THE LOCATION OF DIPLODIA PINEA IN DISEASED AND LATENTLY-INFECTED PINUS NIGRA

ABSTRACT OF DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Agriculture at the University of Kentucky

By

Jennifer Lee Flowers

Lexington, Kentucky

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

Lexington, Kentucky

2005

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ABSTRACT OF DISSERTATION

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*Diplodia pinea* causes Diplodia tip blight on more than 30 different pine species. During the past 10 years, Diplodia tip blight has emerged as a serious problem in landscape and Christmas tree farms in this region. Surveys of diseased and symptomless Austrian pines revealed that latent infections of symptomless shoots by *D. pinea* were common. Latent infections may account for the recently observed rapid decline of mildly diseased pines in our region. To investigate the colonization habits of *D. pinea* within its host, molecular cytology was attempted and traditional histology was performed on naturally infected, diseased and asymptomatic Austrian pine tissues. I devoted much effort to developing a transformation system for *D. pinea*. Ultimately I did not succeed in this goal, but I was able to develop a highly efficient protocol for *Agrobacterium tumefaciens*-mediated transformation of another pathogenic fungus, *Colletotrichum graminicola*, in the process. The work that I did should help in future efforts to transform *D. pinea*, something that will be essential if it is to become a tractable system for the study of fungal latency. Traditional histological methods were more successful, and provided important information about the nature of latent infections. Very sparse epiphytic and subcortical fungal growth was observed in healthy shoots, however, no fungal tissues were present within the shoots. In diseased and latently infected shoots, crevices created between the needle bundles and the shoots were filled with fungal material, and hyphae were observed colonizing the needle sheaths. Hyphae were also observed breaching the shoot epidermal layer in these crevices and colonizing the underlying periderm. *D. pinea* colonization was extensive in all tissues of diseased shoots early in symptom development. In contrast, localized pockets of degradation were observed in the periderm and adjacent cortical cells located around areas of needle attachment in asymptomatic, latently infected shoots. The mechanism that operates to prevent expansion of these infected pockets in the latently infected shoots is still unclear. Obvious signs of pine defense mechanisms were only observed in 2 shoots. My observations were consistent with the idea that colonization progresses into the vascular tissues, and that this results in symptom development. Vascular colonization may occur more readily if the
host is stressed. My research lays the groundwork for future efforts to understand the nature of the transformation from latent to pathogenic infection.

**KEYWORDS:** *Diplodia pinea, Sphaeropsis sapinea, Pine Tip Blight, Latent Infections, Austrian Pine*

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LOCALIZATION OF *DIPLODIA PINEA* IN DISEASED AND LATENTLY-INFECTED *PINUS NIGRA*

By

Jennifer Lee Flowers
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The Graduate School
University of Kentucky
2005
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CHAPTER 1

*Diplodia pinea*, The Causal Agent of Pine Tip Blight

Diplodia tip blight, also known as pine tip blight or Sphaeropsis tip blight, is a serious disease of conifers worldwide. *Pinus* species are particularly susceptible, but the causal pathogen, *Diplodia pinea*, also infects *Cedrus, Abies, Larix, Thuja, Juniperus, Picea,* and *Pseudotsuga*. Typical tip blight symptoms include stunted shoots with necrotic, stunted needles; resinous cankers; and a general decline of the tree. *D. pinea* can also cause seed rot, seedling collar rot, blue stain of cut timber, and branch dieback. Interestingly, *D. pinea* can be isolated from surface-disinfested shoots of symptomless pines, as well as from apparently healthy shoots on diseased pines. These latent infections are very common in landscape *Pinus* plantings in Kentucky and elsewhere, but their precise nature, and their role in the Diplodia tip blight disease cycle, are unknown. The goal of my dissertation research was to use cytological approaches to compare latent and pathogenic *D. pinea* infections of Austrian pine (*Pinus nigra*), in an effort to improve our understanding of both types, and in particular to better understand how the latent infections develop and are maintained.

1.1 Taxonomy:

Over the years, this pathogen has had many names, including at least 23 synonyms. In 1842, Desmazieres first identified this fungus growing on dead *P. sylvestris* needles in France as *Sphaeria pinea* (Desmazieres 1842). Twenty-three years later, the genus was changed to *Diplodia* based on morphology of the conidia, which were described as brown with a single septum and measuring 35-40 µm X 16-18 µm (Kickx 1867). The reported habitat for *Diplodia pinea* at that time was limited to dead needles of *P. sylvestris* and *P. mugo* in Belgium, France, and Italy. In the late 1800's two other synonyms were used in the United States: *Diplodia megalospora* was described on the cones of *P. taeda* in South Carolina (Berkeley 1874), and *Sphaeropsis pinastri* on dead *P.
*sylvestris* twigs in New Jersey (Cooke and Ellis 1878-1879). In these and other early
descriptions the fungus was not found in living tissue or causing any disease symptoms.

The first reports of *D. pinea* causing a significant disease on conifers were
published by the Royal Botanical Gardens in Kew, England (Anon 1907-1908; Bancroft
1911). According to these reports, *D. pinea* was responsible for diseases observed in
seedling and adult *P. radiata* and *P. mugo var. mughus*. Wound inoculation studies of
the recovered pathogen revealed that *P. sylvestris* was also highly susceptible (Anon
1907-1908). In the late 1920's and 1930's, *D. pinea* was reported as a dangerous parasite
of pines, especially of *P. sylvestris* and *P. nigra*, in Russia and Austria. At this time, the
pathogen was also described as "common" in the British Isles, however, tip blight disease
incidence was not reported. The first mention of *D. pinea* as a parasite causing needle
necrosis and stem cankers in the United States was from New Jersey in 1917 (Schwarze
1917). In the late 1930's *D. pinea* was reported by Hedgcock as a weak pathogen on 11
species of both hard and soft pines from 14 states in the central and eastern United States
(Hedgcock 1932). By 1943, 23 states, including Kentucky, had reported pine infections
by *D. pinea*. Even in these early reports, exotic pines were found to be more susceptible
than native pines (Waterman 1943). In the early 1980's, Sutton reviewed the taxonomy
and renamed this pathogen *Sphaeropsis sapinea* (Fr.:Fr.) Dyko & Sutton (Sutton 1980).
The name *S. sapinea* has been used since then until recently. The argument to change the
name back to *D. pinea* was first published in 2003 (de Wet et al. 2003). It was proposed
that the characteristics that Sutton used to change the name, including the number and
timing of septation, were not sufficient in separating this species from the genus *Diplodia*
(de Wet et al. 2003). Moreover, *S. sapinea* consistently grouped with other *Diplodia*
species in phylogenies (Burgess et al. 2001b; de Wet et al. 2003). Although sexual
reproduction has never been observed for this species, it is phylogenetically related to
fungi that have *Botryosphaerea* teleomorphs (de Wet et al. 2003). Based on these
arguments, tip blight disease experts have reverted back to using the name *D. pinea* this
year.
1.2 Population Diversity:

*D. pinea* is a cosmopolitan, asexual fungus that can survive on living or dead host material. Vegetative compatibility studies from around the world have demonstrated that *D. pinea* is genotypically diverse (Smith et al. 2000; Burgess et al. 2001a). The pathogen is generally described as having fast growing, fluffy, white to grey mycelium on agar media (FIGURE 1.1a) (Palmer et al. 1987). However, some strains that were isolated from tip-blight diseased trees produced black hyphae growing appressed to the agar (Palmer et al. 1987). Based on these differences, two *D. pinea* morphotypes, A (white and fluffy) and B (black and appressed), were described (Smith and Stanosz 1995). Observations with the scanning electron microscope (SEM) revealed that the conidia of the A morphotype were typically smooth in contrast to the pitted conidia of the B morphotype (Wang et al. 1985; Wang and Blanchette 1986; Swart et al. 1993a; de Wet et al. 2002).

These morphological distinctions between the A versus B morphotypes were later found to be inconsistent, making it difficult to categorize them by their appearance alone (Swart et al. 1993a; Smith and Stanosz 1995). Analyses of gene sequences, isozymes, random amplified polymorphic DNA (RAPD), and simple sequence repeat (SSR) markers were used to clarify the relationship between the morphotypes (Palmer et al. 1987; Smith and Stanosz 1995; de Wet et al. 2000; Burgess et al. 2001b). Sequencing of the internal transcribed spacer 1 (ITS 1) of the rDNA revealed that the A and B morphotypes differed at only two positions (de Wet et al. 2000; Flowers et al. 2003). RAPD analysis of *D. pinea* isolates from around the world revealed that a third type, morphotype C, was present in Indonesia (de Wet et al. 2000; de Wet et al. 2002). The colony morphology and ITS sequence of the C morphotype were indistinguishable from the A group, but the conidia were larger than those of the other two morphotypes (de Wet et al. 2000). Cluster analyses of RAPD banding patterns separated the A and B morphotypes into two distinct groups, with subgroups representing various hosts and geographic locations (Stanosz et al. 1996). Parsimony analysis of SSR markers
demonstrated that each of the three morphotypes represented a distinct clade, with C more closely related to A (Burgess et al. 2001b).

The A morphotype appears to be more prevalent than the B or C morphotypes in many parts of the world. Both the A and B morphotypes were found associated with main stem cankers and top kill dieback of *P. resinosa* in Minnesota and Wisconsin (Palmer 1991). However, most isolates from 19 *Pinus, Cedrus, Larix, Picea, and Pseudotsuga* species from Africa, Australasia, Europe, and North America belonged to the A morphotype (Stanosz et al. 1999). Isolates with the B morphotype have been recovered from *Pinus* and *Cedrus* across their native range in Mexico, Europe, and the United States (Stanosz et al. 1999; de Wet et al. 2000; Burgess et al. 2001b). Nonetheless, preliminary surveys in Ohio found only the A morphotype (Bonello and Blodgett 2003), and only the A morphotype has been isolated from *P. nigra* and *P. sylvestris* in Kentucky (Flowers et al. 2003).

The A morphotype was consistently more virulent than the B morphotype in inoculation studies. Greenhouse grown, wound inoculated *P. sylvestris, P. mugo, P. resinosa, P. menziesii,* and *A. balsamea* were more susceptible to the A morphotype than to the B morphotype (Blodgett et al. 1997b; Blodgett and Stanosz 1999). *P. nigra* wound-inoculated with the A morphotype developed cankers on the trunk and elongating shoots (Blodgett and Bonello 2003). In contrast, the B morphotype only caused trunk cankers in the same study. The A morphotype was more virulent on 9-year-old inoculated pines in a Wisconsin plantation (Blodgett and Stanosz 1997b). Inoculation studies revealed that the B morphotype required a wound to infect coniferous hosts, in contrast to the A morphotype which can infect both intact and wounded host tissue (Palmer et al. 1987). Wound inoculations of *P. patula* seedlings suggested that the C morphotype was more virulent than either the A or B morphotype (de Wet et al. 2002).

Based on the differences in morphology, and phylogenies based on gene sequences, a new species name, *Diplodia scrobiculata*, was assigned to the B morphotype (de Wet et al. 2003). Recent analysis of 7 SSR markers indicated that there was geographic isolation of *D. scrobiculata* populations in different locations in North
America (Burgess et al. 2004). Since the B morphotype is now considered a separate species, while the C morphotype has a very limited geographical range, and because neither of these types occurs commonly in Kentucky, my dissertation is focused only on *D. pinea* (*S. sapinea sensu lato* A morphotype).

1.3 Life Cycle:

*D. pinea* produces black globose pycnidia (FIGURE 1.1b) containing large hyaline to dark brown conidia (FIGURE 1.1c) on dead host tissues (Sutton 1980). Conidial size varies, with lengths ranging from 30-45 µm and widths from 10-18 µm. Some conidia are coenocytic, while others have 1-3 septa. *D. pinea* conidiogenesis is holoblastic, and the conidia darken as they mature (Swart et al. 1993a). Transmission electron microscopy (TEM) studies of *D. pinea* conidia revealed a single cell wall layer differentiated into an outer electron dense zone and an inner hyaline zone (Wang and Blanchette 1986). The outer electron dense zone was not observed in immature hyaline conidia (Wang and Blanchette 1986). In laboratory tests, conidia germinated at temperatures of between 12-36 °C on water agar, with a temperature optimum of 24 °C. Under these conditions, conidia began to germinate in 1.5 hours, and 95% had germinated within 4 hours (Brookhouser and Peterson 1971). In sterile water, a majority of conidia germinated within 3 hours, with a temperature optimum of 30° C (Chou 1982b). In addition to the large, brown macroconidia, smaller hyaline microspores are occasionally observed in pycnidia (Swart et al. 1993b). These spores are about 2.5-3 µm in diameter, and their role, if any, in the disease cycle is unknown.

1.4 Disease Cycle:

Shoots of *Pinus* hosts are most susceptible to infection from bud break until they have elongated and become lignified (Chou 1982b; Chou 1982a; Palmer et al. 1988). Current year needles are also most susceptible during this time, while needles from the previous year appear to be more resistant to new infections (Brookhouser and Peterson 1971). In the northern hemisphere, *D. pinea* conidia are released from March until
November, depending on environmental conditions. Spore trap studies in South Africa 
(Swart et al. 1987a) and the United States (Palmer et al. 1988) indicated that *D. pinea* 
conidia were most prevalent during rainy weeks, and that there was no correlation 
between mean maximum monthly temperatures and spore release. Conidial counts were 
highest after periods of rainfall during the spring and summer months, when prevailing 
temperatures were higher than 20° C (Swart et al. 1987a; Palmer et al. 1988). 
Temperature and humidity are very important during the early stages of infection 
(Brookhouser and Peterson 1971). Successful infection can occur at temperatures 
ranging from 10° C to 40° C, with adequate periods of leaf wetness. At least 3 hours of 
leaf wetness were required at optimum temperatures of between 20° C and 30° C, but 48 
hours of leaf wetness were necessary for successful infection at higher temperatures 
(Chou 1982b).

*D. pinea* is an aggressive wound pathogen. Dieback caused by *D. pinea* 
associated with hail wounds is a common and important disease of commercial pine 
plantations, resulting in substantial timber losses every year (Zwolinski et al. 1990a; de 
Wet et al. 2000; Smith et al. 2002). Infections via pruning wounds have been linked to 
blue stain, branch dieback, and crown wilt (Chou and MacKenzie 1988), and cankers 
caused by *D. pinea* have been associated with bark beetle wounds in Minnesota (Nicholls 
and Ostry 1990). The pathogen was isolated from almost half of the bark beetles trapped 
around *P. nigra* trees in Ohio, suggesting that bark beetles may actually vector the fungus 
(Bonello and Nielsen, unpublished data in Blodgett 2003). Wound infections can occur 
throughout the growing season, in contrast with infections of intact tissues that occur 
most often in the spring and early summer. Thus, wounds are the primary infection court 
for secondary infections.

Symptoms can develop very rapidly after *D. pinea* infection. *Pinus* species 
developed symptoms of necrotic, discolored leaves within 24 hours post wound 
inoculation and several centimeters of the inoculated shoots were sometimes killed within 
5 to 6 days (Brookhouser and Peterson 1971; Chou 1976a; Flowers et al. 2001). More 
typically, dieback of various pine species occurs within 2 to 4 weeks post-spore 
inoculation in the greenhouse or field (Brookhouser and Peterson 1971; Chou 1976b;
Flowers et al. 2001). *D. pinea* cankers (FIGURE 1.1d) are typically larger on branches inoculated in the spring than on branches inoculated in the summer and fall (Swart and Wingfield 1991b). A multitude of symptoms are caused by *D. pinea* infection, including tip and branch dieback (FIGURE 1.1e), canker, crown wilt, and blue stain of cut timber. The most common symptom however, is tip, or shoot blight consisting of stunted, necrotic current-year shoots and needles (FIGURE 1.1f). Resin droplets, the first symptom of shoot blight, occur in early spring on needles when they are still embedded in or have just emerged from the fascicle sheath (FIGURE 1.1g) (Peterson 1977; Palmer and Nicholls 1985). Later, tan or reddish lesions progressing to stunted, necrotic needles develop (Brookhouser and Peterson 1971; Chou 1976b). The entire current year shoot can also become necrotic and stunted. The dead shoot tips either remain upright or bend into a "shepherd's crook", depending on the host species (Chou 1976b). A more serious symptom of *D. pinea* infection can occur when the leader is infected and killed, resulting in "stag-head" and malformation of the tree (Wright and Marks 1970; Chou 1976a; Currie and Toes 1978). Necrosis can move back from the branch tip toward the trunk, sometimes killing the previous year’s growth as well as the current year’s (Johnson et al. 1985). Symptomatic host tissue is typically resin soaked, and adventitious budding is common below the dead tissue (FIGURE 1.1h) (Palmer and Nicholls 1985). *D. pinea* can also cause stem and trunk cankers that are typically associated with hail or insect wounds (Nicholls and Ostry 1990). Crown wilt is another serious symptom, most often observed in New Zealand, when the vasculature is blocked by localized lower stem infections (Palmer and Nicholls 1985; Chou 1987). In the United Kingdom, *D. pinea* was the most commonly found fungus causing blue stain of cut timber, particularly in mechanically harvested stands (Uzunovic et al. 1999). Sap stain can result from the melanin produced by *D. pinea* in infected wood, but it is generally not associated with reduced structural integrity of the timber (Kreber et al. 2001). Seed rot, seedling damping off, and collar rot are also associated with *D. pinea* infection. Collar rot and root disease caused by *Sphaeropsis ellisii* (synonym of *D. pinea*) were reported as early as 1938 on 3-5-year-old *P. resinosa* seedlings in Maryland (Crandall 1938).
The symptoms of *D. pinea* infection are distinct, and the ease of pathogen isolation facilitates diagnosis. Signs of *D. pinea* infection are abundant on dead host tissue and this also aids diagnosis. Pycnidia were formed in as few as 5 days on killed shoots of inoculated seedlings kept in high humidity (Chou 1976b). In the field, pycnidia containing immature spores can be found as early as June on recently killed host tissue (Palmer and Nicholls 1985). Pycnidia are formed on any type of necrotic host tissue, but most particularly on the needles and the cones. On needles, fruiting structures are generally formed beneath the fascicle sheath but they can also be found along the whole length of the leaf (Palmer et al. 1988). Any dead cone can harbor fruiting structures; however, pycnidia-laden mature third-year cones are the most common (FIGURE 1.1i). Pycnidia were observed in 84% of sampled mature *P. resinosa* cones, (Nicholls and Ostry 1990) and are routinely found on *P. nigra* and *P. sylvestris* cones in Kentucky. The abundant sporulation on dead host tissue plays an important role in inoculum buildup in infected stands. Cones from infected windbreaks have been reported to be the most important source of inoculum in forest nurseries (Palmer et al. 1988).

**1.5 The Role of Stress:**

Host stress and its relationship to tip blight disease development has been the topic of many investigations. Studies of the effect of water stress were spurred by the increase in tip blight disease incidence and severity observed in years of drought (Bega et al. 1978; Nicholls and Ostry 1990). In fact, *D. pinea* was not considered an important pathogen of *Pinus* in Hawaii until severe dieback was observed in plantations following a 4-year drought (Bega et al. 1978). Numerous inoculation studies in greenhouses have supported a link between drought stress and disease severity. The degree of stem colonization, and the distance from the inoculation wound at which necrotic needles occurred, increased as soil water potentials decreased (Bachi and Peterson 1985; Blodgett et al. 1997a; Paoletti et al. 2001; Stanosz et al. 2001). The increase in symptom severity was observed regardless of whether the trees underwent water stress before or after inoculation (Paoletti et al. 2001). A 3-year field study of 9 year-old *P. resinosa* trees found that more severe symptoms were observed on trees that did not receive supplemental water or weed control, compared with other trees that were watered and for
which surrounding weeds were treated with herbicide (Blodgett et al. 1997b). The trees that were not watered or treated with herbicide had lower needle water potentials (Blodgett et al. 1997b). Predisposition to severe disease occurred in *P. radiata* seedlings that had a threshold needle water potential of -2.5 MPa (Chou 1987). Even sap stain of cut timber caused by *D. pinea* is reduced when logs are water soaked (Boddy 1994).

More severe symptoms are often observed on trees receiving fertilizer. Field studies found an increase in lesion lengths on *D. pinea*-infected fertilized versus nonfertilized adult *P. resinosa* (Blodgett et al. 2005). Closer examination revealed that mean foliar nitrogen (N) levels were higher in severely infected *Pinus* spp. stands (Van Dijk et al. 1992; Stanosz et al. 2004). Soil analysis of *P. nigra* ssp. *laricio* and *P. resinosa* stands showed higher levels of water soluble ammonia and phosphorous in severely infected versus less infected stands (Van Dijk et al. 1992; Stanosz et al. 2004). Other diseases of *Pinus*, including pitch canker, also occur more frequently on trees that are treated with manure or N fertilizer (Solel and Bruck 1989). Excess N can affect the water balance in trees by increasing the shoot to root ratio and decreasing fine root mass (Van Dijk et al. 1992). This results in reduced water uptake, even under optimum watering conditions, and could mimic drought stress. In addition, fertilized trees were more succulent and had lower lignin concentrations, optimizing conditions for infection (Stanosz et al. 2004; Blodgett et al. 2005). Precisely why disease severity is greater on fertilized trees is unknown, but it probably results from a combination of effects of excess N on host physiology.

Other host and environmental stresses can also result in more severe tip blight symptoms. Severe damage on both native and introduced *Pinus* species has been related to host age (Stanosz et al. 1997). Many susceptible trees, including *P. nigra* and *P. sylvestris* in Kentucky, are planted out of their native range, typically on unsuitable sites. However, trees planted in the landscape are usually free of severe symptoms until they reach cone-bearing age (Peterson and Wysong 1968; Johnson et al. 1985; Flowers et al. 2001). Younger, non-infected, or mildly symptomatic trees are often observed near sexually mature, heavily infected trees (Flowers et al. 2001). Therefore, exposure to inoculum from nearby infected trees does not appear to be solely responsible for the increase in disease severity that is observed at maturity. Perhaps *Pinus* species are more
resistant to natural *D. pinea* infection until they become sexually mature. If so, this is not universally true, since disease severity was reported to actually decrease in *P. radiata* stands after the trees were 7 to 8 years old (Chou 1977). Furthermore, *Pinus* species of all ages are susceptible to artificial inoculation and can become naturally infected via conidia (Chou 1977). Thus, *D. pinea* became an important pathogen of younger trees in New Zealand with increased thinning and pruning of timber stands (Chou 1976a).

Pruning has been associated with increased disease severity (Chou 1976a); however, it is not clear if the stress caused by pruning to the tree, or the opportunistic use of pruning wounds as infection sites for *D. pinea* (or a combination of the two) is responsible for the increase in tip blight symptoms. Host location also can have an effect on *D. pinea* disease severity. Increased disease severity on second-year tissues were noticed in years following harsh winters in Nebraska and North Dakota (Johnson et al. 1985). In the same region, trees located along the edges of stands exhibited more severe disease (Johnson et al. 1985). New Zealand and South African *Pinus* stands located in enclosed valleys with high humidity and little temperature variation had a higher disease rating than stands on open land (Chou 1976a; Chou 1977; Swart and Wingfield 1991a). Similarly, less disease damage was observed in stands at altitudes higher than 260 meters (Zwolinski et al. 1990b).

### 1.6 Economic Impact:

*D. pinea* has an extensive host range including species in the genera *Cedrus, Abies, Larix, Thuja, Juniperus, Picea, Pseudotsuga,* and *Pinus* (Palmer and Nicholls 1985). Diplodia tip blight disease occurs in both hemispheres between the latitudes of 30° north and 50° south, across six continents (Gibson 1979; Swart and Wingfield 1991a). Diplodia tip blight has had a large economic impact on pine related industries. The greatest losses to the timber industry have been in South Africa, and New Zealand; however, Australia, Europe, Hawaii, and the northern United States have also seen significant losses (Bega et al. 1978; Van Dijk et al. 1992; de Wet et al. 2000; Burgess et al. 2001a). Despite many efforts to reduce its impact, tip blight is considered the most economically important pine disease in South Africa, and results in substantial losses.
estimated at $3.8 million U.S. annually (Zwolinski et al. 1990a; de Wet et al. 2000; Smith et al. 2002). Because of tip-bl rift associated losses, many South African *Pinus radiata* stands have been clear cut or abandoned (Lundquist 1987). In one study, losses increased with tree age and site index, and were due primarily to the decreasing value of timber caused by sap stain, reduced salable wood, and the cost of emergency timber sales. Other losses were incurred because of premature reestablishment costs due to site preparation, planting, and weeding (Zwolinski et al. 1990b; Kreber et al. 2001). Similar losses are also reported in New Zealand and Australia (Burgess et al. 2001a). For example, infections reduced timber increments by 40% and marketable volume by 63% in New Zealand *P. radiata* stands. A 1980's epidemic in the Netherlands left over 35% of *P. nigra* ssp. *laricio* and *P. nigra* stands, and 32% of *P. sylvestris* stands, infected (Van Dijk et al. 1992). Also in the 1980's, *D. pinea* infections caused serious economic losses to native and exotic pine nurseries and plantations in the North Central United States (Brookhouser and Peterson 1971; Johnson et al. 1985). Although tip blight did not always kill seedlings in the forest nurseries, all symptomatic trees were culled because they were not marketable (Palmer and Nicholls 1985). In Kentucky, Sphaeropsis tip blight does not have a large impact on the timber industry but it has caused significant damage on exotic pines, such as *P. sylvestris* and *P. nigra* in the landscape and in Christmas tree plantations. *P. nigra* is still planted in the landscape, however, symptomatic trees typically have to be removed once they sexually mature because they are no longer aesthetically pleasing. Christmas tree growers in the region are scaling back *P. sylvestris* plantings due in part to *D. pinea*. Official loss estimates are not yet available, but comments from the individual growers suggest that they are highly significant.

### 1.7 Management

Few controls are available for effective management of *D. pinea*. Mycelia are very sensitive to benomyl and propiconazole *in vitro* (Swart et al. 1987b). In Wisconsin nursery trials, the systemic fungicides benomyl and thiophanate-methyl, and the protectant fungicide chlorothalonil, were the most effective for tip blight disease control
Benomyl, coupled with copper sulfate, was also successful in reducing *D. pinea* infections of new *P. nigra* shoots in Long Island. However, multiple applications were needed to achieve adequate control and aesthetically pleasing trees (Schweitzer and Sinclair 1976). Bordeaux mixture applied at a high rate between mid-May and mid-June also reduced infection rates of *P. nigra*, *P. sylvestris*, and *P. ponderosa* in the central United States (Peterson and Wysong 1968; Peterson 1977). Applications of a mixture of methyl bisthiocyanate and 2-n-octyl-4-isothiazolin-3-one to cut timber prevented *D. pinea* sapstain (Kreber et al. 2001).

Fungicides are effective, but expensive, and so they are most frequently utilized in seedling nurseries or other high-value situations.

Cultural controls are also commonly recommended, and these are based on our rather imperfect understanding of the *D. pinea* disease cycle. Reducing host stress by planting a well-adapted species on the site, and by providing supplementary water, may reduce symptom severity (Nicholls and Ostry 1990; Blodgett et al. 1997b). Pruning of heavily diseased branches is recommended for aesthetic reasons and to reduce sources of inoculum. Pruning should be done only in the winter months, however, to help prevent wound infections (Swart et al. 1987a; Nicholls and Ostry 1990; Swart and Wingfield 1991a). Pruning can also cause stress to the tree and this should be balanced with the potential beneficial results. Diseased windbreaks should be removed, and replaced by resistant tree species, since infected windbreaks are responsible for most of the *D. pinea* inoculum in nurseries and on Christmas tree farms (Stanosz and Smith 1996). These control options are more effective when combined, and are the most efficient way to reduce diseases caused by *D. pinea* (Stanosz and Smith 1996). Nonetheless, they usually just slow the progression of the disease, and do not entirely stop it: these recommendations were followed by University of Kentucky groundskeepers and it has still been necessary to remove most of the *P. nigra* in the landscape here. Since 1992, well over half of the *P. nigra* trees on campus have been removed due to severe tip blight disease (Flowers et al. 2001).
**1.8 Host-Pathogen Interactions:**

We know very little about the interaction that occurs between *D. pinea* and its hosts on a cellular level. In one report, more extensive colonization, resulting in more severe symptoms, was observed when temperatures were between 20 °C and 25 °C during initial infection periods (Chou 1982b). This temperature range was also in the optimum for hyphal growth in the laboratory, suggesting a correlation between virulence and fungal growth rate (*D. pinea* is a remarkably fast growing organism for a pathogenic fungus) (de Wet et al. 2002). As further evidence, a positive correlation was also found between radial growth rates of different *D. pinea* isolates *in vitro* and the mean extent of cambium discoloration they caused in inoculated *P. radiata* (Swart et al. 1991).

Various chemical factors have been implicated in disease resistance. Lignin concentrations were significantly higher at inoculation sites and had a significant effect on restricting *D. pinea* canker size in the first week post inoculations. However, the correlation between lignin concentration and reduced canker length did not continue in the following weeks (Bonello and Blodgett 2003). Salicylic acid (SA) was not detected in *D. pinea*-inoculated seedlings, suggesting that SA-mediated systemic acquired resistance does not occur in this interaction (Bonello and Blodgett 2003). Monoterpenes and phenolics are the main class of metabolites associated with resistance to pathogens in *Pinus* (Mirov 1967). In New Zealand, some disease-free trees in severely infected stands had high levels of monoterpenes in the cortical tissues. One particular monoterpene, Δ^3^ carene, reduced *D. pinea* germination by 70% *in vitro* when it was applied at half the concentration found in *P. radiata* cortical cells (Chou and Zabkiewicz 1976). Monoterpenes also inhibited spore germination and radial mycelial growth *in vitro* in another study at saturated atmosphere levels (Blodgett and Stanosz 1997a). In contrast to these studies however, monoterpene concentrations were positively correlated with symptom severity in inoculated *P. resinosa* incubated in a growth chamber (Blodgett and Stanosz 1998). Stilbenes are phenolic secondary metabolites that have been implicated in decay resistance in trees (Celimene et al. 2001). Stilbenes extracted from *Pinus*, including pinosylvin and its derivatives, reduced *D. pinea* germination rates and mycelial growth *in vitro*. However, *D. pinea* was relatively tolerant to these compounds in
comparison with other fungi (Blodgett and Stanosz 1997a; Celimene et al. 2001). In one study, an increase in total soluble phenolics was detected in *P. resinosa* inoculated with *D. pinea*, when compared to wounded controls (Blodgett et al. 2005). However, an earlier report by the same author found that phenolic concentrations did not change after inoculation (Blodgett and Stanosz 1998). Because of conflicting information in the literature, the effects of monoterpenes and phenolics *in planta* on symptom severity or *D. pinea* restriction are not clear (Blodgett and Stanosz 1998). Although *D. pinea* appears to be sensitive to many host secondary metabolites *in vitro*, questions remain about the role of these metabolites in resistance *in planta*.

Several metabolites that are produced by *D. pinea* have been identified as potential virulence factors. Sapinofuranones A and B, two *D. pinea* metabolites organically extracted from culture filtrates, exhibited high phytotoxicity on host and non-host plants (Evidente et al. 1999). More specifically, toxicity was particularly high in the bark tissues (Evidente et al. 1999). However, as is the case with host metabolites, proof for a direct role of these fungal compounds in the disease interaction is lacking.

Two totiviruses have been found that infect *D. pinea* (SsRV1 and SsRV2). The genome of each virus is approximately 5 kb, and the sequence of each is similar to the *Helminthosporium victoriae* 190S dsRNA virus, which confers hypovirulence on its host (Preisig et al. 1998). Ten percent of South African isolates tested were positive for dsRNA in this initial report, and dsRNA infected *D. pinea* isolates have been found in other parts of the world, including Kentucky (Preiseg et al. 1998; Flowers, unpublished data). However, in contrast to the *Helminthosporium* totivirus, there did not appear to be any correlation between viral infection and pathogenicity in *D. pinea* greenhouse inoculation experiments (Wu et al. 1989; Preisig et al. 1998; Steenkamp et al. 1998).

1.9 Cytology of Infection and Colonization

Infections can occur on the leaf, primary leaf, fascicle, or stem, but penetration of most of these tissues has never been directly observed (Brookhouser and Peterson 1971; Chou 1976b; Chou 1978). Observations using light and electron microscopy revealed that *D. pinea* spore germination occurred within 3 to 6 hours of being deposited on the
needle surface (Chou 1978). Fluorescent labeling and plastic needle imprints of spore-
inoculated needles suggested that *D. pinea* germ tubes entered the needle stomates
(Brookhouser and Peterson 1971). However, most of the germ tubes did not grow
directly towards the stomates, and some were even observed growing over guard cells
without entering the stomatal cavity, suggesting that stomatal penetration occurs more by
chance than design (Brookhouser and Peterson 1971). Germination rate, germ tube
length, and germ tube orientation did not appear to be affected by the host species or the
age of the needle (Brookhouser and Peterson 1971; Chou 1978). By 24 hours after
inoculation of elongating shoots with *D. pinea* spores, abundant external hyphae were
observed, generally growing transversely along the long axis of the stem, and commonly
forming hyphal aggregates closely appressed to the host surface (Chou 1978). Infection
of non-lignified stems appeared to occur between anticlinal epidermal walls, in
association with these hyphal aggregates (Chou 1978). Host cell walls exhibited
clarification of microfibrillar structures, loss of electron-opacity, and an accumulation of
granular material in the vicinity of hyphae within 6 hours (Chou 1978). *D. pinea* was
observed growing intercellularly and intracellularly within host cells (Luchi et al. 2005).
Growth of *D. pinea* in its host preceded the formation of darkly colored necrotic areas in
shoots (Bachi and Peterson 1985), however, Chou reported that dead host cells were
often seen in the absence of the pathogen (Chou 1976a). Colonization below the
inoculation point was rarely observed (Chou 1987). Based on isolation studies of
diseased shoots, *D. pinea* is thought to colonize all shoot tissues (Chou 1976b; Flowers et
al. 2001). Observations of inoculated seedlings using light microscopy suggested that *D.
pinea* hyphae had entered the needle trace phloem and passed into the stem conductive
tissue (Rees and Webber 1988). Interestingly, significant variation in the rate of *D. pinea*
colonization was observed, even when different branches on the same whorl were
inoculated with the same *D. pinea* isolate (Chou and MacKenzie 1988), and the pathogen
appeared to be discontinuous in the host tissue (Flowers et al. 2003).

In addition to shoots and needles, *D. pinea* also colonizes cone tissues, and could
be recovered from the pith, seeds, seed wings, and ovuliferous scales of mature seed
cones (Smith et al. 1996; Smith et al. 2002). In contrast to isolation studies performed in
our lab on *P. nigra* (Flowers et al. 2001), *D. pinea* was never isolated from first year *P. patula*, *P. nigra*, and *P. sylvestris* cones in South Africa and Nebraska (Peterson 1977; Smith et al. 2002).

In South Africa, *D. pinea* was found to rapidly attack seedling radicles and cause damping off (Rees and Webber 1988). Observations of these seedlings using light microscopy revealed hyphae entering and colonizing the periderm (cells lying beneath the epidermis) (Rees and Webber 1988). In severely diseased seedlings, hyphae were located throughout the tissues, growing along parenchyma rays, and invading the xylem (Rees and Webber 1988). Proliferation of cortical cells caused the common purple appearance of infected seedling stems (Rees and Webber 1988). Collar rot is prevalent in *P. resinosa* nurseries in Wisconsin (Stanosz and Cummings Carlson 1996). Blackened cortical cells and darkly stained xylem without any resin soaking were observed in these seedlings. Recently planted seedlings died before spring growth occurred, while the spring growth of established seedlings became chlorotic, wilted, and died (Stanosz and Cummings Carlson 1996). Although collar rot is important, tip blight symptoms typically cause more damage than collar rot in pine nurseries (Palmer and Nicholls 1985). Unlike the reports in South Africa, no extensive colonization of seedling roots by *D. pinea* was observed in the United States (Stanosz and Cummings Carlson 1996). Interestingly, although there was an early account of root disease of *P. resinosa* in Maryland, root disease of mature trees has not been observed in recent times in the United States and has only been reported from South Africa on mature *P. elliottii*, and *P. taeda* (Wingfield and Knox-Davies 1980). Conversely, collar rot, although common in nurseries in the United States, has never been observed in South Africa (Swart and Wingfield 1991a).

**1.10 Latent infections:**

*D. pinea* is frequently isolated from apparently healthy host tissue (Stanosz et al. 1997; Flowers et al. 2001; Stanosz et al. 2001). These latent infections occur in otherwise healthy seedling and adult trees, as well as in asymptomatic tissues of *D. pinea* diseased trees. *D. pinea* was isolated from a majority of asymptomatic *P. resinosa* seedling shoots in forest nurseries (Stanosz et al. 2001). Surveys of adult trees suggested
that nearly half of the apparently healthy *P. nigra* and *P. sylvestris*, and over a quarter of the apparently healthy *P. resinosa* and *P. banksiana*, had latent *D. pinea* infections (Stanosz et al. 1997; Flowers et al. 2001). More surprisingly, over half of the asymptomatic shoots on diseased *P. nigra* trees were found to be infected with latent *D. pinea* (Flowers et al. 2001). *D. pinea* was most commonly isolated from asymptomatic *P. nigra* shoots, and previous year needles, but asymptomatic current year needles, buds, immature cones, and male flowers also harbored the pathogen (Flowers et al. 2001). Isolation studies from asymptomatic *P. resinosa* and *P. banksiana* found that latent *D. pinea* infections were most commonly associated with the stems and previous year needles; however, no latent infections were found in current year needles (Stanosz et al. 1997). Further dissection studies of latently infected shoots found that *D. pinea* was localized in the shoot cortex, outside the vascular cambium (Flowers et al. 2001). All *D. pinea* isolates from latent infections that have been tested have been completely virulent in greenhouse inoculation studies (Stanosz et al. 1997; Flowers et al. 2001). Why *D. pinea* infection does not always result in symptom development is unknown. Latent infections involving other tree pathogens, such as *Hypoxylon atropunctatum* of oaks, and *Botryosphaeria dothidea* of apples, a fungus closely related to *D. pinea*, have been well documented (Bassett and Fenn 1984; Kim et al. 2001).

Although it has never been directly addressed in experiments, it is an interesting and highly significant possibility that latent *D. pinea* infections could become pathogenic when the host is stressed or reaches physiological maturity (Stanosz et al. 2001). This possibility could explain why disease symptoms seem to develop so quickly during droughts, since drought conditions are not very conducive to fungal spore release and infection. Only a few experiments have been reported testing the potential role of water stress in the shift from latent to pathogenic *D. pinea* infections (Bachi and Peterson 1985; Stanosz et al. 2001). Asymptomatic seedlings from a nursery known to harbor latently infected host material were subjected to different watering regimes in greenhouse experiments. Symptoms of *D. pinea* collar rot developed in more seedlings under the driest regime compared to the watered controls (Stanosz et al. 2001). Experiments with other fungi that have latent phases in angiosperms also suggest that water stress plays a role in switching from latent to pathogenic (in this case sap staining) growth (Boddy
1994). Taken together, this limited evidence suggests that drought stress or other stresses on the host can regulate a switch from latent to pathogenic growth. If this is true, then it becomes very important to understand the nature of latent infections, in particular, how they originate, and how they are maintained.

1.11 Overview of My Dissertation Research

As part of my research for my Masters thesis, I developed a *D. pinea*-specific nested-polymerase chain reaction (PCR) technique to detect latent infections in asymptomatic host tissues (Flowers et al. 2003). At the time, Kentucky was undergoing a drought and tip blight had reached epidemic proportions in the region (Flowers et al. 2001). The question of whether latent infections could transform into pathogenic ones became a compelling one, since it would explain why trees were becoming severely affected so quickly, in many cases despite receiving routine applications of fungicide.

My dissertation research was carried out with *P. nigra*, commonly named Austrian or black pine. *P. nigra* is a widely distributed species from the mountainous regions of southern Europe and Asia minor, with outliers in the Mediterranean islands and northern Africa (Mirov 1967). This species was first introduced in the United States in 1759 for use in ornamental and windscreen plantings (Houston and McClenahen 1996). Today, *P. nigra* is the most widely planted ornamental conifer in the central United States, (Bonello and Blodgett 2003) and it is also commonly used along highways in Indiana, Ohio, West Virginia, and Kentucky because of its high tolerance to salt spray (Houston and McClenahen 1996). As early as 1908, Hedgcock noted that *D. pinea* was the suspected cause of a severe disease of *P. nigra* (Hedgcock 1932). An Ohio inoculation study suggested that *P. nigra* seed sources exhibited variation in resistance to diseases caused by *D. pinea*, although all were susceptible (Bonello and Blodgett 2003).

The goal of my dissertation research was to gain a better understanding of the cytological nature of latent versus pathogenic infections of *P. nigra* by *D. pinea*. Two approaches, molecular cytology and more traditional histological methods, were taken in order to achieve this goal. Chapters 2 and 3 describe the work I did to develop a transformation method for *D. pinea*. Although in the end I did not succeed in transforming the fungus, the work I did will move that goal much closer. I did develop
optimized parameters for Agrobacterium tumefaciens mediated transformation of another plant-pathogenic fungus, Colletotrichum graminicola, which was used as a control in my experiments, and this is described in Chapter 2. Although the molecular cytology approach utilizing the green fluorescent protein (GFP) is still in the future, I succeeded in characterizing latent versus pathogenic infections using more traditional histological methods, which are described in Chapter 4. I found that latent D. pinea infections were localized in the periderm and in a few of the adjacent cortical cells usually around the needle scales at the leaf axis. In contrast, pathogenic infections were localized throughout the shoot tissues even very early in symptom development. Another intriguing observation was that latently infected, dormant terminal buds had localized D. pinea infections of single auxillary buds that appeared to originate from the distal bud scale and not from the D. pinea infected branch on which the bud was set. Despite my unsuccessful attempts to transform D. pinea with the gfp gene, I was still successful in accomplishing my research goal with more traditional histological methods. In addition to being the first researcher to investigate latent D. pinea in P. nigra using microscopy, I was the first to observe latent colonization of any fungal pathogen in conifer shoot tissues.

References
Anon (1907-1908) A pine disease (Diplodia pinea). Journal of Building and Agriculture (Great Britain) 14:164-166
Blodgett JT, Stanosz GR (1997a) Differential inhibition of *Sphaeropsis sapinea* morphotypes by a phenolic compound and several monoterpenes of red pine. Phytopathology 87:606-609
Blodgett JT, Stanosz GR (1997b) *Sphaeropsis sapinea* morphotypes differ in aggressiveness, but both infect nonwounded red or jack pines. Plant Disease 81:143-147
Blodgett JT, Stanosz GR (1999) Differences in Aggressiveness of *Sphaeropsis sapinea* RAPD marker group isolates on several conifers. Plant Disease 83:853-856
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 12:438-441  
Cooke MC, Ellis JB (1878-1879) New Jersey fungi. Grevillea 7:4-10  
Crandall BS (1938) A root and collar disease of pine seedlings caused by Sphaeropsis ellisi. Phytopathology 28:227-229  
Hedgcock GG (1932) Notes of the distribution of the fungi associated with diseases on conifers. Plant Disease Reporter 16:28-42  
Kickx J (1867) Flora cryptogamique de Flandres 1. In, p 397


Nicholls TH, Ostry ME (1990) *Sphaeropsis sapinea* cankers on stressed red and jack pines in Minnesota and Wisconsin. Plant Disease 74:54-56


Palmer MA, McRoberts RE, Nicholls TH (1988) Sources of inoculum of *Sphaeropsis sapinea* in forest tree nurseries. Phytopathology 78:831-835

Palmer MA, Nicholls TH (1985) Shoot blight and collar rot of *Pinus resinosa* caused by *Sphaeropsis sapinea* in forest tree nurseries. Plant Disease 69:739-740


Schwarze CA (1917) The parasitic fungi of New Jersey


Stanosz GR, Cummings Carlson J (1996) Association of mortality of recently planted seedlings and established saplings in red pine plantations with Sphaeropsis collar rot. Plant Disease 80:750-753


Stanosz GR, Smith DR, Guthmiller MA, Stanosz JC (1997) Persistence of *Sphaeropsis sapinea* on or in asymptomatic shoots of red and jack pines. Mycologia 89:525-530

Stanosz GR, Swart WJ, Smith DR (1999) RAPD marker and isozyme characterization of *Sphaeropsis sapinea* from diverse coniferous hosts and locations. Mycological Research 103:1193-1202


Swart WJ, Wingfield MJ, Grant WS (1993a) Comparison of *Sphaeropsis sapinea* and *Sphaeropsis sapinea* f. sp. cupressi. Mycological Research 97:1253-1260


Waterman AM (1943) Diplodia pinea, the cause of a disease of hard pines. Phytopathology 33:1018-1031


Figure 1.1: Color plates of Diplodia tip blight disease on P. nigra. A) 7 day old PDA culture of D. pinea; B) D. pinea pycnidia on sterile needle from 2 week old water agar culture (red arrow heads); C) D. pinea conidia, bar equals 30 µm; D) resinous shoot canker (between the 2 red arrow heads), photo courtesy of Dr. J. Hartman; E) two Diplodia tip blight diseased trees from the University of Kentucky campus showing symptoms of shoot, branch, and crown dieback; F) current year shoot with tip blight symptoms; G) resin droplets (red arrow head) on young needle fascicles; H) adventitious shoots (red arrow heads) below necrotic shoot; I) D. pinea pycnidia on cone.