2007

THE ROLE OF SHEARING IN THE TRANSMISSION OF DIPLODIA PINEA IN SCOTS PINE CHRISTMAS TREES IN KENTUCKY

Amy Bateman
University of Kentucky, ambate2@uky.edu

ABSTRACT OF THESIS

THE ROLE OF SHEARING IN THE TRANSMISSION OF *DIPLODIA PINEA* IN SCOTS PINE CHRISTMAS TREES IN KENTUCKY

Diplodia tip blight is an important disease of pines, especially Scots pine Christmas trees in Kentucky. The hypothesis for my thesis work was that *D. pinea* could be acquired and transmitted on the tools during annual shearing of the Christmas trees. Samples taken from tools after shearing on two different Christmas tree farms in Kentucky in 2005 and 2006 yielded *D. pinea* colony forming units, but in very low quantities; typically less than 10 CFUs per collection. Diplodia-associated dieback from the sheared tips was never found in the field, suggesting that transmission and subsequent infections were not occurring via these sheared tips. Controlled infections indicated that a minimum of 100 spores was necessary to create symptomatic infections on sheared tips. Lysol® Disinfectant Spray did not remove *D. pinea* from tools when sprayed on them after shearing, but it did effectively prevent spore germination *in vitro*. Observations of Diplodia lesion development on one Scots pine Christmas tree farm in Kentucky during the springs of 2006 and 2007 suggested that *D. pinea* infections occurred primarily via the bases of needle bundles on elongating shoots. The most likely source of inoculum was dead infested pine tissues within and beneath the canopy. The use of a protectant fungicide may have resulted in an observed dramatic decrease of disease on this farm.

KEYWORDS: *Sphaeropsis sapinea, Pinus sylvestris,* Shearing, Fungal Transmission, Disinfestant

Multimedia Elements Used: JPEG (.jpg)

Amy Bateman

October 8, 2007

THE ROLE OF SHEARING IN THE TRANSMISSION OF *DIPLODIA PINEA* IN SCOTS PINE CHRISTMAS TREES IN KENTUCKY

By

Amy Bateman

Lisa Vaillancourt
Co-Director of Thesis

John Hartman
Co-Director of Thesis

Lisa Vaillancourt
Director of Graduate Studies

October 8, 2007

RULES FOR USE OF THESES

Unpublished theses submitted for the Master's degree and deposited in the University of Kentucky Library are as a rule open for inspection, but are to be used only with due regard to the rights of the authors. Bibliographical references may be noted, but quotations or summaries of parts may be published only with the permission of the author, and with the usual scholarly acknowledgments.

Extensive copying or publication of the thesis in whole or in part also requires the consent of the Dean of the Graduate School of the University of Kentucky.

A library that borrows this thesis for use by its patrons is expected to secure the signature of each user.

Name	Date

THESIS

Amy Melissa Bateman

The Graduate School

University of Kentucky

2007

THE ROLE OF SHEARING IN THE TRANSMISSION OF *DIPLODIA PINEA* IN SCOTS PINE CHRISTMAS TREES IN KENTUCKY

THESIS

A thesis submitted in partial fulfillment of the
requirements for the degree of Master of Science in the
College of Agriculture
at the University of Kentucky

By

Amy Bateman

Lexington, Kentucky

Co-Directors: Dr. Lisa Vaillancourt, Associate Professor of Plant Pathology
And: Dr. John Hartman, Extension Professor of Plant Pathology

Lexington, Kentucky

2007

I dedicate this thesis to my grandparents, Roger and Shirley Gerwitz, and my parents, John and Terry Bateman, for they filled my life with plants, and if it were not for that, I never would have selected this life path.

ACKNOWLEDGMENTS

The following thesis would not have been possible without the insight, support, and contributions of many people. First, I would like to thank my thesis advisors, Dr. Lisa Vaillancourt and Dr. John Hartman, for providing me with the opportunity to branch out and develop a project that would allow me to interact with the people of Kentucky. I would also like to thank my third committee member, Dr. Deborah Hill, for providing me assistance and insight with my research.

I am also very grateful to the Kentucky Christmas tree growers who worked with me over the course of this study. Without their cooperation, I would never have been able to do a project focusing on Christmas trees. I would also like to thank all those who provided me with technical support; Doug Brown for his help and maintenance of my trees in the Quonset hut, Etta Nuckles for her help and support with laboratory equipment and experiments, Rhonda Van Dyke for her statistical consultations, my French exchange students, Celine Moser, Nathalie Goldbert, and Hugues Chopy for their outstanding help while I was collecting samples, and some of my fellow graduate students, Kate Martin and Maria Holdcroft for their assistance in the field.

In addition to those above who helped me, I am grateful to my family and friends for their much-needed support throughout my time at the University of Kentucky. I am indebted to parents, John and Terry Bateman, who always supported my decision to study science and who always told me that no matter how difficult the challenge, to just take it one day at a time.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF FILES

File Name	File Type	File Size
Figure 1-1a.jpg	JPEG image	2 MB
Figure 1-1b.jpg	JPEG image	1.5 MB
Figure 1-1d.jpg	JPEG image	724 KB
Figure 1-1e.jpg	JPEG image	732 KB
Figure 1-1f.jpg	JPEG image	944 KB
Figure 1-2.jpg	JPEG image	1.7 MB
Figure 2-1.jpg	Adobe Photoshop JPEG file	220 KB
Figure 2-2.jpg	JPEG image	732 KB
Figure 2-3a.jpg	JPEG image	668 KB
Figure 2-3b.jpg	JPEG image	668 KB
Figure 2-3c.jpg	JPEG image	712 KB
Figure 2-3d.jpg	JPEG image	728 KB
Figure 2-3e.jpg	JPEG image	720 KB
Figure 2-3f.jpg	JPEG image	656 KB
Figure 2-4a.jpg	JPEG image	824 KB
Figure 2-4b.jpg	JPEG image	724 KB
Figure 2-4c.jpg	JPEG image	712 KB
Figure 2-4d.jpg	JPEG image	784 KB
Figure 2-4e.jpg	JPEG image	792 KB
Figure 2-4f.jpg	JPEG image	692 KB
Figure 2-7a.jpg	Adobe Photoshop JPEG file	268 KB
Figure 2-7b.jpg	JPEG image	724 KB
Figure 2-7c.jpg	JPEG image	548 KB
Figure 2-7d.jpg	JPEG image	216 KB
Figure 2-8a.jpg	Adobe Photoshop JPEG file	700 KB
Figure 2-8b.jpg	JPEG image	616 KB
Figure 2-8c.jpg	JPEG image	2.1 MB
Figure 2-8d.jpg	JPEG image	752 KB
Figure 2-10a.jpg	JPEG image	768 KB
Figure 2-10b.jpg	JPEG image	744 KB
Figure 3-1a.jpg	Adobe Photoshop JPEG file	168 KB
Figure 3-1b.jpg	Adobe Photoshop JPEG file	532 KB
Figure 3-1c.jpg	JPEG image	204 KB
Figure 3-2a.jpg	JPEG image	84 KB
Figure 3-2b.jpg	Adobe Photoshop JPEG file	640 KB
Figure 3-2c.jpg	JPEG image	192 KB
Figure 3-3.jpg	JPEG image	56 KB
Figure 3-4.jpg	JPEG image	32 KB
Figure 3-5.jpg	JPEG image	212 KB
Figure A3-2.jpg	JPEG image	180 KB
Figure A4-1a.jpg	JPEG image	1.2 MB
Figure A4-1b.jpg	JPEG image	1.5 MB

Figure A4-1c.jpg	JPEG image	740 KB
Figure A4-2a.jpg	JPEG image	780 KB
Figure A4-2b.jpg	JPEG image	732 KB
Figure A4-2c.jpg	JPEG image	768 KB
Figure A4-3.jpg	JPEG image	736 KB
Figure A4-4a.jpg	JPEG image	732 KB
Figure A4-4b.jpg	JPEG image	788 KB
Figure A4-5.jpg	JPEG image	760 KB

CHAPTER ONE:

DIPLODIA TIP BLIGHT DISEASE IN KENTUCKY:

INTRODUCTION AND BACKGROUND

Diplodia tip blight (also known as pine or Sphaeropsis tip blight) is an important disease of pines, especially of exotic two-needle pines, worldwide (20, 21, 23, 56, 63). In Kentucky, it has been most damaging on Austrian pines (*Pinus nigra*) and Scots pines (*Pinus sylvestris*) in landscape settings and on Christmas tree plantations. For the past fifteen years, plant pathologists at the University of Kentucky (U.K.) have been surveying and studying the local impact of this disease. In this chapter, I will review these and other prior studies, and discuss their relevance to my Master's thesis project on Diplodia blight of Scots pine Christmas trees. This chapter is adapted from a series of articles I wrote for the Kentucky Pest News in 2007 (5-13).

Diplodia Tip Blight Symptoms

Austrian pines are a popular choice for Kentucky landscape plantings because of their dense, green foliage and symmetrical shape. A healthy group of these trees can form an attractive year-round screen. Tip blight symptoms on Austrian pines first appear on the elongating shoots (candles) in late April or early May. The needles begin to turn a straw color, and to exude droplets of resin, even before they emerge from the needle sheaths (50, 51). Most of the diseased needles are killed before they are fully elongated. Symptoms on Austrian pines typically progress from the shoot tip downward. In less than a week, all of the needles on an infected shoot will typically turn brown and die, and

the shoot will become stunted, necrotic, and brittle from excessive resin production (50) (Figure 1.1.D). The necrotic needles and resin sometimes give the dead tips a gray color.

As the fungus progresses from the branch tip back towards the trunk, older needles become straw colored and die (Figure1.1.D). This generally happens later in the year or during the following year. Continued infection and colonization by the fungus produces branch dieback, and ultimately results in death of the tree (41). Diplodia tip blight symptoms typically occur first in the lower branches of the tree, and progress upward each year (Figure 1.1.B). On landscape Austrian pines in Kentucky, disease symptoms generally begin to appear only after the trees have reached cone-bearing age, typically 12-13 years old.

Diplodia tip blight has been devastating to Austrian pines on the U.K. campus. In 1994, there were 563 Austrian pines on campus, and more than a third of these appeared to be completely healthy, with no symptoms of tip blight (36). By 2007, only 166 Austrian pines remained on campus and most of these trees had severe symptoms of tip blight.

Tip blight disease progresses rapidly, and can kill an Austrian pine tree within only a few years. For example, in 2003 a group of 40 lightly-to-moderately diseased Austrian pines was surveyed on the southeast side of the U.K. campus. By 2006, 18 of these trees were dead, and the rest were so diseased that it was difficult or impossible to observe any asymptomatic shoots. Periodic droughts that have occurred in Kentucky, especially the severe drought of 1999, may have contributed to the rapid progress of the Diplodia tip blight epidemic on the U.K. campus. Prior research has shown that the incidence and severity of tip blight disease increase when trees are stressed, including by

drought (3, 16, 17). Tip blight is killing Austrian pines throughout Kentucky. While traveling, it is easy to spot the dead or dying brown branches of Austrian pines along our roadways. Austrian pines are often chosen for the landscape because they grow so quickly, but unfortunately, Diplodia tip blight disease can damage and kill them even faster.

Scots pines are one of the most popular Christmas tree species in Kentucky, and have been grown here for decades. Scots pines are a particularly desirable species for use as Christmas trees because they are attractive and popular with consumers; the seedlings are inexpensive; short-needled varieties have been developed specifically for Christmas tree use; and Scots pines take less time to grow to a sellable size than other Christmas tree species (43). Unfortunately, Diplodia tip blight has recently become a very serious problem for Scots pine Christmas tree growers in Kentucky. Most Christmas trees are sold before they begin to produce cones. However, in contrast to Austrian pines, on Scots pine Christmas trees Diplodia tip blight typically produces symptoms well before cone-bearing age, including on seedlings that are only one or two years old (Figure 1.2). Especially during the past five years, Diplodia tip blight has devastated the Scots pine Christmas tree industry in Kentucky. In fact, this disease has been so damaging and so difficult to manage that some growers have stopped planting Scots pines. It is not clear why Diplodia tip blight disease has recently become so severe on Scots pine Christmas trees, but the drought that has contributed to the devastation of Austrian pines in local landscapes may also have played an important role here, since most Christmas tree growers do not irrigate their trees.

The symptoms of Diplodia tip blight on Scots pines are different from those on Austrian pines. Instead of blighted, stunted shoot tips, on Scots pine the infected shoots tend to elongate more, and then they curve over, forming a "shepherd's crook" (Figure 1.1.C) (33). The shoot from the tip to the base of the crook dies and becomes straw colored, but the shoot below the crook remains alive and green for at least the first year after infection. Since the same fungus causes Diplodia blight on both Austrian and Scots pines (31), the difference in symptoms suggests that there may be differences in the infection and/or colonization process on these two tree species. As in Austrian pines, tip blight disease will often progress down the branches of Scots pines in subsequent years and kill older needles, as well as cause branch dieback and cankers on the branches and trunk (Figure 1.1.A). Cankers appear to be more common on Scots pines than on Austrian pines, and they often become coated with exuded resin, which dries and produces unsightly white patches and droplets on the bark. Trunk cankers are particularly damaging and will lead ultimately to the death of the tree. However, because of the significant aesthetic damage caused to the foliage by Diplodia tip blight, most infected Christmas trees are removed before the disease kills them.

Taxonomy and Life Cycle of Diplodia pinea

The Deuteromycete fungus *Diplodia pinea* (Desmaz.) J. Kickx fil. causes Diplodia tip blight on Austrian and Scots pines. *D. pinea* also infects numerous other conifer species including *Cedrus* (cedars), *Abies* (firs), *Larix* (tamaracks), *Thuja* (cypresses), *Juniperus* (junipers), *Picea* (spruces), and *Pseudotsuga* (Douglas-fir), but infections of these species are relatively rare in Kentucky. *D. pinea* was identified and

named in 1842 in France (27), and was first reported in Kentucky in the early 1940s (62). The fungus was known as *D. pinea* until 1980, when the name was changed to *Sphaeropsis sapinea*, based on morphological characteristics of the spores (58). In 2003, it was proposed that the name should be changed back to *D. pinea,* based on the phylogenetic relationship of the fungus to other *Diplodia* species (25). The name change is still being discussed and is not yet official, so either name is currently valid.

D. pinea reproduces by forming asexual spores in pycnidia. The spores are ovoid, and range from hyaline to dark brown depending on maturity (58). To some observers, the spores resemble microscopic baking potatoes. The pycnidia are only produced on dead host tissue, and can be seen with the naked eye, appearing like black pepper sprinkled on cone scales and dead needles (Figures 1.1.E and F). It is not uncommon to see dead cones and needles covered with pycnidia littering the ground beneath infected trees. The pycnidia release spores in warm, rainy weather from March through November, although the majority of spores are produced during the spring and summer (18). The spores are dispersed by rain-splash and windblown rain (18).

Austrian and Scots pine shoots are most susceptible to *D. pinea* infection in the spring, when they are elongating and not yet lignified (22, 23, 50, 62). The newly emerging needles can also be infected, and are much more susceptible than the previous year's needles (18, 62). Spores of *D. pinea* germinate and the hyphae penetrate directly into the host tissue (30). Previous investigations had suggested that *D. pinea* infects pine shoots primarily via needle stomata (18), but a histological study done here at U.K. demonstrated that *D. pinea* typically penetrates Austrian pine shoots directly through the shoot epidermis into the unlignified parenchyma tissues below. Infection occurs

primarily at the bases of needle bundles, where spores and hyphae of *D. pinea* collect in the spaces between the needle sheaths and the needles and shoots (30). As the fungus colonizes the host, it quickly kills the host cells, resulting in the typical necrotic, blighted symptoms (30). Resin production is a common defense reaction of pines, although research suggests that, once inside the tree, the fungus is not inhibited by resin (30). After the fungus has killed the host tissue, it produces pycnidia that overwinter and generate spores during the following spring (51). It has been reported that infected windbreak trees are an important source of *D. pinea* inoculum in forest nurseries (49). Windbreak trees could also be a source of *D. pinea* infection on Christmas tree farms. Seedling damage/death could also result if seedlings purchased by Christmas tree growers come from nurseries that are surrounded by infected windbreaks.

D. pinea infections can occur at temperatures ranging from 10-40˚C, with an optimum of between 20-30˚C (23). Adequate leaf wetness (at least 3 hours) and humidity are also necessary for infection (23). *D. pinea* is most active, aggressive, and destructive in the spring in Kentucky. Under optimal conditions, symptoms including necrosis, discoloration, or resin droplets can occur within 24 hours of infection (18, 20). In addition to infecting young, non-lignified tissue, *D. pinea* also commonly infects wounds, such as those caused by hail (24, 26, 52, 53, 63).

Latent D. pinea *Infections*

D. pinea can produce asymptomatic latent infections of many pine species, including Austrian and Scots pine (54, 57). Latent infections are typically detected by culturing the fungus from asymptomatic, surface-disinfested host tissues. This process

takes many days, and also destroys the tissue so that no further studies can be done. An improved method for detection and identification of latent infections was developed at U.K. (29). For this protocol, nested polymerase chain reaction (PCR) is used to isolate and amplify specific portions of DNA that are unique to *D. pinea*. A band of a specific size in the electrophoresis gel is diagnostic for the presence of *D. pinea* in the sample. The entire nested PCR process only takes a few hours. Also, only a very small sample of pine tissue is needed, so the PCR method of detection is less invasive and damaging to the tree. At the same time, it is also highly sensitive and can detect very small amounts of *D. pinea* DNA.

Tissue isolations and the PCR assay were used to study the occurrence and nature of latent infections of Austrian and Scots pines in Lexington landscapes (29, 31). Latent *D. pinea* infections were very common in landscape Austrian and Scots pines in Lexington and on the U.K. campus (29, 31). As the percentage of diseased tips on a tree increased, so did the percentage of latently infected asymptomatic tips. Three-year-old Austrian pines were inoculated in the greenhouse with *D. pinea* isolates that had been recovered either from symptomatic or from asymptomatic tips. Both types were able to produce symptomatic infections on the inoculated pines. In a few cases, inoculation with either type of fungus produced no symptoms, but when the inoculated stems were cultured, they were found to have latent, asymptomatic infections (31). Thus, the determination of whether an infection will be latent or symptomatic apparently does not depend on genetic differences among *D. pinea* isolates, but rather on unknown features of the individual host-pathogen interaction.

Results of dissection studies done at U.K. demonstrated that *D. pinea* could be recovered equally from all of the tissues of symptomatic diseased shoots (bark, phloem, xylem, and pith), but was present mainly in the outer portions (bark and phloem) of asymptomatic, latently infected shoots (31). In further studies, diseased, healthy, and latently-infected Austrian pine shoot segments were fixed in resin, sectioned, stained, and then examined under the microscope. Diseased and asymptomatic shoot samples were collected from elongating, unlignified shoots in May, and asymptomatic samples were also collected from lignified shoots in August and January. Samples were cut in half, and one half was used for microscopy while the other half was assayed by nested PCR to determine if *D. pinea* was present (30). In diseased tissue (PCR-positive, symptomatic), large quantities of fungal hyphae were observed, while in healthy (PCR-negative, asymptomatic) tissue, no hyphae were visible. This correlation suggests that the fungus visible in the diseased tissue was *D. pinea*. In the diseased samples, the host tissues were severely degraded. The necrosis/tissue degradation always preceded fungal colonization, suggesting that the fungus might use toxins to kill pine tissue. In diseased samples, *D. pinea* hyphae could be seen throughout the pine tissue, including within the vascular tissues and pith. This extensive colonization was found even in very mildly symptomatic shoots, which might explain why Austrian pine shoot tips often die so rapidly from tip blight disease.

In latent infections (PCR-positive, asymptomatic) of both elongating and lignified shoot tissue, *D. pinea* was found in localized pockets of degraded cells in the cortex. Tissue damage and fungal hyphae were not found in the xylem and tracheid tissues or in the pith. In a few samples, necrophylactic periderms were observed separating the

degraded colonized cortex from the cambium. These lignified layers were not found in all latent infections, but their precursor may have been present since it cannot be seen with the microscopic procedure used for the study. This result suggests that pine defensive reactions are important in production and maintenance of latent infections. The results also suggest that a suppression of defenses, which is known to occur in stressed pines, might lead to more symptomatic infections or even conversion of previously latent to symptomatic infections if the pathogen is able to breach the defensive barriers (16, 30, 48, 55).

D. pinea could be brought onto Christmas tree farms as undetected latent infections on seedlings that have been produced in infested areas. Most growers do not irrigate their farms, and Kentucky has had numerous droughts over the past 10 years, so newly planted seedlings tend to experience transplant shock and drought stress. Stress is known to exacerbate Diplodia tip blight, and may cause latent infections to "break out" and become symptomatic (16, 55). Seedlings harboring latent infections may become symptomatic quickly, succumbing to the disease while still very young, and produce spores that can be spread throughout the farm.

Management of Diplodia Tip Blight

During the past ten years, Diplodia tip blight has decimated the Austrian pine population in Lexington landscapes and on the U.K. campus. The damage caused by this disease to Scots pines on Christmas tree farms has also been significant, and has the potential to be economically even more devastating than the epidemic on landscape

pines. The Christmas tree industry is based on aesthetics, and so just a few diseased tips can put a significant dent in the profit margin for growers.

Management recommendations for Diplodia tip blight during the 1990s, when the U.K. epidemic began, included application of protectant fungicide sprays during the spring when infection primarily occurs, and aggressive pruning of diseased shoots to remove sources of inoculum (37). The trees on the U.K. campus were pruned and treated with fungicides in accordance with the recommendations, but to no avail, as the trees continued to decline and die (35, 37). Research done at U.K. since the epidemic began may explain the lack of efficacy of these control measures. Protectant fungicides probably do not penetrate down into the crevices between the needle sheaths and shoots where fungal propagules accumulate, and where infection occurs (30). Application of protectant fungicides and pruning visibly diseased shoots would not eliminate the latent fungus present in apparently healthy tissues of the trees (30, 31) It is possible that the latent fungal mycelium can become "active" at a later time (55). In fact, it is possible that aggressive pruning of diseased branches may do more harm than good because removal of so much foliage causes stress, and stress is known to worsen the disease (48). Pruning also causes wounds, and *D. pinea* is known to be an efficient colonizer of wounds (24, 26, 53).

Based on new research findings from U.K. and elsewhere, alternative management practices were investigated by U.K. plant pathologists for control of Diplodia tip blight on landscape Austrian pines. In 1999, a four-year study utilizing systemic fungicide injections of both diseased and healthy Austrian pines on campus was begun. The goal of the study was to determine whether injected fungicides could prevent

new *D. pinea* infections, and eliminate latent infections, or at least prevent them from causing symptomatic infections. The fungicides tested were oxycarboxin (Carboject), debacarb (Fungisol), and tebuconazole (Tebuject). Cambistat (paclobutrazol) is a plant growth regulator that has been reported to act as a weak fungicide (40). In 2003, U.K. plant pathologists also began a three-year study to test the effects of Cambistat application on diseased Austrian pines. Unfortunately, neither injections of fungicides nor treatment with Cambistat were effective for management of Diplodia tip blight (12).

In general, the future for Austrian and Scots pines in Kentucky does not look promising. In the absence of practical, effective management strategies, the main advice that is offered now is not to plant Austrian and Scots pines in Kentucky. This is not very satisfying, given the aesthetic and economic value of these trees, and the general lack of comparable alternatives.

One management tactic that may have some effect is to reduce stress, especially drought stress (16, 55). Unfortunately, drought stress is very common in Kentucky, and has been a particular problem for the past 10 years. Most Christmas tree farms do not routinely irrigate their trees, although they usually water newly planted seedlings. Irrigating the trees might decrease the impact of this disease, but installation of irrigation systems requires a significant capital input, difficult for many of the smaller Christmas tree farms, and irrigating may contribute to other disease problems (e.g. Phytophthora root rot or foliar blights) that could ultimately be just as serious as Diplodia tip blight. Chemical controls (fungicides) may be too expensive to be economically viable for most Christmas tree growers, and their lack of efficacy against Diplodia tip blight on the Austrian pines on campus suggests they wouldn't be very useful in any case.

It is a mystery why tip blight disease infects Scots pine Christmas trees when they are still so young, while it is generally not a problem on Austrian and Scots pines in landscape settings until they bear cones (31). Diplodia tip blight disease would not be as much of a problem for Christmas tree growers if it could be delayed until Scots pines began to bear cones (8-10 years), because most Christmas trees are cut and sold by this time (43). Every summer Scots pine Christmas trees are sheared to create the classic conical Christmas tree shape, and to encourage the development of new buds that will increase the density of the foliage. If the trees have Diplodia tip blight symptoms, it is possible that spores and/or fungal hyphae could be transmitted to wounded, uninfected tips on the same or other trees on the blades of the tools during the shearing process. In the absence of visible symptoms, it is also possible that fungal hyphae could be picked up and spread from latent infections. Perhaps this explains the occurrence of tip blight disease symptoms on younger trees in Christmas tree farms. This question has never been rigorously investigated, but because it is known that other diseases can be carried on pruning tools (1, 4, 28), it has been recommended that growers disinfest their shearing tools with bleach or alcohol (37). Very few growers actually follow this recommendation due to the inconvenience and time required, and because of the potential damage to tools. Could the tip blight epidemic on Christmas tree farms be related to shearing, and could disinfesting tools reduce the severity and incidence of tip blight disease? The major goal of my Master's thesis work was to address these questions. Since there were few data available, and none for our local area, I also set out to survey tip blight disease progress in local Scots pine Christmas tree farms, and to try to understand how and when the

disease develops on these trees, with the ultimate goal of developing improved management recommendations for our local Christmas tree growers.

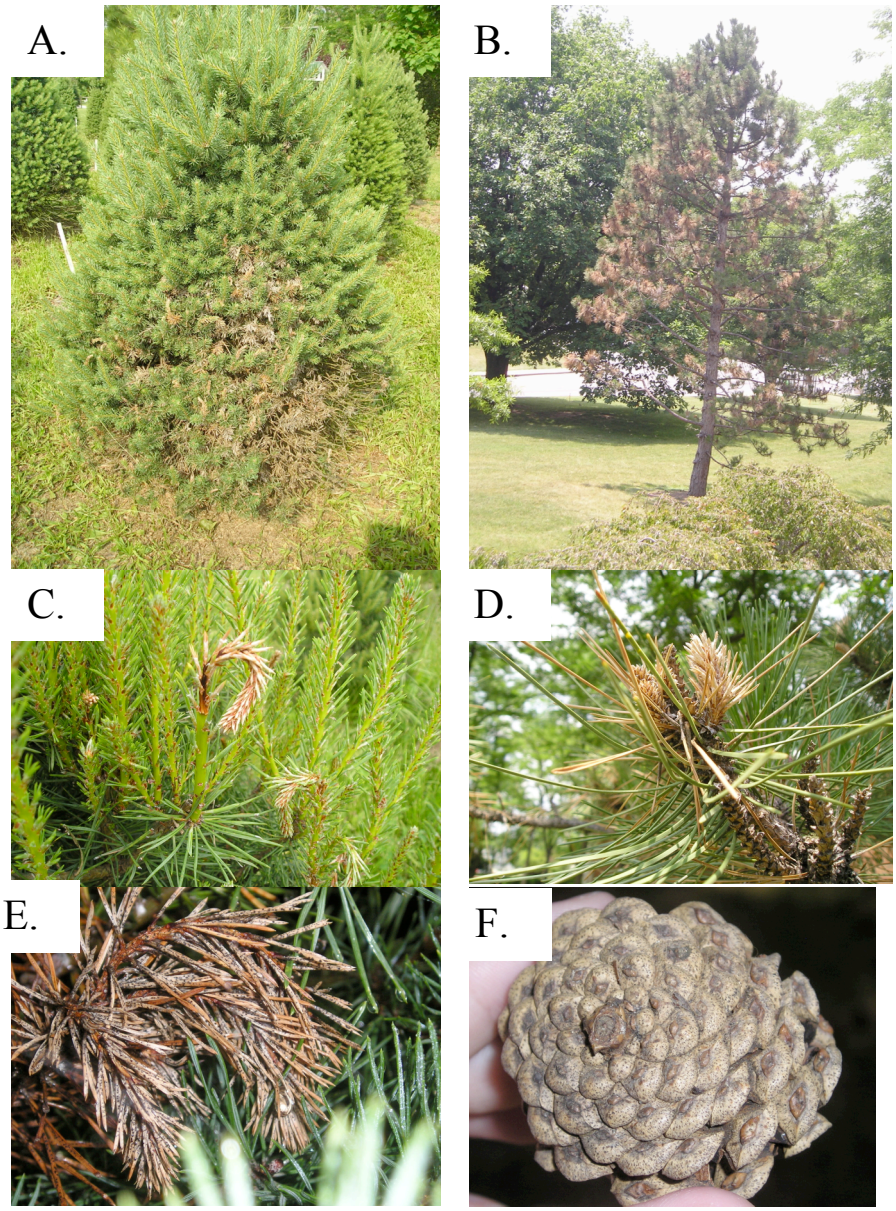


Figure 1.1. *Diplodia pinea* infections on Scots (*Pinus sylvestris*) and Austrian (*Pinus nigra*) pines. **A.** Damage caused by *D. pinea* on a Scots pine Christmas tree. **B.** Damage caused by *D. pinea* on an Austrian pine on the University of Kentucky campus. **C.** Shepherd's crooking on a Scots pine. Only the tissue from the base of the lesion to the shoot apex is dead, with stunted needles. **D.** Shoot and needle stunting on an Austrian pine. The entire new shoot is stunted and dead, and the damage extends to some of last year's needles. **E.** Pycnidia on a dead Scots pine shoot. The shoot died during the spring from tip blight, and by the following September it was covered in pycnidia. The pycnidia shown here are swollen and exuding spores due to exposure to water. **F.** Pycnidia on a 3-year-old Austrian pinecone. The pycnidia here are not swollen because they were dry.

Figure 1.2. Diplodia tip blight on a Scots pine Christmas tree seedling.

CHAPTER TWO:

SCOTS PINE SHOOT PHENOLOGY AND THE DEVELOPMENT OF DIPLODIA TIP BLIGHT DISEASE ON SCOTS PINE CHRISTMAS TREES IN KENTUCKY

Introduction

Scots (also known as Scotch) pines are one of the most popular Christmas tree species in Kentucky. More than 75% of Kentucky Christmas tree farms grow Scots pines, and they have been grown here for decades (42). Scots pines are a particularly desirable species for use as Christmas trees because they are attractive and popular with consumers; the seedlings are inexpensive; short-needled varieties have been developed specifically for Christmas tree use; the trees grow well even on relatively poor sites; and Scots pines take less time to grow to a sellable size than other Christmas tree species (43). Unfortunately, Diplodia tip blight has recently become a very serious problem for Scots pine Christmas tree growers.

Diplodia pinea (Desmaz.) J. Kickx fil. (syn. *Sphaeropsis sapinea* (Fr.:Fr.) Dyko & Sutton in Sutton) is the causal agent of Diplodia tip blight. In Kentucky, this fungus causes serious damage only to Austrian and Scots pines (*Pinus nigra*, and *P. sylvestris*, respectively), both two-needle varieties that are exotic to the region. Diplodia tip blight disease results in necrosis and stunting of elongating shoots, and repeated infections lead to branch dieback and ultimately to death of the tree (18, 20). In an industry based on aesthetics, loss of shoots and branches results in loss of a sellable Christmas tree, even if the tree survives the disease. Unlike on Austrian pines and landscape Scots pines (31), on

Scots pine Christmas trees Diplodia tip blight produces symptoms prior to cone bearing age, and can even cause severe blighting on 1- to 2-year-old seedlings (Figure 1-2).

Diplodia tip blight was first noticed on Kentucky Christmas tree farms a decade ago, but during the past five years it has really devastated the local Scots pine Christmas tree industry (P. Kovalic, personal communication). In fact, this disease has been so damaging and so difficult to manage that some growers have stopped planting Scots pines. It is not clear why Diplodia tip blight disease has recently become such a severe problem.

Since there were few data available, and none for our local area, I decided to survey tip blight disease progress on a local Scots pine Christmas tree farm, to try to understand how and when the disease develops on these trees. If my hypothesis that *D. pinea* was being transmitted via shearing tools was correct, I expected to see tip blight symptoms associated frequently with sheared tips. Understanding more about the process of disease development on Scots pine might allow us to devise improved management recommendations for our local Christmas tree growers.

Materials and Methods

This study was conducted between April 2006 and June 2007. It took place on a choose-and-cut Christmas tree farm in Fayette County (Farm 1), which was not part of the shearing study that is described in Chapter 3 of this thesis. Farm 1 has been growing Scots pines since 1993, and did not have a serious problem with Diplodia tip blight disease until 2002. Appendix 1 of this thesis contains more detailed information about

Farm 1. The map in Figure 2.1 illustrates the number and arrangement of Scots pine trees in my one-acre experimental plot on the 7-acre farm during the summer of 2005.

In the early spring of 2006, twenty trees were selected for detailed examination from within the experimental plot (Figure 2.1). The trees were chosen to represent a variety of disease severities, based on disease ratings I did in 2005 (see below). Ten trees that had been asymptomatic in 2005, and 10 that had been symptomatic, were used. Some of the trees observed in 2006 were harvested and sold during the 2006 Christmas season, or were removed by the grower because of their poor health during the fall and winter of 2006. New trees, comparable in age and health to those removed, were added to the group in 2007 to maintain the number of trees in the study at 20 (Figure 2.1).

Scots Pine Shoot Phenology

During both years of the study, the lengths of 10 randomly selected elongating shoots were recorded for each of the 20 trees once each week, starting at bud break or just after, and continuing until late May, when the shoots were nearly completely elongated. Different shoots were measured each week. The length from the base to the apex of the shoot was measured in millimeters. Needle growth and development on the shoots was observed and recorded at the same weekly intervals, along with other notable features of shoot development.

Diplodia Tip Blight Development

The percentage of symptomatic tips on each Scots pine tree in the experimental plot was recorded during the late spring, two to three weeks prior to shearing, in 2005.

The percentage of symptomatic tips on each tree was also recorded prior to shearing at the end of the study, in June 2007. Each year, individual Diplodia tip blight cankers on each of the 20 study trees were observed at weekly intervals, and stages of development were described. In 2007, the distance of each Diplodia shoot canker from the shoot node in millimeters was recorded.

Detection of Diplodia pinea *Spores in Rain Water*

To determine the number of *D. pinea* spores carried by rain water, two spore traps were hung on each of the 20 trees in the study group to collect rain water during the spring of 2007. Traps consisted of a 15 mL polypropylene tube fastened with twist ties to pine shoot tips (Figure 2.2) (15). Heavy rainfall or extended periods of rainfall were needed in order to obtain water in the traps. Traps were collected after each such rain event (7 events total), and brought back to the laboratory for analysis. Water collected in the traps was mixed with potato dextrose agar (Difco Laboratories, Sparks, MD, USA) acidified with 85% lactic acid at 1 ml/liter (APDA), and supplemented with ampicillin (0.1 mg/mL) to suppress bacterial growth. Fungal colonies with the white, fluffy appearance of *D. pinea* were transferred to water agar containing autoclaved pine needles for positive identification. *D. pinea* produced pycnidia and characteristic spores on the pine needles within 2-3 weeks.

Results

Scots Pine Shoot Phenology

On Farm 1, Scots pine buds broke dormancy and began to elongate when daily high temperatures averaged 17˚C. In 2006, this average temperature did not occur until the beginning of April, but in 2007 bud break occurred at the end of March, due to the unusually warm weather. Many of these elongating shoots were later killed when the farm experienced hard frosts each night from April 5 until April 9 (Figure 2.3).

I observed that Scots pine shoot tips often retained the bud sheaths until the shoots had reached up to 50 mm in length (Figure 2.4.A-C). The sheaths expanded as the shoots elongated, and could eventually extend to cover up to 20 mm of the shoot. The bud sheath was usually sloughed off as the needles began to expand and emerge from the needle sheaths (Figure 2.4.C and D). On nearly half of the trees I observed, the bud sheath formed a collar around the middle of some of the expanding shoots even after the needles began to emerge (Figure 2.4.B). These collars were finally shed later in the spring due to weathering or to the force of the expanding needles beneath.

During the six-week study period in 2006 (comprising the week of April 10 until the week of May 14), shoots elongated from an average of 13 mm up to 94 mm (Figure 2.5.A). This is an average elongation rate of 1.9 mm per day. In 2007, shoots were measured beginning during the week of April 3 until the week of May 28. During this time, shoots elongated from an average of 30 mm to an average of 108 mm (Figure 2.5.A). This is an average elongation rate of 1.2 mm per day. Satterthwaite t-tests were used to compare overall average shoot lengths in 2006 to shoot lengths in 2007, and there was no significant difference (p=0.72; Figure 2.5.A). There was also no significant

difference (p=0.22) between the lengths of shoots from symptomatic trees and from asymptomatic trees.

Diplodia Tip Blight Disease Development

The Diplodia disease survey I did in 2005 demonstrated that there was a very high degree of symptomatic infection in the experimental plot. When the survey was repeated two seasons later, in 2007, disease severity was much lower (Figure 2.6).

In both 2006 and 2007, I observed the first symptomatic tips during the first week of May (Figure 2.4.A). I did not find any new symptomatic tips after the fourth week of May. I never observed symptoms obviously originating from the cut end of a previously sheared tip. I did frequently see symptomatic tips on secondary shoots that had developed from the axial buds of sheared shoots. However, there was no significant difference (n=39, p=0.59) between the numbers of diseased shoots arising from buds on sheared shoots versus on unsheared shoots.

Recognizable symptoms of Diplodia tip blight on Scots pines typically began as tiny water-soaked lesions at the needle bases of elongating shoots (Figure 2.7.A). These water-soaked lesions usually lasted only a day or two before they transformed into dark, necrotic cankers. Canker development occurred so quickly that water-soaked lesions were very hard to find, thus the necrotic cankers were the symptoms I usually noticed first on the expanding shoots. The necrotic lesions typically expanded first on one side of the shoot, and then finally surrounded the entire shoot to form a girdling canker (Figure 2.7.B and C). The girdling canker continued to expand up toward the shoot tip, but never extended down below the needle where the symptoms first began (Figure 2.7.D). Shoots

with girdling cankers rapidly became desiccated and turned reddish-brown to black above the canker, but remained green below the canker (Figure 2.8). The cankered region became resinous and brittle. The cankering stunted the shoot, and resin droplets were often seen exuding from the canker at needle junctions. The correlation between the distance of the canker from the shoot base and the total length of the shoot was significant, however, the correlation was not strong (n=34, r=0.4764, p<0.01; Figure 2.9).

Infection of the newly elongating shoots almost always occurred before the needles had fully emerged from the needle sheaths (Figure 2.8). The needles emerging from the cankered region often stayed green for a few days before they began to show symptoms (Figure 2.8.A and B). The infected Scots pine needles then quickly became straw colored and were stunted (Figure 2.8.C and D).

The characteristic symptom of Diplodia tip blight on Scots pines was shepherd's crooking. The diseased shoots curled over either at the bases of the cankers (Figure 2.8.B and C) or further up, while the asymptomatic portions of the shoots below the cankers did not curve (Figure 2.8.D). Shoots always bent so that the cankers were on the inside of the curve. On a few shoots, canker development occurred at the base, causing the entire shoot to die. In those cases, only a slight bend of the shoot occurred.

Symptomatic tips were found throughout the tree, but there were noticeably more of these at the base of the tree than nearer the top. In some cases symptomatic tips were found within a few centimeters of dead, diseased needles that were littering the inner branches of the trees (Figure 2.10).

Between April 5 and April 10 in 2007, an unusual cold snap occurred throughout central Kentucky. Record low temperatures were recorded at the NOAA Weather Station

at the Lexington Bluegrass Airport, and lows fell below freezing each night during this five-day period. Before this extended cold event occurred, temperatures had been abnormally warm and candles were actively expanding on the Scots pine Christmas trees (Figure 2.5). During the cold snap, candle elongation was arrested (Figure 2.5). By April 10, freeze damage could be seen on some of the candles. Freeze-damaged candles were flaccid and soft to the touch, but still green with no necrotic spots or lesions (Figure 2.3.A). When temperatures returned to normal, undamaged shoots began to elongate again (Figure 2.5). However, shoot tips damaged by the freeze did not resume elongation. In the weeks after the freeze, the freeze-damaged tips retained their bud sheaths. Needles and stems under the sheaths turned a light straw color (Figure 2.3.B and C). As the spring progressed, damaged candles either did not elongate at all (Figure 2.3.C and D), or only elongated at the base (Figure 2.3.E and F). In either case, the tips of the candles under the sheaths gradually turned from light straw colored to brown. Resin exudation was never observed from freeze-damaged tips.

Detection of Diplodia pinea *Spores in Rain Water*

During the spring of 2007, a total of 280 spore traps were deployed and collected during 7 different rain events. Two traps, both collected on April 20, 2007, contained one *D. pinea* spore each. None of the other traps contained *D. pinea*.

Discussion

It has been reported that *D. pinea* infection of Scots pine shoots can occur by various routes, but that wounded shoots are especially susceptible (31, 43). My

hypothesis for my thesis research was that Diplodia tip blight disease was being spread from tree to tree on Christmas tree farms via shearing tools, and that this accounted for the relative severity of the disease on the Christmas tree plantations, even on younger trees that are normally more resistant to the disease. If that was the case, I expected to see the disease developing frequently at the tips of previously sheared shoots. In fact, I never observed this, either on Farm 1 or on the other two farms that I studied (see chapter 3 of this thesis).

D. pinea was shown in a histological study to infect Austrian pines (*Pinus nigra*) by penetrating directly through the shoot epidermis at the bases of newly emerging needles (30), and it has also been reported that infection of Scots pine shoots can occur at needle bases (43). I was able to observe the earliest symptom of *D. pinea* infection, a tiny water-soaked lesion, only three times because the disease progressed so rapidly. In each of those three cases, however, the infection appeared to initiate from the base of a needle bundle (Figure 2.7.A). When I dissected young necrotic lesions, I observed in every case a distinct boundary between the necrotic area expanding up toward the shoot apex, and apparently healthy tissue below (Figure 2.7.D). This boundary always coincided with the location of a needle bundle base. The same type of boundary was also observed in mature lesions (Figure 2.7.C). My observations suggest that inoculation at the needle bases was probably the most common means of shoot infection on Farm 1 in 2006 and 2007.

I observed that young necrotic *D. pinea* lesions typically developed from the infection focus up toward the shoot apex. At first, the spreading necrosis was observed only on one side of the shoot, presumably the side where infection took place (Figure

2.7.B). As the lesion continued to develop, the shoot began to curl, eventually resulting in the characteristic shepherd's crook symptom. Curling always occurred toward the lesion. Thus, the vast majority of curved shoots curled down because most lesions formed on the outer side of the shoot, but in a few shoots the necrotic lesion formed on the inner side, and in these cases the shoot curled up. Shepherd's crooking of *D. pinea*-infected Scots pine shoots has been described previously (31). In that article it was proposed that destruction of meristematic tissues by the fungus occurs on only one side of the expanding shoot, while the other side continues to grow, and that this differential growth rate causes the shoot to curve over with the necrotic lesion on the inside of the curve (Figure 2.7.C).

The necrotic *D. pinea* lesions eventually expanded to encircle the entire shoot and form a girdling canker, causing the portion of the shoot distal to the canker to become desiccated and die. In some preliminary culturing experiments that I did, *D. pinea* could only rarely be cultured from newly desiccated tips or from green tissues below the canker, but the fungus was always present in the canker itself. Thus, it seems likely that the death of the shoots results primarily from the girdling, rather than fungal colonization, which only occurs later. The dead shoot tips appeared stunted, and eventually became brittle and infused with resin.

In the spring of 2007, a rare weather phenomenon occurred in central Kentucky. We experienced above-normal temperatures in March that caused the buds of the Scots pine Christmas trees to break early (Figure 2.5). An unusually harsh cold snap then occurred from April 5 to April 10, resulting in significant freeze damage to some of the elongating shoots. Freeze-damaged shoots curved over and became desiccated, and they

appeared superficially very similar to Diplodia tip-blight infected shoots. However, whereas shepherd's crooking caused by Diplodia tip blight resulted from asymmetrical shoot growth, crooking caused by freezing resulted from generalized damage to the structural components of the shoots. Crooked freeze-damaged shoots were flaccid, soft to the touch, and did not exude resin. Tip-blighted shoots, in contrast, were brittle, rigid, and usually exuded large quantities of resin. Like the tip-blight infected shoots, many of the freeze-damaged shoots only died at the tips while the basal portion of the shoot remained alive. Subsequent shearing will remove the dead top portion, and the trees will presumably be left relatively unscathed by the freeze.

The symptoms of Diplodia tip blight on Austrian and Scots pine shoots in Kentucky are quite different. On Austrian pines, infected candles are usually completely killed before they elongate significantly (50). Shepherd's crooking of the type I typically saw on infected Scots pines is not usually found on Austrian pines, though there is one published description of asymmetric infection of elongating Austrian pine candles which subsequently survived to produce crooked, but living shoots (31). We have never seen this type of symptom on Austrian pines in Kentucky. On the other hand, I did sometimes observe symptoms on Scots pines that were reminiscent of Kentucky Austrian pine symptoms. In those cases, it appeared that the canker had formed at the base of the elongating shoot, rather than further up, and had girdled and killed the entire Scots pine shoot relatively quickly. The type of symptom produced (stunted, blighted tips, versus a shepherd's crook) may be a function of genetic and/or environmental factors that qualitatively and quantitatively affect host resistance. If the infection proceeds relatively slowly, the shoot will elongate either until the lesion can be contained (in which case a

crooked live shoot will be the result), or until a girdling canker is finally formed (resulting in death of the crooked tip). This suggests that Austrian pine shoots in Kentucky are generally more susceptible than Scots pine shoots to *D. pinea,* or perhaps that these landscape Austrian pines are exposed to more inoculum.

My observations of Diplodia tip blight disease development on Farm 1 did not provide obvious support for my hypothesis that shearing tools play a role in dissemination of the disease. If *D. pinea* spores are not being spread on shearing tools, then it is possible that they are being carried by rainwater (18). During both years of my study, I observed the first symptoms of Diplodia tip blight during the first week of May. The degree of development of the cankers at that time suggested that infection had taken place during the last weeks of April. During the spring of 2007, I collected rainwater in traps during April and May to see if spores were being washed down past the elongating tips. Only two *D. pinea* spores were collected, on April 20, ten days before symptoms were first noted. Thus, the rainfall on April 20 in 2007 may have carried spores that initiated the lesions I observed in early May on Farm 1. However, very few spores were recovered from the rain traps, suggesting that *D. pinea* inoculum may not originate primarily from rainwater. It has previously been reported that significant numbers of *D. pinea* spores can be collected during rain events (18). For those studies, spores were collected on greased slides placed under diseased trees, where they could also collect spores that splashed up from debris on the ground. In contrast, my traps only collected spores that were carried in the raindrops, or washed down from the outside of the canopy.

Christmas trees are sheared every year after the second or third growing season, which removes most of the diseased tips from the previous year, eliminating a major

source of inoculum. However, I noticed that there were many naturally abscised needles caught in the inner branches and covering the ground beneath the trees, and I observed abundant pycnidia on these in April during both years of my study. Pycnidia could also be found on diseased tips that had been missed during shearing (Figure 2.10). I suspect that these needles and tips serve as the primary sources of inoculum during the spring, and that the spores are disseminated mostly locally (within a single tree or between two closely spaced trees). As rainwater or dew runs down the shoot, it can collect in the crevices created at the junctions between the shoot and needle bundles, and deposit any Diplodia spores it is carrying. Evidence supporting this idea includes the fact that most of the symptomatic tips were found near the bottom of the trees. This could be a result of inoculum being disseminated from the infected needles under the tree up to the lower branches by rain splash. In the future, it would be interesting to try using the greased slides to see if significant numbers of spores are splashed up from this debris. Infected tips located higher up in the tree could result from spores being splashed from the infected dead needles and shoots inside of the tree onto the outer branches. Scots pine candles grow upward, (Figure 2.4) and at a slight angle away from the tree, with the outer (abaxial) side of the shoot closer to the ground. I observed that the majority of Diplodia tip blight lesions developed initially on the outer side of the shoot. I believe that this is because, as water drips down the shoot, it will tend to flow toward the side closest to the ground, and so more water and spores collect on the outer (lower) side.

Between the spring of 2005 and the spring of 2007, there was a dramatic decrease in the severity of tip blight disease (percentage of blighted tips) on Farm 1 (Figure 2.6). Although I did not record disease severity in 2006, the grower has told me that he

believes that levels of tip blight were less in 2006 than in 2005, but more than in 2007 (D. Barker, personal communication). Coincidentally, Farm 1 was treated with Cleary's 3336™WP (thiophanate ethyl) fungicide (1-8oz package/25 gal water) for the first time during the spring of 2005, and the treatment regimen was repeated in 2006 and in 2007. The first spray was applied (using the same sprayer used to apply colorant) just as the shoots were beginning to elongate (mid-April), and then another application was made 7-10 days later. This coincided with the primary period of infection of Scots pine shoots, based on my observations in 2006 and 2007.

Fungicide sprays began on Farm 1 in 2005, but there was a very high level of disease on the new shoots that year (Figure 2.6). Each year, dead tips are removed from the trees by shearing and dead branches are removed by selective pruning, reducing the amount of overwintering Diplodia inoculum. I don't have data for disease severity on Farm 1 in 2004, but it is possible that disease levels were even higher that year, and that there was a lot of overwintering inoculum in 2005. This may have caused the chemical treatments to be somewhat less effective that year. The combination of shearing and pruning with fungicide treatments may have decreased the amount of inoculum available each year so that the number of new infections also decreased, until in 2007 there was very little visible disease on the farm. Protectant fungicide sprays have been applied to Austrian pines on the U.K. campus to combat Diplodia tip blight, but they were generally ineffective (34). Complete coverage of the tree may be necessary in order to penetrate into the crevices between the shoots and needle bundles where infections occur. This is an easier task with Scots pine Christmas trees, which are rarely over 2 m tall, than with landscape Austrian pines which can range from 6-10 m tall.

Further research is needed to test the efficacy of Cleary's 3336™ applications for Diplodia tip blight control on Scots pine Christmas trees, but my observations on Farm 1 give me hope that good sanitation practices combined with fungicide treatments can be used to combat this disease, and can turn even a heavily diseased farm into a profitable operation with very little disease in just a few years.

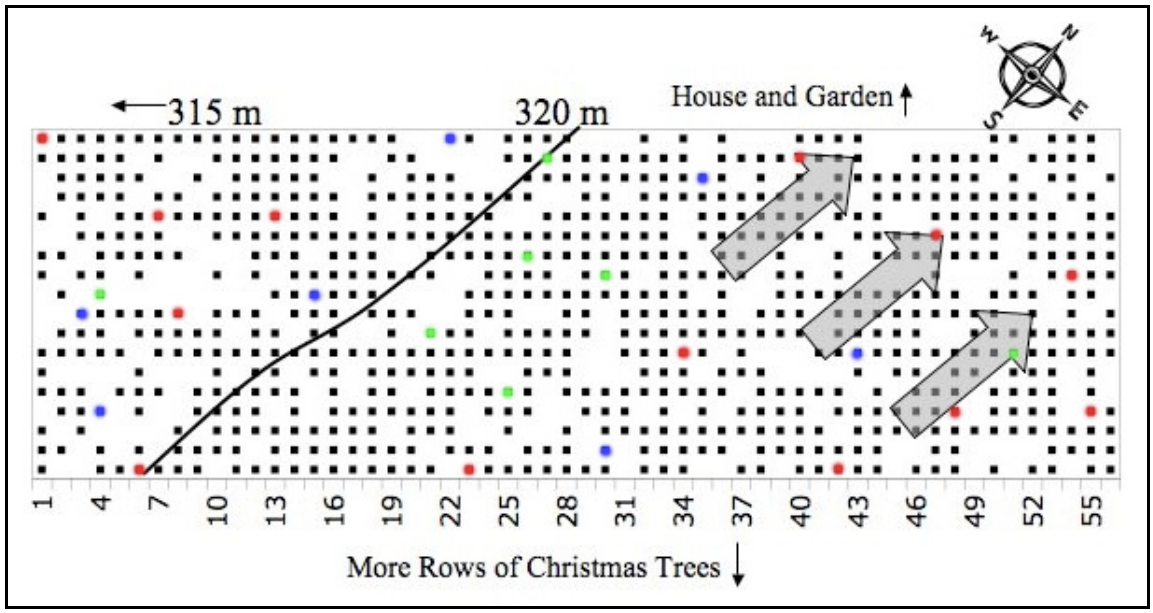


Figure 2.1. Topography map for Farm 1 with land elevations and prevailing wind directions (indicated by the large gray arrows). Black squares represent Scots pine Christmas trees. Blank spots represent other tree species or an empty space. Red squares are trees that were used in both years of study (2006-2007). Blue squares are trees used only in 2006, and green squares are trees used only in 2007.

Figure 2.2. A spore trap hung beneath an elongating Scots pine Christmas tree candle in the spring of 2007.

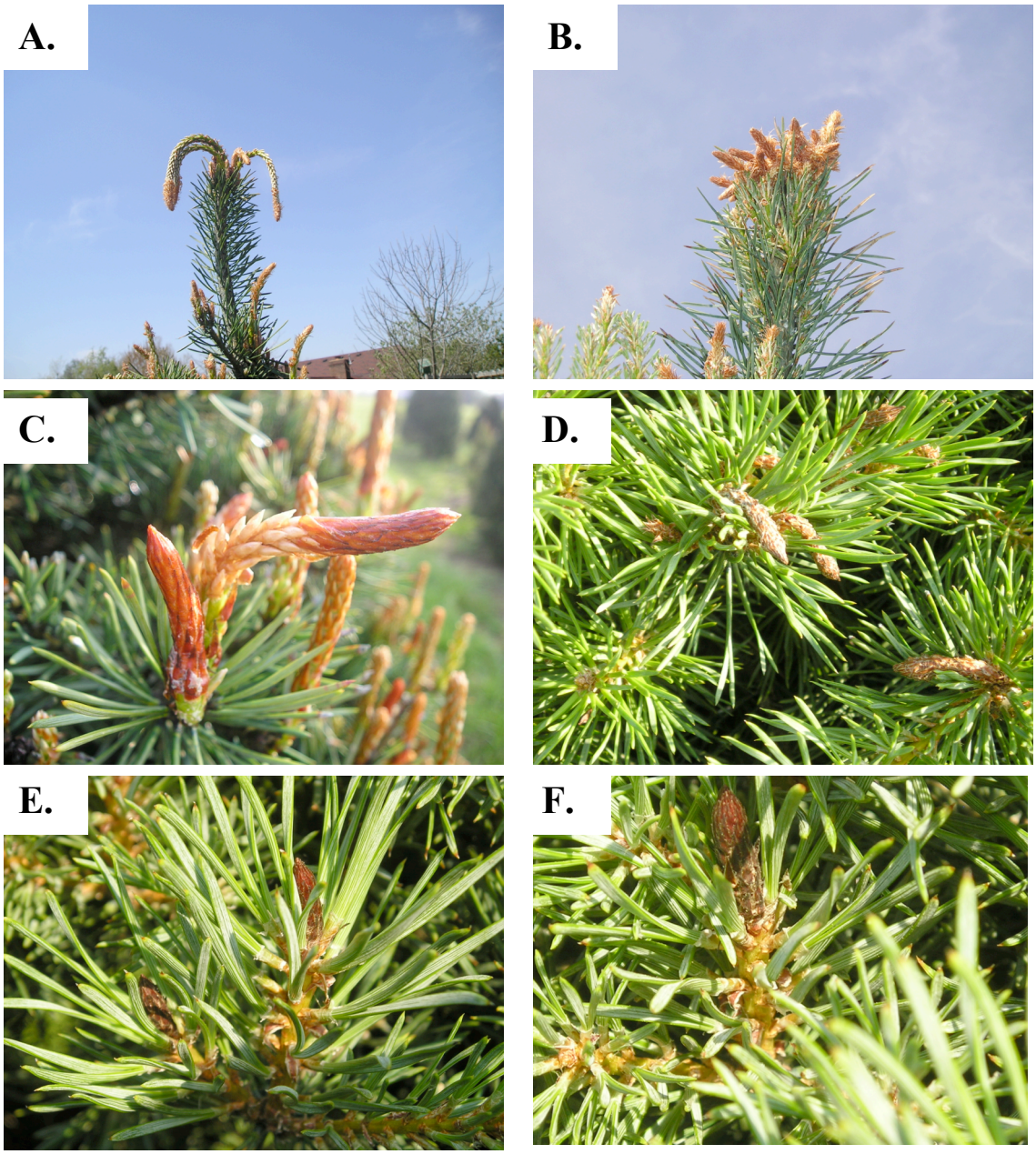


Figure 2.3. Freeze damage on Scots pine shoots in the spring of 2007. Freeze damage caused tips to become flaccid (**A.**) and stop growing (**B-F.**). Candles never lost their sheaths, even after undamaged candles did. Freeze damage tips either stopped growing completely (**D.**) or just the tips stopped growing while the base of the candle continued to elongate throughout the spring (**E-F.**)

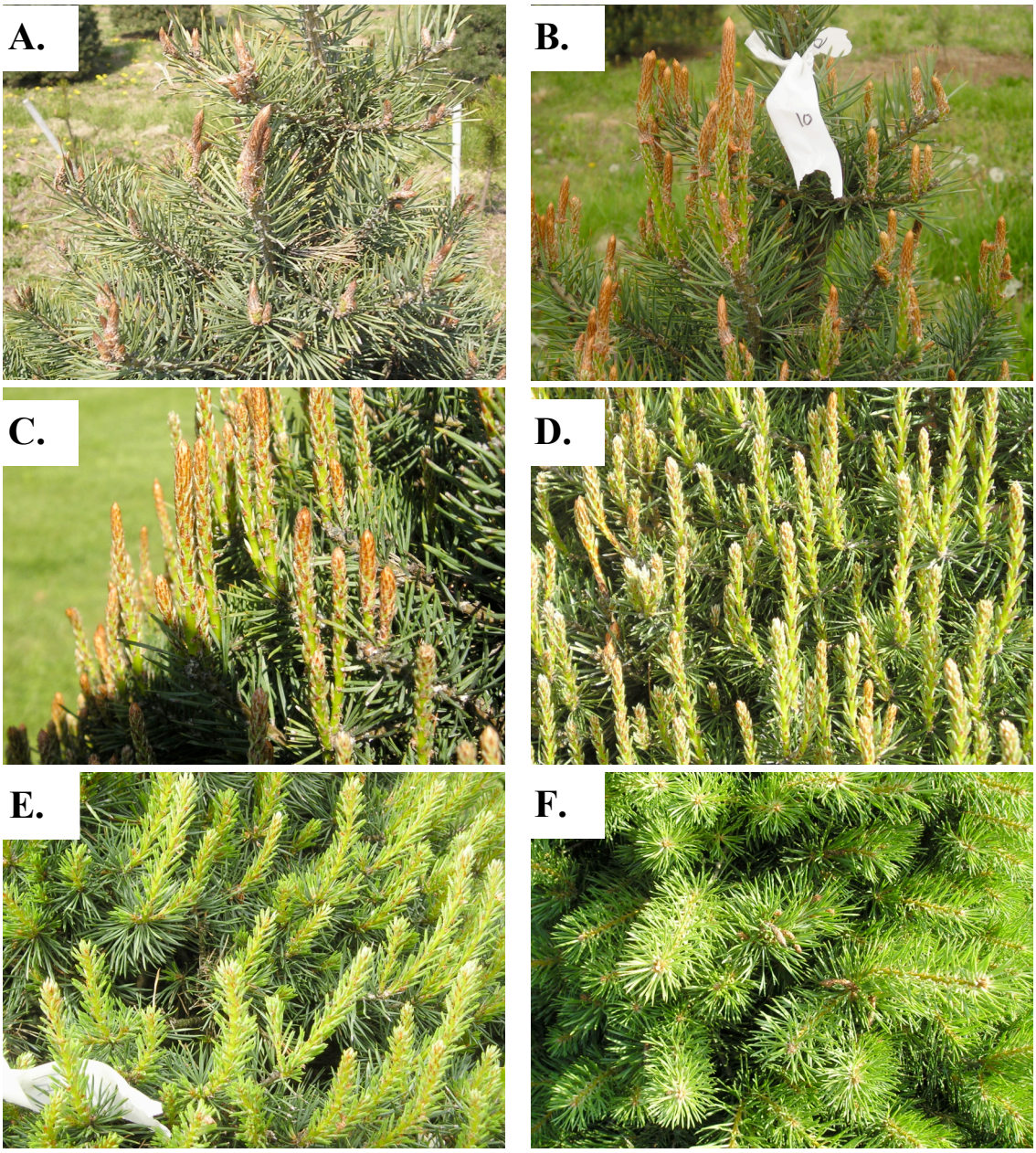
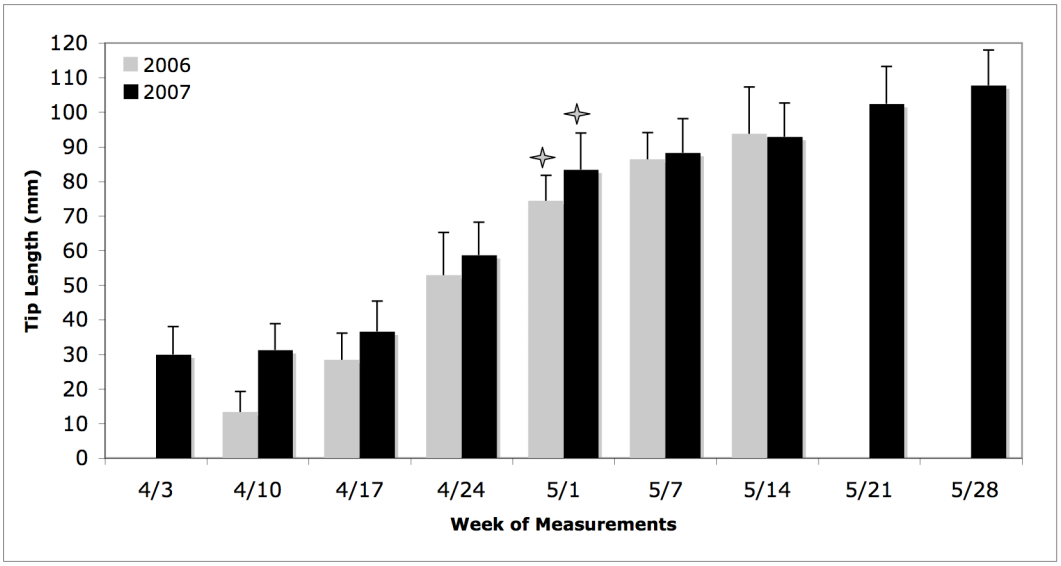


Figure 2.4. Scots pine Christmas tree shoot elongation in the spring. Representative weekly development on April 13 (**A**), April 20 (**B**), April 27 (**C**), May 4 (**D**), May 12 (**E**), and June 8 (**F**). All photographs were taken in 2006 with the exception of F taken in 2007.

A.



B.

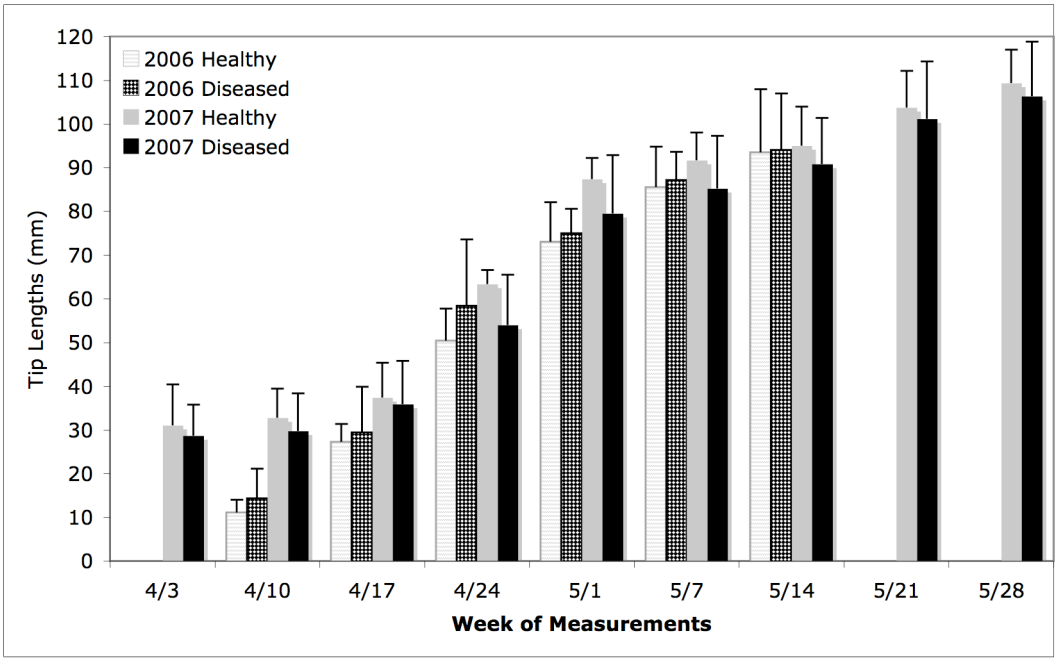


Figure 2.5. Scots pine Christmas tree shoot elongation. **A.)** Average lengths of Scots pine Christmas tree shoots on Farm 1 in 2006 and 2007. Stars indicate weeks when Diplodia tip blight symptoms were first noted. **B.)** Average shoot lengths of asymptomatic (healthy) and symptomatic (diseased) trees in 2005.

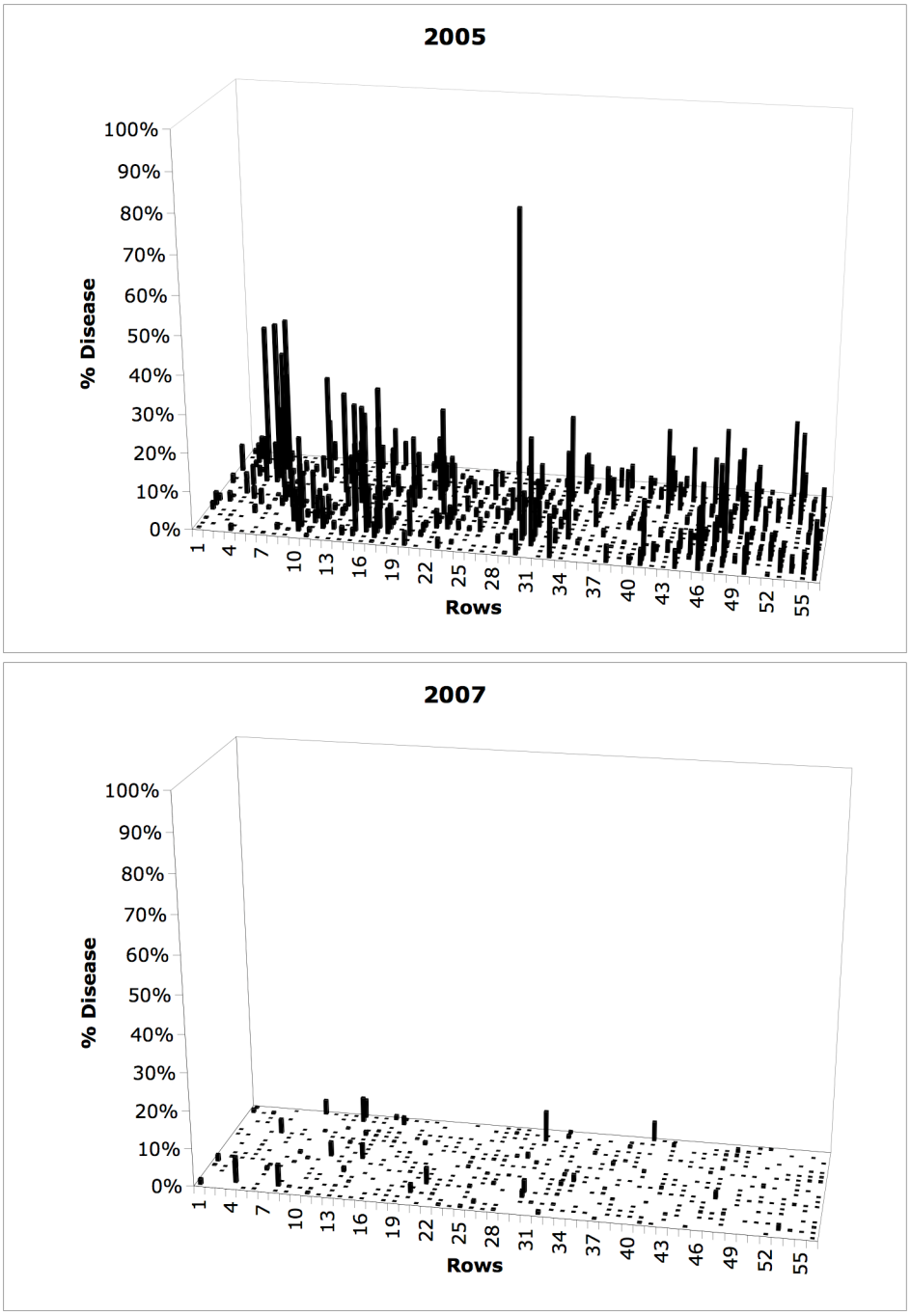


Figure 2.6. Diplodia tip disease severity for each Scots pine Christmas tree in the experimental section of Farm 1 in 2005 and 2007. Each point or bar represents an individual Scots pine tree.

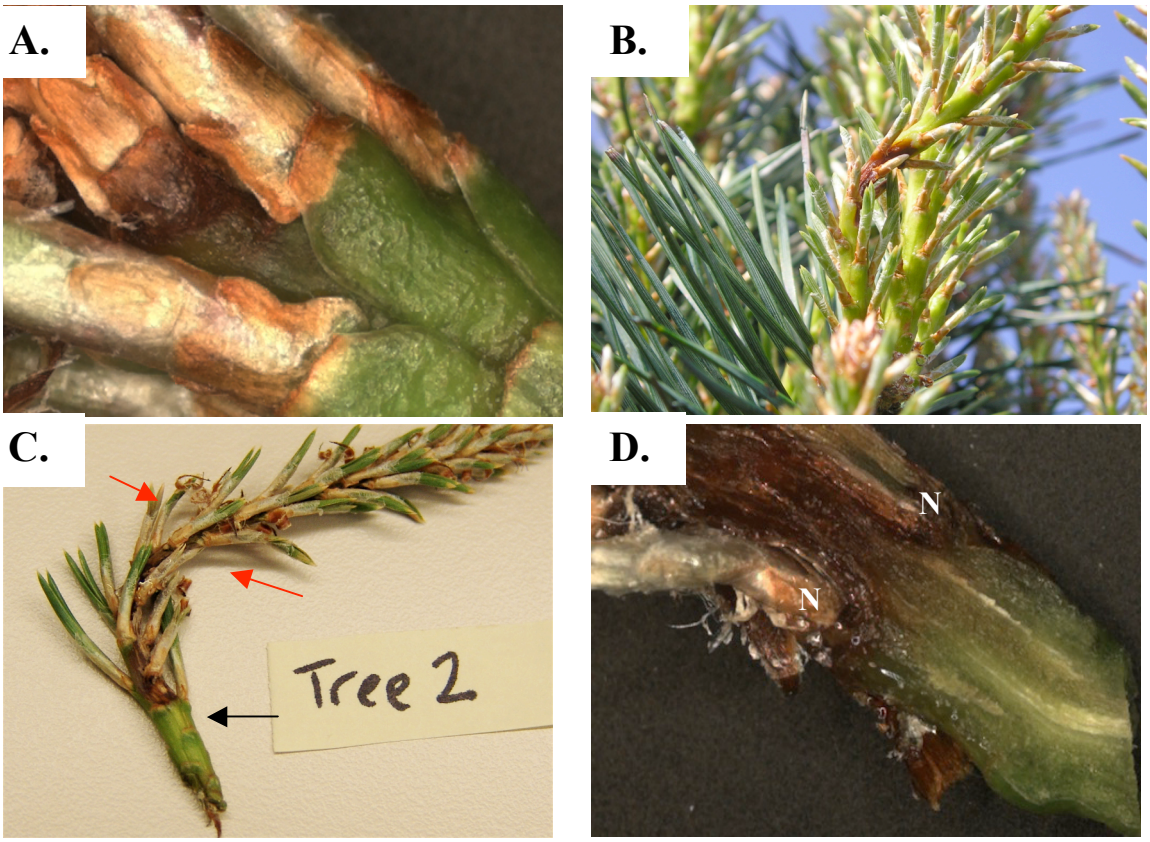


Figure 2.7. Location of infection and necrotic lesion formation by *Diplodia pinea* on Scots pine Christmas trees. **A.)** Water-soaking and necrotic lesion formation at a needle base. **B.)** Canker formation along one side of the shoot causing the shoot to begin to crook over. **C.)** Canker formation along only one side of the shoot. Black arrow indicates the base of the canker where it is limited by a needle junction. Red arrows indicate needles that are beginning to turn straw color. **D.)** Dissection of the base of the necrotic lesion on a Scots pine tip infected with *Diplodia pinea*. "N" are needles. Necrosis does not extend below needle-shoot junction.

Figure 2.8. Symptoms of Diplodia tip blight on Scots pine Christmas trees. Brown to black necrosis of the shoot often occurs before needles emerge from their sheaths (**A.**) and precedes needle discoloration. The necrotic canker develops at a needle base (**B.**) and progresses up to the tip while the shoot below the canker stays alive (**B-D.**). Shepherd's crooking often occurs at the diseased-healthy tissue junction (**C.**), but sometimes the bend occurs further up the shoot (**D.**).

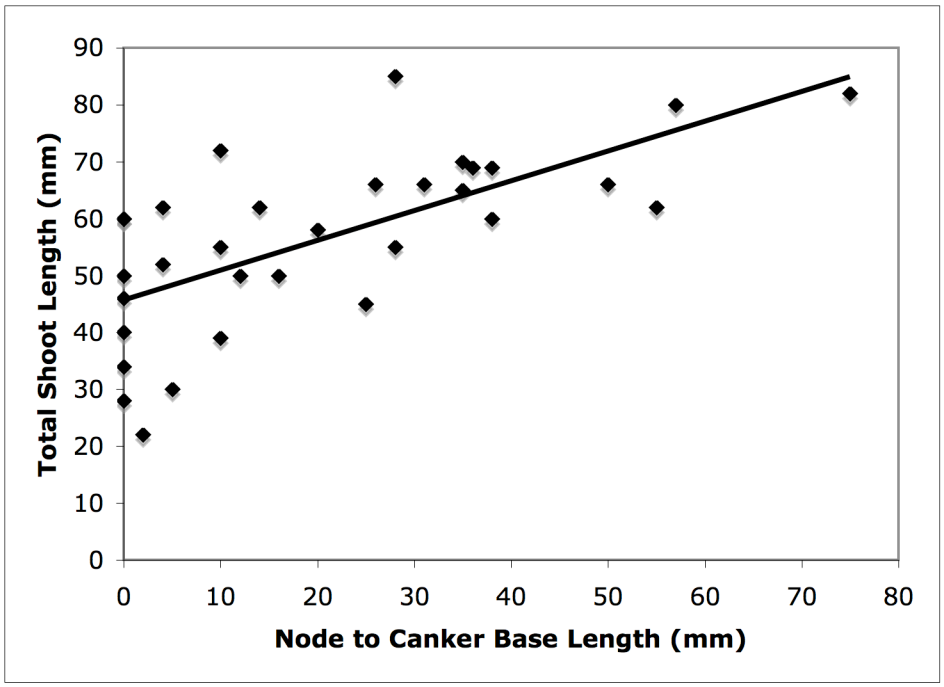


Figure 2.9. Correlation analysis between the distance of Diplodia tip blight cankers from the shoot base (node) to the total shoot length for 34 symptomatic tips in 2007. There is a significant positive correlation (r=0.4764, p<0.01).

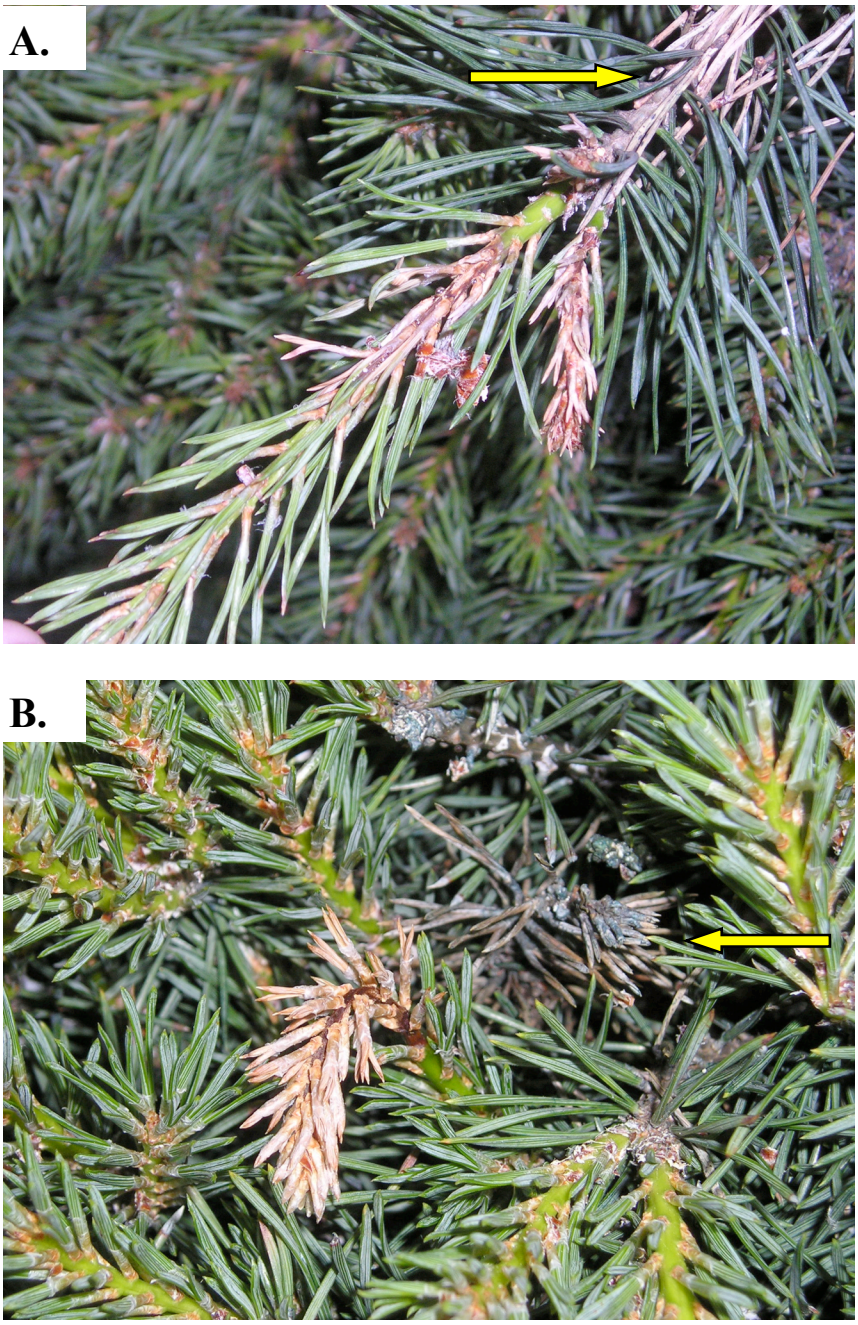


Figure 2.10. Location of infected dead needles in relation to new Diplodia tip blight infections on Scots pine Christmas trees. Yellow arrows in both pictures point to old, dead needles infected with *D. pinea* within the trees. In close proximity to these dead needles, new symptomatic *D. pinea* infections have formed on elongating shoots.

CHAPTER THREE:

THE RELATIONSHIP BETWEEN SHEARING AND THE TRANSMISSION OF *DIPLODIA PINEA* ON CHRISTMAS TREE FARMS

Introduction

In Kentucky, Scots pines (*Pinus sylvestris*) have traditionally been the most popular Christmas tree species to grow and sell. More than 75% of Kentucky Christmas tree farms grow primarily, or only, Scots pines (42). The local Christmas tree industry is based on choose-and-cut farms, and although it accounts for less than 1% of the total agricultural crop sales in the state (61), it is a valuable source of supplemental income for many landholders. Many Christmas tree farms promote family-friendly Kentucky agritourism by offering products and services (such as wreaths, hayrides, and gift shops) in addition to Christmas trees. As well as providing a better quality product, the availability of locally grown Christmas trees, as opposed to trees grown out-of-state and trucked in, helps to keep more of our money in Kentucky.

During the past ten years, Diplodia tip blight disease has become a serious problem on Scots pine Christmas trees in Kentucky. *Diplodia pinea* (Desmaz.) J. Kickx fil. (syn. *Sphaeropsis sapinea* (Fr.:Fr.) Dyko & Sutton in Sutton) is the causal agent of Diplodia tip or shoot blight on more than 30 species of pines as well as cedars, spruces, and firs. Tip blight disease is found worldwide, and can result in severe economic losses (56, 59, 63). In Kentucky, *D. pinea* is mainly found on the exotic two-needled pines, *Pinus nigra* (Austrian pine), and *Pinus sylvestris* (Scots pine).

D. pinea infection typically occurs on newly elongating shoots in the spring, and via wounds throughout the season. Symptoms of Diplodia tip blight disease include needle blight, tip blight, resinous cankers on the main trunk and/or branches, branch dieback, and "shepherd's crooks" of the elongating shoots. Symptoms can occur within days of the infection under ideal conditions (50), and progress rapidly thereafter. In recent years Diplodia tip blight has been so devastating to Scots pines grown for Christmas trees in Kentucky that many Christmas tree farmers are beginning to switch to other tree species (P. Kovalic and D. Barker, personal communication).

Christmas trees are sheared annually in Kentucky once they are two or three years old. Shearing takes place during June and July when shoots are fully elongated, but haven't yet hardened off. Shearing is necessary to produce the typical conical shape desirable for a Christmas tree. Buds form on the cut branches and give rise to new shoots the following year, resulting in an attractive, full tree. Shearing begins in the third year and continues until the tree is sold (38). The possibility that shearing a diseased tree can contribute to dispersal of *D. pinea*, and that shoot tips wounded by shearing are a primary infection court for the fungus, has been proposed previously (1, 4), but has not been investigated.

It has been recommended that Christmas tree growers routinely disinfest their shearing tools in order to prevent the spread of infectious agents (2, 60). Recommended disinfestation protocols include alcohol and bleach dips or washes. However, most growers do not follow these recommendations because of the inconvenience and potential damage to the tools. Spraying tools with household Lysol® Disinfectant Spray has been suggested as a more convenient, less damaging alternative (60).

The purpose of the study reported in this chapter of my thesis was to test the hypothesis that *D. pinea* can be acquired on shearing tools, and that it can be transmitted by contaminated tools from tree to tree during the shearing process. I also tested the efficacy of Lysol® spray as a disinfestant for shearing tools.

Materials and Methods

Experimental Design

Two choose-and-cut Christmas tree farms were selected for my study, which began in May of 2005 and was concluded in June of 2007. Farm 2 is located in Clark County, KY, and Farm 3 is in Fayette County, KY. Both farms have been growing Scots pines for at least 15 years, and neither had a serious problem with tip blight disease until about five years ago. The maps in Figures 3.1.A. and 3.2.A. illustrate the number and arrangement of Scots pine trees on each farm at the beginning of the study. On Farm 2, the mean age of the Scots pines in the first year of the study was 5 years (ranging from 2 to 8). On Farm 3, the mean age of the trees in the first year of the study was 5 years (ranging from 2 to 9). Appendix 1 of this thesis contains detailed information about each of the two farms.

Fifty-four experimental groups, each consisting of a row of six adjacent trees, were identified on Farm 2, and 52 groups were identified on Farm 3. The first tree in each group (the lead tree) was asymptomatic or had varying tip blight disease severities (ranging from 0.1 to 28.5% on Farm 2, and from 0.1 to 40% on Farm 3), while the five other trees in each group were either healthy, or, in a few cases, lightly diseased (generally fewer than 1% diseased tips, but 24 trees had up to 6% diseased tips) (Figures

3.1 B and 3.2 B). The lead tree was always the first tree in each group to be sheared. Adjacent trees in each group were sheared in order of their proximity to the lead tree. Because trees in many of the groups were removed for sale during the winter of 2005, only 27 of the same groups on Farm 2, and only 21 groups on Farm 3, could be used for the second year of the study. New groups were identified to replace some of the missing groups in 2006 so that each farm had a total of 35 study groups in 2006. Disease severities on the lead trees in 2006 ranged from 0 to 32.2% on Farm 2, and from 0 to 8.7% on Farm 3 (Figures 3.1 C and 3.2 C).

Latent and Symptomatic Diplodia Infection Ratings

The percentage of symptomatic tips on every Scots pine tree on each farm was recorded in late spring, approximately one month prior to shearing, during each year of the study. To calculate the amount of latent infection, shoot tips were collected from the lead trees of each experimental group during shearing. In 2006, ten tips (nine asymptomatic, and one symptomatic, if available) were collected. Sheared tips were taken back to the laboratory and a nested polymerase chain reaction (PCR) protocol (29) was used to detect latent *D. pinea*. The same DNA samples were amplified with pine actin primers (46) as a positive control for DNA quality. Negative controls in each experiment included samples with no DNA added, and samples prepared from greenhouse-grown trees that were known to be uninfected.

Detection of Fungal Propagules on Shearing Tools

Trees on Farm 2 were sheared with a Beneke Rotary Tree Trimmer. On Farm 3, trees were sheared manually with long-handled hedge shears. The build-up of resin during shearing made it impossible to collect fungal propagules by simply washing them off of the tools, and so tool cutting surfaces were sampled by using a tape press method. Sterile adhesive tapes (SealPlate™, Research Products International Corp., Mt. Prospect, IL, USA) were cut into fourths (6x4 cm), with the paper backing kept in place until the time of collection to reduce contamination. To collect samples, the adhesive side of the tape was pressed very firmly against the blade surface, then the tape was peeled off and the backing was replaced to protect the samples while they were transported back to the laboratory. Two samples were collected from different parts of the blades immediately after shearing the lead tree, and also after shearing the second, fourth, and sixth tree in each group (Figure 3.3). In accordance with the usual practices on these farms, cutting tools were not routinely cleaned or sterilized during the shearing process, other than occasionally scraping off accumulated resin by using a pocketknife. On Farm 2, the cutting tool was used frequently to cut weeds around the trees during shearing.

Tape press samples were taken back to the lab and stored at 4˚C until they could be processed (no more than two weeks). In 2005, one of the tape press samples from each pair collected was cut in half, and half was examined with the light microscope to detect the presence of *D. pinea* spores, which were easily recognized by their distinctive size, shape, and color. The other halves of the tapes, and the intact tape from each pair, were placed individually into Petri plates and covered with a layer of cooled (45-50˚C) potato dextrose agar (Difco Laboratories, Sparks, MD, USA) acidified with 85% lactic

acid at 1 ml/liter (APDA) (Figure 3.4). The plates were incubated at 24˚C for up to five days. Development of fungal mycelium from the tapes up through the APDA was observed daily. Colonies with the distinctive appearance of *D. pinea* (white, fluffy, and very fast growing) were subcultured onto 2% water agar plates containing pieces of autoclaved pine needles to support production of pycnidia and conidia. After two weeks, needles were examined and colonies that had given rise to these fruiting structures were confirmed as *D. pinea*.

Testing the Efficacy of Lysol® Household Spray for Disinfesting Shearing Tools

Original scented Lysol® Brand II Disinfectant spray (active ingredients: alkyl dimethyl benzyl ammonium saccharinate, 0.106%, and ethanol, 79.646%; Reckitt Benckiser Inc., Wayne, NJ, USA), purchased at the local supermarket, was used for this experiment. The experimental groups of trees on each farm were divided into two sets, matched as much as possible for the age of the trees and for the degree of infection on the lead tree. For one set, shearing tools were sprayed to runoff with Lysol® after the lead tree was sheared, while for the other set the tools were not treated. After the Lysol® dried, tape press samples were collected from the treated and from the untreated tools, and processed as described above. T-tests were used to compare the *Diplodia* pinea colony-forming units (CFUs) recovered from treated versus untreated tools.

In vitro assays were used to quantify the sensitivity of *D. pinea* conidia to Lysol® Disinfectant Spray. Conidia were produced on autoclaved pine needles on water agar, using a modification of a protocol described previously (30). The infested needles were scraped with sterile scalpels, and the scrapings were suspended in 7 mL of sterile MilliQ

water. Suspensions were mixed on an orbital rotator (Orbitron Rotator I, Boekel Scientific, Feasterville, PA, USA) at room temperature. After 2-3 hours, the suspensions were filtered through cheesecloth to remove excess plant material, and the filtrate was centrifuged at 3000 rpms (2000G) for 10 minutes in a Beckman GS-6R tabletop centrifuge. The conidial pellet was washed twice and then resuspended in sterile MilliQ water. Conidial concentration was determined by counting with a hemocytometer. Spore suspensions were used immediately for experiments (17). Ethanol, isopropanol, bleach, and Lysol® spray at concentrations of 5, 10, and 25% were used for the experiments. Twenty-microliter drops of disinfestant solutions containing $1x10^4$ conidia/mL were applied to Teflon coated glass slides (8-well printed slides, Electron Microscopy Sciences, Ft. Washington, PA, USA). Slides were placed into humidity chambers and incubated at 25˚C for 12 hr. Percent spore germination was determined for the first 100 spores observed in each well. The experiment was repeated twice, once with two replicates and once with three replicates.

To test the effect of exposure time to Lysol® on spore germination, spores were collected as described above and exposed to a solution of 25% Lysol® for 30 seconds, 1 minute, 5 minutes, 10 minutes, 30 minutes, and overnight (approximately 15 hours). Water controls were included for all time points. Immediately after each timed treatment, spore suspensions were washed 5 times in 50 mL sterile MilliQ water to remove the Lysol®, and then adjusted to $1x10^4$ spores/mL. Twenty-microliter drops were applied to Teflon slides and incubated overnight in a humidity chamber as described above. The experiment was repeated three times, twice with six replicates including the water controls, and once with six replicates and no water controls. Linear mixed models were

used to compare the average germination rates. The data were adjusted for unequal group size (Tukey-Kramer) and model adequacy checking was performed.

Tool Transmission Studies

I used the Pearson test for correlation to determine whether there was a statistically significant association between the amounts of visible or latent infection on the lead trees and the number of CFUs recovered from the tools used to shear those trees. I used a similar analysis to characterize the relationship between the number of CFUs recovered from the tools and the amount of disease that developed during the following season on trees that were sheared with those tools. Linear mixed model statistical analyses were used to determine whether there was a difference in the amount of disease that developed on the last tree in a group to be sheared versus on the first tree (adjacent to the lead tree). The data were adjusted (Tukey-Kramer) and model adequacy checking was performed.

I performed another tool transmission study using a group of 100 4-year-old Austrian pines (*Pinus nigra*) obtained from Musser Forests Inc., Indiana, PA, USA. The trees were potted in Organic Summer Potting Mix (Bio-Comp Inc., Edenton, NC, USA) in Treepots™ "Short Ones" pots (Stuewe & Sons Inc., Corvallis, OR, USA), and had been maintained inside a Quonset hut (Pro Greenhouse model # 102848, Clear Span, Dyersville, IA, USA) for 3 years on the U.K. campus. During the spring and summer, the doors on either end of the hut were left open, and they were kept closed in the fall and winter months. One pair of current-year shoots in the same whorl was selected on each tree for treatment during the spring of 2006. Sterile scalpel blades (size 22, Feather

Safety Razor Co. Ltd., Medical Division, Osaka, Japan) were infested with *D. pinea* inoculum by using them to slice five times into diseased and sporulating Austrian pine shoots collected from several mature trees on the U.K. campus. The tip of one elongating shoot on each healthy tree in the Quonset hut was sliced off (simulating shearing) with an infested scalpel, while the other shoot in each pair was cut with a sterile scalpel blade taken right from the package. Each blade was used only once, and after use was put individually into a Petri dish and brought back to the laboratory, where it was covered with a layer of cooled APDA. *D. pinea* CFUs arising from the blades were identified and counted as described above. The treated Austrian pine trees were maintained in the Quonset hut for the entire season, and symptom development was evaluated on the cut shoot tips during the following May and June (2007). Approximately 1 cm of each cut shoot tip was removed in June and returned to the laboratory for nested PCR analysis to detect the presence of *D. pinea*. A logistic regression was used to evaluate the probability of obtaining a positive PCR result for an individual shoot tip based on the number of *D. pinea* CFUs recovered from the scalpel used to cut the tip.

Analysis of the Role of Resin in Diplodia Tip Blight Symptom Development

Scots pine 2-2 bare-root tree seedlings were obtained from Porcupine Hollow Tree Farm (Central Lake, MI, USA) in October 2005 and in October 2006, potted in Organic Summer Potting Mix (Bio-Comp Inc., Edenton, NC, USA) in Treepots™ "Short Ones" pots (Stuewe & Sons Inc., Corvallis, OR, USA), and overwintered in the Quonset hut. During the following spring, four fully elongated candles in the same whorl on each plant were selected for testing. The top 2-4 cm of each shoot was removed with a sterile

scalpel blade to simulate shearing, and the needles were also removed from each cut tip down to about 1 cm below the cut. Ten µl of a suspension of $1x10^6$ *D. pinea* conidia per ml of water was taken up in a 200 µL plastic pipette tip (DotScientific, Inc., Burton, MI, USA), and a loaded pipette tip was then placed over the cut end of each shoot so that the spore suspension inside covered the wounded surface (Figure 3.5). The pipette tip was parafilmed to create a humidity chamber. *D. pinea* conidial suspensions were applied to the cut tips in each whorl at 0, 6, and 24 hr after cutting. A pipette tip containing water was applied to the remaining shoot tip in each whorl at 0 hr as a control. After 24 hr, the pipette tips and parafilm were removed, and symptom development was observed for three weeks. In 2006, a Horsfall-Barratt scale was used to visually estimate the amount of disease that developed on each shoot. The scale uses logarithmic rather than linear increments, based on the Weber-Fechner law that says that the human eye can more precisely estimate high and low values (disease severity) than mid-range values (32, 39). In 2007, the lengths in millimeters of the necrotic lesions that formed on the shoot tips were compared for each timing treatment using a mixed linear model. I compared the differences in the subplot factor, and treatment timing.

Analysis of the Role of Spore Concentration in Symptom Development

Scots pines (purchased and grown as described above) were used for this experiment. Four tips from the same whorl were selected for treatment on each seedling. The tips were cut with a scalpel blade as above, and 10 µL of *D. pinea* spore suspensions containing $1x10^6$, $5x10^4$, $1x10^3$, or 0 spores per ml (water control), were applied to each

of the four tips immediately after cutting, using the pipette method described above. Statistical analysis was performed as described above.

Results

Latent and Symptomatic Diplodia Infection Ratings

The Diplodia tip blight disease severity on each Scots pine tree on Farm 2 and Farm 3 was measured (as the percentage of symptomatic tips) during each of the three years of the study (Figures 3.6 and 3.7). In all three years, there was significantly more disease on Farm 2 than on Farm 3 ($p<0.01$ for 2005, 2006, and 2007). Only Farm 2 had a significant change in average disease severity from year to year. Between 2005 and 2006, the average increased significantly (2005: $\bar{x}=1.32\pm4.94$; 2006: $\bar{x}=3.55\pm7.39$; $p<0.01$). Disease severities then significantly decreased between 2006 and 2007 (2007: $\bar{x}=1.51\pm4.23$; $p<0.01$). There was no significant difference in the levels of disease in 2005 versus 2007 ($p=0.99$). The average disease severity on Farm 3 decreased each year, but the changes were not significant (Appendix 2, Table A2.1).

There was a significant negative correlation between disease severity and tree age on Farm 3 in 2006 ($r=-0.12$, $p=0.01$), and a significant positive correlation in 2007 ($r=0.13$, $p=0.02$). On Farm 2, there was a significant positive correlation between disease severity and tree age only in 2007 ($r=0.11$, $p=0.03$). Correlations in other years for both farms were not significant (Table 3.1)

There was no relationship between disease severity and the degree of latent infection on individual lead trees in 2006 on either farm ($p=0.43$ and $p=0.18$ for Farms 2 and 3, respectively). On average, 2.4 (±2.32) of the 10 asymptomatic shoots sampled on

each of the lead trees on Farm 2, and 1.71 (±2.08) of the 10 asymptomatic shoots sampled on each of the lead trees on Farm 3, were latently infected in 2006.

Detection of Fungal Propagules on Shearing Tools

D. pinea propagules were recovered from shearing tools during each year of the study on both farms. In 2005, one half of one tape from each pair collected was visually inspected under a microscope, while the other half was cultured. There was no significant correlation between the number of *D. pinea* spores observed on one half, and the CFUs recovered from the other half, on Farm 2 (r=0.24, p=0.73), but there was a significant positive correlation on Farm 3 (r=0.35, p<0.01). There were significant positive correlations for both farms when the number of spores on the visually inspected halves was compared with the total CFUs recovered from both tapes in each pair (Farm 2: r=0.21, p<0.01; Farm 3: r=0.48, p<0.01).

The number of CFUs recovered from the shearing tool was significantly correlated with disease severity on the lead tree that had been sheared with that tool on Farm 3 in both 2005 and 2006 (p<0.01, r=0.47; p=0.02, r=0.38, respectively; Appendix 2, Table A2.2). The degree of latent infection of each lead tree was also correlated with the CFUs obtained from the tools after shearing that tree on Farm 3 in 2006 (p=0.01, r=0.44) (Appendix 2, Table A2.3). No significant correlations were observed for Farm 2 (Appendix 2, Table A2.3).

Testing the Efficacy of Lysol® Household Spray for Disinfesting Shearing Tools

For both farms, in both years, treatment of tools with Lysol® spray did not significantly alter the number of *D. pinea* propagules recovered from the tools (p=0.94; Appendix 2, Table A2.4).

I tested the efficacy of Lysol® for prevention of *D. pinea* spore germination *in vitro*, and compared it with other recommended disinfestants (ethanol, bleach, and isopropanol) at different concentrations (5, 10, and 25%). Significantly fewer spores germinated in Lysol® than in ethanol and isopropanol at 5 and 10%. There was no significant difference among these three disinfestants at 25%. Spores germinated at statistically the same rate in all three concentrations of Lysol®. The efficacy of Lysol® as a disinfestant was most similar to bleach across all the concentrations tested (Figure 3.8; Appendix 2, Table A2.5).

I also tested the effect of exposure time on the ability of Lysol® to prevent germination of *D. pinea* in vitro. Even after only 30 seconds of exposure, the germination rate was significantly reduced (p<0.01). Increasing the exposure time continued to decrease the germination rate up until 5 minutes, when maximum efficacy was achieved (Figure 3.9; Appendix 2, Table A2.6).

Tool Transmission Studies

On Farm 2, there was a significant correlation between the *D. pinea* CFUs recovered from tools in 2006 and the disease severity in 2007 on the trees that had been sheared with those tools (p=0.02, r=0.22). There was no significant correlation in

2005/2006. On Farm 3, the correlation was significant in both 2005/2006 (p=0.01, r=0.19) and in 2006/2007 (p<0.01, r=0.32) (Appendix 2, Table A2.7).

In both 2005 and 2006, the CFUs recovered from tools on both farms were not significantly different between the first and the last tree to be sheared in each group (Appendix 2, Table A2.8). There was no correlation between the percent disease on each tree was compared with the number of CFUs acquired on the tools the previous year after shearing the previous tree in the group (Appendix 2, Table A2.9).

As a further test for the potential of shearing for transmission of *D. pinea*, healthy Austrian pine shoots were cut with scalpel blades that had been purposely infested with *D. pinea* inoculum. The amount of inoculum that was transferred to the blades was initially evaluated by slicing into heavily diseased Austrian pine shoots, on which *D. pinea* was sporulating, between 1 and 15 times (Figure 3.10). Healthy Austrian pine shoots that were cut with blades infested by slicing diseased tissues five times in 2006 did not develop symptoms of tip blight up through the late spring of 2007, when the experiment was ended. *D. pinea* can produce latent, asymptomatic infections in Austrian pines (31), so each shoot was tested for the presence of latent infections in 2007 using the nested PCR technique. None of the 100 shoots that had been cut with sterile blades had detectable latent infections. Of the 100 shoots that had been sheared with infested blades, six were latently infected with *D. pinea*. The probability of latent infection increased significantly as the degree of infestation of the blades used for shearing increased; specifically, the probability increased by a factor of 2.53 for each additional CFU.

Analysis of the Role of Resin in Diplodia Tip Blight Symptom Development

Shoot necrosis (dieback) resulted when *D. pinea* spores were applied to Scots pine shoot tips after shearing; no necrosis occurred on the water controls. In both 2006 and 2007, the amount of tip dieback decreased as the time elapsed since shearing increased from 0 to 24 hours (Figure 3.11; Appendix 2, Table A2.10). In 2006, all except the 0 hr and 6 hr treatments were significantly different. In 2007, these differences were significant for all treatments ($p<0.01$).

Analysis of the Role of Spore Concentration in Symptom Development

I compared the amount of tip dieback that resulted from application of $1x10^4$, 5000, 100, or 0 *D. pinea* spores to Scots pine shoot tips immediately after shearing. The length of the necrotic lesion produced increased significantly as the number of spores applied increased (Figure 3.12; Appendix 2, Table A2.11). Only 22 of the 24 shoots (91.7%) that were inoculated with 100 spores developed tip blight symptoms. All of the shoots inoculated with higher spore concentrations became symptomatic.

Discussion

The two Christmas tree farms that I chose for my study differed in many ways. Farm 2 is much larger than Farm 3 (7 acres as opposed to 2 acres). Farm 2 sheared their trees earlier each year, and utilized a mechanical trimmer, whereas Farm 3 sheared trees by hand with a pair of hedge trimmers. Farm 2 is on a slope and is generally drier, whereas Farm 3 is in a low, relatively flat area surrounded by tall trees that cut the wind and increase the humidity. Farm 2 is in a relatively rural area, but Farm 3 has been

completely surrounded by Lexington subdivisions for about ten years. During all three years of my study, Farm 2 had significantly higher levels of tip blight disease than Farm 3. The operator of Farm 2 first noticed tip blight symptoms on his farm in 2004 (the year before I began my study). He shared with me his suspicion that tip blight might have been brought onto his farm by workers contracted to shear the trees the previous year. In contrast, the operators of Farm 3 first noticed tip blight symptoms on their farm many years earlier, in 1995 or 1996, and they reported that the disease has been present ever since. Both farms usually remove heavily diseased trees (those with more than 30-40% diseased branches) and selectively prune blighted shoots and branches to improve appearance and reduce inoculum. Neither farm uses chemical fungicides. On Farm 3, there are several older Scots pines at the edge of the farm with active tip blight infections: however, these trees are downwind of most of the trees that are being grown on the farm for sale (Figure 3.2.A).

The average tip blight disease severity on Farm 2 increased significantly between 2005 and 2006, but then decreased between 2006 and 2007. In contrast, disease levels on Farm 3 were basically unchanged during the three years of the study. A decrease in disease severity will result if diseased tips removed by shearing are not replaced by new infections of elongating tips the following year. If new infections do not occur, it is possible to get rapid and drastic reductions in disease severity levels in a very short time (e.g. on Farm 1, see chapter 2 of this thesis). One reason for the difference between Farm 2 and Farm 3 may have been the amount of inoculum present. If a large quantity of inoculum was introduced on Farm 2 in 2003, this could have resulted in the rapid development of a Diplodia tip blight epidemic in 2004-2006 that was beginning to be

brought under control by 2007. In contrast, the longer, slower epidemic on Farm 3 may have originated from the introduction of much smaller quantities of inoculum, perhaps from infected landscape Austrian and Scots pines in the surrounding subdivisions. Farm 3 also may have experienced fewer new infections than Farm 2 because of less favorable weather conditions (e.g., less rain) during the early spring when most infections would have been occurring. Clark County did receive more rain in April and May of 2006 than Fayette County (see Appendix 1 of this thesis). However, weather patterns are sporadic in central Kentucky (e.g. one part of a county may receive a couple centimeters of rain while the weather station records only a trace), so I cannot really tell from the NOAA data if the weather patterns differed on the two farms. Farm 2, because of its topography, was generally drier than Farm 3, and this may have caused the trees to experience more water stress, making them more susceptible to symptomatic Diplodia tip blight than the trees on Farm 3 (16, 48, 55). Another difference between the two farms is tree spacing. Trees on Farm 3 are spaced evenly, but on Farm 2 some trees are closer together than usual, perhaps enabling the fungus to spread more quickly from nearby infected trees. Since Farms 2 and 3 usually share orders of tree seedlings, I can eliminate this possibility as a source for the differences I observed.

The growers usually removed symptomatic trees and tissues quickly, but I wondered about the role of asymptomatic, latent infections in the spread and development of tip blight disease on the Christmas tree farms. My study demonstrated that most of the lead trees on both farms were latently infected, though at a relatively low level. Flowers et al. (31) found that there was a significant positive correlation between the visible disease severity and the degree of latent infection of landscape Austrian and

Scots pines. However, I did not see a similar correlation in my study. Because heavily diseased trees are removed from Christmas tree farms promptly, nearly all of the trees in my study had relatively low levels of disease (less than 20%). Many of the trees in the Flowers study were much more severely infected, and this might explain the difference in our results. I did find a significant correlation between the degree of latent infection and the number of CFUs recovered from tools used to shear those trees. However, I believe that this correlation is an artifact that results from the relatively small number of my samples that were either infected or contaminated because the r-values were rather low.

If my hypothesis was correct, and *D. pinea* inoculum was being picked up on tools during shearing, I expected to see a positive correlation between the amount of disease on the lead tree and the amount of inoculum recovered from the tool after shearing that tree. In both years of my study, Farm 3 had a significant correlation, but Farm 2 never did. Farm 3 sheared their trees later in the summer than Farm 2. At least once during the multi-day process each year, trees on Farm 3 were sheared when they were wet, and when pycnidia exuding spores were easily visible on diseased shoots. Farm 2 never sheared wet trees while I was present, nor did I ever notice pycnidia exuding spores while shearing at Farm 2. Another factor might have been the tools used. Farm 2 used a rotary motorized shearer, which would have cut through the diseased tips much more quickly than the hand pruners used on Farm 3. There may not have been enough contact time between the blade and diseased shoots to pick up spores, or the centrifugal force generated by the blade might have thrown them off before they could adhere. The potential role of the type of shearing tool in accumulation of *D. pinea* propagules is an important question for future study.

I wanted to know if the inoculum acquired on the tools consisted of conidia versus mycelium. In 2005 I examined one half of one tape press from each pair under the light microscope, and cultured the other half. It was difficult to positively identify and count conidia because the tapes often also contained pieces of rust and plant material. The rust fragments sometimes resembled conidia, and plant material occluded my view of portions of the sample, perhaps causing me to miss spores on my slides. If I were going to do this again, I would try staining the tape samples with a stain specific for fungi, such as calcofluor. I expected to see a significant positive correlation between the number of visible *D. pinea* propagules on one half of the tape and the CFUs recovered from the other half, but I saw this only for Farm 3. Perhaps there was no correlation for Farm 2 because more spores were located on one half of the tape than the other, due to the asymmetrical shape of the shearing blade. One side of the blade tooth is longer then the other, so when the tapes were applied and then cut in half, one half of the tape had contact with more of the blade then the other half. The lack of correlation could also have been a function of the lower number of CFUs recovered from the tools on Farm 2, because when the conidial counts were compared to the CFUs recovered from both tapes in each pair together, there were significant positive correlations for both farms. This evidence suggests that the majority of recoverable inoculum on the tools consisted of conidia, and not mycelium.

Lysol® spray was not effective as a disinfestant for shearing tools. My *in vitro* spore germination assays showed that Lysol®, even at a concentration of only 5%, was a highly effective disinfestant if the spores were exposed to it for a long period of time (overnight). However, when sprayed on tools in the field, Lysol® evaporated very

quickly and the tools were usually completely dry within 30 seconds. *In vitro*, Lysol® began to have a significant effect on spore germination after 30 seconds of exposure, but it did not reach maximum efficacy until 5 minutes. Thus, the ineffectiveness of Lysol® on the tools in the field could be because the Lysol® dried so quickly. Another possibility may be related to the resin and plant material that accumulate on the tools during shearing. This material may coat and embed the spores, and create a protective barrier against the Lysol®.

Although my research established that shearing tools could pick up *D. pinea* inoculum from diseased trees, there was no evidence in support of the hypothesis that the inoculum could be transmitted to healthy trees and cause disease. Previous studies have demonstrated that *D. pinea* aggressively infects through wounds and can cause shoot dieback within weeks (24, 31, 44). However, I found diseased tips only on elongating shoots, and I never once saw dieback associated with the sheared tips during three years of study on three different farms. More evidence against transmission by the tools is the number of CFUs that were recovered from tools used to shear the first tree versus the last tree in each experimental group. If transmission were occurring, I expected the number of recoverable CFUs to decrease, but it actually remained about the same. Perhaps the spores become trapped in the resin on the blades and don't easily come off again during shearing. If transmission were occurring, I also expected there to be a significant positive correlation between the CFUs recovered from tools and the disease severity the following year on the trees sheared with those tools. In fact, for both farms in both years (with the exception of Farm 2 in 2005-2006) there was a significant correlation, but I believe that

these correlations are statistical artifacts that result from the low levels of disease and small numbers of CFUs recorded for the majority of the samples.

I did a more direct, controlled test of tool transmission by shearing Austrian pines with blades that I had purposely infested with *D. pinea*. Symptoms of tip blight did not develop during the year that I sheared, or during the following spring. I tested each cut shoot for the presence of latent infections, and of the 100 shoots cut with infested scalpel blades, six were latently infected with *D. pinea*. None of the 100 shoots cut with sterile blades had latent infections. I found that the more CFUs there were on a scalpel blade, the more likely it was that the blade would produce a latent infection. None of the infested blades had more than 10 recoverable CFUs after shearing. I wondered if there was a minimum number of spores needed to create symptomatic infections on Scots pines, and so I applied various dilutions of *D. pinea* spores to sheared Scots pine shoots. Significantly more necrosis developed as inoculum increased, demonstrating that the effectiveness of host defenses in limiting colonization of sheared shoots is related to the amount of inoculum initially present. Symptoms did not develop in every case when I applied only 100 spores to the wound, suggesting that 100 spores is minimal for production of a symptomatic infection on a freshly wounded tip. The relationship between spore numbers and latent versus symptomatic infections would be interesting to study further.

The shearing process creates open wounds that are potential infection points, but the sheared branches quickly exude resin that hardens to seal the wounds. It has been demonstrated that resin exudation is a primary defense mechanism against fungal or insect invasion (14). I applied spore suspensions of *D. pinea* to Scots pine shoots at

various times after shearing. Less disease developed when the resin was allowed to harden for 24 hours. However, even when the resin cap was still in a liquid state, six hours after shearing, it apparently decreased the number of spores that germinated and/or decreased the number of successful penetrations into the shoot. In this experiment, 10,000 spores were applied to each tip. This is probably more spores than any shoot would naturally encounter.

These data together lead me to propose that shoots will be least likely to become infected during shearing if they are cut at a time when inoculum levels are minimal (not during or immediately after a rainfall, for example) and when conditions are optimal for production of and drying of resin (e.g. early on a dry day).

My study provided no support for my original hypothesis that *D. pinea* can be transmitted via infested shearing tools, although it did demonstrate that shearing tools can pick up inoculum from diseased trees. My work provides no basis, therefore, to recommend that tools be routinely disinfested during shearing. This is especially true when we consider the lack of available disinfestants that are both convenient to apply and relatively harmless to the tools. Lysol® spray certainly cannot be recommended because it appeared to have no effect on *D. pinea* spores on shearing tools.

Table 3.1. Correlation between the percent of symptomatic tips and the tree age on Scots pine Christmas trees from two Kentucky farms.

	Farm 2				Farm 3			
Year	Mean Age (yr)	Mean % Disease	*r*-value	*p*-value	Mean Age (yr)	Mean % Disease	*r*-value	*p*-value
2005	5.38	1.32	-0.07	0.09	4.59	0.38	0.04	0.41
2006	6.13	3.55	0.08	0.10	5.10	0.19	-0.12	0.01
2007	7.15	1.51	0.11	0.03	6.04	0.09	0.13	0.02
All Years	6.09	2.12	-0.05	0.05	5.17	0.24	-0.002	0.95

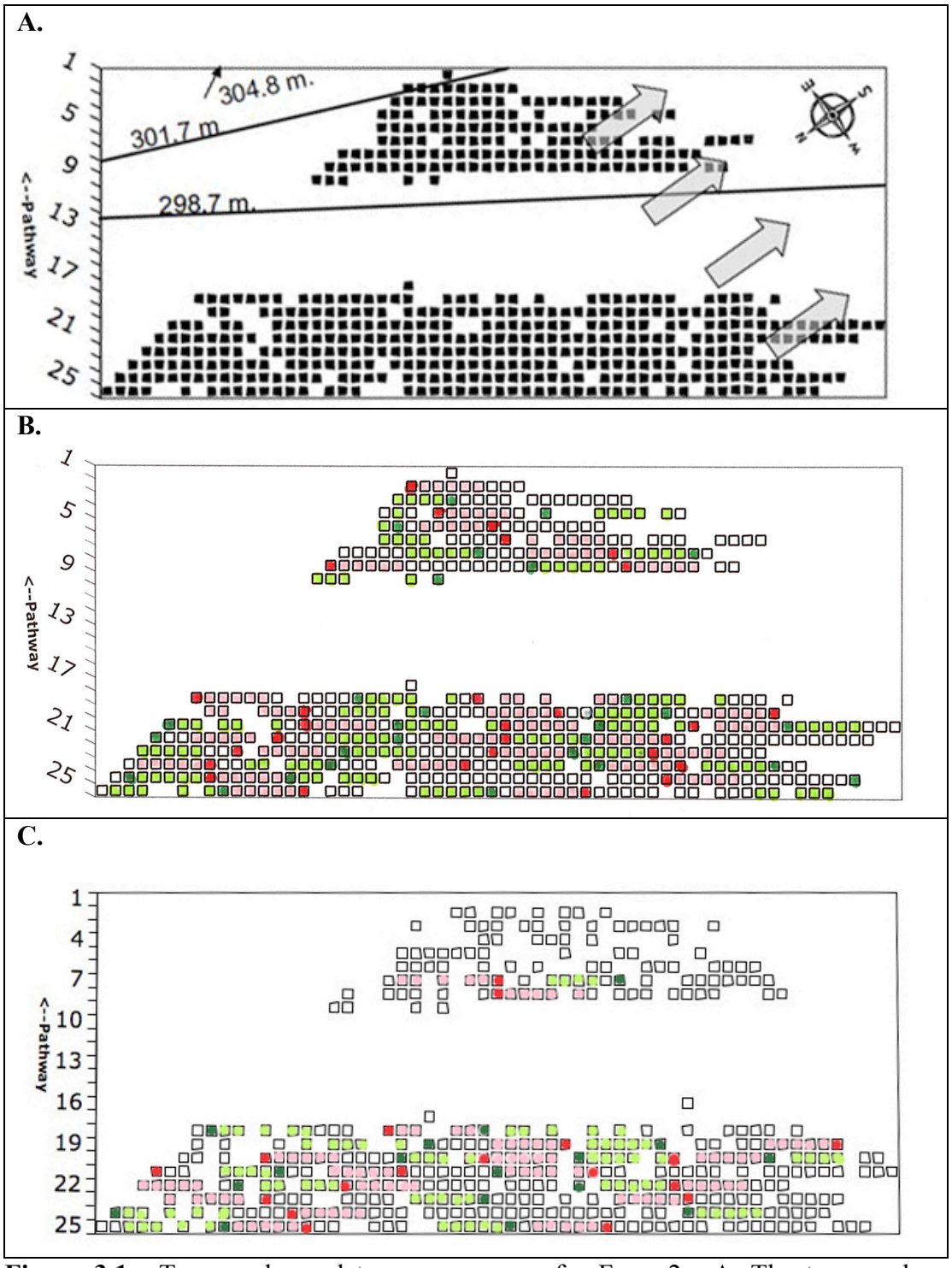


Figure 3.1. Topography and tree group maps for Farm 2. **A.** The topography map indicates elevations, as well as prevailing wind directions (arrows). Tree groups in 2005 are shown in maps **B.**, and groups in 2006 are shown in map **C.** The dark squares (red or dark green) represent the lead trees, with the following trees in that group indicated as lighter colored squares (pink or light green). White squares are Scots pines that were not used. Not all groups could be used again in 2006, and so new groups were created to replace them.

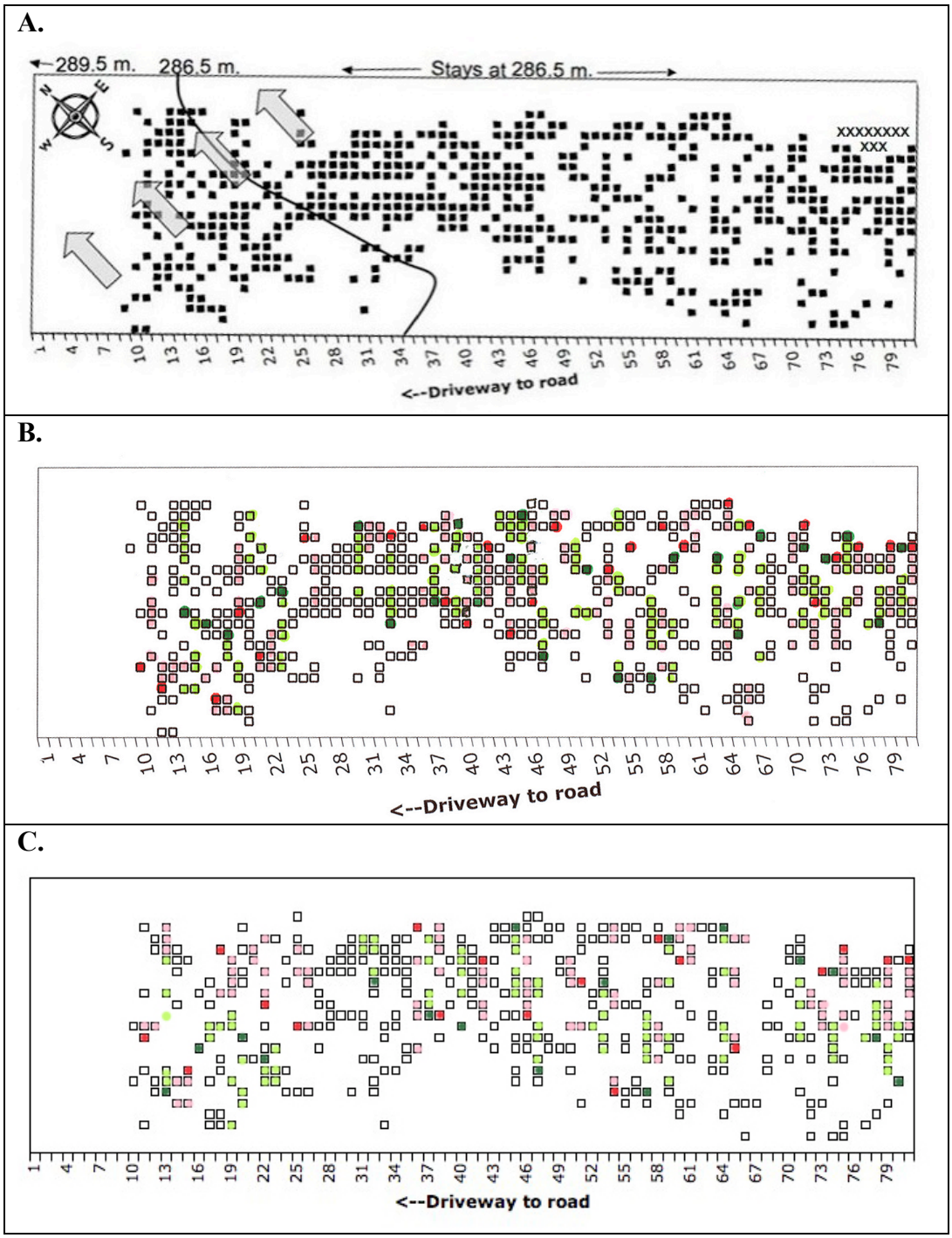


Figure 3.2. Topography and tree group maps for Farm 3. **A.** The topography map indicates elevations, as well as prevailing wind directions (arrows). "XXX" indicates older Scots pines, which are not being grown for sale, that have symptomatic *D. pinea* infections. Tree groups in 2005 are shown in maps **B.**, and groups in 2006 are shown in map **C.** The dark squares (red or dark green) represent the lead trees, with the following trees in that group indicated as lighter colored squares (pink or light green). White squares are Scots pines that were not used. Not all groups could be used again in 2006, and so new groups were created to replace them.

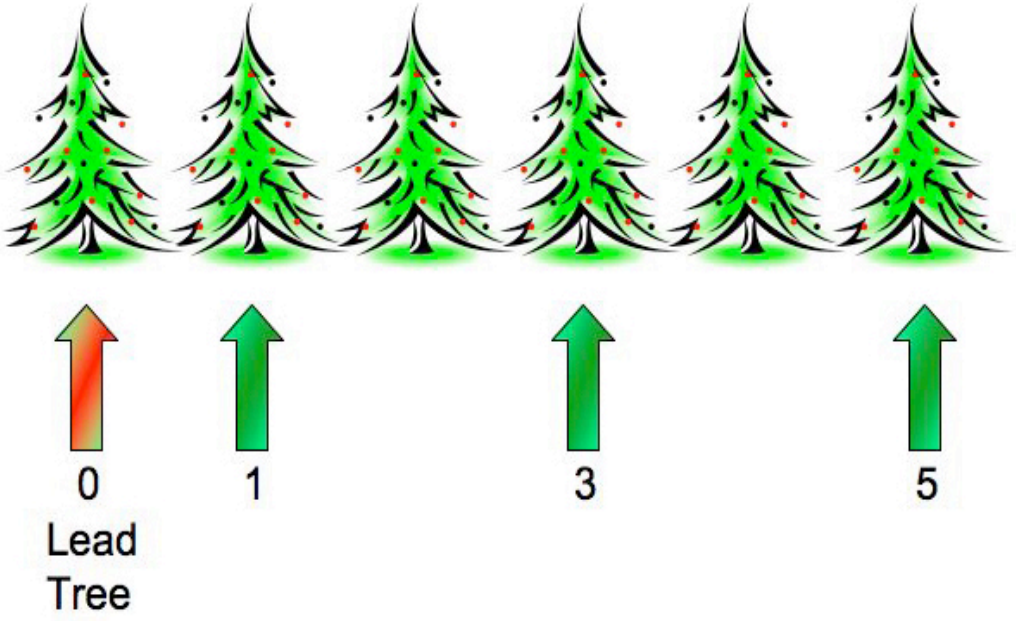


Figure 3.3. A schematic of the shearing order of Scots pines Christmas trees. Tape press collection points from the tools are indicated by red or green arrows.

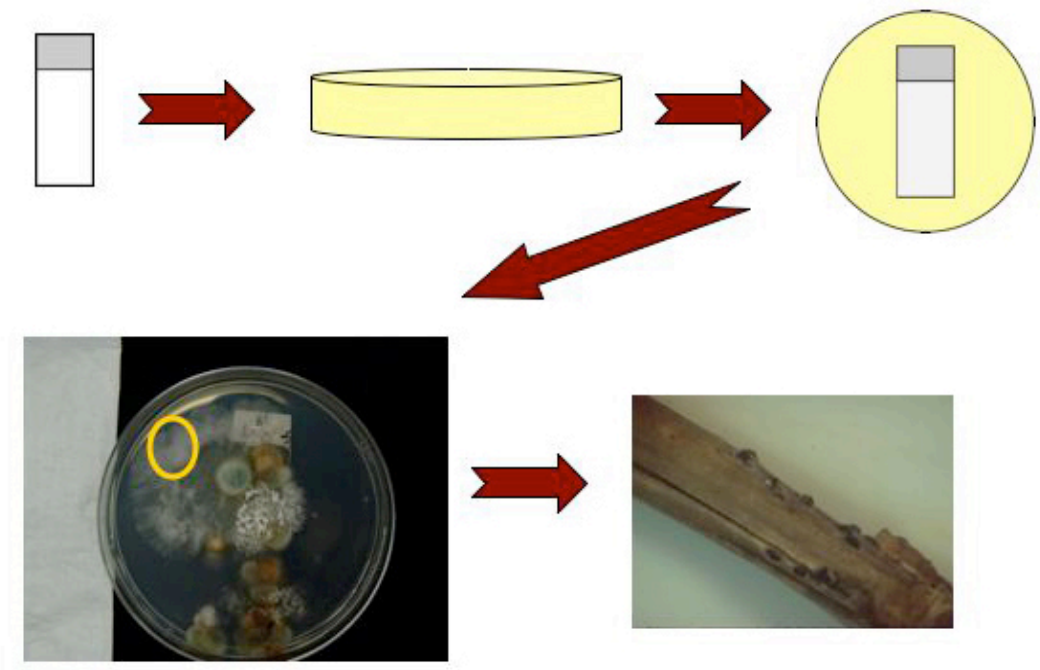


Figure 3.4. A schematic representation of laboratory procedures to confirm the presence of *D. pinea* on shearing tools. Tape presses collected off shearing tools were placed in Petri dishes and covered with acidified PDA. Fungal colonies that resembled *D. pinea* were subcultured onto water agar plates containing autoclaved pine needles. The small yellow circle highlights a fungal colony that was suspected to be *D. pinea.* Spores produced in the pycnidia that formed on the autoclaved needles were used to identify fungal colonies as *D. pinea*.

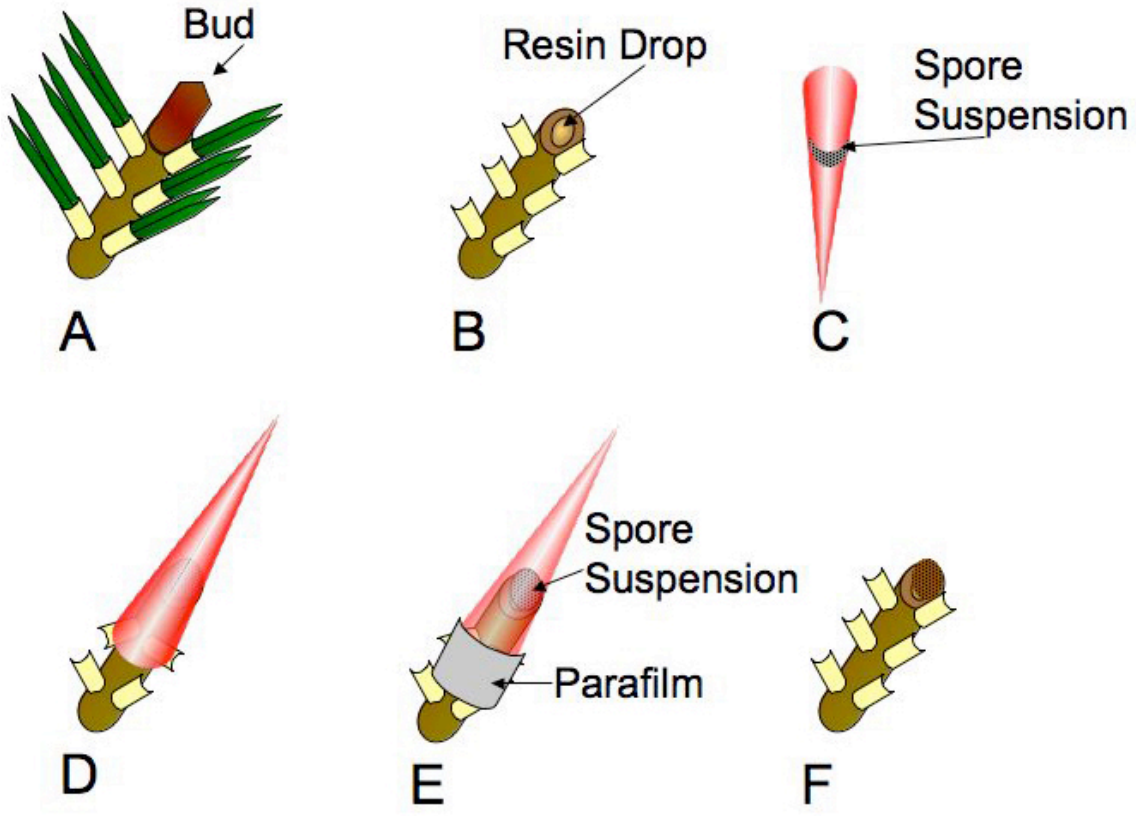
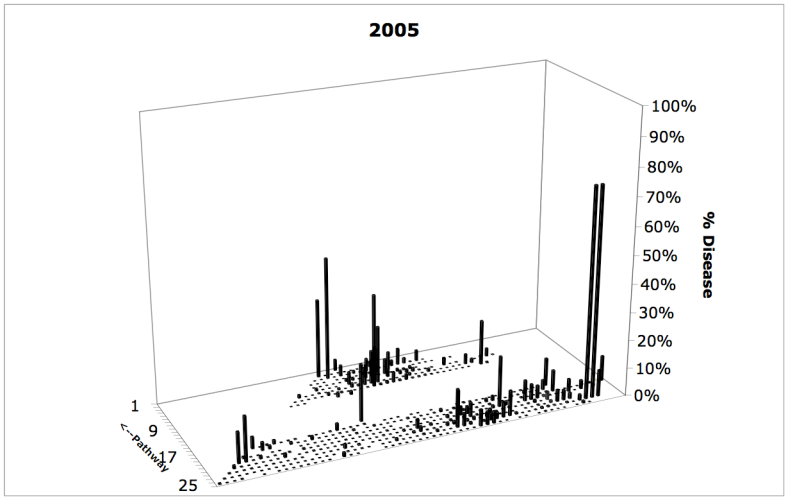
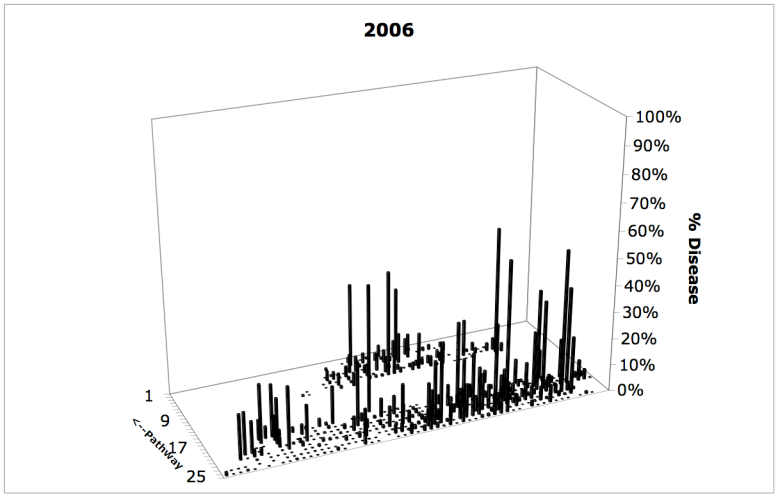


Figure 3.5. Depiction of procedures used to artificially inoculate sheared tips of trees. Tips of branches (**A.**) were cut off and the needles removed just below the cut end (**B.**). After cutting the tips, resin droplets began to form on the cut end. Spore suspensions of *D. pinea* were added to the tip via a 200µL plastic pipette tip so that the suspension would drop onto the cut end (**C.-E.**). Pipette tips were parfilmed onto the shoot to create a humidity chamber around the inoculated end (**E.**). After 24hr, pipette tips and parafilm were removed, leaving the spore suspension behind (**F.**).

A.



B.



C.

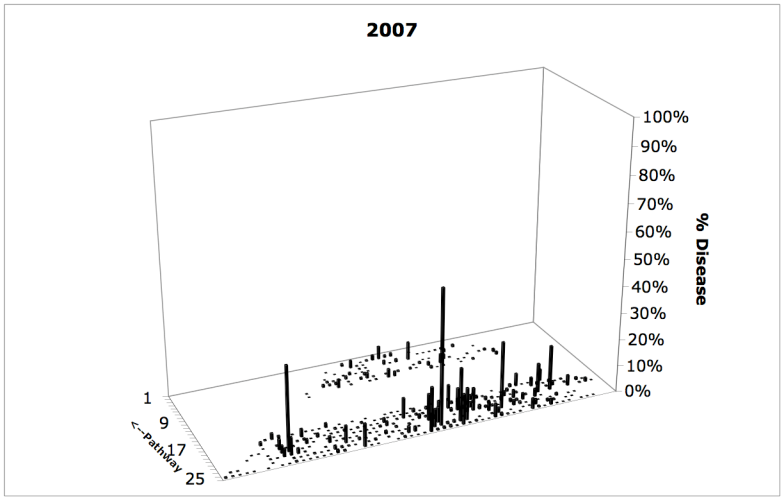
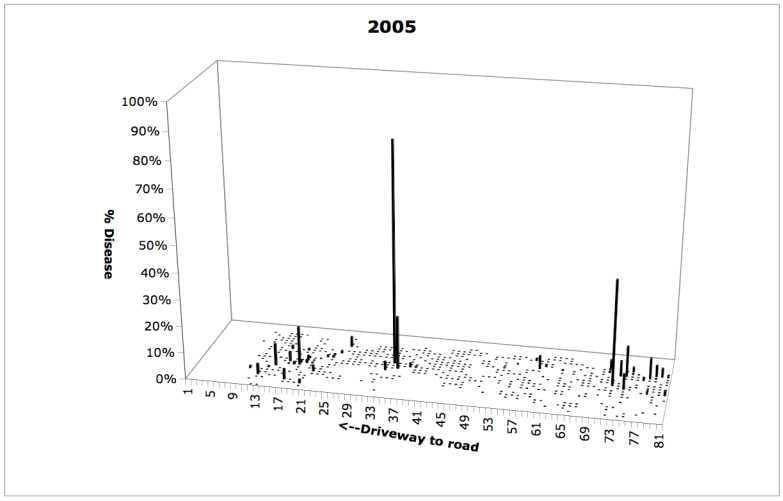
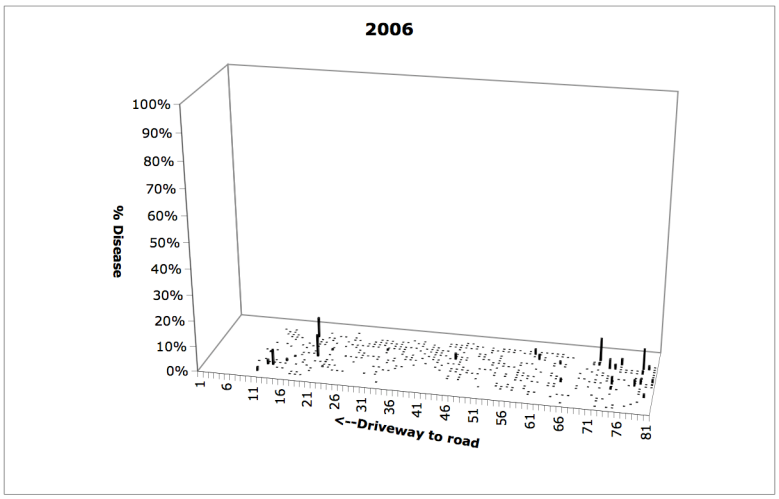


Figure 3.6. Disease percentage maps for Farm 2, years 2005 (**A.**), 2006 (**B.**), and 2007 (**C.**).

A.



B.



C.

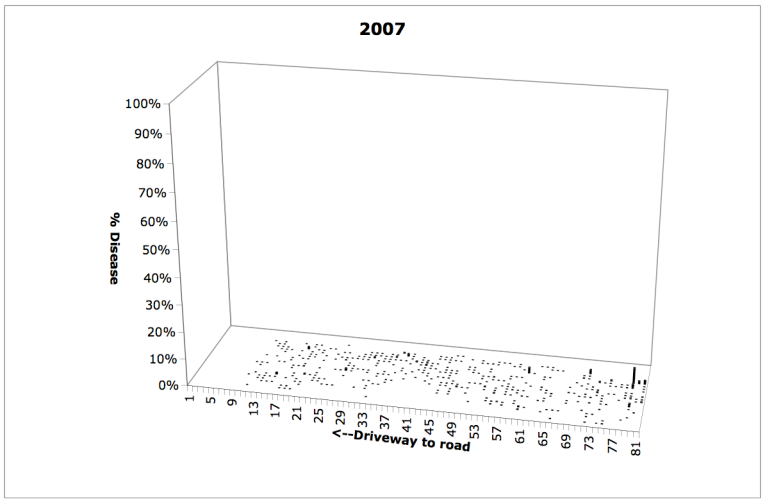


Figure 3.7. Disease percentage maps for Farm 3, years 2005 (**A.**), 2006 (**B.**), and 2007 (**C.**).

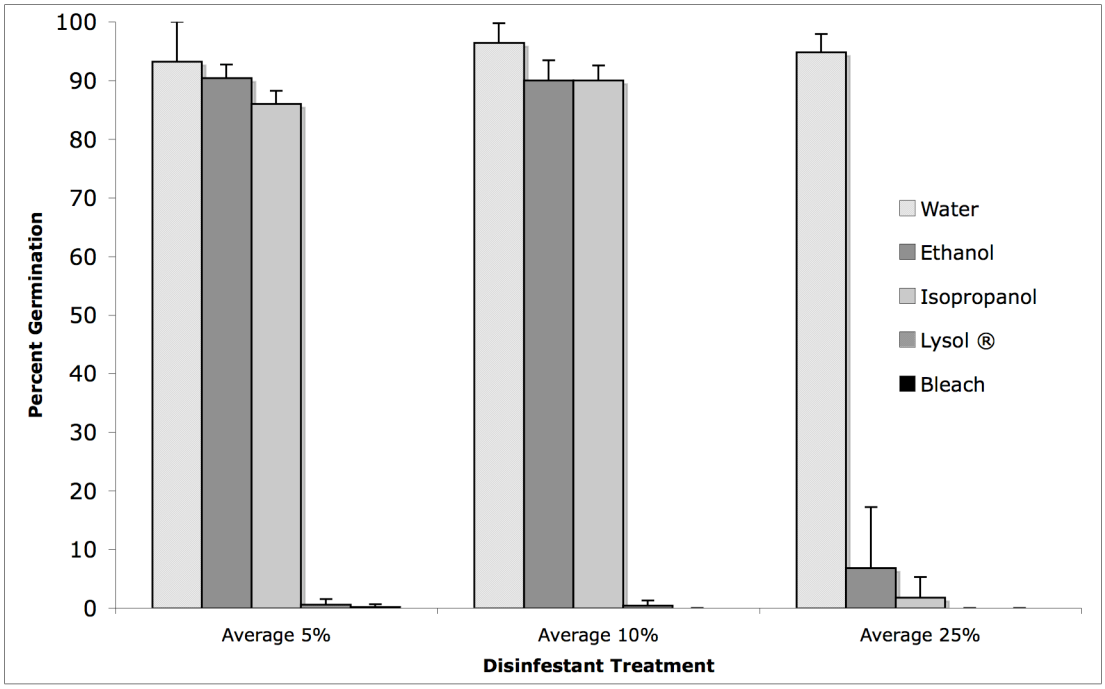


Figure 3.8. Average germination rate of *D. pinea* spores after 12 hr exposure to various concentrations of different disinfestants.

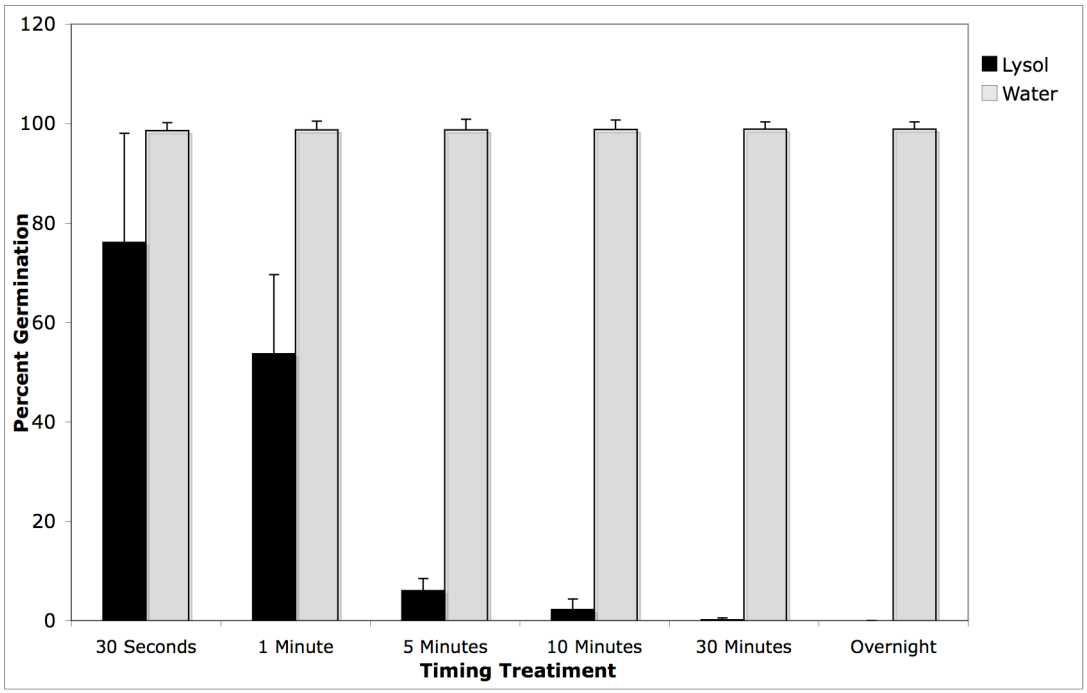


Figure 3.9. Average germination rate of *D. pinea* spores after exposure to 25% Lysol® Disinfectant Spray for various amounts of time.

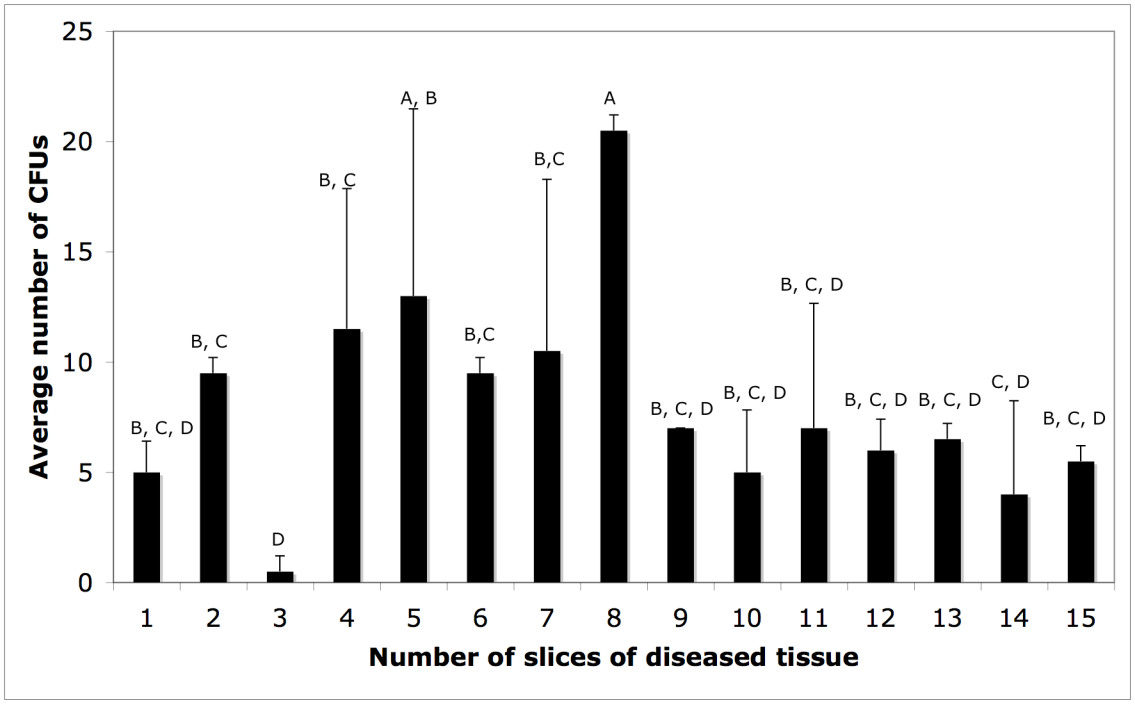
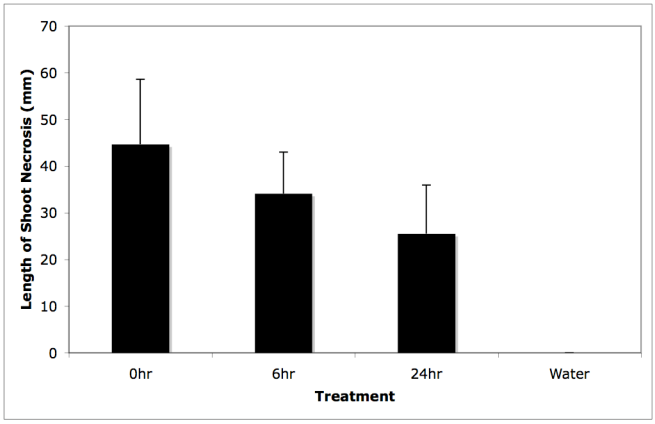
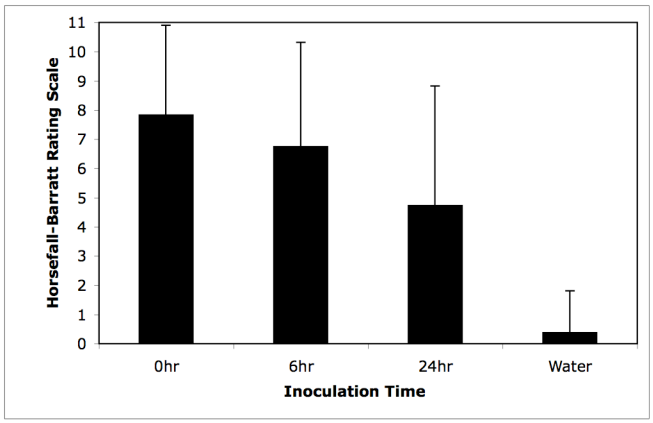


Figure 3.10. Average number of CFUs acquired on sterile scalpel blades after slicing into diseased Austrian pine tissue a varying number of times. Treatments with the same letters are not significantly different.

A.



B.



C.

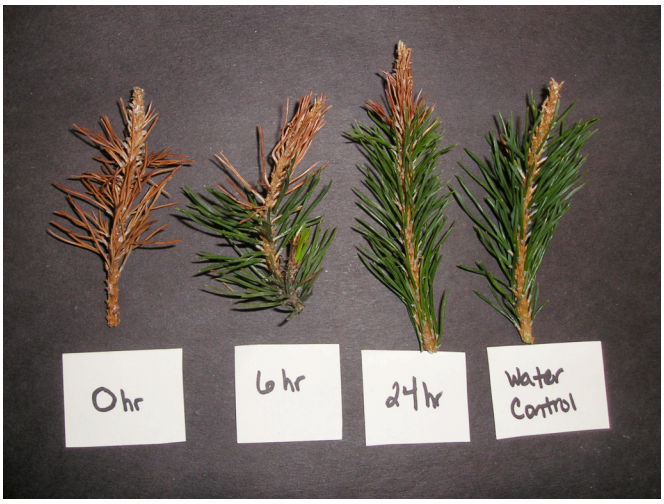
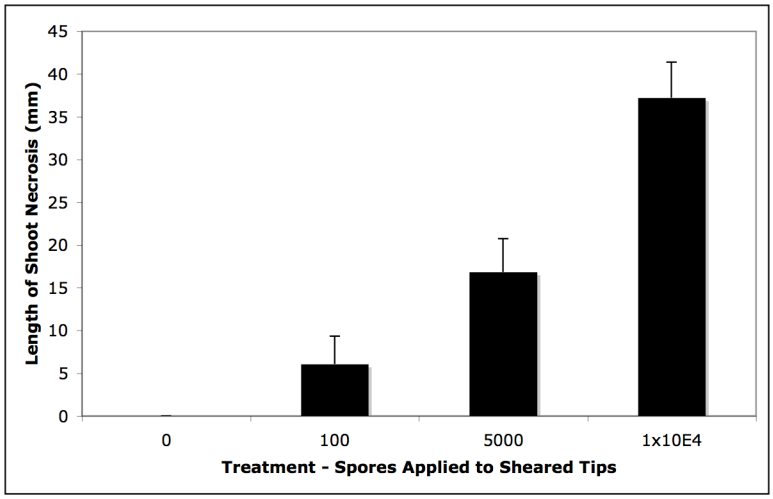


Figure 3.11. Shoot necrosis development on Scots pine seedling inoculated with *D. pinea* at varying times after shearing. In 2007 (**A.**), the lengths of shoot necrosis were measured, but in 2006 (**B.**), the Horsfall-Barratt rating scale was used to estimate the degree of necrosis that developed on the shoots. In 2007, all treatments were significantly different from each other ($p<0.01$). Shoot necrosis from the cut tip back toward the shoot base was visibly different between treatments (**C.**).

A.



B.

Figure 3.12. Length of shoot necrosis development after inoculating shoots of Scots pine seedlings with varying concentrations of *D. pinea* spores. All treatments were significantly different from each other ($p<0.01$).

CHAPTER FOUR:

MANAGEMENT RECOMMENDATIONS FOR DIPLODIA TIP BLIGHT ON SCOTS PINE CHRISTMAS TREES

Previous recommendations for Diplodia tip blight disease management in landscapes and on Christmas tree farms have included: use of good sanitation practices (removal of diseased trees and branches promptly, and disposal of infested material, especially cones, from underneath the trees); application of protectant fungicides to the trees in spring before symptoms develop; avoiding pruning or shearing while the trees are wet; and sanitizing shearing tools (2-45).

The Christmas tree growers I worked with during my study routinely removed severely diseased trees and branches. Sanitation is easier to practice on a Christmas tree farm where aesthetically damaged trees are virtually worthless, than in a landscape where each individual tree has more than just monetary value. Removal of infected material would be expected to reduce inoculum and infection levels, since my data suggest that most inoculum is dispersed locally, and not over long distances in rainfall. Annual shearing of the Christmas trees removes most of the newly infected tips each year, so the fungus does not have an opportunity to move back along the branches to colonize the older growth. Removing the diseased tips also eliminates a potential inoculum source for the following year. I often found tip blight symptoms developing in close proximity to infested material that had been left to overwinter within and under the tree, including detached tips produced from shearing, naturally senescent needles, and attached tips that had been missed during shearing (see Chapter 2 of this thesis). If this diseased material

could be removed each winter, it would decrease the inoculum available in the spring to infect new shoots, and I would expect this to decrease disease severity. However, physical removal of all the infested material from within and under the trees would be very difficult. This problem would be a good candidate for a biological control approach, if an agent could be identified that could help degrade the dead material, or even parasitize the pycnidia so that they could not release spores.

Tip blight disease levels can be rapidly reduced, even on a badly affected Christmas tree farm, if diseased tips can be removed while new infections are prevented. My data suggested that infection of sheared tips is unlikely unless the number of spores transferred to those tips is extremely high, and the tips themselves are freshly cut and haven't had an opportunity to create a protective resin cap. Thus, decreasing the probability that these two things will occur should decrease the likelihood of infection of sheared tips. Promoting conditions that are unfavorable to sporulation can minimize inoculum levels. Cutting weeds and grass around the trees and spacing trees further apart will reduce humidity and promote drying of foliage, both of which should reduce sporulation. Since pycnidia are easily visible with a hand-lens, I would recommend that the grower scout for these directly, and avoid shearing when sporulation is actively occurring. It will also be best to shear when resin flows most readily, e.g. in the morning after the dew is gone, on a dry day when the resin will dry relatively quickly to protect the shoot.

Even though I was able to culture *D. pinea* from shearing tools, I would not recommend that growers routinely disinfest their tools. On the Kentucky Christmas tree farms I studied, having inoculum on the tools did not appear to lead to transmission and

subsequent infection of other trees or shoots, probably because the inoculum loads were always very low. In my opinion, the time and inconvenience required to disinfest tools outweighs the potential benefit, especially since spores that are encased in resin on tools may be resistant to most disinfestants. The choice of shearing tool may be more important: the rotary shearer used by Farms 1 and 2 may have a reduced potential to catch and carry plant material and spores. Many growers (though not those studied here) use a dual-action mechanical hedge trimmer to shear trees. This type of instrument is likely to retain a lot of plant (and fungal) material between the blades, and may therefore have more potential to carry inoculum from tree to tree. More research on this important topic would be desirable.

All new tip blight infections occurred on unsheared tips in my study. Aside from reducing inoculum, it may be possible to reduce the number of newly infected tips by using fungicides. Protectant fungicides are expensive, and probably not an economically viable option for regular use by most Christmas tree growers. They were suggested for use on landscape trees in the 1990s, but this recommendation has since been abandoned due to their lack of effectiveness in controlling tip blight on the U.K. campus (35, 37). However, Farm 1 began to use protectant fungicide sprays (Cleary's 3336™) in 2005 (see Chapter 2 of this thesis). By 2007, the disease severity on Farm 1 had been dramatically reduced. I believe the fungicides applied to the trees on the U.K. campus were not effective because it was not possible to achieve complete coverage. Furthermore, the trees on campus were generally much more heavily infected, and so there were many inoculum sources within and especially under the trees (i.e. infected cones and needles; see Chapter 1 of this thesis). I propose that spraying the trees with

Cleary's 3336™ reduced the number of new infections that occurred on Farm 1. Since there were not as many infected tips, fewer infected tips were left to overwinter on the tree or on the ground. This decreased the amount of inoculum available the next spring, and so even fewer infections occurred. The experience of Farm 1 suggests that limited use of fungicides, even for just a few years, might be useful as a curative for a badly infected farm, if used in combination with good sanitation. Routine use of the chemicals over the long term may not be necessary if inoculum levels can be kept under control. This is definitely a question that deserves further investigation.

Overall, my work has given me some hope that we can successfully manage this disease on Christmas tree farms with a combination of sanitation and chemical controls. Because the trees are not grown for as long as landscape pines, it is necessary to maintain their aesthetic beauty only for a few years. This is a different scenario from landscape trees that need to stay aesthetically pleasing and healthy for many decades. Given the value of Scots pine as a Christmas tree species in Kentucky, it will be worthwhile to continue to test these recommendations.

APPENDIX ONE:

CHRISTMAS TREE FARMS USED FOR THIS THESIS

Three different Christmas tree farms were used for my Master's thesis study of Diplodia tip blight on Scots pine Christmas trees. All three of these central Kentucky farms belong to the Kentucky Christmas Tree Association. Even though they all grow Scots pines for choose-and-cut Christmas tree production, each grower uses different cultural practices, and the environmental conditions under which the trees are grown also differ. A summary of climatic conditions from 2005-2007 is provided in Figure A.1.2. This appendix provides detailed cultural and environmental information about each of these three farms.

BARKER'S CHRISTMAS TREE FARM

Barkers's Christmas Tree Farm (Farm 1), owned and operated by Mr. Dale Barker, is a seven-acre urban choose-and-cut operation located in Fayette County, Kentucky. The predominant soil type is Maury silt loam with a pH of 6.2. The typical soil profile is silt loam from the surface to a depth of 40 cm, silty loam from 40-73 cm, silty clay from 73-106 cm, and clay from 106-190 cm.

Barker's Christmas Tree Farm was established in 1993. It generally takes 6-7 years to grow a Scots pine to a sellable size on this farm. Each fall, trees of a sellable size are sprayed with a false colorant to make the trees a vibrant green color. Approximately 300 Scots pines are harvested each year for use either as Christmas trees or to make wreaths. About 55% of the 7000 trees on this farm are Scots pines.

Mr. Barker purchases seedlings primarily from the Kentucky Department of Forestry. Seedlings are typically 2 years old when purchased.

Unlike some of the other Christmas tree growers, Mr. Barker has planted only the French Highland Scots pine variety on his farm. In addition to Scots pines, the farm also produces white pine (*Pinus strobus*), Douglas-fir (*Pseudotsuga menziesii*), Concolor fir (*Abies concolor*) and Canaan fir (*Abies balsamea* var. *phanerolepis*).

Management

Planting

Planting usually takes place spring or in the fall. Trees are planted on 1.8 m x 1.8 m centers, with rows spaced 1.8 m apart. When planting trees in pre-existing rows after trees have been cut for sale, the stumps of the removed trees are first cut to ground level, and then new seedlings are planted about 30 cm away from the old stumps in late winter or early spring.

Weed and Insect Control

The primary method used for weed control is mowing. Chemical weed control is also used. Mr. Barker uses DuPont ™ Oust® XP herbicide and Goal™ 2XL herbicide to control weeds. Insecticides are not used by Barker's Christmas Tree Farm.

Shearing

The recommendation in Kentucky is that Scots pine Christmas trees be sheared between mid-June and early July (depending on tree development). Trees on Barker's

Christmas Tree Farm were sheared according to this recommended schedule in 2005 and 2006. Mr. Barker (with the help of friends and family) shears his Scots pines with a gas-powered Beneke trimmer. Hand pruners are used to remove malformed shoots and leaders as needed. Mr. Barker cleans the blades of his tools by scraping them with a knife, or by washing them with soap and water, at least once each day, and more often if they become gummed up with resin.

Diseases

Symptoms of Diplodia Tip Blight were first noticed by Mr. Barker in 2002 and have been observed every year since. Cleary's 3336™WP fungicide (1-8 oz package/25 gal water) was applied (using the same sprayer used to apply colorant) to the trees for disease control starting in the spring of 2005. The fungicide is sprayed twice per year, once new growth is starting in the spring and again 7-10 days later.

KOVALIC'S CHRISTMAS TREE FARM

Kovalic's Christmas Tree Farm (Farm 2), owned and operated by Mr. Peter Kovalic, is a seven-acre urban choose-and-cut operation located in Clark County, Kentucky. The predominant soil type is Hampshire silt loam with a pH of 5.5. The typical soil profile is silt loam from the surface to a depth of 15 cm, silty clay loam from 15-33 cm, silty clay from 33-152 cm, with unweathered bedrock starting at a depth of 152 cm.

Kovalic's Christmas Tree Farm was established in 1990. It generally takes 7-10 years to grow a Scots pine to a sellable size on this farm. Each fall, trees of a sellable

size are sprayed with a false colorant to make the trees a vibrant green color. Approximately 200 Scots pines are harvested each year for use either as Christmas trees or to make wreaths. The trees typically sell for between $35 and $45 each. About 40% of the 7500 trees on this farm are Scots pine.

Mr. Kovalic purchases seedlings primarily from Carino Nurseries (Indiana, PA), but some have also come from Flickinger's Nursery (Sagamore, PA) and the Peterson's Riverview Nursery (Allegan, MI). Purchased seedling lots are often shared between the Kovalic's Christmas Tree Farm and the Christmas Memories Tree Farm (see below). The seedlings are typically 2-4 years old when purchased. Two-year-old seedlings have been grown in only one planting bed prior to harvest for sale (2-0), whereas four-year-old seedlings have already been transplanted once by the nursery, when they were two years old (2-2).

Mr. Kovalic has planted an array of Scots pine varieties over the years, including Pennspanish II, East Anglia, French Highland, and Belgian. In addition to Scots pines, Kovalic's Christmas Tree Farm also produces white pine (*Pinus strobus*), red pine (*Pinus resinosa*), Norway spruce *(Picea abies*), white spruce (*Picea glauca*), blue spruce (*Picea pungens*), Douglas-fir (*Pseudotsuga menziesii*) and Canaan fir (*Abies balsamea* var. *phanerolepis*).

Management

Planting

Planting usually takes place in the spring or in the fall. Trees are planted on approximately 1.8 m x 1.8 m centers, with rows spaced 1.5-1.8 m apart. When planting

trees in pre-existing rows after trees have been cut for sale, the stumps of the removed trees are first cut to ground level, and then new seedlings are planted about 30 cm away from the old stumps during late winter or early spring. Trees are not routinely irrigated.

Weed and Insect Control

The primary method used for weed control is mowing, which is accomplished with a riding mower. Chemical weed control (i.e. Roundup®) is also used. The broad-spectrum pesticide Sevin® (carbaryl) is used to control redheaded pine sawflies and bagworms. Affected trees are sprayed at a rate of 1oz per gallon of water as needed.

Shearing

The recommendation is that Scots pine Christmas trees in Kentucky should be sheared between mid-June and early July (depending on tree development). During both years of my thesis study (2005 and 2006), Scots pines at Kovalic's Christmas Tree Farm were sheared between the end of June and the second week of July, at the latest. Mr. Kovalic (with the help of friends and family) shears his Scots pines with a gas-powered Beneke trimmer. Hand pruners are used to remove malformed shoots and leaders as needed. Mr. Kovalic cleans the blades of his tools by scraping them with a knife, or by rinsing them with kerosene, at least once each day, and more often if they become gummed up with resin.

Diseases

Symptoms of Diplodia tip blight were first noticed by Mr. Kovalic in 2004 and have been observed every year since. Even though the farm has experienced losses due to disease, he has opted not to use fungicides on the trees.

CHRISTMAS MEMORIES TREE FARM

Christmas Memories Tree Farm (Farm 3), owned and operated by Dr. Bill and Mrs. Fredda Moody, is a two-acre urban choose-and-cut operation located in Fayette County, Kentucky. The predominant soil type is Maury silt loam with a pH of about 6.2. The typical soil profile is silt loam from the surface to a depth of 40 cm, silty loam from 40-73 cm, silty clay from 73-106 cm, and clay from 106-190 cm. About 15% of the soils on the farm are a Huntington silt loam, with a typical soil profile of silt loam from the surface to 28 cm, and silty clay loam from 28- 190 cm.

The Christmas Memories Tree Farm was established in the spring of 1986, when 1500 tree seedlings were planted. Approximately 2/3 of the seedlings were Scots pines (*Pinus sylvestris*), and the rest were white pine (*Pinus strobus*). It generally takes 5-8 yrs to grow a Scots pine to a sellable size on this farm. The first nine Christmas trees were sold in 1991. Trees have been sold annually since then, with the exception of 1998. In that year there were not enough trees available to sell because of droughts in previous years, and because very few seedlings were planted in 1992 to replenish the stock. Approximately 200 trees are sold each Christmas, and about half of these are Scots pines. The trees typically sell for between $35 and $45 each. Overall, about 50% of the trees on this farm are Scots pines.

The Moodys purchase seedlings primarily from Carino Nurseries (Indiana, PA), but some have also come from Flickinger's Nursery (Sagamore, PA) and the Peterson's Riverview Nursery (Allegan, MI). Seedlings are typically 2-4 years old when purchased. Two-year-old seedling have been grown in only one planting bed before harvest (2-0), whereas four-year-old seedlings have been transplanted once by the nursery, at two years old (2-2). The Moody's have planted an array of Scots pine varieties over the years, including Pennspanish II, Lake Superior Blue II, East Anglia, French Highland, and Spanish Guadarrama. In addition to Scots and white pines, they also produce Douglas-fir (*Pseudotsuga menziesii*) and Canaan fir (*Abies balsamea* var. *phanerolepis*).

Management

Planting

Planting usually takes place during late March and early April, but on a few occasions seedlings have been planted in the fall. If new rows of trees are being established, survey flags are placed on 1.8 m x 1.8 m centers during the preceding spring, and Roundup® is used to treat an area of approximately 0.37 square meter surrounding each flag to remove grass and weeds. A spade is used to loosen the soil and make the planting holes, and a seedling is planted at the site of each flag, with careful attention being paid to root placement and soil coverage. When planting trees in pre-existing rows after trees have been cut for sale, the stumps of the removed trees are first cut to ground level, and then new seedlings are planted about 30cm away from the old stumps in late winter or early spring. In some cases, Farm 3 is now producing the third generation of trees grown on the same spot. Starting in 2004, seedlings were fertilized at planting by

adding a 10-gram fertilizer tablet (20-10-5) to the planting hole, but older trees have never been fertilized. Trees are not routinely irrigated.

Weed and Insect Control

The primary method used for weed control is mowing. Mowing is done by using a Honda riding mower or a DR all-terrain bush mower. Chemical weed control (i.e. Roundup®) is only used prior to planting (see above). The broad-spectrum pesticide, Sevin® (carbaryl), is used to control sawflies and bagworms. Affected trees are sprayed at a rate of 1 ½ - 2 oz per gallon of water in early spring and fall for sawflies, and in June for bagworms.

Shearing

During both years of my thesis study (2005 and 2006), Scots pines at the Christmas Memories Tree Farm were not sheared until mid-July. The Moodys utilize a two-person, multi-step shearing process. First, they trim the leader and top whorl of each tree. At this time, new leaders are also trained in the case of damage to or malformation of the original leader. The remainder of each tree is sheared once top-trimming of all trees is completed. During my study in the 2005 and 2006 seasons, the shearing was done by hand using long-handled hand shears. The Moodys clean the blades of their tools at least once each day, and more often if they become gummed up with resin. Rubbing alcohol is used to help remove resin from the tools.

Diseases

The Moodys lose an estimated 1-2% of their trees, most of them seedlings, as a result of disease and drought each year. This number does not include symptomatic trees that do not die, but may be affected enough to decrease their desirability to buyers. New seedlings are planted the following fall or spring to replace trees that die. Christmas Memories Tree Farm has not been as seriously affected by Diplodia tip blight as many other farms in Kentucky. Symptoms of the disease first appeared in either 1995 or 1996, and have been observed every year since. Even though the Moodys have experienced losses due to disease, they have opted not to use fungicides on their trees.

Table A1.1 Summary of information for the three Christmas tree farms used in this thesis.

Farm	Location	Size (Acres)	Year Farm was Established	Year DTB Symptoms First Noted	Scot Pine Varieties Grown
1	Fayette County, KY	7	1993	2002	French Highland
2	Clark County, KY	7	1990	2004	French Highland, East Anglia, Belgian, Pennspannish
3	Fayette County, KY	2	1986	1995	Pennspanish II, Lake Superior Blue II, East Anglia, French Highland, and Spanish Guadarrama

Farm	Fungicides used	Insecticides Used	Herbicides Used	Mowing Frequency	Watering Frequency
1	Cleary's 3336™ (thiophanate methyl)	None	DuPont™ Oust® XP, Goal™ 2XL	6 times/yr	Seedlings only when needed
2	None	Sevin (carbaryl) 1oz/gal for bagworm and red-headed pine sawfly	Roundup®	Every 2-3 weeks during growing season	Seedlings only during severe drought
3	None	Sevin (carbaryl)	Roundup®	2-4 times/growing season	Seedlings only during severe drought

Farm	Frequency of Cleaning Shearing Tools	Products used to clean tools	Predominant Soil Conditions	Other Tree Species Grown
1	Daily	None	Maury silt loam, pH 6.2	White pine, Canaan fir, Concolour fir, Douglas-fir
2	Daily	Kerosene	Hampshire silt loam, pH 5.5	White pine, Red pine, Canaan fir, Douglas-fir, Norway spruce, White spruce, Blue spruce
3	Daily	Rubbing alcohol	Maury silt loam, pH 6.2	White pine, Douglas-fir, Canaan fir

Farm	Timing of Scots Pine Shearing in 2005 and 2006	Age of Trees at Sale	Typical Number of Scots Pines Sold Each Year
1	Late June, early July	6-7 years	300
2	Late June, early July	7-10 years	200
3	Mid- to late July	5-8 years	200

Table A1.2. Climatic Profiles of Farms 1, 2 and 3 from 2005 – 2007[1]

Farms 1 and 3						Farm 2				
		High (˚C)	Low (˚C)	Precipitation (mm)			High (˚C)	Low (˚C)	Precipitation (mm)
2005	Jan.	6.78	-0.72	166.88		2005	Jan.	X	X	X
	Feb.	8.94	-0.56	66.80			Feb.	X	X	X
	Mar.	9.78	-0.22	159.77			Mar.	X	X	X
	Apr.	19.33	7.61	88.14			Apr.	X	X	X
	May	22.72	10.00	67.06			May	X	X	36.07
	Jun.	29.67	18.06	57.91			Jun.	X	X	X
	Jul.	30.67	20.28	77.47			Jul.	31.94	18.83	96.01
	Aug.	31.67	19.94	154.94			Aug.	33.06	17.50	103.12
	Sep.	28.22	15.83	22.61			Sep.	X	X	X
	Oct.	20.22	8.72	23.62			Oct.	21.00	4.78	24.89
	Nov.	14.39	2.33	44.96			Nov.	15.67	-3.06	61.98
	Dec.	4.44	-4.06	60.96			Dec.	3.89	-6.56	95.00
2006	Jan.	10.56	0.72	202.44		2006	Jan.	12.28	-2.28	137.41
	Feb.	6.83	-3.00	259.59			Feb.	6.89	-6.72	133.10
	Mar.	12.17	1.50	105.92			Mar.	12.78	-2.94	71.37
	Apr.	20.78	9.22	115.57			Apr.	21.72	4.83	127.25
	May	22.33	11.50	94.49			May	23.17	8.00	96.01
	Jun.	27.11	15.89	59.18			Jun.	28.61	13.06	78.49
	Jul.	30.28	19.39	139.19			Jul.	31.44	16.50	96.77
	Aug.	30.00	20.28	88.65			Aug.	X	X	X
	Sep.	22.72	13.00	260.35			Sep.	23.67	9.50	264.16
	Oct.	17.22	6.61	159.77			Oct.	17.83	2.83	134.62
	Nov.	13.61	3.72	60.20			Nov.	14.22	-0.61	49.53
	Dec.	10.61	0.22	85.09			Dec.	10.78	-2.78	51.82
2007	Jan.	7.00	-1.50	95.76		2007	Jan.	8.11	-6.00	220.22
	Feb.	2.11	-7.11	212.60			Feb.	X	X	X
	Mar.	17.17	5.39	60.45			Mar.	17.94	1.06	59.69
	Apr.	17.11	6.06	124.97			Apr.	X	X	X
	May	26.56	13.28	30.73			May	X	X	X

[1] Data obtained from NOAA's National Climatic Data Center records for the Lexington Bluegrass Airport Station (used for Farms 1 and 3) and Mount Sterling Station (used for Farm 2).
[2] "X" indicates missing data.

APPENDIX TWO:

STATISTICAL EVALUATIONS FROM FARMS 2 AND 3

This appendix contains all the statistical evaluations for Chapter 3.

Table A2.1. Comparison of the average Diplodia tip blight disease percentage for all Scots pine Christmas trees in the study plots of Farms 2 and 3 in 2005, 2006 and 2007. **A**) Average disease percentage for each farm for each year. **B**) Adjusted *p*-values (Tukey-Kramer) for the differences of LS-means when comparing farm and year.

A.

Year	Farm 2		Farm 3	
	Mean % Disease	Standard Deviation	Mean % Disease	Standard Deviation
2005	1.32	4.94	0.38	2.43
2006	3.55	7.39	0.19	0.97
2007	1.51	4.23	0.09	0.51

B.

	Farm 2 – 2005	Farm 2 – 2006	Farm 2 – 2007	Farm 3 – 2005	Farm 3 – 2006	Farm 3 – 2007
Farm 2 – 2005		<0.0001	0.9885	0.0032	0.0005	0.0001
Farm 2 – 2006	<0.0001		<0.0001	<0.0001	<0.0001	<0.0001
Farm 2 – 2007	0.9885	<0.0001		0.0014	0.0002	<0.0001
Farm 3 – 2005	0.0032	<0.0001	0.0014		0.9831	0.8970
Farm 3 – 2006	0.0005	<0.0001	0.0002	0.9831		0.9992
Farm 3 – 2007	0.0001	<0.0001	<0.0001	0.8970	0.9992	

Table A2.2. Correlation between the percent of symptomatic tips and the number of *D. pinea* colony forming units (CFUs) acquired on the shearing tools after shearing said tree in the group.

	Tree#	Farm 2					Farm 3			
		Mean % Disease	Mean CFUs	*r*-value	*p*-value		Mean % Disease	Mean CFUs	*r*-value	*p*-value
2005	0	3.08	0.30	-0.05	0.71		2.40	0.79	0.47	<0.01
	1	0.34	0.16	-0.05	0.74		0.03	0.46	0.12	0.40
	3	0.23	0.26	0.11	0.41		0.00	0.38	*	*
	5	0.31	0.30	0.31	0.02		0.00	0.23	-0.06	0.68
2006	0	4.81	0.46	0.27	0.11		1.54	0.69	0.38	0.02
	1	2.62	0.23	0.06	0.73		0.01	0.77	-0.07	0.69
	3	1.40	0.17	0.21	0.21		0.01	0.66	0.92	<0.01
	5	0.94	0.26	0.36	0.03		0.16	0.71	0.11	0.53

*= Percent disease was 0 for all cases and therefore values could not be computed.

Table A2.3. Correlations between the percent of Diplodia tip blight symptomatic tips and the percent of latently infected tips (out of ten tips), and the average number of *D. pinea* colony forming units (CFUs) and the number of latently infected tips (out of ten) on Tree 0 in each group of Scots pine Christmas trees on two Kentucky Farms in 2006.

Farm 2					Farm 3			
Mean % Disease	Mean # of Latent Tips Out of 10	*r*-value	*p*-value		Mean % Disease	Mean # of Latent Tips Out of 10	*r*-value	*p*-value
4.81	2.40	0.14	0.43		1.54	1.71	0.23	0.18
Mean CFUs	Mean # of Latent Tips Out of 10	*r*-value	*p*-value		Mean CFUs	Mean # of Latent Tips Out of 10	*r*-value	*p*-value
0.46	2.40	0.16	0.36		0.69	1.71	0.44	0.01

Table A2.4. Comparison of the mean number of *D. pinea* CFUs obtained from tools treated with Lysol® or not treated with Lysol®. CFU counts only compared for Tree 0, since it was after shearing this tree that Lysol® was applied to the tools.

Year	Farm 2			Farm 3		
	Mean CFUs from Lysol tools	Mean CFUs from non-Lysol tools	*p*-value	Mean CFUs from Lysol tools	Mean CFUs from non-Lysol tools	*p*-value
2005	0.33	0.19	0.10	0.47	0.42	0.71
2006	0.34	0.22	0.38	0.64	0.76	0.68

Table A2.5. Comparisons of the percent germination of *D. pinea* spores exposed to different concentrations of disinfestants overnight. *P*-values were adjusted (Tukey-Kramer) for the differences of LS-means when comparing treatments (**A.**). Mean percent germination rates are found in table **B**.

A.

		5%					10%				
		W	E	I	L	B	W	E	I	L	B
5%	W		0.95	0.20	<0.01	<0.01	1.00	0.92	0.92	<0.01	<0.01
	E	0.95		0.99	<0.01	<0.01	0.27	1.00	1.00	<0.01	<0.01
	I	0.20	0.99		<0.01	<0.01	0.01	0.99	0.99	<0.01	<0.01
	L	<0.01	<0.01	<0.01		1.00	<0.01	<0.01	<0.01	1.00	1.00
	B	<0.01	<0.01	<0.01	1.00		<0.01	<0.01	<0.01	1.00	1.00
10%	W	1.00	0.27	0.01	<0.01	<0.01		0.22	0.21	<0.01	<0.01
	E	0.92	1.00	0.99	<0.01	<0.01	0.22		1.00	<0.01	<0.01
	I	0.92	1.00	0.99	<0.01	<0.01	0.21	1.00		<0.01	<0.01
	L	<0.01	<0.01	<0.01	1.00	1.00	<0.01	<0.01	<0.01		1.00
	B	<0.01	<0.01	<0.01	1.00	1.00	<0.01	<0.01	<0.01	1.00	
25%	W	1.00	0.89	0.12	<0.01	<0.01	1.00	0.84	0.83	<0.01	<0.01
	E	<0.01	<0.01	<0.01	0.39	0.19	<0.01	<0.01	<0.01	0.27	0.13
	I	<0.01	<0.01	<0.01	1.00	1.00	<0.01	<0.01	<0.01	1.00	1.00
	L	<0.01	<0.01	<0.01	1.00	1.00	<0.01	<0.01	<0.01	1.00	1.00
	B	<0.01	<0.01	<0.01	1.00	1.00	<0.01	<0.01	<0.01	1.00	1.00

		25%				
		W	E	I	L	B
5%	W	1.00	<0.01	<0.01	<0.01	<0.01
	E	0.89	<0.01	<0.01	<0.01	<0.01
	I	0.12	<0.01	<0.01	<0.01	<0.01
	L	<0.01	0.39	1.00	1.00	1.00
	B	<0.01	0.19	1.00	1.00	1.00
10%	W	<0.01	<0.01	<0.01	<0.01	<0.01
	E	0.84	<0.01	<0.01	<0.01	<0.01
	I	0.83	<0.01	<0.01	<0.01	<0.01
	L	<0.01	0.27	1.00	1.00	1.00
	B	<0.01	0.13	1.00	1.00	1.00
25%	W		<0.01	<0.01	<0.01	<0.01
	E	<0.01		0.77	0.13	0.13
	I	<0.01	0.77		1.00	1.00
	L	<0.01	0.13	1.00		1.00
	B	<0.01	0.13	1.00	1.00	

W=Water Control; E=Ethanol; I=Isopropanol; L=Lysol®; B=Bleach

B.

	Water Control	Ethanol	Lysol®	Bleach	Isopropanol
5%	93.2	90.4	0.6	0.2	86
10%	96.4	90.0	0.4	0	90
25%	94.8	6.8	0	0	1.8

Table A2.6. Comparisons of the percent germination of *D. pinea* spores exposed to 25% Lysol® for different amounts of time. *P*-values were adjusted (Tukey-Kramer) for the differences of LS-means when comparing treatments (**A.**) Mean percent germination rates are found in table **B.**

A.

	30sec	1min	5min	10min	30min	Overnight
30sec		<0.01	<0.01	<0.01	<0.01	<0.01
1min	<0.01		<0.01	<0.01	<0.01	<0.01
5min	<0.01	<0.01		0.21	<0.01	<0.01
10min	<0.01	<0.01	0.21		0.70	0.54
30min	<0.01	<0.01	<0.01	0.70		1.00
Overnight	<0.01	<0.01	<0.01	0.54	1.00	

B.

	30 Seconds	1 Minute	5 Minutes	10 Minutes	30 Minutes	Overnight
Lysol	76.06	53.71	6.00	2.24	0.18	0
Water	98.58	98.75	98.75	98.83	98.92	98.92

Table A2.7. Correlation between the percent of symptomatic tips and the number of *D. pinea* colony forming units (CFUs) acquired on the shearing tools after shearing said tree in the group.

Yr Pair	Tree #	Farm 2			
		Previous Year Mean CFUs	Following Year Mean % Disease	*r*-value	*p*-value
2005-2006	0	0.29	4.5	0.02	0.88
	1	0.19	2.78	-0.02	0.90
	3	0.26	2.88	0.08	0.62
	5	0.29	1.41	-0.07	0.66
	All	0.26	2.87	0.01	0.86
2006-2007	0	0.46	3.5	0.23	0.23
	1	0.23	1.62	-0.09	0.68
	3	0.17	0.57	0.52	<0.01
	5	0.26	1.01	0.15	0.46
	All	0.28	1.62	0.22	0.02
Yr Pair	Tree #	Farm 3			
		Previous Year Mean CFUs	Following Year Mean % Disease	*r*-value	*p*-value
2005-2006	0	0.78	0.98	0.41	0.02
	1	0.46	0.06	0.18	0.25
	3	0.31	0.01	0.29	0.05
	5	0.22	0.29	0.1	0.05
	All	0.44	0.29	0.19	0.01
2006-2007	0	0.69	0.45	0.95	<0.01
	1	0.77	0.03	0.26	0.13
	3	0.66	0.03	-0.12	0.52
	5	0.74	0.04	0.001	0.99
	All	0.71	0.12	0.32	<0.01

Table A2.8. Comparisons of the number of CFUs picked up off the shearing tools for Farms 2 and 3 between trees. *P*-values were adjusted (Tukey-Kramer) for the differences of LS-means when comparing trees.

Farm 2								
	2005				2006			
Tree Number	0	1	3	5	0	1	3	5
0		0.39	0.77	1.00		0.22	0.12	0.28
1	0.39		0.57	0.39	0.22		0.76	0.88
3	0.77	0.57		0.77	0.12	0.76		0.64
5	1.00	0.39	0.77		0.28	0.88	0.64	
Farm 3								
	2005				2006			
Tree Number	0	1	3	5	0	1	3	5
0		0.09	0.13	<0.01		0.85	0.95	0.95
1	0.09		0.42	0.22	0.85		0.80	0.90
3	0.13	0.42		0.67	0.95	0.80		0.90
5	<0.01	0.22	0.67		0.95	0.90	0.90	

Table A2.9. Correlations between the number of CFUs on the shearing tools one year and the percent disease that developed on the next tree sheared with said tools the following year.

Year Pair	Previous Tree's Mean CFUs the Previous Year	Following Year's Mean Percent Disease	*r*-value	*p*-value
Farm 2				
2005-2006	0.25	2.37	0.20	0.02
2006-2007	0.29	0.94	-0.07	0.53
Farm 3				
2005-2006	0.52	0.12	-0.04	0.65
2006-2007	0.71	0.03	0.12	0.23

Table A2.10. Comparison of the length of necrosis on Scots pine shoots inoculated with 10µL of $1x10^6$ *D. pinea* spores/mL at different times after shearing in 2006 (**A.**) and 2007 (**B.** and **C.**). In 2006, the Horsfall-Barratt rating scale was used to estimate the degree of necrosis that developed on the shoots, and the means of these ratings were compared between treatment groups. *P*-values for 2007 were adjusted (Tukey-Kramer) for the differences of LS-means when comparing treatments (**B.**). Mean shoot necrosis lengths from 2007 are found in **C.**

A.

Inoculation time (hr) after shearing	Inoculation time (hr) after shearing			
	0hr	6hr	24hr	Water Control
0hr		0.13	<0.01	<0.01
6hr	<0.01		<0.01	<0.01
24hr	<0.01	<0.01		<0.01
Water Control	<0.01	<0.01	<0.01	

B.

Inoculation time (hr) after shearing	Inoculation time (hr) after shearing			
	0hr	6hr	24hr	Water Control
0hr		<0.01	<0.01	<0.01
6hr	<0.01		<0.01	<0.01
24hr	<0.01	<0.01		<0.01
Water Control	<0.01	<0.01	<0.01	

C.

	Inoculation time (hr) after shearing			
	0hr	6hr	24hr	Water
Mean length of shoot necrosis (mm)	44.7	34.1	25.5	0

Table A2.11. Comparison of the length of necrosis on Scots pine shoots inoculated with 10µL of different concentrations of *D. pinea* spores. *P*-values were adjusted (Tukey-Kramer) for the differences of LS-means when comparing treatments (**A.**) Mean shoot necrosis lengths (mm) are in **B**.

A.

		Concentration of spores applied to sheared tip (spores/mL)			
Concentration of spores applied to sheared tip (spores/mL)		$1x10^6$	$5x10^4$	$1x10^3$	0 (water)
	$1x10^6$		<0.01	<0.01	<0.01
	$5x10^4$	<0.01		<0.01	<0.01
	$1x10^3$	<0.01	<0.01		<0.01
	0 (water)	<0.01	<0.01	<0.01	

B.

	Concentration of spores applied to sheared tip (spores/mL)			
	$1x10^6$	$5x10^4$	$1x10^3$	0 (water)
Mean shoot necrosis length (mm)	37.25	16.83	6.08	0

APPENDIX THREE:

PREPARATION OF RNA FROM *PINUS NIGRA* TISSUES

Introduction

Diplodia tip blight is an important disease of pines worldwide. Recommended management tactics have proven to be generally ineffective for controlling this disease over the long term (34). Like many other fungal pathogens of trees, *Diplodia pinea* produces both symptomatic and asymptomatic (latent) infections (31). Latent infections occur when the fungus penetrates and colonizes host tissue, but symptoms do not develop.

Since latent infections do not result in necrotic symptoms or fungal reproduction, identifying *Pinus* and *D. pinea* genes that are involved in establishing and maintaining latent as compared to pathogenic infections could help to develop more effective means for disease management. For example, it may be possible to genetically promote production of host transcripts that maintain the pathogen in its latent form, or to develop chemical therapies targeting fungal gene products that are essential for transitioning from latent to pathogenic development.

One of the projects I initially considered for my Master's thesis research was to use a protocol called suppressive subtractive hybridization (SSH) (47) to identify RNA transcripts (both plant and fungal) that are uniquely expressed during latent infection, and not during pathogenic growth, or vice versa. For this project, it was proposed that samples would be collected from uninfected asymptomatic shoots, latently-infected asymptomatic shoots, and symptomatic shoots. Latently infected asymptomatic shoots

would be differentiated from uninfected shoots by use of a nested polymerase chain reaction (PCR) technique that is specific for *D. pinea* (29). Messenger RNA (mRNA) populations from the three samples would be isolated and compared by using SSH. In fact, it is actually rather difficult to isolate good-quality RNA from pine tissues because these tissues contain large quantities of contaminating polysaccharides, polyphenolic compounds and RNases (19). In this appendix, I describe a protocol that I developed, based on a modification of published methods, for production of high-quality total RNA from *P. nigra* tissues.

Methods

Pine Tissue Samples and Storage

During the spring of 2005, I collected samples from 20 Austrian pines from the University of Kentucky campus. Four elongating shoots were removed from each tree for sampling. At least one symptomatic shoot and one asymptomatic shoot were collected from each tree, if possible. Subsamples of bark and phloem were removed from each shoot using a cork borer and stored individually in coin bags to be further processed using the nested PCR protocol for detection of *D. pinea* (29). The samples were surface disinfested in 10% bleach for 2 minutes, rinsed twice in sterile MilliQ water (for 2 minutes each), dried on sterile paper towels, lyophilized, and have been stored at -80˚C. The remainder of each shoot was sealed in a labeled plastic bag and flash frozen in liquid nitrogen for future RNA extraction. The frozen shoot samples have also been stored at -80˚C.

RNA Isolation

I was able to obtain high-quality total RNA from Austrian pine needles, buds, and greenhouse-grown shoots using a modified method of Chang et al. (19). One modification was reducing the amount of pine sample used from 3 g to 1.5 g. I also removed the chloroform:isoamyl alcohol (IAA) extraction step following the addition of 500 µL of 0.5% SDS to the pellet and, instead immediately adding two volumes of chilled ethanol to the RNA/SDS mixture and precipitating the sample at -80˚C for 30 minutes as described in Chang et al. (19). Samples were centrifuged and pellets dried as published. Then the pellet was resuspended in 500 µL DEPC-treated sterile MilliQ water and stored at 4˚C overnight. After 24-48 hr, samples were removed from 4˚C and were extracted at that point with chloroform-IAA. The protocol was finished as published by Chang et al. (19). Figure A3.1 summarizes the difference between the two protocols. The concentration of RNA in each sample was determined using the spectrophotometer. To determine the size of the extracted RNA fragments, and to check for degradation, samples were electrophoresed through a 1.2% formaldehyde gel.

Results

My first attempts to extract RNA from *P. nigra* needle and bud tissue using the method of Chang et al. (19) were unsuccessful. The RNA appeared to be degraded and yields were low. Then, during one RNA prep experiment, I made an error and skipped the step involving extraction with chloroform, finishing the protocol as described. When I realized my error, I attempted to repair it by going back and repeating the protocol from the missed chloroform extraction step. I was able to obtain good yields of intact RNA

samples using this modified protocol (Figure A3.2). This method was repeated 7 times with different needle, bud, and shoots samples in order to ensure repeatability. Yields (μg/μL) are displayed in Table A3.1.

Discussion

The first step in determining what genes are uniquely expressed during latent infections of *D. pinea* in *P. nigra* is to isolate RNA. This task is notoriously difficult due to the presence of lots of contaminating secondary compounds, and because of this, commercial RNA extraction kits cannot be used. When I first attempted to extract RNA from Austrian pine needle and bud tissue using the method of Chang et al. (19), I could not obtain quality RNA. By modifying the method (originating serendipitously from an errors I made during the extraction process) it appears that I was able to remove excess secondary compounds that were contaminating the samples and thus obtain high quality intact RNA samples. Pine tissues are known to contain a lot of polysaccharides, phenolic compounds, and RNases (19). By precipitating in ethanol before the second chloroform extraction, I believe that I was able to remove the RNA from these compounds, and when this more purified RNA suspension was then extracted with chloroform, there were fewer contaminating proteins and these were removed more efficiently (19). Also, by reducing the sample size I used, I believe that the extraction improved because of the decreased amount of total secondary compounds present, thus making the extraction and purification steps more efficient.

I eventually abandoned this project as I developed other avenues of research that were of more interest to me and that were more likely to produce meaningful results in

the short term. However, this preliminary work I did on RNA extraction should be useful if this project is revisited in the future.

Table A3.1. Yields of RNA extracted using a modified protocol from Chang et al.

	Concentration of RNA (μg/μL)		
Experiment #	Needles	Buds	Shoots[1]
1	1.54	2.06	--
2	3.38	17.34	--
3	7.26	10.17	--
4	2.18	13.44	8.06
5	9.56	20.52	4.80
6	5.24	8.64	12.82
7	14.92	11.08	9.52

[1]Shoots were not sampled until the fourth experiment

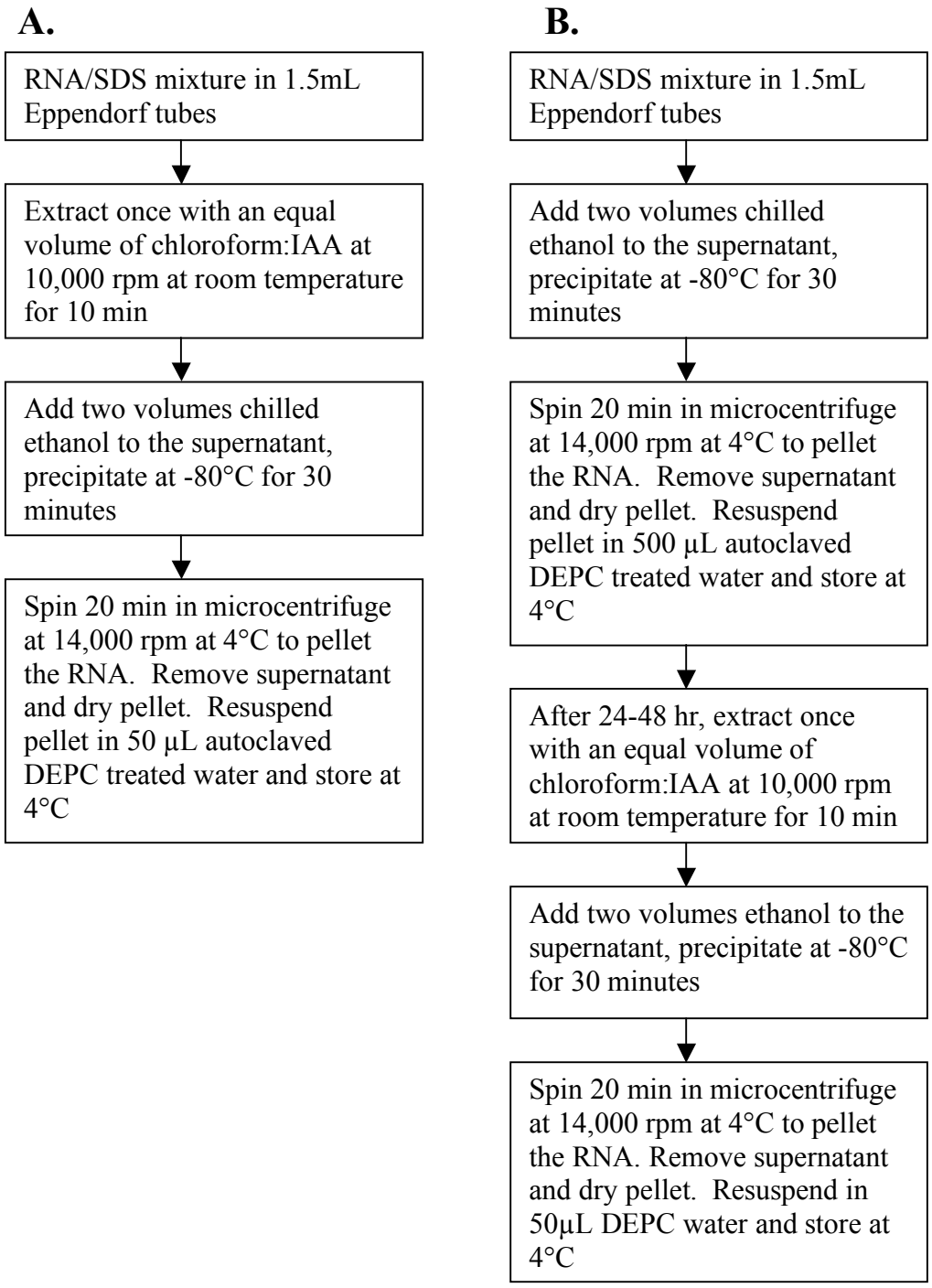


Figure A3.1. Flow chart depicting the differences between the original protocol (**A.**) described in Chang et al. and the modified protocol described here (**B.**). Flow chart does not outline entire protocol, but only from the step before the modification.

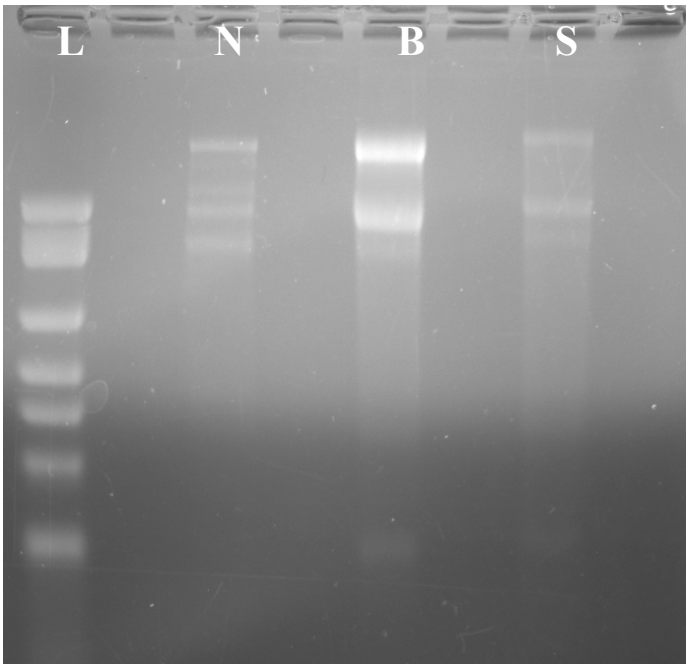


Figure A3.2. RNA extractions from Austrian pine (*Pinus nigra*) needles (N), buds (B), and shoots (S) (L is the 1kb RNA ladder). The two major bands in the bud sample are the 28S (top) and 18S ribosomal RNA. These same bands are present in the needle and shoot samples. Extra bands are to be expected in needle and shoot samples (J.D. Puryear, Texas A&M, personal communication).

APPENDIX FOUR:

OTHER PROBLEMS OBSERVED ON CHRISTMAS TREES IN KENTUCKY

Throughout my study on Diplodia Tip Blight, I also observed other problems, both biotic and abiotic, on Christmas trees grown on my three experimental farms. These problems occurred not only on Scots pines, but also on other tree species, such as white pine (Figures A4.1-A4.5). This appendix presents a series of photos illustrating these biotic and abiotic diseases.

Figure A4.1. Phytophthora root rot (*Phytophthora* spp.) on white pine.

Figure A4.2. Redheaded sawfly (*Neodiprion lecontei*) on Scots pine.

Figure A4.3. Bagworm (*Thyridopteryx ephemeraeformis*) on Scots pine.

Figure A4.4. Pine Needle Scale (*Chionaspis pinifoliae*) on Scots pine.

Figure A4.5. Roundup® damage on white pine.

REFERENCES

1. Anonymous. 2003. EPPO Standards - Diagnostic protocols for regulated pests - PM7/14(1) *Ceratocystis fimbriata* f. sp. *platani*. *European Plant Protection Organization Bulletin* 33: 249-55

2. Anonymous. 2004. Study Manual for Private Applicator Certification: Christmas Tree. ed. CDoE Protection

3. Bachi PR, Peterson JL. 1985. Enhancement of *Sphaeropsis sapinea* stem invasion of pines by water deficits. *Plant Disease* 69: 798-9

4. Barbosa CJ, Pina JA, Perez-Panades J, Bernad L, Serra P, et al. 2005. Mechanical Transmission of Citrus Viroids. *Plant Disease* 89: 749-54

5. Bateman AM. 2007a. Diplodia tip blight on Austrian pines in Kentucky. *Kentucky Pest News* KPN-1125: 4

6. Bateman AM. 2007b. Diplodia tip blight on Scots pines in Kentucky. *Kentucky Pest News* KPN-1126: 4

7. Bateman AM. 2007c. *Diplodia pinea,* the causal agent of Diplodia tip blight. *Kentucky Pest News* KPN-1127: 4

8. Bateman AM. 2007d. Latent infections of Diplodia tip blight. *Kentucky Pest News* KPN-1128: 4

9. Bateman AM. 2007e. Diplodia tip blight cytology. *Kentucky Pest News* KPN-1129: 4-5

10. Bateman AM. 2007f. Transmission of Diplodia tip blight on Scots pine Christmas trees. *Kentucky Pest News* KPN-1130: 6-7

11. Bateman AM. 2007g. Inoculations of Scots and Austrian pines with *Diplodia pinea*. *Kentucky Pest News* KPN-1131: 4

12. Bateman AM. 2007h. Managing Diplodia tip blight. *Kentucky Pest News* KPN-1132: 4-5

13. Bateman AM. 2007i. Fate of Austrian and Scots pines in Kentucky. *Kentucky Pest News* KPN-1133: 4-5

14. Berryman A. 1972. Resistance in conifers to invasion by bark beetle-fungus associations. *Bioscience* 22: 598-602

15. Bertrand PF, English H. 1976. Release and dispersal of conidia and ascospores of *Valsa leucostoma*. *Phytopathology* 66: 987-91

16. Blodgett JT, Kruger EL, Stanosz GR. 1997. Effects of moderate water stress on disease development by *Sphaeropsis sapinea* on red pine. *Phytopathology* 87: 422-8

17. Blodgett JT, Stanosz GR. 1997. *Sphaeropsis sapinea* and water stress in a red pine plantation in central Wisconsin. *Phytopathology* 87: 429-34

18. Brookhouser LW, Peterson G. 1971. Infection of Austrian, Scots, and Ponderosa Pines by *Diplodia pinea*. *Phytopathology* 61: 409-14

19. Chang S, Puryear J, Cairney J. 1993. A simple and efficient method for isolating RNA from pine trees. *Plant Molecular Biology Reporter* 11: 113-6

20. Chou CKS. 1976a. A shoot dieback in *Pinus radiata* caused by *Diplodia pinea*; 1. Symptoms, disease development, and isolation of pathogen. *New Zealand Journal of Forestry Science* 6: 72-9

21. Chou CKS. 1976b. A shoot dieback in *Pinus radiata* caused by *Diplodia pinea*; 2. Inoculation studies. *New Zealand Journal of Forestry Science* 6: 409-20

22. Chou CKS. 1982a. *Diplodia pinea* infection of *Pinus radiata* seedlings: Effect of temperature and shoot wetness duration. *New Zealand Journal of Forestry Science* 12: 425-37

23. Chou CKS. 1982b. Susceptibility of *Pinus radiata* seedlings to infection by *Diplodia pinea* as affected by pre-inoculation conditions. *New Zealand Journal of Forestry Science* 12: 438-41

24. Chou CKS, MacKenzie M. 1988. Effect of pruning intesity and season on Diplodia pinea infection of Pinus radiata stem through pruning wounds. *European Journal of Forest Pathology* 18: 437-44

25. de Wet J, Burgess T, Slippers B, Preisig O, Wingfield BD, Wingfield MJ. 2003. Multiple gene genealogies and microsatellite markers reflect relationships between morphotypes of *Sphaeropsis sapinea* and distinguish a new species of *Diplodia*. *Mycological Research* 107: 557-66

26. de Wet J, Wingfield MJ, Coutinho TA, Wingfield BD. 2000. Characterization of *Sphaeropsis sapinea* isolates from South Africa, Mexico, and Indonesia. *Plant Disease* 84: 151-6

27. Desmazieres JB. 1842. Neuvieme notice sur quelques plantes cryptogames, la plupart inedites, recemment decouvertes en France, et qui vot paraitre en nature dans la collection publiee par l'auteur. . *Annales des sciences naturelles II* 17: 91-118

28. Dochinger LS. 1956. Maple wilt progress report. *The Shade Tree* 29: 1-3

29. Flowers J, Hartman J, Vaillancourt L. 2003. Detection of Latent *Sphaeropsis sapinea* Infections in Austrian Pine Tissues Using Nested-Polymerase Chain Reaction. *Phytopathology* 93: 1471-7

30. Flowers JL, Hartman JR, Vaillancourt LJ. 2006. Histology of *Diplodia pinea* in diseased and latently infected *Pinus nigra* shoots. *Forest Pathology* 36: 447-59

31. Flowers JL, Nuckles E, Hartman JR, Vaillancourt LJ. 2001. Latent Infection of Austrian and Scots Pine Tissue by *Sphaeropsis sapinea*. *Plant Disease* 85: 1107-12

32. Forbes GA, Korva JT. 1994. The effect of using a Horsfall-Barratt scale on precision and accuracy of visual estimation of potato late blight severity in the field. *Plant Pathology* 43: 675-82

33. Haddow WR, Newman FS. 1942. A disease of the Scots pine (*Pinus sylvestris* L.) caused by the fungus *Diplodia pinea* Kickx associated with the pine spittle-bug (*Aphrophora parallela* Say). *Transcripts of the Royal Canadian Institute* 24: 1-19

34. Hartman JR, Flowers JL, Vaillancourt LJ, Magnin J-B, Hart J, Hanks L. 2001. Injections with Fungicides for Management of Pine Tip Blight: A Continuing Study. *University of Kentucky Nursery Landscape Program Research Report* PR-450: 22-3

35. Hartman JR, Flowers JL, Vaillancourt LJ, Schira M, Hart J, Hanks L. 2002. Injections with Fungicides for Management of Pine Tip Blight - A Four-Year Study. *University of Kentucky Nursery Landscape Program Research Report* PR-468: 24-7

36. Hartman JR, Poupard B, Vaillancourt LJ. 1998. Preliminary Results on Isolation of *Sphaeropsis sapinea* from Landscape Austrian Pines. *University of Kentucky Nursery Landscape Program Research Report* PR-409: 28-9

37. Hartman JR, Witt M, Hershman D, McNeil R. 1989. Woody Plant Disease Control Guide for Kentcuky. *Cooperative Extension Service* University of Kentucky Publication ID-88

38. Hill DB. 1991. Pruning and shearing. *Kentucky Christmas Tree Production Workbook* FOR-26

39. Hollis JP. 1984. The Horsfall-Barratt grading system. *Plant Pathology* 33: 145-6

40. Jacobs KA, Berg LC. 2000. Inhibition of fungal pathogens of woody plants by the plant growth regulator paclobutrazol. *Pest Management Science* 56: 407-12

41. Johnson DW, Peterson G, Doreset RD. 1985. Diplodia Tip Blight of Ponderosa Pine in the Black Hills of South Dakota. *Plant Disease* 69: 136-7

42. KCTA. 2006. Kentucky Christmas Tree Association Tree Farms <http://www.kychristmastrees.com/treefarmsalpha.html>.

43. Koelling MR. 2005. Scotch Pine. In *http://www.christmastree.org/trees/scotch.cfm*, ed. NCT Association

44. Luchi N, Ma R, Capretti P, Bonello P. 2005. Systemic induction of traumatic resin ducts and resin flow in Austrian pine wounding and inoculation with *Sphaeropsis sapinea* and *Diplodia scrobiculata*. *Planta* 221: 75-84

45. Munck IA, Stanosz GR. 2007. Excised shoots of top-pruned red pine (*Pinus resinosa*), a source of inoculum of the shoot blight pathogen *Diplodia pinea*. *Phytopathology* 97: S81

46. Nairn CJ, Haselkorn T. 2005. Three loblolly pine CesA genes expressed in developing xylem are orthologous to secondary cell wall CesA genes of angiosperms. *New Phytologist* 166: 907-15

47. Nesbo CL, Nelson KE, Doolittle WF. 2002. Suppressive Subtractive Hybridization Detects Extensive Genomic Diversity in Thermotoga maritima. *J. Bacteriol.* 184: 4475-88

48. Nicholls TH, Ostry ME. 1990. *Sphaeropsis sapinea* cankers on stressed red and jack pines in Minnesota and Wisconsin. *Plant Disease* 74: 54-6

49. Palmer MA, McRoberts RE, Nicholls TH. 1988. Sources of inoculum of *Sphaeropsis sapinea* in forest tree nurseries. *Phytopathology* 78: 831-5

50. Palmer MA, Nicholls TH. 1985. Shoot blight and collar rot of *Pinus resinosa* caused by *Sphaeropsis sapinea* in forest tree nurseries. *Plant Disease* 69: 739-40

51. Peterson G. 1977. Infection, Epidemiology, and Control of Diplodia Blight of Austrian, Ponderosa, and Scots Pines. *Phytopathology* 67

52. Smith H, Coutinho TA, Wolfaardt FW, Wingfield MJ. 2002. Relative susceptibility of northern and southern provenances of *Pinus greggii* to infect *Sphaeropsis sapinea*. *Forest Ecology and Management* 166: 331-6

53. Smith H, Wingfield MJ, Coutinho TA. 2002. The role of latent *Sphaeropsis sapinea* infections in post-hail associated die-back of *Pinus patula*. *Forest Ecology and Management* 164: 177-84

54. Smith H, Wingfield MJ, Crous PW, Coutinho TA. 1996. *Sphaeropsis sapinea* and *Boytryosphaeria dothidia* endophytic in *Pinus* spp. in South Africa. *South African Journal of Botany* 62: 86-8

55. Stanosz GR, Blodgett JT, Smith DR, Kruger EL. 2001. Water stress and *Sphaeropsis sapinea* as a latent pathogen of red pine seedlings. *New Phytologist* 149: 531-8

56. Stanosz GR, Cummins Carlson J. 1996. Association of mortality of recently planted seedlings and established saplings in red pine plantation with Sphaeropsis collar rot. *Plant Disease* 80: 750-3

57. Stanosz GR, Smith DR, Guthmiller MA, Stanosz JC. 1997. Persistence of *Sphaeropsis sapinea* on or in asymptomatic shoots of red and jack pines. *Mycologia* 89: 525- 30

58. Sutton BC. 1980. *The Coleomycetes.* Kew, Surrey, England: Commonwealth Mycology Institute

59. Swart WJ, Wingfield MJ. 1991. Biology and control of *Sphaeropsis sapinea* on *Pinus* species in South Africa. *Plant Disease* 75: 761-5

60. Teviotdale BL. 1991. Further tests on effectiveness of disinfectants for preventing transmission of fireblight. *KAC Plant Protection Quarterly* 1: 5-6

61. USDA-NASS. 2006. 2002 Census of Agriculture. ed. USDA: USDA National Agricultural Statistics Service

62. Waterman AM. 1943. *Diplodia pinea*, the cause of a disease of hard pines. *Phytopathology* 33: 1018-31

63. Zwolinski JB, Swart WJ, Wingfield MJ. 1995. Association of *Sphaeropsis sapinea* with insect infestation following hail damage of *Pinus radiata*. *Forest Ecology and Management* 72: 293-8

VITA

Amy M. Bateman

DATE AND PLACE OF BIRTH
September 19, 1982 Buffalo, New York, USA

EDUCATIONAL INSTITUTION ATTENDED AND DEGREES AWARDED
2000-2004
Hartwick College, Oneonta, New York
B.A.: Biology

PROFESSIONAL POSITIONS HELD
2000-2004
Student Supervisor, Hartwick College Library
Oneonta, New York

Spring 2003
Volunteer Teaching Assistant, BIOL 223 Horticulture, Hartwick College
Oneonta, New York

2004-present
Graduate Research Assistant, Plant Pathology, University of Kentucky
Lexington, Kentucky

Summer 2005 and Summer 2006
Graduate Mentor for International Exchange Students, Plant Pathology, University of Kentucky
Lexington, Kentucky

Fall 2006
Teaching Assistant, PPA 400G Principles of Plant Pathology, University of Kentucky
Lexington, Kentucky

SCHOLASTIC AND PROFESSIONAL HONORS
2003
ASIANetwork Freeman Student-Faculty Fellowship

2004-2007

Kentucky Opportunity Fellowship, University of Kentucky

2006

Graduate Student Support Grant from the Graduate School to fund travel to an American Phytopathological Society professional meeting, University of Kentucky

PROFESSIONAL PRESENTATIONS

Oral Presentations

Introduction to Tip Blight Disease in Scots Pine Christmas Trees, Kentucky Christmas Tree Association Annual Meeting, Lexington, Kentucky, 2005.

Diplodia Tip Blight, Buckeye Yard and Garden Line Diagnostic Arborist Event, Lexington, Kentucky, 2005.

Population Structure and Patterns of Evolution of the Dogwood Anthracnose Fungus, Discula destructiva, University of Kentucky, Lexington, Kentucky, 2005.

Diplodia Tip Blight in Scots Pine Christmas Trees, Kentucky Landscape Industries Conference, Louisville, Kentucky, 2006.

Diplodia Tip Blight: The Grinch That Stole Christmas, Beta Beta Beta Biological Honor Society, Hartwick College, Oneonta, New York, 2006.

Update on Diplodia Tip Blight on Kentucky Christmas Trees, Buckeye Yard and Garden Line Diagnostic Arborist Event, Lexington, Kentucky, 2006.

The Effect of Shearing on the Transmission of Diplodia pinea through Christmas Tree Farms, Kentucky Landscape Industries Conference, Louisville, Kentucky, 2007.

Poster Presentations

The Effect of Shearing on Transmission of *Diplodia pinea* within Scots Pine Christmas Tree Farms. American Phytopathological Society Annual Meeting, Quebec City, Quebec, Canada, 2006.

The Effect of Shearing on Transmission of *Diplodia pinea* within Scots Pine Christmas Tree Farms. Ornamental Workshop on Diseases and Insects, Hendersonville, North Carolina 2006.

PROFESSIONAL PUBLICATIONS

Extension Publications

Bateman, A., Hartman, J., Vaillancourt, L., Godbert, N., Moser, C. 2005. Preliminary Evaluation of the Transmissibility of *Dipoldia pinea* During the Shearing of Scots Pine Christmas Trees. *Nursery and Landscape Program: 2005 Research Report.* Agricultural Experimental Station, University of Kentucky. PR-520 pp. 29-30.

Hartman, J., Dixon, E., Bateman, A., Godbert, N., Moser, C. 2005. Evaluation of Cambistat Treatments for Woody Plant Diseases. *Nursery and Landscape Program: 2005 Research Report.* Agricultural Experimental Station, University of Kentucky, PR-520 pp. 25-26.

Bateman, A., Hartman, J., Vaillancourt, L., Godbert, N., Moser, C., Chopy, H. 2006. Evaluation of the Transmissibility of *Diplodia pinea* During the Shearing of Scots Pine Christmas Trees – 2006. *Nursery and Landscape Program: 2006 Research Report*. Agricultural Experimental Station, University of Kentucky. PR-537 pp. 26-28.

Bateman, A. 2007. Diplodia tip blight on Austrian pines in Kentucky. *Kentucky Pest News*. Agricultural Experimental Station, University of Kentucky. KPN-1125 p. 4.

Bateman, A. 2007. Diplodia tip blight on Scots pines in Kentucky. *Kentucky Pest News*. Agricultural Experimental Station, University of Kentucky. KPN-1126 p. 4.

Bateman, A. 2007. *Diplodia pinea*, the causal agent of Diplodia tip blight. *Kentucky Pest News*. Agricultural Experimental Station, University of Kentucky. KPN-1127 p. 4.

Bateman, A. 2007. Latent infections of Diplodia tip blight. *Kentucky Pest News*. Agricultural Experimental Station, University of Kentucky. KPN-1128 p. 4.

Bateman, A. 2007. Diplodia tip blight cytology. *Kentucky Pest News*. Agricultural Experimental Station, University of Kentucky. KPN-1129 pp. 4-5.

Bateman, A. 2007. Transmission of Diplodia tip blight on Scots pine Christmas trees. *Kentucky Pest News*. Agricultural Experimental Station, University of Kentucky. KPN-1130 pp. 6-7.

Bateman, A. 2007. Inoculations of Scots and Austrian pines with *Diplodia pinea*. *Kentucky Pest News*. Agricultural Experimental Station, University of Kentucky. KPN-1131 p. 4.

Bateman, A. 2007. Managing Diplodia tip blight. *Kentucky Pest News*. Agricultural Experimental Station, University of Kentucky. KPN-1132 pp. 4-5.

Bateman, A. 2007. Fate of Austrian and Scots pines in Kentucky. *Kentucky Pest News*. Agricultural Experimental Station, University of Kentucky. KPN-1133 pp. 4-5.

Abstracts

Bateman, A.M., Hartman, J.R., Vaillancourt, L.J. 2006. The effect of shearing on the transmission of *Diplodia pinea* within Scots pine Christmas tree farms. *Phytopathology* 96:S10 (abstract).

Amy Bateman