DIPLODIA PINEA, *DIPLODIA SCROBICULATA* AND *LASIODIPLODIA THEOBROMAE* IN SOUTH GEORGIA PINE PLANTATIONS AND THEIR RESPECTIVE ROLES IN CAUSING TIP DIEBACK IN SOUTHERN PINE SPECIES

by

EMILY SUSANNA HOUSE

(Under the Direction of Jean Williams-Woodward)

ABSTRACT

Diplodia pinea and *D. scrobiculata* damage a myriad pine species. Previous reports suggest that *Lasiodiplodia theobromae* also causes tip dieback of slash and loblolly pines. A survey of two South Georgia pine plantations for the presence and distribution of these three pathogens was conducted during 2005 and 2006. *Diplodia pinea*, *D. scrobiculata,* and *L. theobromae* were isolated at equal rates from all shoots. No significant relationship exists between tree age and the isolation of these fungal species. Questions of reliability surround two methods used in the identification of these fungal species. Isolates obtained during the survey were used to assess pathogenicity on slash, loblolly, shortleaf, and Virginia pine seedlings. Slash and loblolly pines were susceptible to isolates of *D. pinea, D. scrobiculata,* and *L. theobromae*. Shortleaf pine was susceptible to isolates of *D. pinea* and *D. scrobiculata*. Virginia pine was susceptible to isolates of *D. pinea* and *L. theobromae*.

INDEX WORDS: *Diplodia pinea*, *Diplodia scrobiculata*, *Sphaeropsis sapinea*, *Lasiodiplodia theobromae*, tip dieback, loblolly pine, slash pine, Virginia pine, shortleaf pine, *Pinus taeda, Pinus elliottii, Pinus virginiana, Pinus echinata*

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B.A., Northwestern University, 2000

A Thesis Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA

2007

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August 2007

TABLE OF CONTENTS

CHAPTER 1

INTRODUCTION

Diplodia pinea (Desm.) J. Kickx (formerly *Sphaeropsis sapinea* (Fr.) Dyko & B. Sutton morphotype A) and *Diplodia scrobiculata* J. de Wet, B. Slippers & M.J. Wingfield (formerly *Sphaeropsis sapinea* (Fr.) Dyko & B. Sutton morphotype B) are closely related fungal species that cause disease on a myriad of pine species. First described in 1909 as a pathogen in South African pine plantations, *Diplodia spp.* cause symptoms including shoot blight, stem canker, crown wilt and collar rot (Nichols and Ostrey, 1990; Palmer, 1991; Palmer et al., 1985; USDA Forestry Service, 2005). *Diplodia spp.* gain entry into trees during the spring via elongating needles (Brookhouser and Peterson, 1971; Peterson, 1977) or wound sites, such as those caused by hail (Smith et al., 2002). Initial infection of a mature tree may result in the dieback of only a few shoots. After years of infection, however, large branches or entire trees may be lost (Flowers et al., 2001). The pathogen over-winters as pycnidia embedded in the previous year's diseased shoots, in mature pinecones, or as an endophyte living undetected in shoot tissues (Palmer et al., 1988; Smith et al., 1996; Flowers et al., 2001).

Prior to 1995, *S. sapinea* isolates were characterized based on morphology, but this has shifted to molecular methods in recent years. Taxonomy of *Botryosphaeria* and its anamorphs continues to change. In 2003, de Wet et al. (2003) compared the A-, B-, and C-morphotypes of *S. sapinea* by studying six protein coding genes and six microsatellite regions present in them. They concluded that all three morphotypes were distinct from *Botryosphaeria spp.*, but that they behave identically to the established genus *Diplodia* in that they produce ellipsoid, thick-walled, septate conidia that darken with age. Based on this finding, the authors suggested that genus

designation be changed from *Sphaeropsis* to *Diplodia*. The authors further suggested that the B-morphotype be given the species name *Diplodia scrobiculata* J. de Wet, B. Slippers & M.J. Wingfield due to its genetic distinction from the A- and C-morphotypes. Furthermore, it was suggested that the A- and C-morphotypes re-assume the previously assigned species name *Diplodia pinea* (Desm.) J. Kickx (de Wet et al., 2003).

In 2006 Smith and Stanosz (2006) introduced another molecular tool for the study of *Dipodia spp.*. Three primers—two forward, one reverse—were developed that targeted the mitochondrial small subunit ribosome gene (SSU rRNA). Primer pair DpF/BotR produces a 700 base pair band in the presence of *D. pinea* DNA, but no band in the presence of *D. scrobiculata*; primer pair DsF/BotR produces a 700 base pair band in the presence of *D. scrobiculata* DNA, but no band in the presence of *D. pinea*. Primer pairs were designed such that neither would amplify the DNA of *Botryosphaeria obtusa*, *B. quercum*, *B. rhodina*, *B. ribis*, *B. tsugae*, B. *mutila*, B. *dothidea*, *Diplodia pinea* f. sp. *cupressi*; they were also designed such that neither pair amplifies the DNA of common pine inhabitants *Fusarium tricinctum*, *Cladosporium sp*., *Chaetomium sp*., *Schlerophoma pythiophylla*, *Epicoccum sp*., *Alternatia sp.*, *Pestalotia sp*., *Trichoderma sp*., *Sirococcus conigenus*, and *Pseudorobillarda sp*..

Nuclear rDNA Internal Transcribed Spacers (ITS) have also been used to compare various anamorphs of *Botryosphaeria sp.*. Jacobs and Rehner (1998) used ITS-4 and -5 primers to amplify ITS 1 and 2 regions of 22 strains of botryosphaeriaceous fungi. Among those strains compared were *Fusicoccum ssp*., *Diplodia quercina*, *Diplodia mutila*, *Sphaeropsis sp*., *Botryosphaeria sp*., *Sphaeropsis sapinea*, and *Lasiodiplodia theobromae*. The article demonstrated that these ITS regions were useful in distinguishing among these anamorphs. Six phylogenetic groups were identified: three of the groups were composed solely of *Fusicoccum*

species; one was composed solely of *L. theobromae*; one was composed of *S. sapinea* and *Sphaeropsis sp.*; and one was composed of *Sphaeropsis sp*., *D. mutila*, *D. quercina*, and *Botryosphaeria sp.* Additionally, this paper noted the tendency of *Sphaeropsis sp.* and *Diplodia sp.* to exhibit both conidial pleomorphy and morphological plasticity in culture (Jacobs and Rehner, 1998). Variations among isolates of *S. sapinea* were previously noted by Morelet and Chandelier (1993). In 2003, variability in growth rate, growth form, and mycelial color were noted again (Kay et al., 2002).

Multiple studies have attested to the broad geographical and host ranges of *Diplodia spp.*. Stanosz et al. (1999) documented the presence of the pathogen on genera including *Pinus, Cedrus, Larix, Picea and Pseudotsuga* and in areas including Africa, Australia, Asia, Europe, and North America. Chronicled in that paper was the discovery of *D. scrobiculata* on hosts and in geographic locales where it had never before been found. It was recovered from *P. halepensis* (Aleppo pine)*, P. elliottii* (slash pine)*, P. sylvestris* (Scots pine)*, P. virginiana* (Virginia pine)*,* and *P. taeda* (Loblolly pine) in countries including France, Spain, Israel, Italy, and U.S states such as Georgia, Florida, Kentucky, and West Virginia. Prior to this study, the B-morphotype was only known to exist on *P. resinosa* (red pine) and *P. banksiana* (jack pine) growing in the North Central region of the United States. The A-morphotype was found to be present in an even broader range of areas. In the U.S., it was detected in Wisconsin, Pennsylvania, Michigan, Illinois, Idaho, New York, Hawaii, and Minnesota (Stanosz et al., 1999).

Although *D. scrobiculata* was long considered to be less pathogenic than *D. pinea*, studies have indicated that the relative pathogenicity of the two species is specific to pine species and to the presence or absence of stressors such as drought and wounding events (Blodgett and Bonello, 2003). Drought stress also can increase disease development (Bachi and Peterson, 1985;

Johnson et al., 1997; Blodgett et al., 1997; Paoletti et al., 2001). Latent infections may be important in the eventual development of severe *Diplodia* tip blight (Flowers et al., 2001; Smith et al., 2002; Stanosz et al., 1997; Stanosz, et al., 2005; Stanosz et al., 2001).

Diplodia spp. have the ability to cause substantial damage to pines. That ability is often enhanced by drought conditions or wounding events. Given Georgia's economic dependence on forestry and South Georgia's tendency for long periods of drought interspersed with tumultuous storms, the presence of *Diplodia spp.* in the state's pine plantations needs to be more fully investigated. The aim of the first part of this study was to evaluate the levels at which *D. pinea* and *D. scrobiculata* currently inhabit slash pines in southern Georgia pine plantations. In this study, two slash pine plantations were surveyed for the presence of *Diplodia spp.*. Symptomatic and asymptomatic shoots were collected from 5-, 10-, and 15-year-old trees. Cultures isolated from collected pine tissues were characterized based on SSU rRNA gene sequence using primers described in Smith and Stanosz (2006) and based on sequencing of ITS 1 and 2 regions.

Lasiodiplodia theobromae, another anamorph of *Botryosphaeria,* which has often been dismissed as a legitimate pine pathogen, also was included in this study. Some have suggested that it is capable of causing tip die back in slash and loblolly pine. South et al. (2002) offered infection by *L. theobromae* as a possible cause for tip dieback of young loblolly pines seen in the eastern U.S.. Rowan implicated it in tip dieback of slash and loblolly pines (Rowan, 1982). Inoculation studies performed at Clemson University demonstrated the ability of *L. theobromae* to cause dieback in 2-year-old loblolly pines (Jolley and Hedden, 2001). Because it was recovered so frequently from diseased slash pines during the aforementioned surveys, its role in causing that dieback needs to be assessed.

Several pine species are quite important to the economy and history of the southeastern United States; among them are loblolly, slash, shortleaf (*Pinus echinata*), and Virginia (*Pinus virginiana*) pine. Loblolly and slash pines are particularly valuable as pole, lumber, timber, and pulp wood. Shortleaf pine is used for sub-flooring, interior finishes, pulpwood, and as a landscape tree. In nature, it is particularly important to wildlife. Virginia pine is used for pulp and firewood and shows particular promise as a tree capable of growing on strip-mined areas during reclamation projects (USDA Forestry Service: *P. echinata*; USDA Forestry Service: *P. elliottii*; USDA Forestry Service: *P. taeda*; USDA Forestry Service: *P. virginiana*) .

According to the USDA Forest Service, loblolly and shortleaf pines provided 56% of Georgia's total softwood output in 2003, while longleaf and slash pines accounted for 39%. Softwood is responsible for approximately 75% of pulpwood, saw-log, and veneer-log production in Georgia; these three products make up 94% of total roundwood production in the state (Johnson and Wells, 2005). IMPLAN models have estimated that, in 2004, forestry created direct impacts in Georgia responsible for the employment of over 67,000 people who were paid a total of over $3 billion to produce over $14 billion worth of product (Riall, 2006). When multiplier effects were included, total economic impact of forestry on Georgia's economy were increased to over 144,000 people paid approximately $6.3 billion to produce more than $22 billion worth of product. It was estimated that the forestry industry contributed $155 million in net revenue to the state's government (Riall, 2006).

Based on the importance of slash, loblolly, shortleaf, and Virginia pines to Georgia's economy, and the ability of *Diplodia spp.* to cause damage to many pine species, it is important that the susceptibilities of these four important southern pine species to *D. pinea* and *D. scrobiculata* be evaluated.

CHAPTER 2

LITERATURE REVIEW

Characterizing the Pathogen

Diplodia pinea (Desm.) J. Kickx (formerly *Sphaeropsis sapinea* (Fr.) Dyko & B. Sutton morphotype A) and *Diplodia scrobiculata* J. de Wet, B. Slippers & M.J. Wingfield (formerly *Sphaeropsis sapinea* (Fr.) Dyko & B. Sutton morphotype B) are closely related fungal species that cause disease on a myriad of pine species. First described in 1909 as a pathogen in South African pine plantations, *Diplodia spp.* cause symptoms including shoot blight, stem canker, crown wilt and collar rot (Nichols and Ostrey, 1990; Palmer, 1991; Palmer et al., 1985; USDA Forestry Service, 2005). *Diplodia spp.* are capable of gaining entry into trees during the spring via elongating needles (Brookhouser and Peterson, 1971; Peterson, 1977). However, wound sites, such as those caused by hail, can also provide an infection court (Smith et al., 2002). Initial infection of a mature tree may result in the dieback of only a few shoots. After years of infection, however, large branches or entire trees may be lost (Flowers et al., 2001). The pathogen can over-winter as pycnidia embedded in the golden-brown remains of last years diseased shoots, which are retained on the tree, at least partially, by characteristic resin flow during active infection (Palmer et al., 1988). It can also persist in mature pinecones (Palmer et al., 1988; Smith et al., 1996) or as an endophyte living undetected in shoot tissues (Flowers et al., 2001).

The recent name change is testament to the controversy surrounding the classification of these fungi. Prior to 1995, *S. sapinea* isolates were characterized based on morphology. The morphotype classification scheme was honed by Wang et al. (1985) and by Palmer et al. (1987). Basing their conclusions on 30 isolates originating from ten different countries, Wang et al..

(1985) found the A-morphotype produced smooth conidia while the B-morphotype produced pitted conidia. Samples analyzed by Palmer et al. (1987) were collected from naturally infected pines living in the North Central region of the United States. The isolates were divided into two groups based primarily on cultural characteristics. The A-morphotype produced fluffy, white to gray-green mycelia, with conidia ranging in size from 34 x 13 µm to 39 x 13 µm. Light was required for sporulation. The B-morphotype produced white to black mycelia that were tightly appressed to the agar with conidia typically 34 x 12 µm. Sporulation occurred under both light and dark conditions. Both the A- and B-morphotypes grew most rapidly at 25°C (Palmer et al., 1987).

By the mid 1990s, the descriptions of *S. sapinea* began to reflect advances in molecular biology. In 1995, Smith and Stanosz (1995) used Randomly Amplified Polymorphic DNAs (RAPDs) to uncover the genotypic basis for the phenotypic differences observed among isolates of *S. sapinea*. Their research included the analysis of 32 isolates (16 possessing A-type cultural characteristics and 16 possessing B-type characteristics). Isolates were collected from diseased pines growing in Michigan, Wisconsin, and Minnesota. Hosts included *Pinus banksiana* (eight B-type), *P. nigra* (three A-type), *P. resinosa* (ten A-type; eight B-type), *P. strobes* (one A-type), and *P. sylvestris* (two A-type). RAPD analysis yielded different banding patterns for each morphotype.

RAPDs were used extensively during the next several years as researchers attempted to clarify host range, geographic range, and relative susceptibilities. Blodgett and Stanosz (1999) inoculated seedlings of six conifer species (*Pinus sylvestris, P. mugo, P. resinosa, Picea pungens, Pseudotsuga menziesii*, *and Abies balsamea*) with *S. sapinea* isolates belonging either

to the A- or B-type. Except on *Picea pungens*, they found that A-type isolates caused more extensive disease than the B-type isolates. Additionally, *P. sylvestris* was most susceptible to the A-type while *Abies balsamea* was least susceptible (Blodgett et al., 1999).

Stanosz et al. (1999) used RAPDs to analyze 79 isolates of *S. sapinea* collected from a broad range of genera, including *Pinus, Cedrus, Larix, Picea and Pseudotsuga*. Samples came from Africa, Australia, Asia, Europe, and North America. Nineteen isolates belonged to RAPD B-type; the remaining 60 belonged to RAPD A-type. Of special interest was the discovery of RAPD B-type on hosts and in geographic locales where it had never before been found. B-type isolates were recovered from *Cedrus deodora, C. atlantica, Pinus halepensis, P. elliottii, P. sylvestris, P. virginiana,* and *P. taeda* in countries including France, Spain, Israel, Italy, and U.S states such as Georgia, Florida, Kentucky, and West Virginia. Prior to this study, the B-type was only known to exist on *Pinus resinosa* and *P. banksiana* growing in the North Central region of the United States. The A-type was found in even more locales, including South Africa, Australia, France, Netherlands, New Zealand, United Kingdom, Zambia, Honduras, Israel, Canada, Mexico, China, Australia, India, and others. It also was recovered in U.S. states of Wisconsin, Pennsylvania, Michigan, Illinois, Idaho, New York, Hawaii, and Minnesota (Stanosz et al., 1999).

De Wet et al. (2000) demonstrated that *S. sapinea* isolates did not always fit neatly into one of the two accepted RAPD types. Using samples collected from South Africa, Mexico, and Indonesia, analysis grouped samples into three RAPD types: A, B, and C. The C-type was found only in Mexico and Indonesia, and it possessed conidia that were longer than those of A- and B-types (de Wet et al., 2000).

Hausner et al. (1999) previously suggested that limiting *S. sapinea* to only two types was premature. While studying *S. sapinea* isolates from eastern Canada, they noticed that some isolates possessed cultural characteristics that were intermediate between the A- and B-types. They referred to the deviants as the I-morphotype. Using Restriction Fragment Length Polymorphisms (RFLPs), they demonstrated a genetic difference among the three groups.

By 2000, four genetic sub-types of *S. sapinea* had been suggested (A, B, C, and I). The A-, B-, and C- types were distinguished from each other by RAPD analysis. The I- type was distinguished from the A- and B-types by RFLP analysis. Neither of these analytical methods, however, had been used to distinguish the C-type from the I-type. More importantly, both of these methods possess innate weaknesses. RAPD analyses identify dominant molecular markers; results are either positive or negative based on the presence or absence of a specific band. As such, experimenter error can lead to false negatives. RFLP analyses identify co-dominant molecular markers. False negatives are not as much of a problem, but reading and reproducing results from these finicky analyses are. Additionally, because RFLPs are not PCR-based, they require relatively large amounts of sample DNA.

The use of Simple Sequence Repeat PCR (SSR-PCR) to identify and classify morphotypes of *S. sapinea* was suggested by Burgess et al. (2001). Diagnosis using SSR-PCR is preferable to diagnosis with RAPDs or RFLPs because it amplifies co-dominant markers, and requires little DNA. Their research led to the development of 11 primer sets each capable of amplifying a single microsatellite region of the *S. sapinea* genome. Three of these primer sets amplified regions that were polymorphic among the A-, B-, and C-morphotypes. Seven of the 11 primer sets amplified regions that were identical for the I-morphotype and *Botryosphaeria*

obtusa, the teleomorph of *Sphaeropsis.* For this reason, the authors suggested that the I-type no longer be considered a morphotype of *Sphaeropsis.*

Burgess et al. (2001) used the 11 SSR-PCR primers that they developed to form hypotheses about the phylogenetic relationship among isolates in their study. Parsimony analysis suggested that the A- and C-morphotypes were more closely related to one another than they were to the B-morphotypes. The B-morphotype was also found to be the most genetically diverse of the morphotypes.

Building on this base, de Wet et al. (2003) attempted to further elucidate relationships among the A-, B-, and C-morphotypes. Phylogenies were suggested based on comparison among the isolates of six protein coding genes and six microsatellite regions. Isolates of *S. sapinea* included in the study originated from midwestern U. S. states (one A- and one B-type isolate from *P. resinosa*, one B-type isolate from *P. banksiana*), California (two B-type isolates from *P. resinosa*), Australia (one A-type isolate from *P. radiata*), Mexico (two B-type isolates from *P. patula*), Italy (one B-type isolate from *Pinus sp*.), and Indonesia (two C-type isolates from *P. patula)*. Four isolates of the closely related *B. obtusa* (originating from Canada and South Africa) also were included in the analysis. Results supported those of Burgess et al. (2001) in that the A- and C-morphotypes were more closely related to each other than to the B-morphoptype. All three morphotypes were distinct from *Botryosphaeria spp.*, but were clonal anamorphs of it (de Wet et al., 2003). There are three main anamorphic genera of *Botryosphaeria spp.* (*Diplodia, Lasiodiplodia,* and *Fusicoccum*). All three morphotypes behave identically to the established genus *Diplodia* in that they produce ellipsoid, thick-walled, septate conidia that darken with age. For this reason, the authors suggest that the genus designation be changed from *Sphaeropsis* to *Diplodia*. The authors further suggested that the B-morphotype be given the species name

Diplodia scrobiculata J. de Wet, B. Slippers & M.J. Wingfield due to its genetic distinction from the A- and C-types. Finally, they suggested that the A- and C-morphotypes re-assume the previously assigned species name *Diplodia pinea* (Desm.) J. Kickx (de Wet et al., 2003).

Smith and Stanosz (2006) introduced yet another molecular tool aimed at rapid and accurate diagnosis of *Diplodia spp*. infection in pines. Three primers (two forward, one reverse) were developed that targeted the mitochondrial small subunit ribosome gene. The reverse primer, BotR, anneals to the DNA of both *D. pinea* and *D. scrobiculata*. Forward primer DpF anneals to *D. pinea* DNA; forward primer DsF anneals to *D. scrobiculata* DNA. Primer pair DpF/BotR produces a 700 base pair band in the presence of *D. pinea* DNA, but no band in the presence of *D. scrobiculata*; primer pair DsF/BotR produces a 700 base pair band in the presence of *D. scrobiculata* DNA, but no band in the presence of *D. pinea*. Primer pairs were designed such that neither would amplify the DNA of *B. obtusa*, *B. quercum*, *B. rhodina*, *B. ribis*, *B. tsugae*, *B. mutila*, *B. dothidea*, *D. pinea* f. sp. cupressi, as well as the DNA of common pine inhabitants *Fusarium tricinctum*, *Cladosporium sp*., *Chaetomium sp*., *Schlerophoma pythiophylla*, *Epicoccum sp*., *Alternatia sp.*, *Pestalotia sp*., *Trichoderma sp*., *Sirococcus conigenus*, and *Pseudorobillarda sp*..

Nuclear rDNA Internal Transcribed Spacers (ITS) also have been used to compare various anamorphs of *Botryosphaeria spp.* Jacobs and Rehner (1998) successfully used ITS 4 and 5 primers to amplify ITS 1 and 2 regions to distinguish 22 strains of botryosphaeriaceous fungi. Among those strains compared were *Fusicoccum* sp., *Diplodia* sp., *Sphaeropsis* sp., and *Lasiodiplodia theobromae* (teleomorph = *Botryosphaeria rhodina*). Additionally, the tendency of *Sphaeropsis* sp. and *Diplodia* sp. to exhibit both conidial pleomorphy and morphological plasticity was noted (Jacobs and Rehner, 1998).

Host Range and Susceptibility

Jack (*Pinus banksiana*), red (*P. resinosa*), Austrian (*P. nigra*), Scots *(P. sylvestris*), Gregg's (*P. greggii*), Aleppo (*P. halepensis*), Monterey (*P. radiata*), and Mexican Weeping (*P. patula*) pines are susceptible to *S. sapinea (*Blodgett and Stanosz, 1997; Blodgett and Bonello, 2003; Chou, 1987; Flowers et al., 2001; Paoletti et al., 2001; Smith et al. 2002). Symptom development and severity, however, depend on factors such as pathogen morphotype, host pine species, wounding events, water stress, and fertility. Some trees, under certain conditions, are symptomless, although they are capable of spreading disease to more tender tissues on themselves or neighboring trees.

Physical damage can predispose pine trees to infection by *S. sapinea*. In South Africa, summer hailstorms have been associated with an increased incidence of dieback on *P. patula* and *P. radiata* plantations. The link between hailstorms and *S. sapinea* dieback has long been known. *Sphaeropsis sapinea* was first reported on *P. radiata* in South Africa in 1909 (at the Fort Cunynghame State Forest in the Eastern Cape Province). By the 1920s, South Africans began planting *P. patula* instead of *P. radiata* in hopes that it would be less susceptible to post-hailstorm *S. sapinea* infection. Within 15 years, their hopes were dashed. Although *P. patula* continues to be planted at rates rivaling *P. radiata*, it too suffers greatly from post-hailstorm *S. sapinea* infection (Smith et al., 2002).

Smith et al. (2002) theorized that the predictable development of *S. sapinea* infections on hail damaged pines could be explained by the pathogen being present on the pine prior to weather-related damage. In the course of their study, they collected 30 current-year seed cones, 30 two-year-old seed cones (actively expanding), 30 three-year-old seed cones (mature, but unopened), and 30 current-year pollen cones from a 14-year-old stand of *P. patula* in the

Mpumalanga Province, South Africa. The mature seed cones were divided into four parts (seed, seed wings, ovuliferous scales, and pith tissue) and plated separately. For the two-year-old cones, all parts except seed wings were plated. Current year cones (pollen and seed) were simply cut in half before plating. No *S. sapinea* was recovered from any of the current year cones. It was recovered, however, from 20% of the expanding two-year-old cones and from 60% of the mature, unopened three-year-old cones. In the two-year-old cones, the pathogen was only recovered from the ovuliferous scales (not from the seed or pith tissues), while in the three-year-old cones, the pathogen was present in all four of the cone parts. Clearly, after one year, seed cones of *P. patula* became a reservoir for *S. sapinea* (Smith et al., 2002).

This finding supported previous findings by Stanosz et al. (1997) and Smith et al. (1996). Stanosz et al. (1997) showed that asymptomatic, nursery-grown *P. resinosa* seedlings have a latent *S. sapinea* infection rate of 27.5% and that forest-grown trees had similar levels of latent infection in stems (previous and current year's growth) and needles (last year's growth only). Smith et al. (1996) found that mature, unopened seed cones of *P. patula* and *P. radiata* also had high rates of latent infection, whereas *P. taeda* and *P. elliottii* cones were not similarly infested.

A severe hailstorm in the Sabie region of the Mpumalanga Province in South Africa during the summer of 1995 provided Smith et al. (2002) with a chance to further bolster their argument that latently infected seed cones were responsible for much of the post-hailstorm dieback seen in *P. patula* plantations. In the first set of experiments, three plantations representing tree ages of five-, 14-, and 25-years were involved. The 14- and 25-year-old trees bore seed cones of all three ages. The five-year-old trees were too young too have any mature (three-year-old) seed cones. Three months after the storm, wood, pith and cortex tissue exhibiting symptoms associated with *Sphaeropsis* canker and dieback were collected and plated

from trees in all three age groups. Mortality rates within each age group also were noted. Results from the first set of experiments demonstrated a strong correlation between tree age and mortality following hailstorms. The 25-, 14-, and 5-year-old stands experienced mortality rates of 92%, 34%, and 9%, respectively. This phenomenon may be explained by the relative abundance of mature seed cones on older trees compared to younger trees. Sixty percent of three-year-old seed cones and 20% of second-year seed cones were found to be infected. No current-year seed cones were infected.

In the second set of experiments, branches with attached cones were collected from hail-damaged 14-year-old trees, hail-damaged five-year-old trees and 13-year-old trees that had not been damaged by hail. Attempts were made to isolate the pathogen from the pith tissues of the seed cones, from the pith tissue of the branch, and from the stipe that connected the branch and the cone (Smith et al., 2002).

The authors found that branch pith tissues collected from hail-damaged 14-year-old trees exhibited dark staining while pith tissues from non-damaged 13-year-old trees did not. From the hail-damaged, stained trees, *S. sapinea* was collected from the seed cone pith, the cone stipe, and the pith of the attached branch. From the unstained, non-damaged trees, *S. sapinea* was isolated only from the seed cone pith and the cone stipe. These findings suggest that the physical damage caused by hail enables *S. sapinea* to shift from latent pathogen of cones to active pathogen of stems and, eventually, needles. Interestingly, from the 5-year-old trees, *S. sapinea* could be isolated from cankers surrounding hail wounds, but could not be isolated from any pith tissues (Smith et al., 2002).

In the North Central region of the United States, both the A- and B-morphotype are known to infect native pine species. In the late 1990s, Blodgett and Stanosz (1997) conducted a

study in which young seedlings of jack pine (*P. banksiana*) and red pine (*P. resinosa*) were inoculated with isolates of each morphotype in an effort to determine both the relative aggressiveness of the morphotypes and the ability of each morphotype to colonize nonwounded tissue. The isolates in this study were originally collected from Michigan, Minnesota, and Wisconsin. Two experiments were conducted. In the first experiment, one-year-old seedlings of both red and jack pine (4-weeks post transplant) were inoculated with monoconidial suspensions of the pathogen. Specifically, five isolates of each morphotype were grown on 1.5% water agar (WA) plates in which red pine twig sections were embedded. The plates were flooded with sterile deionized water and the pycnidia, which had formed on the twigs, were scraped to release conidia. The monoconidial suspensions were collected and concentrations were adjusted to approximately 5×10^{3} conidia ml^{-1}. Seedlings were held upside down and covered with plastic such that only the top 2-cm of their elongating, asymptomatic shoots would be subjected to the conidial spray, and covered entirely with plastic for 4-days post inoculation to ensure that relative humidity would remain high.

After five days, necrotic symptoms typical of *Sphaeropsis* infection (i.e., dead tips, crooked shoots, necrotic needles, stem cankers) were visible on both the A- and the B-morphotype inoculated seedlings. For red pines, seedlings infected with the A-morphotype experienced greater disease frequency than those infected with the B-morphotype (97% and 18% respectively). For jack pine, the same trend held true (42% and 6%, respectively). In comparing the pathogenicity of the two morphotypes on each of the two pine species, red pines suffered significantly greater disease incidence than jack pines when exposed to the A-morphotype ($P < 0.001$). When exposed to the B-morphotype, the difference in disease incidence on the two pines was present, but less pronounced ($P = 0.059$). Disease severity (as measured by percentage

of necrotic needles) was greater on both types of seedlings when infected with A-morphotype isolates than when infected with B-morphotype isolates. A-morphotypes also caused more severe symptoms on red pines than on jack pines ($P < 0.001$), but inoculation with B-morphotypes did not cause a statistically significant difference between the two pine species.

In the second Blodgett and Stanosz (1997) experiment, two-year-old seedlings of both red and jack pine were wounded prior to being inoculated by removing a single fascicle 2-cm from the elongating shoot apex by making a scalpel cut flush with the stem. A mycelial plug of one of 17 monoconidial isolates (nine A-type, eight B-type) was affixed to each seedling such that the fungus and the wound were in direct contact. Six days later, the plugs were removed.

With one exception, results were similar to those obtained from the nonwounded seedling tests. Symptoms typical of *Sphaeropsis* infection were present six days post inoculation. Both A- and B-morphotypes caused disease in each type of pine. The A-morphotype caused greater incidence of disease than the B-morphotype on both red pine (100% and 21%, respectively) and jack pine (99% and 53%, respectively). Inoculation with the A-morphotype produced symptoms (necrotic needles) at greater distance from the point of inoculation than inoculation with the B-morphotype for both red pines (6.6 and 8.8 cm compared to 0.4 and 0.1 cm) and for jack pines (5.8 and 6.5 cm compared to 1.7 and 3.0 cm). Disease incidence in the two species of pine was not significantly different when inoculated with A-type isolates ($P = 0.958$), but it was significantly different when inoculated with B-types. In fact, when wounded prior to inoculation, jack pines were actually more susceptible to *S. sapinea* than red pines ($P < 0.001$). In the non-wounded inoculations, red pines appeared more susceptible.

After 8 weeks, *S. sapinea* of the applied morphotype could be recovered from both nonwounded red and jack pines, even in asymptomatic seedlings. Recovery occurred more

frequently from red pines than from jack pines. After four weeks, *S. sapinea* of the applied morphotype could again be recovered from the inoculated, wounded seedlings. However, A-morphotypes could be recovered at greater distances from the wound site than the B-morphotypes. The distance from the inoculation site at which the pathogen could be recovered was positively correlated to the distance at which symptoms had been observed ($r = 0.94$; $P<0.001$).

Blodgett and Stanosz (1997) demonstrated that both A- and B-morphotypes can infect non-wounded tissues of red and jack pine. It also suggested that the A-morphotype is more aggressive than the B-morphotype, but that the relative performance of pine species in response to *S. sapinea* inoculation can change based on which morphotype is used and the presence or absence of wounding events. Based on the recovery *of S. sapinea* from asymptomatic stems, the pathogen appears to exist undetected on the stems of red and jack pines which enables it to invade tissues immediately following physical damage. Said damage could occur during insect feeding, windstorms, or hailstorms (Blodgett and Stanosz, 1997).

Blodgett and Bonello (2003) began to characterize the pathogenicity of the A- and B-morphotypes of *S. sapinea* on Austrian pine (*P. nigra*). Two separate experiments were conducted: one in which shoot-tips were wounded and inoculated and one in which stems were wounded and inoculated. In both experiments, 5-year-old nursery grown trees were transferred to individual pots and placed in a greenhouse. The trees were approximately 35 inches tall with a stem diameter of 1.4 inches.

In the first experiment, a single fascicle was removed 2-cm from the shoot apex. A 4-mm agar plug, colonized either with the A-type or the B-type, was placed mycelia-side down atop the wound. Parafilm held the plug in place for 3-days before being removed. Symptoms were

assessed 4-weeks following inoculation. In the second experiment, a sterile 1-cm cork borer was used to remove a small amount of bark/phloem from an area 10-cm above the soil line. Inoculations proceeded as in the first experiment, however, the parafilm was not removed until symptoms were assessed at 6 weeks.

The A- and B-type isolates caused disease on wounded *P. nigra* stems and shoots. The A-type produced 100% infection on the stems and on the shoots. The B-type produced 100% infection on the stems, but only 60% infection on the shoots. Stem cankers caused by the A-type generally produced less resin and were two-fold longer than those caused by the B-type. Symptoms associated with shoot cankers were less pronounced when caused by the A-type than when caused by the B-type. Shoot cankers were longer when caused by the A-type than the B-type. In fact, shoot cankers caused by the B-type were not significantly longer than those caused by the control. Interestingly, the pathogen was recovered from 100% of inoculated trees regardless of morphotype, symptom severity, or wound site. This fact demonstrates the ability of *S. sapinea*, particularly the B-morphotype, to create asymptomatic infections in Austrian pines (Blodgett and Bonello, 2003).

Flowers et al. (2001) offered further evidence that Austrian pines, as well as Scots pines (*P. sylvestris*), are often latently infected by *S. sapinea*. In the first phase of their study, over 800 asymptomatic shoots were collected from 96 Austrian pines and 24 Scots pines. From the 96 Austrian pines, 211 shoots were sampled from completely asymptomatic trees and 554 shoots were sampled from trees having some blighted shoots. From the 24 Scots pines, 17 shoots were sampled from completely asymptomatic trees and 32 shoots were sampled from trees having some blighted shoots. A few blighted shoots also were collected from the diseased trees as a means of future comparison. Shoots were surface disinfested, and stems, needles, buds, and

female and male cones were plated separately. Male cones, as well as first- and second-year female cones, were cut in half vertically before being plated. Individual scales from unopened third-year mature cones were plated without any further dissection.

Bark samples also were collected from four Austrian pines and one Scots pine. Samples were taken both from branches bearing blighted shoots and from branches bearing only asymptomatic shoots. Samples were collected aseptically using either a #2 or #4 cork borer. They were then dissected to create three subsamples: outer bark (mostly periderm); cortex (inner portion of the periderm, the cortex and a portion of primary phloem); phloem (the remaining primary phloem and secondary phloem). The outer bark, cortex and phloem subsamples were surface disinfested and plated on acidified potato dextrose agar (PDA).

Sphaeropsis sapinea was commonly cultured from the shoots of apparently healthy (asymptomatic) Austrian and Scots pines, 39% and 70%, respectively. The pathogen was equally likely to be recovered from shoot, buds, or needles of symptomatic Austrian pine trees. For asymptomatic trees, however, the pathogen was significantly more likely to be cultured from the shoot than from needles or buds. *Sphaeropsis sapinea* was also commonly cultured from the bark and phloem of asymptomatic Austrian pines and from the bark, phloem, xylem and pith of symptomatic Austrian pines.

There was a tendency for blighted trees to be older than asymptomatic trees. For Austrian pines, asymptomatic trees ranged from 6-17 years of age while blighted trees ranged from 10-25 years of age. Similarly, age range for asymptomatic Scots pines ranged from 3-12 years while blighted trees ranged in age from 7-20 years. Additionally, the pathogen was recovered more frequently from apparently healthy shoots when those shoots were collected from trees on which greater than 20% of total shoots were blighted. This finding affirms those of Smith et al. (2002)

that mature seed cones, which are found in greatest abundance on older trees, serves as a primary site for the pathogen to persist and serve as a source of secondary inoculum for future infections.

Another portion of the Flowers et al. (2001) study involved 90 asymptomatic 3-year-old Austrian pines. Stems, needles, and buds from 15 of the seedlings were cultured in an effort to ensure that the seedlings were in fact free of *S. sapinea*. The remaining 75 seedlings were either inoculated or used as controls during the fall of 1999. Eleven isolates of *S. sapinea* were used in the inoculations: two from latently infected Scots pines, two from visibly diseased Scots pines, four from latently infected Austrian pines, and three from visibly diseased Austrian pine. Each isolate was used to inoculate six different seedlings. The remaining nine seedlings were used as uninoculated controls. To inoculate the seedling, a shallow wound was made 5-cm below the tip of the terminal shoot. Parafilm held a colonized PDA plug close to the wound. The parafilm and plugs were removed 24 hours after inoculation. Seedlings were kept in a greenhouse for 11 weeks before being rated for disease development.

After 11 weeks, 17 of the 66 inoculated seedlings had not developed tip blight symptoms, of which 10 had been inoculated with isolates originating from asymptomatic pine tissues. The other seven had been inoculated with isolates originating from symptomatic pine tissues. To ascertain if these 17 seedlings were latently infected, ten of the 17 asymptomatic seedlings were dissected and cultured. *Sphaeropsis sapinea* was recovered in nine of ten cases. Six of the nine isolates originated from asymptomatic shoots of infected pine tissues.

Flowers et al. (2001) demonstrated that *S. sapinea* can be a latent pathogen of Scots pines and Austrian pines, and that, under certain conditions, a portion of those isolates can switch from latent pathogen to active pathogen (and presumably vice versa). The study also supports earlier findings that latent infections are typically limited to the shoots, bark, and outer phloem of

healthy pine trees while it inhabits the shoot, bark, wood, buds and needles of visibly diseased pines (Flowers et al., 2001).

It has been suggested that susceptibility of a pine to *S. sapinea* can depend not only on pathogen morphotype and host species, but also on host provenance. *Pinus greggii* is native to Mexico, but South African plantation owners have considered planting it (rather than *P. radiata* or *P. patula)* due to its purported resistance to *S. sapinea* infection. In a study by Smith et al. (2002), *P. greggii* seedlings were planted in South Africa and evaluated for *S. sapinea* infection following summer hail storms. Thirteen tree families from southern Mexico (representing three provenances) and 51 tree families from northern Mexico (representing six provenances) were represented in the study. *Pinus patula* was included in the study as a negative control for tolerance and *P. taeda* and *P. elliottii* were included as positive controls for tolerance.

The authors found that provenance played a significant role in susceptibility. Susceptibility of tree families from southern Mexico differed significantly from one another along provenance lines. All southern provenance families exhibited significantly more dieback than *P. elliottii* (one of the positive controls for tolerance). These same southern provenance families did not respond differently from *P. patula* (the negative control for tolerance). These results indicate that the southern families evaluated were susceptible to *S. sapinea*. Susceptibility of tree families from northern Mexico did not differ significantly from one another along provenances lines. As a whole, trees from northern provenances did exhibit less die back than trees from southern provenances. The northern trees outperformed *P. patula* and performed equally well to *P. elliottii*. These data suggest that South African growers, as well as all growers, should consider provenance when considering seed source.

Furthermore, the authors found that northern provenance *P. greggii* families outperformed *P. taeda,* the other positive control for tolerance. In fact, even the southern provenance *P. greggii* families outperformed *P. taeda* in four of 13 cases. These results bring into question the level of resistance previously reported for *P. taeda.*

A second set of experiments outlined in the Smith et al. (2002) paper may explain this anomaly. These experiments involved the inoculation of unwounded tree limbs with an isolate of *S. sapinea* previously confirmed to be pathogenic. All *P. greggii* families represented in the initial experiments were represented in these inoculation experiments. *Pinus patula*, *P. taeda*, and *P. elliottii* were also included. With the exception of pine families from one of the three southern provenances, the authors found no statistical difference in lesion length between the northern provenance *P. greggii*, the southern provenance *P. greggii, P. patula, P. elliottii*, and *P. taeda.* These findings bring into question the methods by which tolerance and susceptibility of pines to *S. sapinea* have previously been evaluated.

Effects of Drought Stress on Disease Development

Field observations and greenhouse experiments suggest that drought stress can increase incidence of and worsen severity of pine infection by *S. sapinea*. In a study by Bachi and Peterson (1985), 3-year-old Austrian and Scots pines inoculated with *S. sapinea* developed longer stem cankers if, following inoculation, they were subjected to water stress. Chou (1987) found that crown wilt severity in Monterrey pines (*P. radiata)* was directly related to greatly reduced pre-dawn water potential in the pines' needles. Johnson et al. (1997) showed that the duration of a drought affects disease development in Scots pine (*P. sylvestris*) seedlings. In that study, seedlings were inoculated with *S. sapinea* before being subjected to drought stress for varying periods of time. At the end of the six week trial, those seedlings having low needle water

potential for 14-21 days after inoculation exhibited greater incidence of die back and significantly longer cankers than seedlings having low needle water potential for four days or less.

In a study by Paoletti et al. (2001), disease severity, as measured by canker length, was nearly identical in Aleppo pine (*P. halpensis*) seedlings inoculated prior to five months of water stress and in those seedlings inoculated following 12 months of water stress. To conduct this experiment, 150 *P. halepensis* seedlings were transplanted into plastic pots and either covered with a UV-B permeable plastic tarp designed to prevent rainwater from reaching them or left uncovered. A subset of the seedlings growing under the plastic and a subset of the seedlings left uncovered were wounded using a 3-mm diameter cork borer and inoculated with 3-mm diameter agar plug of a monoconidial isolate of *S. sapinea* (A-morphotype) prior to the manipulation of water levels.

Inoculated seedlings were divided into four groups and each group was watered to field capacity every 7, 14, 21, or 28 days. After five months, canker lengths of the inoculated seedlings were measured.

At the same time non-wounded and non-inoculated trees were subjected to the same watering regimes and this was continued for seven more months, for a total of 12 months. At the end of 12 months, the non-inoculated trees were re-hydrated to field capacity every day for 15 days. The seedlings were then wounded and inoculated as described previously. For the next five months, all seedlings were watered weekly. At the end of 5 months, canker length was measured on each of the seedlings.

Results suggested that severe water stress increased the sensitivity of Aleppo pine to infection by *S. sapinea*. Seedlings that were watered every 28 days (the least frequent watering

schedule in this study) developed the largest cankers in both the pre- and post-water stress inoculations. All seedlings inoculated with *S. sapinea,* regardless of watering regime, developed cankers. However, the pathogen did the most damage on seedlings that were deprived of water either before or after inoculation; and increased susceptibility of the pines to the pathogen persisted, at least for 15 days, after an adequate water supply has been restored.

In a paper by Blodgett et al. (1997), the authors explored whether or not *S. sapinea* morphotype (A or B) would affect disease development in water stressed red pine (*P. resinosa*) seedlings. Two-year-old seedlings were brought into a greenhouse and watered to field capacity every three days for one month. For the next six weeks, seedlings were either watered to field capacity daily (non-stressed) or watered to field capacity only when needle water potential dropped below -1.64 MPa (stressed). After two weeks under these regimes, tender shoots were inoculated with isolates of either A- or B-morphotype. Observations were made 4-weeks later. The authors found that seedlings inoculated with A-morphotype isolates exhibited significantly more dieback (measured in cm from inoculation site) when subjected to water stress than when provided with ample water. No significant difference was seen, however, between water stressed and non-stressed seedlings that had been inoculated with B-morphotype isolates. Possibly contributing to this lack of significance, B-morphotype isolates caused a minimal amount of dieback in both the stressed and non-stressed seedlings.

A second, similar experiment was conducted to verify results. Again, seedlings were brought into a greenhouse and watered to field capacity every three days for one month. At the end of that month, differing watering regimes were instituted; seedlings were either watered to field capacity daily or not watered again for the remainder of the study. After one week, all seedlings were transferred to growth chambers. After one week more, tender shoots were

inoculated with an A-morphotype isolate. Seedlings were returned to the growth chambers. Four weeks later, symptoms were observed. The authors again found that inoculated seedlings displayed significantly more dieback when subjected to water stress than when provided with ample water.

Aware of the prevalence of asymptomatic *S. sapinea* infections, Stanosz et al. (2001) explored how host water stress affected the pathogen's shift from latent to active invader. In the first of two greenhouse experiments, non-inoculated 2-year-old red pine seedlings were divided into six treatment groups. One group was watered to field capacity every other day. Each of the remaining five groups was watered only when pre-dawn needle water potential dipped to -1.1, -1.7. -2.0, -2.5, or -3.2 MPa. At the end of four weeks, shoot displaying visible signs of *S. sapinea* infection (i.e. tip dieback, resin flow, pycnidia) were surface disinfested and plated on WA. Mortality rates ranged from 8% in the non-stressed seedlings to 50% in the most stressed seedlings. Statistical analysis verified a real difference in mortality rate among the treatment levels ($P<0.001$). Total active infection rates of seedlings (living or) ranged from 42% in the non-stressed seedlings to 72% in the most stressed seedlings. Again, statistical analysis verified a real difference in active infection rates among the treatment levels ($P<0.001$).

In a second experiment, the authors used a complete factorial design to determine the combined effect of fungicide treatment and water deprivation on disease development. The non-inoculated, 2-year-old, red pine seedlings were either 1) watered to field capacity every other day (non-stressed) and sprayed weekly with benomyl; 2) watered to field capacity every other day (non-stressed) but never sprayed with benomyl; 3) watered but was sprayed weekly with benomyl; or 4) neither watered nor sprayed with benomyl. Once the non-watered seedlings reached a mean needle water potential of -2.8 MPa, all seedlings were watered to field capacity

every other day for two weeks. At the end of two weeks, shoots displaying visible signs of *S. sapinea* infection (i.e. tip dieback, resin flow, pycnidia) were surface disinfested and plated on WA. The authors found that water-stressed seedlings exhibited significantly higher levels of mortality than did non-stressed seedlings. Of the 400 non-stressed seedlings, only 13 died. Of the 400 stressed seedlings, 195 died. More importantly, they recovered *S. sapinea* from 182 of 400 stressed seedlings, but from only 39/400 non-stressed seedlings. Among water-stressed seedlings, treatment with benomyl was associated with a decreased in active infection rate from 66% to 25% ($P<0.001$). Among non-stressed seedlings, treatment with benomyl was associated with a decrease in active infection rate from 17% to 3% ($P<0.001$).

CHAPTER 3

SURVEY OF TWO SOUTH GEORGIA PINE PLANTATIONS FOR THE PRESENCE OF *DIPLODIA* SPP.

INTRODUCTION

Diplodia pinea (Desm.) J. Kickx (formerly *Sphaeropsis sapinea* [Fr.] Dyko & B. Sutton morphotype A) and *Diplodia scrobiculata* J. de Wet, B. Slippers & M.J. Wingfield (formerly *Sphaeropsis sapinea* [Fr.] Dyko & B. Sutton morphotype B) are closely related fungal species that cause disease on a myriad of pine species. First described in 1909 as a pathogen in South African pine plantations, *Diplodia spp.* cause symptoms including shoot blight, stem canker, crown wilt and collar rot (Nichols and Ostrey, 1990; Palmer, 1991; Palmer et al., 1985; USDA Forestry Service, 2005). *Diplodia spp.* are capable of gaining entry into trees during the spring via elongating needles (Brookhouser and Peterson, 1971; Peterson, 1977). However, wound sites, such as those caused by hail, can also provide an infection court (Smith et al., 2002). Initial infection of a mature tree may result in the dieback of only a few shoots. After years of infection, however, large branches or entire trees may be lost (Flowers et al., 2001). The pathogen can over-winter as pycnidia embedded in the golden-brown remains of last years diseased shoots, which are retained on the tree, at least partially, by characteristic resin flow during active infection (Palmer et al., 1988). It can also persist in mature pinecones (Palmer et al., 1988; Smith et al., 1996) or as an endophyte living undetected in shoot tissues (Flowers et al., 2001).

Prior to 1995, *S. sapinea* isolates were characterized based on morphology. Two morphotypes were initially described. The A-morphotype produces fluffy, white to gray-green mycelia, with smooth conidia ranging in size from 34 x 13 µm to 39 x 13 µm (Wang et al., 1985;

Palmer et al., 1987). The B-morphotype produces white to black mycelia that were tightly appressed to the agar with pitted conidia typically 34 x 12 μm (Wang et al., 1985; Palmer et al., 1987). Two additional morphotypes, I (Hausner et al., 1999) and C (de Wet et al., 2000), have also been proposed. However, these two morphotypes are no longer recognized as unique entities.

Over the past 20 years, identification of *Diplodia spp*. has shifted from morphological to molecular. Randomly Amplified Polymorphic DNAs **(**RAPDs), Restriction Fragment Polymorphisms (RFLPs), and Simple Sequence Repeat PCR (SSR-PCR) have been used to identify and classify the various morphotypes (Smith and Stanosz, 1995; Hausner et al., 1999; Burgess et al., 2001). Burgess et al. (2001) used SSR-PCR to prove that morphotype I was nearly identical to *Botryosphaeria obtusa,* the teleomorph of *Sphaeropsis*, and therefore no longer a viable morphotype. De Wet et al. (2003) compared the A-, B-, and C-morphotypes by studying six protein coding genes and six microsatellite regions, and concluded that all three morphotypes behave identically to the established genus *Diplodia,* producing ellipsoid, thick-walled, septate conidia that darken with age. Based on this finding, it was suggested that the genus designation be changed from *Sphaeropsis* to *Diplodia,* and that the A- and C-morphotypes re-assume the previously assigned species name *Diplodia pinea* (Desm.) J. Kickx. Furthermore, the B-morphotype was given the species name *Diplodia scrobiculata* J. de Wet, B. Slippers & M.J. Wingfield due to its genetic distinction from the A- and C- types (de Wet et al., 2003).

Smith and Stanosz (2006) introduced another molecular tool for the study of *Dipodia spp.*. Three primers were developed that targeted the mitochondrial small subunit ribosome gene (SSU rDNA). Primer pair DpF/BotR produces a 700 base pair band in the presence of *D. pinea* DNA, but no band in the presence of *D. scrobiculata*; primer pair DsF/BotR produces a 700 base

pair band in the presence of *D. scrobiculata* DNA, but no band in the presence of *D. pinea*. Primer pairs were designed such that neither would amplify the DNA of closely related species *Botryosphaeria obtusa*, *B. quercum*, *B. rhodina*, *B. ribis*, *B. tsugae*, *B. mutila*, *B. dothidea*, and *Diplodia pinea* f. sp. *cupressi* nor the DNA of common pine inhabitants *Fusarium tricinctum*, *Cladosporium sp.*, *Chaetomium sp.*, *Schlerophoma pythiophylla*, *Epicoccum sp.*, *Alternatia sp.*, *Pestalotia sp.*, *Trichoderma sp.*, *Sirococcus conigenus*, and *Pseudorobillarda sp.*.

Nuclear rDNA Internal Transcribed Spacers (ITS) also have been used to compare various anamorphs of *Botryosphaeria.* Jacobs and Rehner (1998) used ITS 4 and 5 primers to amplify ITS 1 and 2 regions of 22 strains of botryosphaeriaceous fungi. Among those strains compared were *Fusicoccum sp.*, *D. quercina*, *D. mutila*, *Sphaeropsis sp.*, *Botryosphaeria sp.*, *S. sapinea*, and *Lasiodiplodia theobromae*. Six phylogenetic groups were identified: three of the groups were composed solely of *Fusicoccum* species; one was composed solely of *L. theobromae*; one was composed of *S. sapinea* and *Sphaeropsis sp.*; and one was composed of *Sphaeropsis sp.*, *D. mutila*, *D. quercina*, and *Botryosphaeria sp.* The authors noted that both *Sphaeropsis sp.* and *Diplodia sp.* exhibited conidial pleomorphy and morphological plasticity in culture (Jacobs and Rehner, 1998).

Multiple studies have attested to the broad geographical and host ranges of *Diplodia spp.*. Stanosz et al. (1999) documented the presence of the pathogen on genera including *Pinus, Cedrus, Larix, Picea and Pseudotsuga* and in areas including Africa, Australia, Asia, Europe, and North America. In addition, *D. scrobiculata* was discovered on hosts and in geographic locales where it had never before been found. It was recovered from *P. halepensis* (Aleppo pine), *P. elliottii* (slash pine), *P. sylvestris* (Scots pine), *P. virginiana* (Virginia pine), and *P. taeda* (loblolly pine) in countries including France, Spain, Israel, Italy, and U.S states such as Georgia,

Florida, Kentucky, and West Virginia. Prior to this study, *D. scrobiculata* (syn. B-morphotype) was only known to exist on *Pinus resinosa* (red pine) and *P. banksiana* (jack pine) growing in the North Central region of the United States. *Diplodia pinea* (syn. A-morphotype) was found to be present in an even broader range of areas. In the U.S., it was detected in Wisconsin, Pennsylvania, Michigan, Illinois, Idaho, New York, Hawaii, and Minnesota (Stanosz et al., 1999).

Although *D. scrobiculata* was long considered to be less pathogenic than *D. pinea* (Blodgett et al., 1999; Smith et al., 2002; South et al., 2002), studies have indicated that the relative pathogenicity of the two species is specific to pine species and to the presence or absence of stressors such as drought and wounding events. Jack pine is more susceptible to infection by *D. pinea* or *D. scrobiculata* if wounded prior to inoculation. Red pine, however, has no higher infection rate if wounded prior to inoculation with either of the pathogens. Comparatively, jack pine is more susceptible than red pine to *D. scrobiculata* infection when wounded prior to inoculation, but is less susceptible if not wounded. (Blodgett and Bonello, 2003).

Drought stress also plays a role in disease development. Three-year-old Austrian pines inoculated with *Diplodia spp.* developed longer stem cankers if, following inoculation, they are subjected to water stress (Bachi and Peterson, 1985). The same trend held true for disease development in Scots pine seedlings (Bachi and Peterson, 1985; Johnson et al., 1997). Red pine seedlings inoculated with *D. pinea* exhibit significantly more dieback when subjected to water stress than when provided ample water. Red pine seedlings inoculated with *D. scrobiculata*, however, showed no significant difference in canker length between stressed and non-stressed seedlings (Blodgett et al., 1997). Canker length caused by *Diplodia spp*. on Aleppo pine was directly related to the extent of water stress imposed on the seedling, regardless of whether that

stress occurred pre- or post-inoculation (Paoletti et al., 2001). In Hawaii, in the 1970s, 20 year-old Slash, and Loblolly pines developed damaging *Diplodia spp.* infections only after enduring a 3-4 year drought (Bega et al., 1978).

Latent infections also play a role in the eventual development of severe *Diplodia* tip blight. Smith et al. (2002) considered asymptomatic infection of 25-, 14-, and 5-year-old stands of *Pinus patula* (Mexican weeping pine) in South Africa. They found that mature unopened third year cones were more frequently infected than expanding second year cones, and that first year cones showed no infection at all. Older trees in the study showed higher mortality rates, assumably because they contained a greater number of older cones (Smith et al., 2002).

Scots and Austrian pine in Kentucky were often latently infected by *Diplodia spp.* (Flowers et al., 2001). The fungus was cultured from 70% of the asymptomatic Scots pine shoots and from 39% of the asymptomatic Austrian pine shoots. For asymptomatic trees, the pathogen was best isolated from the shoots; for diseased trees, isolation was equally likely from shoots, buds, or needles. Diseased trees were, on average, older than asymptomatic trees. Asymptomatic infections were most common in shoots collected from trees having greater than 20% of their total shoots blighted (Flowers et al., 2001).

Stanosz et al. (1997) documented that asymptomatic, nursery grown red pine seedlings have a latent *Diplodia sp.* infection rate of 27.5% and that forest grown trees had similar levels of latent infection in the previous year's stems and needles and the current year's stems. A survey of Midwestern red pine nurseries uncovered asymptomatic infection rates up to 88%, with the highest rates associated with seedlings grown near red pine wind breaks (Stanosz, et al., 2005). Water stress has been implicated in the shift of *Diplodia spp.* from latent to active

pathogen. In one study, active infection rates of seedlings (living or dead) ranged from 42% in the non-drought stressed seedlings to 72% in the most stressed seedlings (Stanosz et al., 2001).

Reports from China have indicated that *Diplodia spp.* is a major cause of tip dieback of slash and loblolly pine grown in the Hubei, Hunan, and Jiangsu provinces of China (Su et al., 1991; Yang et al., 1988; Shen, 1990). In South Africa and Swaziland, it has been associated with a damaging root disease of slash and loblolly pines (Wingfield and Knox-Davies, 1980). Despite these findings, loblolly and slash pine have been viewed as relatively resistant to aerial infection by *Diplodia spp.*. Though not the intent of the study, Smith et al. (2002) found that although loblolly and slash pine were included in their study as positive controls for tolerance, they proved susceptible in some cases. Loblolly pine, for instance, were more susceptible to *D. pinea* than many of the *P. greggii* (Greg's pine) families in the study. In another experiment, no statistical difference was found among lengths of lesions caused by *D. pinea* on the northern provenance Greg's pine, the southern provenance Greg's pine, Mexican weeping pine, loblolly pine, and slash pine even though Mexican weeping pine is commonly regarded as being quite susceptible to *D. pinea* (Smith et al., 2002).

Forestry is a critical component of Georgia's economy. IMPLAN models have estimated that, in 2004, forestry in Georgia was responsible for the employment of over 67,000 people who were paid a total of over $3 billion to produce over $14 billion worth of product (Riall, 2006). When multiplier effects were included, total economic impact of forestry on Georgia's economy were increased to over 144,000 people paid approximately $6.3 billion to produce more than $22 billion worth of product. It was estimated that the forestry industry contributed $155 million in net revenue to the state's government (Riall, 2006).

Diplodia spp. have the ability to cause substantial damage to pines. That ability is often enhanced by drought conditions or wounding events. Given Georgia's economic dependence on forestry and southern Georgia's tendency for long periods of drought interspersed with tumultuous storms, the presence of *Diplodia spp.* in the state's pine plantations needs to be more fully investigated. The aim of this study is to evaluate the levels at which *D. pinea* and *D. scrobiculata* currently inhabit slash pine in southern Georgia pine plantations.

MATERIALS AND METHODS

Two pine plantations near Fargo and Hoboken, Georgia were surveyed during the spring of 2005 and 2006. During 2005, the sampling was preliminary. Twelve sites were identified that were known to have diseased trees. Shoots exhibiting symptoms characteristic of infection by *Diplodia spp.* were collected from slash (*P. elliottii*), loblolly (*P. taeda*), and/or longleaf (*P. palustris*) pine. During March and April 2006, the same two plantations were sampled, but sampling was more directed. At each location, three areas affected by tip blight were identified. Within each area, three plots each of 5-, 10-, and 15-year old slash pine were selected. In each plot, 10 diseased trees were sampled along a single 50-75 meter transect. Due to the thickness of underbrush, those transects typically ran along dirt service roads. However, every effort was made to sample from trees that were, at least, 2-3 rows in from the road. Two shoots (one symptomatic and one asymptomatic) were collected from each diseased tree selected. One shoot also was collected from an asymptomatic tree neighboring each diseased tree. The total number of shoots collected in 2006 was 540. During both years, samples were transported on ice from the field to laboratory on the same day that they were collected, and were stored at 8°C until processed.

Samples were processed in essentially the same way both years. For each shoot, the outer bark was removed to expose any darkening pith tissue. Five tissue pieces measuring approximately 3 x 3-mm were excised from each sample. Each piece of tissue was surface sterilized by being soaked in succession in 95% ethanol for 10 seconds, 10% Clorox for 30-60 seconds, and sterile de-ionized water for 30-60 seconds. The tissue was allowed to dry on sterile paper towels before being plated onto potato dextrose agar (PDA) (Difco Laboratories, Sparks, MD). Mycelia having the appearance of *Diplodia spp.* were transferred to new PDA until pure cultures were obtained.

Due to known variation of the morphological characteristics within species of *Diplodia* (Jacobs and Rehner, 1998; Morelet and Chandelier, 1993; Kay et al., 2002), and due to the difficulty obtaining mature pycnidia, molecular methods were employed to characterize cultures. Isolates were grown in 1.5 mL eppendorf tubes (Sarstedt Newton, NC) of potato dextrose broth (PDB) (Difco Laboratories, Sparks, MD) for 2-3 weeks. The resulting mycelial mass was transferred to a 2.0 mL bead beating tube (BioSpec Products, Inc., Bartlesville, OK) along with two 5-mm solid glass beads (Fisher Scientific, Pittsburgh, PA). DNA was extracted using the QIAGEN DNeasy Plant Mini Kit (QIAGEN Inc., Valencia, CA), and was stored at -20°C.

Two PCR protocols were used to characterize the samples. The first protocol is based on the use of primers designed by Smith and Stanosz (2006) to differentiate between *Diplodia pinea* and *Diplodia scrobiculata*. For the duration of this paper, it will be referred to as the Smith/Stanosz protocol. In this protocol, DNA from each sample was amplified in two separate PCR reactions. In the first reaction, DpF, a forward primer, and BotR, a reverse primer, produce a 700 base pair band if *D. pinea* is present. In the second reaction, DsF, a forward primer, and BotR, a reverse primer, produce a 700 base pair band if *D. scrobiculata* is present. Primer

sequences are presented in Table 3.1. Reactions were performed using PuReTaq Ready-To-Go PCR beads (GE Healthcare, formerly Amersham Biosciences, Piscataway, NJ). Per the manufacturer's instructions, the following were added to each 50 µL tube: 21 µL HPLC-grade H_20, 1 µL 20 µM forward primer (DpF or DsF), 1 µL 20 µM reverse primer (BotR), and 2µL of fungal DNA at a concentration between 10 and 25 ng/µL for a total reaction volume of 25µL. DNA was amplified either in an Eppendorf Mastercycler Gradient or an Eppendorf Mastercycler Personal thermocycler (Eppendorf North America Inc., New York, NY). The following thermal profile was used: 1 cycle of 5 min at 95°C followed by 30 cycles of 1 min at 95°C, 1 min at 53°C, 1 min at 72°C, followed by 1 cycle of 5 min at 72°C (Smith and Stanosz, 2006). The presence and size of amplicons was verified on 1.5 % certified molecular biology-grade agarose gels (BioRad Laboratories, Inc., Hercules, CA) run in Tris-Acetate-EDTA (TAE) buffer. Gels were run for 45 minutes at 100 volts.

Every sample that produced *Diplodia* sp. bands in the Smith/Stanosz protocol and the majority of those that did not, were subjected to a second protocol based on Jacobs and Rehner (1998). That protocol will be referred to as the ITS protocol for the duration of this paper. Nuclear rDNA internal transcribed spacers (ITS) 1 and 2 were amplified using universal primers ITS-5 and ITS-4. Primer sequences are presented in Table 3.1. Reactions were performed using PuReTaq Ready-To-Go PCR beads (GE Healthcare, formerly Amersham Biosciences, Piscataway, NJ), and per the manufacturer's instructions, the following were added to each 50 µL tube: 21 µL HPLC-grade H_20, 1 µL 20 µM forward primer (ITS-4), 1 µL 20 µM reverse primer (ITS-5), and 2µL of fungal DNA at a concentration between 10 and 25 ng/µL for a total reaction volume of 25µL. DNA was amplified either in an Eppendorf Mastercycler Gradient or an Eppendorf Mastercycler Personal thermocycler (Eppendorf North America Inc., New York,

NY). The following thermal profile was used: 1 cycle of 5 min at (94)°C followed by 35 cycles of 30 sec at (94)°C, 30 sec at (50)°C, 1 min at (72)°C, followed by 1 cycle of 10 min at 72°C (Jacob and Rehner, 1998). Samples were purified using a QIAquick PCR Purification Kit (QIAGEN Inc., Valencia, CA). Sequencing of the amplicons was completed by Integrated Biotech Laboratories (IBL) at the University of Georgia, Athens. Sequence results were read using Chromas Lite (v. 2.01; Technelysium Pty Ltd, www. technelysium.com.au). Sequences were identified using BLASTn analysis available on the website of the National Center for Biotechnology Information (NCBI). Multiple sequence alignment was performed using ClustalW (EMBL-EBI, Hinxton, UK). In that analysis, *Diplodia pinea* and *Diplodia scrobiculata* type cultures were included as references (obtained from Glen Stanosz, University of Wisconsin, Madison, under USDA APHIS PPQ Permit #P526P-06-00193). Frequency of recovery of *Diplodia sp.* and *Lasiodiplodia sp.* were analyzed using SAS Contingency Table Analysis (v. 8.02; SAS Institute, Cary, N.C.); Fisher's Exact Test two-sided *P*-values are reported unless otherwise noted.

RESULTS

Preliminary sampling during 2005 yielded 19 isolates having the appearance of *Diplodia* spp. The Smith/Stanosz method and the ITS protocol were performed on all 19. The Smith/Stanosz method characterized three of the collected isolates as *D. scrobiculata* (B2-S1, B2-S2, D3-S1) and one as *D. pinea* (E2-S3). All three isolates characterized as *D. scrobiculata* using the Smith/Stanosz method were identified as *Sphaeropsis sapinea* using the ITS protocol followed by BLAST analysis. The one isolate characterized as *D. pinea* by the Smith/Stanosz method was identified as *Botryosphaeria obtusa* using BLAST analysis. The remaining 15

isolates were identified as either *Lasiodiplodia theobromae* or its teleomorph, *Botryosphaeria rhodina,* when subjected to ITS sequencing (Table 3.2).

Although sampling was extensive during 2006, relatively few botryosphaeriaceous isolates were collected from the South Georgia sites: 76 from Fargo and 67 from Hoboken. The isolates were collected in nearly equal proportions from symptomatic shoots on diseased trees, asymptomatic shoots on diseased trees, and asymptomatic shoots on healthy trees neighboring diseased trees. Similarly, the age and location of the trees from which samples were collected did not seem to affect the number of isolates collected (Table 3.3). *Botryosphaeria spp*. and *Lasiodiplodia spp*. are, however, common inhabitants of pines, and both are morphologically similar to *Diplodia spp.* In fact, many of the isolates collected proved not to be *Diplodia spp.* when identified using molecular techniques.

DNA extracted from all 143 botryosphaeriaceous isolates collected from Fargo and Hoboken during 2006 was analyzed using the Smith/Stanosz method. Eleven samples were identified as *D. pinea*; two were identified as *D. scrobiculata*. Both of the *D. scrobiculata* samples were from 15-year-old symptomatic shoots on diseased trees at the Fargo location. Of the eleven *D. pinea* isolates identified, two were collected from asymptomatic shoots on diseased trees at the Fargo location; one tree was 15-years-old, the other was 5-years-old. The remaining nine *D. pinea* isolates came from the Hoboken location; one from a 15-year-old tree, five from 10-year-old trees, and three from 5-year-old trees. Four of the nine isolates were collected from symptomatic shoots on diseased trees, two from asymptomatic shoots on diseased trees, and three from asymptomatic shoots on trees neighboring diseased trees (Table 3.4).

Eleven of the 13 samples deemed *Diplodia spp*. by the Smith/Stanosz method were successfully analyzed using the ITS protocol. Of the two isolates identified as *D. scrobiculata*

using the Smith/Stanosz method, BLAST analyses characterized one (S-C5D) as *S. sapinea* and the other (S-E4D) as *D. scrobiculata*. Of the 11 isolates identified as *D. pinea* using the Smith/Stanosz method, BLAST analysis characterized seven as *Botryosphaeria obtusa* (S-B6H, V-C2H, V-D2D, V-F8, V-G10H, V-I4D, V-D1D), two as *S. sapinea* (V-D4, V-F9D), and two did not produce meaningful results (S-E5H, V-C8) (Table 3.4). *Botryosphaeria obtusa* is considered to be the teleomorph of *Diplodia spp*.. BLAST results of *B. obtusa* likely reflect a choice made by those making entries into the database to use the teleomorphic name rather than the anamorphic name. However, due to the recent division of *Diplodia spp.* into two species, *D. pinea* and *D. scrobiculata*, the use of the teleomorphic name in the past creates ambiguity for those using the database at present. Similarly, entries made in the past as *Sphaeropsis sapinea*, a previous anamorphic name for *Diplodia spp*., do not reflect the recent division of *Diplodia spp*. into two species and are, therefore, only somewhat informative.

In addition to the isolates deemed by the Smith/Stanosz method to be *D. pinea* or *D. scrobiculata*, the ITS protocol was employed and fragments sequenced for a sampling of 84 of the remaining 130 botryosphaeriaceous isolates collected from the Hoboken and Fargo locations during 2006 that had not produced a positive result using the Smith/Stanosz protocol. BLAST analysis suggested that 69 of these 84 samples were *Lasiodiplodia theobromae*. This identification was confirmed by performing multiple sequence analysis of ITS regions 1 and 2 of all 69 isolates. Of the remaining 15 isolates, one was identified as *Fusicoccum viticlavatum* and fourteen were either unreadable or did not produce meaningful results (Table 3.5).

ClustalW analyses were performed in an effort to elucidate the relationships among *Diplodia* type cultures originating from Wisconsin, *Diplodia spp*. isolates collected from south Georgia during this study, and *L. theobromae* isolates collected from south Georgia during this

study. In all analyses, ITS regions 1 and 2 were compared. In the first analysis (results not shown), 97 botryosphaeriaceous isolates collected from Georgia were compared to *Diplodia spp*. type cultures. Among the 97 Georgia isolates included in this study were the 4 isolates collected during 2005 and the 13 isolates collected during 2006 which were positively identified as *Diplodia spp*. using the Smith/Stanosz method. Also among those 97 Georgia isolates were the 69 isolates collected during 2006 which had not produced a positive result using the Smith/Stanosz method, but which were identified as *L. theobromae* based on BLAST analysis of ITS regions 1 and 2. The phylogram produced using ClustalW grouped all of the *Diplodia spp*. type cultures together. All four of the *D. scrobiculata* isolates collected from Georgia and seven of the 12 *D. pinea* isolates collected from Georgia grouped with those type cultures. The five remaining *D. pinea* isolates collected from Georgia grouped among the *L. theobromae* isolates collected from Georgia.

A second ClustalW analysis was conducted in which only 22 of the botryosphaeriaceous isolates collected from Georgia were compared to *Diplodia spp*. type cultures. These 22 isolates were a subset of isolates included in the previous, larger analysis. Among the 22 Georgia isolates included in this analysis were the 4 isolates collected during 2005 and the 13 isolates collected during 2006 which were positively identified as *Diplodia spp*. using the Smith/Stanosz method. Also included in the analysis were five of the 69 isolates collected during 2006 which had not produced a positive result using the Smith/Stanosz method, but which were identified as *L. theobromae* based on BLAST analysis of ITS regions 1 and 2. The phylogram produced using ClustalW was analogous to the phylogram discussed above. All of the *Diplodia* type cultures grouped together. All four of the *D. scrobiculata* isolates collected from Georgia and seven of the 12 *D. pinea* isolates collected from Georgia grouped with those type cultures. Of the five

remaining *D. pinea* isolates collected from Georgia, three grouped among the *L. theobromae* isolates collected from Georgia and two grouped between the *Diplodia* type cultures and the *L. theobromae* isolates collected from GA (Figure 3.1).

The age of shoots (5-, 10-, and 15-years-old) did not significantly impact the frequency at which *Diplodia sp*., as defined by the Smith/Stanosz method, or *L. theobromae*, as identified by the ITS protocol, were recovered. This finding held true when the three age groups were analyzed separately, ($P = 0.8644$ and $P = 0.9266$, respectively), when they were analyzed as young (5-years-old) or older (10- and 15-years-old) ($P = 0.7487$ and $P = 1.00$, respectively), and when they were analyzed as younger (5- and 10-years-old) or old (15-years-old) ($P = 0.7303$ and $P = 0.7397$, respectively) (Table 3.6). Similarly, the proximity of a shoot to a symptomatic shoot [symptomatic shoot on a symptomatic tree (D), asymptomatic shoot on a symptomatic tree (H), asymptomatic shoot on an asymptomatic neighboring tree (N)] did not significantly impact the frequency at which *Diplodia sp.* or *L. theobromae* were isolated, $P = 0.8073$ and $P = 0.8029$, respectively. Nor did the health of the tree from which the shoot was collected [symptomatic (D) and asymptomatic (H) shoots from symptomatic trees (D_t), neighboring (N) shoots from asymptomatic neighboring trees (N_t)] significantly impact the frequency at which *Diplodia sp.* or *L. theobromae* were isolated, $P = 0.7487$ and $P = 1.00$, respectively. Finally, neither *Diplodia* spp. nor *L. theobromae* were recovered any more frequently from symptomatic shoots than from asymptomatic shoots [symptomatic (D) versus asymptomatic (H + N)] ($P = 0.5399$ and $P = 0.5429$, respectively) (Table 3.7).

DISCUSSION

Diplodia pinea and *D. scrobiculata* have been found on slash pines in southern Georgia. *D. scrobiculata* was recovered only from symptomatic shoots on 10- and 15-year-old trees, while

D. pinea was recovered from symptomatic and asymptomatic shoots on 5-, 10-, and 15-year-old trees. Studies have suggested that older Scots, Austrian, and Mexican weeping pine are more frequently infected by *Diplodia sp*. than their younger counterparts (Flowers et al., 2001, Smith et al., 2002). In this study, however, no significant relationship was found between the age of a tree and the frequency with which the pathogen was recovered from it. Previous reports also have shown that the shoots of Scots and Austrian pine exhibit higher rates of latent infection when on trees exhibiting greater than 20% of active shoot dieback (Flowers et al., 2001). In this study, however, no significant relationship was found between infection of a visibly healthy shoot and its proximity to a diseased tree. Finally, no relationship was found between the appearance of tip dieback symptoms on a shoot and the presence of *D. pinea* or *D. scrobiculata* on that shoot. These results may have been skewed by the low rate at which *Diplodia* spp. were recovered from Georgia pines.

Lasiodiplodia theobromae was recovered more frequently from pines in this study than were either *Diplodia* species. It was found on all three ages of trees surveyed, as well as on both asymptomatic and symptomatic trees. However, no significant relationship was found between the age of a shoot and the frequency of recovery or between the health of a shoot and the frequency with which it was recovered. Based on data presented here, *L. theobromae* does not appear to be the cause of the observed tip dieback.

The overall frequency at which *Diplodia* spp. were recovered was low. Of the 143 botryosphaeriaceous isolates recovered during 2006, only 13 proved to be *Diplodia* sp. (5.3% for Fargo; 13.4% for Hoboken). It is feasible, however, that the pathogen was present more or even much more frequently than the low numbers in this study would suggest. Had methods been in place allowing shoots to be screened directly for the pathogen, rather than relying on isolation of

the pathogen from the plant's tissue, it is possible that more infections would have been detected and that statistical results would have been different. Even so, wounding events have been shown to increase susceptibility of some pines to infection by *Diplodia sp.* (Blodgett and Stanosz, 1997; Blodgett and Bonello, 2003); and in many pines, drought stress has been shown to worsen the effects of the pathogen (Bachi and Peterson, 1985; Johnson et al., 1997; Blodgett et al., 1997; Paoletti et al., 2001). Weather events could create an environment in which even the low frequency of detected infections could spawn problems for southern Georgia slash pine growers.

Multiple sequence analysis of ITS regions 1 and 2 demonstrated close relationships between 12 of 17 *Diplodia spp*. isolates collected from Georgia during 2005 and 2006. These 10 isolates were very similar to known *Diplodia* sp. type cultures. Furthermore, they produced expected results when subjected to ITS sequencing and BLAST analysis. The 5 remaining *Diplodia spp.* isolates, all identified as *D. pinea* by the Smith/Stanosz method, grouped either among *L. theobromae* isolates collected from Georgia or between the *Diplodia* type cultures and the *L. theobromae* isolates collected from GA. Two of these *D. pinea* isolates had not produced meaningful results when subjected to BLAST analysis of ITS regions 1 and 2; three of them were identified either as *B. obtusa* or *S. sapinea*.

This lack of agreement among methods used to identify botryosphaeriaceous isolates in this study is troubling. The work of Jacobs and Rehner (1998) suggests that ITS regions 1 and 2 of *L. theobromae* and *Diplodia spp*. are different enough to allow for reliable molecular distinction between the two. It seems that either that assessment was inaccurate, or the Smith/Stanosz primers DpF and BotR are mistakenly amplifying isolates of *L. theobromae*, which is unlikely given that the specificity of these primers was tested against *Botryosphaeria rhodina*, the teleomorph of *L. theobromae*. It appears more likely that due to the close

relationship between *Diplodia spp*. and *L. theobromae*, comparison of ITS regions is not a sufficient tool for definitive identification of these species. Further analysis is needed in order to confirm the true identity of those five needs isolates deemed *D. pinea* by the Smith/Stanosz method, but that did not group along with type cultures of *Diplodia* during multiple sequence analysis of ITS regions 1 and 2. It also will be important to perform inoculation studies in order to confirm that the *Diplodia* s*pp*. present in southern Georgia pine plantations are, in fact, capable of causing disease on pines grown in Georgia.

Table 3.1. Nucleotide sequences of primers used in this study

Primer*	Nucleotide sequence (5'—3')
forward primer DpF	CTTATATATCAAACTATGCTTTG-TA
forward primer DsF	CTTATATATCAAACTAATGTTTG-CA
reverse primer BotR	GCTTACACTTTCATTTATAGACC
forward primer ITS-5	GGAAGTAAAAGTCGTAACAAGG
reverse primer ITS-4	TCCTCCGCTTATTGATATGC

* Sequences were synthesized at the Molecular Genetics Instrumentation Facility, The University of Georgia, Athens.

Table 3.2. Molecular characterization of 19 botryosphaeriaceous isolates collected from southern Georgia during 2005.

Isolate	SSU rDNA[1]	ITS sequencing[2]
E2-S3	*D. pinea*	*Botryosphaeria obtusa*
B2-S2 (#1)	*D. scrobiculata*	*Sphaeropsis sapinea*
B2-S1	*D. scrobiculata*	*Sphaeropsis sapinea*
D3-S1 (#1)	*D. scrobiculata*	*Sphaeropsis sapinea*
A4-S2		*Lasiodiplodia theobromae*
C1-F1		*Lasiodiplodia theobromae*
D3-S4		*Lasiodiplodia theobromae*
D1-F1		*Lasiodiplodia theobromae*
A2-F1		*Lasiodiplodia theobromae*
D2-S3		*Lasiodiplodia theobromae*
D2-S2		*Lasiodiplodia theobromae*
E1-S1		*Lasiodiplodia theobromae*
A1-S1		*Lasiodiplodia theobromae*
B3-S1		*Lasiodiplodia theobromae*
B3-S5		*Lasiodiplodia theobromae*
D3-S6		*Lasiodiplodia theobromae*
E2-S1		*Lasiodiplodia theobromae*
F1-3e		*Lasiodiplodia theobromae*
D2-S2		*Lasiodiplodia theobromae*

[1] Based upon SSU rDNA method described by Smith and Stanosz, 2006.
[2] BLAST results of ITS 1 and 2 region sequences based upon Jacobs and Rehner, 1998.

Table 3.3. Number of botryosphaeriaceous isolates obtained from symptomatic shoots (D), asymptomatic shoots (H), and asymptomatic shoots on neighboring healthy trees (N) in a 2006 survey of pine plantations in Fargo and Hoboken, Georgia

FARGO, GA March 2006					**HOBOKEN, GA April 2006**			
AREA 1	D[1]	H[2]	N[3]		AREA 1	D[1]	H[2]	N[3]
5 yr	1	3	2		5 yr	1	4	2
10 yr	3	1	2		10 yr	1	2	3
15 yr	4	3	2		15 yr	3	1	2
SUM	8	7	6		SUM	5	7	7
AREA 2	D	H	N		AREA 2	D	H	N
5 yr	2	1	3		5 yr	0	5	5
10 yr	4	3	2		10 yr	3	3	3
15 yr	3	3	3		15 yr	2	4	2
SUM	9	7	8		SUM	5	12	10
AREA 3	D	H	N		AREA 3	D	H	N
5 yr	3	1	3		5 yr	3	6	0
10 yr	5	4	4		10 yr	2	1	0
15 yr	4	5	2		15 yr	3	3	3
SUM	12	10	9		SUM	8	10	3
TOTAL	29	24	23		TOTAL	18	29	20

[1] D = symptomatic shoot on diseased trees
[2] H = asymptomatic shoot on diseased trees
[3] N = asymptomatic shoot on healthy tree neighboring a diseased tree

Table 3.4. Thirteen isolates collected from Fargo and Hoboken, Georgia during 2006 that were identified as *D. pinea* or *D. scrobiculata* based on SSU rDNA analysis

Isolate	Collection Site	Age of tree (yr)	D/H/N[1]	SSU rRNA[2]	ITS sequencing[3]
S-C5D	Fargo, GA	15	D	*D. scrobiculata*	*Sphaeropsis sapinea*
S-B6H	Fargo, GA	5	H	*D. pinea*	*Botryosphaeria obtusa*
S-E5H	Fargo, GA	15	H	*D. pinea*	No meaningful result
S-E4D	Fargo, GA	15	D	*D. scrobiculata*	*Diplodia scrobiculata*
V-C2H	Hoboken, GA	10	H	*D. pinea*	*Botryosphaeria obtusa*
V-C8	Hoboken, GA	10	N	*D. pinea*	No meaningful result
V-D1D	Hoboken, GA	10	D	*D. pinea*	*Botryosphaeria obtusa*
V-D2D	Hoboken, GA	10	D	*D. pinea*	*Sphaeropsis sapinea*
V-D4	Hoboken, GA	10	N	*D. pinea*	*Sphaeropsis sapinea*
V-F9D	Hoboken, GA	5	D	*D. pinea*	*Sphaeropsis sapinea*
V-G10H	Hoboken, GA	5	H	*D. pinea*	*Botryosphaeria obtusa*
V-I4D	Hoboken, GA	15	D	*D. pinea*	*Botryosphaeria obtusa*
V-F8	Hoboken, GA	5	N	*D. pinea*	*Botryosphaeria obtusa*

[1] Isolates were collected either from a symptomatic shoot (D), from an asymptomatic shoot on a diseased tree (H), or from an asymptomatic shoot on a tree neighboring a diseased tree (N).
[2] SSU rDNA results obtained using method described by Smith and Stanosz, 2006
[3] ITS 1 and 2 sequence results using BLAST analysis.

Table 3.5. Frequency of recovery of non-*Diplodia*, botryosphaeriaceous isolates from slash pine shoots collected in two southern Georgia pine plantations during 2006.

Analysis Result[1]	Isolates[2]
Lasiodiplodia theobromae	69
Fusicoccum viticlavatum	1
No significant result	14

[1] Isolates were identified using ITS sequencing and BLAST analysis followed by multiple sequence analysis using ClustalW.

[2] Number of isolates recovered on potato dextrose agar from discolored pith tissue on symptomatic and asymptomatic slash pine shoots.

Table 3.6. Relative frequency[1] at which *Diplodia sp*. and *Lasiodiplodia theobromae* were isolated from southern Georgia pine plantations during 2006 according to tree age.

Isolate	Three age groups (yrs)			Two age groups (yrs)			
Diplodia sp.	5	10	15	Older (>5)[2]	Young (5)[2]	Old (15)[3]	Younger (<15)[3]
Diplodia sp.	23.1	46.2	30.8	76.9	23.1	30.8	69.2
Non-*Diplodia sp.*	30.4	45.6	24.1	69.6	30.4	24.1	76.0
P-value[4]	0.8644			0.7487		0.7303	
L. theobromae							
L. theobromae	24.6	49.3	26.1	75.4	24.6	26.1	73.9
non-*L. theobromae*	23.1	46.2	30.8	76.9	23.1	30.8	69.2
P-value[4]	0.9266			1.0		0.7397	

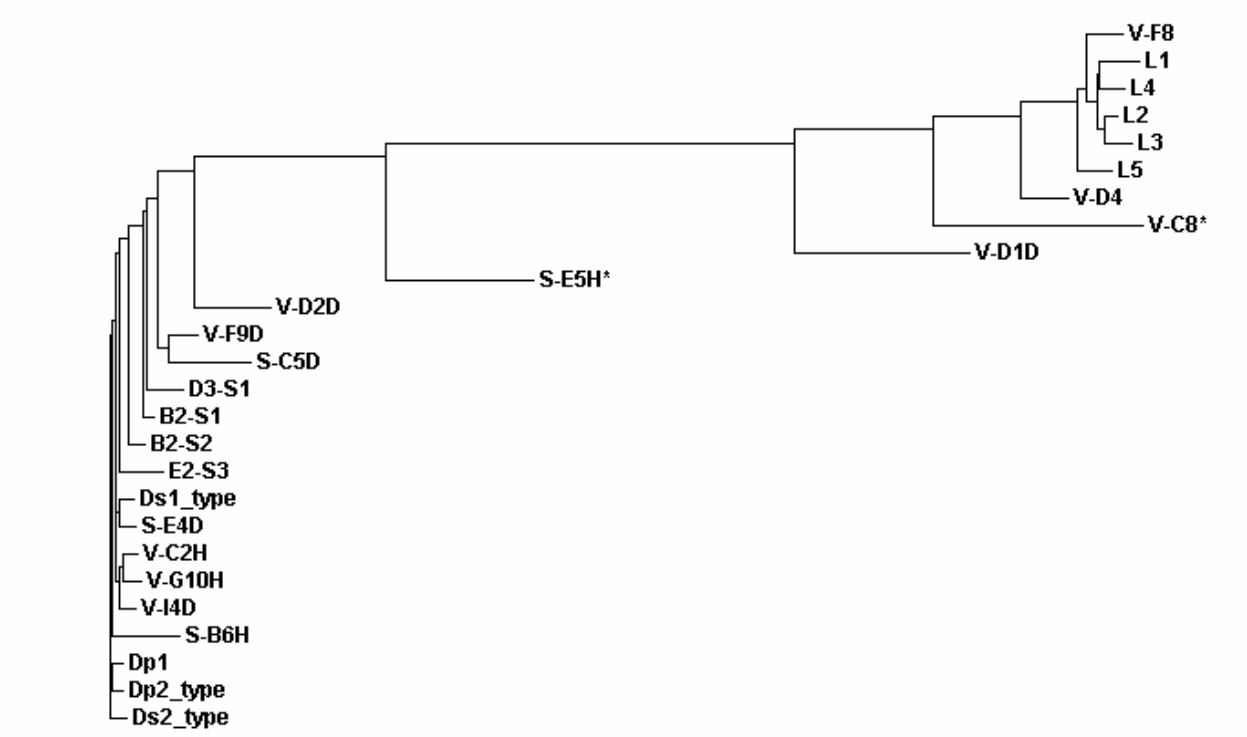
[1] Frequencies are reported as percentages for each isolate-type within each of three tree age classifications.
[2] Older = 10- and 15-yrs-old; Young = 5-yrs-old
[3] Old = 15 yrs old; Younger = 5- and 10-yrs old
[4] Data was analyzed by SAS Contingency Table Analysis; Fisher's Exact Test two-sided *P*-values are reported.

Table 3.7. Relative frequency[1] at which *Diplodia sp*. and *Lasiodiplodia theobromae* were isolated from southern Georgia pine plantations during 2006 according to shoot source.

Isolate	Proximity of shoot to disease[2]			Health of tree[3]		Symptomatic vs. Asymptomatic[4]	
Diplodia sp.	D	H	N	D_t	N_t	S	A
Diplodia sp.	46.2	30.8	23.1	76.9	23.1	46.2	53.9
Non-*Diplodia sp.*	35.4	87.1	88.9	69.6	30.4	35.44	64.6
P-value[5]	0.8073			0.7487		0.5399	
L. theobromae							
L. theobromae	36.2	36.2	27.5	72.5	27.5	63.8	36.2
non-*L. theobromae*	46.2	30.1	23.1	76.9	23.08	46.2	53.9
P-value[5]	0.8029			1.00		0.5429	

[1] Frequencies are reported as percentages for each isolate-type within each of three shoot source classifications.
[2] D = symptomatic shoot on diseased tree; H = asymptomatic shoot on a diseased tree; N = asymptomatic shoot on a tree neighboring a diseased tree
[3] D_t = any shoot from diseased tree (D and H); N_t = asymptomatic shoot from tree neighboring diseased tree (N)
[4] S = symptomatic shoots (D); A = asymptomatic shoots (H + N).
[5] Data was analyzed by SAS Contingency Table Analysis; Fisher's Exact Test *P*-values are reported unless.

Figure 3.1. Phylogram representing sequence similarities between ITS regions 1 and 2 of two *D. pinea* type cultures[1], two *D. scrobiculata* type cultures[2], and 22 botryosphaeriaceous isolates collected from GA during 2005 and 2006: 5 *D. scrobiculata* isolates[3], 12 *D. pinea* isolates[4], and 5 *L. theobromae* isolates[5]



[1] *D. pinea* type cultures: Dp1_type, Dp2_type
[2] *D. scrobiculata* type cultures: Ds1_type, Ds2_type
[3] *D. scrobiculata* isolates from Georgia: B2-S1, B2-S2, D3-S1, S-C5D, S-E4D
[4] *D. pinea* isolates from Georgia: E2-S3, S-B6H, V-F9D, V-C2H, V-D2D, V-F8, V-G10H, V-I4D, V-D4, V-D1D, V-C8*, S-E5H*
[5] *L. theobromae* isolates from Georgia: L1, L2, L3, L4, L5
*Isolates identified as *D. pinea* using the Smith/Stanosz method, but that did not produce meaningful results based on ITS sequencing and BLAST analysis

CHAPTER 4

PATHOGENICITY OF *DIPLODIA PINEA*, *DIPLODIA SCROBICULATA* AND LASIODIPLODIA *THEOBROMAE* ON FOUR SOUTEHRN PINE SPECIES

INTRODUCTION

Diplodia pinea (Desm.) J. Kickx (formerly *Sphaeropsis sapinea* (Fr.) Dyko & B. Sutton A-morphotype) and *Diplodia scrobiculata* J. de Wet, B. Slippers & M.J. Wingfield (formerly *Sphaeropsis sapinea* (Fr.) Dyko & B. Sutton B-morphotype) are closely related fungal species that cause disease on a myriad of pine species. *Diplodia spp.* cause symptoms including shoot blight, stem canker, crown wilt and collar rot. Though capable of gaining entry into pines via elongating needles, wound sites also provide an infection court (Brookhouser and Peterson, 1971; Peterson, 1977; Smith et al., 2002). Initial infection of a mature tree may result in the dieback of only a few shoots. However, after years of infection, large branches or entire trees may be lost (Flowers et al., 2001). The pathogen can over-winter as pycnidia in or on the previous year's diseased shoots, or as an endophyte of mature pinecones or shoots (Palmer et al., 1988; Smith et al., 1996; Flowers et al., 2001).

Prior to 1995, *S. sapinea* isolates were characterized based on morphology (Wang et al., 1985; Palmer et al., 1987). The A-morphotype produced fluffy, white to gray-green mycelia, with smooth conidia ranging in size from 34 x 13 µm to 39 x 13 µm. The B-morphotype produced white to black mycelia that were tightly appressed to the agar with pitted conidia averaging 34 x 12 µm. During the 1990s, molecular techniques were employed to describe three morphotypes (A, B, and C) of *S. sapinea* (Smith and Stanosz, 1995; Stanosz et al., 1999; de Wet et al., 2000; Burgess et al., 2001). By 2003, Simple Sequence Repeat (SSR) PCR was used to

argue for the changing of the genus designation from *Sphaeropsis* to *Diplodia*. The A- and C-morphotypes became *Diplodia pinea* and the B-morphotype became *D. scrobiculata* (Burgess et al., 2001; de Wet et al., 2003)

Smith and Stanosz (2006) developed a pair of simple PCR-based tests to identify *D. pinea* and *D. scrobiculata.* Three primers (two forward, one reverse) were developed that targeted the mitochondrial small subunit ribosome gene. Primer pair DpF/BotR produces a 700 base pair band in the presence of *D. pinea* DNA, but no band in the presence of *D. scrobiculata*; primer pair DsF/BotR produces a 700 base pair band in the presence of *D. scrobiculata* DNA, but no band in the presence of *D. pinea*.

Nuclear rDNA Internal Transcribed Spacers (ITS) also have been used to compare various anamorphs of *Botryosphaeria sp.*. ITS regions 1 and 2 could differentiate between anamorphs of *Botryosphaeria sp*., including *Fusicoccum spp*., *Sphaeropsis sapinea*, and *Lasiodiplodia theobromae*. (Jacobs and Rehner, 1998).

Jack (*Pinus banksiana*), red (*P. resinosa*), Austrian (*P. nigra*), Scots (*P. sylvestris*), Aleppo (*P. halepensis*), Monterey (*P. radiata*), and Mexican weeping (*P. patula*) pines are all susceptible to infection by *Diplodia* sp. (Blodgett and Bonello*,* 2003; Blodgett and Stanosz*,* 1997; Chou, 1987; Flowers et al*.,* 2001; Paoletti et al., *2001;* Smith et al. 2002*).* Symptom development and severity, however, depend on factors such as pathogen species, host pine species, wounding events, and water stress (Bachi and Peterson, 1985; Blodgett and Bonello, 2003; Blodgett and Stanosz, 1997; Johnson et al., 1997; Paoletti et al., 2001; Smith et al., 2002). These factors must be considered on a case by case basis in order to fully understand the threat posed to a given pine species by *D. pinea* or *D. scrobiculata*.

Blodgett and Stanosz (1997) found that red pine seedlings inoculated with isolates of *D. pinea* showed near 100% infection whether wounded prior to inoculation or not. However, the absence of wounding significantly lowered the susceptibility of jack pine to *D. pinea* (from 99% to 42%) and to *D. scrobiculata* (from 53% to 6%) within the same study. Regardless of wounding, both jack and red pine exhibited higher rates of infection when inoculated with *D. pinea* as opposed to *D. scrobiculata*, although *D. scrobiculata* was capable of infecting both pine species (Blodgett and Stanosz, 1997).

Blodgett, Kruger & Stanosz (1997) also found that red pine seedlings inoculated with *D. pinea* exhibited significantly more dieback (measured in cm from inoculation site) when subjected to water stress than when provided with ample water. No significant difference was seen, however, between water stressed and non-stressed seedlings inoculated with *D. scrobiculata* isolates. The authors noted that the low overall disease caused by *D. scrobiculata* in this study may have contributed to the lack of statistical significance (Blodgett, Kruger & Stanosz, 1997). Similarly, *Diplodia* sp. disease severity was directly proportional to the extent of water stress imposed on Aleppo pine seedlings (Paoletti et al., 2001). Although all seedlings inoculated with *Diplodia sp.* developed cankers, the longest cankers were seen on seedlings that were deprived of water, regardless of whether this deprivation occurred before or after inoculation.

Austrian pine exhibits varying susceptibility to *Diplodia sp.* depending on water stress and plant part inoculated. Austrian pine seedlings inoculated with *D. pinea* developed longer stem cankers if, following inoculation, they were subjected to water stress (Bachi and Peterson, 1985). Blodgett and Bonello (2003) demonstrated that stems of Austrian pine inoculated with either *D. pinea* or *D. scrobiculata* exhibited 100% infection. However, inoculation of wounded

shoots resulted in 100% infection when inoculated with *D. pinea*, but only 60% infection when inoculated with *D. scrobiculata*. Of interest was that pathogens were recovered from 100% of inoculated trees regardless of species, symptom severity, or wound site. This demonstrates the ability of *Diplodia sp.,* particularly *D. scrobiculata,* to create asymptomatic infections in Austrian pine (Blodgett and Bonello, 2003).

In another experiment, asymptomatic 3-year-old Austrian Pines were inoculated with one of eleven isolates of *Diplodia sp.* originating from asymptomatic and symptomatic Scots and Austrian pines. Of the 66 inoculated seedlings, 17 did not develop tip blight symptoms. Ten of the 17 asymptomatic seedlings were dissected and cultured to ascertain if they were latently infected. *Diplodia sp.* was recovered in nine of ten seedlings. This study demonstrates that *Diplodia sp.* can be a latent pathogen of Scots and Austrian pine; and that, under certain conditions, a portion of those isolates can switch from a latent pathogen to active pathogen and presumably vice versa (Flowers et al., 2001).

Studies in which plant tissues are wounded prior to inoculation are meant to mimic natural environment damage. In South Africa, summer hailstorms have been associated with an increased incidence of *Diplodia sp.* dieback on Mexican weeping pine and Monterey pine plantations (Smith et al., 2002).

Lasiodiplodia theobromae, another anamorph of *Botryosphaeria* has often been dismissed as a legitimate pine pathogen. However, it has been cited as a possible cause for tip dieback of slash and loblolly pines in the eastern U.S. (South et al., 2002; Rowan, 1982). Pathogencity of *L. theobromae* to cause dieback in 2-year old loblolly pines has been demonstrated (Jolley & Hedden, 2001). It also constituted a large percentage (48.95%) of the

botryosphaeriaceous fungi recovered from surveys within two southern Georgia slash pine plantations (see Chapter 3).

Historically, loblolly (*P. taeda*) and slash (*P. elliottii*) pine were considered relatively resistant to aerial infection by *Diplodia spp*. (Smith et al., 1996; Smith et al., 2002; South et al., 2002). However, *Diplodia spp.* has been shown to be a major factor of tip dieback of slash and loblolly pine grown in the Hubei, Hunan, and Jiangsu provinces of China (Su et al., 1991; Yang et al., 1988; Shen, B. K., 1990). In Hawaii, 20-year-old slash, and loblolly pines developed damaging *Diplodia spp.* infections after enduring a 3-4 year drought (Bega et al., 1978). Though not the intent of the study, Smith et al. (2002) also found that loblolly and slash pines, included in their study as positive controls for tolerance, were susceptible, in some cases, to infection by *Diplodia sp*..

Several pine species are quite important to the economy and history of the southeastern United States; among them are loblolly, slash, shortleaf (*P. echinata*), and Virginia (*P. virginiana*) pine. Loblolly and slash pines are particularly valuable as pole, lumber, timber, and pulp wood (USDA Forestry Service: *P. elliottii*; USDA Forestry Service: *P. taeda*;). Shortleaf pine is used for subflooring, interior finishes, pulpwood, and as a landscape tree. In nature, it is particularly important to wildlife (USDA Forestry Service: *P. echinata*). Virginia Pine is used for pulp and firewood and shows particular promise as a tree capable of growing on strip-mined areas during reclamation projects (USDA Forestry Service: *P. virginiana*).

According to the USDA Forest Service, loblolly and shortleaf pines provided 56% of Georgia's total softwood output in 2003 while longleaf and slash pines accounted for 39%. Softwood is responsible for approximately 75% of pulpwood, saw-log, and veneer-log production in Georgia; these three products make up 94% of total roundwood production in the

state (Johnson and Wells, 2005). IMPLAN models have estimated that, in 2004, forestry created direct impacts in Georgia responsible for the employment of over 67,000 people who were paid a total of over $3 billion to produce over $14 billion worth of product (Riall, 2006). When multiplier effects were included, total economic impact of forestry on Georgia's economy were increased to over 144,000 people paid approximately $6.3 billion to produce more than $22 billion worth of product. It was estimated that the forestry industry contributed $155 million in net revenue to the state's government (Riall, 2006).

Based on the importance of slash, loblolly, shortleaf, and Virginia pines to Georgia's economy, the ability of *Diplodia spp.* to cause substantial damage to other pine species (Bega et al., 1978; Blodgett and Stanosz, 1997; Chou., 1987; Palmer, 1991),previous studies which have found this pathogen on pines in Georgia (Stanosz et al., 1999), and sampling done prior to this experiment which found *D. pinea* and *D. scrobiculata* in two South Georgia pine plantations (Chapter 3), it is important that the susceptibilities of these four important pine species to *D. pinea* and *D. scrobiculata* be evaluated. In this study, slash, loblolly, shortleaf, and Virginia pine seedlings were inoculated with isolates of *D. pinea, D. scrobiculata, and L. theobromae* in an effort to assess isolate pathogenicity and pine species susceptibility.

MATERIALS AND METHODS

Ten *D. pinea, D. scrobiculata*, and *L. theobromae* isolates collected in Georgia from symptomatic slash and loblolly pine shoots in 2005 were used in this study (Table 4.1). Isolates were identified using a PCR-based method described by Smith and Stanosz (2006), as well as BLAST analysis of ITS regions 1 and 2 (Jacobs and Rehner, 1998). As controls, two *D. pinea* (A1, A2) and two *D. scrobiculata* (B1, B2) type cultures (obtained from Glen Stanosz,

University of Wisconsin, Madison, under USDA APHIS PPQ Permit #P526P-06-00193) were also included.

To obtain DNA from the isolates, a small amount of mycelia was transferred from PDA culture to 1.5 mL eppendorf tubes (Sarstedt, Newton, NC) containing potato dextrose broth (PDB) (Difco Laboratories, Sparks, MD). Care was taken not to allow any agar into the broth. The broth culture was grown for 2-3 weeks. The resulting mycelial mass was transferred to a 2.0 mL bead beating tube (BioSpec Products, Inc., Bartlesville, OK) along with two 5-mm solid glass beads (Fisher Scientific, Pittsburgh, PA). DNA was extracted according to the protocol outlined in the QIAGEN DNeasy Plant Mini Kit (QIAGEN Inc., Valencia, CA), and was stored at -20°C.

The first protocol used to identify isolates was based on the work of Smith and Stanosz (2006). It uses primers designed to differentiate between *Diplodia pinea* and *D. scrobiculata*. For the duration of this paper, it will be referred to as the Smith/Stanosz protocol. In this protocol, DNA from each sample was amplified in two different PCR reactions. In the first reaction, DpF, a forward primer, and BotR, a reverse primer, produce a 700 base pair band if *D. pinea* is present. In the second reaction, DsF, a forward primer, and BotR, a reverse primer, produce a 700 base pair band if *D. scrobiculata* is present. Primer sequences can be found in Table 4.2. Reactions were performed using PuReTaq Ready-To-Go PCR beads (GE Healthcare, formerly Amersham Biosciences, Piscataway, NJ). Per the manufacturer's instructions, the following were added to each 50 µL tube: 21 µL HPLC-grade H_20, 1 µL 20 µM forward primer (DpF or DsF), 1 µL 20 µM reverse primer (BotR), and 2µL of fungal DNA at a concentration between 10 and 25 ng/µL for a total reaction volume of 25µL. DNA was amplified either in an Eppendorf Mastercycler Gradient or an Eppendorf Mastercycler Personal thermocycler (Eppendorf North

America Inc., New York, NY). The following thermal profile was used: 1 cycle of 5 min at 95°C followed by 30 cycles of 1 min at 95°C, 1 min at 53°C, 1 min at 72°C, followed by 1 cycle of 5 min at 72°C (Smith and Stanosz, 2006). The presence and size of amplicons was verified on 1.5 % certified molecular biology-grade agarose gels (BioRad Laboratories, Inc., Hercules, CA) run in Tris-Acetate-EDTA (TAE) buffer. Gels were run for 45 minutes at 100 volts.

The second protocol used to identify isolates was based on the on the work of Jacobs and Rehner (1998). For the duration of this paper, it will be referred to as the ITS protocol. Nuclear rDNA internal transcribed spacers (ITS) 1 and 2 were amplified using universal primers ITS-5 and ITS-4. Primer sequences can be found in Table 4.2. Reactions were performed using PuReTaq Ready-To-Go PCR beads (GE Healthcare, formerly Amersham Biosciences, Piscataway, NJ). To each 50 µL tube was added: 21 µL HPLC-grade H_20, 1 µL 20 µM forward between 10 and 25 ng/µL for a total reaction volume of 25 µL. DNA was amplified either in an Eppendorf Mastercycler Gradient or an Eppendorf Mastercycler Personal thermocycler (Eppendorf North America Inc., New York, NY) using a thermal profile of: 1 cycle of 5 min at (94)°C followed by 35 cycles of 30 sec at (94)°C, 30 sec at (50)°C, 1 min at (72)°C, followed by 1 cycle of 10 min at 72°C (Jacob and Rehner, 1998). Samples were purified using a QIAquick PCR Purification Kit (QIAGEN Inc., Valencia, CA). Sequencing of the amplicons was completed by Integrated Biotech Laboratories (IBL) at the University of Georgia, Athens. Sequence results were read using Chromas Lite (v. 2.01; Technelysium Pty Ltd, www. technelysium.com.au). Sequences were identified using BLASTn analysis available on the website of the National Center for Biotechnology Information (NCBI).

A monoconidial culture of each isolate was obtained by transferring mycelia from each isolate onto water agar plates (20 g agar/L) (Difco Laboratories, Sparks, MD) containing a 2.5-

cm section of an autoclaved slash pine needle slightly embedded into the medium. Plates were incubated at room temperature and subjected to 12 hours of light followed by 12 hours of dark. After 2 weeks, mature pycnidia developed on the pine needle. A single pycnidium was removed from each culture and placed onto a clean glass slide. A drop of sterile water was added and a sterile razor was used to rupture the pycnidium. Using a sterile loop, the contents were streaked onto potato dextrose agar (PDA) (Difco Laboratories, Sparks, MD). After several days, individual germinating spores were transferred onto fresh PDA.

Four pine species common to Georgia; slash (*P. elliottii*), loblolly (*P. taeda*), shortleaf (*P. echinata*), and Virginia (*P. virginiana*) were obtained from the Georgia Forestry Commission seedling nursery in Byromville, GA. In February 2006, 1-yr-old seedlings of each pine species were planted into 25-cm x 5.5-cm cone containers (Stuewe and Sons, Inc., Corvallis, OR) containing ProMix BX General Purpose Growing Mix (Premier Horticulture, Quakertown , PA), and irrigated regularly throughout the spring and summer months. In early October 2006, 240 seedlings of each species were selected to be used in the study. Half of those trees selected (120 of each species) remained in the greenhouse (A), and the other half were transferred to a second greenhouse (B) approximately 2 miles away. Seedlings in greenhouse A were inoculated during the second week of October 2006. Seedlings in Greenhouse B were inoculated during the third week of October 2006. Following inoculation, all seedlings were irrigated once per week to induce mild drought stress.

Inoculations were performed using a split-plot design in which isolate was the main plot factor and pine species was the sub-plot factor. Eight replications of each isolate and pine species combination were performed in each of the two greenhouses. Each replication included 15 groups of four single-plant pine seedlings (one of each of the four pine species). Each group

of 15 was inoculated with a different isolate; the order in which isolates were used was chosen at random within each replication. The order in which seedlings were inoculated was chosen at random within each sub-plot.

One-week-old cultures of each isolate on PDA were used as inoculum. Non-colonized PDA was used as a negative control. In preparation for inoculation, a mark was made on each seedling 8-cm from the soil line. Immediately preceding inoculation, a sterile 3-mm cork borer was lightly pressed into the seedling at that mark to remove a disc of outer bark. A 3-mm agar plug of the chosen isolate was placed mycelia side down atop the wound and held securely in place with parafilm. Parafilm was not removed until the end of the study. Seven weeks after inoculation, seedlings were clipped at the soil line, bagged individually, and stored at 8°C until processed in the laboratory. Stem diameter at the point of inoculation and the total length of pith discoloration above and below the site of inoculation was measured using a digital micrometer.

One replication from each experiment was selected in an effort to complete Koch's postulates. For seedlings within those replications, the outer bark was removed to expose any darkening of pith tissue. Five tissue pieces measuring approximately 3 x 3-mm were obtained for each sample. Each piece of tissue was surface sterilized in succession in 95% ethanol for 10 seconds, 10% Clorox for 30-60 seconds, and sterile de-ionized water for 30-60 seconds. The tissue was allowed to dry on sterile paper towels before being plated onto unamended PDA. Mycelia having a botryosphaeriaceous appearance were transferred to fresh PDA until pure cultures were obtained. Cultures were grouped according to isolate and replication. Two pure cultures from each group were transferred to 1.5 mL eppendorf tubes (Sarstedt, Newton, NC) containing potato dextrose broth (PDB) (Difco Laboratories, Sparks, MD), and allowed to grow for 2-3 weeks. DNA was extracted and processed as previously described.

Results were analyzed using SAS two way analysis of variance (ANOVA) for split-plot designs (v. 8.02; SAS Institute, Cary, N.C). It was performed separately on data from each greenhouse study. Means were separated using Tukey's Studentized Range Test (P=0.05) in which canker length was the dependent variable.

RESULTS

In all of the analyses, a decision was made to assign a canker length value of 200-mm to all seedlings which died during the experiment. This decision was based on the fact that 200-mm was close to the upper limit of canker lengths observed in seedlings that were visibly severely diseased, though still living, at the end of the 7-week period.

Two way analysis of variance (ANOVA) for split-plot was performed separately on data from each greenhouse study; canker length was the dependent variable, the main plot was isolate, the sub-plot was pine species, and the replications were blocks. For both of the experiments, the *P*-values for model (>0.0001, <0.0001), pine (>0.0001, <0.0001), and isolate*pine (>0.0001, 0.0042) were significant. Based on proven similarities between the two experiments, the data sets were combined for further analysis.

A one way ANOVA was performed on the combined data for each of the isolates used to inoculate the pines to determine which of the isolates produced different results on different pines. Canker length was the dependent variable. Only eight of 14 isolates produced models that were valid. That is, eight of the isolates behaved differently on the four pines while six isolates and the negative control did not. The six isolates producing cankers that were not significantly different among the pine species were: A4-S2 (*L. theobromae*), D2-S2 (*L. theobromae*), D2-S3 (*L. theobromae*), A2-F1 (*L. theobromae*), A1 (*D. pinea*), and E2-S3 (*D. pinea*) (Table 4.3).

Isolate A1 (*D. pinea*) was equally pathogenic on all four pine species; whereas, the other five isolates, appear to be non-pathogenic.

For the eight isolates producing significant models, Tukey's Studentized Range (HSD) Test was performed to further characterize the relative pathogenicities of a given isolate on each pine species. Stem cankers formed by isolate B1 (*D. scrobiculata*), B2-S2 (*D. scrobiculata*), S-C5D (*D. scrobiculata*), and D3-S4 were significantly longer on slash and loblolly pine than on shortleaf and Virginia pine. Isolate A2 (*D. pinea*) produced stem cankers significantly longer, on slash and loblolly pine than on Virginia pine. Isolate B2 (*D. scrobiculata*) developed cankers significantly longer on slash pines than on shortleaf and Virginia pine. While isolate C1-F1 (*L. theobromae*) developed significantly longer stem cankers on loblolly than on shortleaf and Virginia pine. Stem cankers caused by isolate D1-F1 (*L. theobromae*) were significantly longer on slash than on loblolly, shortleaf, and Virginia pine (Table 4.3).

Because there was a significant interaction between pine and isolate, a one way ANOVA test was performed on the combined data for each of the four pine species to determine how each isolate behaved within each pine species. All four models were determined to be valid ($P<0.001$) and isolate type was a significant predictor of canker length in all four cases. Tukey's Studentized Range (HSD) Test was performed to further characterize the relative pathogenicities of the isolates on each of the pine species.

Virginia and shortleaf pine were each susceptible to only three of the isolates used in the study. Virginia pine was susceptible to both *D. pinea* type cultures (A1, A2) and to one *L. theobromae* isolate (D3-S4), but was not susceptible to any of the *D. scrobiculata* isolates. One of the *D. pinea* type cultures (A1) caused cankers significantly longer than those caused by the *L. theobromae* isolate. Shortleaf pine was susceptible to both *D. pinea* type cultures (A1, A2) and to

one *D. scrobiculata* type culture (B1), but was not susceptible to any of the *L. theobromae* isolates. There was no statistical difference among canker lengths caused by these three isolates on shortleaf pine (Table 4.4).

Loblolly pine was susceptible to eight of the 14 isolates used in this study. They were susceptible to both *D. pinea* type cultures (A1, A2), to one *D. scrobiculata* type culture (B1), to both *D. scrobiculata* isolates collected from Georgia (B2-S2, S-C5D) and to three of the seven *L. theobromae* isolates collected from Georgia (D3-S4, C1-F1, D2-S2). The three type cultures (A1, A2, B1), the two *D. scrobiculata* isolates collected from Georgia (B2-S2, S-C5D), and one of the *L. theobromae* isolates (D3-S4) caused the longest cankers in the pines. Of the two Georgia *D. scrobiculata* isolates, B2-S2 caused cankers significantly longer than those caused by two of the three pathogenic *L. theobromae* isolates (C1-F1, D2-S2), while S-C5D caused cankers significantly longer than only one of them (D2-S2) (Table 4.4).

Slash pine was susceptible to 10 of 14 isolates used in this study, including both *D. pinea* type cultures (A1, A2), one *D. scrobiculata* type culture (B1), both *D. pinea* isolates collected from Georgia (B2-S2, S-C5D), and five of the seven *L. theobromae* isolates collected from Georgia (D3-S4, C1-F1, D2-S2, D1-F1, D2-S3). Again, the three type cultures (A1, A2, B1), the two *D. scrobiculata* isolates collected from Georgia (B2-S2, S-C5D), and one of the *L. theobromae* isolates (D3-S4) caused the longest cankers in the pines. Of the two Georgia *D. scrobiculata* isolates, B2-S2 caused cankers significantly longer than those caused by four of the pathogenic *L. theobromae* isolates (C1-F1, D2-S2, D1-F1, D2-S3), while S-C5D caused cankers significantly longer than only one of them (D2-S3) (Table 4.4).

Isolates A1 and A2 (both type cultures of *D. pinea*) were the only two isolates that, in all four pine species, caused stem canker lengths significantly longer than those caused by the

negative control. Isolates B1 (*D. scrobiculata*) and D3-S4 each caused canker lengths significantly longer than the control in three of four cases; B1 was not pathogenic on Virginia pine while D3-S4 was not pathogenic on Shortleaf pine. Isolates B2-S2 (*D. pinea*), S-C5D (*D. scrobiculata*), C1-F1 (*L. theobromae)*, and D2-S2 (*L. theobromae*) were significantly pathogenic only on Slash and Loblolly Pines. Isolates D2-S3 (*L. theobromae*) and D1-F1 (*L. theobromae*) were significantly pathogenic only on Slash Pines. A2-F1 (*L. theobromae*), A4-S2 (*L. theobromae*), E2-S3 (*D. pinea*), and B2 (*D. scrobiculata*) did not behave significantly different from the control on any of the four pine species tested (Table 4.4).

In 12 of 14 cases (Remaining isolates currently being sequenced at IBL), the fungal species that was inoculated onto the pines was recovered from it, suggesting that that it was responsible for the cankers observed.

DISCUSSION

Based on this study, Virginia and shortleaf pines are generally less susceptible to infection by *D. pinea, D. scrobiculata*, and *L. theobromae* than are slash and loblolly pines. However, certain *D. pinea* isolates, such as type culture A1 used in this study, were equally deadly on all four pine species. Virginia pines were unique among the pine species tested in that they showed no susceptibility to the *D. scrobiculata* isolates included in this study. Shortleaf pines were unique in that they showed no susceptibility to the *L. theobromae* isolates included in this study. Slash and loblolly pines demonstrated nearly identical patterns of susceptibility to *D. pinea*, *D. scrobiculata*, and *L. theobromae* isolates, although slash pine was susceptible to two isolates that did not cause significant disease on loblolly. This study has confirmed that *L. theobromae* is capable of causing disease on slash, loblolly, and Virginia pines at levels rivaling that of *D. pinea* and *D. scrobiculata*. This finding adds credence to the arguments made by other

authors that *L. theobromae* is capable of causing tip dieback on loblolly and slash pines (South et al., 2002; Rowan, 1982; Jolley & Hedden, 2001).

No overriding statements can be made, however, about the susceptibilities of slash, loblolly, Virginia, and shortleaf pine to *D. pinea* or *D. scrobiculata*, and *L. theobromae* based on this study, as isolates within the same pathogen species behaved differently from one another on a given pine species. For instance, of the three *D. pinea* isolates in the study, two caused visible disease on all four pine species, whereas the other appeared to be non-pathogenic on all pine species. Similarly, in slash and loblolly pine, three of the four *D. scrobiculata* isolates in the study were pathogenic, while the other was not. Isolate D3-S4, *L. theobromae*, was pathogenic on three of the pine species included in this study, but other *L. theobromae* isolates, such as A2-F1, did not cause cankers on any of the pines. Perhaps in the future, genetic distinctions among the isolates of each of the three fungal species will be identified and used to create tests that accurately predict the pathogenicity of a given isolate.

Historically, seemingly slight changes can make a large difference in the response of pine species to inoculation with *D. pinea* and *D. scrobiculata*. As was the case with Austrian pine (Blodgett and Bonello, 2003), inoculating shoots rather than stems of the seedlings in this study might have produced different results. Or, as was the case with jack pine (Blodgett and Stanosz, 1997), not wounding seedlings prior to inoculation might cause them to be less susceptible to the isolates in the study. Effects of various levels of drought stress on the susceptibility of a pine also may need to be considered. Aleppo and red pines have been shown to experience differing levels of disease severity based on the extent of water stress present (Blodgett, Kruger and Stanosz, 1997; Paoletti et al., 2001). The same may hold true for loblolly, slash, Virginia, or shortleaf pines.

Table 4.1. Origin and molecular description of isolates used in greenhouse inoculations of slash, loblolly, Virginia, and shortleaf 1-yr-old pine seedlings.

Isolate[1]	**Origin of Isolate**	**SSU rDNA**[2]	**ITS 1 and 2 sequencing**[3]
A1	University of Wisconsin	*D. pinea*	*D. pinea*
A2	University of Wisconsin	*D. pinea*	*D. pinea*
B1	University of Wisconsin	*D. scrobiculata*	*D. scrobiculata*
B2	University of Wisconsin	*D. scrobiculata*	*D. scrobiculata*
A4-S2	Georgia	No bands	*L. theobromae*
A2-F1	Georgia	No bands	*L. theobromae*
B2-S2	Georgia	*D. scrobiculata*	*S. sapinea*
C1-F1	Georgia	No bands	*L. theobromae*
D1-F1	Georgia	No bands	*L. theobromae*
D2-S2	Georgia	No bands	*L. theobromae*
D2-S3	Georgia	No bands	*L. theobromae*
D3-S4	Georgia	No bands	*L. theobromae*
E2-S3	Georgia	*D. pinea*	*B. obtusa*
S-C5D	Georgia	*D. scrobiculata*	*S. sapinea*
CONTROL	N/A	N/A	N/A

[1] Type cultures (A1, A2, B1, B2) were obtained from Glen Stanosz of the University of Wisconsin, Madison, under USDA APHIS PPQ Permit #P526P-06-00193. Non-colonized potato dextrose agar served as the negative control.

[2] SSU rDNA identification based upon Smith and Stanosz, 2006.

[3] BLAST identification of ITS 1 and 2 region sequence based upon Jacobs and Rehner, 1998.

Table 4.2. Nucleotide sequences of primers used in this study

Primer*	Nucleotide sequence (5'—3')
forward primer DpF	CTTATATATCAAACTATGCTTTG-TA
forward primer DsF	CTTATATATCAAACTAATGTTTG-CA
reverse primer BotR	GCTTACACTTTCATTTATAGACC
forward primer ITS-5	GGAAGTAAAAGTCGTAACAAGG
reverse primer ITS-4	TCCTCCGCTTATTGATATGC

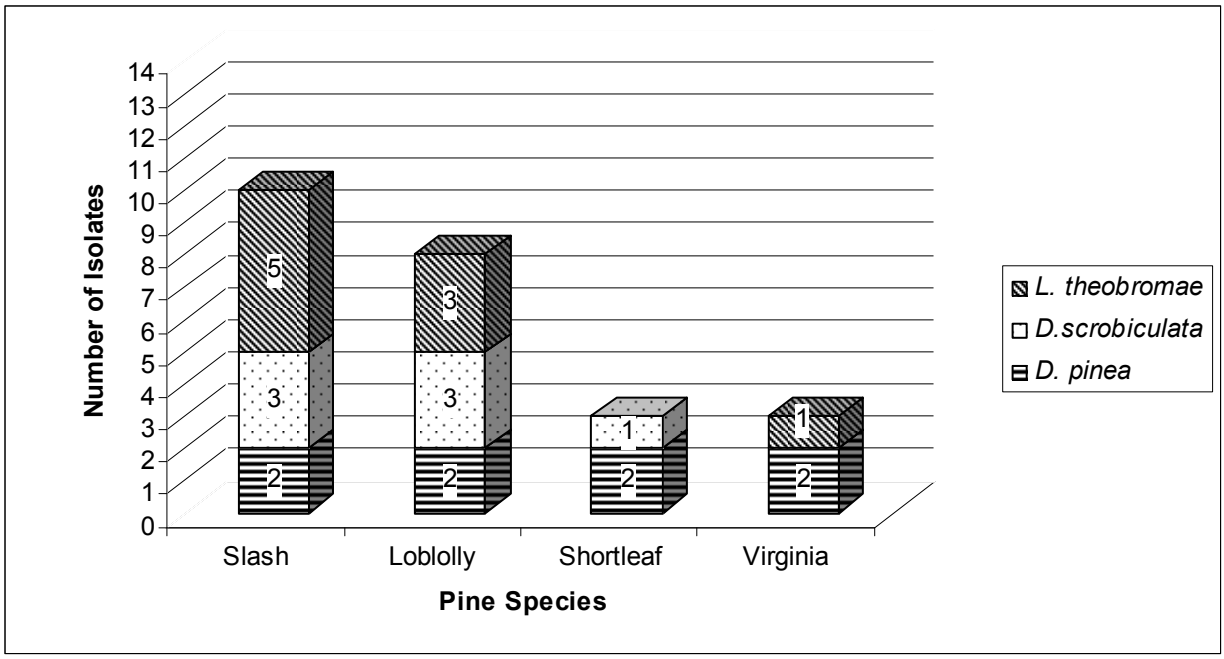
* Sequences were synthesized at the Molecular Genetics Instrumentation Facility, The University of Georgia, Athens.

Table 4.3. Average length of pith discoloration (mm) caused by isolates of *Diplodia pinea*, *D. scrobiculata*, and *Lasiodiplodia theobromae* on slash, loblolly, shortleaf, and Virginia pines.

	Average length of discolored pith (mm) by isolate (designation)[1]				
Pine species[2]	*D. pinea* (A1)	*D. pinea* (A2)	*D. scrobiculata* (B1)	*L. theobromae* (D3-S4)	*D. scrobiculata* (B2-S2)
Slash	200.0 a[3]	200.0 a	200.0 a	187.0 a	189.7 a
Loblolly	200.0 a	200.0 a	188.2 a	188.8 a	179.4 a
Shortleaf	159.3 a	167.3 ab	123.7 b	60.2 b	60.1 b
Virginia	170.4 a	118.9 b	61.9 b	87.4 b	38.2 b
Mean	*182.4*	*171.6*	*143.5*	*130.9*	*116.9*
Pine species	*D. scrobiculata* (S-C5D)	*L. theobromae* (C1-F1)	*L. theobromae* (D2-S2)	*L. theobromae* (D1-F1)	*L. theobromae* (A2-F1)
Slash	162.7 a	102.2 ab	111.2 a	112.7 a	48.3 a
Loblolly	151.3 a	112.7 a	83.3 a	32.8 b	61.6 a
Shortleaf	82.2 b	68.0 b	43.1 a	38.0 b	39.3 a
Virginia	33.3 b	43.9 b	19.2 a	13.1 b	17.5 a
Mean	*107.4*	*81.7*	*64.2*	*49.2*	*41.7*
Pine species	*L. theobromae* (D2-S3)	*L. theobromae* (A4-S2)	*D. scrobiculata* (B2)	*D. pinea* (E2-S3)	Negative Control
Slash	73.0 a	25.2 a	70.0 a	29.7 a	8.2
Loblolly	40.6 a	50.8 a	37.0 ab	23.8 a	8.8
Shortleaf	15.7 a	50.5 a	16.1 b	22.8 a	32.1
Virginia	11.2 a	11.1 a	11.4 b	10.3 a	7.9
Mean	*35.1*	*34.4*	*33.6*	*21.7*	*14.3*

[1] Average of eight single-plant replications of each pine species and isolate combination.
[2] Pine species: slash (*P. elliottii*), loblolly (*P. taeda*), shortleaf (*P. echinata*), and Virginia (*P. virginiana*)
[3] Numbers followed by the same letter within each isolate group are not significantly different based upon Tukey's studentized range test (P=0.05)

Table 4.4. Number of isolates included in the study that were pathogenic (as measured by average length of discolored pith) on slash, loblolly, shortleaf, and Virginia pines.



CHAPTER 5: CONCLUSIONS

Diplodia pinea, *D. scrobiculata*, and *L. theobromae* were recovered from slash pine in southern Georgia during 2005 and 2006 surveys. *D. scrobiculata* was recovered only from diseased shoots on 10- and 15-year old trees, while *D. pinea* was recovered from diseased and healthy shoots on 5-, 10-, and 15-year old trees. *Lasiodiplodia theobromae* was recovered much more frequently from pines in this study than either *Diplodia* species. Both *Diplodia* sp. and *L. theobromae* were isolated at equal frequencies from symptomatic and asymptomatic shoots preventing any definitive conclusion regarding cause of the dieback to be made. Additionally, no significant relationship was found between the age of a tree and the isolation of *D. pinea, D. scrobiculata,* or *L. theobromae*.

The overall frequency at which *Diplodia sp.* were recovered was low. It is feasible that the pathogen was present more, or even much more, frequently than the low numbers this study would suggest. Had methods been in place allowing shoots to be screened directly for the pathogen, as opposed to having to isolate the pathogen from the plant's tissue, it is possible that more infections would have been detected and that statistical results would have been different. Even so, wounding events have been shown to increase susceptibility of some pines to infection by *Diplodia sp.* (Blodgett and Stanosz, 1997; Blodgett and Bonello, 2003); and in many pines, drought stress has been shown to worsen the effects of the pathogen (Bachi and Peterson, 1985; Johnson et al., 1997; Blodgett et al., 1997; Paoletti et al., 2001). Weather events could create an

environment in which even the low frequency of detected infections could spawn problems for southern Georgia slash pine growers.

Multiple sequence analysis of ITS regions 1 and 2 demonstrated close relationships between 12 of 17 *Diplodia* sp. isolates collected from Georgia during 2005 and 2006. The 5 remaining *Diplodia sp.* isolates, all identified as *D. pinea* by the Smith/Stanosz method, grouped either among *L. theobromae* isolates or between isolates of *L. theobromae* and *Diplodia* type cultures. This finding suggests that sequencing of ITS regions 1 and 2 may not be sufficient for differentiating between isolates of *L. theobromae* and *Diplodia* spp.. Further analysis will be necessary in order to confirm this conjecture.

An inoculation study was performed during the fall of 2006. Based on that study, Virginia and shortleaf pines are generally less susceptible to infection by *D. pinea, D. scrobiculata*, and *L. theobromae* than are slash and loblolly pines. However, certain *D. pinea* isolates, such as type culture A1 used in this study, were equally deadly on all four pine species. Virginia pines were unique among the pine species tested in that they showed no susceptibility to the *D. scrobiculata* isolates included in this study. Shortleaf pines were unique in that they showed no susceptibility to the *L. theobromae* isolates included in this study. Slash and loblolly pines demonstrated nearly identical patterns of susceptibility to *D. pinea*, *D. scrobiculata*, and *L. theobromae* isolates used in the study, though slash pine was susceptible to two isolates that did not cause significant disease on loblolly pine. This study has confirmed that *L. theobromae* is capable of causing disease on slash, loblolly, and Virginia pines at levels rivaling that of *D. pinea* and *D. scrobiculata*. This finding adds credence to the arguments made by other authors that *L. theobromae* is capable of causing tip dieback on Loblolly and Slash Pines (South et al., 2002; Rowan, 1982; Jolley & Hedden, 2001).

No overriding statements can be made, however, about the susceptibilities of slash, loblolly, Virginia, and shortleaf pines to *D. pinea* or *D. scrobiculata*, and *L. theobromae* (= based on this study as isolates within the same pathogen species behaved differently from one another on a given pine species. For instance, of the three *D. pinea* isolates in the study, two caused visible disease on all four pine species. The other did not cause visible disease on any of the pine species. Similarly, in slash and loblolly pines, three of the four *D. scrobiculata* isolates in the study caused visible disease while the other did not. Isolate D3-S4, *L. theobromae*, was pathogenic on three of the pine species included in this study, but other *L. theobromae* isolates, such as A2-F1, did not cause cankers on any of the pines. Perhaps in the future, genetic distinctions among the isolates of each of the three fungal species will be identified and used to create tests that accurately predict the pathogenicity of a given isolate.

Historically, seemingly slight changes can make a large difference in the response of pine species to inoculation with *D. pinea* and *D. scrobiculata*. As was the case with Austrian pine, inoculating shoots rather than stems of the seedlings in this study might have produced different results (Blodgett and Bonello, 2003). Or, as was the case with jack pine, not wounding seedlings prior to inoculation might cause them to be less susceptible to the isolates in the study (Blodgett and Stanosz, 1997). The effects of various levels of drought stress on the susceptibility of a pine also may need to be considered. Aleppo and red pines have shown differing levels of disease severity based on the extent of water stress present (Blodgett, Kruger and Stanosz, 1997; Paoletti et al., 2001). The same may hold true for loblolly, slash, Virginia, or shortleaf pines. In the future, the presence of pathogenic isolates of *D. pinea*, *D. scrobiculata*, or *L. theobromae* may need to be considered as forest managers choose plantation sites and pine species.

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