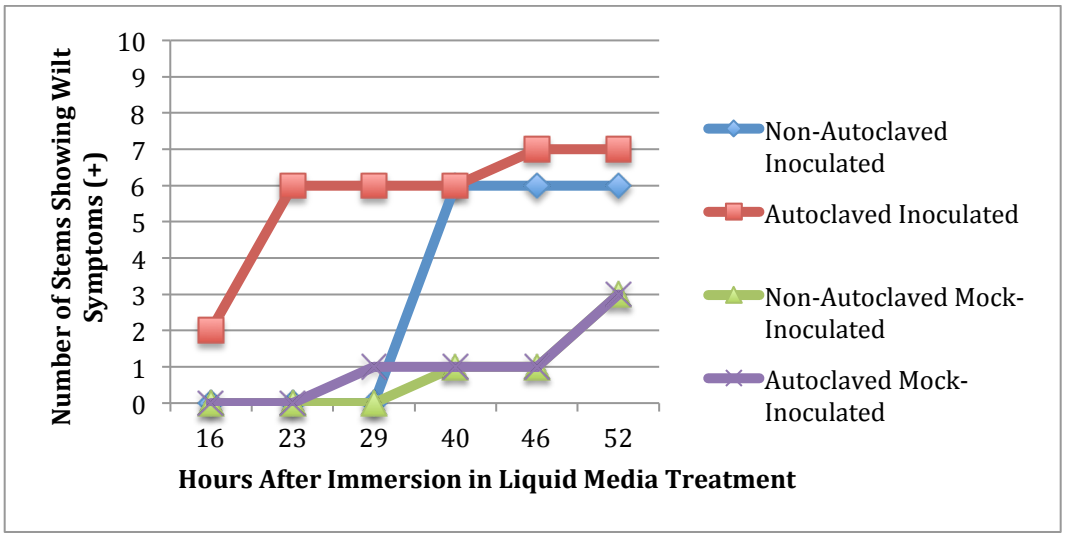


Figure 4.3. Comparison of non-wilted (-) (left) to wilted (+) (right) leaves of pagoda dogwood after 52 hours in non-autoclaved mock-inoculated liquid media (left) and non-autoclaved inoculated liquid media (right). Inoculated media contained fungal exudates of *C. corni*.

Table 4.1. Proportions of pagoda dogwood stems with wilted leaves (+) after being immersed in four different liquid media treatments. Inoculated media contained fungal exudates of *C. corni*. Observations were taken at 6 different time points. Stems with any leaves wilted were scored as a (+).

	Mock-Inoculated (Controls)		Inoculated w/ *C. corni*		Not Autoclaved		Autoclaved	
Time After Immersion[W]	**Not Autoclaved**	**Autoclaved**	**Not Autoclaved**	**Autoclaved**	**Mock-Inoculated[X]**	**Inoculated[Y]**	**Mock-Inoculated**	**Inoculated**
Hour 16	0.0a[Z]	0.0a	0.0a	0.2a	0.0a	0.0a	0.0a	0.2a
Hour 23	0.0a	0.0a	0.0a	0.6b	0.0a	0.0a	0.0a	0.6b
Hour 29	0.0a	0.1a	0.0a	0.6b	0.0a	0.0a	0.1a	0.6a
Hour 40	0.1a	0.1a	0.6a	0.6a	0.1a	0.6a	0.1a	0.6a
Hour 46	0.1a	0.1a	0.6a	0.7a	0.1a	0.6a	0.1a	0.7b
Hour 52	0.3a	0.3a	0.6a	0.7a	0.3a	0.6a	0.3a	0.7a

[W] Number of hours after stems of pagoda dogwood were placed in different liquid media treatments

[X] Liquid media inoculated with sterile malt extract agar plugs

[Y] Liquid media inoculated with C. corni

[Z] Differences in proportions in rows within paired columns (columns adjacent to columns of the same color) with the same letter are not considered statistically significant ($p>0.05$) using Fisher's exact test

Chapter 5. Genetic Diversity of *Cryptodiaporthe corni* Wehm. and Placement Within the Cryphonectriaceae Family

Introduction

The fungus *Cryptodiaporthe corni* Wehm. is the causal agent of golden canker disease of *Cornus alternifolia* L. (pagoda dogwood). It is believed to only infect pagoda dogwood and can be found throughout the range of the host (Redlin and Rossman, 1991). The fungus is capable of living as a parasite, saprophyte, and endophyte (Redline and Rossman, 1991; Chapter 2; Chapter 3).

Cryptodiaporthe corni is in the order Diaporthales, which consists of nine different families (Rossman et al., 2007). Members of the Diaporthales are known for having dark perithecia imbedded in stroma or plant tissue. Genera within the group are differentiated by differences in shape and position of the perithecia, differences in ascospores, and differences in stromatic tissues (Barr, 1978).

The genus *Cryptodiaporthe* Petrak is in the Gnomoniaceae family, and is comprised of 56 species (Sogonov et al., 2008). Species within the genus can be found in both hemispheres, but a majority are found in Europe and North America (Shearer et al., 1995; Punithalingam & Booth, 1977; Sieber et al., 1990; Gremmen, 1978; Petrak, 1921, Reviewed by Barr, 1978; Wehmeyer, 1933; Barr, 1978). A wide variety of woody plants are host to the different species. These include horse-chestnut (*C. aesculi* (Fuckel) Petr.), willow (*C. salicella* (Fr.) Petr.), beech (*C. galericulata* (Tul. & C. Tul.) Wehm.), and others (Barr, 1978 & Petrak, 1921, reviewed by Barr, 1978). In this genus ascospores are two celled, separated

by a septum and each cell is equal in size. The stroma is composed of loosely woven cells as opposed to tightly packed cells and the lower portion of the stroma is weakly developed. Also, within the plant tissue there are no blackened stromatic zones (Wehmeyer, 1933; Petrak, 1921, reviewed by Barr, 1978; Micales & Stipes, 1987). Based on phylogenetic analysis by Sogonov et al. (2008) the type species of *Cryptodiaporthe* (*Cryptodiaporthe aesculi*) grouped with the genus *Plagiostoma*, suggesting that *Cryptodiaporthe* be changed to *Plagiostoma*. Studies of the large subunit nuclear ribosomal DNA (LSU) region of *Cryptodiaporthe corni* by Castlebury et al. (2002) and Gryzenhout et al. (2006) placed this fungus in the Cryphonectriaceae family instead of the Gnomoniaceae family.

Placement of *Cryptodiaporthe corni* in Gnomoniaceae has been questioned (Castlebury et al., 2002). After phylogenetic analysis of the LSU region of a large number of isolates from the Diaporthales, *C. corni* appeared to be more appropriately assigned to the *Cryphonectria-Endothia* complex (Castlebury et al., 2002). Gryzenhout et al. (2006) performed further phylogentic analysis using the LSU region with results leading to creation of the Cryphonectriaceae family containing the genera/species in the *Cryphonectria-Endothia* complex. Members of this family, including *C. corni*, have orange pigment in their stromatic tissue, which reacts and turns purple in the presence of KOH. This reaction allows fungi in this group to be differentiated from other families in the Diaporthales (Gryzenhout et al., 2006). Since *C. corni* has a unique morphology and did not directly group with any of the other genera in

Cryphonectriaceae, these investigators suggested that *C. corni* needs new generic placement in the Cryphonectriaceae family. Because their research had access to only two isolates, they recommended additional studies involving many isolates be undertaken before the new genus name can be given (Gryzenhout et al., 2006). The primary objective for this study was to determine the genetic diversity of *C. corni* throughout its range in the United States with a large number of isolates to better understand the taxonomic placement of the fungus in the Cryphonectriaceae family.

Materials and Methods

Isolates

Cornus alternifolia samples for this study were obtained from across the eastern United States including: Connecticut, Delaware Iowa, Illinois, Maine, Michigan, Minnesota, North Carolina, Pennsylvania, South Dakota, and Wisconsin (Table 5.1). Isolates of *C. corni* were obtained from branch samples showing visible signs of golden canker disease except for the isolate collected from William O' Brien State Park, Minnesota (MNA1003) (Chapter 2), which was collected from an asymptomatic stem. The isolate from an asymptomatic plant was used to determine if the fungus growing asymptomatically was genetically different from those found on diseased plants. Bark segments from the advancing canker or from pycnidia on the surface of the canker were plated onto Petri dishes containing acidified malt extract agar (2 mL 85% lactic acid, 15 g malt extract, 15 g agar, and 1 L deionized water).

Samples were monitored for fungal growth for one month. If a Petri dish contained a fungus, which had mycelium resembling *Cryptodiaporthe corni*, a small portion of the mycelium was subcultured and plated onto a 100 x 15 mm Petri dish with media consisting of malt extract agar (15 g malt extract, 15 g agar, and 1 L of deionized water). Once the fungus was isolated in pure culture it was identified by morphological characteristics, such as orange stromatic tissue and lack of aerial mycelium, and by the use of KOH that specifically stains the mycelium of fungi in the Cryphonectriaceae family (Gryzenhout et al., 2006). Orange stromatic tissue of *Cryptodiaporthe corni* turns purple in the presence of 3% KOH solution (Redlin & Rossman, 1991).

DNA Isolation

The pure cultures were transferred to yeast malt extract agar (0.5 g yeast extract, 15 g malt extract, 15 g agar, and 1 L deionized water) to promote the growth of aerial mycelium. A CTAB procedure was used to extract DNA from each isolate. Aerial mycelium from ¼ of the Petri dish was harvested and suspended in 500 μL CTAB lysis buffer with glass beads and vortexed for 1 minute. The lysate suspension was centrifuged for 1 minute at 10,000 RPM and then removed to a clean tube where it was incubated for 15 – 20 minutes at 65° C. Subsequently, 500 μL chloroform/phenol/isoamyl was added to the tube, which was shaken and then centrifuged for 5 minutes at 13,000 RPM. The supernatant was removed to a new tube and isopropanol was added (2/3 the volume of the supernatant). It was incubated 5 minutes at room temperature, followed by centrifuging for 7 minutes at 15,000 RPM. The isopropanol was removed and the

pellet was washed with 500 μL of ice cold 70% EtOH. Next, it was centrifuged for 3 minutes at 15,000 RPM and the EtOH was removed. The rinsing of the pellet was repeated twice. The pellet was allowed to air dry and was rehydrated with 100 μL sterile, nuclease-free water. All other fungal sequences utilized in this study were obtained from GenBank®. Isolates used for the analysis of the Cryphonectriaceae family were based on studies by Begoude et al. (2010) and Chen et al. (2011). For analysis of the Diaporthales, sequences from studies by Castlebury et al. (2002) and Cheewangkoon et al. (2010) were utilized.

DNA Amplification

Four regions were investigated in this study including: the internal transcribed spacer (ITS) region, the β-tubulin region 1 (Bt1), the β-tubulin region 2 (Bt2), and the large subunit nuclear ribosomal DNA region (LSU). To examine the ITS region primers ITS1 and ITS4 were used (White et al., 1990). The Bt1a and Bt1b primers were used for the β-tubulin region 1 and Bt2a and Bt2b were used for β-tubulin region 2 (Glass and Donaldson, 1995). To amplify the LSU rDNA region the primers LR0R and LR5 were used (Rehner and Samuels, 1994; Vilgalys and Hester, 1990). The gene regions were amplified in a PTC-200 Thermal Cycler (MJ Research, Watertown, MA, USA). Amplification reaction mixtures consisted of 12.5 μL amplitaq master mix, 9.5 μL water, 1 μL of each primer (5 μM), 0.5 μL BSA (bovine serum albumin), and 1 μL DNA template. The PCR protocols were based on Arenz and Blanchette (2009) for amplification of the ITS and LSU gene regions. For the Beta tubulin gene regions the following

protocol was used: 94° C for 1 minute, 30 cycles of 94° C for 1 minute, 60° C for 1 minute, and 72° C for 1 minute. Following amplification, a mixture for sequencing was created using 4.9 μL water, 0.75 μL PCR product, and 0.42 μL 10 μM primer. Forward and reverse sequencing for all amplified products was done using an ABI 3730xl DNA sequencer (Applied Biosystems, Foster City, CA, USA). A consensus sequence was made using Chromas software (Technelysium Ltd., Helensvale, Australia).

Analysis

Phylogenetic analysis was performed on the four different gene regions: ITS, Beta tubulin 1, Beta tubulin 2, and LSU. The ITS, Beta tubulin 1, and Beta tubulin 2 gene regions were used for comparison of the *Cryptodiaporthe corni* isolates with the Cryphonectriaceae family. The LSU region was used to compare the *Cryptodiaporthe corni* isolates to other species in the Diaporthales order. Sequences were aligned using ClustalW in MEGA 5.05 (Tamura et al., 2011). A partition homogeneity test (Farris et al., 1994) was performed in PAUP version 4.0b10 (Swofford, 2000) to determine if there was a conflict between the Beta tubulin 1, Beta tubulin 2, and ITS gene regions being combined for analysis.

For analysis both a distance-based method (neighbor-joining (NJ)) and a character-based method (maximum parsimony (MP)) were used. Both the NJ and MP analysis were performed using PAUP version 4.0b10 (Swofford, 2000). For the MP analysis a heuristic search was used with stepwise addition and the branch-swapping algorithm utilized was tree-bisection-reconnection and the branches collapsed if maximum branch length was 0 (Begoude, et al., 2010). The

"maxtrees" was set to 1000 trees with the "MulTrees" option in effect. Gaps encountered during analysis were considered as missing (Begoude, et al., 2010). Both the MP and NJ analysis included 1000 bootstrap replications to assess the confidence levels of branches. *Diaporthe ambigua* Nitschke was used as an outgroup for analysis of the ITS, Beta tubulin 1, and Beta tubulin 2 gene regions (Begoude et al., 2010) and *Gaeumannomyces graminis* (Sacc.) Arx & D.L. Oliver served as an outlier for analysis of the LSU region (Castlebury et al., 2002).

Results

Results from the partition homogeneity test showed a significant p-value for the use of all three gene regions for both analysis of the *Cryptodiaporthe* isolates (P = 0.05) and analysis of the Cryphonectriaceae family (P = 0.01). Since there was a significant conflict between the different partitions neither of the combined phylogenetic trees were used. Statistics for the analysis for the different gene regions can be found in Table 5.2.

The greatest variation found among the newly sequenced isolates of *Cryptodiaporthe corni* was in the analysis using the Bt1 gene region (Fig. 5.1). When the outgroup was removed, there were 12 base pairs out of 442 that showed variation. Analysis of the ITS region showed the least amount of variation and none of the branch nodes had confidence levels above 50% for either the MP or NJ bootstrap analysis (Fig. 5.3). This region only had 6 base pairs showing variation out of 474. There was very little clear consensus on what isolates grouped together when all three gene regions were assessed together. Only the DES1001 and ILS1001 isolates grouped together in all three gene regions. Only

one branch had confidence levels above 70% for both MP and NJ bootstrap analysis and this was for the isolates MNA1003 and NCS1001 when the Beta tubulin 2 gene region was analyzed (Fig. 5.2).

In the analysis of the Cryphonectriaceae family, the isolates of *Cryptodiaporthe corni* branched separately from any of the other genera in the family. The confidence levels at the node separating the *C. corni* isolates from the other genera based on the bootstrap replications were 100% for both the NJ and MP for Beta tubulin 1, Beta tubulin 2, and ITS gene regions (Fig. 5.4, 5.5, and 5.6).

The isolates of *Cryptodiaporthe corni* grouped with the Cryphonectriaceae family in the analysis of the Diaporthales based on the LSU gene region (Fig. 5.7). The node that separated the Cryphonectriaceae from other families in the Diaporthales had a MP bootstrap confidence level less than 70%, but a NJ bootstrap confidence level of 98%. Within the Cryphonectriaceae family, the *C. corni* isolates were distinct from other groups with a confidence level of 100% for MP and 83% for NJ. The five other species of *Cryptodiaporthe*/*Plagiostoma* analyzed all grouped in the Gnomoniaceae family including the type species *Plagiostoma euphorbiae* (Fuckel) Fuckel. Confidence levels for both the NJ and MP at the node separating the Gnomoniaceae and the Melanconidaceae families from any other family in the Diaporthales was 100%.

Discussion

The taxonomic placement of fungi changes as new methods of analysis such as molecular methods are used. One of the most commonly used molecular methods is sequencing of conserved DNA regions and comparing them to sequences of other species to determine their phylogenetic relationships. For fungi, the internal transcribed spacer (ITS) region is one of the most frequently utilized conserved regions for phylogenetic analysis. The ITS region has proven to be useful in distinguishing between species. Although there have been studies indicating that the ITS sequence variation can be used to detect intraspecies diversity (Gandolfi et al., 2001; Korabecna, 2003), its utility often appears limited (Nilsson et al., 2008). For this study, analysis of ITS sequence variation did group isolates, but none of them had confidence levels above 50% for either the MP or NJ bootstrap analysis. Analysis of the Beta tubulin gene regions were better for distinguishing isolates into different groups based on the number of nodes with confidence levels above 50% for bootstrap analysis.

Based on the analysis of all three gene regions there is some genetic diversity among isolates of *Cryptodiaporthe corni* obtained from the United States. However, that genetic diversity appears to be relatively small based on the number of base pairs which showed variation: 12 of 442 bp for Beta tubulin 1, 9 of 345 bp for Beta tubulin 2, and 6 of 471 bp for ITS. Isolates from the eastern United States grouping with Midwest isolates was dependent on which analysis was used. A previous study showed that this fungus was capable of living in asymptomatic plant material as an endophyte (Chapter 2). With nursery material

being shipped throughout the United States, it is not surprising the fungus appears panmictic in the United States; such genetic mixing has been observed for pathogens of other nursery crops in North America (Whitaker et al., 2007).

By observing the phylogenetic tree based on the LSU gene region of isolates in the Diaporthales it is clear that *Cryptodiaporthe corni* is quite different from other species of *Cryptodiaporthe/Plagiostoma*. The new isolates obtained for this study were very similar to the isolate used by Castlebury et al. (2002) and Gryzenhout et al. (2006) and grouped in a similar way relative to the other species in the Diaporthales. Since all of the isolates of *Cryptodiaporthe corni* clustered separately from any of the other species in either the Cryphonectriaceae family and the order Diaporthales, it does not appear that there are multiple species responsible for causing Golden Canker in the United States.

There is strong evidence based on the phylogenetic analysis in this study and that of others that *Cryptodiaporthe corni* represents a new genus within the Cryphonectriaceae family (Castlebury et al., 2002; Gryzenhout et al, 2006). Also, although the morphological characteristics of *C. corni* do not group with any specific genus in the family, it does share the characteristics of the Cryphonectriaceae family (Redlin and Rossman, 1991; Gryzenhout et al, 2006). Based on its unique morphology, such as its conidiomata, which are irregularly subspherical to flattened (Redlin and Rossman, 1991), it should be placed in a new genus in the Cryphonectriaceae family. Before a new genus can be described, further observations and categorization of the different morphological characteristics of both the anamorphic and teleomorphic stages should be done.

Table 5.1. Description and geographical origin of isolates of *C. corni* collected from pagoda dogwood used in this study.

Isolate Number[Z]	State	City/Location
CTS1001	Connecticut	Redding
DES1001	Delaware	Greenville
DES1002	Delaware	Newark
ILS1001	Illinois	Chicago
IAS1001	Iowa	Ames
IAS1002	Iowa	Dallas Center
MES1001	Maine	Boothbay
MIS1001	Michigan	Ontonagon
MNS1001	Minnesota	Carlos Avery WMA
MNS1002	Minnesota	Great River Bluffs State Park
MNA1003	Minnesota	William O' Brien State Park
NCS1001	North Carolina	Ashville
PAS1001	Pennsylvania	Philadelphia
SDS1001	South Dakota	Freeman
WIS1001	Wisconsin	Burlington

[Z] The first two letters in the isolate number are abbreviations for the state collected from and the third letter represents whether the host plant was symptomatic or asymptomatic (S = symptomatic, A = asymptomatic).

Table 5.2. Statistical summary for phylogenetic analysis of the four different gene regions.

Cryptodiaporthe corni Analysis

	Bt1	Bt2	ITS
Aligned Characters	442	384	474
Constant Characters	378	283	383
Parsimony Uninformative Characters	2	5	2
Parsimony Informative Characters	62	96	89
Tree Length	73	108	95
Consistency Index (CI)	0.9589	0.9815	1.0000
Homoplasy Index (HI)	0.0411	0.0185	0.0000
Retention Index (RI)	0.9643	0.9813	1.0000
Rescaled Consistency Index (RC)	0.9247	0.9631	1.0000

Cryphonectriaceae Analysis

	Bt1	Bt2	ITS
Aligned Characters	453	426	547
Constant Characters	263	168	279
Parsimony Uninformative Characters	6	22	30
Parsimony Informative Characters	184	236	238
Tree Length	768	836	782
Consistency Index (CI)	0.5065	0.6136	0.6036
Homoplasy Index (HI)	0.4935	0.3864	0.3964
Retention Index (RI)	0.8551	0.9124	0.8856
Rescaled Consistency Index (RC)	0.4331	0.5599	0.5345

Diaporthales Analysis

	LSU
Aligned Characters	1157
Constant Characters	563
Parsimony Uninformative Characters	22
Parsimony Informative Characters	572
Tree Length	1191
Consistency Index (CI)	0.7229
Homoplasy Index (HI)	0.2771
Retention Index (RI)	0.9650
Rescaled Consistency Index (RC)	0.6976

Figure 5.1. Neighbor-joining tree created from Beta tubulin 1 gene sequences to compare isolates of *Cryptodiaporthe corni*. Confidence levels above 50% based on 1000 bootstrap replications are listed for maximum parsimony (top) and neighbor-joining (bottom). The species for sequences obtained from Genbank are followed by the strain. Two isolates of *Diaporthe ambigua* were used as an outgroup.

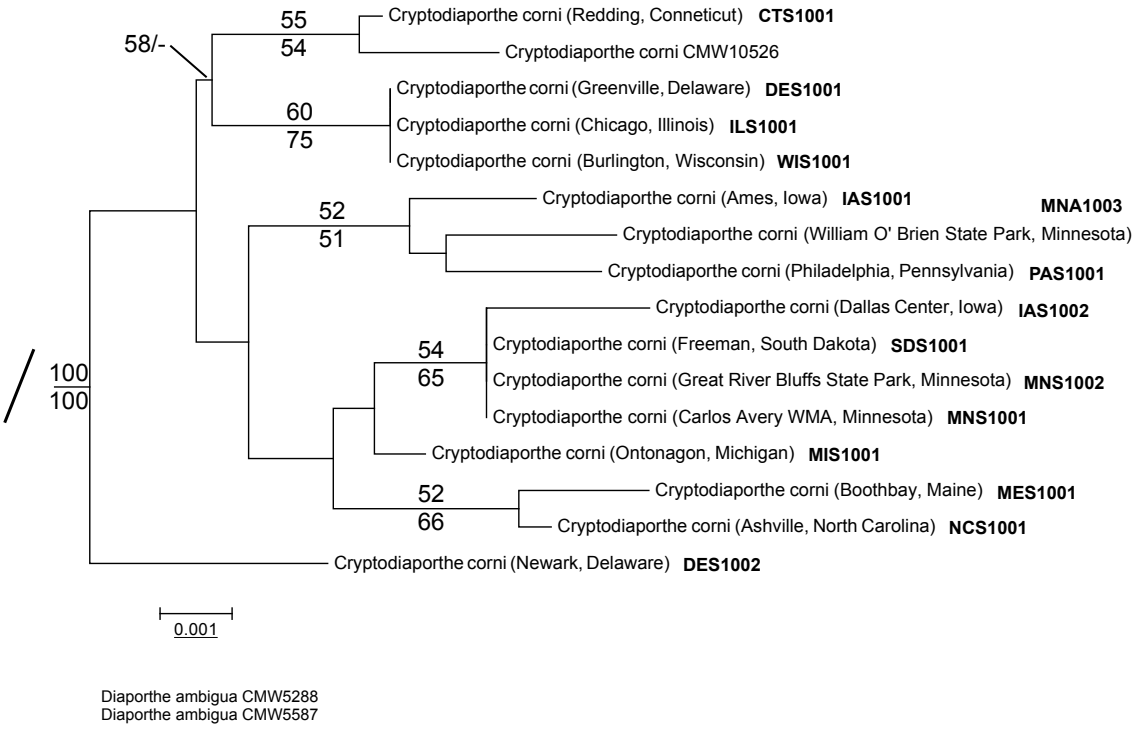


Figure 5.2. Neighbor-joining tree created from Beta tubulin 2 gene sequences to compare isolates of *Cryptodiaporthe corni*. Confidence levels above 50% based on 1000 bootstrap replications are listed for maximum parsimony (top) and neighbor-joining (bottom). The species for sequences obtained from Genbank are followed by the strain. Two isolates of *Diaporthe ambigua* were used as an outgroup.

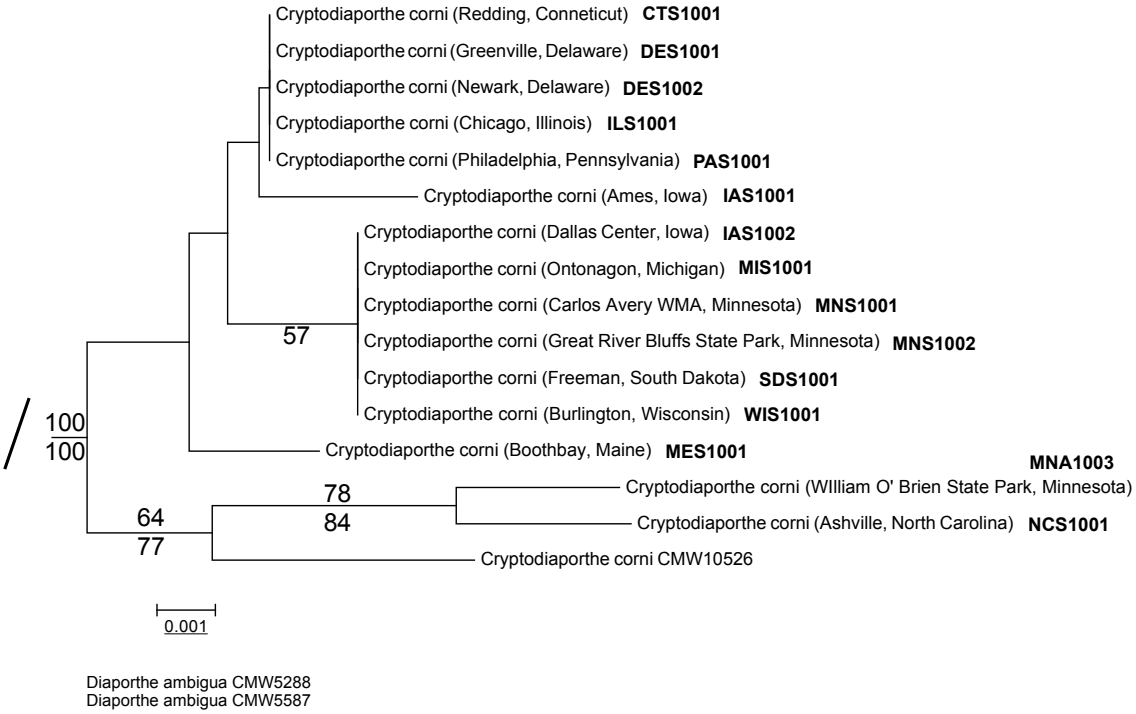


Figure 5.3. Neighbor-joining tree created from ITS gene sequences to compare isolates of *Cryptodiaporthe corni*. Confidence levels above 50% based on 1000 bootstrap replications are listed for maximum parsimony (top) and neighbor-joining (bottom). The species for sequences obtained from Genbank are followed by the strain. Two isolates of *Diaporthe ambigua* were used as an outgroup.

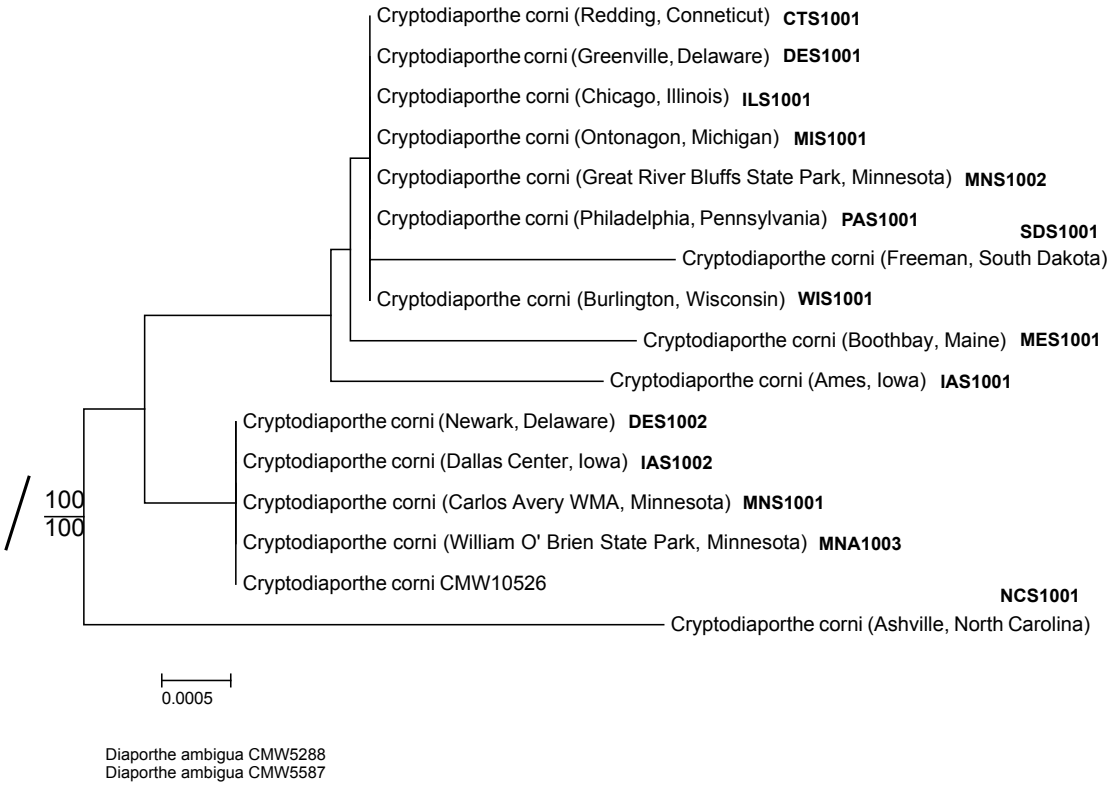


Figure 5.4. Neighbor-joining tree created from Beta tubulin 1 gene sequences to compare isolates in the Cryphonectriaceae family. Confidence levels 70% and above based on 1000 bootstrap replications are listed for maximum parsimony (top) and neighbor-joining (bottom). The species for sequences obtained from Genbank are followed by the strain. Two isolates of *Diaporthe ambigua* were used as an outgroup.

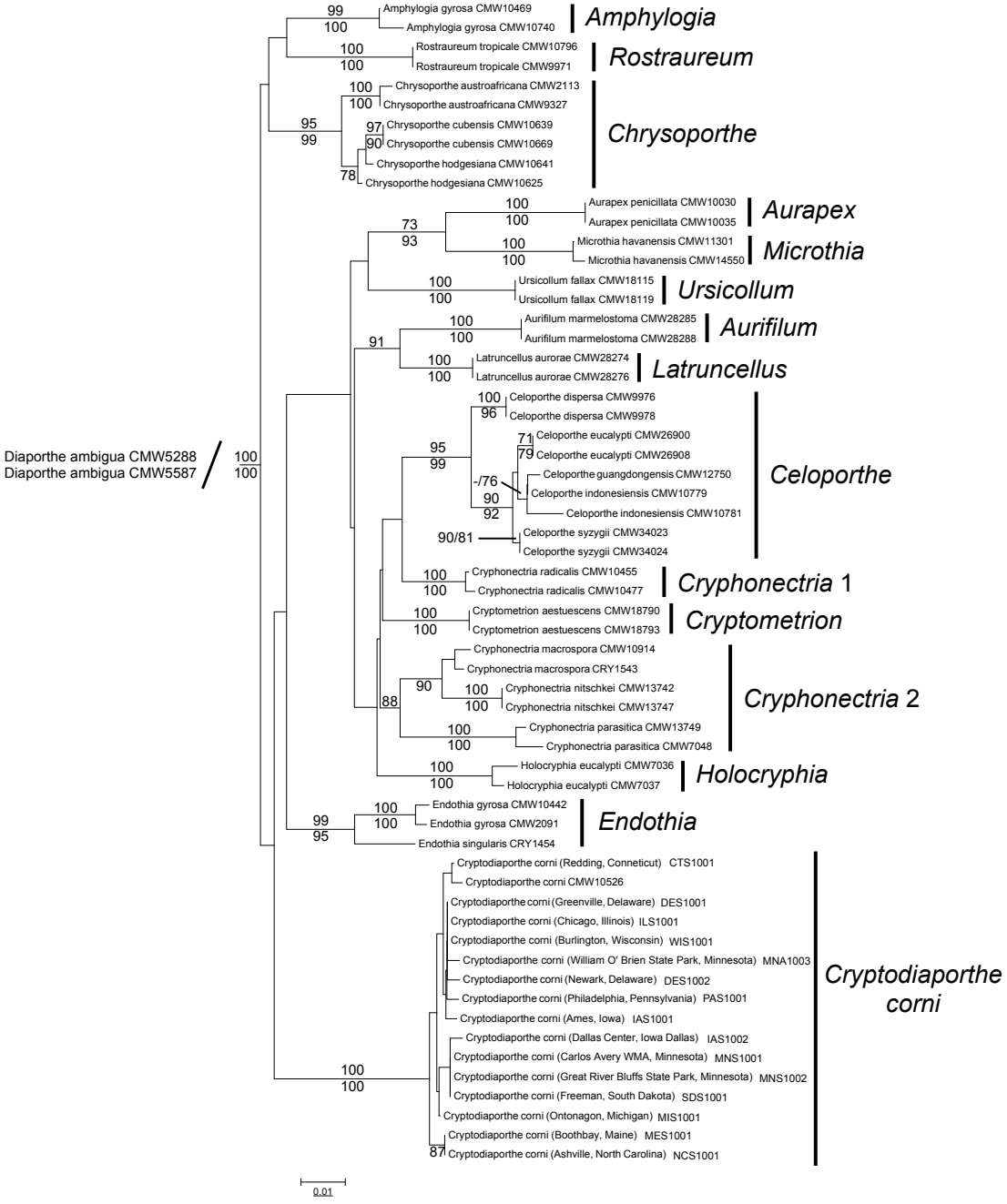


Figure 5.5. Neighbor-joining tree created from Beta tubulin 2 gene sequences to compare isolates in the Cryphonectriaceae family. Confidence levels 70% and above based on 1000 bootstrap replications are listed for maximum parsimony (top) and neighbor-joining (bottom). The species for sequences obtained from Genbank are followed by the strain. Two isolates of *Diaporthe ambigua* were used as an outgroup.

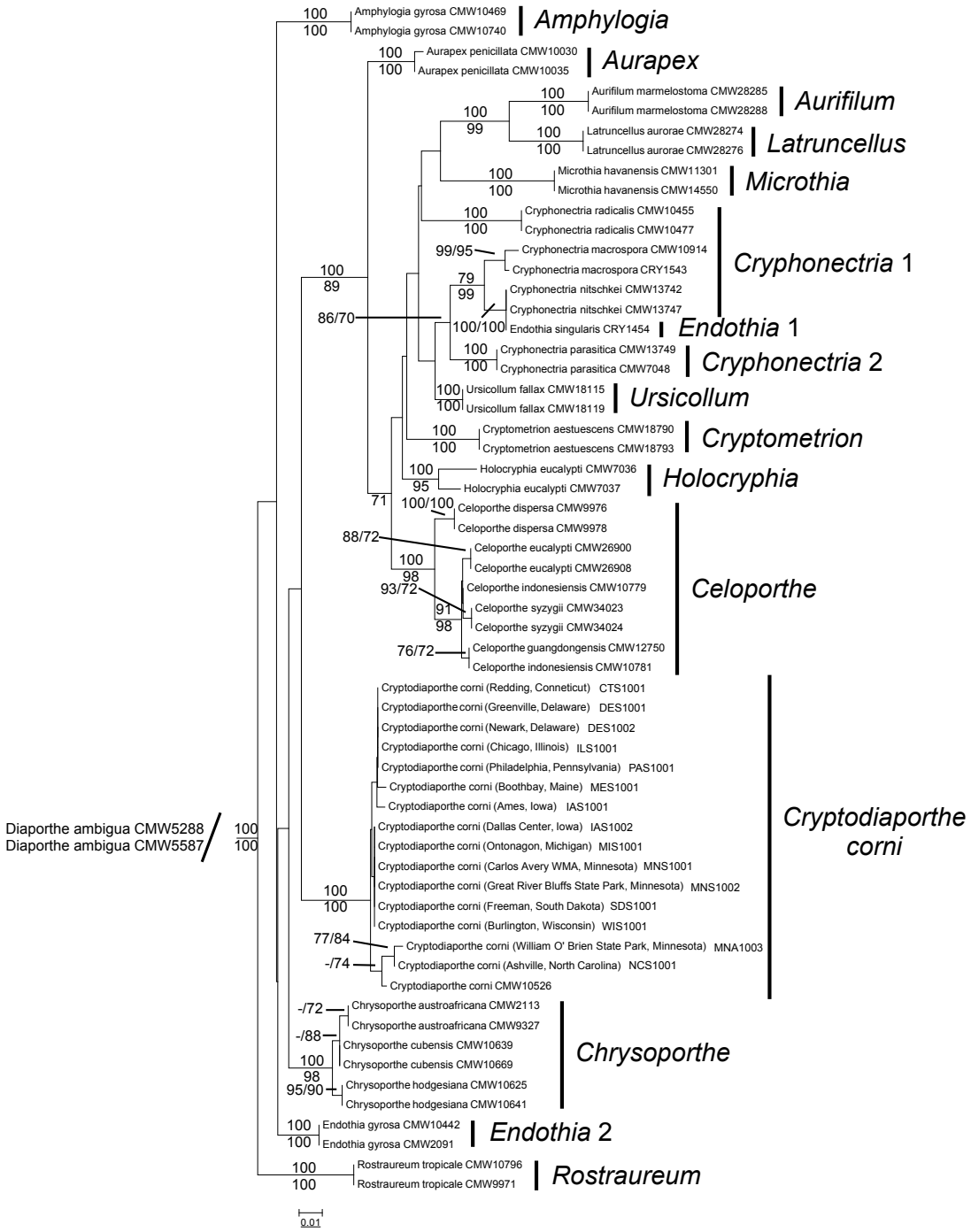


Figure 5.6. Neighbor-joining tree created from ITS gene sequences to compare isolates in the Cryphonectriaceae family. Confidence levels 70% and above based on 1000 bootstrap replications are listed for maximum parsimony (top) and neighbor-joining (bottom). The species for sequences obtained from Genbank are followed by the strain. Two isolates of *Diaporthe ambigua* were used as an outgroup.

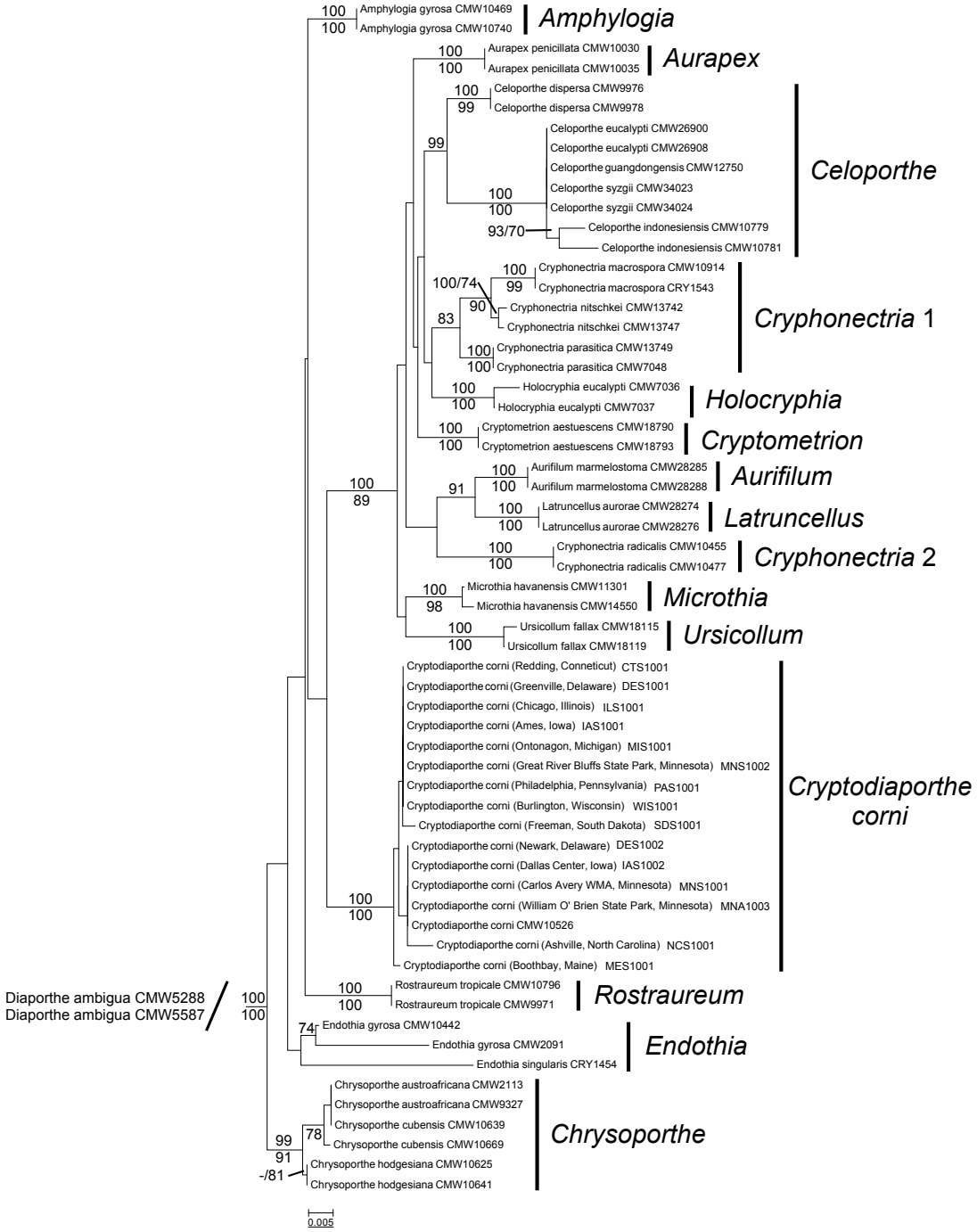
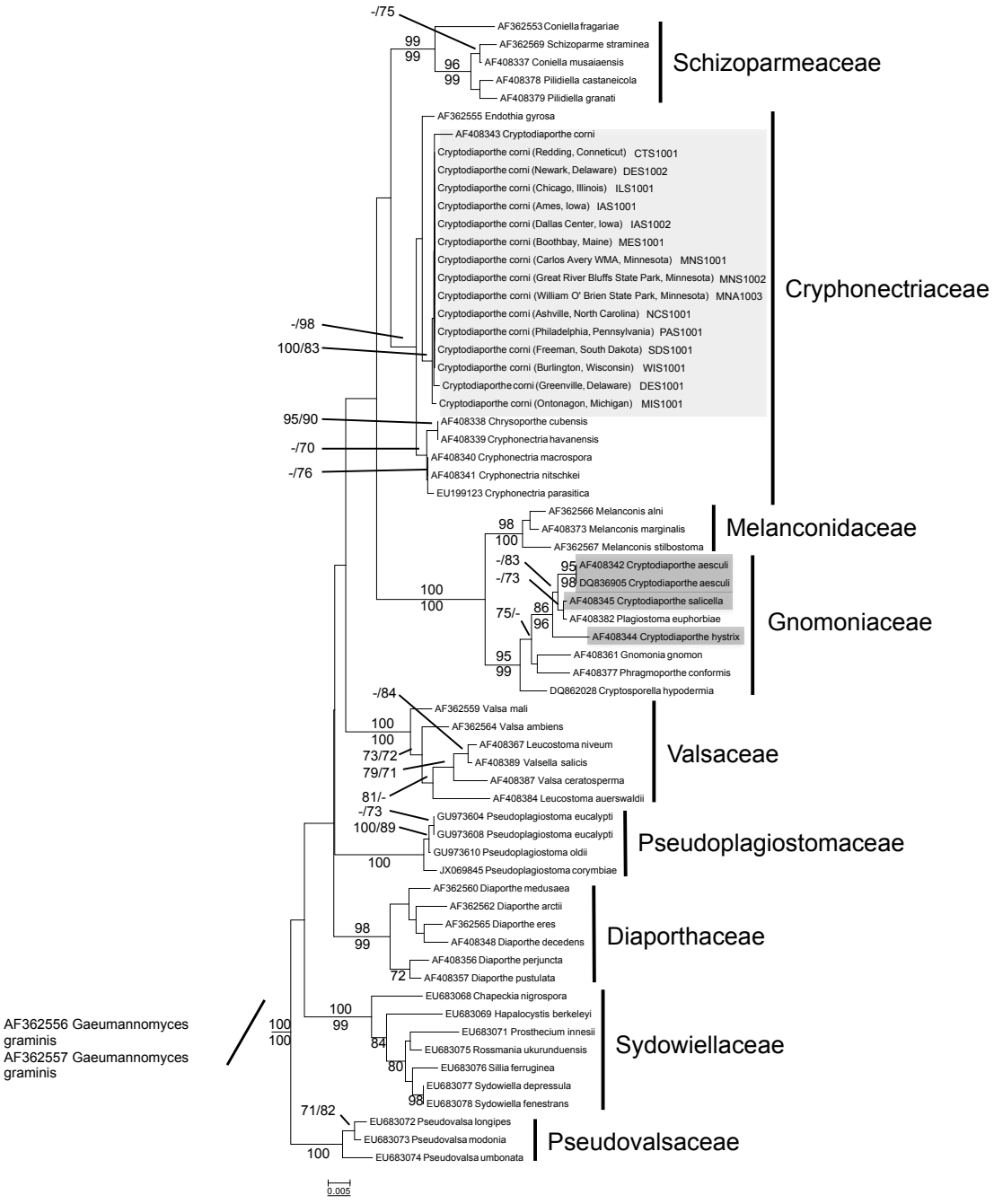


Figure 5.7. Neighbor-joining tree created from LSU gene sequences to compare isolates in the Diaporthales. Confidence levels 70% and above based on 1000 bootstrap replications are listed for maximum parsimony (top) and neighbor-joining (bottom). Isolates of *Cryptodiaporthe corni* are highlighted in light gray and other species of *Cryptodiaporthe* are highlighted in dark gray. The species for sequences obtained from Genbank are preceded by the accession number. Two isolates of *Gaeumannomyces graminis* were used as an outgroup.



Bibliography

Addicott, F.T. 1982. Abscission. University of California Press, Berkeley, CA.

Appel, D.N. and Stipes, R.J. 1984. Canker expansion on water-stressed pin oaks colonized by *Endothia gyrosa*. Plant Disease 68: 851-853.

Arenz, B. and Blanchette, R.A. 2009. Investigations of fungal diversity in wooden structures and soils at historic sites on the Antarctic Peninsula. Canadian Journal of Microbiology 55: 46-56.

Bachi, P.R. and Peterson, J.L. 1985. Enhancement of *Sphaeropsis sapinea* stem invasion of pines by water deficits. Plant Disease 69: 798-799.

Bagga, D.K. and Smalley, E.B. 1974a. The development of Hypoxylon canker of *Populus tremuloides* role of interacting environmental factors. Phytopathology 64: 658-662.

Bagga, D.K. and Smalley, E.B. 1974b. Variation of *Hypoxylon pruinatum* in cultural morphology and virulence. Phytopathology 64: 663-667.

Ball, J. 2010. Grant County (tar spot and golden canker on pagoda dogwood). South Dakota State University. Pest Update 8: 25.

Barr, M.E. 1978. The Diaporthales in North America with emphasis on Gnomonia and its segregates. Mycologia Memoirs 7: 1-232.

Begoude, A.D.B., Gryzenhout, M., Wingfield, M.J., and Roux, J. 2010. *Aurifilum*, a new fungal genus in the Cryphonectriaceae from *Terminalia* species in Cameroon. Antonie van Leeuwenhoek 98: 263-278.

Behnke, M. 1980. General resistance to late blight of *Solanum tuberosum* plants regenerated from callus resistant to culture filtrates of *Phytophthora infestans*. Theory of Applied Genetics 56: 151-152.

Bennett, A.R. and Hindal, D.F. 1989. Mycelial growth and oxalate production by five strains of *Cryphonectria parasitica* in selected liquid culture media. Mycologia 81: 554-560.

Blodgett, J.T., Kruger, E.L., and Stanosz, G.R. 1997. Effects of moderate water stress on disease development by *Sphaeropsis sapinea* on red pine. Phytopathology 87: 422-428.

Borras, O., Santos, R., Matos, A.P., Cabral, R.S., and Arzola, M. 2001. A first attempt to use a *Fusarium subglutinans* culture filtrate for the selection of pineapple cultivars resistant to fusariose disease. Plant Breeding 120: 435-438.

Capretti, P. & Battisti, A. 2007. Water stress and insect defoliation promote the colonization of *Quercus cerris* by the fungus *Biscogniauxia mediterranea*. Forest Pathology 37: 129-135.

Castlebury, L.A., Rossman, A.Y., Jaklitsch, W.J., and Vasilyeva, L.N. 2002. A preliminary overview of the Diaporthales based on large subunit nuclear ribosomal DNA sequences. Mycologia 94: 1017-1031.

Chapela, I.H. and Boddy, L. 1988. Fungal colonization of attached beech branches. II. Spatial and temporal organization of communities arising from latent invaders in bark and functional sapwood, under different moisture regimes. New Phytologist 110: 47-57.

Cheewangkoon, R., Groenewald, J.Z., Verkley, G.J.M., Hyde, K.D., Wingfield, M.J., Gryzenhout, M., Summerell, B.A., Denman, S., Toanun, C., and Crous, P.W. 2010. Re-evaluation of *Cryptosporiopsis eucalypti* and *Cryptosporiopsis*-like species occurring on Eucalyptus leaves. Fungal Diversity 44: 89-105.

Chen, S.F., Gryzenhout, M., Roux, J., Xie, Y., Wingfield, M.J., and Zhou, X. 2011. Novel species of *Celoporthe* from *Eucalyptus* and *Syzygium* trees in China and Indonesia. Mycologia 103: 1384-1410.

Cullina, W. 2002. Native trees, shrubs, & vines: a guide to using, growing, and propagating North American woody plants. Houghton Mifflin Harcourt, Boston, MA 98.

Desprez-Loustau, M.L., Marcais, B., Nageleise, L.M., Piou, D., and Vannini, A. 2006. Interactive effects of drought and pathogens in forest trees. Annual Forest Science 63: 597-612.

Dirr, M.A. 1998. Manual of woody landscape plants, their identification, ornamental characteristics, culture, propagation, and uses, 5th Ed. Stipes Publishing LLC.

Farris, J.S., Kallersjo, M., Kluge, A.G., and Bult, C. 1994. Testing significance of incongruence. Cladistics 10: 315-319.

Fazio, J.R. 2011. How to prune young shade trees. Arbor Day Foundation, Nebraska City, NE. Tree City USA Bulletin 1.

Flowers, J., Nuckles, E., Hartman, J., and Vaillancourt, L. 2001. Latent infection of Austrian and Scots pine tissues by *Sphaeropsis sapinea*. Plant Disease 85: 1107-1112.

Gandolfi, A., Bonilauri, P., Rossi, V., and Menozzi, P. 2001. Intraindividual and intraspecies variability of ITS1 sequences in the ancient asexual *Darwinula stevensoni* (Crustaceae: Ostracoda). Heredity 87: 449-455.

Glass, N.L. and Donaldson, G.C. 1995. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous Ascomycetes. Applied Environmental Microbiology 61: 1323-1330.

Godoy, G., Steadman, J.R., Dickman, M.B., and Dam, R. 1990. Use of mutants to demonstrate the role of oxalic acid in pathogenicity of *Sclerotinia sclerotiorum* on *Phaseolus vulgaris*. Physiological and Molecular Plant Pathology 37: 179-191.

Grabowski, M. 2009. Golden canker on pagoda dogwood. University of Minnesota. Yard and Garden News.

Gremmen, J. 1978. Research on dothichiza-bark necrosis (*Cryptodiaporthe populea*) in poplar. European Journal of Forest Pathology 8: 362-368.

Gryzenhout, M., Tarigan, M., Clegg, P.A., and Wingfield, M.J. 2010. *Cryptometrion aestuescens* gen. sp. nov. (Cryphonectriaceae) pathogenic to Eucalyptus in Indonesia. Australasian Plant Pathology 39: 161-169.

Gryzenhout, M., Myburg, H., Wingfield, B.D., and Wingfield, M.J. 2006. Cryphonectriaceae (Diaporthales), a new family including *Cryphonectria*, *Chrysoporthe*, *Endothia*, and allied genera. Mycologia 98: 239-249.

Gryzenhout, M., Wingfield, B.D., and Wingfield, M.J. 2009. Taxonomy, phylogeny, and ecology of bark-inhabiting and tree-pathogenic fungi in the Cryphonectriaceae. The American Phytopathological Society, St. Paul, MN.

Hoegger, P.J., Rigling, D., Holdenrieder, O., and Heiniger, U. 2002. *Cryphonectria radicalis*: Rediscovery of a lost fungus. Mycologia 94: 105-115.

Hudelson, B. 2004. Golden canker (*Cryptodiaporthe corni*). University of Wisconsin. Extension Bulletin, XHT1125.

Hyde, K.D. and Soytong, K. 2008. The fungal endophyte dilemma. Fungal Diversity 33: 163-173.

Jacobi, W.R. 1984. Optimal conditions for in vitro growth, asexual spore release, and germination of *Thyronectria austro-americana*. Phytopathology 74: 566-569.

Jacobi, W.R. 1989. Resistance of honeylocust cultivars to *Thyronectria austro-americana*. Plant Disease 73: 805-807.

Johnson, J.A. and Whitney, N.J. 1989. An investigation of needle endophyte colonization patterns with respect to height and compass direction in a single crown of balsam fir (*Abies balsamea*). Canadian Journal of Botany 67: 723-725.

Korabecna, M., Liska, V., and Fajfrlik, K. 2003. Primers ITS1, ITS2, and ITS4 detect the intraspecies variability in the internal transcribed spacers and 5.8S rRNA gene region in clinical isolates of fungi. Folia Microbiology 48: 233-238.

Kotze, J.J. 1935. Forest fungi: The position in South Africa. British Empire Forestry Conference 1935. Government Printer, Pretoria.

Kuo, M.S., Yoder, O.C., and Scheffer, R.P. 1970. Comparative specifity of the toxin of *Helminthosporium carbonum* and *Helminthosporium victoriae*. Phytopathology 60: 365-368.

Laughton, E.M. 1937. The incidence of fungal disease on timber trees in South Africa. South African Journal of Science 33: 589-604.

Lloyd, F.E. 1914. Injury and abscission in *Impatiens sultani*. Sixth Annual Report of the Quebec Society for the Protection of Plants 72-79

Lloyd, F.E. 1916. Abscission in *Mirabilis jalapa*. Botanical Gazette 61: 213-230.

Luckhoff, H.A. 1964. Diseases of exotic plantation trees in the Republic of South Africa. FAU/IUFRO Symposium on Internationally Dangerous Forest Diseases and Insects-Oxford 1964.

Micales, J.A. and Stipes, R.J. 1987. A reexamination of the fungal genera *Cryphonectria* and *Endothia*. Phytopathology 77: 650-654.

Mostert, L., Crous, P.W., and Petrini, O. 2000. Endophytic fungi associated with shoots and leaves of *Vitis vinifera*, with specific reference to the *Phomopsis viticola* complex. Sydowia 52, 46-58.

Nilsson, R.H., Kristiansson, E., Ryberg, M., Hallenberg, N., and Larsson, K.H. 2008. Intraspecific ITS variability in the kingdom Fungi as expressed in the international sequence databases and its implications for molecular species identification. Evolutionary Bioinformatics 4: 193-201.

Old, K.M., Gibbs, R., Craig, I., Myers, B.J., and Yuan, Z.Q. 1990. Effect of drought and defoliation on the susceptibility of Eucalypts to cankers caused by *Endothia gyrosa* and *Botryospaeria ribis*. Australian Journal of Botany 38: 571-581.

Pataky, N. 2010. *Cryptodiaporthe* canker of pagoda dogwood. University of Illinois. Home, Yard & Garden Pest Newsletter, Issue 7.

Petrak, F. 1921. Mykologische notizen. II. Annales Mycologici 19: 17-128.

Petrini, O. 1991. Fungal endophyte in tree leaves. In: Microbial ecology of leaves (eds. J.H. Andrews and S.S. Hirano) Springer, New York: 179-197.

Pijut, P.M., Domir, S.C., Lineberger, R.D., and Schreiber, L.R. 1990. Use of culture filtrates of *Ceratocystis ulmi* as a bioassay to screen for disease tolerant *Ulmus americana*. Plant Science 70: 191-196.

Pukacki, P.M. and Przybyl, K. 2005. Frost injury as a possible inciting factor in bud and shoot necrosis of *Fraxinus excelsior* L. Journal of Phytopathology 153: 512-516.

Punithalingam, E. and Booth, C. 1977. A new species of *Cryptodiaporthe* on pimento. Kew Bulletin 31: 465-467.

Ramsfield, T.D., Ball, R.D., Gardner, J.F., and Dick, M.A. 2010. Temperature and time combinations required to cause mortality of a range of fungi colonizing wood. Canadian Journal of Plant Pathology 32: 368-375.

Redlin, S.C., Rossman, A.Y. 1991. *Cryptodiaporthe corni* (Diaporthales), cause of *Cryptodiaporthe* canker of pagoda dogwood. Mycologia 83: 200-209

Redlin, S.C., Stack, R.W. 2001. *Cryptodiaporthe* canker of pagoda dogwood in North Dakota. Plant Disease 85: 1290.

Rehner, S.A. and Samuels, G.J. 1994. Taxonomy and phylogeny of *Gliocladium* analyzed from nuclear large subunit ribosomal DNA sequences. Mycology Research 98: 625-634.

Rossman, A.Y., Farr, D.F., and Castlebury, L.A. 2007. A review of the phylogeny and biology of the Diaporthales. Mycoscience 48: 135-144.

Schoeneweiss, D.F. 1975. Predisposition, stress, and plant disease. Annual Review of Phytopathology 13: 193-211.

Schoeneweiss, D.F. 1981. The role of environmental stress in diseases of woody plants. Plant Disease 65: 308-314.

Seixas, C.D.S., Barreto, R.W., Alfenas, A.C., and Ferreira, F.A. 2004. *Cryphonectria cubensis* on an indigenous host in Brazil: a possible origin for Eucalyptus canker disease. Mycologist 18: 39-45.

Shearer, B.L., Fairman, R.G., and Bathgate, J.A. 1995. *Cryptodiaporthe melanocraspeda* canker as a threat to *Banksia coccinea* on the south coast of western Australia. Plant Disease 79: 637-41.

Sieber, T.N., Sieber-Canavesi, F., and Dorworth, C.E. 1990. Simultaneous stimulation of endophytic *Cryptodiaporthe hystrix* and inhibition of *Acer macrophyllum* callus in dual culture. Mycologia 82: 569-575.

Sinclair, W.A. and Lyon, H.H. 2005. Diseases of trees and shrubs, second edition. Comstock Publishing, Ithaca, NY.

Sogonov, M.V., Castlebury, L.A., Rossman, A.Y., Mejia, L.C., and White, J.F. 2008. Leaf-inhabiting genera of the Gnomoniaceae, Diaporthales. Studies in Mycology 62: 1-79.

Stanosz, G.R. 1998. The "golden canker" of pagoda dogwood. Wisconsin Department of Natural Resources. Wisconsin Urban and Community Forests 6: 2.

Steiner, L.M. 2011. Landscaping with native plants of Minnesota, 2nd edition. Voyageur Press, Minneapolis, MN 162.

Stermer, B.A., Scheffer, R.P., and Hart, J.H. 1984. Isolation of toxins of *Hypoxylon mammatum* and demonstration of some toxin effects on selected clones of *Populus tremuloides*. Phytopathology 74: 654-658.

Swart, W.J., Conradie, E., Wingfield, M.J., and Venter, W.B. 1992. Effects of water stress on the development of cambial lesions caused by *Cryphonectria cubensis* on *Eucalyptus grandis*. Plant Disease 76: 744-746.

Swart, W.J., Wingfield, M.J., and Knox-Davies, P.S. 1987. Factors associated with *Sphaeropsis sapinea* infection of pine trees in South Africa. Phytophylactica 19: 505-510.

Swofford, D.L. 2000. PAUP*: Phylogenetic analysis using parsimony (*and other methods). Version 4.01 b. Sunderland, Massachusetts: Sinauer Associates.

Takai, S. and Richards, W.C. 1978. Cerato-ulmin, a wilting toxin of *Ceratocystis ulmi*: Isolation and some properties of cerato-ulmin from the culture of *C. ulmi*. Phytopathology 91: 129-146.

Takai, S., Richards, W.C., and Stevenson, K.J. 1983. Evidence for the involvement of cerato-ulmin, the *Ceratocystis ulmi* toxin, in the development of Dutch elm disease. Physiological Plant Pathology 23: 275-280.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S. 2011. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Molecular Biology and Evolution 28: 2731-2739.

Tawaraya, K., Hashimoto, K., and Wagatsuma, T. 1998. Effect of root exudate fractions from P-deficient and P-sufficient onion plants on root colonisation by the arbuscular mycorrhizal fungus *Gigaspora margarita*. Mycorrhiza 8: 67-70.

USDA, Accessed 2013. *Cornus alternifolia* L. f. alternate leaf dogwood. United States Department of Agriculture. http://plants.usda.gov/java/profile?symbol=COAL2&mapType=nativity&photoID=coal2_002_ahp.tif

USGS, Accessed 2012. Native range of *Cornus alternifolia*. United States Geological Survey. http://esp.cr.usgs.gov/data/atlas/little/cornalte.pdf

Verhoeff, K. 1974. Latent infections by fungi. Annual Review of Phytopathology 12: 99-110.

Vermeulen, M., Gryzenhout, M., Wingfield, M.J., and Roux, J. 2011. New records of the Cryphonectriaceae from southern Africa including *Latruncellus aurorae* gen. sp. nov. Mycologia 103: 554-569.

Vilgalys, R. and Hester, M. 1990. Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several *Cryptococcus* species. Journal of Bacteriology 172: 4238-4246.

Wegulo, S. and Gleason, M. 2001. Fungal cankers of trees. Iowa State University. Pest Management, 5-1.

Wehmeyer, L.E. 1933. The genus *Diaporthe* Nitschke and its segregates. Ann Arbor, Michigan: University of Michigan.

Wennstrom, A. 1994. Endophyte: The misuse of an old term. Oikos 71, 535-536.

Whitaker, V.M., Hokanson, S.C., and Bradeen, J. 2007. Distribution of rose black spot (*Diplocarpon rosae*) genetic diversity in Eastern North America using amplified fragment length polymorphism and implications for resistance screening. Journal of American Society of Horticultural Science 132: 534-540.

White, I.G. 1955. Toxin production by the oak wilt fungus, *Endoconidiophora fagacearum*. American Journal of Botany 42: 8: 759-764.

White, T.J., Bruns, T., Lee, S., Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis, M.A., Gelfand, D.H., Sninsky, J.J., White, T.J., eds. PCR protocols: a guide to methods and applications. San Diego: Academic Press, 315-322.

Wilson, D. 1995. Endophyte – the evolution of a term, and clarification of its use and definition. Oikos 73: 274-276.

Wingfield, M.J. and Knox-Davis, P.S. 1980. Observations on diseases in pine and Eucalyptus plantations in South Africa. Phytophylactica 12: 57-63.