Pathogenicity and Virulence of Root-Inhabiting Ophiostomatoid Fungi on *Pinus* Species of the Southeastern United States

by

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Abstract

Root- inhabiting ophiostomatoid fungi cause root disease in conifer hosts around the world. A group of ophiostomatoid fungi in the genus *Grosmannia* Goid. and their *Leptographium* Lagerb. & Melin anamorphs have been recently associated with declining loblolly (*Pinus taeda* L.) and longleaf (*P. palustris* Mill.) pine in the southeastern United States. This dissertation establishes the potential for ophiostomatoid fungal species to cause disease in healthy southern pine. In addition, virulence differences, among the primary ophiostomatoid fungi, were investigated. Six inoculation tests were conducted exploring the relationship root-inhabiting ophiostomatoid fungi have with southern *Pinus* species. Qualitative and quantitative measurements of the lesion reaction were used to assess the pathogenicity to host species and virulence among fungal species.

In young pine hosts, *Grosmannia huntii* (R.C. Rob. Jeffr.) Zipfel, Z.W. de Beer & M.J. Wingf., *Leptographium procerum* (Kendrick) M.J. Wingfield, *L. terebrantis* S.J. Barras & T.J. Perry and *L. serpens* (Goidanich) Siemaszko caused dark lesions surrounding the point of inoculation. *Grosmannia huntii* caused the largest average lesion in loblolly and slash (*P. elliottii* Engelm.) pine. Longleaf pine seedlings and young longleaf pine trees appear to be more resistant to infection and damage, compared to other southern pine species.

All ophiostomatoid fungi caused damage in large, mature tree roots following inoculations. Common observations included a darkened, pitch-filled lesion, often accompanied by a severe primary resin response eight weeks following inoculation. *Grosmannia huntii* caused the largest lesions and root damage. *Leptographium serpens* was the second most virulent pathogen tested, while *L. terebrantis* and *L. procerum* caused less damage.

Each root-inhabiting ophiostomatoid fungus is capable of causing local disease symptomlogy and is pathogenic to the southeastern *Pinus* species tested. *Grosmannia*

huntii and *L. serpens* are most virulent among the fungi and have the greatest potential for root damage following inoculation.

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Chapter 1

Introduction and Literature Review

1.1. Southern Forest Timber Production

The southeastern region represents the main softwood producing area of the United States and accounts for approximately 64% of the total timber harvested in the nation (Smith et al. 2001). Timberland area has risen throughout the southeastern United States since 1987 despite recent land ownership patterns, which have shifted timberland from forest industry to non-industrial private lands. In spite of the recent trend, approximately 55% of the total forest industry timberlands are in the southeastern region (Smith et al. 2001). Growing-stock volume has increased by approximately 5 percent in the southeastern U.S. since 1987, corresponding to approximately 3 percent on a per acre basis. Loblolly and shortleaf pine are the primary timber species grown in the southeastern U.S, representing the second largest softwood group by volume (Smith et al. 2001).

1.2. Loblolly Pine (*Pinus taeda* L.)

1.2.1. Importance

Loblolly pine is the most cold hardy and prolific southern pine, able to colonize an extremely diverse range of sites. It is the leading timber species in the United States and dominates on approximately 13.4 million ha throughout the southeastern forests (Shultz 1997). The native range of loblolly pine extends from southern New Jersey south to Florida and west to eastern Texas. It also extends north into the coastal plain throughout the Piedmont Plateau and into the Appalachian Highlands (Baker and Langdon 1990). Prior to European settlement, loblolly pine predominated on an estimated 2 million ha and was considered to be a minor component of the natural ecosystem (Shultz 1999). After settlement, much of the southeastern forest was cleared

for agricultural uses, including the extensive cotton (*Gossypium* sp.) industry that existed through the 1880's. After the dramatic fall of the cotton industry, due in part to the introduction of the boll weevil (*Anthonomus grandis grandis* Boheman), much of the piedmont and coastal plain were left abandoned (Shultz 1997). Following the long period of exploitive agriculture, planting loblolly pine became the most common way to transform severely eroded soils into productive forests (Shultz 1999). Loblolly pine volume increased from approximately 0.6 billion m^3 in the late 1940's to 1.4 billion m^3 in 1989. Loblolly pine now comprises over 50% of the dominant and co-dominant growing-stock in the southeastern United States (Shultz 1997), much of it growing on poor sites and eroded soils.

1.2.2. Biology

The unique biology of loblolly pine is responsible for its current dominance on many sites throughout its range. Seed production is variable depending on physiographic region. Loblolly pine is a consistent prolific producer of seed along the coastal plain, becoming a more periodic producer as you move inland (Baker and Langdon 1990). Loblolly pine seed goes through a dormant stage prior to germination, which lasts longer than any other southern pine. The dormant seed is especially susceptible to predation by many seed pests. However, once established loblolly pine trees grow rapidly and consistently throughout a stand. In natural stands, differences in growth rates exist and individual trees best suited for their microsite conditions express dominance at an early age (Baker and Langdon 1990). In contrast, planted stands consisting of genetically improved seedlings tend to have much less growth variation, leading to a relatively uniform stand with few clear dominant individuals. Loblolly pine is a medium-lived tree and normally does not live past 200 years, with the oldest known individual being 245 years old in North Carolina. It produces a relatively short taproot and generally favors production of and extensive network of shallower lateral roots that have the tendency to graft in dense plantations (Baker and Langdon 1990).

1.2.3. Insect and Pest Tolerance

Loblolly pine is susceptible to many damaging pests throughout its range. The southern pine beetle (*Dendroctonus frontalis* Zimmermann) is the most serious pest of loblolly pine (Baker and Langdon 1990). The beetle caused an estimated 900 million dollars worth of damage between 1960 and 1990 (Price et al. 1992) and continues to kill mainly off-site pines (Baker and Langdon 1990). Other pine beetles readily attack and cause damage to loblolly pine trees, including *Ips* species bark beetles (Connor and Wilkinson 1983), and the black turpentine beetle (*Dendroctonus terebrans* (Olivier))(Smith and Lee 1972). Loblolly pine is susceptible to many damping-off fungi in the seedling stage (Hartley 1921). It is also very susceptible to fusiform rust (*Cronartium fusiforme* f.sp. *fusiforme (*Hedg. & Hunt) Burdsall & G. Snow (Phelps and Czabator 1978) and the root disease pathogen *Heterobasidion annosum* (Fr.) Bref. (Robbins 1984), among other pathogens (Hepting 1971).

1.3. Longleaf Pine (*Pinus palustris* Mill.)

1.3.1. Importance

Longleaf pine once occupied a range extending from southern Virginia south to central Florida and west to eastern Texas along the gulf coastal plain (Boyer 1990). In 1995, longleaf pine resided on only approximately 1.2 million ha of its original estimated 38 million ha (Brockway et al. 2005). Exploitation coupled with lack of planned regeneration contributed to a nearly continuous decline in longleaf pine forests since colonial times (Boyer 1990). Traditionally, longleaf pine was used as the major source of naval stores and saw timber. Ecologically, longleaf pine supports an extremely diverse community (Brockway et al. 2005). Among the common herbaceous associates of longleaf pine are wiregrass (*Aristida stricta* Michx. and *A. beyrichiana* Trin. & Rupr.) and bluestem grasses (*Andropogon* species), which were traditionally found throughout large portions of its range (Peet 2006). The longleaf pine ecosystem is required for several endangered and sensitive animal species, including the red-cockaded woodpecker (*Picoides borealis* Vieillot)(Fig 1.1) and gopher tortoise (*Gopherus polyphemus* Daudin). Interest in restoration of the longleaf pine ecosystem coupled with the forest health

concerns associated with loblolly pine has contributed to an increased interest in planting longleaf pine (Johnson 1999).

Fig. 1.1. Red cockaded woodpecker (*Picoides borealis* Viellot) cavity in longleaf pine.

1.3.2. Biology

Longleaf pine biology is unique among the common southern *Pinus* species Seeds germinate soon after being dispersed, avoiding many of the common seed pests (Boyer 1990). After germination, seedlings exhibit a stemless, 'grass stage' condition that may last one to multiple years depending on the growing conditions (Fig 1.2). Extensive root systems develop while seedlings are in the grass stage. Stemless seedlings with greater than 0.8 cm root-collar diameters are highly tolerant to surface fires common in the southeastern pine forests (Boyer 1990). Unlike loblolly pine, longleaf pine individuals express dramatically different growth rates, which lead to a wide range of size classes. Longleaf pine can grow as well or better than other major southern pines on a wide range of sites, once height growth has begun (Boyer 1990). However, poor survival after artificial regeneration coupled with the inability to predict emergence from the grass stage prevented longleaf from becoming the dominant timber species (Wakeley 1954). Longleaf pine has the biological potential to live for 500 years, however due to constant disturbance; most do not reach 300 years (Brockway et al. 2005). Compared to loblolly pine, it produces a more massive taproot that can often reach 3.7 m (Boyer 1990).

Fig. 1.2. Longleaf pine seedling in the characteristic 'grass stage'.

1.3.3. Insect and Pest Tolerance

Longleaf pine is less susceptible to many common damaging biotic agents compared to other southern pines, including both fusiform rust and the southern pine beetle (Snow et al. 1990). Southern pine beetle, one of the most destructive pests of southeastern pines, is significantly less damaging to stands mixed with longleaf pine (Hedden and Lorio 1985). However, brown-spot needle blight *Mycosphaerella dearnessii* M.E. Barr causes serious damage to seedlings, primarily in the absence of fire. Longleaf pine is susceptible to pitch canker disease of pine, however is much more resistant than both loblolly and slash pine (Dwinell et al. 1985). Longleaf pine is often found to be resistant to successful insect attack, due in part to its resin production capabilities (Hodges et al. 1979).

1.4. Slash Pine (*Pinus elliottii* Englem.)

1.4.1. Importance

Traditionally grown for timber and naval stores, slash pine is one of the most important pine species grown in the southeastern United States. Of the main southeastern pines, slash pine has the smallest native range. It extends from southern South Carolina south, throughout Florida and west to the eastern tip of Louisiana. Naturally, slash pine was found only to occur in the lower coastal plain on poorly drained flatwoods and

stream edges (Barnett and Sheffield 2004). Due to its excellent timber quality, it was planted extensively on marginal sites. However, exceptionally poor growth off-site has relegated it to relatively deep poorly drained soils in the coastal plain. Despite its shortfalls, 69% of the current slash pine stands are planted, making it the primary species on approximately 10.4 million acres (Barnett and Sheffield 2004). Slash pine is most often found in mixed stands, making it a major component of three forest cover types (Longleaf Pine-Slash Pine (83), Slash Pine (84), and Slash Pine-Hardwood (18)) and associated with ten others (Lohrey and Kossuth 1990).

1.4.2. Biology

Two varieties of slash pine are recognized (*P. elliottii* var. *elliottii Englem.*, *P. elliottii* var. *densa* (Little & Dorman) Gaussen) in the southeastern United States. The less common, South Florida slash pine variety (*P. elliottii* var. *densa*) experiences a "grass" seedling stage similar to longleaf pine. It also often exhibits a split stem structure leading to a flat top or rounded crown as it gets older (Barnett and Sheffield 2004). The more common, *Pinus elliottii* var. *elliottii* is the primary variant recognized in the southeastern United States. With adequate soil moisture, seeds germinate rapidly, most after two weeks (Lohrey and Kossuth 1990). Slash pine has rapid height and diameter growth in its early ages. Almost three-quarters of the 50-year volume yield is produced by age 30, making it acceptable on short rotations under intensive management. Provenance tests have shown that seed source is an extremely important variable to consider when planting slash pine (Lohrey and Kossuth 1990). Results from provenance tests suggest several distinct ecotypes exist, with significant genetic variation within the species (Snyder et al. 1967). Slash pine genetic improvement for disease resistance has shown increases in survival and volume (Dhakal et al. 1996).

1.4.3. Insect and Pest Tolerance

Although slash pine is generally known to be moderately tolerant to most forest pests, like loblolly pine, fusiform rust continues to plague slash pine growers (Lohrey and Kossuth 1990). Pitch canker caused by *Fusarium circinatum* Nirenberg & O'Donnell causes damage to slash pine in nurseries, seed orchards, and plantations (McRae et al.

1985). However, genetic improvements in planting stock have alleviated disease incidence and severity (Oak et al. 1987). Unlike loblolly pine, slash pine is tolerant of southern pine beetle attacks, probably due to its high pitch production (Barnett and Sheffield 2004). Southern cone rust (*Cronartium strobilinum* Hedgc. & Hahn) has been a serious problem in seed orchards where seed production is the main management objective (Mathews 1964). In terms of root disease, annosus root rot (*Heterobasidion annosum*) has proven to cause severe damage, particularly in soils with a heavy clay component (Lohrey and Kossuth 1990).

1.5. Decline Diseases

The decline disease concept was first defined and characterized by Manion (1981). Decline diseases involve a series of factors (stressors) that collectively contribute to a slow decline in tree health leading to mortality. Factors associated with decline diseases are grouped into one of three roles (predisposing, inciting, or contributing). Predisposing factors place an underlying, constant stress on tree individuals. Inciting factors act in aggravating the stress level and tip the tree towards poor health, while contributing factors act in finishing decline process ultimately leading to premature mortality. Stress factors may be biotic or abiotic in origin.

Predisposing factors are almost exclusively abiotic stressors, although abiotic stressors may occur as any of the three factor types. Abiotic predisposing factors are often site related and may include certain topographic features (Eckhardt and Menard 2008), poor drainage (Hennon et al. 1992) heavily eroded or shallow and rocky soils (Manion 1991), or overstocking (Heitzman et al. 2007). Other abiotic stressors that may act as either inciting or contributing factors include moisture stress (Jurskis 2005), highway deicing salts (Horsley et al. 2002**;** Simini and Leone 1982), atmospheric deposition (Duchesne et al. 2002), and logging damage (Edger et al. 1976), among others (Manion 1991).

Biotic factors may work in conjunction or alone to increase the tree stress level and ultimately lead to mortality. Examples of common biotic factors, which are generally considered inciting or contributing factors, include, insect defoliation (Starkey et al. 2004) root disease (Leaphart and Copeland 1957). It is common for one biotic

stress to allow for the successful colonization of another biotic pest, as illustrated by the well-known relationship between Scolytid bark beetles and their associated fungi (Paine et al. 1997).

Examples of classic decline disease systems exist throughout the United States and the world (Millers et al. 1989). Both Maple and Ash have a history of decline in the northeastern United States (Castello et al. 1985). In both systems, declining trees are closely tied to site factors that place a predisposing stress on individuals (Nolet et al. 2007). A complex group of abiotic and biotic stressors are common, leading to the premature mortality of both species (Horsley et al. 2002). Major oak declines have been well documented since the late 1890's (Millers et al. 1989). Generally, a combination of host maturity, complex site and climate factors, defoliating and boring insects, as well as root and canker disease fungi have been identified as inciting and contributing factors in oak decline (Starkey et al. 2004). Finally, pine decline throughout the United States has been noted in several separate systems. Western Pole Blight (Leaphart and Copeland 1957) and ponderosa pine (*P. ponderosa* C. Lawson) root disease (Livingston and Mangini 1981) in the western states , red pine (*P. resinosa* Aiton) decline in the lake states (Klepzig et al. 1991), eastern white pine (*P. strobus* L.) decline (Dochinger 1967), littleleaf disease and loblolly pine decline (Eckhardt et al. 2007) in the southeast are all examples of separate decline disease syndromes.

1.6. Southern Forest Decline

Timber mortality (as percentage of growing-stock) has increased substantially and steadily since 1976, greater than any other region over this time (Smith et al. 2001). Increases in southern pine mortality have certainly been affected by increases in frequency, severity, and distribution of southern pine beetle outbreaks over the past 30 years (Belanger et al. 1993). However, other forest health concerns have been observed, including nearly consistent documentation of premature mortality in shortleaf and loblolly pines (Brown and McDowell 1968; Campbell and Copeland 1954; Hess et al. 1999; Eckhardt et al. 2007).

1.6.1. History of Southern Pine Decline

Premature mortality in loblolly pine, named "loblolly pine die-off", was first observed in 1959 on the Oakmulgee and Tuscaloosa Ranger Districts of the Talladega National Forest in Alabama (Brown and McDowell 1968). Early observations suggested the premature mortality occurred most frequently in trees over 50 years old. These observations followed documentation of premature mortality in shortleaf pine (*P. echinata* Mill.)(Siggers and Doak 1940) and the discovery of the fine root pathogen, *Phytopthora cinnamomi* Rands consistently associated with roots of dying shortleaf pine. The observations in shortleaf pine subsequently led to the description of littleleaf disease of shortleaf pine (Campbell and Copeland 1954)(Fig 1.3). However, early observations in "loblolly pine die-off" suggested littleleaf disease was not the cause of the mortality, due to the inability to isolate *P. cinnamomi* from declining stands (Brown and McDowell 1968) and more extensive root deterioration than observed in littleleaf disease (Roth 1954). Early investigators were unable to conclusively determine the causal factor for the observed mortality in loblolly pine.

Fig. 1.3. Shortleaf pine on poorly drained site afflicted by littleleaf disease.

Hess et al. (1999) re-examined "loblolly pine die-off" and suggested that *P. cinnamomi* was the causal agent in decline following isolation from fine roots of diseased trees. Investigation of other root pathogens, including ophiostomatoid fungi and *H. annosum* were conducted and led to limited results, with ophiostomatoid fungi being found on only 47% of the plots and no evidence of *H. annosum*. Although evidence suggests *P. cinnamomi* is associated with "loblolly pine die-off" (Hess et al. 1999), loblolly pine appears to be more tolerant than shortleaf pine (Campbell and Copeland 1954). Littleleaf disease and the causal agent, *P. cinnamomi* is limited by certain soil parameters including adequate internal drainage (Copeland and McAlpine 1955), making the disease most problematic on the Piedmont Plateau region. Eckhardt et al. (2007) established plots throughout the southeastern United States in multiple physiographic regions in order to more conclusively establish the causal factor in "loblolly pine die-off" (Fig. 1.4). The occurrence of declining and dying loblolly pines in areas outside the Piedmont Plateau suggested something other than *P. cinnamomi* was contributing to the observed mortality. Eckhardt et al. (2007) reached markedly different conclusions from those reached by Hess et al. (1999). Root-inhabiting ophiostomatoid fungi and their insect vectors were found to be consistently associated with deteriorating crowns across all physiographic regions (Eckhardt 2004). *Phytopthora cinnamomi* was isolated from soil in only 12.8

Fig. 1.4. Dead and dying saw timber-sized loblolly pine from an unknown cause.

percent of the plots. Studies that followed have provided supporting evidence to the theory that ophiostomatoid fungi and their vectors are more consistently observed in dying stands compared to *P. cinnamomi* (Menard 2007).

Recently, the premature mortality in loblolly pine has been characterized as a decline disease syndrome (Eckhardt et al. 2007). Loblolly pines are predisposed to biotic inciting factors by topographical features, including increased slope and southern or southwest facing aspects (Eckhardt and Menard 2008). It has been hypothesized that stress associated with unfavorable microsite conditions attracts root and lower stem feeding bark beetles (Coleoptera: Curculionidae) (Eckhardt et al. 2004b). Root and lower stem infesting insects found associated with declining loblolly pine include *Hylastes salebrosus* Eichhoff, *H. tenius* Eichhoff, *Pachylobius picivorus* (Germar), and *Hylobius pales* (Herbst). *Hylastes* species use roots of both dead and living conifers for maturation feeding (Fig. 1.5) and breeding activities, rarely causing significant damage (Milligan 1978). Collectively, *P. picivorus* and *H. pales* are known as pine regeneration weevils, often causing extensive mortality following artificial seedling regeneration (Edmonds et al. 2000). Pine regeneration weevils are also pests of mature trees, by feeding on branches (Nord et al. 1984) and ovipositing in roots (Drooz 1985).

Each of the insects associated with loblolly pine decline also commonly vector root-inhabiting ophiostomatoid fungal associates with anamorphs in the genus *Leptographium* (Eckhardt et al. 2007).

Fig. 1.5. *Hylastes salebrosus* creating a gallery in loblolly pine root.

1.7. *Leptographium* species and Other Associated Ophiostomatoid Fungi

Leptographium species are anamorphs of the genus *Grosmannia* (Zipfel et al. 2006), formerly *Ophiostoma* (Harrington 1987). Morphologically, *Leptographium* species are known to produce darkly pigmented, mononematous conidiophores (Kendrick 1962). At the apex of the conidiophore, a series of numerous sporogenous cells reside in a mass of slimy amerospore conidia, which arise from the sporogenous cells and are suspended in a mucilaginous drop. *Leptographium* is most commonly distinguished from other closely related genera by the conidiogenesis process and sporogenous cell type, proposed by Hughes (1953) for all hyphomycetes. *Leptographium* species are also characterized by the presence of cellulose, rhamnose, and chitin in their cell walls as well as tolerance to the antibiotic cycloheximide (Spencer and Gorin 1971).

The taxonomy of *Leptographium* has a storied past, not unlike most fungal groups (Jacobs and Wingfield 2001). Nomenclatural differences in the past led to disagreement among scientists on the correct name (Kendrick 1962). However, the similarities of morphology and ecology have led to including all fungi of the type into *Leptographium*. Some *Leptographium* species have been associated at times through history with several other closely related genera including, *Verticicladiella*, *Scopularia*, and *Phialocephala* (Jacobs and Wingfield 2001). However, several characteristics are shared among *Leptographium* species. Common features shared by *Leptographium* species include an intimate relationship with bark beetle vectors (Harrington 1988), presence in living plant tissue, as well as having telomorphs in the genus *Grosmannia* (Zipfel et al. 2006).

The distribution of *Leptographium* is worldwide where appropriate hosts are present (Jacobs and Wingfield 2001) with many new discoveries in recent years (Masuya et al. 2004; Zhou et al. 2008). Most *Leptographium* species are found inhabiting conifers, with only few known to colonize hardwood hosts (Jacobs et al. 2006). Consequently, many more known species are found in the northern hemisphere, where conifer forests are more widespread. Pine forests predominate throughout much of the southeastern United States along the coastal plain and Piedmont Plateau, where timber production is emphasized. Four dominant pine species exist through portions of the southeastern United States. *Leptographium* species are commonly found parasitizing

southern pines. Five ophiostomatoid fungi with *Leptographium* anamorphs have been successfully isolated from pine roots in the southeastern states including, *L. procerum* (W.B. Kendr.) M.J. Wingf. (Horner and Alexander 1983) *L. truncatum* (Jacobs and Wingfield 2001, previously *L. lundbergii* Strydom et al. 1997) *L. terebrantis* Barras and Perry (Barras and Perry 1971) *L. serpens* (Goid) Siemaszko (Eckhardt et al. 2007) *G. huntii* (R.C. Rob. Jeffr.) Zipfel, Z.W. de Beer & M.J. Wingf (Zanzot 2009)

Although many root-inhabiting ophiostomatoid fungi are non-pathogenic and saprophytic (Wingfield et al. 1988), some species are pathogenic and are associated with disease complexes often involving insect vectors (Jacobs and Wingfield 2001). Black stain root disease, caused by *L. wageneri* (W.B. Kendr.) M.J. Wingf. is considered a virulent primary pathogen of several western conifer species (Cobb 1988). *Leptographium wageneri* is unique among *Leptographium* species. It colonizes only the current years' xylem tracheids, which quickly girdles infected tissue (Hessburg and Hanson 1987). In contrast, most other *Leptographium* species colonize phloem, xylem tracheids as well as ray parenchyma cells, causing a wedge-shaped appearance in the wood (Jacobs and Wingfield 2001). Black stain root disease, like all other diseases caused by ophiostomatoid fungi, is associated with insect transmission (Hansen et al. 1988). Two root weevils (*Pissodes fasciatus* LeConte, J.L. and *Steremnius carinatus* (Bohemon)) and one root bark beetle (*Hylastes nigrinus* (Mannerheim)) carry and transmit the fungus between hosts (Witcosky et al. 1986). Transmission occurs during maturation feeding and breeding activities, common actions associated with root and lower stem feeding insects. Insect vectors burrow through the soil and are attracted to host volatiles produced by injured or diseased roots (Witcosky and Hansen 1985). *Leptographium wageneri* may move short distances through root grafts (Jacobs and Wingfield 2001), however, contact between hosts is not necessary for spread (Hessburg and Hansen 1986). Symptoms other than characteristic black stain in the xylem include, chlorotic shortened needles, reduced terminal growth, and premature needle cast (Cobb 1988). It is common for young seedlings and saplings to die after only a few months. Large trees can die in two years or may survive for up to ten years. More commonly, bark beetles kill removed trees before pronounced foliar symptoms become present. Root diseases associated with other ophiostomatoid fungi have very similar

characteristics; however black stain root disease is the most damaging (Wingfield et al. 1988).

1.7.1. *Leptographium procerum* (W.B. Kendr.) M.J. Wingf.

Leptographium procerum is commonly found inhabiting pine roots throughout much of the United States and other countries including, Canada, Europe, New Zealand, and South Africa (Jacobs and Wingfield 2001). It has been isolated from a variety of conifer species including each of the four primary southern pines, *Pinus echinata* Mill. (Alexander et al. 1988), *P. elliottii* (Barnard et al. 1991), *P. palustris* (Otrosina et al. 1999), and *P. taeda* (Alexander et al. 1988).

The morphology of *L. procerum* is distinct from others found in the southeastern states. It generally produces pigmented dark grey to olive colored colonies (as do most *Leptographium* species), sometimes found with darker concentric rings encircling the center (Jacobs and Wingfield 2001). The edge of the colony margin is characteristically smooth, produced from dense clusters of hyphae growing at approximately the same rate. Conidiogenous cells are discrete and generally have between two and four per branch (Jacobs and Wingfield 2001). Conidia are hyaline and aseptate and generally ovoid in shape. When the conidial mass is initially produced it appears clear to slightly white, while over time appearing to be white to cream colored after drying considerably. *Leptographium procerum* conidiophores are commonly joined to one another through the presence of a rhizoid-like structure (Kendrick 1962)(Fig 1.6).

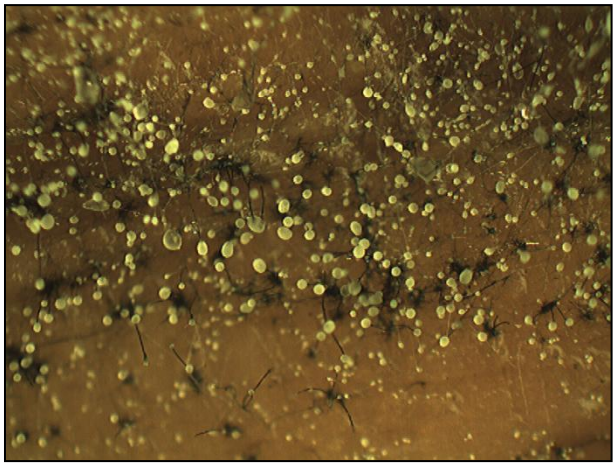

Fig. 1.6. *Leptographium procerum* conidiophores joined by rhizoids.

Leptographium procerum has been isolated from a wide variety of insect vectors throughout the world (Jacobs and Wingfield 2001). In the southeastern United States, it has been found associated with damaging above-ground bark beetles like *Dendroctonus frontalis* (Otrosina et al. 1997), *D. terebrans* (Olivier), and *Ips typographus* L. (Harrington 1988) among others. It has also been isolated from damaging stem and root feeding weevils like *H. pales* (Lackner and Alexander 1982), *P. picivorus* (Wingfield 1983) and *P. nemorensis* Germar. (Nevill and Alexander 1992b). Finally, *L. procerum* is commonly found on root bark beetles around the world including *H. tenuis* and *H. salebrosus* (Eckhardt et al. 2007) found in the southeastern United States.

The pathogenicity of *L. procerum* is relatively well known. As stated previously, *L. procerum* has been associated with many conifer species in several different systems. The wide occurrence of disease association with *L. procerum* has prompted the investigation of its pathogenicity on many different host species, most notably, eastern white pine (Nevill, and Alexander 1992b)(Wingfield 1986), lodgepole pine (Bertagnole et al. 1983), loblolly pine (Eckhardt et al. 2004a), Douglas-Fir (*Pseudotsuga menziesii* (Mirb.)) (Harrington and Cobb 1983), longleaf pine (Otrosina et al. 2002), and red pine (Klepzig, et al. 1996). Although some results illustrate *L. procerum* is capable of severe infections (Eckhardt et al. 2004a; Lackner and Alexander 1981a), most authors have suggested it to be a mild wound pathogen (Klepzig et al. 1996; Nevill et al. 1995; Wingfield 1986). Some evidence suggests pathogenicity differences exist among isolates (Lackner and Alexander 1983), however, similar results were not supported in Wingfield (1986) when eight different isolates were tested. Previous discrepancies in the pathogenicity of *L. procerum* can be attributed to inoculation technique (Wingfield 1986). Several authors found *L. procerum* to severely infect pines using a root dip inoculation technique using either conidial suspensions (Lackner and Alexander 1982) or mycelial slurry (Eckhardt et al. 2004a). In contrast, *L. procerum* appears to be mildly pathogenic when using stem wound inoculations (Nevill et al. 1995; Otrosina et al. 2002; Wingfield 1986). Root inoculations seem appropriate, based on its ability to persist in forest soil, however soilborne propogules were determined to be unimportant in its spread (Lewis at al. 1987).

1.7.2. *Leptographium terebrantis* S.J. Barras and T.J. Perry

Like *L. procerum*, *L. terebrantis* is commonly found associated with dying pine trees throughout the United States and Canada (Harrington 1988). It has been isolated from dying Scots pine (*P. sylvestris* L.) in Massachusetts (Highley and Tattar 1985), red pine in Wisconsin (Klepzig et al. 1991), and eastern white pine (Harrington 1988). In the western states and Canada it has been found in pinyon pine (*Pinus edulis* Engelm.), Douglas-fir (Harrington 1988), and lodgepole pine in British Columbia (Morrison and Hunt, 1988). Three of the main southeastern pine species, loblolly (Eckhardt et al. 2007), longleaf (Otrosina et al. 2002), and slash (Eckhardt observations) pines have been infected by *L. terebrantis* in the ecosystem.

The morphology of *L. terebrantis* has been described as the "typical *Leptographium*" by Jacobs and Wingfield (2001). It is not unlike many other *Leptographium* species with no readily distinguishable characteristics. Conidiophores may occur singly or in groups, often occurring on aerial mycelium. Unlike *L. procerum*, *L. terebrantis* has no rhizoid-like structures joining conidiophores. *Leptographium terebrantis* is most often identified based on its branching, which differs slightly from other closely related species (Jacobs and Wingfield 2001).

Leptographium terebrantis has been isolated from a wide variety of insect vectors within the United States. It has been associated with *D. terebrans* (Barras and Perry 1971) and *D. frontalis* (Otrosina et al. 1997) feeding above ground. Similar to *L. procerum*, it has been isolated from weevil species like *H. pales* and *P. picivorus* as well as root bark beetles *H. tenuis* and *H. salebrosus* in the southeastern United States (Eckhardt et al. 2007).

Leptographium terebrantis is found in several pine systems throughout the United States and the pathogenicity has been well studied in the past. Susceptible hosts include, loblolly pine (Eckhardt et al. 2004a; Nevill et al. 1995), Douglas-fir (Harrington and Cobb 1983), red pine (Klepzig et al. 1996), eastern white pine (Wingfield 1983; Wingfield 1986), longleaf pine (Otrosina et al. 2002), ponderosa pine (Owen et al. 1987; Parmeter et al. 1989, 1992), lodgepole pine, western white pine (*P. monticola* Douglas ex. D. Don), western hemlock (*Tsuga heterophylla* (Raf.) Sarg.), mountain hemlock (*Tsuga mertensiana* (Bong.) Carriere), white spruce (*Pica glace* (Moench) Voss),

subalpine fir (*Abies lasiocarpa* (Hooker) Nuttall), grand fir (*Abies grandis* (Dougl. ex D. Don) Lindl.), western larch (*Larix occidentalis* Nuttall), western red cedar (*Thuja plicata* Donn ex D. Don)(Morrison and Hunt 1988), and Japanese black pine (*P. thunbergii* Parl.)(Rane and Tattar 1987). *Leptographium terebrantis* has been shown to be moderately pathogenic to some hosts (Eckhardt et al. 2004a; Nevill et al. 1995; Wingfield 1983), primarily in the eastern states. However, *L. terebrantis* was found to be weakly or non-pathogenic to many western conifer species (Harrington and Cobb 1983; Morrison and Hunt 1988). Evidence suggests that *L. terebrantis* has extremely variable pathogenicity based primarily on the pine host (Morrison and Hunt 1988). Although the pathogenicity of *L. terebrantis* to loblolly pine is relatively well established (Eckhardt et al. 2004a; Nevill et al. 1995), its pathogenicity to the other primary southern pine species is uncertain.

1.7.3. *Leptographium serpens* (Goid.) M.J. Wingf.

Unlike *L. procerum* and *L. terebrantis*, the role of *L. serpens* (previously *Verticicladiella alacris* Wingfield and Marasas [1981]) is relatively unknown within the United States. Reports of *L. serpens* inhabiting pine roots are much less common when compared to *L. procerum* and *L. terebrantis*. Within the United States, *L. serpens* has been found in Christmas tree plantations (Nevill and Alexander 1992b) and air pollution sensitive *P. strobus* in Virginia (Lackner and Alexander 1981b). It has also been isolated from 42% of trees expressing crown symptoms in loblolly pine in Alabama and was subsequently linked to loblolly pine decline (Eckhardt et al. 2007). Despite its relatively sparse occurrence afflicting pines in the United States, *L. serpens* has been shown to cause distinct infection centers in South Africa (Wingfield and Knox-Davies 1980).

The morphology of *L. serpens* is distinctly different from species previously described. Colonies produce profuse mycelium, much less dense when compared to both *L. procerum* and *L. terebrantis*. Hyphae often grow serpentine-like, although Kendrick (1962) suggested this character is not consistent and not necessarily unique to the species. The conidiophores are also slightly different with cells produced at a 30-40° angle with respect to the main stipe (Fig 1.7). The pigmenting of the stipe is also not consistent and all color is lost by the apex of the stipe (Kendrick 1962).

Fig. 1.7. *Leptographium serpens* sporulating on pine tissue.

Leptographium serpens has been found to be vectored by insect species throughout the world, including the United States. Unlike *L. procerum* and *L. terebrantis*, *L. serpens* appears to only be vectored by root bark beetles (Jacobs and Wingfield 2001). In the southeastern United States, Eckhardt et al. (2007) found that *Hylastes tenuis* and *H. salebrosus* transported *L. serpens* consistently, while *Hylobius* pales and *P. picivorus* did not. *Hylastes angustatus* (Herbst.)(Wingfield et al. 1988), *H. ater* (Paykull)(Wingfield and Gibbs 1991), and *H. linearis* Erichson (Wingfield and Knox-Davies 1980) have been known to vector *L. serpens* in other parts of the world.

The pathogenicity of *L. serpens* is largely unknown (Wingfield et al. 1988). The lack of pathogenicity data reflects the lack of consistent isolation from diseased trees. Few studies have included *L. serpens* in inoculation experiments (Eckhardt et al. 2004a; Wingfield and Knox-Davies 1980; Zhou et al. 2002). In limited root inoculation tests, *L. serpens* was found to produce dark lesions extending an average of 20 cm after six months (Wingfield and Knox-Davies 1980). A similar technique was used to inoculate pine branches in South Africa (Zhou et al. 2002) and loblolly pine stems in the United States (Eckhardt et al. 2004a). *Leptographium serpens* produced lesions between 1.5

and 3.7 cm after six weeks in South Africa, however the authors concluded that *L. serpens* was non-pathogenic to the *Pinus* species tested (Zhou et al. 2002). Eckhardt et al. (2004a) found similar results with *L. serpens* producing lesions averaging 3.00 cm after three weeks. The three inoculation tests used similar techniques on different regions of the tree. Despite differences in experiment length, their cumulative results suggest *L. serpens* grows most successfully in pine roots, where it is most commonly isolated (Eckhardt et al. 2007; Wingfield and Knox-Davies 1980). Since limited pathogenicity tests have been undertaken causing conflicting results, Wingfield et al. (1988) suggests the pathogenicity of *L. serpens* has not been conclusively established.

1.7.4. *Grosmannia huntii* (R.C. Rob. Jeffr.) Zipfel, Z.W. de Beer & M.J. Wingf.

Grosmannia huntii (formerly *Ophiostoma huntii* [Zipfel et al. 2006]), is much less well known when compared to the other species mentioned. The sexual (*G. huntii*) and asexual (*L. huntii*) states were first described in lodgepole pine attacked by mountain pine beetle in British Columbia, Canada (Robinson-Jeffery and Grinchenko 1964). In the United States, it has also been collected in New York, Colorado, Oregon, Washington, and Arizona (Davidson and Robinson-Jeffrey 1965). Although *G. huntii* has been consistently isolated from insect vectors in Georgia (Zanzot et al. 2010) and other areas of the southeast (Eckhardt unpublished), very few reports have been made within the United States. *Grosmannia huntii* is much more commonly observed when compared to *L. huntii*. *Grosmannia huntii* was isolated from diseased red pine roots in Wisconsin (Klepzig et al. 1991). It has also been isolated from pine roots in the southeastern states including from loblolly (Menard 2007) and longleaf (Zanzot 2009) pine.

Like other blue-stain species, *G. huntii* colonies initially grow hyaline, pigmenting with time. Also, pigmentation is lost with continued subculturing (Jacobs and Wingfield 2001). *Grosmannia huntii* may be confused with *L. serpens* due to the presence of serpentine-like hyphae under some circumstances (Fig 1.8). *Grosmannia huntii* has also been reported producing abundant aerial hyphae (Jacobs and Wingfield 2001).

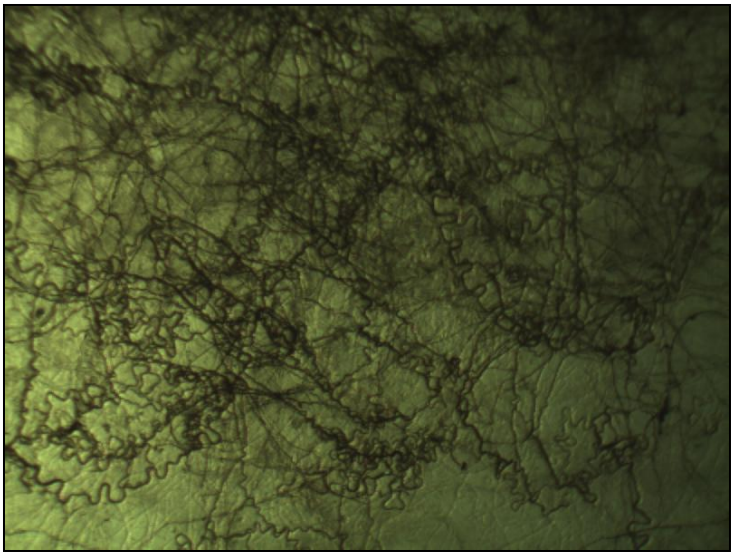

Fig. 1.8. Serpentine-like growth of *Grosmannia huntii*.

Colonies produce diffuse mycelium unlike both *L. procerum* and *L. terebrantis*. Conidiophores can arise singly or more commonly in groups on aerial or surface mycelium (Jacobs and Wingfield 2001). Rhizoid-like structures are absent. Conidia are hyaline and aseptate, ovoid in shape with rounded apices and truncate bases. Perithecia commonly present, have black bases ornamented with small hyphal hairs. Perithecial necks are smooth, commonly found with a slight taper, and ostiolar hyphae are absent. Ascospores are characteristically hat-shaped, aseptate, and hyaline (Robinson-Jeffrey and Grinchenko 1964).

Grosmannia huntii has been isolated from a wide variety of insect vectors in the United States. It has been transported by the mountain pine beetle (*Dendroctonus ponderosae* Hopkins) (Robinson-Jeffrey and Grinchenko 1964), *Ips pini* (Say)(Davidson and Robinson-Jeffrey 1965), *Tomicus piniperda* (L.)(Gibbs and Inman 1991), and *Hylastes* species in the southeast (Zanzot et al. 2010). The presence of *G. huntii* on a wide variety of insect vectors around the United States, represents the potential for widespread movement into new hosts.

1.8. Pathogenicity and Virulence of Ophiostomatoid Fungi

Precise and accurate usage of biological vocabulary is essential to successful communication in science. It has been suggested that the terms “pathogenicity” and

"virulence" have been commonly misused in the past, particularly, as they relate to plant pathogens (Shaner et al. 1992). Before a review of the pathogenicity and virulence of ophiostomatoid fungi, it seems pertinent to discuss the correct usage of the terms in question in order to ensure an accurate depiction of the science. The lack of standard nomenclature as it relates to pathogenicity and virulence of plant pathogens has contributed to confusion. Therefore, before using each of the words extensively throughout this section, it is necessary to provide a clear, concise definition for the way in which each word will be used.

1.8.1. Pathogenicity

Pathogenicity is commonly used to describe the ability of a pathogen to cause disease on a given host (Agrios 2005). For example, when working with obligate pathogens, all species are pathogenic due to the fact that their survival is dependent on infection of their host. However, in non-obligate parasites, it has been suggested that pathogenicity is not an attribute of a species, but rather of a single isolate, due to genetic variation and subsequent phenotypic plasticity of the pathogen (Nelson et al. 1970). Omdall et al. (1995) found that several isolates of *Armillaria ostoyae* were not pathogenic to certain conifer host species, while several others were pathogenic. Each pathogen species is different and should be treated accordingly with respect to potential variations in pathogenicity. As recommended by Shaner et al. (1992), pathogenicity will be used as the ability of a fungal species or isolate to cause disease, a qualitative trait of the species or isolate.

1.8.2. Virulence

The terms virulence and antonym, avirulence were first used by Flor (1942) to describe the continuous nature of damage caused as a result of host reaction to the pathogen, *Melampsora lini* (Ehrenb.) Lév. These terms are most commonly used as adjectives that describe the quantity of pathogenicity. Disease severity is synonymous with virulence, a continuum from less to more virulent. Since many studies with ophiostomatoid fungi compare pathogens directly, the level of virulence is a relative measure between the pathogen species in question under a certain set of environmental

and host conditions. I prefer to use virulence as a relative measure within each study, due to the variability in environmental conditions that exist between experiments. Avirulence, as suggested by Shaner et al. (1992) is valueless, since it implies a virulence of zero, and "nonpathogenic" is more correctly used to describe the inability of a parasite to cause disease.

1.8.3. Pathogenicity and Virulence Testing of Ophiostomatoid Fungi

Inoculation experiments are most commonly used to assess the pathogenicity and virulence of ophiostomatoid fungi to their plant hosts (Ben Jamaa et al. 2007; Bertagnole et al. 1983; Cobb and Platt 1967; Harrington and Cobb 1983). Observations on the success and potential fitness of the fungus (Hessburg and Hansen 2000) as well as the subsequent host reaction following infection (Cook and Hain 1985), can be easily made during inoculation experiments. Inoculation studies also facilitate the completion of Koch's postulates, used to confirm the causal relationship between disease and associated microorganisms (Agrios 2005). Other types of experiments may test particular portions of the reaction between pathogen and host. For example, pine oleoresin pressure (Lorio and Hodges 1968) and monoterpene composition (Bridges 1987) are often measured to assess potential host defense against invading pests. Also, fungal physiological bioassays' provide fungal growth characteristics and give an indication to parasitic abilities (Leaphart 1956). Fungal growth is measured in the presence of common host secondary defense metabolites found in oleoresin (Himejima et al. 1992; Kopper et al. 2005; Paine and Hanlon 1994). All other types of experiments attempt to replicate the reaction or certain portions of the reaction under controlled conditions. However, inoculation experiments' provide the most direct opportunity for observation and measurement of the host-pathogen reaction.

Ophiostomatoid fungi have been inoculated in a wide variety of conifer hosts representing trees in the seedling, sapling, and full grown, mature tree stages (Kirisits and Ofeenthaler 2002; Kuroda 2005; Owen et al. 1987). The vast majority of ophiostomatoid species are found inhabiting large, mature trees in the forest. Following isolation from diseased tissue, many researchers have chosen to inoculate identically-sized hosts in which the fungus was found (Långström et al. 2001; Rice et al. 2007). These studies

provide the opportunity to observe a clear replication of local symptomology, following inoculation. However, there are several reasons why inoculation of large, mature trees may not be appropriate or possible. The disease triangle clearly depicts three main factors leading to disease, including the pathogen, host, and environment (Tainter and Baker 1996). Inoculation experimentation attempts to observe and characterize the reaction between two factors, the pathogen and host. Therefore, a carefully controlled environment is necessary to provide the greatest opportunity for an accurate characterization of the relationship. Full grown, mature trees in the forest often have significant inter-tree variability (Lieutier et al. 1993), caused primarily by microsite factors (Lorio and Hodges 1968). In an attempt to carefully control environmental conditions, inoculations have commonly been made on either sapling or seedling-sized hosts. Although sapling and seedling inoculations allow for more environmental control over the experiment, the observations may be less accurate. For example, the primary host defense against invading microorganisms is the ability to synthesize and deliver the potent antimicrobial compounds found in oleoresin (Nebeker et al. 1993). Traumatic resin ducts, formed in the xylem tissue, allow for efficient delivery of the oleoresin compound. Young trees (i.e. sapling and seedling-sized) do not have the well-developed system found in older, larger individuals. Sandnes and Solheim (2002) found variation in the hypersensitive response to exist among individuals of different sizes within a stand. Also, it is well known that host defense systems in conifers are dependent on host vigor (Raffa and Beryman 1982a) and carbohydrate reserves (Nebeker et al. 1993), both of which are lower in sapling and seedling-sized individuals. Therefore, sapling and seedling-sized individuals are inherently more susceptible to certain disease-causing agents. Results and observations from sapling and seedling inoculations must be carefully interpreted when assessing pathogenicity and virulence.

1.8.4. Tree Seedling Inoculations

Tree seedling inoculation experiments are most commonly used to assess the pathogenicity (Wingfield 1986) and in some cases the relative virulence (Krokene and Solheim 1998a), of ophiostomatoid species to their hosts. Stem inoculations of host seedlings are commonly performed due to the ease of inoculation (Nevill and Alexander

1992a). In some instances, toothpicks (Wingfield 1986) or wooden blocks (Owen et al. 1987) are colonized with mycelium before inoculation in small wounds. However, more commonly mycelium grown on artificial media agar is aseptically placed in wounds directly (Nevill et al. 1995; Paine 1984a; Rane and Tattar 1987). Root inoculations of host seedlings have also been performed using several different methods. Roots have been dipped with or without wounding in fungal mycelium (Hessburg and Hansen 2000) or spore suspensions (Lackner and Alexander 1982). The taproots of host seedlings have also been inoculated with or without wounding (Harrington and Cobb 1983). Inoculum has been administered with both colonized wooden blocks (Witcosky and Hansen 1985) and colonized artificial nutrient agar (Webber and Hansen 1990).

Several consistent symptoms have been observed following inoculation of host seedlings. In some instances, fungal pathogens have caused mortality in wounded and unwounded inoculations (Harrington and Cobb 1983). However in many cases, mortality following inoculation was not significant (Nevill and Alexander 1992a; Wingfield 1983; Wingfield 1986). Foliar symptoms associated with ophiostomatoid fungal infection includes needle chlorosis (Rane and Tattar 1987), shortened needles, and in some instances wilting (Webber and Hansen 1990). Unwounded inoculations with *L. wageneri* var *pseudotsugae* have shown that hyphae enter plant organs through wounds and natural openings and appear to be incapable of penetrating cortical cells (Hessburg and Hansen 2000). Also, conidia appear to only germinate and develop germ tubes on wounded roots, with no germination on unwounded tissue (Diamandis et al. 1997). These observations support the ecology of ophiostomatoid fungi as wound pathogens. Following entrance into the host, discoloration of the inner bark (Rane and Tattar 1987), xylem tracheids (Harrington and Cobb 1983) and phloem (Krokene and Solheim 1998b) was consistently observed leading to development of darkened, resin-soaked lesions (Wingfield 1986). Penetration of vascular cells resulted in a hypersensitive response from the host through evidence of visual occlusion (Nevill et al. 1995). The combined lesion and occlusion development in the vascular cells has been shown to limit water conduction (Paine 1984a), leading to a decrease in plant water potential (Rane and Tattar 1987) and an overall increase in plant water stress. Inoculation of plant host seedlings

with ophiostomatoid fungi consistently results in a distinct set of symptoms associated with vascular blockage, in some cases leading to mortality.

1.8.5. Large, Mature Tree Inoculations

Inoculation studies involving larger, mature trees are commonly used to test the pathogenicity and virulence of ophiostomatoid fungi (Krokene and Solheim 1998b; Solheim et al. 1993). Host response to fungal inoculation is commonly observed (Cook and Hain 1987), leading to local tissue symptomology associated with ophiostomatoid fungal infection (Popp et al. 1995a). The primary inoculation method involves a single wound, using the cork-borer method (Wright 1933), followed by insertion of actively-growing fungal mycelia.

Fig. 1.9. Wound inoculation with actively growing fungal mycelium in longleaf pine stem as described by Wright (1933).

Ophiostomatoid fungal transmission often requires an insect vector, generally a conifer-feeding member of the Curculionidae (Coleoptera)(Jacobs and Wingfield 2001). Artificial wound inoculations attempt to replicate wounding and subsequent natural inoculation that follows bark beetle attack. In contrast, other root pathogens spread from tree to tree through the soil and must penetrate the constitutive physical defenses of the root without the assistance of an insect. For example, *H. annosum* has been shown to invade root tissue through four pathways, including direct penetration of cortical cells by a single hyphal tip following enzymatic degradation of the cell wall (Werner et al. 2005). Following introduction to the root by insects, ophiostomatoid fungi must germinate on

susceptible tissues in order for inoculation to be successful. However, few inoculation studies have used spore suspensions in wound inoculations (Lackner and Alexander 1982; Otrosina et al 2005). More commonly, the inoculum source is actively growing mycelium (Klepzig et al. 2005; Krokene and Solheim 1998a), in either an artificial media broth (Cook and Hain 1987) or agar disks (Fernandez et al. 2004), often containing spores (Croisé et al. 1998a). Artificial wound inoculations have also been performed in order to simulate a mass inoculation following a bark beetle vector mass attack (Croisé et al. 2001; Krokene and Solheim 1998b). Similar to single inoculations, the cork-borer method is most commonly used when determining threshold attack densities, however up to 800 holes m^{-2} are required (Lee et al. 2006). The bark flap method (Strobel and Sugawara 1986) has been shown to be three times faster and therefore, may be an efficient alternative (Kim et al. 2008).

Artificial wound inoculations may be performed on tree roots (Bertagnole et al. 1983), or more commonly on tree stems (Bois and Lieutier 2000). Many bark beetle-fungus associations involve damaging beetle species that attack above-ground portions of the tree (Schowalter and Filip 1993). Fungal associates are introduced upon attack of stem tissue, penetrating the vascular tissues (Solheim 1995). In these instances, fungal inoculation of tree stems is appropriate. However, some common bark beetle species attack predominantly below-ground root structures (Wood 1982). Commonly, they transport blue-stain fungal associates to their gallery and feeding sites (Witcosky 1985). In the past, some studies have inoculated tree stems (Eckhardt et al. 2004a) and branches (Zhou et al. 2002) with root-inhabiting fungal associates of below-ground vectors. However, some other studies have inoculated mature tree roots with root-inhabiting fungal associates (Otrosina et al. 2002; Wingfield and Knox-Davies 1980). Despite the complexity and additional effort required, root inoculations may be more appropriate when testing the pathogenicity and virulence of root-inhabiting ophiostomatoid fungi.

A common host response and local tissue symptomology is observed following inoculation of mature trees. Invasion of the phloem causes rapid desiccation and necrosis of the cells followed by the release of terpenes and polyphenols (Raffa and Berryman 1982b). Blue-stain fungal infection commonly induces a significant host response in the sapwood, known as the hypersensitive reaction (Beryman 1972). Upon tissue invasion,

resin is synthesized and deposited in infected and non-infected tissue surrounding the invasion. Conifer secondary chemicals penetrate cells, ultimately killing both infected and non-infected neighboring tissue. As the invading fungus advances, tissue is impregnated with fungitoxic and fungistatic secondary compounds. The resulting lesion is darkened necrotic phloem tissue with pitch-soaked sapwood surrounding the point of inoculation and infection. With the exception of *L. wageneri* and few others, blue-stain fungi invade the living and non-living sapwood in a wedge-shaped appearance (Parmeter et al. 1992), often impeded by ray cell formed in secondary tissues. Lesions also extend both laterally and vertically or longitudinally from the inoculation point on the surface of phloem (Bleiker and Uzunovic 2004). Lesion formation and the impregnation of functional xylem with secondary compounds ultimately cause a loss of hydraulic conductivity (Joseph et al. 1998). Once inoculated fungi have successfully moved radially, surrounding the organ, tissue conductivity falls to zero and the organ becomes non-functional. Mass inoculations have shown the loss of tissue conductivity leads to foliar symptoms including, shortened chlorotic needles, thinning crowns, and mortality (Fernandez et al. 2004).

Chapter 2

Variation in Virulence among Four Root-Inhabiting Ophiostomatoid Fungi on *Pinus taeda* L., *P. palustris* Mill., and *P elliottii* Engelm. Seedlings

2.1. Abstract

Ophiostomatoid fungi have been recently implicated in root disease of pines in the southeastern United States. To determine their virulence, inoculation studies were conducted on loblolly (*Pinus taeda*), longleaf (*Pinus palustris*), and slash pine (*Pinus elliottii*). One year-old bareroot seedlings were wound-inoculated with one of four prominent North American ophiostomatoid fungal species. After three months, a darkened lesion, extending from the point of inoculation was observed for all species. *Grosmannia huntii* (*L. huntii*) caused the greatest lesion and occlusion length in loblolly pine and slash pine. *Leptographium procerum* and *L. terebrantis* caused similar lesion and occlusion lengths and were the smallest among the fungal treatments. These studies indicate clear virulence differences among the four North American fungi. *Grosmannia huntii*, previously not known to be pathogenic, caused significant damage compared to other well-known *Leptographium* species. Finally, lesion and tissue occlusion lengths were significantly smaller in longleaf pine for all fungal species when compared to loblolly and slash pine.

2.2. Introduction

Root-inhabiting ophiostomatoid fungi have caused disease in many pine systems throughout the world (Wingfield et al. 1988). In some instances, ophiostomatoid fungi act as primary pathogens, causing mortality to their host. *Leptographium wageneri* (W.B. Kendr.) M.J. Wingf, a virulent primary pathogen, causes extensive pine mortality

throughout the northwestern United States (Cobb 1988). In other cases, ophiostomatoid fungi may only act as stressors, in a larger complex, that ultimately leads to tree mortality (Otrosina et al. 2002). Root-inhabiting ophiostomatoid fungi have been identified as contributors to pine decline in several systems, including red pine (*Pinus resinosa* Ait) in the great lake states (Klepzig et al. 1991), eastern white pine (*P. strobus* L.) in the northeastern United States (Dochinger 1967) and most recently, loblolly pine (*P. taeda* L.) in the southeastern United States (Eckhardt et al. 2007).

Several ophiostomatoid fungi with *Leptographium* anamorphs, including *L. procerum* (Kendrick) M.J. Wingfield, *L. terebrantis* S.J. Barras & T.J. Perry, *L. serpens* (Goidanich) Siemaszko, and *Grosmannia huntii* (R.C. Rob. Jeffr.) Zipfel, Z.W. de Beer & M.J. Wingf. (*L. huntii* M.J. Wingfield) have been isolated from the roots declining pine throughout the southeastern United States, including Georgia (Menard et al. 2006), Alabama (Eckhardt et al. 2007), and South Carolina (Otrosina et al. 2002). *Leptographium procerum*, *L. terebrantis*, and *L. serpens* have been consistently associated with symptomatic loblolly pine (Eckhardt et al. 2007). Each *Leptographium* species has been isolated from longleaf pine (*P. palustris* Mill.) root tissue (Zanzot 2009). *Grosmannia huntii* (formerly *Ophiostoma huntii* [Zipfel et al. 2006]) has recently been isolated from loblolly root tissue and bark beetles breeding in pine hosts (Matusick and Eckhardt unpublished data). While root disease and decline associated with *Leptographium* species have not been well documented in slash pine (*P. elliottii* Engelm.), *L. procerum* has been isolated from stump (Barnard et al. 1991) and root tissues (Horner and Alexander 1983).

Leptographium procerum and *L. terebrantis* are two well known ophiostomatoid fungal root pathogens of North American pines (Wingfield et al. 1988). *Leptographium procerum* is the causal agent of procerum root disease in eastern white pine (Dochinger 1967) and is associated with various conifer species around the world (Jacobs and Wingfield 2001), particularly *Pinus* species within the United States (Alexander et al. 1988). In inoculation studies, *L. procerum* has been reported to be weakly virulent (Wingfield 1983). *Leptographium terebrantis* is only found in North America and has been associated with various diseases of pine (Jacobs and Wingfield 2001). *Leptographium terebrantis* consistently causes resin-soaking (Nevill et al. 1995),

sapwood discoloration (Rane and Tattar 1987), and long vertical lesions in *Pinus* hosts (Wingfield 1986). *Leptographium terebrantis* is considered a moderate to severe pathogen, often causing mortality (Harrington and Cobb 1983). In loblolly pine seedling inoculations, Eckhardt et al. (2004a) found *L. procerum* to readily infect root tips and cause root and foliar dieback. In the same study, *L. terebrantis* caused darkly stained lesions measuring 20 mm after four months.

Leptographium serpens and *G. huntii* have been less commonly reported in North American pines. However, Eckhardt et al. (2007) isolated *L. serpens* from loblolly pine where it was associated with decline symptoms and root-feeding insect vectors. *Grosmannia huntii* has previously been found invading *Pinus* hosts of more northern latitudes in North America (Olchowecki and Reid 1974) and has been closely associated with damage caused by insects. *Hylastes porculus* Erichson vectors the fungus in red pine stands (Klepzig et al. 1991) and mountain pine beetle (*Dendroctonus ponderosae* Hopk.) in lodgepole pine (*P. contorta* Douglas var. *latifolia* Engelmann)(Solheim 1995). Despite some evidence that *L. serpens* is moderately to severely virulent to *Pinus* species (Eckhardt et al. 2004a), some consider *L. serpens* to be a weak pathogen in South Africa (Zhou et al. 2002). Unlike other *Leptographium* species, no information is available pertaining to the virulence of *G. huntii*.

Past studies with *Leptographium* species in the southeastern United States have mainly focused on inoculations with *L. procerum* and *L. terebrantis* on loblolly (Lackner and Alexander 1981a) and longleaf (Otrosina et al. 2002) pine. More recently, *L. serpens* has been included in inoculations of loblolly pine (Eckhardt et al. 2004a). However, the relative virulence of ophiostomatoid fungi in the southeastern United States is not known. These studies were initiated in order to test the hypothesis that root-inhabiting ophiostomatoid fungi are equally virulent to southern pine hosts.

2.3. Materials and Methods

Isolates of *L. procerum*, *L. terebrantis*, *L. serpens*, and *G. huntii* were obtained from either loblolly or longleaf pine roots (Table 2.1) exhibiting decline disease symptoms. Primary lateral roots were excavated and tissues were obtained using methods described in Eckhardt et al. (2007). All isolates used in the inoculation tests

were in the anamorphic state, were from single-spore isolations and have been used in other studies (Eckhardt et al. 2004a, 2008).

Bareroot seedlings of loblolly pine, slash pine and longleaf pine were obtained from the Smurfit Stone Rock Creek Nursery near Brewton, Alabama. A total of 250 bareroot seedlings of each species were planted with ProMix BX® (Premier Tech, Quebec, Canada) peat-based potting mix in one-gallon plastic pots one week following lifting in December 2007. A two-factor experiment, including three pine hosts and the four fungal species, was housed in an outdoor screen facility on the Auburn University campus. The building environment was homogeneous, unobstructed from sunlight and accessible to natural precipitation. After eleven weeks, prior to imposing the inoculation treatment, dead trees were removed from each group leaving a total 225 seedlings of each pine species respectively to be used in the experiment. In December of 2008, the study was repeated using a total of 225 seedlings per species (or 200 seedlings after culling).

The four fungal treatments and an unwounded control were randomly assigned to an equal number of seedlings. Each fungal isolate was placed on 2% malt extract agar (MEA) two weeks prior to inoculation. Seedlings assigned to the four fungal treatments were wound inoculated in the lower stem approximately 2 cm from the soil line. A small (1 cm) vertical slit was made with a sterile razor blade extending into the vascular tissues, followed by placing a 3 mm diameter plug of colonized MEA in the wound (Fig. 2.1). The inoculation was wrapped in moist cotton and sealed with Parafilm®, as described by Eckhardt et al. (2004a).

Table 2.1. Fungal isolates used in pine seedling inoculation experiment

Fungal Species	Isolate no./ ATCC accession no.	Collection Site	Host Source
G. huntii	LLP-R-02-100/ MYA-3311	Fort Benning Military Reservation, GA	Longleaf Pine Root
L. serpens	LOB-R-00-309/ MYA-3315	Westervelt Company Land, AL	Loblolly Pine Root
L. terebrantis	LOB-R-00-805/ MYA-3316	Talladega National Forest, Oakmulgee Ranger District, AL	Loblolly Pine Root
L. procerum	LOB-R-00-456/ MYA-3313	Talladega National Forest, Oakmulgee Ranger District, AL	Loblolly Pine Root

Note: All fungal isolates were obtained from trees exhibiting symptoms characteristic of root disease

Fig. 2.1. Loblolly pine seedling wound inoculated with *L. terebrantis* in the lower stem.

At the culmination of the study, a subset of five seedlings was randomly selected from each treatment x pine group for pine needle water potential measurements. Measurements were made at predawn and midday on one seedling from each group for five straight days in 2007 using a pressure chamber (Model 670, PMS Instrument Inc. Albany, OR). In 2008 measurements were made over a 3 day period. Two fascicles from the first flush of the current year on each plant were measured, and then averaged to obtain one value for each tree at both predawn and midday.

Twelve weeks after inoculation, seedlings were destructively sampled and the final root collar diameter was measured. The lateral and fine roots were removed from the seedlings and the biomass was weighed after drying for 3 days at 70° C. The stem and taproot biomass was determined after lesion and occluded tissue measurements. Living seedlings were inspected for the presence of cambial lesions and the lesion length and length of occluded (blocked) vascular tissue was measured. Lesion length was considered the total length of darkly pigmented tissue and may or may not have extended the total length of the wound. To determine the length of occluded tissue, the living shoot was placed in a FastGreen stain (FastGreen FCF; Sigma Chemical Co.) and water

solution (0.25 g/liter) (adapted from Nevill et. al. [1995]). After three days, the length of stem tissue not stained by solution was recorded. A 1 cm segment of stem tissue at the lesion margin was removed and placed on CSMA (MEA containing 800 mg/l of cycloheximide and 200 mg/l of streptomycin sulfate) to confirm the fungal infection in inoculated trees.

Seedling host response variables were analyzed using a general linear model (GLM) in SAS statistical software (SAS Institute, 9th ed., Cary, NC). All binary response variables, including survival, lesion presence and re-isolation of fungal species were transformed to percentages for each treatment x pine species combination. All continuous response variables, including root collar diameter, stem and fine root biomass, lesion length and occlusion length were analyzed using the seedling as the experimental unit. Stem and fine root biomass values were transformed using the square-root function to ensure a normal distribution. In the model, each experiment (2007, 2008) was considered a replicate (blocked factor). Both testable factors, including pine and fungal species as well as their interaction were included in the linear model. All pair-wise comparisons were analyzed using Tukey's multiple comparison test, followed by contrast statements. When testing water potential measurements, the two years were combined and the day of measurement was considered blocked. Treatment and tree main effects along with their interaction were included as testable factors.

2.4. Results

Within a given pine species, the assigned treatments varied in ther effect on RCD and seedling stem biomass (Table 2.2). Tree species and treatment independently affected the biomass of the fine root tissue. Seedlings inoculated with *G. huntii* had the smallest mean RCD among the treatments in longleaf pine; however, the same affect was not observed in other pines (Table 2.3). Seedlings inoculated with *L. serpens* resulted in slightly more stem mass compared with those inoculated with *G. huntii*. The same difference was not observed in slash and loblolly pines. Longleaf pine seedlings had the greatest lateral and fine root biomass among the tree species (Table 2.4) and seedlings assigned to the *L. procerum* treatment had the highest biomass among the treatments (Table 2.5).

Mortality was observed throughout each experiment in all pine species. Seedling survival was significantly different between pine species (F=15.73, P=0.0003)(Table 2.6). Slash and longleaf pine had less seedling survival when compared to loblolly pine (Table 2.7). Inoculation with fungi did not affect seedling survival (F=0.53, P=0.7177).

Fig. 2.2. Darkened lesion following inoculation with *L. serpens*.

All fungal species caused dark sunken or sometimes slightly raised lesions in all pine species tested (Fig. 2.2). Lesions extended vertically from the wounded area, with little evidence of radial movement. Callus tissue was associated with fungal inoculation, most notably in seedlings treated with *L. procerum* and *L. terebrantis* (Fig. 2.3). Within each pine species, the presence of lesions was different among fungal species (F=5.66, P=0.0066). Lesions were detected in nearly all pines inoculated. However in longleaf pine, *L. procerum* and *L. terebrantis* caused lesions in 80 and 85 percent of seedlings respectively, fewer than other treatment x host combinations.

Fig. 2.3. Callus tissue formed surrounding the point of inoculation with *L. procerum* (Left) and *L. terebrantis* (Right).

The average lesion length varied among fungal species, within a given pine host (F=8.53, P<0.0001). Lesion development following fungal inoculation was poorest in longleaf pine for each species. The inoculation of *G. huntii* into pine seedlings resulted in the largest average lesion lengths, though not different from *L. serpens* in longleaf pine (F=0.10, P= 0.7572). Seedling lesions from *L. procerum* and *L. terebrantis* inoculation were the smallest among *Leptographium* species treated but not different from each other on loblolly and slash pine. Lesions found on *L. terebrantis* inoculations were longer than *L. procerum* in longleaf pine.

Similar to lesion length, *G. huntii* caused the largest occlusions in all pine hosts treated. *Leptographium serpens* caused the second greatest tissue occlusion in each of the pine species. In all pine species, *L. procerum* caused the smallest average tissue occlusion, often not significantly different from *L. terebrantis*. Occlusion length was smallest for each fungal species in longleaf pine.

All fungal species were successfully re-isolated from the inoculated pines with re-isolation percentage affected by the pine host (F=10.52, P=0.0028). Re-isolation was less successful in longleaf pine seedlings when compared to loblolly and slash pines. No

differences were observed in re-isolation among the four fungal species (F=2.77, P=0.0920).

Predawn and midday water potential measurements were not affected by fungal treatment but were different among tree species used (Table 2.8). Longleaf pine seedlings had significantly higher predawn and midday water potential measurements compared to loblolly and slash pine seedlings (Table 2.9).

Table 2.2. Probability of a greater *F*-statistic for seedling health parameters, root collar diameter (RCD), stem biomass, fine root biomass, the transformed variables, square root of stem biomass and fine root biomass.

Source	df	RCD (mm)	Stem Biomass (g)	Square-root Stem Biomass	Fine Root Biomass (g)	Square-root Fine Root Biomass
Replication	1	0.0001	0.2034	0.0679	0.0001	0.0001
Treatment	4	0.0029	0.1039	0.0526	0.0052	0.0011
Tree Species	2	0.0001	0.0001	0.0001	0.0001	0.0001
Treatment x Tree Species	8	0.0001	0.0079	0.0025	0.2497	0.1801
Error	1070					

Table 2.3. Root collar diameter (RCD), stem biomass, and transformed variable square-root of stem biomass for each tree x treatment combination.

Tree Species	Treatment	RCD (mm)	Stem Biomass (g)	Square-Root Stem Biomass
P. taeda	Control	5.40 (1.20)d	6.91 (3.60)	2.55 (0.64)c
	G. huntii	5.31 (1.04)d	6.89 (2.93)	2.56 (0.59)c
	L. serpens	5.35 (1.05)d	6.67 (2.92)	2.52 (0.55)c
	L. terebrantis	5.68 (1.18)d	6.71 (2.77)	2.53 (0.55)c
	L. procerum	5.51 (1.24)d	6.66 (2.66)	2.53 (0.51)c
	Average	5.45 (1.15)	6.77 (2.98)	2.54 (0.57)
P. elliottii	Control	5.40 (1.36)de	7.03 (3.68)	2.55 (0.71)c
	G. huntii	5.31 (1.46)d	7.06 (4.03)	2.54 (0.80)c
	L. serpens	4.66 (1.43)f	5.12 (3.04)	2.16 (0.68)c
	L. terebrantis	4.79 (1.18)ef	5.58 (2.84)	2.27 (0.67)c
	L. procerum	6.00 (1.54)d	7.87 (4.04)	2.72 (0.71)c
	Average	5.29 (1.47)	6.53 (3.68)	2.45 (0.74)
P. palustris	Control	11.09 (2.79)b	10.85 (5.78)	3.18 (0.86)ab
	G. huntii	10.59 (3.38)c	10.23 (4.78)	3.11 (0.78)b
	L. serpens	11.61 (3.34)ab	12.03 (6.26)	3.36 (0.88)a
	L. terebrantis	11.74 (2.94)a	11.19 (4.65)	3.26 (0.74)ab
	L. procerum	11.68 (3.27)a	11.96 (6.21)	3.35 (0.88)ab
	Average	11.34 (3.16)	11.26 (5.62)	3.25 (0.83)

Note**:** Means (followed by standard deviation in parentheses) within a column with the same letter are not significantly different from one another at the 0.05 level.

Table 2.4. Biomass of all lateral and fine roots as well as the square-root of the root biomass for each tree species.

Tree Species	Fine Root Biomass (g)	Square Root FR Biomass
P. taeda	1.91 (1.14)	1.32 (0.40)b
P. elliottii	1.91 (1.14)	1.19 (0.48)c
P. palustris	2.95 (2.24)	1.60 (0.62)a

Note**:** Means (followed by standard deviation in parentheses) within a column with the same letter are not significantly different from one another at the 0.05 level.

Table 2.5. Biomass of all lateral and fine roots as well as the square-root of the root biomass for each treatment.

Treatment	Fine Root Biomass (g)	Square Root FR Biomass
Control	2.33 (1.82)	1.42 (0.56)ab
G. huntii	1.97 (1.47)	1.30 (0.52)b
L. serpens	1.95 (1.60)	1.29 (0.54)b
L. terebrantis	2.17 (1.43)	1.39 (0.48)ab
L. procerum	2.42 (2.05)	1.46 (0.55)a

Note**:** Means (followed by standard deviation in parentheses) within a column with the same letter are not significantly different from one another at the 0.05 level.

Table 2.6. Probability of a greater *F*-statistic for survival, lesion presence, re-isolation, lesion length, and occlusion length following inoculation with four ophiostomatoid fungi.

Source	df[†]	Survival	df[‡]	Lesion	Re-Isolation	df[§]	Lesion Length	Occlusion Length
Replication	1	0.9861	1	0.7654	0.0345	1	0.0001	0.0001
Treatment	4	0.7177	3	0.0087	0.0920	3	0.0001	0.0001
Tree Species	2	0.0003	2	0.0001	0.0028	2	0.0001	0.0001
Treatment x Tree Species	8	0.1491	6	0.0066	0.9339	6	0.0001	0.0001
Error	14		11			853		

[†]Control seedlings were included in the analysis.

[‡]Control seedlings were omitted from the analysis

[§]All living seedlings were included in analysis

Table 2.7. Seedling survival, lesion occurrence, lesion length, sapwood occlusion length and pathogen re-isolation frequency after 12 weeks following inoculation.

Tree Species	Treatment	Survival (%)	Lesion (%)	Lesion Length (mm)	Occlusion Length (mm)	Re-Isolation (%)
	Control	91 (3)	NA	NA	NA	NA
	G. huntii	96 (6)	100 (0)a	21 (7.06)b	33 (9.83)a	89 (9)
P. taeda	*L. serpens*	98 (4)	100 (0)a	19 (5.45)c	28 (7.63)b	81 (2)
	L. terebrantis	99 (2)	100 (0)a	17 (5.17)de	25 (7.41)c	85 (6)
	L. procerum	99 (2)	100 (0)a	15 (4.32)e	22 (6.29)d	91 (13)
	Average	96 (4)a	100 (0)	18 (8.85)	27 (13.14)	86 (8)a
	Control	80 (3)	NA	NA	NA	NA
	G. huntii	84 (16)	100 (0)a	24 (11.34)a	32 (13.18)a	83 (7)
P. elliottii	*L. serpens*	63 (1)	100 (0)a	18 (9.50)cd	25 (13.29)c	79 (15)
	L. terebrantis	81 (21)	98 (0.2)a	13 (6.37)f	19 (9.65)d	88 (14)
	L. procerum	67 (4)	100 (0)a	13 (7.28)f	17 (9.50)de	96 (6)
	Average	75 (12)b	99 (1)	17 (10.17)	23 (13.48)	86 (11)a
	Control	82 (5)	NA	NA	NA	NA
	G. huntii	71 (12)	98 (1)a	10 (6.06)g	16 (9.46)ef	62 (6)
P. palustris	*L. serpens*	86 (6)	98 (3)a	10 (5.22)g	14 (7.20)f	83 (16)
	L. terebrantis	87 (1)	85 (7)b	7 (5.41)h	11 (7.31)g	70 (14)
	L. procerum	92 (1)	80 (7)b	6 (4.69)i	10 (7.11)g	79 (12)
	Average	84 (9)b	90 (9)	8 (5.59)	13 (8.25)	68 (12)b

Note: Means, followed by standard deviation in parentheses within a column with the same letter are not significantly different from one another at the 0.05 level.

Table 2.8. Probability of greater *F*-statistic for predawn and midday water potential measurements.

Source	df	Predawn	Midday
Measurement Day	7	0.0001	0.0001
Treatment	4	0.6830	0.9342
Tree Species	2	0.0001	0.0001
Treatment x Tree Species	8	0.4335	0.9977
Error	128		

Table 2.9. Mean predawn and midday water potential for each tree species.

Tree Species	Needle $\Psi_{predawn}$ (Mpa)	Needle Ψ_{midday} (Mpa)
P. taeda	-0.52a	-1.41a
P. elliottii	-0.49a	-1.26a
P. palustris	-0.39b	-0.94b

Note: Means within a column with the same letter are not different from one another at alpha = 0.05 based on the Tukey-Kramer multiple comparison test.

2.5. Discussion

All ophiostomatoid species tested were capable of successful infection and development of local symptoms in southern pine seedlings; however variation between fungal pathogens within certain hosts existed. *Grosmannia huntii* caused the longest lesions and occluded tissue in loblolly pine slash pines. These studies are first to confirm lesion development and damage following artificial inoculation with *G. huntii*. Lesions following inoculation with *L. terebrantis* and *L. procerum* were consistently smaller than other treatments.

An unwounded control was chosen based primarily on results of previous studies concerning many of the same ophiostomatoid fungi. In pine seedlings, it is well

established that wounding without the introduction of fungi results in only callus tissue, which encloses the wound without formation of a resinous lesion (Eckhardt et al. 2004a; Klepzig et al. 1995; Wingfield 1986). The wounding method used, causes minor, temporary damage and does not contribute to an increased mortality (Chapter 3). In addition, the pathogenicity of many of the same ophiostomatoid fungi has been established previously (Eckhardt et al. 2004a; Nevill et al. 1995). These tests were initiated to test virulence differences among the four ophiostomatoid fungi, with little interest in testing their ability to infect and produce lesions, compared to wounded controls.

Lesions were readily observed surrounding the point of inoculation in seedlings, with few exceptions. Discolored lesions often appeared sunken and commonly extended beyond the wound beneath the surface of the epidermis. Callus tissue (wound periderm) was present in loblolly pine and slash pine seedlings surrounding the wounded site only, clearly not extending to the lesion margin. Similar lesion morphology and occurrence have been observed in previous seedling inoculation studies with many of the same fungal species (Eckhardt et al. 2004a). Lesion occurrence was overall lower in longleaf pine seedlings inoculated with *L. procerum* and *L. terebrantis*. It was apparent that under some circumstances lesions failed to develop following inoculation. Longleaf pine is known to be more resistant to many other insect and disease pests (Snow et al. 1990), but these studies are the first to illustrate resistance to ophiostomatoid fungi. Longleaf pine resin has been shown to inhibit growth of ophiostomatoid fungi in vitro, particularly *L. procerum* (Eckhardt et al. 2008).

Lesions associated with fungal infection extended above and below the point of inoculation. Lesions were primarily oriented longitudinally with poor evidence of radial movement. Radial movement is most characteristic of highly virulent ophiostomatoid fungi such as *L. wageneri* (Cobb 1988) and in some instances *L. terebrantis* (Wingfield 1983). Lesions associated with *G. huntii* were larger than *L. serpens* in loblolly and slash pine seedlings. However, *G. huntii* and *L. serpens* infection were not significantly different in longleaf pine. *Leptographium serpens* has been previously observed causing mortality in *Pinus* species as well as similarly large lesions in controlled experiments (Wingfield and Knox-Davies 1980). These inoculation studies represent the first report

of *G. huntii* causing significant damage in pine tissue, following artificial inoculation. *Grosmannia huntii* is known as a proficient sapstainer in large pine trees and logs (Robinson-Jeffrey and Grenchenko 1964; Kim et al. 2005b); however it has not been shown to be pathogenic. Lesions following *L. terebrantis* and *L. procerum* inoculation were consistently smaller than those formed from *G. huntii* and *L. serpens*. In similar studies with loblolly pine seedlings, *L. terebrantis* was shown to cause larger lesions than *L. procerum* (Eckhardt et al. 2004a; Nevill et al. 1995). Previous studies have found smaller lesions associated with *L. procerum* when compared to other *Leptographium* species (Wingfield 1983). These new inoculation trials support previous findings that consider *L. procerum* a mild pathogen to *Pinus* species (Harrington and Cobb 1983).

Tissue occlusion is often observed associated with invasion by root-inhabiting ophiostomatoid fungal species (Wingfield and Knox-Davies 1980). Occlusion of vascular tissue has been detected in the past as a measure of host response to infection (Nevill et al. 1995). Generally, occlusion length closely mirrors measures of lesion length and gives supportive evidence to the virulence of ophiostomatoid species (Eckhardt et al. 2004a). Occlusion length was greatest in loblolly and slash pine seedlings infected by *G. huntii*. Tissue occlusion was smaller in seedlings inoculated with *L. terebrantis* and *L. procerum*, when compared to *G. huntii* and *L. serpens*. Similar trends were observed by Eckhardt et al. (2004a) when determining the occlusion of several of the same *Leptographium* species to loblolly pine. Comparable occlusion lengths were observed in a previous experiment with *L. serpens* inoculations of longleaf pine seedlings (Chapter 3).

Consistent re-isolation of inoculated ophiostomatoid species confirms the ability to infect and grow within pine host tissue. However, re-isolation of fungal species from longleaf pine seedlings was statistically lower compared to loblolly and slash pine. Longleaf pine is resistant to several insect and disease pests (Snow et al. 1990), including root disease (Hodges 1969). Recent observations confirm that growth of *Leptographium* species are negatively affected by constitutive longleaf pine resin (Eckhardt et al. 2008). Fungal growth was least affected by loblolly pine resin and more impeded when in the presence of longleaf pine resin. The virulence data coupled with previous findings

suggest that longleaf pine resin may restrict movement within longleaf pine tissue, making it more resistant of ophiostomatoid fungal infection and growth.

Despite development of local symptomology, infection and tissue damage as a result of fungal infection did not result in conclusive variation in whole-tree symptomology. In living seedlings, root collar diameter and stem + taproot biomass was relatively consistent across treatments. Although longleaf pine seedlings inoculated with *G. huntii* were found to have a slightly smaller mean RCD, significant variation was observed between the seedling stock used. Since no pretreatment RCD measurements were taken, the slightly smaller average RCD cannot be fully attributed to *G. huntii*. In addition, inoculation with fungi had no significant effect on seedling water potential, suggesting damage was not causing increased tension in the vascular column. The lack of whole-tree symptomology in inoculated trees resulted in mortality values similar to controls. It is probable that if the experiment were longer in duration or more inoculations were imposed on each seedling, whole-tree symptomology would have been detected. Rane and Tattar (1987) found significant losses of xylem pressure potential 15 days following inoculation of *L. terebrantis*. However, two inoculations were performed on either site of the stem, in contrast to just a single inoculation in this study. In addition, previous experiments that have noted significant treatment mortality were left for a longer duration (Harrington and Cobb 1983; Nevill et al. 1995; Wingfield 1983). Seedling measurements were larger for longleaf pine seedlings compared to loblolly and slash pine. These differences can be fully attributed to the unique phenology and growth habits of longleaf pine seedlings.

Grosmannia huntii produced larger lesion and occlusion lengths in loblolly and slash pine seedlings. In contrast, *L. procerum* and *L. terebrantis* caused the smallest average lesion lengths in loblolly and slash pine. Infection and virulence in longleaf pine seedlings were less apparent, when compared to other southern *Pinus* hosts. Despite smaller lesions, and in some cases, poorer infection in longleaf pine, ophiostomatoid fungi are capable of causing local symptomology similar to that observed in loblolly and slash pines. Disease symptomology was restricted to areas surrounding the infection point with poor evidence of significant xylem dysfunction and foliar symptoms after three months. Future studies concerning root-inhabiting ophiostomatoid species and

southern pine mortality should focus on large, mature trees and the role *G. huntii* plays in relation to the other more commonly published species.

Chapter 3

Virulence of *Leptographium serpens* on Longleaf Pine Seedling under Varying Soil Moisture Regimes

3.1. Abstract

Recently, *Leptographium serpens* has been recovered from the roots of declining and dead longleaf pine (*Pinus palustris*) in stands associated with various abiotic stresses. Although most data suggests *L. serpens* is pathogenic to various *Pinus* species, there is little known of its virulence on longleaf pine or its relationship with abiotic stress in causing disease. These trials examined the effects of *L. serpens* infection coupled with drought stress. Trials began with wound inoculations of bareroot longleaf pine seedlings in spring 2006 and 2007 at the seedling stress facility at Auburn University. Soon after inoculation, seedlings were also subjected to adequate moisture, moderate drought, or severe drought. Sixteen weeks after inoculation, longleaf pine survival, *L. serpens* virulence, and seedling growth characteristics were measured. Longleaf pine seedlings inoculated with *L. serpens* had 33% mortality (138/420) which was significantly greater than non-wounded control seedlings (22%, 47/211). Survival and lesion size on longleaf pine suggests that *L. serpens* is moderately pathogenic to longleaf pine seedlings. Separately, moisture stress associated with low soil moisture also contributed to seedling mortality. Results suggest *L. serpens* infection and moisture stress commonly experienced by southern pines act independently on longleaf pine.

3.2. Introduction

Longleaf pine (*Pinus palustris* Mill) was once the main southern pine species found throughout the southeastern United States, encompassing approximately 38 million hectares (Frost 1993). After nearly complete destruction of the longleaf pine ecosystem,

restoration efforts in recent years have increased the planting of longleaf pine on many state and federal lands (Kush et al. 2004). Many factors have contributed to this renewed interest, including the requirement of longleaf pine forest for endangered and sensitive species, specifically the red-cockaded woodpecker (*Picoides borealis* Vieillot)(Alavalapati et al. 2002). Unfortunately, decline and premature tree mortality has recently been observed in longleaf pine stands (Otrosina et al. 1999; Otrosina et al. 2002). The current rate of longleaf pine mortality may affect future restoration efforts.

Studies concerning decline and premature tree mortality in loblolly pine (*P. taeda* L.) have identified several possible contributing factors, including a group of root-inhabiting stain fungi in the genus *Leptographium* (Eckhardt et al. 2007). Despite the common theory that longleaf pine is "resistant" to native insect and diseases (Johnson 1999; Pritchard et al. 1997), it is hypothesized that *Leptographium* species may be contributing to this decline and mortality of longleaf pine (Otrosina et al. 1999; Otrosina et al. 2002).

Leptographium serpens (Goidanich) Siemaszko has been associated with various pine diseases throughout the world (Wingfield et al. 1988), including root disease of two *Pinus* species in South Africa (Wingfield and Knox-Davies 1980). In inoculation tests, *L. serpens* (formerly *Verticicladiella alacris* [Wingfield and Marasas 1981]) was found to cause 20 cm lesions on pine roots after six months (Wingfield and Knox-Davies 1980). In contrast, Zhou et al. (2002) found *L. serpens* to be nonpathogenic to *Pinus* branches in South Africa using a similar inoculation technique. Within the United States, the fungus has been identified as a contributor to loblolly pine decline (Eckhardt et al. 2007). In loblolly pine seedling inoculations, *L. serpens* was more pathogenic than both *L. terebrantis* and *L. procerum* (Eckhardt et al. 2004a), causing 30 mm sapwood lesions after four months.

Many *Leptographium* species have been found associated with pine decline and mortality, usually with one or more contributing site or stand factors (Eckhardt et al. 2007; Klepzig et al. 1991). These syndromes are most often referred to as decline diseases, characterized by many factors contributing to the prolonged death of the tree (Manion 1981). It is possible that multiple inciting biotic and abiotic factors may be acting in conjunction with *Leptographium* to cause the observed longleaf pine mortality.

Inadequate soil moisture is often experienced by southern pines and imposes a significant stress on individual pine trees. Successive, extreme and devastating droughts in recent years (2000 to 2007) along with the pathogen's presence may contribute to the observed tree mortality. These studies seek to determine the virulence of *L. serpens* to longleaf pine seedlings grown in three soil moisture regimes.

3.3. Materials and Methods

In all, 840 bareroot longleaf pine seedlings were used in inoculation trials with *L. serpens*. In January 2006 and 2007, pine seedlings were planted into 12 and 9 raised outdoor planting boxes (Fig. 3.1), respectively (40 per box). Planting boxes containing pure sand were housed under an open outdoor pavilion, which provide ambient sunlight, yet restricting natural precipitation. Seedlings were allowed to acclimate to their soil conditions with regular watering for two weeks prior to inoculation. Stem inoculations were administered with one isolate of *L. serpens* (LOB-R-00-309 / MYA-3315 Westervelt Company Land, AL) grown on 2% malt extract agar (MEA) plates.

Fig. 3.1. Longleaf pine seedlings planted in raised boxes experiencing three separate soil moisture regimes.

Fig. 3.2. Wound inoculation with *L. serpens* in the upper stem.

Within each planting box, twenty seedlings were wound inoculated with a 3-mm-diameter colonized plug of MEA (Fig. 3.2)(adapted from Eckhardt et al. [2004a]). Also within each box, ten seedlings were wounded and ten seedlings were unwounded.

Within each replication (3 boxes), one of three moisture treatments were randomly assigned to each box. The three watering treatments included adequate moisture (26 m^3 m^{-3}), moderate drought (18 m^3 m^{-3}) and severe drought (6 m^3 m^{-3}). Volumetric moisture levels were estimated (adapted from Turtola et al. [2003]), adjusted slightly for field capacity of pure sand and pine species. Soil moisture was regulated using moisture probe sensors, buried 10 cm below the soil line within each box and continuously measuring the dielectric constant. Solenoid valves, attached to each box,

controlled the flow of water. Six irrigation nozzles were placed evenly around the perimeter of each planting box in order to ensure equal water distribution.
Sixteen weeks after inoculation, seedlings were removed from the soil boxes and lesion length was measured on seedlings with identifiable lesions. To measure tissue occlusion length, the bark was removed from stem and root sections above and below the inoculation point. Seedlings were placed in a solution containing FastGreen FCF (Sigma-Aldrich) stain in water (0.25 g/liter) for several days (Nevill et al. 1995)(Fig. 3.3) and the length of the unstained tissue portion represented the occlusion length.

Fig. 3.3. Seedlings placed in FastGreen FCF stain solution.

Re-isolation was attempted on all seedlings with identifiable lesions by removing small portions of tissue surrounding the lesion and plating on MEA containing 800 mg/l of cycloheximide and 200 mg/l streptomycin sulfate. Seedling survival by treatment, dry-weight biomass of the fine roots, stem and taproot, root collar diameter and bud break was also measured.

Statistical analysis was conducted using SAS statistical software (SAS Institute, 9th ed., Cary, NC). Continuous variables were analyzed using a linear model in the General Linear Models procedure. Inoculation, moisture level, and trial year, including

interactions between each factor, were included in the model. The analysis was run separately for each year (2006 and 2007) for those responses that differed between years. Comparisons between inoculation or soil moisture levels were made by the Tukey-Kramer multiple comparison test. All binary variables (mortality and bud break) were transformed to percentages prior to analysis. All percentages were transformed using the square root of the arcsine function to ensure a normal distribution. Transformed binary variables were subsequently used in the linear model for comparison.

3.4. Results

Sixteen weeks after wounding and inoculation with *L. serpens*, longleaf pine mortality was 33% (Table 3.1). A significant difference in mortality was detected between the three inoculation treatments (F=6.61, P=0.0038). Mortality was greater within the wound-inoculated treatment when compared to both the wound (F=7.49, P=0.0098) and non-wounded (F=11.85, P=0.0015) controls. However, no difference was found between the wounded and non-wounded treatments (F=0.50, P=0.4860). Seedlings within the adequate moisture treatment had significantly less mortality than either the severe (F=180.64, P=0.0473) or moderate drought (F=164.82, P=0.0495) treatments (Table 3.2). Mortality differences were not observed between the severe drought and moderate drought treatments (F=0.36, P=0.6552). No significant interaction was detected between inoculation and moisture factors for any response variable measured, including seedling mortality (F=1.63, P=0.1888).

Table 3.1. Seedling mortality, root collar diameter (RCD), stem biomass, and bud break following inoculation with *L. serpens* and controls.

Treatment	Mortality (%)	RCD (mm)	Stem Biomass (g)	Bud Break (%)
Control (n=211)	6 (9)a	11.0 (2.4)a	11.64 (6.87)a	73 (20)a
Wound (n=209)	40 (30)b	10.2 (2.5)a	10.99 (5.71)a	58 (33)a
Inoculated (n=420)	41 (26)b	9.8 (2.5) a	10.47 (6.51)a	66 (24)a

Note: Means (followed by standard deviation in parentheses) followed by the same letter within a column are not different at alpha= 0.05.

Table 3.2. Seedling mortality, root collar diameter (RCD), stem biomass, and bud break in seedlings experiencing three soil moisture treatments.

Treatment	Mortality (%)	RCD (mm)	Stem Biomass (g)	Bud Break (%)
Adequate (n=280)	6 (9)a	11.0 (2.4)a	11.64 (6.87)a	73 (20)a
Moderate Drought (n=280)	40 (30)b	10.2 (2.5)a	10.99 (5.71)a	58 (33)a
Severe Drought (n=280)	41 (26)b	9.8 (2.5) a	10.47 (6.51)a	66 (24)a

Note: Means (followed by standard deviation in parentheses) followed by the same letter within a column are not different at alpha= 0.05.

Seedling health characteristics, including RCW, stem biomass, and bud break were not affected by either *L. serpens* inoculation (Table 3.1) or soil moisture level (Table 3.2). Stem and taproot biomass was significantly greater in seedlings not inoculated by *L. serpens* (F=3.18, P=0.0422). Also, biomass weights and root collar diameter were slightly less in seedlings under the low soil moisture treatment, though not significant.

Longleaf pine seedlings inoculated with *L. serpens* had dark-brown to black, sunken or slightly raised lesions surrounding the inoculation site. Resin was deposited in response to wounding and inoculation. Overall, 94% of living seedlings inoculated with *L. serpens* had lesions, with an average length of 9.3 mm. Lesion length on longleaf

seedlings inoculated in 2006 (11.3 mm) was significantly greater (F=9.06, P=0.0088) than lesions measured in 2007 (8.0 mm)(Table 3.3). Average lesion lengths tended to increase as soil moisture decreased; however treatment means were not statistically significant (F=0.51, P=0.6112)(Table 3.4). Seedlings produced callus surrounding the lesion.

Occluded stem tissue was detected above and below the inoculation site in advance of all lesions. Similar to the lesion lengths, soil moisture had no affect on levels of tissue occlusion observed in longleaf pine seedlings (F=1.78, P=0.2017). Occlusion length did vary between the two study years, with lengths shorter overall in 2007 (F=3.18, P=0.0422).

Table 3.3. Effects of inoculation of *Leptographium serpens* on lesion development, length, host response, and infection.

Treatment	Lesion Presence (%)	Lesion Length[y] (mm) 2006	Lesion Length[y] (mm) 2007	Occlusion Length (mm) 2006	Occlusion Length (mm) 2007	Re-Isolation (%) 2006	Re-Isolation (%) 2007
Control (n=211)	0.0	0.00	0.00	0.00	0.00	0	0
Wound (n=209)	3.9 (19.5)b	1.00 (0)b	6.20 (2.28)b	3.00 (0)b	8.80 (8.23)b	0	0
Inoculated (n=420)	94.0 (23.8)a	11.29 (5.96)a	7.95 (3.05)a	18.99 (8.42)a	13.03 (6.18)a	100 (0)	85.6 (16.44)

Note: Numbers followed by the same letter within a column are not significantly different at *P*=0.05, based on Tukey-Kramer

Note: Variables with both years shown were found to have significant differences by experiment year and analyzed separately

Table 3.4. Lesion presence, length, length of occluded tissues, and *L. serpens* re-isolation following inoculations of seedlings experiencing three soil moisture treatments.

Soil Moisture	Lesion (%)	Lesion Length (mm)	Occlusion Length (mm)	Re-isolation (%)
Adequate (n=280)	95 (5)a	9.0 (4.81)a	17.3 (8.6)a	95.7 (5.0)a
Moderate Drought (n=280)	96 (5)a	9.9 (4.3)a	16.3 (7.0)a	91.8 (10.9)a
Severe Drought (n=280)	94 (7)a	10.0 (6.4)a	13.8 (7.7)a	90.8 (10.4)a

Note: Means (followed by standard deviation in parentheses) followed by the same letter within in a column are not different at *P*=0.05, based on the Duncan grouping multiple comparison test.

3.5. Discussion

These results suggest both *L. serpens* infection and low soil moisture negatively affect longleaf pine seedlings survival independently. Inoculation of longleaf pine seedlings with *L. serpens* and exposure to high levels of moisture stress contributed separately to mortality in longleaf pine seedlings. Previous pine seedling inoculation experiments with *Leptographium* species have resulted in conflicting results with respect to host mortality. Although some studies have reported that *Leptographium* species infection does not result in seedling mortality (Eckhardt et al. 2004a; Nevill et al. 1992a; Nevill et al. 1995), other inoculation trials have reported significant mortality (Harrington and Cobb 1983; Rane and Tattar 1987; Wingfield 1986). In this study, mortality of longleaf pine seedlings was significantly greater in those seedlings that were wounded and then inoculated with *L. serpens*. In addition to inoculation, the amount of soil moisture also contributed to seedling mortality. Seedlings experiencing low soil moisture were more susceptible to mortality compared to seedlings raised in adequate soil moisture conditions. The presence of *Leptographium* species in other *Pinus* species roots has been linked to abiotic stressors (Eckhardt et al. 2007; Klepzig et al. 1991; Otrosina et al. 2002) including, increasing slope (Eckhardt and Menard 2008) and limited moisture (Goheen et al. 1978). However, these trials indicate that soil moisture does not influence the severity of *Leptographium* species infection. Mortality in pine seedlings inoculated with *L. serpens* was not influenced by soil moisture.

The presence of a defined, darkened lesion surrounding the point of inoculation with *L. serpens* is consistent with other seedling inoculation studies (Eckhardt et al. 2004a; Harrington and Cobb 1983; Wingfield 1986). The presence of resin is similar to findings by Zhou et al. (2002) and callus tissue has been observed in inoculations with *L. procerum* (Wingfield 1983). Although *L. serpens* successfully colonized longleaf pine, growth within the seedling tissue was restricted to the wounded area. In most instances, fungal colonization of the seedling was minimal, particularly in seedlings with adequate moisture. Despite successful colonization by *L. serpens*, average lesion length differed between trial year (2006 versus 2007). Smaller lesion lengths and re-isolation success in 2007 could indicate a reduction in virulence that has been reported with another blue-

stain fungus, *Ceratocystis polonica* (Krokene and Solheim 2001). Wounding and mortality of longleaf pine seedlings confirm that *L. serpens* is capable of infecting and killing longleaf pine. However, the amount of mortality and average lesion length on adequately watered seedlings suggests *L. serpens* is a mild to moderate pathogen to healthy longleaf pine seedlings.

Tissue occlusion surrounding the inoculation site was associated with *L. serpens* infection. Occlusion length did not differ among the soil moisture treatments. In contrast, Croisé and Lieutier (1993) found a reduction in host response as water stress increases. Occluded tissues are formed by resin movement into the sapwood surrounding the point of infection. Generally this pathogen-induced defense strategy is considered an advantage; however evidence suggests blockage of the xylem tissues may significantly alter water transport (Horner and Alexander 1985; Joseph et al. 1998). In these trials, mortality was not affected by soil moistures in inoculated seedlings, suggesting xylem blockage was not severe enough to contribute to mortality. The occlusion length difference between study years illustrates a weaker seedling response in 2007, supporting the hypothesis that the *L. serpens* isolate lost virulence between study years.

Infection by *L. serpens* affected the production of new stem and taproot biomass compared to control seedlings. In field observations of pine affected by *Leptographium* species, crown growth often appears stagnant with thin, sparse crowns (Eckhardt et al. 2007; Klepzig et al. 1991; Menard 2007). In loblolly pine, reduced radial growth and crown density has been positively correlated with *Leptographium* infection and root deterioration (Eckhardt et al. 2007). It is thought that *Leptographium* infection significantly alters water and nutrient movement (Horner and Alexander 1985), which leads to the observed reduction in growth.

These inoculation and moisture stress studies indicate that both *L. serpens* infection and low soil moisture contribute to longleaf pine mortality. However, these data show no interaction between inoculation and soil moisture factors. Although both factors negatively affect the survival of longleaf pine seedlings, their affect was independent. On the forest scale, longleaf pine experiencing low soil moisture or *L. serpens* infection are under more stress, with an increased susceptibility to premature mortality. Future studies related to multiple stresses and host reactions are required to

better understand the premature pine mortality observed in the southeastern United States.

Chapter 4

The Pathogenicity and Virulence of Four Ophiostomatoid Fungi on Young Longleaf Pine Trees

4.1. Abstract

Insects feeding on the roots and lower stem of trees commonly vector ophiostomatoid fungi that infect hosts during feeding. In the southeastern United States, insect vectors transfer pathogenic ophiostomatoid fungi that cause disease in southern pines. Potted longleaf pines (*P. palustris*), of similar ages ranging from 58 to 198 cm in height, were inoculated in 2007 and 2008 in order to assess the pathogenicity (and virulence) of *Grosmannia huntii*, *Leptographium procerum*, *L. serpens*, and *L. terebrantis*. Seventeen weeks after inoculation, *L. terebrantis, L. serpens* and *G. huntii* caused significantly larger lesions and more sapwood discoloration than wound controls. *Leptographium terebrantis* caused significantly more sapwood discoloration than all other treatments. Despite significant sapwood occlusion after fungal inoculation, no reductions in needle water potentials were observed between treatments. All fungal species were successfully re-isolated from longleaf pine trees.

4.2. Introduction

Root-colonizing ophiostomatoid fungi in the genus *Grosmannia* (previously *Ophiostoma* Zipfel et al. [2006]) and their anamorphs *Leptographium* (Lagerberg & Melin), cause disease of conifers (Wingfield et al. 1988). *Leptographium wageneri* (W.B. Kendr.) M.J. Wingf., the causal agent of black-stain root disease, extensively colonizes the outer xylem of infested trees and causes mortality in many western conifer species (Cobb 1988). Procerum root disease (caused by *L. procerum* [Kendrick] M.J.

Wingfield), may girdle and kill many conifer species (Alexander et al. 1988), particularly white pine (*Pinus strobus* L.)(Dochinger 1967). *Leptographium* species have also been identified as contributors to decline diseases, specifically red pine (*P. resinosa* Ait) decline (contributed by *L. procerum* and *L. terebrantis* S.J. Barras & T.J. Perry Klepzig et al. 1991) and loblolly pine (*P. taeda* L.) decline (contributed by *L. procerum, L. terebrantis,* and *L. serpens* (Goidanich) Siemaszko Eckhardt et al. 2007).

Hylobius pales Herbst. (Pales weevil) and *Pachylobius picivorus* Germar. (Pitch-eating weevil) commonly feed in the lower stem and near the root collar of sapling-sized pines (Edmonds et al. 2000). In young longleaf pine plantations, weevil damage and wounds in the lower stem and *Hylastes* damage on the lateral roots have been observed (Matusick and Eckhardt unpublished data) and represent potential infection sites for fungi. Natural inoculation of young pines with ophiostomatoid fungi has been illustrated in other pine systems around the world. For example, in *Pinus radiata* D. Don seedlings, *Hylastes ater* (Paykull) has been shown to transmit ophiostomatoid species during feeding (Reay et al. 2002). Nevill and Alexander (1992c) observed the transmission of *L. procerum* to five year-old eastern white pine (*P. strobus* L.) by *Hylobius pales* adults. In addition, *L. procerum* has been isolated from various sapling-sized pines attacked by weevils (Wingfield 1983).

Longleaf pine (*P. palustris* Mill.) once occupied approximately 370,000 km^2 in the southeastern United States, but is now a relatively minor component of the ecosystem, residing on just 2% of its original range (Frost 1993). However, based on seedling production trends, however, planting of longleaf pine has been increasing (Hainds 2002). This is in part due to the species' relative resistance to several insect pests and diseases (Snow et al. 1990) and the habitat it provides for the endangered red-cockaded woodpecker (*Picoides borealis* Vieillot Brockway et al. 2005). Decline and premature mortality of longleaf pine have recently been observed in 30-45 year-old stands. *Leptographium procerum* and *L. terebrantis* have been associated with symptoms (Otrosina et al. 1999), similar to loblolly pine decline (Eckhardt et al. 2007). More recently, *L. serpens* and *G. huntii* (R.C. Rob. Jeffr.) Zipfel, Z.W. de Beer & M.J. Wingf were isolated from roots of symptomatic longleaf pine. *Leptographium* species are

commonly isolated from Curculionid beetles in longleaf pine stands, including *Hylastes* species, *Hylobius pales*, and *Pachylobius picivorus* (Zanzot et al. 2010).

There have been inoculation tests with ophiostomatoid fungi on longleaf pine. *Leptographium serpens* was found to cause mortality in longleaf pine seedlings grown for four months under varying soil moistures (Chapter 3). Also, inoculations with *L. terebrantis* and *L. procerum* caused necrotic lesions in the cambial zone on mature stems and roots (Otrosina et al. 2002). The objective of the current study was to determine the pathogenicity and relative virulence of four ophiostomatoid fungi (*L. procerum*, *L. terebrantis*, *L. serpens*, and *G. huntii*) to longleaf pine saplings. It is hypothesized that establishment of ophiostomatoid fungi can contribute to mortality in longleaf pine.

4.3. Materials and Methods

On November 27, 2006, 200 5-7 year old longleaf pine trees were obtained from a horticulture nursery in central Alabama. Trees ranged in height from 58 to 198 cm with an average root collar diameter of 5.3 cm (+/- 0.7 cm). The trees were potted in 18.9 liter (5-gal.) pots with a mixture of pine bark and sand and grown in full sunlight with access to natural precipitation, supplemented with irrigation as needed throughout the experimental period.

Two weeks prior to inoculations, single isolates of *L. procerum*, *L. terebrantis*, *L. serpens*, and *G. huntii* were placed on 2% malt extract agar (MEA). All isolates were in the anamorphic state and were obtained from roots of symptomatic loblolly or longleaf pines using methods described in Eckhardt et al. (2007) (Table 2.1). The identities of all isolates have been confirmed by Dr. Mike Wingfield at the Forestry and Agricultural Biotechnology Institute, South Africa using morphological and sequence data from the partial ITS operon, the β-tubulin region, and elongation factor-1a.

On April 3, 2007 and April 8, 2008, 100 trees were arranged in a single factor, completely randomized experiment. Each tree was randomly assigned to one of five treatments including wound inoculation with *L. procerum*, *L. terebrantis*, *L. serpens*, and *G. huntii*, as well as a wound control (n=20). Wound inoculations were made approximately 10 cm above the soil line using the cork-borer method (Wright 1933). Either one inoculation or control was performed on each tree (Fig. 4.1). A 14 mm

diameter cork borer was used to remove a plug of bark tissue and a 10 mm diameter plug of actively growing mycelium with minimal sporulation was placed against the exposed cambium. The bark plug was replaced to cover the mycelium and duct tape was wrapped around the point of inoculation to minimize desiccation and contamination.

Fig. 4.1. Potted longleaf pine trees following wound inoculations with four ophiostomatoid fungi and control.

Seventeen weeks after inoculation, predawn and midday needle water potential measurements were taken on a subset of five randomly selected saplings per treatment. Two fascicles were selected from each tree for both predawn and midday water potential measurements using a pressure chamber (Model 670, PMS Instrument Inc. Albany, OR). Needle water potential measurements were averaged to give one predawn and midday value per tree.

Trees were destructively sampled seventeen weeks after inoculation (July 31, 2007 and August 5, 2008) to quantify fungal infection and host response. All bark was removed from the area surrounding the point of inoculation and necrotic lesions were traced on transparent sheets. Next, trees were cut transversely, through the point of

inoculation and total sapwood area and discolored sapwood were traced on transparent sheets. Lesion length and lesion depth were measured. Lesion surface area and total discolored sapwood area were determined using a LASICO planimeter (LASICO Co. Los Angeles, CA). The ratio of discolored sapwood area to total sapwood area was used to calculate the percentage of discolored sapwood for each sample tree. Finally, small tissue samples surrounding the point of inoculation were surface-sterilized and placed on CSMA (MEA containing 800 mg/l of cycloheximide and 200 mg/l of streptomycin sulfate) for re-isolation of fungi.

All continuous variables, including root collar diameter, height, lesion length, lesion depth, lesion surface area, discolored sapwood, and predawn and midday water potential measurements were analyzed with ANOVA using the GLM procedure in SAS (SAS Institute, 9.1 ed., Cary, NC). Each response variable was used in a simple linear model testing inoculation treatment. Experiment year (replication) was also included in the model as a blocked variable. Individual treatments were compared using Tukey's multiple comparison test. The log transformation was made on the lesion area variable due to non-normally distributed data. Re-isolation results (binary) were tested using the logit model in the Genmod procedure in SAS. The model included both inoculation treatment and experiment year similar to the linear model.

4.4. Results

Four trees died during the experiments, one each of the following treatments, *L. terebrantis, L. serpens, G. huntii* and control. Immediately after death, the area surrounding the point of inoculation was uncovered and observed. In each case, no evidence was found to suggest the treatment was responsible for mortality; however *L. terebrantis, L. serpens,* and *G. huntii* were recovered from their respective stems. The cause of death was ultimately not determined. No treatment differences were observed with respect to the growth of root collar diameter ($F = 0.54$, $P = 0.71$) or tree height ($F = 1.21$, $P = 0.31$) (Table 4.1).

Lesion length (F=8.73, P<0.0001) and lesion surface area (F=6.84, P<0.0001) were affected by treatment in the linear model (Table 4.1). Dark brown to black, discolored, resin-filled tissue (lesion) was observed surrounding the point of inoculation

(Fig. 4.2). In contrast, stem tissue surrounding wound controls was lightly colored with a faint brown ring surrounding the wound (Fig. 4.3). *Leptographium terebrantis* and *G. huntii* induced longer lesions than controls. All ophiostomatoid fungi, with the exception of *L. procerum*, caused the development of larger lesions than controls (with respect to total lesion area cm^2) (Table 4.2). All fungi were isolated from tissue surrounding lesions. However, infection was confirmed on only 20 percent of trees inoculated with *L. terebrantis*, *G. huntii*, and *L. serpens* (40 percent on trees inoculated with *L. procerum*).

Fig. 4.2. Stem inoculated with *L. terebrantis*.

Fig. 4.3. Wounded control

Discolored sapwood was observed in all inoculated trees. Stems inoculated with ophiostomatoid fungi generally had darkened, resin-filled sapwood penetrating in a wedge-shaped pattern from the point of inoculation. Cross-sectional discoloration associated with wound controls was generally lighter colored in a similar wedge-shape. *Leptographium terebrantis* and *G. huntii* caused deeper sapwood penetration than controls. Inoculations with *L. terebrantis*, *G. huntii*, and *L. serpens* were associated with greater overall sapwood discoloration (%) than controls. *Leptographium terebrantis* caused deeper lesions and more sapwood discoloration than all other treatments. No differences were observed in needle water potential at predawn (F=1.91, P=0.13) or midday (F=0.65, P=0.63) measurements for any of the treatments.

Table 4.1. Effects attributed to replication and inoculation treatment following tests with longleaf pine trees. Probability of a greater *F*-statistic for sample tree characteristics, lesion measurements, and needle water potential.

Source	df[†]	Root Collar Diameter	Height	df[‡]	Lesion Length	Lesion Depth	Lesion Area	Log (Lesion Area)	Discolored Sapwood	df[§]	Needle $\Psi_{predawn}$ (MPa)	Needle Ψ_{midday} (MPa)
Replication	1	0.0001	0.7832	1	0.0934	0.0084	0.3332	0.0547	0.0001	1	0.0035	0.0152
Inoculation Treatment	4	0.7608	0.2877	4	0.0001	0.0001	0.0001	0.0001	0.0001	4	0.1261	0.6329
Error	199			190						49		

[†] All trees were used in the analysis

[‡] Only living trees were used in the analysis

[§] A subsample of five trees per treatment per year were measured for predawn and midday water potential

Table 4.2. Lesion length, lesion depth, lesion area, cross-sectional sapwood discoloration, fungal re-isolation and needle water potential 17 weeks following inoculation.

Treatment	Lesion Length (cm)	Lesion Depth (mm)	Lesion Area (cm^2)	Log Lesion Area	Discolored Sapwood (%)	Needle $\Psi_{predawn}$ (MPa)	Needle Ψ_{midday} (MPa)	Re-Isolation (%)
L. terebrantis	4.9 (1.9) ab	10 (8) a	10.9 (7.81)	0.96 (0.25) a	12.40 (5.43) a	-0.40	-1.34	20 a
G. huntii	4.9 (2.0) ab	7 (2) bc	10.0 (5.43)	0.94 (0.24)a	9.64 (3.89) b	-0.39	-1.26	20 a
L. serpens	4.0 (1.3) bc	5 (3) cd	8.6 (6.10)	0.88 (0.19) ab	8.59 (4.20) b	-0.45	-1.37	20 a
L. procerum	3.7 (1.3) bc	5 (2) cd	6.9 (3.21)	0.80 (0.18) bc	8.19 (3.48) bc	-0.44	-1.34	40 a
Control	3.2 (1.2) c	4 (2) d	5.4 (2.98)	0.68 (0.20)c	5.94 (2.88) c	-0.45	-1.42	0 b

Note: Means (followed by standard deviation in parentheses) with the same letter within a column are not significantly different from one another at 0.05.

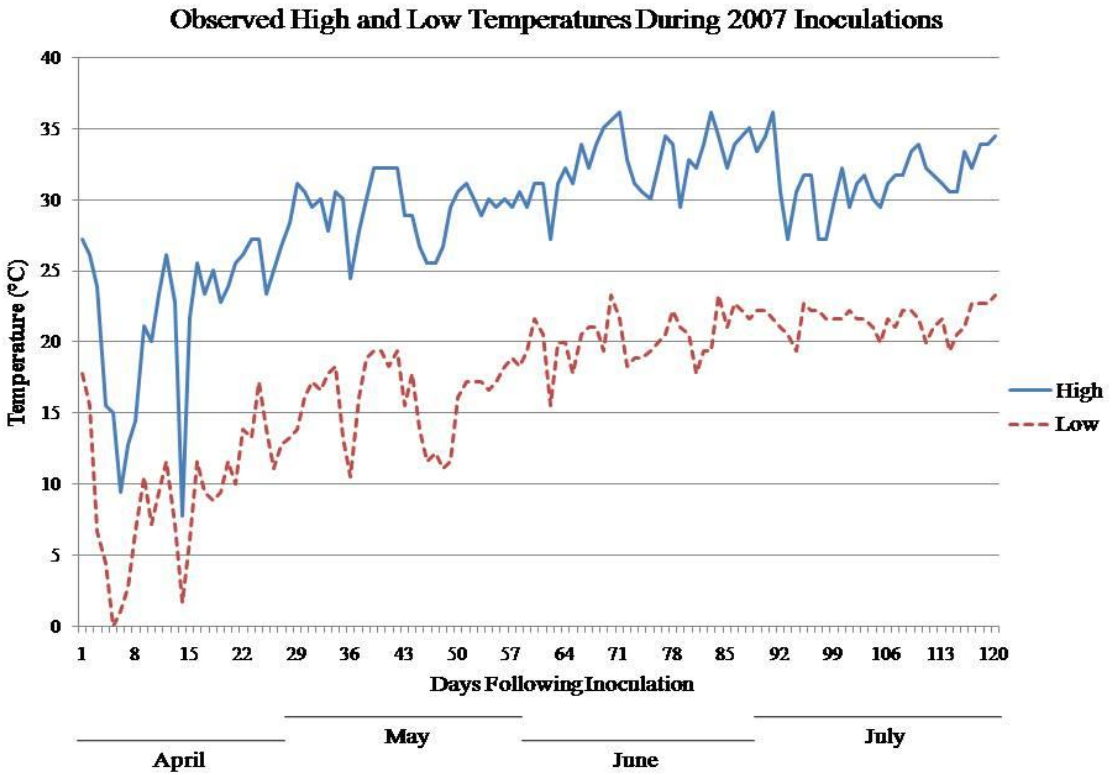

Fig. 4.4. Observed high and low temperatures at Auburn University during the 2007 inoculation test. Data was obtained from Alabama Mesonet Weather Data, located on Auburn University Campus 32.60 N, 85.50 W approximately 1.40 km from the study site (32.59 N, 85.49 W) http://www.awis.com/mesonet/

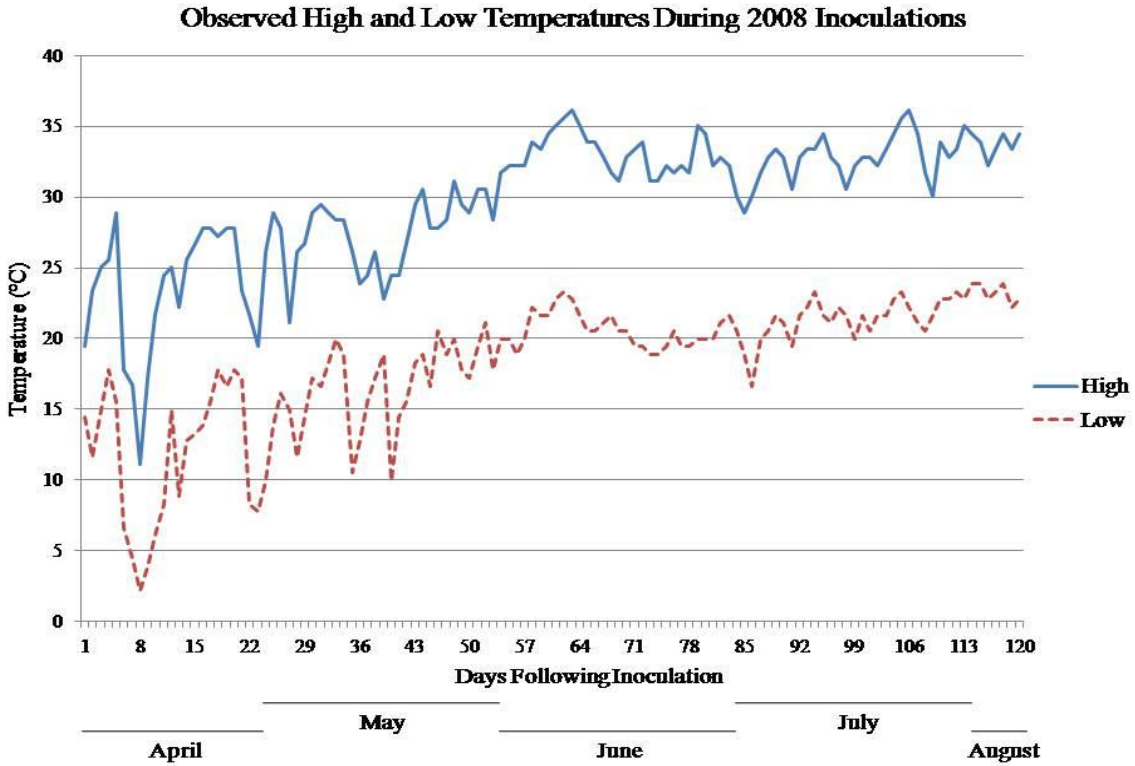

Fig. 4.5. Observed high and low temperatures at Auburn University during the 2008 inoculation test. Data was obtained from Alabama Mesonet Weather Data, located on Auburn University Campus 32.60 N, 85.50 W approximately 1.40 km from the study site (32.59 N, 85.49 W) http://www.awis.com/mesonet/

4.5. Discussion

The tested fungal species differed in their virulence to longleaf pine. *Leptographium terebrantis* caused significantly more sapwood damage than any other treatment, suggesting it has the greatest potential for physiological disruption. In contrast, *L. procerum* appears to be least virulent, producing lesions and sapwood discoloration comparable to controls. Even though most fungal species were able to infect longleaf stem tissue and cause more damage than control inoculations, no foliar symptoms or water stress were detected. Perhaps a mass inoculation of ophiostomatoid fungal species, simulating feeding damage at multiple locations on the stem, would provide a more realistic assessment of potential damage from inoculation.

Common observations made in these studies including, darkened, resin-filled sapwood and discolored inner bark have been noted in other pine hosts (Eckhardt et al. 2004a; Nevill et al. 1995; Rice et al. 2007). Lesion length and lesion area following fungal inoculation is often used to assess the pathogenicity and virulence to pine hosts (Nevill et al. 1995; Parmeter et al. 1989). *Leptographium terebrantis* caused larger lesions than controls and has been observed inducing significant lesion development after inoculation in various *Pinus* species (Nevill et al. 1995; Parmeter et al. 1989; Raffa and Smalley 1988a; Wingfield 1983) including longleaf pine (Otrosina et al. 2002). This represents the first report of *G. huntii* causing damage in pine tissue following inoculation. Although the associations have been made with dying trees and the presence of *G. huntii* (Klepzig et al. 1991; Zanzot 2009), no pathogenicity tests have been previously performed. *Leptographium serpens* also produced lesions that were larger than controls (lesion area), but not longer (lesion length). The fungus has previously been found to cause large lesions and disease in pine stems and roots (Eckhardt et al. 2004a; Wingfield and Knox-Davies 1980). However, some have found it non-pathogenic following artificial inoculation in certain *Pinus* hosts (Zhou et al. 2002). *Leptographium procerum* wounds were similar to the controls. Generally, *L. procerum* has been considered a weak pathogen to *Pinus* species (Harrington and Cobb 1983; Wingfield 1983; Wingfield 1986), in some cases causing larger lesions than controls (Eckhardt et al. 2004a). Successful re-isolation of each of the test fungi confirms their pathogenicity to

young longleaf pine trees. Previous inoculation tests have consistently confirmed infection for the same fungal species (Eckhardt et al. 2004a; Otrosina et al. 1999).

Despite greater sapwood discoloration with *L. terebrantis*, *G. huntii*, and *L. serpens* inoculations, discoloration following inoculation was lower than previously reported using similar hosts and fungi (Joseph et al. 1998; Lieutier et al. 2004). For example, Parmeter et al. (1992) observed 71% sapwood occlusion after inoculation with *L. terebrantis* in ponderosa pine, seventeen weeks following inoculation. The relatively minor sapwood damage could be explained by low temperatures shortly following inoculations in both years (Figs. 4.4 and 4.5). Nebeker et al. (1993) stated that the host response to fungal invasion depended on the physiological activity of the host which is largely influenced by temperature. However, bud break and needle elongation in longleaf pine generally occurs between mid and late April (Sheffield et al. 2003). In both studies, needle elongation was observed shortly following inoculation and trees flushed multiple times throughout each experiment. Some have found that a given hosts' resistance to blue-stain infection is associated with the amount of resin produced (Horntvedt 1988). Significant resin production has been observed following inoculation of large longleaf pine roots (Chapter 7). In these studies, little resin was observed following inoculation, suggesting host response to inoculation was minimal. More likely, the limited sapwood damage is a result of the inability of ophiostomatoid fungi to consistently infect longleaf pine stem tissue. Fungal infection, confirmed by re-isolation data, was less than previous studies (Eckhardt et al. 2004a; Chapters 5-7). Inoculations of longleaf pine seedlings, *L. serpens* was successfully isolated from greater than 85% of trees (Chapter 3). Additionally, infection could be confirmed in over 70% of trees inoculated with each of the four ophiostomatoid fungal species in root inoculations of large longleaf pine (Chapter 7).

Fungal invasion following inoculation and the resulting tissue damage has the ability to significantly alter water conduction (Croisé et al. 2001; Horner and Alexander 1985; Joseph et al. 1998). Dysfunctional sapwood is thought to be important in the development of foliar symptoms in hosts affected by root and stem inhabiting ophiostomatoid fungi (Butnor et al. 2000; Rane and Tattar 1987). The minor sapwood damage following fungal inoculation is consistent with the lack of needle water potential

differences between treatments. Croisé et al. (2001) found a decrease in needle water potential after inoculation with *L. wageneri*, but only after mass inoculation with the fungus coupled with severe water stress. In another experiment, Croisé et al. (1998b) found that trees inoculated at a density of 400 mycelial plugs/m^2 showed external symptoms, including needle yellowing. Single inoculations of trees are generally not used when attempting to detect physiological changes in the host. It is possible that the inoculum dosage was not high enough to cause significant sapwood damage. However, Paine and Stephen (1987a) found that lesion size is not related to the amount of inoculum introduced. In each experiment, fungal species were left to grow (following inoculation) much longer than similar studies. It is more likely that, in these studies, southeastern ophiostomatoid fungal species caused minor physiological disruption in stems of young longleaf pine hosts following inoculation. More tests should be completed before we can determine the likely contribution ophiostomatoid fungi make towards mortality in longleaf pine.

Chapter 5

Variation in Pathogenicity and Virulence of Five Pathogenic Fungi in Healthy Loblolly and Slash Pine Roots

5.1. Abstract

Root disease is an important natural disturbance in pine-dominated ecosystems around the world including the southeastern United States. In some instances, both *Heterobasidion annosum* and root-inhabiting ophiostomatoid fungi have been observed infecting the same dying southern pine individuals. Studies with loblolly and slash pine were conducted in order to determine the relative pathogenicity and virulence of *H. annosum* and four root-inhabiting ophiostomatoid fungi in healthy tree roots, as well as characterize the local symptomology following infection. All fungal species tested were found capable of infecting healthy pine roots and induced lesions following inoculation. In both loblolly and slash pine, *G. huntii* caused larger lesion areas when compared to *H. annosum*, *L. terebrantis*, and *L. procerum*. *Heterobasidion annosum* formed lesions comparable to *L. serpens*, *L. terebrantis*, and *L. procerum* in loblolly pine and was larger than *L. procerum* in slash pine family D. *Leptographium procerum* generally resulted in the smallest lesions when compared to all other ophiostomatoid fungi tested. *Grosmannia huntii* and in some cases *L. serpens*, appear to cause significantly greater lesion development following infection and may be contributing more to the symptomology observed in diseased trees. These studies illustrate that important differences exist in the relative pathogenicity and virulence of the fungi tested. Future work should focus on the interaction between these competing pathogenic root fungi and their collective role in root disease in the southeastern United States.

5.2. Introduction

Root disease of *Pinus* species in the coastal plain physiographic region of the southeastern United States is a concern to forest land managers. Vast areas of genetically similar, densely planted pines that require frequent thinning facilitate the proliferation of Annosus root disease (*Heterobasidion annosum* (Fr.) Bref.) (Stambaugh 1989). Annosus root disease can affect all southern pine species (Robbins 1984); however, research has focused on loblolly (*Pinus taeda* L.) and slash (*P. elliottii* Engelm.) pine due to their role in wood production and increased susceptibility, relative to other species (Hodges 1969). More recently, another disease termed loblolly pine decline (Eckhardt et al. 2007), has been frequently diagnosed in coastal plain areas of Alabama and Georgia (Menard 2007). The hypothesized disease cycle includes several abiotic and biotic factors, including a group of root-inhabiting ophiostomatoid fungi with *Leptographium* anamorphic states.

In the southeastern United States, Annosus root disease causes significant damage to plantation pine stands following thinning. The primary mode of infection is through root grafts (Rishbeth 1951), formed in densely stocked plantations (Shultz and Woods 1967). Infection of living roots is facilitated by the colonization of pine stumps (Driver and Dell 1961), which can become infected following thinning (Driver and Ginns 1969). Colonized stumps represent inoculum reservoirs which allow for the infection of living roots through root grafts (Otrosina 1989). Additionally, insects have been shown to function in the spread of *H. annosum* experimentally with the black turpentine beetle (*Dendroctonus terebrans* (Olivier)) in loblolly pine (Himes and Skelly 1972). Annosus root rot may lead to wind throw, even when pines are exhibiting no observable crown symptoms. In addition, annosus root disease may lead to thinning crowns and shortened needles, often with light green or yellow discolored foliage. Alexander (1989), states that symptoms of severely affected trees are similar to those observed for any stressed pine tree. Other root pathogens cause host tree root deterioration by blocking important conducting tissues, leading to similar crown symptomology to those described for annosus root disease (Jacobs and Wingfield 2001).

Throughout the world, ophiostomatoid fungal species are commonly associated with bark beetles that attack conifer hosts (Paine et al. 1997). Ophiostomatoid fungal associates are thought to facilitate successful colonization of host sapwood tissue by both aggressive and non-aggressive bark beetles (Krokene and Solheim 1998b). *Hylastes* species (Coleoptera: Curculionidae) are generally regarded as non-aggressive root-feeding bark beetles (Wood 1982) that commonly vector a variety of blue-stain fungal species with *Leptographium* anamorphs (Jacobs and Wingfield 2001). In the southeastern states, two *Hylastes* species (Coleoptera: Curculionidae)(*H. salebrosus* Eichhoff and *H. tenuis* Eichhoff) predominate and have been observed in declining pine stands transporting a collection of ophiostomatoid fungi (Eckhardt et al. 2004b). Root-inhabiting ophiostomatoid fungi are often associated with root deterioration and crown symptomology consistent with root disease in pine hosts (Jacobs and Wingfield 2001). Under certain circumstances, root-inhabiting ophiostomatoid fungi have the opportunity to act as primary pathogens (Cobb 1988), disrupting host root functions (Joseph et al. 1998) after entering through wounds created by vectors during maturation feeding activities (Harrington et al. 1985). However, many ophiostomatoid fungi are known to be either secondary or facultative pathogens, causing severe disruption in only stressed hosts (Klepzig et al. 1996; Harrington and Cobb 1983). Eckhardt et al. (2007) found *Hylastes* species and their fungal associates correlated with areas of severe loblolly pine decline in Alabama. *Hylastes* species have been observed feeding on seemingly healthy southern pine roots, providing an opportunity for fungal associates to invade host tissues (Matusick and Eckhardt unpublished data). Ophiostomatoid fungi have been commonly isolated from roots of symptomatic southern pine species. However, researchers have disregarded ophiostomatoid fungi as a product of the dying condition and not a contributing factor (Hess et al. 1999).

Pine tree roots support a wide variety of ectomycorrhizal (Karkouri et al. 2005), pathogenic (Barnard and Gilly 1991), and decay (Vandegrift et al. 2007) fungal species and groups. Therefore, the simultaneous presence of *H. annosum* and ophiostomatoid fungi in roots of declining pines would not be unusual. Otrosina et al. (1999) have isolated both *H. annosum* and ophiostomatoid fungi from declining longleaf pine following prescribed burning. In Virginia, *H. annosum*, *L. serpens* (Goid) M.J. Wingf.,

and *L. procerum* (W.B. Kendr.) M.J. Wingf.(formerly *Verticicladiella procera* W.B. Kendr.) were found invading the roots of air-pollution sensitive white pines (*P. strobus* L.)(Lackner and Alexander 1983). *Heterobasidion annosum* and ophiostomatoid fungi both invade pine stumps soon after cutting (Hunt and Cobb 1982). During a survey of thinned slash pine in Florida, Barnard and Gilly (1991) isolated *H. annosum* and *L. procerum* along with a variety of other pathogenic and saprophytic fungi from stumps and roots of both dead and living trees. Disease caused by *H. annosum* and ophiostomatoid fungi is commonly more severe in weakened, stressed hosts, illustrating their opportunistic nature (Croisé et al. 1998a; Långström et al. 2001; Paine and Stephen 1987b; Towers and Stambaugh 1968). Under many circumstances, both *H. annosum* and ophiostomatoid fungi are successful at colonizing stressed and dying hosts. However, interest lies in their ability to infect and cause disease in seemingly healthy southern pines and the contribution they may make toward root disease symptoms.

Pathogenicity or virulence studies are commonly used to test fungi for their potential to incite disease development in healthy host plants. Seedling, stem, and root inoculations of seemingly healthy plants have been inoculated with *H. annosum* in the past (Johansson et al. 2004). Similarly, a variety of inoculation tests have been performed using a number of ophiostomatoid species with their respective hosts (Wingfield et al. 1988). Seedling inoculation tests are commonly performed in order to readily observe the effect of *H. annosum* and ophiostomatoid fungi on their hosts (Bodles et al. 2007; Harrington and Cobb 1983; Kuhlman 1969; Owen et al. 1987). Several studies have inoculated roots of southern pines with *H. annosum* (Hodges 1969; Hodges and Kuhlman 1974); however, few have tested ophiostomatoid fungi on mature tree roots (Otrosina et al. 1999). While *H. annosum* and ophiostomatoid fungi have been compared directly in inoculation tests in the past (Nagy et al. 2005), no comparative test of pathogenicity and virulence has been conducted in the southeastern United States using susceptible pines. These studies seek to compare the pathogenicity and virulence between four root-inhabiting ophiostomatoid fungi and *H. annosum* on healthy roots of unthinned, field-growth loblolly and slash pine. It is hypothesized that each fungal species can infect and cause damage in pine roots. In addition, we hypothesize the tested fungal species will cause different reactions. Observations will help to determine the

potential damage host roots incur following root infection and elucidate any differences in the host defense reaction to infection characteristic of each fungal species.

5.3. Materials and Methods

Four families of 400 pine trees, including two half-sib families of loblolly and two families of slash pines were used in inoculation tests. All four families were planted adjacent to one another in an abandoned field in Covington County, Alabama in January 1998. A total of fifty random pines from each family were selected for inoculation tests in January 2007, then repeated in January 2008.

Five root-inhabiting fungi: *H. annosum*, *L. procerum*, *L. terebrantis* S.J. Barras & T.J. Perry, *L. serpens*, and *G. huntii* (R.C. Rob. Jeffr.) Zipfel, Z.W. de Beer & M.J. Wingf., were each isolated from pine hosts exhibiting root-disease symptoms in the southeastern United States. A single isolate of each species (Table 5.1) was placed on malt extract agar fourteen days prior to the inoculation. All fungal species were paired with one another, resulting in ten fungal species combinations. Each combination was randomly assigned to five trees within each pine group. Additionally, two control treatments, including a wound only and wound + sterile media, were also assigned to each tree within the study.

Two primary lateral roots were excavated approximately 122 cm (4 feet) from the root collar area. Each fungal species was then randomly assigned to one root. Additionally, each control treatment was randomly assigned to one root, leaving one fungal treatment and one control treatment on each root (Fig 5.1). Approximately 30.5 cm (1 ft) and 91.5 (3 ft) from the root collar area on each root, the control and fungal inoculation wounds were performed, respectively.

Table 5.1. Fungal species, isolate number, site of collection, and host source for isolates used in loblolly and slash pine inoculations

Fungal Species	**Isolate no./ ATCC accession no.**	**Collection Site**	**Host Source**
G. huntii	LLP-R-02-100/ MYA-3311	Fort Benning Military Reservation, GA	Longleaf pine root
L. serpens	LOB-R-00-309/ MYA-3315	Westervelt Company Land, AL	Loblolly pine root
L. terebrantis	LOB-R-00-805/ MYA-3316	Talladega National Forest, Oakmulgee Ranger District AL	Loblolly pine root
L. procerum	LOB-R-00-456/ MYA-3313	Talladega National Forest, Oakmulgee Ranger District AL	Loblolly pine root
H. annosum	LLP-R-01-223/ MYA-3318	International Paper Land AL	Longleaf pine root

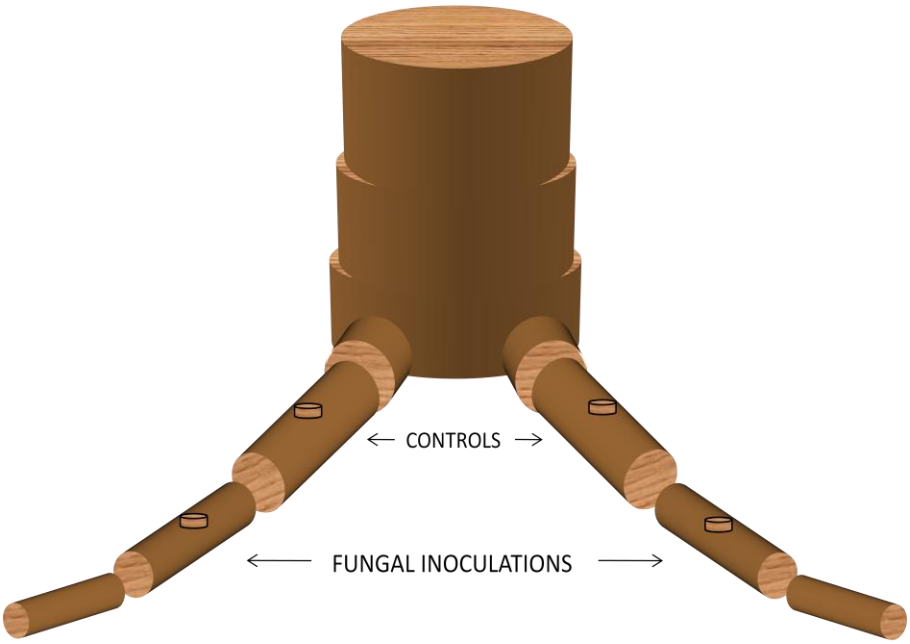

Fig. 5.1. Diagram of each experimental unit (tree). Two controls administered proximal and two different fungal treatments admistered distal.

Wound inoculations were administered based on a method for inoculating mature trees established by Wright (1933). At each inoculation site, a 1.3 cm diameter circular wound was created using a punch in the epidermis (Fig 5.2). A 1 cm diameter plug of actively growing fungal mycelium was aseptically placed on the exposed cambial surface. The removed epidermal tissue was replaced, covering the mycelium, and the inoculation site was sealed with duct tape. Controls were administered in an identical fashion, with the exception of placing sterile malt extract agar in the place of growing mycelium for the wound + sterile media treatment. The wound only control had nothing inserted at the cambial surface. All inoculation points were marked with colored flags and buried (Fig. 5.3).

Fig. 5.2. Wound created in the top of the root prior to administering inoculation.

Fig. 5.3. Loblolly pine stand following root inoculations.

Eight weeks following the inoculation, all treated roots were re-excavated. A section of each root, extending approximately 152 cm (5 ft) from the root collar was severed from the tree and removed (Fig. 5.4). At each inoculation site the bark tissue was removed and the length and width of the darkened lesion recorded. A clear transparent sheet was used to trace the outline of the exposed lesion and a Lasico® planimeter was used to determine the surface area of the lesion tracing (Fig. 5.5). A transverse cut was made through the center of the inoculation point and the depth of discolored sapwood was recorded. Finally, tissue was removed from the area surrounding the inoculation point and placed on malt extract agar for fungal identification.

Fig. 5.4. Treated roots following excavation and removal in 2008.

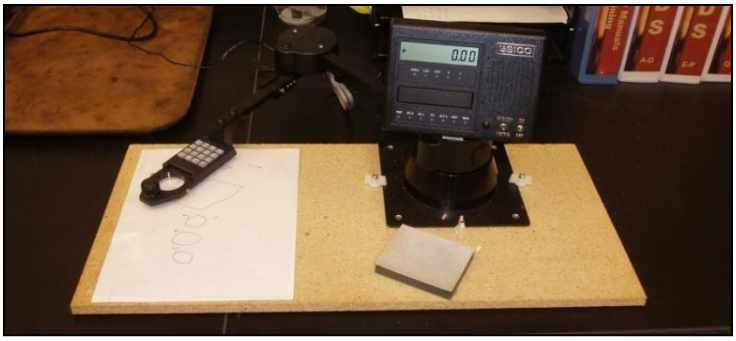

Fig. 5.5. Planimeter used to measure surface area of lesion tracings.

Loblolly and slash pine were analyzed separately for all response variables. First, a comparison was made between the two types of control treatments. Secondly, differences between fungal species and controls were assessed. Comparisons were then made between fungal species, using only those trees that had both fungal treatments paired. Control treatment responses were subtracted from one another on each tree,

resulting in one response. The response values were used in an ANOVA in a general linear model (GLM) procedure in SAS statistical software using experiment year and pine family (for loblolly pine) in the model. The appropriate Estimate statements were included in order to test significance from zero.

Each fungal treatment was compared to the control treatment performed on the same root. The difference was taken then tested using Estimate statements included in an ANOVA in the GLM) procedure. In both families of loblolly pine and one family of slash pine (family C) the two control treatments were found to be similar and grouped. In one family of slash pine (family D), the control treatments were found to be different and analyzed separately. An ANOVA in the GLM procedure was used to determine the effect of pine group on the comparison between fungal and control treatments. Factors in the ANOVA model included experiment year and pine group (for loblolly). Estimate statements were included to test significance. Ultimately, the analyses determined those fungal species that significantly affected the pine root, relative to controls.

Individual fungal treatments were compared on those trees in which they were paired. Fungal treatment responses were subtracted from one another, resulting in a single response per sampling unit (tree). The analysis was conducted for each response variable using an ANOVA in the GLM procedure. Factors included in the model were; 1) experiment year 2) pine group 3) treatment (the value difference between the two paired fungal treatments) and 4) the interaction between treatment and pine group. Estimate statements were included in the GLM in order to assess differences. The analysis represents a direct test between each fungal species.

5.4. Results

Discolored root tissue was observed surrounding areas affected by wound only (Fig. 5.6) and wound + media (Fig. 5.7) controls. In loblolly pine, control treatments did not differ in their effect on lesion length, width, depth, or area (Tables 5.2, 5.3). Roots on which controls were performed were similar in diameter, with an average difference of 0.37 and 0.44 cm in family A and family B respectively. The average difference between control treatments in lesion length, width and depth was less than 0.1 cm in both families. The lesion area difference was also negligible between control treatments. Similar to

loblolly pine, no differences were observed in measurements of lesion length, width, depth, or area in slash pine family C (Tables 5.4, 5.5). However in slash family D, the presence of sterile media was responsible for an increase in all measured lesion parameters.

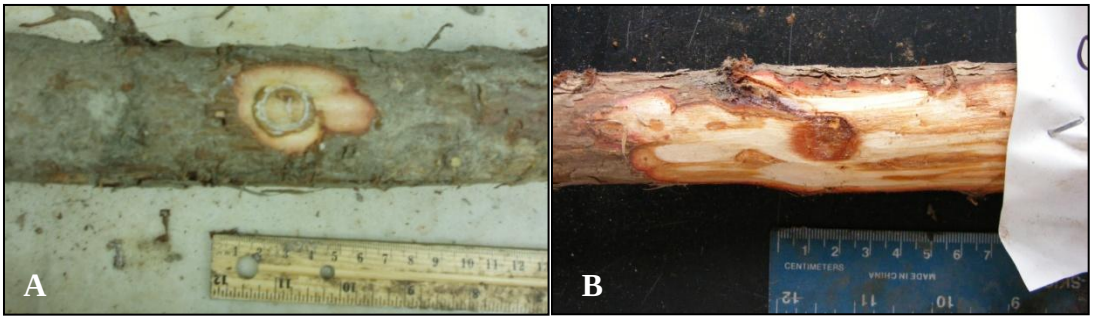

Fig. 5.6. Discolored control response following a wound (A) and wound + media (B) control.

Table 5.2. Probability of a greater *F*-statistic following ANOVA with wound and wound + sterile media controls on loblolly pine.

Source	Lesion Length	Lesion Width	Lesion Depth	Lesion Area
Year	0.5620	0.6998	0.2146	0.1079
Pine Family	0.7848	0.6730	0.8813	0.1695

Table 5.3. Average lesion length, lesion width, lesion depth, and lesion area increases due to the presence of sterile media on loblolly pine.

Response Variable	Increase due to sterile media	*T*-value	*P*-value
Lesion Length (cm)	0.1	0.97	0.33
Lesion Width (cm)	0.0	1.12	0.26
Lesion Depth (cm)	0.0	0.16	0.87
Lesion Area (cm^2)	0.15	1.14	0.25

Note: Results obtained from Estimate statements included in the GLM procedure.

Table 5.4. Probability of a greater *F*-statistic following ANOVA with wound and wound + sterile media controls in slash pine.

Source	Lesion Length	Lesion Width	Lesion Depth	Lesion Area
Year	0.0024	0.0001	0.0007	0.0001
Pine Family	0.0033	0.0016	0.0011	0.0022

Table 5.5. Average lesion length, lesion width, lesion depth, and lesion area increases due to the presence of sterile media in slash pine.

Response Variable	Pine Family	Increase due to sterile media	*T*-value	*P*-value
Lesion Length (cm)	C	0.1 b	0.77	0.4439
	D	0.5 a	4.95	0.0001
Lesion Width (cm)	C	0.0 d	1.42	0.1566
	D	0.2 c	5.90	0.0001
Lesion Depth (cm)	C	0.0 f	0.83	0.4063
	D	0.1 e	5.48	0.0001
Lesion Area (cm^2)	C	0.32 h	1.76	0.0804
	D	1.12 g	6.10	0.0001

Note: Results obtained from Estimate statements included in the GLM procedure.
Note: Means followed by same letter within the column, within response variable are not significantly different at alpha = 0.05.

Generally, fungal inoculation resulted in significantly larger host response and variables when compared to control treatments. The two control treatments in loblolly and slash pine family C were combined in analyses since they were found to be statistically similar in the previous test. In slash pine family D, the wound + media control treatment was found to be significantly larger for all response variables. In

loblolly pine, each fungal treatment caused larger lesion lengths, widths, depths (Fig. 5.7), and areas, when compared to controls (Tables 5.6, 5.7). In slash pine group C, fungal inoculations caused larger lesions compared to controls (with the exception of lesion width)(Tables 5.8, 5.9). Similarly, in slash pine group D, lesion parameters were larger when fungal species were used, with the exception of lesion width with certain fungal species x control combinations (Tables 5.10, 5.11, 5.12).

Fig. 5.7. Cross-sectional discoloration in sapwood of slash pine roots following inoculations with *L. terebrantis* (T), *H. annosum* (A), *L. serpens* (S), *G. huntii* (H), wound + sterile media (M), and wound only (W) controls.

Table 5.6. Probability of a greater *F*-statistic following ANOVA analyses using each fungal species following the subtraction of controls in loblolly pine.

		H. annosum	*G. huntii*	*L. procerum*	*L. serpens*	*L. terebrantis*
Lesion Length						
	Year	0.0505	0.0122	0.3155	0.1580	0.0533
	Pine Family	0.1842	0.8806	0.8458	0.6291	0.0176
Lesion Width						
	Year	0.0445	0.1758	0.0069	0.3661	0.3863
	Pine Family	0.6987	0.1966	0.0332	0.4785	0.8782
Lesion Depth						
	Year	0.0208	0.2492	0.6193	0.0852	0.0005
	Pine Family	0.5683	0.0099	0.6603	0.9472	0.4311
Lesion Area						
	Year	0.0130	0.0122	0.9145	0.1045	0.0417
	Pine Family	0.1563	0.5992	0.1806	0.6898	0.3769

Note: Analyses were conducted using alpha = 0.05.

Table 5.7. Increase in lesion length, width, depth, and area when *H. annosum*, *G. huntii*, *L. procerum*, *L. serpens*, and *L. terebrantis* were present versus when absent (controls) in loblolly pine.

	Lesion Length (cm)	Lesion Width (cm)	Lesion Depth (cm)	Lesion Area (cm^2)
Increase due to *H. annosum*	2.4 (2.2)	0.1 (0.4)	0.3 (0.2)	4.59 (3.97)
T-value	10.21	3.33	11.52	10.71
P-value	0.0001	0.0013	0.0001	0.0001
Increase due to *G. huntii*	6.0 (3.6)	0.8 (0.8)	0.5 (0.3)	12.28 (8.03)
T-value	15.23	9.10	16.48	14.0000
P-value	0.0001	0.0001	0.0001	0.0001
Increase due to *L. procerum*	1.7 (1.0)	0.2 (0.3)	0.2 (0.2)	3.20 (1.98)
T-value	15.53	5.52	8.50	14.32
P-value	0.0001	0.0001	0.0001	0.0001
Increase due to *L. serpens*	4.2 (2.7)	0.7 (0.5)	0.4 (0.2)	8.76 (5.36)
T-value	13.71	11.01	15.51	14.61
P-value	0.0001	0.0001	0.0001	0.0001
Increase due to *L. terebrantis*	2.6 (1.4)	0.34 (0.4)	0.4 (0.3)	4.83 (3.33)
T-value	17.07	7.43	12.25	13.19
P-value	0.0001	0.0001	0.0001	0.0001

Note: Results obtained from Estimate statements included in GLM procedures.

Table 5.8. Increase in lesion length, width, depth, and area when *H. annosum* and *L. huntii* were present versus when absent (controls) in slash pine family C.

	N	Lesion Length (cm)	Lesion Width (cm)	Lesion Depth (cm)	Lesion Area (cm^2)
Increase due to *H. annosum*	42	6.0 (6.5)	0.0 (0.3)	0.5 (0.4)	8.09 (9.91)
T-value		5.95	-1.00	8.15	5.25
P-value		0.0001	0.3224	0.0001	0.0001
Increase due to *G. huntii*	40	15.9 (10.4)	0.4 (0.5)	0.6 (0.3)	23.08 (13.93)
T-value		9.59	5.22	11.59	10.33
P-value		0.0001	0.0001	0.0001	0.0001

Note: Results obtained from Estimate statements included in GLM procedures.
Note: Mean increases due to fungal species are followed by standard deviation.

Table 5.9. Increase in lesion length, width, depth, and area when *L. procerum*, *L. serpens*, and *L. terebrantis* were present versus when absent (controls) in slash pine family C.

	N	Lesion Length (cm)	Lesion Width (cm)	Lesion Depth (cm)	Lesion Area (cm^2)
Increase due to *L. procerum*	39	1.6 (0.8)	0.0 (0.3)	0.2 (0.2)	2.27 (1.27)
T-value		12.65	-0.07	6.02	11.11
P-value		0.0001	0.9442	0.0001	0.0001
Increase due to *L. serpens*	40	8.8 (6.4)	0.3 (0.4)	0.5 (0.3)	15.03 (10.01)
T-value		8.53	5.52	10.34	9.44
P-value		0.0001	0.0001	0.0001	0.0001
Increase due to *L. terebrantis*	39	4.0 (0.3)	0.0 (0.3)	0.6 (0.4)	6.44 (5.19)
T-value		8.41	0.22	8.74	7.64
P-value		0.0001	0.8243	0.0001	0.0001

Note: Results obtained from Estimate statements included in GLM procedures.
Note: Mean increases due to fungal species are followed by standard deviation.

Table 5.10. Increase in lesion length, width, depth, and area when *H. annosum* and *G. huntii* were present versus when absent (controls) in slash pine family D.

		Lesion Length (cm)	Lesion Width (cm)	Lesion Depth (cm)	Lesion Area (cm^2)
Increase due to *H. annosum*	Wound	7.3 (8.7)	0.1 (0.3)	0.5 (0.2)	11.61 (16.57)
	T-value	4.19	1.59	10.16	3.45
	P-value	0.0004	0.1266	0.0001	0.0024
	Wound + Sterile Media	7.0 (7.5)	-0.1 (0.4)	0.6 (0.4)	13.21 (23.48)
	T-value	4.04	-0.74	5.86	2.26
	P-value	0.0011	0.4710	0.0001	0.0395
Increase due to *G. huntii*	Wound	13.3 (7.1)	0.4 (0.6)	0.7 (0.3)	22.62 (14.20)
	T-value	8.77	2.76	10.41	7.31
	P-value	0.0001	0.0119	0.0001	0.0001
	Wound + Sterile Media	8.6 (5.1)	0.3 (0.5)	0.5 (0.4)	15.89 (10.33)
	T-value	6.47	3.00	5.48	5.90
	P-value	0.0001	0.0096	0.0001	0.0001

Note: Results obtained from Estimate statements included in GLM procedures.
Note: Mean increases due to fungal species are followed by standard deviation.

Table 5.11. Increase in lesion length, width, depth, and area when *L. procerum* and *L. serpens* were present versus when absent (controls) in slash pine family D.

				Lesion Length (cm)	Lesion Width (cm)	Lesion Depth (cm)	Lesion Area (cm^2)
Increase due to *L. procerum*	Wound		14	1.1 (0.8)	0.1 (0.3)	0.2 (0.2)	1.27 (1.24)
		T-value		6.33	1.75	3.54	5.03
		P-value		0.0001	0.1052	0.0041	0.0003
	Wound + Media		26	1.1 (1.5)	-0.1 (0.4)	0.2 (0.2)	2.60 (6.07)
		T-value		3.87	-1.64	5.03	2.18
		P-value		0.0007	0.1140	0.0001	0.0391
Increase due to *L. serpens*	Wound		23	8.2 (8.6)	0.5 (0.4)	0.6 (0.2)	10.14 (9.84)
		T-value		4.98	5.24	13.35	5.06
		P-value		0.0001	0.0001	0.0001	0.0001
	Wound + Media		16	8.3 (5.2)	0.3 (0.3)	0.5 (0.3)	10.01 (6.28)
		T-value		6.23	3.37	6.98	6.51
		P-value		0.0001	0.0046	0.0001	0.0001

Note: Results obtained from Estimate statements included in GLM procedures.
Note: Mean increases due to fungal species are followed by standard deviation.

Table 5.12. Increase in lesion length, width, depth, and area when *L. terebrantis* was present versus when absent (controls) in slash pine family D.

			Lesion Length (cm)	Lesion Width (cm)	Lesion Depth (cm)	Lesion Area (cm^2)
Increase due to *L. terebrantis*	Wound	16	8.1 (6.5)	0.3 (0.4)	0.9 (0.6)	14.72 (14.97)
	T-value		4.77	2.92	6.12	3.80
	P-value		0.0003	0.0113	0.0001	0.0019
	Wound + Media	23	4.1 (3.0)	-0.1 (0.5)	0.6 (0.4)	5.64 (4.63)
	T-value		6.59	-0.69	8.26	5.87
	P-value		0.0001	0.4983	0.0001	0.0001

Note: Results obtained from Estimate statements included in GLM procedures.
Note: Mean increases due to fungal species are followed by standard deviation.
Note: Analyses conducted using alpha = 0.05.

Root-inhabiting fungal species varied in their effect on healthy pine roots (Fig. 5.8). Inoculation with *G. huntii* caused the development of lesions that were longer, wider, deeper, and larger (lesion area) than *H. annosum*, *L. terebrantis*, and *L. procerum* in loblolly pine (Tables 5.13, 5.14). Lesions following *L. serpens* inoculations were not different from those observed following *G huntii* inoculation. In slash pine family C, *G. huntii* caused longer lesions than all other treatments (Tables 5.15, 5.16)(Fig. 5.9). Also, lesions were larger than all other treatments with the exception of *L. serpens*. In slash pine family D, *G. huntii* caused longer lesions than *L. procerum* and *L. terebrantis* as well as larger lesions than *H. annosum* (Tables 5.17, 5.18). *Heterobasidion annosum* and *L. serpens* caused lesions of similar size with the exception of lesion width, which was larger in roots inoculated with *L. serpens* in both loblolly (T=-2.78, P=0.0060) and slash pine family C (T=-2.16, P=0.0337) and family D (T=-2.57, P=0.0118). In loblolly pine, *L. terebrantis* lesions were smaller than *L. serpens* but statistically similar to *H. annosum*. *Leptographium terebrantis* lesions were found to be similar to *L. serpens* and *H. annosum* in both slash pine families. Generally, *L. procerum* caused smaller lesions than *G. huntii* and *L. serpens*, but were comparable to *L. terebrantis* and *H. annosum*.

Table 5.13. Probability of a greater *F*-statistic following ANOVA involving fungal treatment combinations (pairings) in loblolly pine.

Source	Lesion Length	Lesion Width	Lesion Depth	Lesion Area
Year	0.3152	0.7737	0.4334	0.5141
Pine Family	0.6375	0.7449	0.6758	0.9715
Treatment Pairing	0.0001	0.0001	0.0001	0.0001
Treatment Pairing x Pine Family	0.6148	0.8422	0.1908	0.4251

Table 5.14. Fungal treatment comparisons in lesion length, width, depth, and area using only those trees in which both fungal species were paired in loblolly pine.

Treatment Pairing				Average Difference (A-B)							
A	-	B	N	Lesion Length (cm)	*P*-value	Lesion Width (cm)	*P*-value	Lesion Depth (cm)	*P*-value	Lesion Area (cm^2)	*P*-value
H. annosum	vs.	*G. huntii*	20	-4.7 (4.0)	0.0001	-0.6 (0.8)	0.0011	-0.3 (0.4)	0.0008	-9.10 (6.03)	0.0001
H. annosum	vs.	*L. procerum*	20	0.9 (3.0)	0.2411	-0.1 (0.6)	0.6085	0.1 (0.2)	0.3398	1.37 (5.46)	0.3772
H. annosum	vs.	*L. serpens*	19	-0.8 (2.8)	0.3012	-0.5 (0.9)	0.0060	-0.1 (0.2)	0.2813	-2.92 (5.02)	0.0668
H. annosum	vs.	*L. terebrantis*	20	0.3 (1.8)	0.6823	-0.1 (0.3)	0.5270	-0.1 (0.2)	0.1956	0.99 (3.71)	0.5216
G. huntii	vs.	*L. procerum*	19	3.4 (4.8)	0.0001	0.8 (1.1)	0.0001	0.2 (0.4)	0.0014	8.96 (13.06)	0.0001
G. huntii	vs.	*L. serpens*	20	0.7 (5.2)	0.3278	0.1 (0.8)	0.4515	0.1 (0.3)	0.2467	1.07 (10.96)	0.4906
G. huntii	vs.	*L. terebrantis*	20	4.5 (3.8)	0.0001	0.5 (0.8)	0.0018	0.2 (0.4)	0.0300	9.89 (6.18)	0.0001
L. procerum	vs.	*L. serpens*	20	-2.2 (1.6)	0.0029	-0.5 (0.5)	0.0066	-0.3 (0.3)	0.0005	-4.48 (2.89)	0.0042
L. procerum	vs.	*L. terebrantis*	20	-1.2 (1.8)	0.1130	-0.3 (0.5)	0.0927	-0.3 (0.4)	0.0001	-2.95 (4.39)	0.0577
L. serpens	vs.	*L. terebrantis*	20	1.5 (2.0)	0.0482	0.2 (0.8)	0.3062	-0.1 (0.4)	0.4131	2.72 (3.94)	0.0800

Note: Results obtained by including Estimate statements in the GLM procedure.
Note: Analyses were conducted using alpha = 0.05.

Table 5.15. Probability of a greater *F*-statistic following ANOVA involving fungal treatment combinations (pairing) in slash pine family C.

Source	Lesion Length	Lesion Width	Lesion Depth	Lesion Area
Year	0.4972	0.3850	0.8428	0.2528
Treatment Pairing	0.0001	0.0001	0.0006	0.0001

Note: Analysis was conducted using alpha = 0.05.

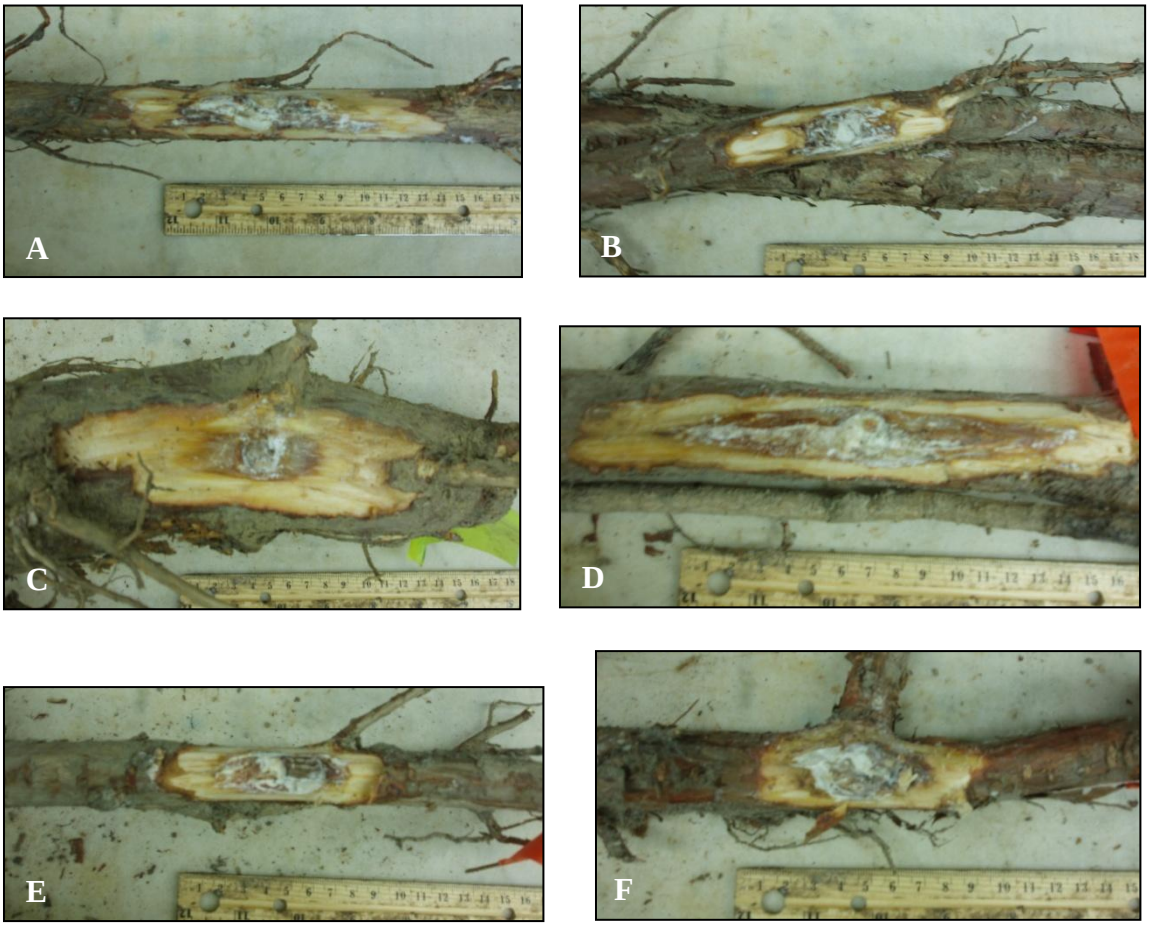

Fig. 5.8. In loblolly pine, typical root damage following inoculation with *L. serpens* (A), *L. procerum* (B), *L. terebrantis* (C), *G. huntii* (D), and *H. annosum* (E/F) in 2008.

Table 5.16. Fungal treatment comparisons in lesion length, width, depth, and area using only those trees in which both fungal species were paired in slash pine family C.

Treatment Pairing				Average Difference (A-B)							
A	-	B	N	Lesion Length (cm)	*P*-value	Lesion Width (cm)	*P*-value	Lesion Depth (cm)	*P*-value	Lesion Area (cm^2)	*P*-value
H. annosum	vs.	*G. huntii*	11	-13.0 (10.3)	0.0001	-0.6 (0.7)	0.0001	-0.1 (0.4)	0.3099	-22.26 (13.01)	0.0001
H. annosum	vs.	*L. procerum*	10	2.6 (3.3)	0.3581	0.1 (0.4)	0.7368	0.1 (0.4)	0.4147	3.97 (4.94)	0.3231
H. annosum	vs.	*L. serpens*	10	0.2 (9.3)	0.9381	-0.3 (0.4)	0.0337	-0.1 (0.4)	0.2543	-3.47 (13.6)	0.3875
H. annosum	vs.	*L. terebrantis*	10	4.1 (8.4)	0.1483	0.0 (0.3)	0.7880	-0.1 (0.4)	0.4627	5.07 (15.98)	0.2078
G. huntii	vs.	*L. procerum*	10	11.2 (6.1)	0.0002	0.4 (0.6)	0.0121	0.4 (0.4)	0.0012	20.64 (9.76)	0.0001
G. huntii	vs.	*L. serpens*	10	7.6 (12.2)	0.0089	0.3 (0.5)	0.0461	0.3 (0.3)	0.0435	4.74 (18.00)	0.2387
G. huntii	vs.	*L. terebrantis*	9	13.6 (16.6)	0.0001	0.1 (0.5)	0.6924	0.0 (0.4)	0.9947	16.51 (19.15)	0.0002
L. procerum	vs.	*L. serpens*	9	-8.5 (5.7)	0.0054	-0.3 (0.4)	0.0532	-0.3 (0.4)	0.0509	-15.01 (11.86)	0.0006
L. procerum	vs.	*L. terebrantis*	10	-0.9 (2.4)	0.7642	0.1 (0.2)	0.3831	-0.4 (0.2)	0.0032	-1.27 (3.54)	0.7513
L. serpens	vs.	*L. terebrantis*	10	3.4 (5.7)	0.2389	0.7 (0.6)	0.0001	0.1 (0.4)	0.2896	5.99 (7.67)	0.1374

Note: Results obtained by including Estimate statements in the GLM procedure.
Note: Analyses were conducted using alpha = 0.05.

Table 5.17. Probability of a greater *F*-statistic following ANOVA involving fungal treatment combinations (pairing) in slash pine family D.

Source	Lesion Length	Lesion Width	Lesion Depth	Lesion Area
Year	0.9896	0.6697	0.7008	0.4268
Treatment Pairing	0.0001	0.0011	0.0001	0.0001

Note: Analysis was conducted using alpha = 0.05.

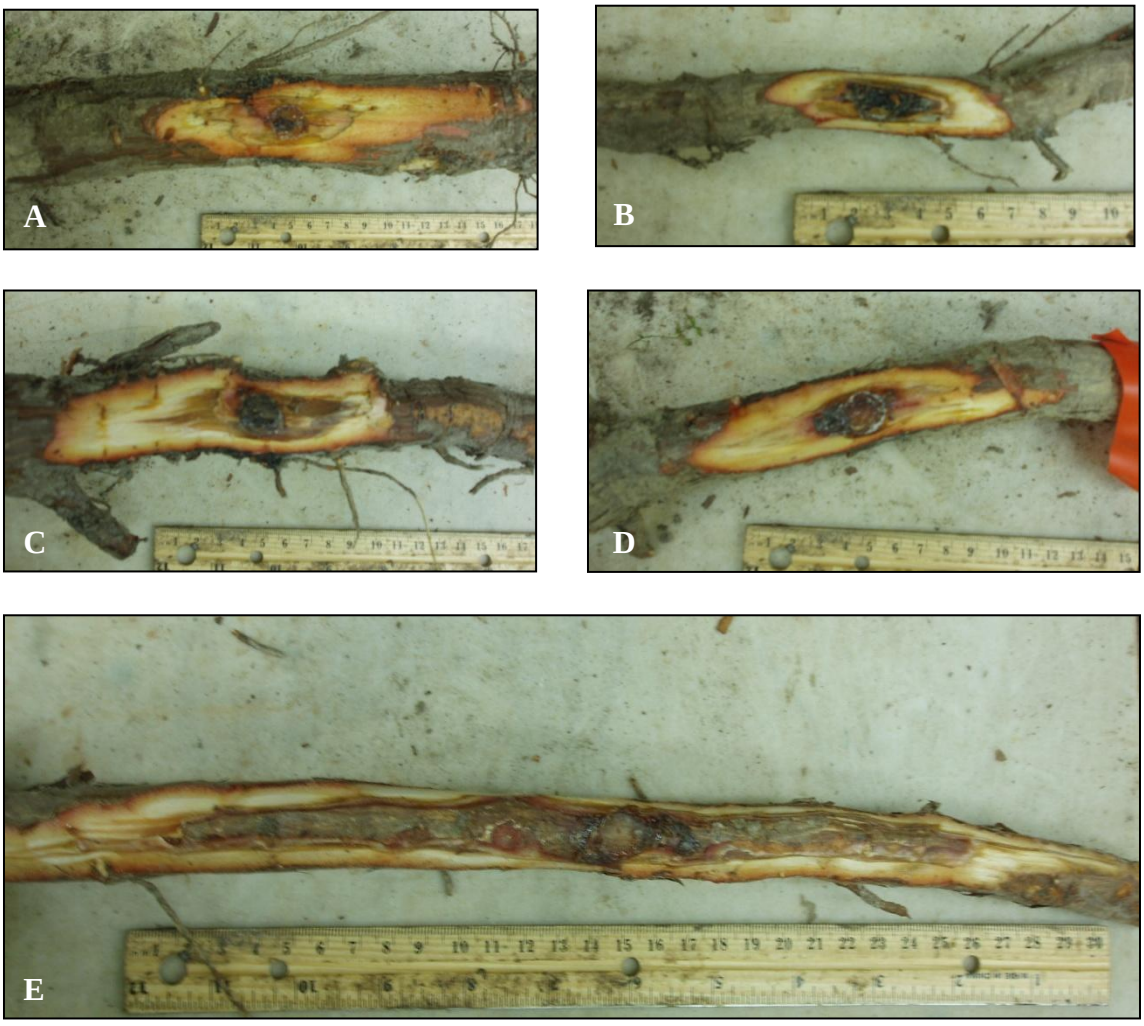

Fig. 5.9. In slash pine, typical root damage following inoculation with *L. serpens* (A), *L. procerum* (B), *L. terebrantis* (C), *H. annosum* (D), and *G. huntii* (E) in 2008.

Table 5.18. Fungal treatment comparisons in lesion length, width, depth, and area using only those trees in which both fungal species were paired in slash pine family D.

Treatment Pairing				Average Difference (A-B)							
A	-	B	N	Lesion Length (cm)	*P*-value	Lesion Width (cm)	*P*-value	Lesion Depth (cm)	*P*-value	Lesion Area (cm^2)	*P*-value
H. annosum	vs.	*L. huntii*	10	-3.4 (8.8)	0.1950	-0.4 (0.6)	0.0043	-0.2 (0.5)	0.1383	-10.45 (19.80)	0.0416
H. annosum	vs.	*L. procerum*	10	7.4 (10.5)	0.0056	-0.1 (0.5)	0.3936	0.4 (0.4)	0.0008	11.98 (19.92)	0.0200
H. annosum	vs.	*L. serpens*	10	-2.3 (7.2)	0.3822	-0.4 (0.3)	0.0118	-0.1 (0.4)	0.4805	1.41 (11.30)	0.7807
H. annosum	vs.	*L. terebrantis*	10	3.7 (9.0)	0.1581	0.2 (0.4)	0.1874	-0.3 (0.4)	0.0196	7.80 (17.23)	0.1264
L. huntii	vs.	*L. procerum*	10	10.8 (7.5)	0.0001	0.3 (0.6)	0.0172	0.4 (0.3)	0.0014	16.61 (10.31)	0.0015
L. huntii	vs.	*L. serpens*	9	1.9 (7.4)	0.5007	0.0 (0.4)	0.8107	0.1 (0.3)	0.4165	4.36 (11.34)	0.4158
L. huntii	vs.	*L. terebrantis*	9	6.8 (7.2)	0.0156	0.0 (0.4)	0.9885	0.0 (0.4)	0.8585	9.43 (16.27)	0.0803
L. procerum	vs.	*L. serpens*	10	-13.7 (10.4)	0.0001	-0.4 (0.4)	0.0023	-0.3 (0.3)	0.0437	-14.22 (19.52)	0.0060
L. procerum	vs.	*L. terebrantis*	10	-6.5 (6.3)	0.0151	-0.2 (0.4)	0.1196	-0.7 (0.5)	0.0001	-13.16 (14.92)	0.0108
L. serpens	vs.	*L. terebrantis*	10	0.2 (6.5)	0.9484	0.1 (0.3)	0.6183	-0.1 (0.5)	0.5830	1.54 (14.58)	0.7620

Note: Results obtained by including Estimate statements in the GLM procedure.
Note: Analyses were conducted using alpha = 0.05.

All fungal species were consistently isolated from roots following inoculations (Table 5.18). In loblolly pine, re-isolation ranged from 67% for *H. annosum* to 95% for *L. procerum*. In slash pine, *H. annosum* was isolated from 78% of samples, while the highest rate of recovery was observed in *L. terebrantis* at 94%.

Table 5.19. Percentage of loblolly and slash pine roots which yielded *H. annosum*, *G. huntii*, *L. procerum*, *L. serpens*, and *L. terebrantis* following inoculation.

	Loblolly Pine		Slash Pine	
Treatment	N	Re-isolation (%)	N	Re-isolation (%)
H. annosum	76	67	77	78
G. huntii	81	80	78	88
L. procerum	81	95	81	89
L. serpens	79	86	82	93
L. terebrantis	78	83	78	94

5.6. Discussion

Lesions were consistently observed in all treatments, including the controls in both root inoculation experiments of loblolly and slash pine. Lesions, consisting of darkened, discolored, pitch-filled tissue surrounding the point of inoculation have been observed in previous inoculations of mature trees (Lee et al. 2006; Wingfield 1986). In wound controls, discolored tissue was lighter in color and less occluded. In contrast, phloem tissue surrounding wound + media controls were darker and pitch-filled, similar to observations when active fungi were present, though considerably smaller. Other studies that included both control types found similar results with respect to differences in their appearance (Rice et al. 2007). In these studies, the addition of sterile media caused larger lesions in slash pine family D only, when compared to wound controls. Since all test trees were located in the same general vicinity (< 40 yards apart), the probability of infection by other pathogenic soil fungi in slash pine family D only, is unlikely. Previous studies have also found variability in the intensity at which pine hosts respond to artificial media (Raffa and Smalley 1988). These observations indicate that genetic differences in slash pine are responsible for the differential response to the sterile

media. As a result, both control types were pooled in tests involving loblolly pine and slash pine in family C, while the control types were kept separate in further analyses with family D.

A significant host reaction, resulting in darkened lesions following artificial fungal inoculation can be considered to be an indicator of fungal virulence (Dunn et al. 2002; Solheim and Krokene 1998). All fungal species used caused significant root lesions in pine hosts. Artificial inoculation with *H. annosum* commonly causes resinosis, and tissue discoloration in loblolly pine (Hodges and Kuhlman 1974). *Leptographium terebrantis* and *L. procerum* have been shown to induce significant stem lesions. Similarly, *L. serpens* has been observed causing a darkened lesion, larger than controls, in loblolly pine (Eckhardt et al. 2004a). *Grosmannia huntii* has not been tested on large mature pines. These studies are first to observed a significant lesion in mature pine hosts. The lesion width following *L. procerum* inoculation was commonly not larger than controls in slash pine. *Leptographium procerum* has previously failed to cause significant damage in host tissue (Nevill et al. 1995; Wingfield 1986) and is generally thought to be mildly pathogenic to pine (Wingfield 1983). *Leptographium procerum* has been isolated from slash pines in the past, including from damaged roots in pine seed orchards (Horner and Alexander 1983). These root inoculations indicate that all fungi tested have the potential to be significant contributors to root damage in loblolly and slash pine.

Pine hosts commonly vary their response to different fungal species following artificial inoculation (Ben Jamaa et al. 2007; Paine et al. 1988). In these studies, with the exception of *L. serpens*, *G. huntii* clearly induced a larger lesions in both pine hosts, compared to other fungal species. *Grosmannia huntii* also caused the largest lesions, among the four ophiostomatoid fungi, in southern pine seedlings (Chapter 2). These studies are first to confirm the relative virulence of *G. huntii* in large tree roots. *Leptographium procerum* appears to be the least virulent, among the fungi tested, and is commonly found to be less virulent than other blue-stain pathogens (Eckhardt et al. 2004a; Nevill et al. 1995; Wingfield 1983; Wingfield 1986). *Heterobasidion annosum*, *L. serpens*, and *L. terebrantis* were generally not different in their effect on loblolly and slash pine roots. *Heterobasidion annosum*, cause of annosum root rot, is considered a

primary pathogen of pines in the southeastern states and around the world. However, data suggests that environmental conditions (Froelich et al. 1966) and host vigor (Gibbs 1967) significantly affects its potential for root infection and damage. Similarly, *L. serpens* (previously *V. alacris* [Wingfield and Marasas 1981]) has been identified and considered the primary cause of pine root disease in South Africa (Wingfield and Knox-Davies 1980). In some hosts, *L. terebrantis* induces a considerable host response and has been implicated in several root disease systems (Jacobs and Wingfield 2001). When ophiostomatoid fungi and *H. annosum* have been compared in the past, ophiostomatoid species have caused comparable and in several instances significantly greater lesions in host tissues (Gibbs 1967; Nagy et al. 2005). In these studies, *G. huntii* was found to be more virulent compared to *H. annosum* and results indicate *G. huntii* and *L. serpens* have the greatest potential for root damage of loblolly and slash pine.

Grosmannia huntii has recently been found infecting southern pine host roots (Zanzot 2009) that were fully occluded with significant tissue damage from resin impregnation (Matusick observations). Results suggest *G. huntii* infection causes an extraordinary lesion, caused by the combination of fungal damage and host hypersensitivity. A recent below ground survey of declining trees in Alabama found *Hylastes* species breeding in galleries surrounded by occluded, blue-stained tissue infested with *G. huntii*. Bark beetles recovered from the galleries were also infested with *G. huntii* (Matusick and Eckhardt unpublished data), and *Hylastes* have recently been observed transporting *G. huntii* in stands of longleaf pine in Georgia (Zanzot et al. 2010). Collectively, these observations indicate that *G. huntii* is a pathogen when found in loblolly and slash pine roots, and should be considered in future studies of root disease in the southeastern states.

Determining one specific causal agent when diagnosing pine root disease in the southeastern United States may be difficult and not necessarily important. *Heterobasidion annosum* is known to act as a primary pathogen under a certain set of conditions (Stambaugh 1989), where it acts as a root rot fungus breaking down secondary, non-functional sapwood, often leading to wind throw of otherwise healthy trees. In contrast, *H. annosum* has also been associated with a more gradual decline in host vigor and health, leading to thinning, discolored foliage (Bega 1963). Root infection

by ophiostomatoid fungi has been associated with host symptomology similar to *H. annosum* (Eckhardt et al. 2007). Declining trees are often found with a host of pathogenic root fungi with varying degrees of pathogenicity and virulence (Barnard et al., 1991; Barnard et al. 1985). Since root pathogens cause more damage in stressed hosts (Gibbs 1967), a cascading effect with respect to host tree health may be observed following the initial infection. In addition, stressed hosts are known to attract bark beetle and weevil vectors of pathogenic ophiostomatoid fungi (Erasmus and Chown 1994; Flechtmann et al., 1999), which may lead to an accumulation of pathogenic root fungi. Determining the specific fungal species that initially infected the host is difficult depending on when the host is sampled (Kim et al. 2005a). Ophiostomatoid fungi are often involved in saprophytic cycles, in which insect vectors and fungal associates rely on dying trees for resources (Wingfield et al. 1988). However, in the southeastern United States, *Hylastes* species and regeneration weevils have been observed feeding on seemingly healthy tree roots (Matusick observations), which may lead to infection if ophiostomatoid fungi are present. However, evidence for this assertion is poor, since most data suggests *Hylastes* species are significantly more attracted to dead and dying root tissue for breeding substrate (Rudinsky and Zethner-Møller 1967) and young newly planted seedlings for maturation feeding (Leahy et al. 2007). More research is needed on the frequency in which vectors feed on healthy root tissue. Regardless of which fungus is primary, and which is secondary, it may be more important to concentrate on the interaction between competing pathogenic root fungi and their cumulative effects on host root decline. A better understanding of the interaction may lead to more accurate tree health diagnoses and more promising land management solutions.

Chapter 6

Root Lesions in Mature Loblolly Pine Following Inoculation with Four Root-Inhabiting Ophiostomatoid Fungi

6.1. Abstract

Loblolly pine decline, characterized by deteriorating root systems leading to shortening and thinning of foliage has been observed throughout portions of the southeastern United States. Several root-inhabiting ophiostomatoid fungi, including *Leptographium procerum*, *L. terebrantis*, *L. serpens*, and *Grosmannia huntii* are associated with lateral root damage on declining loblolly pine. Trees of various ages were inoculated with the four fungi in fall 2006/fall 2007 and spring 2007/spring 2008. Fungal root inoculations were performed on primary lateral roots and the lesion reaction was characterized after eight weeks in order to determine the pathogenicity and virulence of the fungi. All fungi caused a darkened, resin-filled lesion on the surface of the phloem, extending into the xylem that was larger than controls. Only lesions associated with *G. huntii* infection were significantly larger in the spring season, compared to the fall. *Grosmannia huntii* was found to be the most virulent fungus, causing lesions than were longer, deeper, and larger than all other fungal species during the spring and larger than *L. terebrantis* and *L. procerum* in the fall. *Leptographium serpens* was the second most virulent fungal species, causing lesions larger than *L. procerum* and *L. terebrantis* (with the exception of lesion depth) during both seasons. *Leptographium procerum* and *L. terebrantis* lesions were of similar size in the fall; however, *L. terebrantis* was larger in every parameter in the spring. These tests indicate *G. huntii* and *L. serpens* are significant root pathogens, capable of causing considerable damage, while *L. terebrantis* and *L. procerum* are less virulent. It is clear ophiostomatoid fungi can be responsible for significant damage in healthy loblolly pine roots, and depending on the actions of their

vectors, *G. huntii* and *L. serpens* may be responsible for significant root deterioration and tree disease.

6.2. Introduction

Loblolly pine is the leading commercial timber species in the United States (Shultz 1997). It is estimated either directly or indirectly provide 110,000 jobs and $30 billion to the economy (Shultz 1999). Ecologically, loblolly pine-dominated stands support a diverse assemblage of organisms including the endangered the red-cockaded woodpecker (*Picoides borealis* Vieillot). Poor growth and increasing tree decline leading to mortality has been well-documented in loblolly pine over the past 50 years, particularly in saw timber-sized pines (Brown and McDowell 1968; Hess et al. 1999; Ross and Preacher 1971). Observations in affected stands suggest root damage and mortality associated with ophiostomatoid 'blue-stain' fungi contribute to tree decline and mortality (Eckhardt et al. 2007; Menard 2007). Declining loblolly pine has and will continue to have serious economic and ecological implications in the future.

Loblolly pines experiencing decline show shortened chlorotic needles leading to thinning crowns (Roth and Peacher 1971) with a distinct absence of above-ground insect or disease pests (Brown and McDowell 1968). However, damage below ground on lateral (Eckhardt et al. 2007) and fine (Hess et al. 1999) roots has been consistently observed. On lateral roots, feeding and breeding damage by *Hylastes* species (Coleoptera: Curculionidae) bark beetles and two weevil species (*Hylobius pales* (Herbst.) and *Pachylobius picivorous* (Germ.) Coleoptera: Curculionidae) accompanied by resin-soaked lesions have been apparent (Eckhardt et al. 2007; Menard 2007). Early observations note the deterioration of fine roots (Roth and Peacher 1971; Hess et al. 1999), which may be a product of extensive lateral root damage or potentially caused by the fine root pathogen *Phytopthora cinnamomi* Rands (Campbell and Copeland 1954). Several insect-vectored ophiostomatoid fungal species with *Leptographium* anamorphs have been isolated from roots of pine trees in various stages of decline (Eckhardt et al. 2007). *Leptographium procerum* (W.B. Kendr.) M.J. Wingf.(formerly *Verticicladiella procera* W.B. Kendr.) and *L. terebrantis* S.J. Barras & T.J. Perry are isolated from insect damaged or seemingly healthy loblolly pine root tissue. *Leptographium serpens* (Goid)

M.J. Wingf., also successfully isolated from loblolly pine roots, is exclusively associated with insect damage (Eckhardt et al. 2007) and root resinosis (Matusick personal observations). More recently, *Grosmannia huntii* ((R.C. Rob. Jeffr.) Zipfel, Z.W. de Beer & M.J. Wingf (*L. huntii* M.J. Wingfield) has been isolated from insect vectors and loblolly pine roots (Matusick and Eckhardt unpublished data). In each instance that *G. huntii* has been recovered from loblolly pine, severe tissue resinosis and insect damaged tissue is present (Matusick observations). These observations suggest loblolly pine roots support a host of ophiostomatoid fungi associated with a variety of root tissue conditions; however, the degree to which fungal infection causes root deterioration in unknown.

Root decline and tree mortality associated with ophiostomatoid fungi have been observed in pine systems throughout North America (Klepzig et al. 1991; Dochinger 1967; Cobb 1988). In western conifers, *L. wageneri* (W.B. Kendr.) M.J. Wingf. causes extreme resinosis of the xylem tracheids restricting water conduction through affected tissues (Joseph et al. 1998). Also, Procerum root disease (*L. procerum*) causes significant physiological disruption of vascular tissues in eastern white pine (*P. strobus* L.)(Butnor et al. 2000). Following inoculation of lateral pine roots, Klepzig et al. (1996) found *L. terebrantis* to cause resin-soaked lesions extending far past what would be expected from wounded controls. In each of the pine disease systems descbribed, root-inhabiting ophiostomatoid fungi were determined to be either causal or contributing factors, following evidence of vascular root damage in large trees.

To determine the potential root damage caused by ophiostomatoid fungi, artificial root inoculation studies were conducted in loblolly pine stands of various ages during the fall of 2006 and 2007 as well as the spring of 2007 and 2008. The primary objective was to determine the pathogenicity and virulence of *L. procerum*, *L. terebrantis*, *L. serpens*, and *G. huntii* on healthy pine host roots. It is hypothesized that the four fungal species can cause differing degrees of damage following inoculation. These experiments were conducted in order to better determine the role each fungal species contributes to the observed decline.

6.3. Materials and Methods

All fungal isolates were originally isolated from dying loblolly or longleaf pine in the southeastern United States using a procedure described in Eckhardt et al. (2007)(Table 2.1) . Single spore isolates in the anamorphic state (*Leptographium* species) of *L. procerum*, *L. terebrantis*, *L. serpens*, and *G. huntii* (*L. huntii*) were used. The identities were confirmed by Mike Wingfield (Forestry and Agricultural Biotechnology Institute, South Africa) using morphological and sequence data. Two weeks prior to inoculation, all isolates were placed on 2% malt extract agar (MEA). In the fall inoculations, a wound and a wound + sterile MEA control were used for comparison, whereas, in the spring, only a wound control was included due to reservations about maintaining sterility.

Loblolly pine stands were selected in a variety of different age classes; 10 to 20 years, 20 to 30 years, and 30 to 40 years. The inoculation test was repeated within the same stands for the spring 2007 tests, while new stands, within the same age classes were used for fall 2007 and spring 2008 inoculations.

All fungal species were paired, resulting in six total pairings (Table 6.1), in order to more accurately distribute treatments throughout the stand. All pairings were randomly assigned to nine trees within each stand, each season. In addition, control treatments were assigned to each tree. Two primary lateral roots were excavated on each tree to approximately 152 cm (5 ft) from the root collar area. One fungal species assigned to the tree was randomly assigned to each excavated root, along with one control. The control was administered approximately 30 cm (1 ft), with the fungal inoculation administered approximately 91 cm (3 ft) from the root collar area, respectively. Inoculations were conducted using methods described by Wright (1933). A 13 mm cork borer was used to create a wound, the bark plug was removed and a 10 mm plug of actively growing mycelium was aseptically placed on the surface of the exposed root cambium. The bark plug was then placed on the mycelial plug and the site was covered and sealed with duct tape (Fig. 6.1). Controls were performed in a similar fashion with nothing inserted in the wound only control and sterile MEA placed at the cambial surface for the wound + sterile media controls. All treatment sites were marked with colored pin flags then buried.

Table 6.1. Six fungal treatment pairings used for treatment assignment and distribution

Fungal Pairings		
G. huntii	+	*L. procerum*
G. huntii	+	*L. serpens*
G. huntii	+	*L. terebrantis*
L. serpens	+	*L. procerum*
L. serpens	+	*L. terebrantis*
L. terebrantis	+	*L. procerum*

Fig. 6.1. Exposed loblolly pine roots following inoculations.

Eight weeks following the inoculation, all roots were re-excavated, severed from the tree, and then removed from the ground for measurements in the laboratory (Fig. 6.2). All treatment sites were inspected for indications of host response and disease symptoms.

In the laboratory, the bark tissue was removed surrounding all treatment sites and the diameter of each root was measured. At each inoculation site, the length and width of the darkened, discolored tissue was recorded and then traced on clear transparent sheets, measured later for an estimate of lesion surface area (cm^2) using a Lasico® Planimeter (Lasico®, Los Angeles, CA). Roots samples were cut transversely at the point of inoculation to expose the sapwood and the depth of discolored sapwood was measured. In addition, small pieces of root tissue 1 cm from either side of the infection point were

removed and plated on 2% MEA amended with 800 mg/L of cycloheximide and 200 mg/L of streptomycin sulfate (CSMA) to re-isolate the fungi.

It was hypothesized that control treatments (wound only and wound + media) would cause a similar effect when included in fall inoculations. For each tree, the two control treatments were subtracted from one another, leaving only the effects from the media presence. The additional media effects were then used in an ANOVA in the general linear model (GLM) procedure in SAS (SAS institute, 9.1 ed., Cary, NC). Included in the model were blocked variables, experiment year and age class. An additional test (estimate statement), within the GLM, was included to test whether the effects from media were larger than zero on average. All continuous response variables (lesion length, width, depth, and area) were tested in an identical fashion.

Fig. 6.2. Pile of inoculated roots following excavation and removal from the stand

Secondly, it was hypothesized fungal species would cause a larger lesion than controls. For each root, the control treatment was subtracted from the fungal treatment, resulting in the effects from fungal presence. Analyses were conducted for each fungal x

control treatment combination separately, using an ANOVA in the GLM procedure. For those fungal x control treatment combinations involving the wound control, season was included with experiment year (blocked) and age class (blocked) as factors in the model. For those fungal x control combinations involving the wound + media control (used in fall only), season was not included as a factor. Tukey's multiple comparison test was included to decipher seasonal effects for those tests involving controls without media. Additional tests (estimate statements) were included in the GLM in order to determine whether effects from fungi were significant (greater than zero).

Finally, it was hypothesized fungal species would cause variable lesion effects. For each tree (sampling unit), fungal treatments were subtracted from one another, resulting in the difference between treatments. Experiment year (blocked), age class (blocked), season (fall/spring), fungal treatment pairing (6 pairings), and the interaction between season and fungal treatment pairing were included in the GLM. Tukey's multiple comparison test was used as a post hoc analysis of season, fungal treatment pairing, and their interactions. Additional tests (estimate statements) were included in the GLM, to test whether fungal species, within a treatment pairing, were different from one another (difference value was statistically different from zero).

6.4. Results

In the fall inoculations, statistical differences were observed between the two control treatments. The presence of sterile media caused increases in lesion length (0.2 cm), depth (0.1 cm), and area (1.39 cm^2)(Table 6.2).

Table 6.2. Mean increase in response variables, lesion length, width, depth, and area, when sterile media was present

	Mean Increase Due to Sterile Media
Lesion Length (cm)	0.2 (1.3)
P-value	0.0183
Lesion Width (cm)	0.0 (0.3)
P-value	0.1762
Lesion Depth (cm)	0.1 (0.4)
P-value	0.0001
Lesion Area (cm^2)	1.39 (4.27)
P-value	0.0001

Note: Means (followed by standard deviation in parentheses) followed by *P*-value < 0.05 are significantly different from zero.

All fungi inoculated into pine roots caused longer, wider, deeper, and larger lesions on average than the wound only (Table 6.3) and wound + media (Table 6.4) controls. Season contributed to variation in lesion length, depth, and area when testing the additional effects attributed to *G. huntii* (Table 6.5). When *G. huntii* was present, lesion length, depth, and area were larger during the spring inoculations compared to fall (Table 6.6). Lesion parameters were not affected by season for all other fungi tested.

Grosmannia huntii caused significantly longer, deeper, and larger (lesion area) lesions than all other fungal species, with the exception of *L. serpens* (lesion depth and lesion area) and *L. terebrantis* (lesion depth) in the fall inoculations (Table 6.7). In addition, the difference in lesion area between *G. huntii* and all other fungal species was larger in the spring, compared to fall. *Leptographium serpens* caused longer, deeper, and larger lesions than *L. procerum* and longer and larger lesions than *L. terebrantis* (Table 6.8). *Leptographium terebrantis* caused longer, deeper, and larger lesions than *L. procerum* in the spring, but not in the fall. *Grosmannia huntii* and *L. serpens* caused the development of wider lesions than *L. terebrantis* and *L. procerum*, but not each other

(Table 6.9). *Leptographium terebrantis* caused wider lesions compared to *L. procerum*. The frequencies in which fungal species were re-isolated from inoculated roots were similar (Table 6.10).

Table 6.3. Net increase in lesion length, width, depth, and area due to the addition of *G. huntii*, *L. procerum*, *L. serpens*, and *L. terebrantis* compared to wound only.

Fungal Treatment - Control				Net increase due to fungal treatment							
A	-	B	N	Lesion Length (cm)	*P*-value	Lesion Width (cm)	*P*-value	Lesion Depth (cm)	*P*-value	Lesion Area (cm^2)	*P*-value
G. huntii	vs	Wound	227	6.4 (4.9)	0.0001	0.7 (0.7)	0.0001	0.8 (0.5)	0.0001	28.00 (20.97)	0.0001
L. procerum	vs	Wound	237	2.3 (2.0)	0.0001	0.3 (0.5)	0.0001	0.4 (0.3)	0.0001	8.82 (6.73)	0.0001
L. serpens	vs	Wound	238	5.4 (3.4)	0.0001	0.7 (0.7)	0.0001	0.8 (0.5)	0.0001	21.60 (15.27)	0.0001
L. terebrantis	vs	Wound	250	3.4 (2.7)	0.0001	0.4 (0.5)	0.0001	0.7 (0.5)	0.0001	12.93 (9.11)	0.0001

Note: Means (followed by standard deviation in parentheses) followed by *P*-value < 0.05 are significantly larger than zero.

Table 6.4. Net increase in lesion length, width, depth, and area due to the addition of *G. huntii*, *L. procerum*, *L. serpens*, and *L. terebrantis* compared to wound + sterile media.

Fungal Treatment - Control				Net increase due to fungal treatment							
A	-	B	N	Lesion Length (cm)	*P*-value	Lesion Width (cm)	*P*-value	Lesion Depth (cm)	*P*-value	Lesion Area (cm^2)	*P*-value
G. huntii	vs	W + M	89	5.5 (5.2)	0.001	0.7 (0.6)	0.0001	0.7 (0.6)	0.0001	19.64 (18.12)	0.0001
L. procerum	vs	W + M	78	2.5 (2.2)	0.0001	0.3 (0.4)	0.0001	0.3 (0.3)	0.0001	8.61 (8.16)	0.0001
L. serpens	vs	W + M	80	5.1 (3.0)	0.0001	0.5 (0.6)	0.0001	0.7 (0.4)	0.0001	21.01 (13.36)	0.0001
L. terebrantis	vs	W + M	69	3.2 (2.5)	0.0001	0.3 (0.5)	0.0001	0.5 (0.5)	0.0001	11.93 (9.51)	0.0001

Note: W + M stands for wound + sterile media control

Note: Note: Means (followed by standard deviation in parentheses) followed by *P*-value < 0.05 are significantly larger than zero.

Table 6.5. Probability of a greater *F*-statistic from ANOVAs testing fungal species vs. wound only controls.

Fungus-Wound Control	Source	df	Lesion Length	Lesion Width	Lesion Depth	Lesion Area
G. huntii - wound	Experiment Year	1	0.0915	0.0035	0.6398	0.0541
	Age Class	2	0.7274	0.6983	0.0168	0.0962
	Experiment Season	1	0.0020	0.1182	0.0001	0.0001
	Error	222				
L. procerum - wound	Experiment Year	1	0.6008	0.0973	0.0954	0.0004
	Age Class	2	0.2624	0.0349	0.8405	0.0415
	Experiment Season	1	0.6714	0.5811	0.4823	0.5619
	Error	232				
L. serpens - wound	Experiment Year	1	0.0518	0.1226	0.1118	0.0001
	Age Class	2	0.0200	0.6465	0.4857	0.2432
	Experiment Season	1	0.8306	0.5421	0.4630	0.1510
	Error	233				
L. terebrantis - wound	Experiment Year	1	0.7720	0.8482	0.4168	0.0009
	Age Class	2	0.0027	0.7317	0.0047	0.0043
	Experiment Season	1	0.1711	0.2472	0.1638	0.3032
	Error	245				

Table 6.6. Lesion length, depth, and area following as a result of treatment with *G. huntii*.

G. huntii - Wound	N	Lesion Length (cm)	*P*-value	Lesion Depth (cm)	*P*-value	Lesion Area (cm^2)	*P*-value
Fall	68	5.4 (3.9)		0.7 (0.4)		21.55 (12.23)	
Spring	159	7.4 (4.7)	0.0020	1.0 (0.5)	0.0001	32.46 (21.63)	0.0001

Table 6.7. Net increase in lesion length, depth, and area due to fungal species A compared with fungal species B in each season of inoculation.

				Net increase due to fungal treatment A					
A	-	B	N	Lesion Length (cm)	*P*-value	Lesion Depth (cm)	*P*-value	Lesion Area (cm^2)	*P*-value
G. huntii	vs	*L. procerum*							
		Fall	50	3.1 (2.9)abc	0.0001	0.4 (0.5)ab	0.0001	10.48 (11.28)cde	0.0001
		Spring	54	5.3 (4.3)a	0.0001	0.6 (0.5)a	0.0001	26.81 (21.21)a	0.0001
G. huntii	vs	*L. serpens*							
		Fall	54	1.3 (4.6)c	0.0108	0.0 (0.5)cd	0.9208	1.21 (22.04)e	0.5896
		Spring	52	2.4 (5.1)bc	0.0001	0.2 (0.5)bcd	0.0019	14.28 (22.74)bc	0.0001
G. huntii	vs	*L. terebrantis*							
		Fall	54	1.7 (3.9)bc	0.0013	0.0 (0.6)cd	0.4943	5.00 (12.32)cde	0.0257
		Spring	53	4.0 (4.7)ab	0.0001	0.2 (0.6)bcd	0.0008	21.65 (21.43)ab	0.0001

Note: Means (followed by standard deviation in parentheses) followed by *P*-value < 0.05 are significantly greater than zero.

Note: Mean (followed by standard deviation in parentheses) with the same letter within a column are not significantly different based on alpha=0.05 using Tukey's multiple comparison test.

Table 6.8. Net increase in lesion length, depth, and area due to fungal species A compared with fungal species B in each season of inoculation.

				Net increase due to fungal treatment A					
A	-	B	N	Lesion Length (cm)	*P*-value	Lesion Depth (cm)	*P*-value	Lesion Area (cm^2)	*P*-value
L. serpens	vs	*L. procerum*							
		Fall	52	3.0 (2.9)abc	0.0001	0.3 (0.4)abc	0.0001	12.38 (15.36)bcd	0.0001
		Spring	53	2.8 (4.3)bc	0.0001	0.4 (0.5)ab	0.0001	14.88 (12.82)bc	0.0001
L. serpens	vs	*L. terebrantis*							
		Fall	54	2.0 (3.1)bc	0.0001	0.1 (0.6)cd	0.0587	9.90 (14.92)cde	0.0001
		Spring	53	1.8 (3.3)bc	0.0007	-0.1 (0.7)d	0.4664	10.48 (15.72)cde	0.0001
L. terebrantis	vs	*L. procerum*							
		Fall	53	0.9 (2.6)c	0.0959	0.3 (0.5)bcd	0.0005	2.07 (10.82)de	0.3605
		Spring	53	1.7 (2.6)bc	0.0013	0.5 (0.5)ab	0.0001	5.99 (8.26)cde	0.0081

Note: Means (followed by standard deviation in parentheses) followed by *P*-value < 0.05 are significantly greater than zero.

Note: Mean (followed by standard deviation in parentheses) with the same letter within a column are not significantly different based on alpha=0.05 using Tukey's multiple comparison test.

Table 6.9. Net increase in fungal species A compared to fungal species B for lesion width.

A	-	B	N	Lesion Width (cm)	*P*-value
G. huntii	vs	*L. procerum*	108	0.5 (0.7)a	0.0001
G. huntii	vs	*L. serpens*	106	0.1 (0.7)b	0.1516
G. huntii	vs	*L. terebrantis*	107	0.3 (0.7)ab	0.0001
L. serpens	vs	*L. procerum*	105	0.3 (0.7)ab	0.0001
L. serpens	vs	*L. terebrantis*	107	0.3 (0.7)ab	0.0001
L. terebrantis	vs	*L. procerum*	106	0.2 (0.7)ab	0.0006

Note: Means (followed by standard deviation in parentheses) followed by *P*-value < 0.05 are significantly greater than zero.

Note: Mean (followed by standard deviation in parentheses) with the same letter within a column are not significantly different based on alpha=0.05 using Tukey's multiple comparison test.

Table 6.10. Frequency of success in re-isolating *L. huntii*, *L. procerum*, *L. serpens*, and *L. terebrantis* from inoculated roots.

Treatment	N	Re-isolation Success (%)
G. huntii	317	73
L. procerum	315	73
L. serpens	318	71
L. terebrantis	320	72

6.5. Discussion

Grosmannia huntii and *L. serpens* are much more pathogenic to loblolly pine roots compared to *L. procerum* and *L. terebrantis*, with *G. huntii* being the most pathogenic of the four fungal species. *Grosmannia huntii* and *L. serpens* cause an extreme resin response, blue-stained tissue, and large resin-filled lesions (Fig. 6.3).

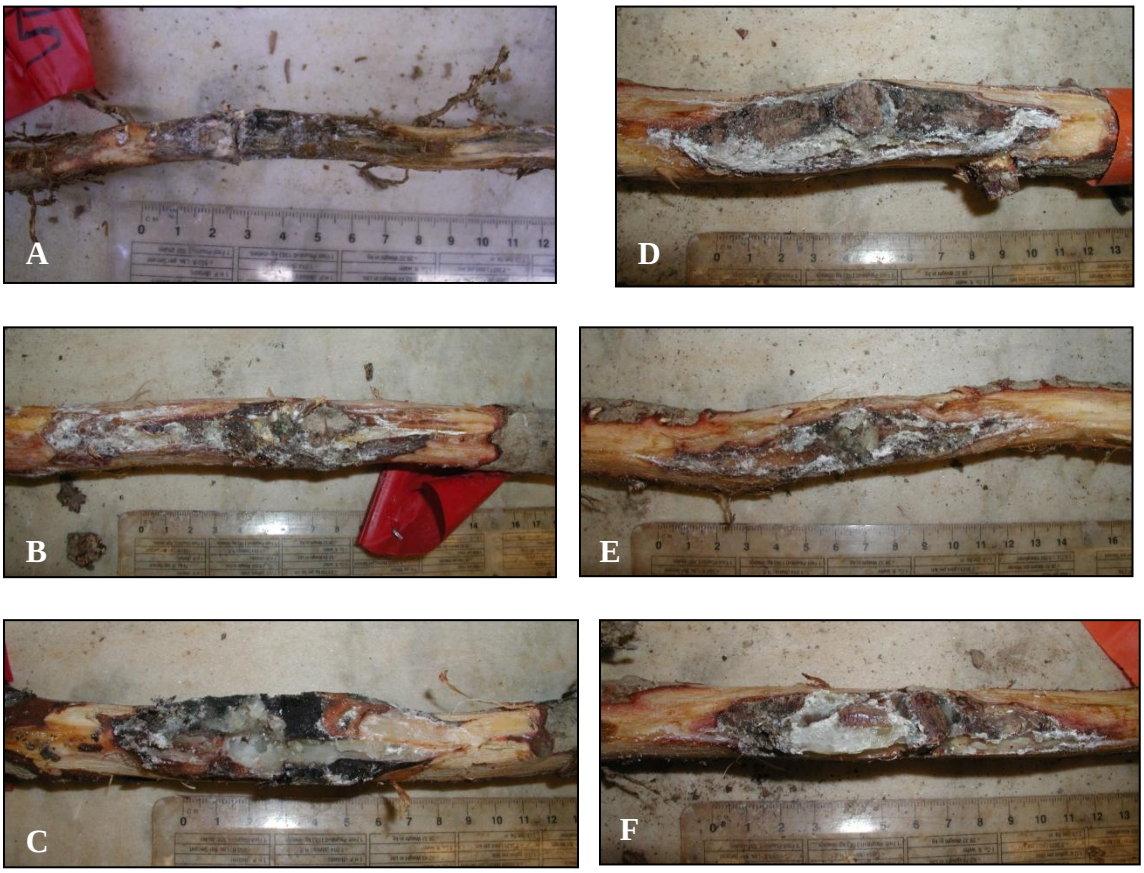

Fig. 6.3. Common root observations following inoculation with *L. serpens* (A, B, C) and *G. huntii* (D, E, F) including an extreme resin response, resin-filled tissue, and blue-staining.

In contrast, *L. procerum* and *L. terebrantis* cause lesions that are qualitatively and quantitatively smaller, have less resin-filled tissue, and induce less host resin production. These results support other studies that have found *L. procerum* to act as a weak pathogen to *Pinus* species (Rane and Tattar 1987; Wingfield 1983: Wingfield 1986), including loblolly pine (Eckhardt et al. 2004a; Klepzig and Walkinshaw 2003). *Leptographium*

terebrantis has been known to be a virulent pathogen in other *Pinus* hosts (Jacobs and Wingfield 2001). However, in loblolly pine it appears to be significantly less pathogenic compared to *G. huntii* and *L. serpens*. In addition, inoculation tests have been performed with *L. serpens* in loblolly pine seedlings and large tree stems in the past (Eckhardt et al. 2004a) and results largely confirm previous the findings, with respect to the relative virulence to other fungal species. Results from these control inoculations suggest the fungi are responsible for considerable damage in pine roots. Lesion reactions extended far past the inoculation point on average with little indication of host success in impeding fungal growth after eight weeks.

Quantitative and qualitative characteristics of the lesion reaction help to elucidate the relationship between specific ophiostomatoid fungi and loblolly pine. *Leptographium serpens* has been much less frequently isolated from declining loblolly pines compared to *L. terebrantis* and *L. procerum*, but is found in the most severely symptomatic trees (Eckhardt et al. 2007). Similar qualitative results have been observed with *G. huntii* in loblolly (Matusick and Eckhardt unpublished data) and longleaf pine (*P. palustris* Mill.)(Zanzot 2009). Large, destructive lesions following inoculation confirm causation with respect to severely damaged roots and the presence of *G. huntii* and *L. serpens*. *Leptographium terebrantis* also caused lesions considerably larger than controls and has been closely associated with the decline condition in the past (Eckhardt et al. 2007). Following inoculation, *L. terebrantis* has been shown causing severe hydrolysis in callus cells accompanied by high phenolic accumulation indicating a severe host response (Klepzig and Walkinshaw 2003). However, it caused lesions significantly smaller than both *G. huntii* and *L. serpens*. *Leptographium procerum* is commonly observed in healthy pine root tissue (Matusick observations), suggesting it can live without initiating a host response possibly as a parasite on non-essential host cell carbohydrates. Asymptomatic loblolly pine resin has also been shown to slow the growth of *L. procerum*, while *G. huntii*, *L. serpens*, and *L. terebrantis* are seemingly unaffected (Eckhardt et al. 2008). Results from controlled root inoculations coupled with data from field observations suggest *G.huntii* and *L. serpens* are primary pathogens of loblolly pine roots, while *L. terebrantis* and *L. procerum* are moderately and mildly pathogenic, respectively.

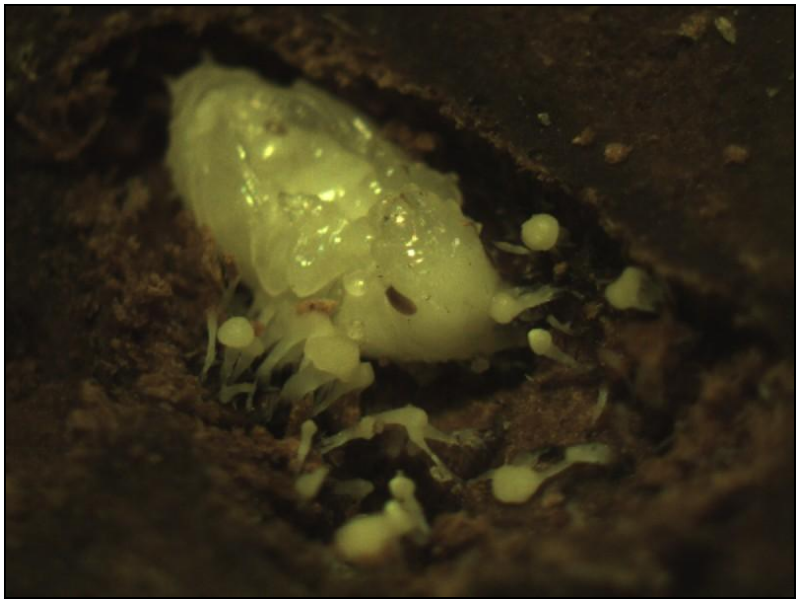

Fig. 6.4. *Hylastes* species pup in loblolly pine root tissue surrounding by a *Graphium* species fungus, closely related to *Leptographium.*

Based on population and genetic data, it has been hypothesized that *G. huntii* and *L. serpens* are more recently introduced in the southeastern United States (Zanzot 2009). The damage resulting from *G. huntii* and *L. serpens* infection is substantial in healthy loblolly pine and has the potential to affect the actions and reproductive success of its primary vectors. *Hylastes tenuis* appears to be the principle vector of *G. huntii* and *L. serpens* (Fig. 6.4)(Zanzot et al. 2010). Vector species, including *H. tenuis,* are generally considered non-aggressive insects, feeding and breeding in stressed or dying hosts (Wood 1982). However, vector actions can be linked to the virulence of their fungal associates (Krokene and Solheim 1998), which may allow *Hylastes tenuis* to become more aggressive in the future. Although *G. huntii* is the most virulent in loblolly pine roots, its potential for damage is regulated by actions of it's' vectors. Future research on vector species is required to better understand the potential for development of bark beetle-fungal associated root disease in the southeastern United States.

Chapter 7

Susceptibility of Longleaf Pine Roots to Infection and Damage by Four Root-Inhabiting Ophiostomatoid Fungi

7.1. Abstract

Restoration of longleaf-pine dominated uplands has become common on many public and private lands throughout the southeastern United States. The once dominant, longleaf pine ecosystem is incredibly important to many now threatened and endangered plant and animal species. Tree mortality in longleaf pine has been observed following attempts to re-introduce prescribed fire. Root-inhabiting ophiostomatoid fungi and their insect vectors have been observed invading roots of symptomatic longleaf pine, however the relationship between ophiostomatoid fungi and longleaf pine roots is largely unknown. In order to assess the pathogenicity and virulence of ophiostomatoid fungi to longleaf pine, two broad tree age classes (20-30 and 40-60 years) were used to inoculate the primary roots with four ophiostomatoid fungal species during the fall (2006/2007) and spring (2007/2008). All fungal species consistently caused resin-filled, discolored lesions on the phloem surface extending to the xylem. The successful inoculation of healthy longleaf pine roots confirms the pathogenicity of *Grosmannia huntii, Leptographium procerum*, *L. serpens*, and *L. terebrantis*. *Grosmannia huntii* was the most virulent fungal species, causing the largest lesions. In contrast, *L. procerum* was found to be the least virulent fungal species tested. Restoration efforts of longleaf pine may be affected by fungal root infection in the future. Future studies should focus on the interaction between stress factors associated with longleaf pine to more clearly define the ecological role of root-inhabiting ophiostomatoid fungi in the ecosystem.

7.2. Introduction

Longleaf pine was once the dominant *Pinus* species on upland sites throughout the southeastern United States. Once occupying 74 million acres (Frost 1993), longleaf pine-dominated land has been reduced to a small fraction of its original range (Outcalt and Sheffield 1996). Longleaf pine is intimately associated with frequent surface fires, which maintain ecosystem structure and function (Gilliam and Platt 1999). Wildfire suppression, the introduction of feral hogs (*Sus scrofa domesticus* L.)(Lipscomb 1989), and inherently slow regeneration (Frost 1993) are some of the many factors contributing to its' drastic reduction. The decline in longleaf pine-dominated forests has resulted in habitat loss for many species, including the red-cockaded woodpecker (RCW)(*Picoides borealis* Vieillot), which is reliant on large living trees for nesting (Conner et al. 2001). Following the federal listing of the RCW, restoration of the longleaf pine ecosystem has become a significant priority on many private, state, and federal lands throughout its original range (Alavalapati et al. 2002).

Fig. 7.1. Longleaf pine stand shortly after a low intensity prescribed burn.

With long needles, thick, flakey bark and a well protected bud, longleaf pine is particularly well adapted for frequent fire disturbance events (Fig. 7.1)(Landers 1991).

Fine fuels produced by longleaf pine help to facilitate frequent burn events (Platt et al. 1988), which aid in nutrient turnover, herbaceous growth, and the proliferation of a diverse assemblage of organisms in the understory (Carter and Foster 2003). The longleaf pine ecosystem has been considered one of the most biologically diverse forested systems on the planet (Peet and Allard 1993) with the RCW among the many species that experienced a severe population decline that paralleled the loss of longleaf pine habitat. Recovery of the RCW is dependent on the successful restoration of the longleaf pine-dominated uplands (Conner et al. 2001). However, restoration efforts have been plagued with a variety of different challenges (Brockway et al. 2005). An unexplained premature mortality in longleaf pine has been observed, especially following the re-introduction of prescribed fire after a period of fire exclusion (Otrosina et al. 2002).

Despite the frequency and severity of disturbance in longleaf-dominated ecosystems, annual mortality rate is relatively low on average (Boyer 1979; Palik and Pederson 1996). Longleaf pine has few serious insect and disease pests, relative to other closely related southeastern pine species (Snow et al. 1990). The southern pine beetle (*Dendroctonus frontalis* Zimmerman) is the largest biotic pest in the southeastern United States and causes catastrophic damage in loblolly (*Pinus taeda* L.) and shortleaf (*Pinus echinata* Mill.) pine. However, longleaf pine is known to be incredibly tolerant of SPB infestation (Friedenberg et al. 2007). The tree is also more tolerant of prominent root disease pests, *Heterobasidion annosum* (Fr.) Bref. (Platt et al. 1965) and *Phytopthora cinnamomi* Rands, compared to other southeastern *Pinus* species (Barnard et al. 1993). Arguably the most damaging disease of longleaf pine is brown spot needle blight (*Mycosphaerella dearnessii* M.E. Barr), causing mortality in young grass-stage seedlings. However, a group of root-inhabiting ophiostomatoid fungi have recently been associated with dying longleaf pines (Otrosina et al. 1999). Additionally, ophiostomatoid fungi, including *Leptographium terebrantis* S.J. Barras and T.J. Perry and *L. procerum* (W.B. Kendr.) M.J. Wingf. were isolated from woody longleaf pine roots following burning (Otrosina et al. 2002) and in a more recent survey of longleaf pine roots at Fort Benning Military Installation, Georgia (Zanzot 2009). Evidence suggests ophiostomatoid fungi

are widespread in woody roots of longleaf pine; however, their role in the longleaf pine ecosystem in not well established.

Grosmannia species (previously *Ophiostoma* Zipfel et al. 2006) and their anamorphs, *Leptographium* species have been identified as causal agents of conifer diseases around the world (Wingfield et al. 1988). Under some circumstances, ophiostomatoid fungi have been considered primary pathogens of conifer hosts (Cobb 1988). The best example is black-stain root disease affecting various conifer species of the western United States, caused by *L. wageneri* (W.B. Kendr.) M.J. Wingf. Infection by ophiostomatoid fungi often result in a pitch-filled resinous lesion, with the potential to cause severe occlusion of affected tissues (Hessburg and Hansen 1987). Significant loss of root conductivity has been detected in naturally infected root sections (Joseph et al. 1998). Recently, mortality in loblolly pine has been closely associated with *L. procerum*, *L. terebrantis* and possibly *L. serpens* (Goidanich) Siemaszko, along with other biotic and abiotic factors (Eckhardt et al. 2007). Loblolly pine decline is hypothesized to be a product of several different stress factors including topography (Eckhardt and Menard 2008), insect pest damage (Eckhardt et al. 2004a), and fungal infection (Eckhardt et al. 2004b). However, roles of each factor have not been conclusively established. Some in the southeastern United States believe ophiostomatoid fungi act as facultative pathogens, causing significant damage in stressed hosts (Otrosina et al. 2002). A similar decline of red pine (*P. resinosa*) also involves root-inhabiting ophiostomatoid fungi and their insect vectors (Klepzig et al. 1991). Klepzig et al. (1996) suggests their damage is a result of a compromised host condition.

Although the pathogenicity of several southeastern ophiostomatoid fungi has been shown experimentally in young longleaf pine trees and certain fungal species were determined to be more virulent (Chapter 4), inoculations of large tree roots provide the most accurate measure of pathogenicity and virulence. A large-scale root inoculation experiment, incorporating a range of tree ages, during two seasons, was established in 2006 and 2007 in order to determine if ophiostomatoid fungi cause significant root damage in longleaf pine. It is hypothesized ophiostomatoid fungi can cause significant damage following inoculation of seemingly healthy longleaf pine roots. The secondary objective is to determine the most damaging root-inhabiting ophiostomatoid species

(among four). It is hypothesized that ophiostomatoid fungi vary in their effect on longleaf pine roots, which will help determine the largest potential threat to longleaf pine in the future. Finally, the time of inoculation will be considered, in order to determine when host trees may incur the most severe root damage.

7.3. Material and Methods

A total of four longleaf pine stands were used in inoculation tests with two pine age groups represented (20-30) and (40-60). All stands were located in the east gulf coastal plain of Alabama in Conecuh and Covington Counties. In fall 2006, inoculations were performed in a 25 and 44 year old longleaf pine stand, located at the Solon Dixon Forestry Center, AL. In spring 2007, an identical inoculation test was conducted using different trees within the same stands. The entire study was then repeated in fall 2007 and spring 2008 in one 27 and 54 year-old stand at the Conecuh National Forest. All stands have been maintained with frequent prescribed fire. All inoculated trees appeared healthy with no crown characters suggesting of root disease or stress. Inoculation tests were conducted on trees of two age classes and during two different seasons in order to obtain the widest possible representation of conditions.

Inoculation tests were performed using a single spore isolate, in the anamorphic state, of each of the following four ophiostomatoid fungi, including *G. huntii* (R.C. Rob. Jeffr.) Zipfel, Z.W. de Beer & M.J. Wingf., *L. procerum*, *L. serpens*, and *L. terebrantis* (Table 2.1). All fungal isolates had been previously collected from roots of southern pines exhibiting local tissue damage and deteriorating crowns using methods described in Eckhardt et al. (2007). Two weeks prior to each inoculation, all isolates were placed on 2% malt extract agar (MEA). One day prior to each inoculation test, a 10 mm diameter punch was used to create plugs along the actively growing portion of the fungal colony margin.

In the fall inoculation tests, the four fungal species were used along with two control treatments (wound only and wound + sterile MEA). In the spring inoculation tests, only one control was used (wound only). The four fungal treatments were paired with one another, resulting in six total fungal pairings with each pairing randomly assigned to nine trees within each stand. In the fall inoculations, both control treatments

were assigned to each sample tree. In the spring inoculations two wound only controls were assigned to each tree.

Two primary lateral roots on each sample tree were excavated approximately 1.5 m (5 ft) from the root collar area. One of the two fungal species (from the fungal pairing) was randomly assigned to each excavated root. The fungal inoculation was performed approximately 91 cm (3 ft) from the root collar area. Additionally, one control treatment was assigned to each root. The control treatment was performed approximately 30 cm (1 ft) from the root collar area. All inoculations were administered using a 13 mm diameter cork borer to create a wound in the top of the root, exposing the cambial layer. Next, the 10 mm diameter plug of actively growing mycelium was placed against the exposed tissue. The plug of bark tissue, removed from the wound, was then placed over the mycelia plug and duct tape was used to cover the inoculation site (Wright 1933). The wound controls were administered in an identical fashion, with nothing and sterile MEA in place of fungal mycelium for the wound and wound + media control respectively. All treatment sites were marked with pin flags prior to burying each root.

Eight weeks after inoculation the roots were re-excavated paying careful attention not to damage the roots during the process. All inoculated roots were severed from the tree and removed from the ground for measurements in the laboratory. The bark was removed surrounding each treatment and the length and width of discolored vascular tissue was recorded along with the root diameter at each treatment point. A clear transparent sheet was used to trace the outline of each discolored lesion and a LASICO® (LASICO Co. Los Angeles, CA) planimeter was used to estimate the total surface area (cm^2) for each discolored lesion (Klepzig and Walkinshaw 2003). Each root was cut transversely through the point of inoculation and the total depth of discolored sapwood was measured from the center of each inoculation site. Small sections of tissue were removed from areas surrounding each inoculation site and placed on CSMA (MEA containing 800 mg/l of cycloheximide and 200 mg/l of streptomycin sulfate) for identification.

The two control wounds were tested for similarities using data from the fall 2006 and 2007 tests. The control treatments were subtracted from each other on each tree, resulting in the added effect of media. The additional media effect was subjected to

ANOVA in the general linear model (GLM) procedure in SAS statistical software. Year and stand age class (20-30) (40-60) were included as blocked factors in the model. Estimate statements, included in the GLM, allowed for testing whether the added effect of MEA was statistically larger than zero.

The control lesion measurement was subtracted from the fungal lesion measurement on each root, resulting in the added effect of fungi tested. Then the added effect of each fungal species separately was subjected to an ANOVA in the GLM procedure. Included in the model were year (blocked variable), stand age class (blocked variable), and season. Appropriate estimate statements were included to test whether the added effect of each fungal species was significantly greater than zero.

The effect of the fungal inoculation on longleaf pine roots were compared using a method described previously. On each tree, the difference between the two fungal isolates was calculated and then subjected to an ANOVA in the GLM procedure for each host response variable. Year (blocked factor), stand age class (blocked factor), season, fungal pairing, and the interaction between season and fungal pairing were included in the model. Estimate statements were included to test whether the difference between the fungi was greater than zero, for each host response variable. The analysis represents a direct comparison between all fungal species, using only those trees in which both fungal species were paired. Seasonal effects were tested for each treatment – control separately as well as for each treatment (wound control, *G. huntii*, *L. procerum*, *L. serpens*, *L. terebrantis*) separately.

7.4. Results

A clearly discolored, pitch-filled lesion was observed surrounding the point of root inoculation on the surface of the phloem, often extending deep into the sapwood with all ophiostomatoid fungi tested on longleaf pine. The pitch response was severe, particularly when *G. huntii* and *L. serpens* were present, creating a large fissure extending the length of the discolored lesion (Fig. 7.2). In contrast, control inoculations resulted in a weak pitch response, with light colored tissue surrounding the treatment point (Fig. 7.3). The presence of sterile media in the wounds caused increases in root lesion size when it was included during the fall inoculations. Lesion length, depth, and area were larger in those wounds that received sterile media (Table 7.1, 7.2). Despite significance,

increases due to the sterile media were negligible considering sources of measurement error and biological significance.

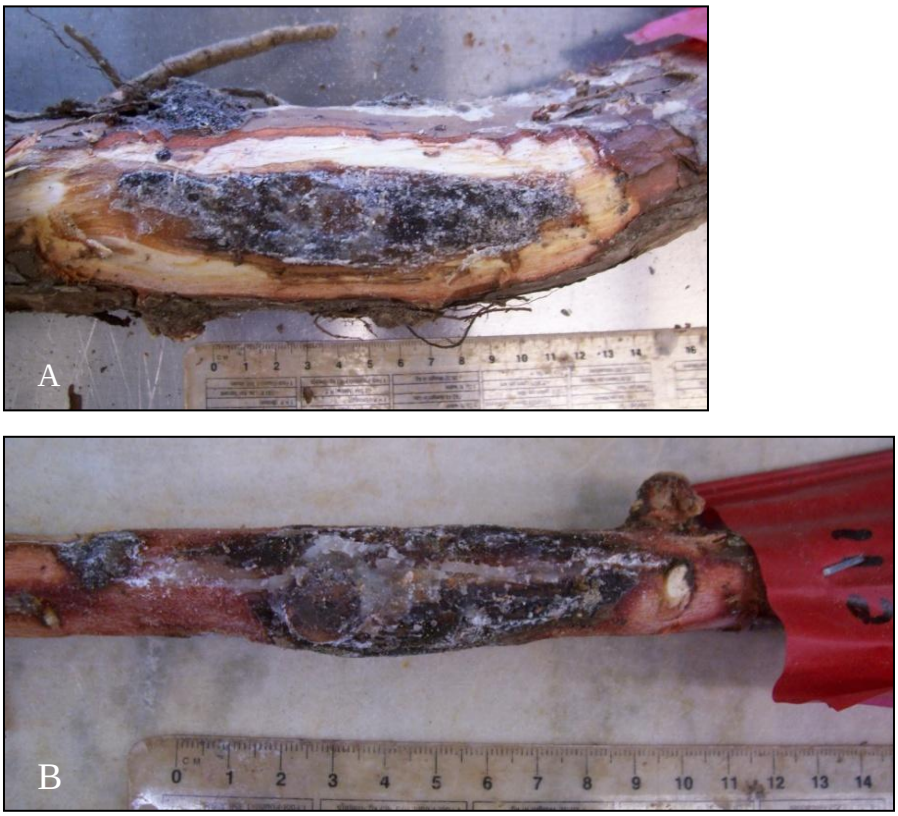

Fig. 7.2. Discolored, pitch-filled lesion and severe pitch response following inoculation with *G. huntii* (A) and *L. serpens* (B).

Fig. 7.3. Light colored wounded tissue surrounding control treatment.

Table 7.1. Probability of a greater *F*-statistic from ANOVA testing the presence of sterile media vs. when in was absent (wound only).

Source	df	Lesion Length	Lesion Width	Lesion Depth	Lesion Area
Experiment Year	1	0.0204	0.3218	0.8248	0.7010
Stand Age Class	1	0.7596	0.8939	0.6274	0.6622
Error	214				

Table 7.2. Average increases in lesion length, width, depth, and area due to the presence of sterile media in controls.

	Mean increase due to sterile MEA
Lesion Length (cm)	0.2 (1.4)
P-value	0.0245
Lesion Width (cm)	0.0 (0.3)
P-value	0.5728
Lesion Depth (cm)	0.1 (0.1)
P-value	0.0117
Lesion Area (cm^2)	0.83 (4.17)
P-value	0.0028

Note: means (followed by standard deviation) with *P*-value ≥ 0.05 are not different from zero.

The root inoculation of *G. huntii, L. serpens*, *L. procerum,* and *L. terebrantis* caused greater lesion lengths, widths, depths, and areas compared to when the fungus was not included (Table 7.3).

Table 7.3. Net increase in lesion length, width, depth, and area when *G. huntii*, *L. procerum*, *L. serpens*, and *L. terebrantis* were present compared to controls.

	Net Increase due to Fungal Species			
	G. huntii	*L. procerum*	*L. serpens*	*L. terebrantis*
Lesion Length (cm)	7.2 (6.2)	2.0 (2.2)	4.4 (3.3)	3.4 (3.6)
P-value	0.0001	0.0001	0.0001	0.0001
Lesion Width (cm)	0.7 (0.7)	0.2 (0.5)	0.6 (0.6)	0.4 (0.6)
P-value	0.0001	0.0001	0.0001	0.0001
Lesion Depth (cm)	0.9 (0.6)	0.3 (0.4)	0.6 (0.5)	0.8 (0.6)
P-value	0.0001	0.0001	0.0001	0.0001
Lesion Area (cm^2)	25.62 (18.07)	6.13 (5.72)	16.21 (11.54)	12.31 (12.08)
P-value	0.0001	0.0001	0.0001	0.0001

Note: means (followed by standard deviation) with *P*-value ≥ 0.05 are not different.

Significant differences were observed between the fungal pairings (Table 7.4) as fungal species tested unequally contributed to root lesion size. *Grosmannia huntii* caused the greatest lesion length and total lesion area when compared to all other fungal species tested (Table 7.5). *Leptographium serpens* inoculation resulted in a larger ($T = 2.32$, $P = 0.0209$) but not longer ($T = 1.56$, $P = 0.1202$) lesion compared to *L. terebrantis*. All other fungal species used caused longer and larger lesions than *L. procerum.* Lesion width followed a similar pattern for the fungi tested. *Grosmannia huntii* resulted in deeper lesions than *L. serpens* and *L. procerum*, but not *L. terebrantis*. *Leptographium terebrantis* caused deeper lesions than *L. serpens* ($T=-2.82$, $P= 0.0050$) and *L. procerum* consistently produced lesions shallower than all other species. Average root diameter, tree diameter, and tree height were similar among fungal pairings. Tree diameters ranged

from 25.4 cm to 26.7 cm on average, while average tree heights ranged from 20.57 m to 21.24 m (Table 7.6).

The time of year (season) in which inoculations were conducted significantly affected lesion development. When wounds were subtracted, fungal lesions were larger (cm^2) on average during the spring season (Tables 7.7, 7.8). In addition, the lesion depth into the sapwood was larger in the spring with *G. huntii* and *L. terebrantis*. Seasonal differences were also observed when each treatment was used individually used (without subtracting the controls) in tests. Root lesion area and the lesion depth were affected by season (Table 7.9). Lesion area was larger during the spring inoculations for each fungal treatment tested on longleaf pine. The mean lesion depth was larger in the spring inoculations for the inoculations involving *G. huntii*, *L. terebrantis*, and *L. procerum* (Table 7.10). In contrast to fungal treatments, the wound control had deeper lesions in the fall, but the overall size was not different between seasons. Root infection was confirmed by re-isolating the fungi. On average, each of the fungal species was re-isolated from greater than 70% of inoculated roots.

Table 7.4. Probability of greater *F*-statistic for ANOVA testing lesion length, width, depth, area, average root diameter, tree diameter, and height.

Source	df	Lesion Length	Lesion Width	Lesion Depth	Lesion Area	Root Diameter	Tree Diameter	Height
Year	1	0.0001	0.0302	0.0426	0.0153	0.7327	0.0001	0.0001
Age Class	1	0.0043	0.0876	0.2006	0.0052	0.2067	0.0001	0.0001
Season	1	0.1123	0.4235	0.5630	0.0882	0.3855	0.9559	0.9665
Fungal Pairing	5	0.0001	0.1327	0.0001	0.0001	0.2923	0.6309	0.2967
Error	419							

Table 7.5. Mean fungal residual (A-B) and probability of greater *F*-statistic for lesion length, width, area, and root diameter.

A	-	B	N	Lesion Length (cm)	*P*-value	Lesion Width (cm)	*P*-value	Lesion Depth (cm)	*P*-value	Lesion Area (cm^2)	*P*-value
G. huntii		*L. serpens*	70	3.0 (6.1)	0.0001	0.3 (0.7)	0.0001	0.4 (0.6)	0.0001	9.21 (16.25)	0.0001
G. huntii		*L. terebrantis*	72	3.2 (4.0)	0.0001	0.4 (0.7)	0.0001	0.1 (0.7)	0.2297	13.37 (16.42)	0.0001
G. huntii		*L. procerum*	73	6.3 (11.9)	0.0001	0.4 (0.7)	0.0001	0.6 (0.6)	0.0001	22.20 (23.02)	0.0001
L. serpens		*L. terebrantis*	71	1.1 (4.5)	0.1202	0.2 (0.8)	0.0100	-0.2 (0.6)	0.0050	4.27 (12.67)	0.0209
L. serpens		*L. procerum*	72	3.0 (3.7)	0.0001	0.5 (0.5)	0.0001	0.3 (0.4)	0.0001	10.69 (11.29)	0.0001
L. terebrantis		*L. procerum*	69	2.4 (3.2)	0.0014	0.3 (0.6)	0.0001	0.5 (0.7)	0.0001	8.65 (11.32)	0.0001

Note: means (followed by standard deviation in parentheses) with *P*-value ≥ 0.05 are not significantly different.

Table 7.6. Mean tree diameter at breast height and total tree height.

A	-	B	N	DBH (in)	Tree Height (ft)	Root Diameter (mm)	*P*-value
G. huntii		*L. serpens*	70	10.3 (2.8)	67.5 (12.1)	0.50 (2.12)	0.0588
G. huntii		*L. terebrantis*	72	10.0 (2.9)	68.1 (11.5)	-0.28 (2.33)	0.2881
G. huntii		*L. procerum*	73	10.3 (2.8)	68.6 (12.0)	-0.26 (1.76)	0.3180
L. serpens		*L. terebrantis*	71	10.5 (3.1)	68.9 (12.4)	0.00 (2.75)	0.9915
L. serpens		*L. procerum*	72	10.0 (2.8)	68.3 (12.3)	0.17 (2.08)	0.5088
L. terebrantis		*L. procerum*	69	10.2 (2.8)	69.7 (13.5)	0.08 (2.24)	0.7696

Table 7.7. Probability of a greater *F*-statistic in four separate ANOVA testing fungal vs. control residuals for lesion length, width, depth, and area.

	Source	df	Lesion Length	Lesion Width	Lesion Depth	Lesion Area
G. huntii - Control						
	Year	1	0.0001	0.0125	0.0072	0.0176
	Age Class	1	0.0001	0.1335	0.4791	0.0010
	Season	1	0.3132	0.9479	0.0001	0.0008
	Error	211				
L. procerum - Control						
	Year	1	0.2072	0.7439	0.2421	0.0626
	Age Class	1	0.9793	0.4006	0.9272	0.0886
	Season	1	0.7025	0.9845	0.1051	0.0015
	Error	210				
L. serpens - Control						
	Year	1	0.1760	0.1661	0.2350	0.0011
	Age Class	1	0.7449	0.0003	0.1305	0.2413
	Season	1	0.5641	0.9129	0.0828	0.0060
	Error	209				
L. terebrantis - Control						
	Year	1	0.2220	0.0006	0.5607	0.0002
	Age Class	1	0.9903	0.5390	0.9500	0.7256
	Season	1	0.6777	0.0315	0.0001	0.0054
	Error	208				

Table 7.8. Net increase in lesion depth and area when *G. huntii*, *L. procerum*, *L. serpens*, and *L. terebrantis* were compared to controls for fall and spring separate and differences in values observed in fall and spring inoculation tests.

Fungal Treatment - Control				Net increase due to fungal treatment			
A	-	B	N	Lesion Depth (cm)	*P*-value	Lesion Area (cm^2)	*P*-value
G. huntii	vs	Control					
		Fall	108	0.7 (0.6)b	0.0001	21.43 (18.26)b	0.0001
		Spring	107	1.0 (0.6)a	0.0001	29.83 (19.11)a	0.0001
L. procerum	vs	Control					
		Fall	108	0.3 (0.4)a	0.0001	4.87 (5.30)b	0.0001
		Spring	106	0.4 (0.3)a	0.0001	7.38 (6.23)a	0.0001
L. serpens	vs	Control					
		Fall	107	0.6 (0.6)a	0.0001	14.00 (9.57)b	0.0001
		Spring	106	0.7 (0.4)a	0.0001	18.41 (13.73)a	0.0001
L. terebrantis	vs	Control					
		Fall	107	0.7 (0.5)a	0.0001	9.98 (9.83)b	0.0001
		Spring	105	1.0 (0.6)b	0.0001	14.64 (14.62)a	0.0001

Note: Means (followed by standard deviation in parentheses) with *P*-value ≥ 0.05 are not different.

Note: Means (followed by standard deviation in parentheses) followed by the same letter within a column are not different (each fungal species tested separately).

Table 7.9. Probability of greater *F*-statistic for lesion length, width, depth, and area in four ANOVAs using data for each fungal species separately.

	Source	df	Lesion Length	Lesion Width	Lesion Depth	Lesion Area
Wound	Year	1	0.0001	0.0307	0.0001	0.0001
	Age Class	1	0.0817	0.6178	0.0807	0.7098
	Season	1	0.1567	0.0001	0.0001	0.2994
	Error	642				
G. huntii	Year	1	0.0001	0.0001	0.5979	0.4496
	Age Class	1	0.0003	0.0997	0.6028	0.0008
	Season	1	0.1215	0.1069	0.0125	0.0005
	Error	214				
L. procerum	Year	1	0.1433	0.1685	0.0001	0.0001
	Age Class	1	0.8558	0.3163	0.9169	0.0831
	Season	1	0.2019	0.4885	0.0057	0.0402
	Error	211				
L. serpens	Year	1	0.2341	0.1982	0.0074	0.0001
	Age Class	1	0.6270	0.0015	0.0005	0.1924
	Season	1	0.9676	0.0300	0.2510	0.0057
	Error	210				
L. terebrantis	Year	1	0.4119	0.2307	0.0007	0.0001
	Age Class	1	0.6218	0.8969	0.8644	0.6240
	Season	1	0.9325	0.8790	0.0090	0.0021
	Error	209				

Table **7.10**. The lesion depth and area observed in fall and spring inoculations for the wound, *G. huntii*, *L. procerum*, *L. serpens*, and *L. terebrantis* treatments, each analyzed separately.

Treatment	N	Lesion Depth (cm)	Lesion Area (cm^2)
Wound			
Fall	217	0.4 (0.3)a	5.04 (3.69)a
Spring	429	0.2 (0.3)b	5.35 (3.89)a
G. huntii			
Fall	109	1.1 (0.5)b	27.14 (17.97)b
Spring	109	1.3 (0.5)a	35.98 (19.73)a
L. procerum			
Fall	108	0.7 (0.3)a	10.35 (5.45)b
Spring	107	0.6 (0.3)b	11.99 (6.79)a
L. serpens			
Fall	107	0.9 (0.4)a	19.37 (10.65)b
Spring	107	1.0 (0.5)a	23.87 (13.96)a
L. terebrantis			
Fall	107	1.0 (0.6)b	15.50 (10.30)b
Spring	106	1.2 (0.6)a	20.57 (14.55)a

Note: Means (followed by standard deviation in parentheses) followed by the same letter within a column are not different (analyzed for each treatment separately).

Table 7.11. The proportion of samples where infection was confirmed for *G. huntii*, *L. procerum*, *L. serpens*, *L. terebrantis*.

Treatment	N	Re-isolation Success (%)
G. huntii	215	70
L. procerum	214	73
L. serpens	213	70
L. terebrantis	212	71

7.5. Discussion

These studies were designed to represent a conclusive test of pathogenicity, including trees from several different age classes, stands, and seasons. Each fungal species consistently infected inoculated roots and affected more tissue than controls, confirming their pathogenicity to longleaf pine roots. Similar conclusions have been made previously with respect to *L. serpens* (Chapter 3**)**, *G. huntii*, *L. procerum*, and *L. terebrantis* in longleaf pine seedlings (Chapter 2). In addition, Otrosina et al. (2002) observed *L. procerum* and *L. terebrantis* causing significant lesion development in roots and stems of longleaf pine.

The darkened lesion reaction is commonly observed in conifer hosts around the world following inoculation with ophiostomatoid fungi (Kuroda 2005; Plattner et al. 2008; Solheim et al. 2001). Increases in oleoresin flow are characteristic of tissues surrounding artificial inoculations (Klepzig et al. 2005; Knebel et al. 2008), particularly following the introduction of highly pathogenic species (Cobb 1988). A darkened lesion forms on the surface of the phloem often extending deep into the xylem parenchyma (Nagy et al. 2005). Identical observations were made following artificial inoculations of longleaf pine roots and are consistent with inoculation of *Pinus* hosts with other pathogenic ophiostomatoid fungi (Lee et al. 2006; Solheim and Krokene 1998). In contrast, control inoculations with sterile agar and control wounds initiate a passive desiccation of damaged tissues immediately surrounding the inoculation point, with little or no lasting damage of conducting tissues (Klepzig and Walkinshaw 2003).

Discolored lesions following inoculation with ophiostomatoid fungi are known to increase with increasing fungal virulence (Owen et al. 1987; Parmeter et al. 1989). It is

thought that ethylene production triggers an increase in monoterpene synthesis, which regulates the size of the resulting lesions (Popp et al. 1995). Of the fungi tested, *G. huntii* was the most virulent on longleaf pine roots. These tests are first to confirm the pathogenicity and virulence of *G. huntii* on large longleaf pine. *Leptographium serpens* was also more virulent than both *L. terebrantis* and *L. procerum* isolates. These findings support previous reports in loblolly pine, which found *L. serpens* to cause larger lesions in seedlings and large tree stems (Eckhardt et al. 2004b). *Leptographium serpens* has been associated with disease of *P. pinaster* Aiton. and *P. radiata* D. Don. in South Africa. In root inoculation tests, *L. serpens* produced lesions extending 20 cm after six months in *P. pinaster* and *P. radiata* (Wingfield and Knox-Davies 1980). Discoloration associated with *G. huntii* and *L. serpens* inoculation extended far from the inoculation point on average and penetrated deep into susceptible sapwood, resulting in severe damage to host tissues. Although no clear host physiological disruption was detected, severe occlusion of roots does lead to a reduction in hydraulic conductivity (Joseph et al. 1998). More than 60% of the cross-sectional sapwood was found to be occluded in several roots samples following inoculation. *Leptographium terebrantis* also caused larger lesion areas than *L. procerum*, which is in line with findings that *L. terebrantis* is more pathogenic than *L. procerum* (Eckhardt et al. 2004b; Klepzig and Walkinshaw 2003; Klepzig et al. 1996; Wingfield 1986). When considering all lesion measurements, *L. procerum* was the least damaging species tested and although commonly isolated from pine roots, should not be considered a primary agent of longleaf root disease.

Grosmannia huntii is more virulent than all other fungal species tested; however, transmission of *G. huntii* to longleaf pine roots is dependent on the actions of their vectors. *Grosmannia huntii* is closely associated with a variety of bark beetle vectors around the world (Jacobs and Wingfield 2001), including root-feeding bark beetles *Hylastes tenuis* Eichhoff and *H. salebrosus* Eichhoff in longleaf pine stands (Zanzot et al. 2010). Despite the association between *G. huntii* and root-feeding bark beetles, longleaf pine roots naturally infected by *G. huntii* have been rare (Zanzot 2009). However survey results could be misleading, due to the sampling of longleaf pine root tissue which has been systematic and unbiased in the past, with most samples taken from healthy root sections (Matusick observations). *Leptographium terebrantis* and *L.*

procerum are commonly isolated from root tissue using this method (Zanzot 2009). However, *G. huntii* causes a distinct set of severe local symptoms and it is likely that biased sampling of damaged, occluded roots would more efficiently detect its presence.

Several studies have illustrated seasonal patterns of lesion size and development following inoculation with ophiostomatoid fungi (Horntvedt 1988; Paine 1984; Reid and Shrimpton 1971). Larger lesion sizes are routinely observed during the growing season, compared to the dormant months (Stephen and Paine 1985). Lesion development is partially a product of starch conversion to resin constituents for defense, which decreases continuously from May to December in loblolly pine (Blanche et al. 1992). Cook et al. (1986) found a clear relationship between lesion size and temperature, with the largest lesions formed during the summer months, decreasing into the fall. Additional support for this hypothesis is that root cells formed shortly following differentiation from the meristem have the greatest potential for xylem resin production (Berryman 1972). These longleaf pine studies illustrate, larger, and with certain fungal species, deeper lesions during the spring ('growing') season. Some authors have suggested lesion area following inoculation of host stems represents the trees ability to defend itself against invading bark beetles and associated pathogens (Paine et al. 1997). However following inoculation of host roots, it is clear that significant sapwood damage results from the hypersensitive lesion response and may lead to significant losses of hydraulic conductivity resulting from tissue occlusion, particularly during periods of strong response. Joseph et al. (1998) observed loss of conductivity in roots naturally affected by black-stain root disease and similar findings have been shown experimentally following inoculation of host stems (Kuroda 2005; Matusick et al. 2008).

On the ecosystem scale, the consequences of infection by root-inhabiting ophiostomatoid fungi in longleaf pine roots are unknown. It has been difficult to determine the amount of longleaf pine mortality attributed to root fungal infection alone. More realistically, root infection by ophiostomatoid fungi should be considered among the group of stress factors that are regularly experienced by longleaf pine. For example, the re-introduction of cyclical burning regimes has occurred throughout many areas of southeastern United States and is known to increase tree stress (Varner et al. 2005). As a result, common bark beetle and weevil vectors are attracted to stands following severe

burns (Sullivan et al. 2003) and introduce root-inhabiting ophiostomatoid fungi (Hanula et al. 2002). Otrosina and Ferrell (1995) suggest common secondary pests (i.e. bark beetles, and root-inhabiting ophiostomatoid fungi) can act as primary pests when stand disturbance regimes are altered. There is clearly the potential for root damage in longleaf pine by ophiostomatoid fungi and restoration of longleaf pine-dominated uplands may be hampered by root disease associated with ophiostomatoid fungi in the future. Further studies on the interactions among stress factors will more clearly define the role of root-inhabiting ophiostomatoid fungi in longleaf pine mortality.

Chapter 8

Summary and Conclusions

8.1. Southern Pine Forests

The southeastern pine-dominated forests are some of the most valuable forests on the planet. Economically, southern pines provide valuable timber and fiber to the United States and the world (Wear and Geis 2002). The region produces more than 60 percent of the nation's timber and is projected to continue its growth (Prestemon and Abt 2002). Several timber products, including saw logs, pulpwood, and plywood are commonly formed from southern pines (Wear and Geis 2002). More recently, biomass has been harvested for fuel wood (Mitchell and Gallagher 2007). Historically, turpentine, rosin, and mineral spirits have been important exports extracted from southern pines (Brockway et al. 2005). The southern pine forests also provide valuable ecosystem services and support a diverse assemblage of organisms, including many threatened and endangered species. The most notable species, endangered from habitat loss and degradation, is the red-cockaded woodpecker (RCW)(*Picoides borealis* Vieillot). As a result of the RCW 's population decline, management on many federal, state, and private lands have focused restoration of the native pine ecosystem.

Disturbance has historically been an important regulating factor in the southern pine ecosystem with fire the most prominent factor influencing the health, diversity, and structure of the forests. Entrenched weather patterns and topography, among other factors, have regulated fire events across the United States. Burning surface fuels provides for effective nutrient turnover, maintaining species diversity, and ensuring ecosystem stability. Extreme weather events, including hurricanes, tornados, and floods cause overstory pine mortality from damaging winds, hail, rain, and lightening. Arguably the most damaging biotic agent in the southeastern pine forests is

feral hogs, which cause root damage to established trees and mortality in seedlings (Lipscomb 1989). The major insect pests include, pine bark beetles, weevils, wood wasps, and sawflies. The southern pine beetle (*Dendroctonus frontalis*Zimmermann) has historically caused pine mortality on thousands of acres per year (Price et al. 1992). Other pine bark beetle groups including *Ips* species and *Dendroctonus terebrans* (Olivier) cause damage on stressed hosts. Southern pine species are susceptible to many disease agents. Stem cankers from fusiform rust and pitch canker can cause mortality (Tainter and Baker 1996). Root diseases are common in the southeastern United States and have historically been caused by two primary root pathogens. *Phytopthora cinnamomi* Rands, the causal agent of littleleaf disease in loblolly and shortleaf pines, infects fine rootlets which contribute to shortened needles, and thinning crowns preceding death (Campbell and Copeland 1954). Annosum root disease (*Heterobasidion annosum* (Fr.) Bref.) affects nearly all southern pine species and causes decay in lateral roots (Robbins 1984). Advanced stages of root rot can result in wind throw of seemingly healthy trees or can cause a more gradual thinning of the crown, reduced radial growth rate and chlorotic needles prior to death (Tainter and Baker 1996). More recently, the presence of root-inhabiting ophiostomatoid fungi have been associated with thinning crowns, chlorotic foliage and reduced radial growth preceding mortality. It is hypothesized that ophiostomatoid fungi, and damage from their insect vectors, are among several factors which collectively cause a gradual health decline of pine (Eckhardt et al. 2007).

8.2. Southern Pine Decline

Pine decline in the southeastern United States is a relatively new phenomenon. The first report of mortality in loblolly pine was in 1959 (Brown and McDowell 1968). Early observations indicated the decline was found across the range of soil types, unlike littleleaf disease, which is more closely associated with poorly drained soils (Copeland 1952). The peculiar absence of above-ground pests suggests that either abiotic or biotic below-ground factors are responsible for the decline. Brown and McDowell (1968) suggested one or more factors, below the soil surface, were contributing to increasing stress in loblolly pine. Eckhardt et al. (2007) has established a working hypothesis for the disease cycle. Abiotic stresses, such as slope and topography are thought to impose

an underlying stress on southern pines (Eckhardt et al. 2007), probably from offsite planting (Roth and Peacher 1971). As a result of abiotic stress, root-feeding bark beetle and weevil species are attracted to affected stands for maturation feeding and breeding in stressed hosts. During their actions, bark beetle and weevil insects transport and can inoculate susceptible hosts with ophiostomatoid fungal associates (Eckhardt et al. 2004b). Eckhardt et al. (2007) proposes a cascading effect occurs, whereby insect and fungal damage increases the attractiveness of the pine host to additional bark beetle insects and fungal pests, which ultimately accumulate to cause mortality. The hypothesis is based on the association between dying trees, certain slope and topography characteristics, high numbers of non-aggressive bark beetles, and the presence of ophiostomatoid fungi in the roots. Similar observations have been made associating abiotic factors (forest fire) and the presence of root-inhabiting ophiostomatoid fungi and their vectors in longleaf pine (Hanula et al. 2002; Otrosina et al. 1999; Sullivan et al. 2003). Experiments have been conducted to determine a cause and effect relationship for several aspects of the working pine decline hypothesis (Eckhardt et al. 2004a; Eckhardt et al. 2004b). However, more testing must be conducted before the decline hypothesis can be confirmed. For example, multiple stress tests could more accurately explain the proposed interacting factors experimentally. In addition, soil alterations from historical site disturbance have been proposed as a potential underlying cause that has yet to be tested empirically and represents a promising missing link considering the scope of the decline in the southeast. This dissertation represents a series of experiments to specifically address one aspect of the decline hypothesis: the ability of root-inhabiting ophiostomatoid fungi to infect and cause disease and damage in pine host tissue.

Inoculation experiments have been performed with ophiostomatoid fungi and conifer hosts around the world, including in North America (Wingfield et al. 1988). Inoculations allow for an accurate characterization of the relationship between fungi and hosts. Following inoculations, ophiostomatoid fungi are generally characterized as either pathogens or non-pathogens. In some circumstances, damage following inoculation can result in mortality of the host (Harrington and Cobb 1983; Lee et al. 2006; Rane and Tattar, 1987). In contrast, when minor damage occurs, ophiostomatoid bark-beetle associates may be found non-pathogenic to certain hosts (Neal and Ross 1999; Parmeter

et al. 1992). Inoculation tests can also provide a virulence comparison between competing ophiostomatoid fungi (Krokene and Solheim,1998a; Rice et al. 2007). It has been hypothesized that aggressive bark beetles associate with more virulent fungi compared to non-aggressive beetles (Krokene and Solheim 1998b). Nonetheless, virulence differences between fungal species and isolates within species are important in understanding the relative threat to pine stands.

8.3. Pathogenicity

Pathogenicity is defined as the ability of a microorganism to cause disease (Shaner et al. 1992). Following inoculations, several variables have been used to assess pathogenicity. First, the development of visual host symptoms leading to mortality provides conclusive evidence of pathogenicity (Lackner and Alexander 1982; Morin et al. 2007; Strobel and Sugawara 1986). However, local tissue symptom development, have been used in addition to or in place of mortality (Nevill and Alexander 1992a; Wingfield and Knox-Davies 1980). Pathogenic ophiostomatoid fungi cause tissue damage surrounding the point of infection, generally referred to as a lesion. In the case of seedlings, the lesion can manifest itself as a darkened, raised, or slightly sunken area surrounding the point of inoculation (Wingfield 1983; Wingfield 1986). In larger tree inoculations, the darkened lesion may be formed on the phloem surface (Popp et al. 1995a), xylem (Hessburg and Hansen 1987), or extend from the phloem into the xylem (Solheim and Krokene 1998). The lesion represents the reaction between fungal infection, fungal growth, and the chemical host response to the invading pathogen. Most ophiostomatoid fungi produce darkly pigmented mycelium which stains infected cells blue (‘blue-stain’) and contributes to the darkened appearance of the lesion (Bertagnole et al. 1983). However, another factor contributing to darkened tissue is the chemical host response (Raffa and Smalley 1995), which is known as a hypersensitive reaction (Cook and Hain 1986). Specific secondary anti-fungal metabolites are synthesized and distributed to cells surrounding the pathogen (Hofstetter et al. 2005; Klepzig et al. 1995). The resulting lesion is often described as resin-soaked, since cells are filled with the chemical mixture. The lesion size is commonly compared to a control lesion in order to determine if the fungus causes damage (i.e. pathogenic) (Eckhardt et al. 2004a).

Pathogenicity is contingent upon successful infection, which is detected by re-isolating the inoculated fungus after an incubation period. Other indicators of pathogenicity may involve measuring the host defense only, including resin quantity (Knebel et al. 2008; Krisits and Offenthaler 2002) or composition (Popp et al. 1995b). In addition, induced host physiological changes at the cellular (Klepzig and Walkinshaw 2003) and whole plant level have been used to assess pathogenicity.

The physiology of the lesion reaction has been studied extensively in pine hosts (Klepzig and Walkinshaw 2003; Lombardero et al. 2000; Popp et al. 1995b). Examination of the inner bark, phloem, and xylem following fungal infection has revealed a consistent set of steps that occur in healthy hosts. Immediately following inoculation, soluble-sugar content decreases as the host increases production of monoterpenes in the inner bark and xylem tissues (Cook and Hain 1987). Axial traumatic resin ducts may form to transport newly synthesized monoterpenes to the infection site (Lombardero et al. 2000). Monoterpene concentrations then increase in the xylem tissues surrounding infection (Klepzig and Walkinshaw 2003; Raffa and Smalley 1995). In addition, the polyphenolic parenchyma cells in the secondary phloem swell and excrete pre-formed and induced monoterpenes and phenolics into the surrounding tissues (Nagy et al. 2005). The lesion response on the phloem surface is known as the hypersensitive response (Berryman 1972). The combined responses of the phloem and xylem produces a three dimensional reaction zone surrounding fungal infection and represents the hosts attempt to stop the invading fungus.

Within the chapters of this dissertation, a number of seedling inoculation tests were conducted that paired the fungus with the host. In each test, consistent infection and lesion formation was observed. In nearly every host tree inoculation, a darkened lesion was observed (Chapters 2 and 3). Lesions generally grew longitudinally through the seedling stems. These observations are consistent with previous inoculations involving other pine hosts (Wingfield 1983; Wingfield 1986). In addition, occlusion of host vascular tissues in advance of the expanding lesion was detected for all fungi tested. Dry, non-conducting cells have been observed above and below the noticeable lesion in other inoculations (Kuroda 2005). Ophiostomatoid fungi are thought to disrupt water and nutrient conduction within the plant. For example, Procerum Root Disease causes a

severe reduction of water conductivity (Butnor et al. 2000) and *Leptographium terebrantis* S.J. Barras and T.J. Perry disrupts phloem and cambial tissues (Rane and Tattar 1987). Resinosis and the resulting occlusion of vascular tissues impede vascular conduction of roots infected by *Leptographium wageneri* (W.B. Kendr.) M.J. Wingf., the causal agent of black stain root disease (Joseph et al. 1998). In addition, metabolites produced by *L. wageneri*, have been shown to inhibit water transport in seedlings (Ayer et al. 1989), but do not cause a significant reduction in needle water potential. In addition, ophiostomatoid fungi, with the exception of *L. serpens* (Goidanich) Siemaszko in longleaf pine (Chapter 3), did not contribute to pine seedling mortality (Chapter 2). It is possible that the seedling inoculation experiment (Chapter 2), if left for a longer period of time, would have observed significant effects on tree mortality. Previous studies that have found significant mortality have left inoculated seedlings for considerably longer (Nevill et al. 1995; Owen et al. 1987; Wingfield 1983; Wingfield 1986). Despite the lack of mortality, consistent infection and lesion formation indicates each of the tested ophiostomatoid fungi are capable of causing disease and local tissue damage in loblolly, longleaf, and slash pine seedlings.

Lesions were also observed following inoculations of small (sapling-sized) longleaf pine trees (Chapter 4). Lesions extended from the surface of the phloem into the xylem. Inner bark tissues were resin-soaked compared to tissues not affected. The lesion reaction in the xylem was wedge-shaped, characteristic of ophiostomatoid fungal colonization (Kuroda 2005; Strobel and Sugawara 1986). With the exception of *L. procerum* (W.B. Kendr.) M.J. Wingf., all ophiostomatoid species caused significantly larger lesions than controls, indicating they are pathogenic to longleaf pine saplings. However, re-isolation of *G. huntii*, *L. serpens*, and *L. terebrantis* was lower than expected (20%) based on results from previous studies in seedlings and large trees. These results suggest *L. procerum* does not induce a lesion reaction to be considered pathogenic to longleaf pine in this stage of development. The pathogenicity of *G. huntii* (R.C. Rob. Jeffr.) Zipfel, Z.W. de Beer & M.J. Wingf., *L. serpens*, and *L. terebrantis* was confirmed, although small longleaf pine trees appear to be more tolerant to infection, compared to larger trees (Otrosina et al. 2002). It is possible that low temperature shortly following inoculation in both years affected fungal survival.

Inoculation of loblolly, longleaf, and slash pine roots resulted in consistent, dramatic lesion formation (Chapters 5-7). Lesions covered the surface of the phloem extending deep into the xylem. The lesion margin on the phloem surface was clearly defined by a dark red to black band separating resin-soaked tissue from healthy. In contrast, the xylem margin was much diffuse with less color contrast. Similar observations were made by Wingfield (1986) who included pictures of the diffuse lesion margin in the sapwood. Roots inoculated with *G. huntii*, *L. serpens*, and in some instances *L. terebrantis* resulted in large fissures (cracks) extending through the point of inoculation in the pine species tested. A large amount of resin was produced at the inoculation site, such that the root became swollen, resulting in tissue breakage. Often impressive massses of resin were observed covering the inoculation site on the outside of the root and were most evident with inoculations involving longleaf and slash pine. Loblolly pine produced less resin in response to wounding and inoculation. Control lesions were lighter in color, not resin-soaked, and dry. In Chapter 5, all fungal species developed significant lesions following root inoculation of loblolly and slash pine trees, including *H. annosum*. In addition, all fungal species were re-isolated from greater than 67% of inoculated trees and were found to be pathogenic to loblolly and slash pine. *Grosmannia huntii*, *L. procerum*, *L. serpens*, and *L. terebrantis* all produced darkened lesions surrounding the point of inoculation larger than both control types in both loblolly and longleaf pine roots (Chapters 6 and 7). For example, lesions were 25.62 cm^2 larger on average when *G. huntii* was present compared to when it was absent in longleaf pine. In addition, all fungi successfully infected more than 70% of inoculated roots. Without doubt *G. huntii*, *L. procerum*, *L. serpens*, and *L. terebrantis* are all pathogenic to loblolly and longleaf pine in both the fall and spring seasons. These experiments were first to report the pathogenicity of *G. huntii* and *L. serpens* in roots of southern pine as well as the pathogenicity of *L. procerum* and *L. terebrantis* in longleaf and slash pine roots.

8.4. Virulence

The definition of virulence can be stated as the amount damage caused by a pathogen in susceptible host tissue (Shaner et al. 1992). Virulence is a quantitative, relative measure of damage and is contingent upon pathogenicity. Virulence is often

reported when comparisons are made between two species or strains of the same species (Ben Jamaa et al. 2007; Rice et al. 2007). Several response variables have been used to assess the virulence of ophiostomatoid fungi. Host mortality, lesion size, and length of occluded tissues, among other variables have been used to assess virulence (Eckhardt et al. 2004a; Klepzig et al. 1996; Krokene and Solheim 1998b; Nevill et al. 1995).

The fungal root pathogens examined in this dissertation were found to be variable in their virulence to southern pine hosts. Overall, *G. huntii* is the most virulent of the fungal species tested in southern pines. In pine seedling inoculations, *G. huntii* caused longer lesions than *L. procerum*, *L. terebrantis*, and *L. serpens* in loblolly and slash pine and *L. procerum* and *L. terebrantis* in longleaf pine (Chapter 2). In Chapter 5, *G. huntii* caused longer and larger lesions on average than *H. annosum*, *L. procerum*, and *L. terebrantis* in loblolly pine and similar results were observed in slash pine. In loblolly pine root inoculations (Chapter 6), *G. huntii* was found to be most virulent among ophiostomatoid fungi. Similar results were observed in longleaf pine root inoculations (Chapter 7). *Leptographium serpens* was the second most virulent pathogen. In root inoculations of loblolly and slash pine (Chapter 5), *L. serpens* was found to be more virulent than *L. procerum*. In root inoculations of both loblolly (Chapter 6) and longleaf pine (Chapter 7), *L. serpens* was more virulent than *L. procerum* and *L. terebrantis*. *Heterobasidion annosum* caused lesions comparable to *L. serpens*, *L. terebrantis*, and *L. procerum* in loblolly pine and larger than *L. procerum* in slash pine roots (Chapter 5). *Leptographium terebrantis* has been reported to be more virulent than *L. procerum* in the past. However, from the collection of tests presented in this dissertation *L. terebrantis* and *L. procerum* cannot be separated, with respect to their virulence. Certain tests determined *L. terebrantis* was more virulent than *L. procerum* (Chapters 6- 7), while other tests did not (Chapters 2 and 4). Following inoculation of small longleaf pine trees, there were no significant differences between lesions (Chapter 4). *Leptographium terebrantis* caused a greater proportion of discolored sapwood and average lesion depth in the sapwood when compared to all other fungal species. With the exception of *L. terebrantis*, there appears to be limited differences in virulence between fungi in small longleaf pine.

Leptographium procerum has been well represented in inoculation tests in the past with a variety of different pine hosts (Eckhardt et al. 2004a; Klepzig et al. 1996; Nevill and Alexander 1992c; Nevill et al. 1995; Wingfield 1983; Wingfield 1986). It is considered the primary causal agent in Procerum Root Disease of eastern white pine (*P. strobus* L.)(Dochinger 1967). In some inoculation tests of healthy white pine, the fungus has caused mortality (Lackner and Alexander 1982). However, others have found *L. procerum* failed to cause sufficient damage (Wingfield 1983; Wingfield 1986). In other pine hosts the fungus is generally thought to be either mildly or weakly pathogenic (Klepzig et al. 1996). In loblolly pine seedlings *L. procerum* was significantly less virulent than other ophiostomatoid fungi tested (Eckhardt et al. 2004a; Nevill et al. 1995). Results and observations from these studies confirm previous suggestions that *L. procerum* is a weak pathogen and likely contributes little to the decline observed.

Overall, *L. terebrantis* appears to be more pathogenic than *L. procerum*. The fungus often caused smaller lesions compared to both *G. huntii* and *L. serpens*, however in certain samples, large destructive lesions were observed. A large amount of variability was observed within all fungal species x host combinations and *L. terebrantis* was no different. *Leptographium terebrantis* consistently caused sapwood discoloration when compared to *L. procerum*, despite having similar lesion sizes on the phloem surface. In most cases, *L. terebrantis* and the most virulent fungus tested, *G. huntii* produced the most sapwood discoloration. Significant sapwood penetration is a common trait of *L. terebrantis* (Parmeter et al. 1989). Tissue damage from *L. terebrantis* infection is known to cause significant losses in conduction and is thought to contribute significantly to mortality in trees (Jacobs and Wingfield 2001; Rane and Tattar 1987). It is generally thought to be a moderate to severe pathogen and has been observed causing larger lesions and more damage than *L. procerum* (Eckhardt et al. 2004a; Nevill et al. 1995; Wingfield 1986). Results and observations from these studies suggest *L. terebrantis* can be responsible for extreme damage on certain pine individuals, but on average is not a consistent threat to healthy trees.

Leptographium serpens is the primary cause of root disease of pines in South Africa (Wingfield and Knox-Davies 1980) and has been observed on insects in eastern white pine stands (Nevill and Alexander 1992b). It was found to be generally more

pathogenic than *L. terebrantis*, which confirms findings by Eckhardt et al. (2004a) in loblolly pine seedlings and tree stems. In previous inoculation tests, the fungus has caused large lesions in pine roots (Wingfield and Knox-Davies 1980) and stems (Eckhardt et al. 2004a), but when tested in branches, lesions were small (Zhou et al. 2002). Results and observations from these studies indicate that *L. serpens* is a severe pathogen to southern pine species. In root inoculations, lesions were consistently large and damaging. Strong pitch responses were consistently observed following inoculation and infected sapwood was darkly stained. Despite its' potential for severe damage in pine tissue, *L. serpens* has not been one of the most commonly isolated ophiostomatoid species from damaged roots in pine decline sites. In a recent survey of longleaf pine roots, *L. serpens* was conspicuously absent, while each of the other ophiostomatoid species were present (Zanzot 2009). *Leptographium serpens* has the potential to become an important root pathogen to pines in the southeastern United States.

A more severe response was observed following inoculation with *G. huntii*, which was the most virulent pathogen tested. The tests described in this dissertation are the first experimental results that confirm *G. huntii* is a severe pine pathogen. *Grosmannia huntii* was the only ophiostomatoid fungus to be more virulent than *H. annosum* in healthy loblolly and slash pine roots. Host damage resulting from *G. huntii* inoculations was consistently high, particularly in pine roots. Of the fungal species tested, *G. huntii* has the greatest potential for severe damage in southern pine roots. Previous studies only reference its' sapstaining ability in cut logs (Davidson and Robinson-Jeffrey 1965), however, the fungus has been observed in declining red pine stands and associated with the insect vector *Hylastes porculus* Erichson (Klepzig et al. 1991). In the southeastern United States *G. huntii* has recently been found associated with the root-feeding bark beetle *H. tenuis* Eichhoff, which appears to be its' primary vector in longleaf pine stands (Zanzot 2009). With the frequency at which *G. huntii* is being transferred by vectors and its potential for causing severe damage, *G. huntii* should be considered the greatest threat to southern pine roots from among the ophiostomatoid fungi tested.

8.5. Multiple Stress Factors and Their Interaction

As mentioned previously, the damaging lesion consistently observed, represents the combination of fungal infection, growth, and the amount of host response. Therefore, not only does lesion size vary by fungal virulence but also by the hosts' condition and physiology. Previous studies have attempted to test interactions between several abiotic factors and the lesion reaction resulting from fungal infection (Croisé et al. 1998; Klepzig et al. 1996; Solheim et al. 1993). In Chapter 3, longleaf pine seedlings were subjected to soil drought treatments in an attempt to find a relationship between drought stress and the virulence of *L. serpens*. No clear relationship between the two stress factors was detected and they were found to act independently. However, some trends observed indicate there may be a relationship that was not significant. The mean lesion length was smaller in trees with adequate moisture, though not significant, suggesting adequately watered pines may have the ability to restrict movement of *L. serpens*. In addition, the mean length of occluded tissues was slightly larger in adequately watered seedlings (though not significant), suggesting healthy seedlings may be capable of a stronger host defense. Using a larger sample size with more replications, establishing a relationship between stress factors may be possible. Some studies have detected a weak relationship between host water stress and sapwood damage following inoculation (Croisé and Lieutier 1993). However, others have failed to detect a relationship and found similar results to those found in Chapter 3 (Croisé et al. 1998a).

In addition to abiotic host stresses, seasonality and host physiology have shown to affect lesion size (Paine and Stephen 1987c; Stephen and Paine 1985). Cook et al. (1986) have shown that that lesions following inoculation with ophiostomatoid fungi have a positive relationship with temperature. The lesion size relationship with temperature can be explained by the physiology of both the pathogen and host. For example, *L. serpens* has an optimal growth temperature of 25° C on 2% MEA. Temperatures above or below the optimum may limit the growth of ophiostomatoid fungi. For example, *L. wageneri* growth in pine roots and stems is slower during periods of warm and cold temperatures (Hessburg and Hansen 1986). Smaller than expected lesions and lower than expected infection in young longleaf pine trees (Chapter 4) could be at least partially explained by low temperature shortly following inoculation. In addition, host defense physiology is

positively correlated with temperature (Blanche et al. 1992). During the growing season, trees can formulate an active response against invading pests, while dormant season tissue is much less efficient at defending itself. During longleaf pine root inoculation tests (Chapter 7), lesion size was smaller for each fungus during the fall season, compared to lesions in spring. Results presented in this dissertation support previous observations with other ophiostomatoid fungi in pine hosts (Horntvedt 1988; Reid and Shrimpton 1971). Actively growing tissue is more efficient at formulating a significant host response.

8.6. The Lesion Reaction

There is legitimate disagreement among scientists pertaining to the interpretation of the lesion. Most scientists have used the lesion to establish fungal pathogenicity (Ben Jamaa et al. 2007; Nevill et al. 1995; Solheim et al. 2001), since lesion size is positively associated with fungal virulence (Basham 1970; Popp et al. 1995a). Fungal growth within pine tissue has been correlated with the size of the lesion reaction and tissue necrosis precedes fungal expansion (Solheim and Krokene 1998). These observations suggest ophiostomatoid fungi cause the lesion reaction. The isolation of fungal metabolites in *L. wageneri* that lead to lesion development has further established causation (Ayer et al. 1989). However, other scientists have chosen to interpret the lesion reaction as the ability of a given host to resist bark beetle and fungal invasion (Cook and Hain 1986; Paine and Stephen 1987b; Paine and Stephen 1987c). These conclusions have been based on the observation that trees capable of resisting bark beetle invasion and establishment are capable of producing a large lesion response, while susceptible trees cannot (Raffa and Berryman 1982a). In above-ground bark beetle-fungal associations the hypersensitive response may be extremely beneficial, stopping invading fungi while only causing minimal damage (proportional to the organ affected, tree stem)(Hain et al. 1983). However, in below-ground systems, bark beetles and fungal associates invade roots, which are generally smaller than tree stems. In roots, the hypersensitive reaction may be relatively more damaging by significantly restricting vascular conduction (Joseph et al. 1998), particularly in small-diameter roots where *Hylastes* species tend to feed (Matusick observations). It is possible that both groups

have a valid interpretation of the lesion reaction, as the trees reaction confers host resistance and indicates fungal virulence. The presence of callus tissue is a good indication that the host reaction has stopped advancement of the fungus (Klepzig and Walkinshaw 2003; Popp et al. 1995b). In contrast, if the fungal infection is not stopped, significant tissue disruption and depletion of host carbohydrates can cause severe damage or mortality (Fernández et al. 2004). The most valid interpretation cannot be fully understood unless trees are inoculated at a biologically correct density or the host is left for sufficient time to observe large-scale host symptoms.

8.7. Final Conclusions and Potential Future Research

Root-inhabiting ophiostomatoid fungi found in pines of the southeastern United States are pathogenic. Upon inoculation of healthy hosts, ophiostomatoid fungi consistently infected and caused a significant host lesion. Although no whole-tree symptoms were observed, local root and stem tissue symptomology was consistent and damaging and long term inoculations would lead to advanced tree symptomology, in particular with *G. huntii*, *L. serpens*, and in some hosts, *L. terebrantis*.

Certain root-inhabiting ophiostomatoid fungi in the southeastern United States are significantly more virulent than others. *Grosmannia huntii* was consistently more virulent than *L. procerum* and in many circumstances *L. serpens* and *L. terebrantis*. When considering this collection of experiments as a whole, *L. serpens* was the second most virulent pathogen to pines. *Leptographium terebrantis* was the third most virulent species and *L. procerum* was the least. These results have important implications for long-term management of southern pines.

With continued restoration of the longleaf pine-dominated uplands and long-term management of loblolly and slash pine, infection by root-inhabiting ophiostomatoid fungi has the potential for an increased role in tree mortality. As discussed previously, *G. huntii* and *L. serpens* have the greatest potential for root damage in southern pines. One hypothesis would be as infections accumulate by *G. huntii* and *L. serpens*, severe root damage and the progression to more advanced symptomology in healthy hosts would be observed. Primarily due to the extreme host response, leading to severe root damage at the local level. Also, the large lesions associated with *G. huntii* and *L. serpens* in pine

roots showed no evidence of containment. However, the experiments described previously were short-term studies and evidence of containment may be found with more long-term studies. The largest threat to older aged southern pines is an accumulation of infections over time, which could lead to a slow progression towards death if fungi are not impeded by the defense systems. *Leptographium terebrantis* and *L. procerum* are less likely to cause sufficient damage leading to advanced symptomology and mortality.

These findings pertaining to the pathogenicity and virulence of root-inhabiting ophiostomatoid fungi merely provide a basis for understanding the potential for root disease and damage. Ultimately, the insect vectors will determine the role of their fungal associates. The limited current knowledge of root feeding bark beetles suggests they are non-aggressive insects, attacking and feeding and breeding in severely stressed hosts (Erasmus and Chown 1994; Flechtmann et al. 1999; Klepzig et al. 1996; Rudinsky and Zethner-Møller 1967; Wood 1982). However, the frequency on which root-inhabiting insect vectors attack healthy hosts is not well understood. Future research should focus on the actions and activities of the bark beetle vectors in order to fully understand the impact of root-inhabiting ophiostomatoid fungi on their hosts and the southeastern pine ecosystem as a whole.

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