Variation in resistance of loblolly pine (*Pinus taeda* L.) and slash pine (*P. elliottii* Englem.) families against *Leptographium* and *Grosmannia* root fungi

by

Amritpal Singh

A thesis submitted to the Graduate Faculty of
Auburn University
in partial fulfillment of the
requirements for the Degree of
Master of Science

Auburn, Alabama
December 6, 2012

Keywords: pine decline, *Leptographium*, *Grosmannia*, *Pinus taeda*, *Pinus elliottii* ophiostomatoid fungi, disease resistance

Approved by

Lori Eckhardt, Chair, Associate Professor of Forestry and Wildlife Sciences
Scott Enebak, Professor of Forestry and Wildlife Sciences
Kathy Lawrence, Associate Professor of Entomology and Plant Pathology
Mary Anne Sword Sayer, Plant Physiologist, U.S. Forest Service Southern Research Station

Abstract

Leptographium Lagerb. & Melin and *Grosmannia* Goid. root-inhabiting fungi are the important contributing biotic factors associated with declining loblolly (*Pinus taeda* L.) and slash pine (*P. elliottii* Englem.) species in the southeastern United States. The research in this thesis is focused on exploring the variation among the commonly out planted genotypes of loblolly and slash pine by the timber industries in the southeastern United States. Artificial inoculation experiments were conducted on the seedlings and the mature tree roots. Following inoculations, lesion parameters were recorded to access the relative virulence of fungal species and to determine the variation in lesion parameters among the families. *Grosmannia* and *Leptographium* species produced dark brown resin filled lesions on the seedling stems and the mature tree roots also exhibited darkened pitch-filled lesions and occlusions around the point of inoculation.

In an effort to study the role of nitrogen and carbon allocation pattern of the genotypes on *Grosmannia huntii* infection, a greenhouse experiment was conducted. Although, the lesion response indicated successful fungal infection, no significant differences in lesion parameters were noticed between normal and high nitrogen treatment levels. Family differences were noticed in stem total phenolic concentration. Also, the positive correlations between stem total phenolic concentration and family morphological traits (total seedling dry weight, fraction of total dry weight allocated to foliage) for several families show that morphological variables may provide a basis for predicting preformed defense potential.

Leptographium terebrantis followed by *G. huntii* caused larger lesions on seedlings stems and tree roots in the present research. Significant differences in lesion parameters were observed among the seedling families in year 2011 and 2012 experiments. Seedlings inoculation experiments identified some families (L-5, L-20, L-8, and, L-13) that developed consistently smaller lesions, while other families (L-1, L-2, L-3 and L-4) were found to have consistently larger lesions. Variability in lesions was observed between the two fungal species from inoculations on the mature tree roots. However, lesion parameters did not vary significantly among the families. Overall results indicated that ophiostomatoid fungi produced local symptomology and are capable of causing damage to the southern pines. Deployment of these families in the field should take into account the relative risk of other abiotic factors and root-feeding beetles associated with the ophiostomatoid fungi.

Acknowledgments

I am sincerely thankful to my major professor Dr. Lori Eckhardt for providing me an opportunity to conduct this research and her guidance throughout the project period. I would like to thank my committee members Dr. Scott Enebak, Dr. Kathy Lawrence and Dr. Mary Anne Sword Sayer for their valuable comments, suggestions, academic, research, and moral support. I am greatly thankful to Dr. Greg Somers and Dr. Fikret Isik for statistical assistance.

My special thanks are due to our former research associate Ms. Rebecca Kidd and our present research associate Mr. Daniel Anderson. I am indebted to Daniel for providing me every kind of support as mentor and friend. My sincere thanks are due to my fellow graduate students Ben, Matt, Yuan and Andy for their companionship and help. I would also like to thank all the undergraduate students and lab technicians (John and Alyssa) who helped me on the project. I am thankful to the Forest Health Cooperative for funding this research. Finally, I express my love and appreciation to my friends, family and late mother; without them I would not be capable of finishing this endeavor.

Table of Contents

List of Tables

List of Figures

Chapter One

Introduction and Review of Literature

1.1. Land Use History

The diverse and dynamic nature of the southeastern forests is a result of land use history that includes significant man-made and natural disturbance (Wear and Greis 2002) over the past 300 years. Before European settlement, the southeastern United States had diverse vegetation that included savannas, barren lands and swamps with mixed and sparse tree stands consisting of oak-pine canopy (Wear and Greis 2002). Across most of the landscape, frequent, low-intensity, fires occurred naturally that perpetuated an understory dominated by herbaceous vegetation (Trani et al. 2001). Natural disturbances such as flooding, and fires caused by lightening altered forest succession and yielded an ever-changing forest landscape (Lorimer 2001). In the 19th and early 20th centuries, large scale disturbances took place as forests were cleared and land was brought under agriculture. Cultivation of crops led to soil erosion and nutrient depletion in the soils and agriculture was eventually abandoned. The absence of vegetation perpetuated erosion until federal and state agencies initiated reforestation programs with loblolly pine (*Pinus taeda* L.) as the species of choice (US Forest service 1988). This species was favored by its ease of establishment, rapid juvenile growth and good response to silvicultural treatments (Baker and Langdon 1990).

1.2. Present Scenario

Between the mid-1950s and early 1960s, efforts of federal and state agencies curtailed the soil erosion problem but by 1982 with the harvest of fourth and fifth rotations, reduced rates of stemwood growth were observed (Wear and Greis 2002; Gadbury et al. 2004). Also, during earlier rotations, reductions in growth had been observed in southeastern United States in both natural and intensively managed plantations (Bechtold et al. 1991; Haywood 1994). By the sixth rotation, growth reductions were considered significant when compared to the fourth rotation. Growth decline is still probable in certain forest settings under changing climate conditions such as severe drought experienced by the southern states in recent years (Klos et al. 2009). During a period of surveys from 1953 to 1999, along with growth fluctuations, mortality had also increased due to various causes. Disease and weather conditions contributed to more than 60 percent of this mortality with stand origin the most significant factor as 92 percent of the mortality was found in natural stands (Wear and Greis 2002).

At present, large parts of southern forests are managed for timberland with loblolly pine and shortleaf pine (*P. echinata* Mill.) dominating on 55 million acres which is about one-fourth of all southern forests (Smith et al. 2007). The area under southern pine plantation management has increased 10-fold since 1953 and it is expected to increase (Haynes et al. 2007). While shortleaf pine is more widely distributed than loblolly pine, loblolly pine is the backbone of the southern pine industry, covering 80 percent of commercial forest area in the South (Smith et al. 2007) and with a geographical range extending across 15 states in the south and mid-Atlantic region (Baker and Langdon 1990). Loblolly pine reproduces and grows rapidly in diverse

conditions and it's per hectare yield is large and it also provides versatile marketable products, making it the most favorable pine species in the south (Shultz 1997).

1.3. Loblolly Pine (*Pinus taeda* L.) Biology and Genetics

Depending upon the region, type of soil, slope, aspect and topography, loblolly pine is found in association with a variety of conifer and hardwood species. Factors such as temperature, soil type, fertilization, day length, moisture and genetics affect the growth rate of loblolly pine. Growth decreases as hardwood over story composition increases. In addition to vegetative competition, edaphic and climatic factors may have adverse effects on growth (Baker and Langdon 1990).

Loblolly pine can be subjected to a number of abiotic and biotic stresses. Abiotic factors such as wind, flooding, and high and freezing temperatures can result in decreased vigor and tree growth. Abiotic factors have been shown to predispose trees to organisms like insects and fungi. These biotic agents, especially bark beetles have resulted in a substantial amount of tree mortality (Baker 1972). The important bark beetle species include pine engraver beetles (*Ips* spp.), regeneration weevils (*Hylobius* spp. and *Pachylobius* spp.) that cause damage to young seedlings, *Hylastes* spp., and the southern pine beetle (*Dendroctonus frontalis* Zimmermann). Several outbreaks of southern pine beetle have been recorded from 1999 to 2003 damaging millions of acres of state, federal, industrial and private forests (Thatcher and Barry 1982). In addition to beetles, loblolly pine is susceptible to fungal diseases almost at every life stage. Fusiform rust (*Cronartium quercum* f.sp. *fusiforme* Hedg. & Hunt) is the most devastating stem disease of loblolly pine (Phelps and Czabator 1978) along with heterobasidion root rot

(*Heterobasidion irregulare* nom. nov. Garbelotto & Otrosina) which can cause up to 30 percent stand mortality in severe cases (Robbins 1984). In some areas, pitch canker has caused significant mortality (*Fusarium circinatum* Nirenberg and O' Donnell) as well (Barnard and Blakeslee 1987).

Tree breeding programs have resulted in both qualitative and quantitative improvements in loblolly pine through the selection of superior trees with rapid growth rates, desirable wood properties and disease resistance (Shultz 1997). Various deployment techniques used in tree breeding programs have resulted in different genetic diversity levels in the planting stocks. Open pollinated families provide a good planting stock in terms of genetic gain and diversity (McKeand et al. 2003). About 840 million loblolly pine seedlings were planted in the south in years 2007, 2008 and 2009 that included 95% open pollinated families and 5% specific crosses and clones (McKeand et al. 2009). Most tree breeding efforts in the past were focused on genetic gains in growth and fusiform rust resistance (Schultz 1997). Many diseases other than fusiform rust are also genetically controlled and opportunities exist to select for resistance (Shultz 1997). For example, loblolly xylem decay is inversely proportional to specific gravity. Therefore, selecting for high specific gravity decreases susceptibility to xylem decay (Schmidtling and Amburgey 1982). Similarly, the differences in resistance to *Hypoderma lethale* Dearn., causal organism of hard pine gray blight in loblolly pine progeny test plantations, were found to be heritable (Kraus and Hunt 1971).

1.4. Forest Declines

Forest declines involve a sequence of orderly events eventually leading to premature tree mortality. The theory of tree decline proposed by Sinclair and Hurdler (1988) describe decline as premature loss of vigor on one of the following bases (i) continuous stress from a single factor, e.g. pin oak decline due to iron deficiency (Neely 1976) and sugar maple decline as a result of deicing salt uptake (Westing 1969), (ii) major damage or wounding by environmental stress that leads to attack from secondary factors like insects and diseases, e.g. oak decline due to primary defoliation which attracts secondary biotic agents (Houston 1981), (iii) premature senescence of groups of trees attributed to the lack of diversity in age or genetic makeup as described by Mueller-Dombois (1983) in ohia (*Metrosideros collina* J.R & G. Forst.) decline, (iv) two or more simultaneous agents that act interchangeably and lower resistance and/or tolerance thus leading to decline. These factors were described by Sinclair (1967) as predisposing, inciting and contributing factors with a key concept being that introduction of a new abiotic/ biotic factor or changing one of the factors accelerates the decline. The concept was later modified by Manion (1981) who proposed that the three specifically ordered abiotic and biotic factors that play a role in tree decline are predisposing, inciting and contributing and developed a model called as the disease decline spiral model. Predisposing factors place the tree under constant stress thus leading to diminished health and vigor; inciting factors increase the severity of the stress and incite the tree toward decline, while contributing factors finish the decline resulting in premature mortality.

Predisposing factors are abiotic in origin and are related to the site conditions like topography, slope, elevation (Eckhardt et al. 2007), soil fertility (Saxe 1993), thinning and

logging damage and extreme climactic conditions like drought and early or late frost (Schutt and Cowling 1985), tree age, genetic potential and species planted outside their native range (Edmonds et al. 2000). Predisposing factors allow the development of biological stress and make the tree vulnerable to secondary agents such as insects and disease pathogens thus leading to premature mortality. Sometimes a single factor such as the presence of a susceptible host over a large area is sufficient enough to place the tree under stress. The tree responds by reducing growth, and shedding leaves and branches, with recovery depending upon the severity, duration of stress and extent to which the damage has progressed (Millers et al. 1989). Severe and prolonged stress can lead to invasion of secondary agents resulting in tree mortality.

Biotic factors can act as either inciting or contributing factors and it is the interaction of biotic and abiotic factors that can result in tree mortality. Some of the more common biotic factors that act as contributing or inciting factors in decline are root diseases (Leaphart and Copelend 1957) like armillaria root rot and saprophytic decay, wood boring insects (Wermelinger et al. 2008), bacterial canker (Scortichini 2002), and defoliation of trees by insects (Starkey et al. 2004). In some instances, two biotic stresses work in association with each other such that one leads to successful colonization by the other biotic agent, thus hastening the tree decline. As an example, scolytid beetles and specific fungi have mutualistic beneficial relationships. Beetles carry the fungi on their body and inoculate the fungi into the trees. Mycelium penetration of fungi into host tissue leads to premature tree mortality (Paine et al. 1997).

Several reports of forest declines have been recorded from eastern hardwood forests with classical examples for various oak species including white oak, northern red oak, scarlet oak and black oak. Generally, the interactions of abiotic factors like soil type, topography, weather conditions and biotic factors such as insects and diseases have been identified as components that lead to oak decline (Millers et al. 1989). Decline of scarlet oak and red oak in the eastern United States was concluded to involve leaf roller defoliation that led to diminished growth due to a shortage of carbohydrate reserves followed by secondary factors such as root rot, drought and frost (Staley 1965). White ash has a decline history in the United States with drought being attributed as main predisposing factor that results in attack by canker fungi and other additional stresses such as air pollution, mycoplasma like organisms (MLO's), viruses and leaf infections (Castelo et al. 1985; Hibben and Silverborg 1978). Other hardwood species for which declines have been observed include maple, beech, birch, yellow poplar and sweetgum (Millers et al. 1989). Conifers declines have also been reported in the United States with some prominent examples such as red pine (*P. resinosa* Aiton) decline in Wisconsin (Klepzig et al. 1991), ponderosa pine (*P. ponderosa* P. & C. Lawson) root disease/decline in New Mexico (Livingston et al. 1983), pole blight of western white pine (*P. monticola* Dougl. Ex D. Don) (Leaphart and Copeland 1957), and lastly loblolly pine (*P. taeda* L.) decline in southeastern United States (Eckhardt et al. 2007).

1.5. Southern Pine Decline

Decline in loblolly pine was first noticed in 1959 in the Talladega National Forest on the Oakmulgee and Tuscaloosa Ranger districts in Alabama (Brown and McDowell 1968). Referred to as, 'loblolly pine die-off', the disease was associated with saw timber size trees over 50 years

of age (Hess et al. 2002). Incidences of mortality were reported during the early 1970s with symptoms such as sparse and chlorotic crowns, fine and lateral root deterioration, and poor growth that were attributed to unusual physiological and environmental conditions (Roth and Peacher 1971). Earlier studies of declining shortleaf pine on the Oakmulgee Ranger district indicated that the pathogen responsible was littleleaf disease, *Phytophthora cinnamomi* Rands. (Campbell and Copeland 1954). The role of *P. cinnamomi* in the decline of shortleaf pine was validated by the consistent recovery of *P. cinnamomi* from declining shortleaf pine trees on poorly drained sites with poor fertility. After 10 years of evaluation, however, the pathogen associated with 'loblolly pine die-off' could not be identified, in part, because *P. cinnamomi*, *Heterobasidion irregulare*, and *Phythium* spp. were inconsistently isolated from the declining loblolly pine trees (Brown and McDowell 1968).

In the early 2000s more occurrences of loblolly pine decline were reported and Hess et al. (2002) further examined the conditions associated with this early tree mortality and its differences from littleleaf disease across a range of sites. In those studies, a number of fungi were recovered from the soil root zone including, *P. cinnamomi* and *Leptographium* Upadhyay & Kendr., the latter of which was found to be associated with roots damaged by root feeding bark beetles. Again, like the studies in the mid 1950's no clear link could be established between either *P. cinnamomi* or *Leptographium* and the premature mortality of loblolly pine (Hess et al. 2002). The spread of littleleaf disease and recovery of *P. cinnamomi* have been limited by soil type as reported by Roth (1954) with disease incidence and recovery of the pathogen being high from heavy textured, moist soils which are typical of the piedmont region, compared to light textured, dry soils (Copeland and MacAlpine 1955). But, loblolly pine decline, unlike littleleaf

disease, was occurring on soils not within the piedmont region. Extension of the loblolly pine decline range beyond the clayey soils of the piedmont region indicated the involvement of factors other than *P. cinnamomi* and littleleaf disease.

This led to establishment of a 3-year study by Eckhardt et al. (2007) to better understand the pine decline complex and to investigate what role the fungi and root feeding bark beetles and soil conditions play in pine decline. Root feeding bark beetles such as *Hylastes salebrosus* Eichhoff., *H. tenuis* Eichhoff., *Pachylobius picivorus* Germar. and *Hylobius pales* Herbst. were recovered in higher numbers from symptomatic plots than from the non-symptomatic plots with healthy trees. *Leptographium* species including *L. procerm* (W.B. Kendr.) M.J. Wingf., *G. alacris* T.A. Duong, Z.W. de Beer & M.J. Wingf. sp. nov. and *L. terebrantis* S.J. Barras and T.J. Perry were recovered from the root systems of the unhealthy trees. Also, it was established that increased slope and south/southwest facing aspects predispose the trees to decline (Eckhardt and Menard 2008). Further, the role of *Hylastes* spp. as *Leptographium* vectors in decline was evaluated under artificial conditions leading to the conclusion that the *Hylastes* species were capable of transmitting the fungi, and the fungi in return enhance the reproduction rate of the *Hylastes* beetles (Eckhardt et al. 2004b).

Presently, premature mortality in loblolly pine is described as a decline disease syndrome that involves complex interactions of biotic and abiotic factors (Eckhardt et al. 2007). Abiotic factors like topography, increased slope, and southwest facing aspects predispose the pines to biotic factors such as bark beetles (Eckhardt and Menard 2009). Root and lower stem feeding bark beetles are attracted due to stress induced in the trees by abiotic factors (Eckhardt et al.

2007), the feeding activity of the beetles help in movement of the fungus from infected to healthy trees (Paine and Hanlon 1994). The fungus, in turn, enhances the beetle brood by providing a food source for the larvae and also by making conditions favorable for insect activity (Eckhardt et al. 2004b). This insect-fungal interaction that leads to expansion of the decline disease complex is understood as a mutualism in which the fungi and the beetles benefit each other (Paine et al. 1997). However, in certain conditions the fungus alone can contribute to decline by blocking the vascular tissues, hence preventing translocation of water and minerals (Eckhardt et al. 2004a).

1.6. Ophiostomatoid Fungi

Ophiostomatoid species have different morphological features and are found in diverse ecological niches. Previous fungal classification systems were based on anamorph and teleomorph structures and the taxonomic classification of ophiostomatoid fungi has been confusing (Harrington 1987; Zipfel et al. 2006; Spatafora and Blackwell 1994). Different phylogenetic groups have been defined recently for ophiostomatoid species by comparing DNA sequencing profiles. This effort groups ophiostomatoid species into three distinct lineages (i) the telomorph genus *Grosmannia* Goid., with *Leptographium* Lagerb &Melin, as anamorphs, (ii) the telomorph genus *Ceratocystiopsis* Upadhyay & Kendr., with *Hyalorhinocladiella* Upadhyay & Kendr., as anamorphs and (iii) the telomorph genus *Ophiostoma* Syd. & Syd., with *Sporothrix* Hektoen & C.F. Perkins. or *Pesotum* Hektoen & C.F. Perkins, as anamorphs (Zipfel et al. 2006). The taxonomic classification of fungi belonging to genera *Ceratocystis* Ellis &Halstead and *Ophiostoma* has been debatable since they were described. Phylogenetic studies during the last decade have confirmed a polyphyletic origin of these two genera and *Ceratocystis* had been

separated from *Ophiostoma* on the basis of anamorph morphology, antibiotic cycloheximide sensitivity, conidia development, and cell wall composition (Spatafora and Blackwell 1994; Hausner et al. 1992). While both cellulose and rhamnose are important cell wall components of *Leptographium*, *Ceratocystis* species lack cellulose and rhamnose in their cell walls (Spencer and Gorin 1974; Harrington 1981).

Previously considered as anamorphs of *Ophiostoma*, *Leptographium* species have now been grouped as anamorphs of the genus *Grosmannia* that includes 27 species (Zipfel et al. 2006). The system of classification proposed by Hughes (1953) on the basis of spore production method separates the *Leptographium* from the other related genera. The unique method of *Leptographium* conidial development involves formation of darkly pigmented conidiophores that bear sporogenous apparatus of one to six multiplicative series of metulae having a large number of sporogenous cells (Kendrick 1962). Conidia are produced as slimy amerospores in a mucilaginous drop usually on the sporogenous apparatus. Formation of sticky spores make these fungi adapted to insect transmission especially bark beetles (Harrington and Cobb 1983). *Leptographium* species are cycloheximide insensitive unlike *Ceratocystis* species as reported by Harrington (1981) and this property has been exploited for isolations of *Leptographium* by using selective meda (Hicks et al. 1980; Wingfield 1983).

Blue staining of plant tissues is a characteristic of ophiostomatoid fungi. The fungi grow and sporulate on the wood surface leading to superficial discoloration. Dark pigmented hyphae also penetrate into the wood leaving a blue color and degrade the wood quality leading to economic losses (Uzunovic et al. 1999; Seifert 1993). In addition to their color, many

ophiostomatoid species are pathogenic and are associated with bark beetles (Wingfield et al. 1988). *Ophiostoma ulmi* (Buisman) Melin & Nannf., a causal organism of Dutch elm disease (DED), spread by elm bark beetles (Pecknold 1978), is responsible for nearly eliminating American elm (*Ulmus Americana* L.) from North America. *Leptographium wageneri* (W.B. Kendr.) M.J. Wingf. causes a unique black stain root disease in western conifers (Cobb 1988) that is also associated with root feeding insects such as *Hylastes* species. The uniqueness about *L. wageneri* is that it can spread to smaller distances through soil without root to root contact. However, a natural opening or a wound is required for the entry of the pathogen into the host tissue where the fungus colonizes the outer xylem and grows rapidly (Hansen et al. 1988). This disease infects all stages of growth (i.e., trees, saplings and seedlings). Mortality occurs rapidly once the xylem is infected due to the systemic nature of the disease (Cobb 1988).

1.7. *Leptographium* species associated with southern pine decline

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

Leptographium procerum was first identified on jack pine (*P. banksiana* Lamb.) as *Verticicladiella procera* (Kendrick 1962). Earlier classification was based on annelidic, sympodial and phialidic conidial development in which the *Leptographium, Verticicladiella* and *Phialocephala* were considered as distinct genera. More recently, the genus, *Verticicladiella* was included in the genus *Leptographium* due to the presence of sympodial and annellidic conidial development in both genera (Wingfield 1985). Its wide distribution has resulted in *L. procerum* being recovered from other conifer species including longleaf pine (*P. palustris* Mill.) (Otrosina et al. 1999), loblolly pine (Eckhardt et al. 2004b) and slash pine (Barnard et al. 1991).

Colonies of *L. procerum* on nutrient media are fibrillose and diffuse with regular margins. Hyphae are septate, smooth walled and light brown in color with aerial mycelia seen occasionally. Numerous conidiophores are produced forming concentric rings in the colony and rhizoid like structures are visible at the base of the conidiophores (Kendrik 1962) (Figure 1.1). A large number of insect vectors have been associated with dissemination of *L. procerum* as reported by its recovery from bark beetles such as *Dendroctonus frontalis* (Otrosina et al. 1997), *Haylestes* spp. (Wingfield and Gibbs 1991; Eckhardt et al. 2007), *H. pales*, *P. picivorus* and *H. radices* (Buchanan), and declining stands of red pine (*P. resinosa* Aiton) in Wisconsin (Klepzig et al. 1991).

The virulence of *L. procerum* has been tested on different pine species. *Leptographium procerum* as well as three other species produced resin filled lesions on healthy roots of lodgepole pine (*P. contorta* Douglas ex Louden) (Bertagnole et al. 1983). In virulence trials on seedlings of eastern white pine (*P. strobus* L.) and loblolly pine, *L. procerum* caused significant mortality when the root dip inoculation method was used (Lackner and Alexander 1982). In addition to white pine and loblolly pine, *L. procerum* has been shown to be pathogenic on longleaf pine (Otrosina et al. 2002), ponderosa pine (*P.* ponderosa Dougl. ex Laws.), Douglas fir (*Pseudotsuga* spp.) (Harington and Cobb 1983) and loblolly pine (Eckhardt et al. 2004a). While *L. procerum* is pathogenic to a wide range of species, its virulence is less than that of other *Leptographium* species and is considered a mild pathogen (Eckhardt et al. 2004a; Matusick and Eckhardt 2010a).

Figure 1.1. Conidiophores with conidia of *Leptographium procerum.*

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

Leptographium terebrantis was first recovered from black turpentine beetles, *D. terebrans* (Olivier), in infested loblolly pine stands (Barras and Perry 1971). This fungus has been found in association with red pine (*P. resinosa* Ait.) in Wisconsin (Klepzig et al. 1991), the roots of longleaf pine (Otrosina et al. 2002) and from the root samples and insects captured from several loblolly pine plantations across the southeastern United States (Eckhardt et al. 2007).

Leptographium terebrantis produces mononematous, penicillate conidiophores which are typical of *Leptographium* species. Conidia are formed in succession which places the fungus into the annelosporae in the classification of Barron (1968). Conidia have a truncated base and are produced basipetally with visible annellations. The hyphae are hyaline with dark green color, aerial hyphae are seen occasionally, and numerous conidiophores are formed covering the entire colony. Annellospores are produced in a gelatinous sticky mass (Barras and Perry 1971).

Leptographium terebrantis has been found in association with many insects especially root feeding bark beetle and weevil species. Found for the first time from *D. terebrans* (Barras and Perry 1971), it has been isolated from *D. frontalis* (Otrosina et al. 1997), *Haylestes* spp. and weevil species like *H. pales* and *P. picivorus* (Klepzig et al. 1991; Eckhardt et al. 2007).

Using loblolly pine, several studies have explored the relative virulence of *L. terebrantis* and other *Leptographium* species (Wingfield 1983; Nevill et al. 1995; Eckhardt et al. 2004a; Matusick and Eckhardt 2010a). Wingfield (1983) found that *L. terebrantis* caused significant mortality in loblolly pine seedlings. Furthermore, others have reported larger sapwood discoloration and lesions from inoculations with *L. terebrantis* on longleaf pine saplings when compared to other *Leptographium* species including *G. alacris*, *G. huntii* and *L. procerum* (Matusick and Eckhardt 2010b). In addition to loblolly pine and longleaf pine, other conifer species tested for the virulence of *L. terebrantis* include red pine (Klepzig et al. 1996), white pine (Wingfield 1986) and ponderosa pine (Owen et al. 1987).

1.7.3. *Grosmannia alacris* T.A. Duong, Z.W. de Beer & M.J. Wingf. sp. nov.

Grosmannia alacris (Duong et al. 2012), previously named *L. serpens*, was first identified as *Verticicladiella alacris* (Goid.) (Kendrik 1962). The genus *Verticicladiella* was later reduced to *Leptographium* on the basis of conidial development as both the genera exhibited an annellidic and sympodial proliferation of conidia (Wingfield 1985). *Grosmannia alacris* has been isolated from eastern white pine (Lackner and Alexander 1981) and has been reported as causing root disease of *P. pinaster* (Ait.) and *P. radiata* (D. Don) in South Africa

(Wingfield and Knox-Davies 1980). More recently, *G. alacris* has been linked to southern pine decline in the southeastern United States (Eckhardt et al. 2007).

On nutrient media, *G. alacris* grows as a profuse, 'chaetura black'mycelium with a relatively slow growth rate as compared to either *L. terebrantis* or *L. procerum*. Hyphae are hyaline to brown and aerial hyphae are absent. Conodiophores are mononematous, emerge from the center of the colony, and are inclined at 30 to 40 degree angles to the stipe. Conidia are produced at the apex of the conidiophore in a mucilaginous drop as reported by Kendrik (1962) and while serpentine hyphae can be produced, they are not the distinct identification characteristic of this species. Like other *Leptographium* species, *G. alacris* has been found in association with bark beetles. *Hylastes tenuis* and *H. salebrosus* often carry this fungus and help in its transport from tree to tree (Eckhardt et al. 2007). In South Africa, *Hylastes agustatus* (Herbst.), *H. linearis* (Erichson.) and *H. ater* (Payk.) were found carrying *G. alacris* (Wingfield and Knox-Davies 1980; Wingfield and Gibbs 1991). However, attempts to recover the fungus from regeneration weevil species (*Hylobius pales* and *P. picivorus*) have been unsuccessful (Eckhardt et al. 2007).

Several inoculation studies have been conducted using *G. alacris* with significantly longer lesions observed on loblolly pine seedlings and mature trees compared to those produced by *L. terebrantis* and *L. procerum* (Eckhardt et al. 2004a). In a comparative virulence study done on four South African *Pinus* spp., *G. alacris* caused significantly longer lesions but was found to be less pathogenic than *L. lundbergi* (Lagerb.) with none of the species reported as a serious threat to the *Pinus* spp. (Zhou et al. 2002). In a recent study on loblolly pine, slash pine

and longleaf pine, *G. alacris* produced lesions that were consistently longer than those produced by *L. procerum* (Matusik and Eckhardt 2010a).

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

Grosmannia huntii, formely *Ophiostoma huntii* (Zipfel et al. 2006) was first described after isolation from lodgepole pine stands that were infested with mountain pine beetle in British Columbia, Canada. Sexual (*G. huntii*) and asexual (*L. huntii*) stages were identified from perithecia (Figure 1.2) and conidia, respectively (Robinson-Jeffery and Grinchenko 1964). To date *G. huntii* has also been recovered from longleaf pine in the southeastern United States (Zanzot et al. 2010).

Colonies of *G. huntii* on nutrient media are shiny and olive brown with aerial hyphae often observed. If present, submerged hyphae are sinuous and two types of aerial hyphae are visible (i) hyaline, smooth, and having uniform width, (ii) light brown with irregular thickenings giving rise to conidiophores. Conidiophores are mononematous with obclavate conidia displaying penicillate branches. Unlike, *L. procerum* rhizoids are not produced at the base of the conidiophore. Hyphae can be serpentine as those in *G. alacris*. Perithecia, often produced in cultures are black, spherical with long necks and an ostiole. Evanescent asci with eight ascospores are rarely observed but ascospores are hyaline, aseptate, thick-walled and unicellular (Robinson-Jeffery and Grinchenko 1964). *Grosmannia huntii* was isolated and described from mountain pine beetles by Robinson-Jeffery and Grinchenko (1964). Other beetle species observed as carrying *G. huntii* included *Tomicus piniperda* L. (Gibbs and Inman 1991) and *Hylastes* species (Zanzot et al. 2010). *Grasmannia huntii* has only recently been reported to be

one of the most pathogenic among all other *Leptographium* species in the southeastern United States (Matusick and Eckhardt 2010a).

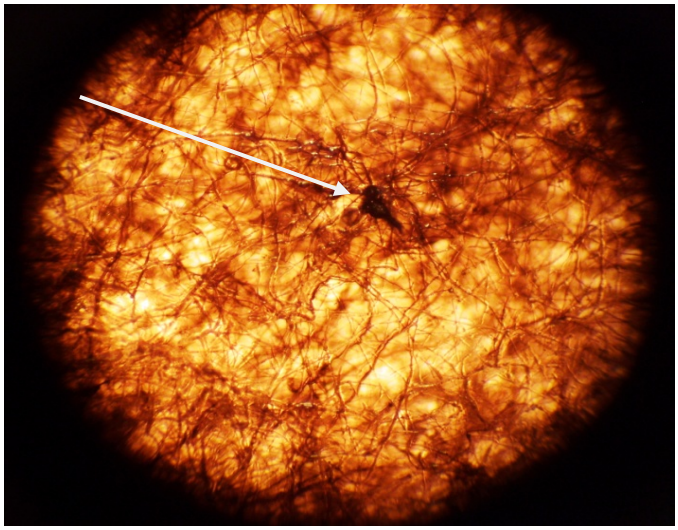

Figure 1.2. Perithecium of *Grosmannia huntii* as seen on a culture plate.

1.8. Virulence testing of Ophiostomatoid fungi (Inoculation Experiments)

The primary mode of spread and infection of ophiostomatoid fungi is by beetles and weevils (Coleoptera). To simulate natural inoculation by beetles and assess relative virulence of ophiostomatoid fungi, artificial inoculation experiments have been widely used (Wingfield 1983; Bertagnole et al. 1983; Harrington and Cobb 1983; Eckhardt et al. 2004a; Matusick and Eckhardt 2010a). The choice of inoculation depends on the age of the plant material and has included seedling, sapling and mature trees (Owen et al. 1987; Matusick et al. 2010; Matusick and Eckherdt 2010a; Matusick and Eckhardt 2010b). Ophiostomatoid fungi primarily infect roots of mature trees; therefore, in order to mimic the beetle attack and fungal inoculation, similar sized trees under the same conditions are used in artificial inoculation experiments (Långström et al. 2001; Rice et al. 2007). However, in mature tree inoculation experiments control over

environmental factor of the disease triangle, which is important in the infection process, is not possible due to microsite conditions that affect the tree's defense mechanisms. For example, in 40 year old loblolly pines, daily oleoresin exudation pressure was found to be reduced in trees grown on flat and wet sites compared to trees grown on low mounds (Lorio and Hodges 1968). Also, variation in resistance to *Ceratocystis polonica* (Siem.) C. was found within Norway spruce (*Picea abies* L.) stands among different tree sizes (Sandnes and Solheim 2002). Moreover, inter-tree variability was found in the defense reaction of Scots pines as a result of inoculation with fungi and the tree's defense capability was not related to lesion length or total resin produced in the reaction zone (Lieutier et al. 1993).

Controlled environment is thus important for simulating the actual disease and infection conditions. Seedling and sapling inoculation experiments provide more control over the environment and have been used to assess the pathogenicity of ophiostomatoid fungi. Sapling size trees were found to be suitable for the study of conifer defense mechanisms based on oleoresin production (Lewinsohn et al. 1991). Although environmental control is possible in seedling and sapling inoculations, the accuracy of the experiment may be reduced because of an underdeveloped defensive system in these stages of growth. Conifers have a well-developed resin duct systems through which oleoresin is mobilized to the insect attacking sites thus providing defensive action to pitch out the beetles. The duct system depends upon the age and growth rate and is less developed in young tree stages, making the seedlings the most susceptible stage of growth (Nebeker et al. 1993). Thus, careful interpretation of the results is necessary when dealing with seedling virulence studies.

By far the most common inoculation system involves the seedling stem and subsequent host response. Under aseptic conditions, colonized mycelium is placed in a slanting cut made in the seedling stem (Nevill et al. 1995; Eckhardt et al. 2004a; Matusick and Eckhardt 2010a). Various substrates have been used to grow the mycelium and accumulate inoculum such as nutrient agar media, wooden blocks and toothpicks (Nevill et al. 1995; Owen et al. 1987; Wingfield 1986). Seedling root inoculations involving several methods have also been performed in virulence studies. Wounded and unwounded inoculations have also been used on the tap roots of seedlings (Harrington and Cobb 1983). Root- dips in mycelium have been done to assess the entry point of the fungus into the root system and to compare the effect of the fungus on the dormant and actively growing seedlings (Hessburg and Hansen 2000).

Successful fungal colonization can be assessed following plant inoculations by observing the symptomology and host response. These host responses include mortality (Harrington and Cobb 1983; Wingfield 1983), localized symptoms in the form of dark resinous lesions (Nevill et al. 1995; Eckhardt et al. 2004a; Matusick and Eckhardt 2010a), necrosis of inner bark, chlorotic needles and decreased water potential (Rane and Tatter 1987). In addition, the occlusion of vascular tissues has also been measured (Eckhardt et al. 2004a; Matusick and Eckhardt 2010a) as well as changes in monoterpene composition and carbohydrate composition in loblolly pine (Cook and Hain 1985). Microscopic examination of the infection process following unwounded root inoculations with *L. wageneri* var. *pseudotsugae* revealed that pathogen hyphae enter the root system through natural openings and wounds (Hessburg and Hansen 2000) and also the conidial germination was observed on wounded roots but not on unwounded roots (Diamandis et al. 1997), supporting the fact that *Leptographium* requires a wound for infection. Following

entry into the host, hyphae grow into tracheids resulting in staining and discoloration of the xylem and formation of dark resinous lesions (Harington and Cobb 1983).

1.9. Disease resistance in response to physiology

1.9.1. Natural and Inducible defense

The two main types of defense mechanisms in plants are (i) pre-formed defense that exists naturally in the absence of any beetle attack with specialized secretory cells responsible for production of defense chemicals. (ii) Induced defense mechanisms are activated only after beetles attack the tree and involve the activation of genes that lead to increased production of specific compounds. The secretory cells are a part of every organ and provide first line resistance against beetles and fungi. An aspect of preformed defense in which the level of activity of defensive structures remain the same before and after the attack is called constitutive defense, as an example, bark thickness in conifers can be regarded as an efficient constitutive defense against bark beetle species, such as *Pityogenes chalcographus* L. and *Ips acuminatus* Gyll. on spruce and pine in Europe respectively (Lieutier 2002). The three types of induced defenses have been described, and these include induced resin flow that was described and concluded by Ruel et al. (1998) in loblolly pine as a tree reaction induced by wounds; hypersensitive reaction as noticed by Reid et al. (1967) leading to enriched concentration of terpene and phenolic compounds at the point of aggression that results in death of the affected tissues; and delayed resistance involving long term complex changes due to cell division and differentiation (e.g. wound periderm that develops due to bark beetle and fungi attack) (Berryman and Ferrell 1988).

1.9.2. Natural defense chemicals

Secondary metabolites such as terpenoids, phenolics and alkaloids are an important part of tree defense and are known to influence insect resistance in plants. These metabolites can be constitutive, i.e. present in the plant previously, or induced by insect attack (Karkan and Baldwin 1997). Much attention has been given in recent years to increased concentrations of metabolites in response to insect attack with a higher concentration of tannins found in oak due to gypsy moth feeding (Rossiter et al. 1998) and increased terpenoids and phenolics as a result of bark beetle attack in pines (Raffa 1991). The distribution of secondary plant metabolite has been explained on the basis of two hypotheses: the evolutionary approach, that aims to understand the secondary metabolites patterns among different plant species with different evolutionary histories, different habitats, plant appearance (Feeny 1976) and availability of resources (Coley et al. 1985); and the ecophysiological approach that is focused on studying the carbon and nutrient availability (Bryant et al. 1983), balance between growth and differentiation (Lorio 1986), and source/sink regulation (Hinkanen and Haukioja 1998).

1.9.3. Growth-differentiation balance hypothesis

The growth-differentiation balance concept was given by Loomis (1932) as a basis to explain plant behavior. Plant development can be divided into three overlapping stages: cell division, cell enlargement and cell differentiation. The first two stages are regarded as elements of growth and the third one is related to morphological changes due to prevailing chemical conditions in the cells involved. For growth-differentiation balance, differentiation is defined as the sum of chemical and morphological changes that occur in the maturing cells such as thickening of cell walls and leaf cuticle, protoplasm hardening, and formation of gums, resins

and essential oils (Loomis 1932). Under normal conditions, when all the factors such as water, oxygen, sugar and inorganic nutrients are favorable, growth is favored over differentiation. But under stress conditions, when any of the above mentioned factors is limiting, differentiation is favored over growth and the tissues have a higher content of lignin and wax. In the case of severe water deficiency, both growth and differentiation rates are reduced. As an example, southern pines are exposed to water deficit and it has been a topic of interest. But little attention has been paid to water deficit as a beneficial factor as it leads to the formation of oleoresins as the products of differentiation that are regarded as the source of resistance to beetle attack (Lorio 1986).

1.9.4. Genetic control of physiological characteristics that affect Carbon fixation

Physiological processes associated with growth include photosynthetic capability, carbon assimilation (Ledig and Perry 1969) and nitrogen use efficiency (Li et al. 1991). Relative photosynthetic capability has been suggested as a basis for selection of trees for superior growth rate (Richardson 1960; Kozlowski 1969). This idea is based on the point that carbon accumulation increases as the rate of photosynthesis exceeds respiration which in turn increases the capacity of the tree for wood production (Campbell and Rediske 1966). Also, an increase in net photosynthetic rate and growth has been noticed in loblolly pine clones in response to fertilization with physiological and growth differences observed among the clones (King et al. 2008). Further, canopy level characteristics such as photosynthetically active radiation (PAR), leaf area index (LAI) and radiation use efficiency have been investigated in loblolly pine families and were found to be responsible for differences in family performance (McCrady and Jokela 1997).

Because nitrogen availability generally limits forest productivity in the absence of fertilization, greater improvement in productivity is possible by selecting genotypes for high nitrogen use efficiency or the gain in biomass per unit of nitrogen utilized (Li et al. 1991). Nitrogen is an important factor that determines the growth of forests (Allen et al.1987). Tree breeders select for superior growth rate on the basis of shoot elongation under varying nitrogen concentrations to maximize nitrogen use efficiency (Li et al. 1991). Response to nitrogen is measured in terms of various factors like carbon allocation to the roots, shoot elongation, leaf conductance and photosynthetic rate (Li et al. 1991; Samuelson 2000). Stability in family variation for physiological traits has been observed in response to nitrogen treatment and allocation patterns have been found to be different under high and low nitrogen treatments. Generally, biomass allocation is greater to the fine roots under low nitrogen conditions, and above ground biomass is under high nitrogen treatment (Li et al. 1991).

1.9.5. Genetic control of morphological characteristics that affect carbon allocation

Carbon allocation has a critical role in cycling of carbon in the forest ecosystem. The three main components of carbon allocation in forest ecosystem as described by Litton et al. (2007) are: (i) biomass, mass or amount of material, (ii) carbon flux, the rate of carbon movement to different components per unit time, (iii) carbon partitioning, the fraction of carbon flow/flux to a particular component that is measured as fraction of gross primary productivity (GPP). Carbon flux to all the components such as foliage, stem, and roots increases with increase in GPP. However, resource and environmental factors differentially affect carbon partitioning to above and below ground plant parts, as increased allocation to above ground parts and decreased allocation to below ground parts have been noticed with an increase in resource

availability and stand age. But the allocation of carbon to crown/foliage has been found to be less sensitive as compared to stemwood, and roots (Litton et al. 2007).

Several studies have been done to assess the effect of genetics and fertilization on carbon allocation (Stovall et al. 2012; Bongarten and Teskey 1987; Charles et al. 2012; Tyree et al. 2009; Retzlaff et al. 2001). Patterns of dry weight allocation to various plant parts in loblolly pine seedlings from diverse geographic regions have been studied and found that seedlings from coastal regions allocated more dry weight to above ground portions and less to below ground portions than the seedlings from continental regions (Bongarten and Teskey 1987). Also, significant family variation in morphological characteristics of spruce seedlings was found, thus making early genetic selection possible for superior root systems (Charles et al. 2011). Furthermore, in two loblolly pine clones, shifts in carbon partitioning in response to fertilization varied between the clones (Stovall et al. 2011). However, in a study done on juvenile loblolly pine trees, carbon partitioning was found to be similar in both fast and slow growing families from different regions without any effect of fertilization (Retzlaff et al. 2001).

1.9.6. Genetic control of defense chemicals

Phenolics play a role in imparting disease resistance to plants. Some phenolics act as inhibitors and are formed previously, while some others are formed in response to the infection by the pathogen (Nicholson and Hammerschmidt 1992). It has been reported that the production of phenolics is greater under increased CO_2 and decreased nitrogen availability in above (primary and fascicular needles) and below (lateral roots) ground plant parts (Gebauer et al. 1998). Also, an increased concentration of defense chemicals (phenolics) has been reported

under low nitrogen and high CO_2 leading to the conclusion that elevated CO_2 and low nitrogen may play a role in plant-pathogen interactions and decomposition rates (Booker and Maier 2001). These results indicate that under optimum levels of nitrogen, plants tend to be more resistant to pathogens.

Genetic differences in the production of total phenolics and tannins have been investigated in several studies using loblolly pine (Sword et al. 1998) and slash pine (Saxon et al. 2004). It was concluded that the foliage concentration of tannins decreases with fertilization (Sword et al. 1998). Also, the concentration of total phenolics and condensed tannins was found to be higher in a genotype resistant to fusiform rust and the concentration of total phenolics but not the condensed tannins varied with elevated CO_2 and nitrogen levels (Saxon et al. 2004). However, in a recent study conducted with loblolly pine clones, full sib families and half sib families, little variation was found in the concentration of phenolics and tannins among the genotypes (Aspinwall et al. 2011).

Chapter Two

Variation in resistance of loblolly pine (*Pinus taeda* L.) and slash pine (*P. elliotii* Englem.) families against *Leptographium* and *Grosmannia* root fungi

2.1. Abstract

Pine decline poses a serious threat to forest sustainability in the southeastern United States. Complex interactions of biotic and abiotic factors are involved that include root-feeding bark beetle vectors and their associated fungal genera, *Leptographium* and *Grosmannia*. A screening study was conducted to determine the relative resistance of loblolly (*Pinus taeda* L.) and slash pine (*P. elliottii* Englem.) seedling families when challenged with *Leptographium* and *Grosmannia* species. In year one, bare root seedlings from loblolly and slash pine families (23 and 5 respectively) were screened for resistance using an artificial inoculation method. In year two, containerized seedlings from loblolly and slash pine families (27 and 2 respectively) were screened. Seedling responses such as lesion presence, lesion length, occlusion of vascular tissues and seedling survival were measured twelve weeks after inoculations. Seedling stems exhibited dark brown lesions and resinous occluded tissues. Year one results indicated that the seedling families had variable responses to different *Leptographium* and *Grosmannia* species leading to identification of some families (L-5, L-20, L-8, and, L-13) that developed consistently smaller lesions, while other families (L-1, L-2, L-3 and L-4) were found to have consistently larger lesions. The year two screening identified family L-42 as having consistently smaller lesions

among all families tested. These responses indicate that family genetics could be used for deployment into high risk areas to mitigate the potential for pine decline.

2.2. Introduction

The diverse and dynamic nature of southeastern forests is the result of variation in land use history (Wear and Greis 2002). Before European settlement, the southeastern United States was under natural forest conditions with mixed and sparse tree stands consisting of an oak-pine canopy and naturally occurring fires controlling the understory vegetation. In the 19th and early 20th centuries there were large-scale disturbances in forest ecosystems throughout the region. The forests were cleared for agriculture, and cultivation of row crops led to soil erosion and nutrient depletion. Agriculture was abandoned but the problem of erosion persisted due to the absence of vegetation. With the action of federal and state agencies, loblolly pine (*Pinus taeda* L.) was widely planted in the region to prevent further soil erosion. This particular pine species exhibits rapid juvenile growth; responds well to silvicultural treatments and can be easily established (Baker and Langdon 1990).

Since 1953, there has been a continuous increase in pine plantation establishment in the southeastern United States (Haynes et al. 2007). At present, large portions of southeastern United States forests are managed for wood products, including pulp, saw timber and poles. Loblolly and slash pine are two important tree species grown in this region. Loblolly pine continues to be the most favored species for timber production in the Southeast consisting of 80% of the commercial forest area (Smith et al. 2007). The forest industry within the southeastern United States provides 110,000 jobs directly or indirectly and contributes $30

billion to the economy of the region. Beside timber production, loblolly pine offers a variety of other environmental related benefits like soil erosion control, habitat for wildlife, and recreational activities (Schultz 1999).

Recently, premature mortality has been observed and characterized as a decline syndrome (Eckhardt et al. 2007). The decline concept was first described by Manion (1981) and involves a number of factors that together contribute to mortality and these factors have been broadly classified as predisposing, inciting and contributing. Predisposing factors bring the tree under constant stress. Inciting factors act for a short period of time and increase the severity of the stress and contributing factors adds to decline and in the end, result in premature mortality. Decline in loblolly pine was first noticed in 1959 in the Talladega National Forest on the Oakmulgee and Tuscaloosa Ranger districts in Alabama (Brown and McDowell 1968). At that time, the premature tree mortality was coined "loblolly pine die-off". Loblolly pine is host to a number of beetle species that vector the fungi associated with pine decline. There is a complex interaction of these insects and fungi that together contribute to the observed pine decline (Eckhardt et al. 2007). Among the biotic factors, *Leptographium* has a contributing role in pine decline. Earlier, several attempts were made to identify the fungi associated with decline. Potential root pathogens studied were *Phytophthora cinnamomi* Rands., *Heterobasidium irregulare* nom. nov. Garbelotto & Otrosina and *Leptographium* spp. It is now well understood that ophiostamatoid fungi are an important component of pine decline (Hess et al. 2005; Eckhardt et al. 2007; Eckhardt and Menard 2009). Earlier studies also showed that ophiostomatoid root fungi cause significant damage in many pine ecosystems. *Leptographium wageneri* (W.B. Kendr.) M.J. Wingf, was found to be associated with black stain root disease in

conifers of the northwestern United States (Cobb 1988). *Leptographium procerum* (Kendrick) M. J. Wingfield, was recovered from the roots and stumps of slash pine plantations (Barnard et al. 1991). Among others, red pine (*P. resinosa* Ait*)* decline in Wisconsin (Klepzig et al. 1991), eastern white pine (P. *strobus* L.) decline (Dochinger 1967) and *Leptographium* spp. recovery from southern pine plantations under southern pine beetle attack (Otrosina et al. 1997) are a few examples.

Several studies have focused on testing the relative virulence of *Leptographium* species in pine (Wingfield 1983; Harrington and Cobb 1983; Eckhardt et al. 2004a; Matusick and Eckhardt 2010a). *Leptographium procerum* and *L. terebrantis* S. J. Barras & T. J. Perry are well studied and have been included in several virulence experiments. *Leptographium procerum* was found to cause procerum root disease in eastern white pine (Dochinger 1967). However, in other virulence studies, *L. procerum* was found to be a mild pathogen. *Leptographium procerum* did not cause significant damage, while *L. terebrantis* caused extensive mortality of white pine seedlings (Wingfield 1983). *Leptographium procerum* was found to be less virulent to southern pine species than other *Leptographium* species commonly found associated with southern pines (Eckhardt et al. 2004a; Matusick and Eckhardt 2010a). *Leptographium terebrantis* is considered to be a mild to moderate pathogen. It has consistently produced resin soaked lesions (Nevill et al. 1995) and sapwood discoloration in pines (Matusick and Eckhardt 2010b). *Leptographium terebrantis* was frequently isolated from study seedlings of ponderosa pine (*P. ponderosa* P. & C. Lawson) and Douglas-fir (*Pseudotsuga* spp.) but was not linked to black-stain root disease (Harrington and Cobb 1983).

Among other species of *Leptographium*, *G. alacris* (formely *L. serpens*) T.A. Duong, Z.W. de Beer & M.J. Wingf. sp. nov., and *Grosmannia huntii* (R. C. Rob. Jeffr.) have been found to be associated with southern pines. *Grosmannia alacris* has been recovered from loblolly pine roots infested with *H. salebrosus* and *H. tenius* insect vectors (Eckhardt et al. 2007). *Grosmannia alacris* along with *L. lundbergii* produced the longest lesions in a study conducted on both loblolly pine seedlings and mature trees (Eckhardt et al. 2004a). *Grosmannia alacris* and two other *Leptographium* species (*Ophiostoma ips* and *L. lundbergi*) were not considered serious pathogens of pine forests in South Africa based on virulence trials (Zhou et al. 2002). Morphological distinction between *G. alacris* and *G. huntii* was difficult with both species having distinctive serpentine hyphae. Recently, the presence of *G. huntii* in the southeastern United States was confirmed using DNA sequence data (Zanzot 2009) and it was recovered from longleaf pine (*P. palustris* Mill.) plantations in Georgia (Zanzot et al. 2010). The virulence of ophoistomatoid involved in southern pine decline was tested recently on southern pine species and resulted in *G. huntii* having the longest lesions in all three pine species in the study (Matusick and Eckhardt 2010a).

Previous studies have been focused on testing the relative virulence of *Leptographium* root fungi on a particular pine species (Wingfield 1983; Harrington and Cobb 1983; Eckhardt et al. 2004a; Bertagnole et al. 1983; Nevill 1995). Recently, the relative virulence of the *Leptographium* root fungi and comparative resistance/tolerance of the three main southern pine species have shown that different *Leptographium* species vary in their virulence among southern pines. Also, longleaf pine and slash pine were found to be more tolerant to *Leptographium* than loblolly pine (Matusick and Eckhardt 2010a).

Loblolly and slash pine are prone to a wide variety of insect pests and diseases that include fusiform rust (*Cronartium quercum* [Berk.] Miyabe. Ex Shirai. f. sp. *fusiforme*). Resistance to fusiform rust along with increases in growth rates has been incorporated into genetically improved seedlings by tree breeding efforts. As a result of these breeding programs, the infection rate of fusiform rust has been decreased to 20-25% in the improved seedlots (Li et al. 1999). However, studies have yet to focus on the relative virulence of *Leptographium* species among commonly deployed families of either loblolly or slash pine. These studies examined within species variability for resistance to *Leptographium* root fungi among the loblolly pine and slash pine families deployed by forest industries throughout the southeastern United States.

2.3. Materials and Methods

In year one, seed from 28 families (23 loblolly and 5 slash) representing some of the more commonly out-planted genotypes were grown in a common nursery bed at the Glennville Regeneration center in Georgia. Seedlings were lifted in early January 2011 and re-potted at Auburn University in an outdoor screening facility. The family screening was continued for a second year with a different set of genotypes using containerized seedlings from 27 loblolly pine and 2 slash pine families which were delivered to the Auburn University Forest Health Dynamics Laboratory in early January of 2012 and re-potted. Trade gallon pots filled with ProMix BX® (Premier Tech, Quebec, and Canada) peat based potting media were used for planting the seedlings. To maintain anonymity, each family was assigned a unique code for data reporting and analysis. In both years, seedlings were placed under natural environmental conditions with access to natural precipitation, sunlight and temperature with irrigation to prevent moisture stress. The experiment was set up as a randomized complete block design with three

blocks/replications in the first and second year. Pre-treatment and final (just before harvesting) root collar diameter and height measurements were taken to monitor growth. To determine the pre-pot family morphology, in year two, ten seedlings per family were subsampled before re-potting and after root collar diameter and height measurements, each seedling was separated into fine roots, coarse roots, stem and foliage. These parts were oven dried separately to calculate the dry biomass. Initial root to shoot ratio was calculated from the dry weight measurements.

Inoculation treatments were applied two months after re-potting the seedlings. In the year one screening, six treatments were assigned to randomly selected seedlings within each family. Ten seedlings per family per block were selected for each treatment. The treatments included inoculations with one of four fungal species: *L. procerum, L. terebrantis, G. alacris* and *G. huntii*. Wounded and wound+media treatments served as the controls. In the year two screening, the number of treatments was decreased to four based on year one results. Four treatments included *G. huntii, L. terebrantis*, wound control and wound+media control. Two weeks before inoculations, each fungus was cultured on 2% malt extract agar (MEA). Pure isolates of the fungal species were used for inoculations. The wound inoculation method included making a vertical cut with sterile razor blade in the lower stem of the seedling about 2 cm from the soil line (Nevill et al. 1995; Eckhardt et al. 2004a) (Figure 2.1). A 3 mm diameter plug of colonized mycelium was placed in the slit and the wound was wrapped with moist cotton and sealed with Parafilm®.

Figure 2.1. Loblolly pine seedling inoculated in the stem with *Leptographium.*

After destructively harvesting the seedlings, final seedling growth measurements and seedling survival were recorded. On each seedling stem, seedling responses to the fungal inoculations were measured. These included lesion presence/absence, lesion length, lesion width, lesion depth and occlusion of vascular tissues. Lesion length consisted of dark brown tissue which either equaled or extended to both sides of the length of the inoculation wound. For determining occlusion length, the roots were separated from the shoots and the living shoots were placed in a solution of FastGreen stain (FastGreen FCF; Sigma Chemical Co.) and water (0.25g/L of water) (Nevill et. al. 1995) (Figure 2.2). After three days of allowing capillary action, the length of unstained stem tissue was recorded as occlusion length. To confirm if the fungal infection occurred with the inoculations, re-isolation of associated fungi was attempted by excising a 1 cm of stem section surrounding the lesion and plating it on CSMA nutrient medium (Malt extract agar with 800 mg/l of cycloheximide and 200 mg/l of streptomycin sulfate). In

year two, four seedlings per family per treatment per replication were processed for biomass measurements by separating each seedling into foliage, stem, fine roots and coarse roots.

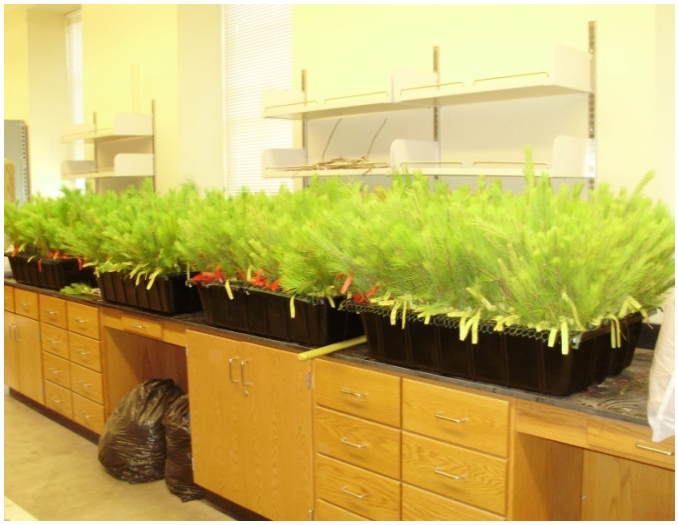

Figure 2.2. Seedlings placed in fast green solution for recording vascular tissue occlusion.

Seedling response variables to the artificial inoculations were analyzed using SAS statistical software (SAS Institute, 9.2 ed., Cary, NC). Both generalized linear fixed model and linear mixed models were fit to the data to compare the treatments and families. In the fixed model, each block was considered a replicate. The families, fungal treatments and their interaction were included in the model under fixed effects. A set of ten seedlings per family in each replication was used as an experimental unit for all the continuous response variables while running the analysis for the year one screening and a set of sixteen seedlings was used as an experimental unit in the year two screening. Lesion length was found to be the strongest response variable. For comparison among the pine families, therefore, lesion length was used as the response variable. Families were assigned into groups based on their average lesion length and analysis was conducted using contrast statements to find significant differences between the groups. The contrast statements were used on the main effects and interaction effects. Binary

response variables like survival, lesion presence and re-isolation were analyzed by the GENMOD procedure in SAS using logistic ANOVA for the second year screening as it was noticed that unlike the first year, mortality was found to be significant.

In the mixed model, family and family x treatment interaction were kept random, while the treatments were kept under fixed effects. All the continuous variables were analyzed considering the seedling as an experimental unit. Both main effects and two way interaction effects were included in the model. Covariance parameter estimates for each response variable (lesion length, occlusion length, lesion width, and lesion depth) were used to determine the variation among the families and the families were ranked on the basis of covariance parameter estimates for lesion length. Fungal treatments were compared by type 3 fixed effects.

2.4. Results

2.4.1. Year One

Inoculation of the 28 different pine families with the four different fungi resulted in less than 7% seedling mortality. A total of 94% of the seedlings survived the inoculations. Seedling mortality was observed in loblolly pine families L-14 and L-22 which had comparatively less survival than the other loblolly pine families tested. All fungi used in the trial caused dark brown lesions along seedling stems. Lesions extended vertically beyond the inoculation site along both sides of the seedling stem (Figure 2.3). Evidence of radial movement of the fungus was observed more so in seedlings challenged with *G. huntii.* As a comparison, lesions did not occur beyond the inoculation site in either of the wound or wound+media controls and they were not significantly different from each other ($F = 0.07$, $P = 0.8231$). However, lesion length was found

to be significantly longer for all four fungi tested compared to the two control treatments (Table 2.1). While lesions occurred in all seedlings inoculated with the fungal species, their presence as indicated from the fixed model results was not significantly different among the fungal treatments and families (Table 2.2).

Figure 2.3. Brown lesions reported on seedlings stems following inoculations.

Table 2.1. Pairwise comparison of treatments for lesion and occlusion length for loblolly pine families.

Treatment	Lesion length	Occlusion length
G. huntii vs. *L. procerum*	0.0073	0.0794
G. huntii vs. *G. alacris*	0.1277	0.1368
G. huntii vs. *L. terebrantis*	<0.0001	<0.0001
L. procerum vs. *G. alacris*	0.2349	0.7735
L. procerum vs. *L. terebrantis*	<0.0001	<0.0001
G. alacris vs. *L. terebrantis*	<0.0001	<0.0001
Wound vs. Wound-Media	0.8231	0.7986
Wound vs. *G. huntii*	<0.0001	<0.0001
Wound vs. *L. procerum*	<0.0001	<0.0001
Wound vs. *G. alacris*	<0.0001	<0.0001
Wound vs. *L. terebrantis*	<0.0001	<0.0001
Wound-Media vs. *G. huntii*	<0.0001	<0.0001
Wound-Media vs. *L. procerum*	<0.0001	<0.0001
Wound-Media vs. *G. alacris*	<0.0001	<0.0001
Wound-Media vs. *L. terebrantis*	<0.0001	<0.0001

Note: t- value <0.05 shows a significant difference at alpha=0.05.

Of the six seedling variables measured, lesion length was considered the primary seedling response variable. Average lesion length was found to be significantly different among the families ($F = 2.87$, $P < 0.0001$) and among the fungi tested ($F = 49.56$, $P < 0.0001$) (Table 2.2). Average lesion length was not significantly different among the fungal treatments within each family ($F = 0.60$, $P = 0.9878$) (Table 2.2). Similar to lesion length, occlusion length was also significantly different among the families ($F = 2.63$, $P = 0.0004$) (Table 2.2) and among the fungi ($F = 57.60$, $P < 0.0001$) (Table 2.2) but not within each family ($F = 0.84$, $P = 0.7744$) (Table 2.2). When average lesion length was plotted for each fungus separately, it was observed that seedlings inoculated with *G. huntii* formed three distinct family groups with the intermediate group not significantly different from either extreme group (Figure 2.4). When seedlings were challenged with *G. alacris*, families L-1, L-2, L-3, L-4, and L-21 were found to have significantly longer lesions than all other families examined (Figure 2.5). Likewise, when *L.*

terebtantis was used, the group of families with intermediate lesion length was found to be significantly different from both the extreme groups having smallest and largest lesion length (Figure 2.6). There was no difference in lesion length among families tested when *L. procerum* was used (Figure 2.7). However, the overall family ranking trend of *L. procerum* was found to be similar to the other three fungal species. With respect to the other seedling characteristics, while the mean RCD was found to be different among the families tested, it was not affected by the fungal inoculations. Similar to mean RCD, mean height was also not affected by the fungal inoculations but it varied significantly among the families (Table 2.3).

Table 2.2. Probability of a greater F-value for lesion presence, occlusion presence, lesion length and occlusion length of loblolly pine families.

Effect	DF	Lesion presence	Occlusion presence	Lesion length	Occlusion length	Lesion width	Lesion depth
Block (B)	2	0.6096	0.6096	<0.0001	<0.0001	<0.0001	0.0159
Family (F)	21	0.7447	0.7447	<0.0001	0.0004	0.0020	0.1123
Trt (T)	3	0.6901	0.6901	<0.0001	<0.0001	<0.0001	<0.0001
TxF	63	0.7428	0.7428	0.9878	0.7744	0.7981	0.5950

Note: P- value <0.05 shows a significant difference at alpha=0.05.

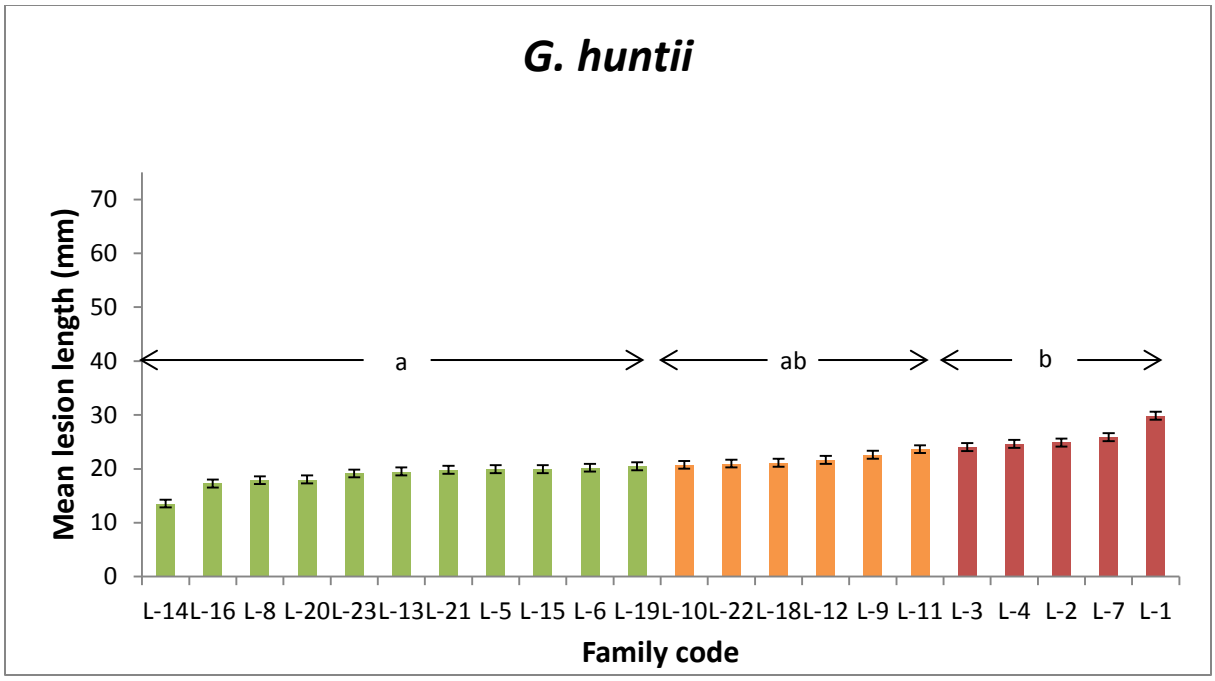

Figure 2.4. Average lesion length for loblolly pine families following inoculations with *Grosmannia huntii* from the year one family screening.

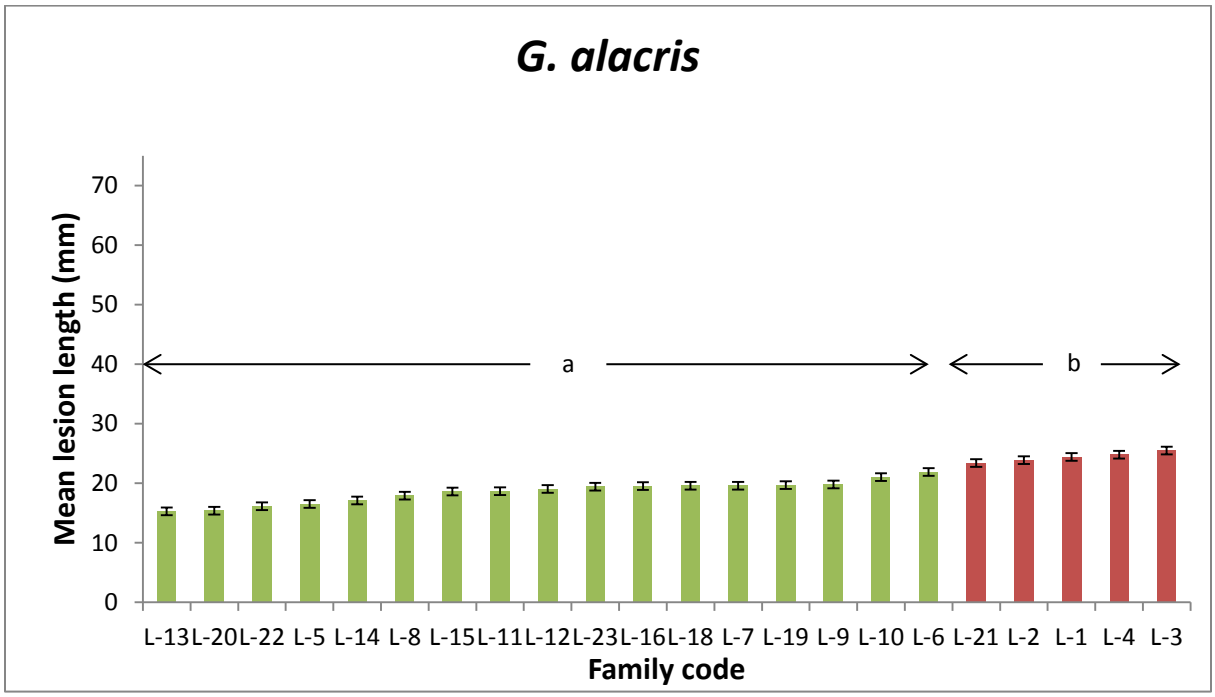

Figure 2.5. Average lesion length for loblolly pine families following inoculations with *Grosmannia alacris* from the year one family screening.

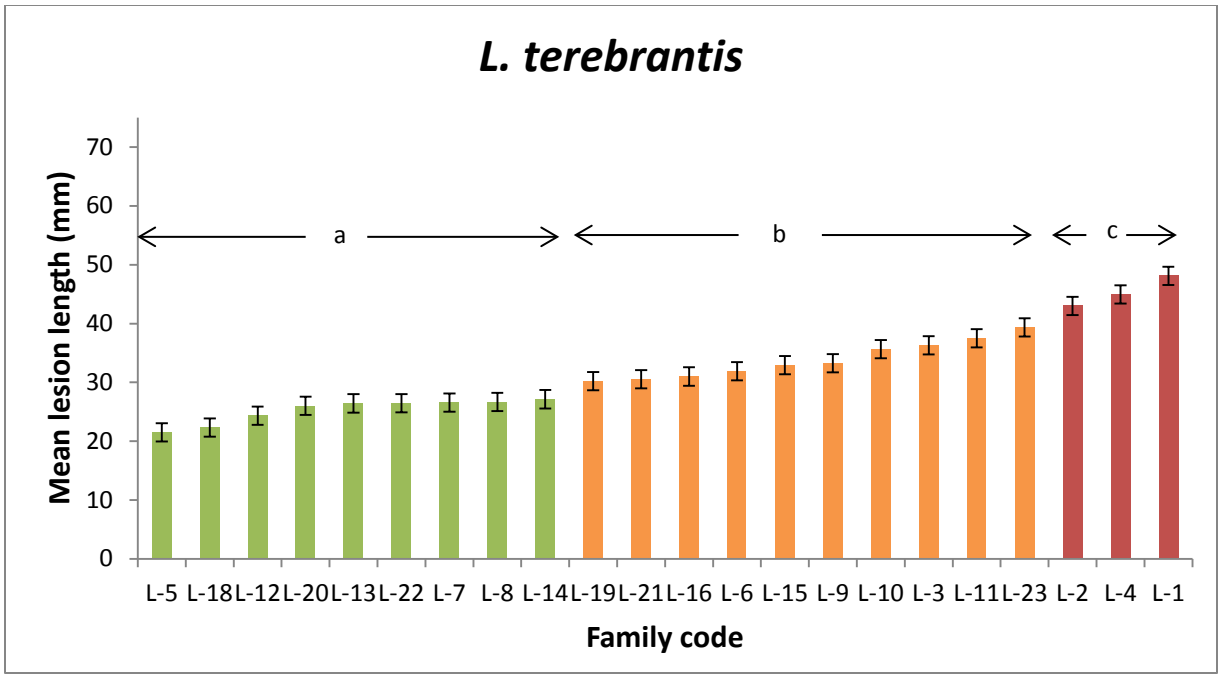

Figure 2.6. Average lesion length for loblolly pine families following inoculations with *Leptographium terebrantis* from the year one family screening.

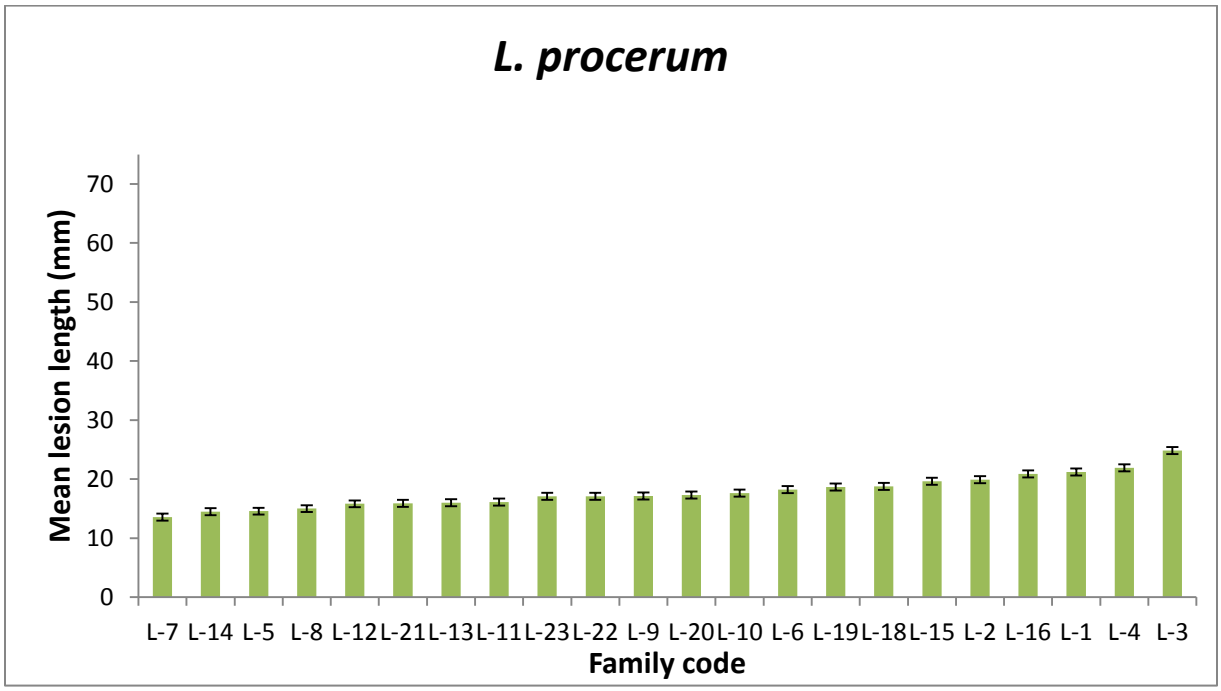

Figure 2.7. Average lesion length for loblolly pine families following inoculations with *Leptographium procerum* from the year one family screening.

Table 2.3. Probability of a greater F-value for final RCD and total height of loblolly pine families.

Effect	DF	RCD	Total height
Block (B)	2	<0.0001	<0.0001
Family (F)	21	<0.0001	<0.0001
Trt (T)	5	0.9913	0.3328
TxF	105	0.2419	0.7119

Note: P- value <0.05 shows significant differences at alpha=0.05.

The interaction (TxF) was not significant for lesion or occlusion length, indicating that the four fungi used in the inoculations did not vary in their virulence within a particular seedling family. Therefore, the 23 loblolly pine families were ranked for their overall response to the artificial inoculations by pooling the fungal species together (Figure 2.5). Group wise comparison using average lesion length among the seedling families sorted the loblolly pine families into three distinct groups. Family response (lesion length) to the fungal inoculations within each group was found to be different from the other two groups (Figure 2.5). In the slash pine families, fungal species used significantly affected both the lesion and occlusion length overall ($F = 16.09$, $P < 0.0001$) (Table 2.4). One slash pine family S-1 had significantly longer lesions than families S-3 and S-5 caused by *L. terebrantis* (Figure 2.6). No other differences were observed on slash pine families for other variables measured: lesion and occlusion presence or lesion and occlusion length. Average lesion length, occlusion length, percentage survival and percentage re-isolation for loblolly pine families and slash pine families are shown in Table 2.5 and 2.6 respectively.

Table 2.4. Probability of a greater F-value for lesion and occlusion length of slash pine families.

Effect	DF	Lesion presence	Occlusion presence	Lesion length	Occlusion length
Block (B)	2	0.1332	0.1335	0.0031	0.2419
Family (F)	4	0.3843	0.3802	0.2153	0.4478
Trt (T)	3	0.5402	0.5355	<0.0001	<0.0001
TxF	12	0.5191	0.5228	0.8039	0.8762

Note: P- value <0.05 shows significant differences at alpha=0.05.

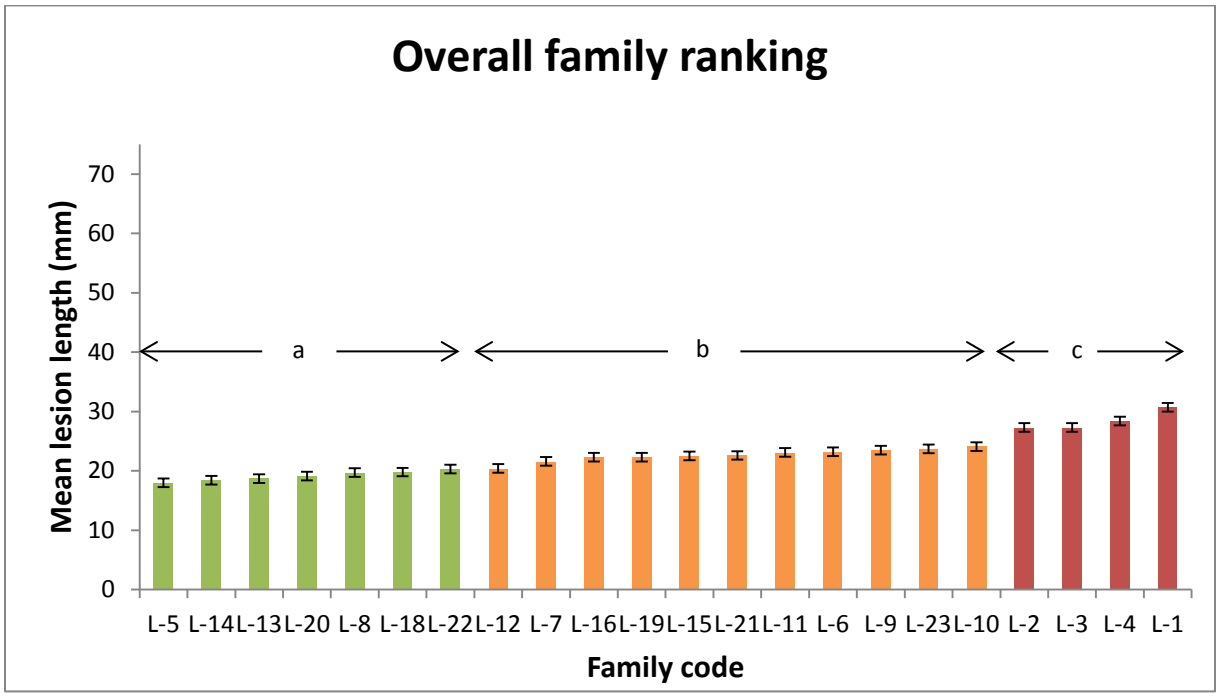

Figure 2.8. Average lesion length for loblolly pine families following inoculations with four ophiostomatoid fungal species from the year one family screening.

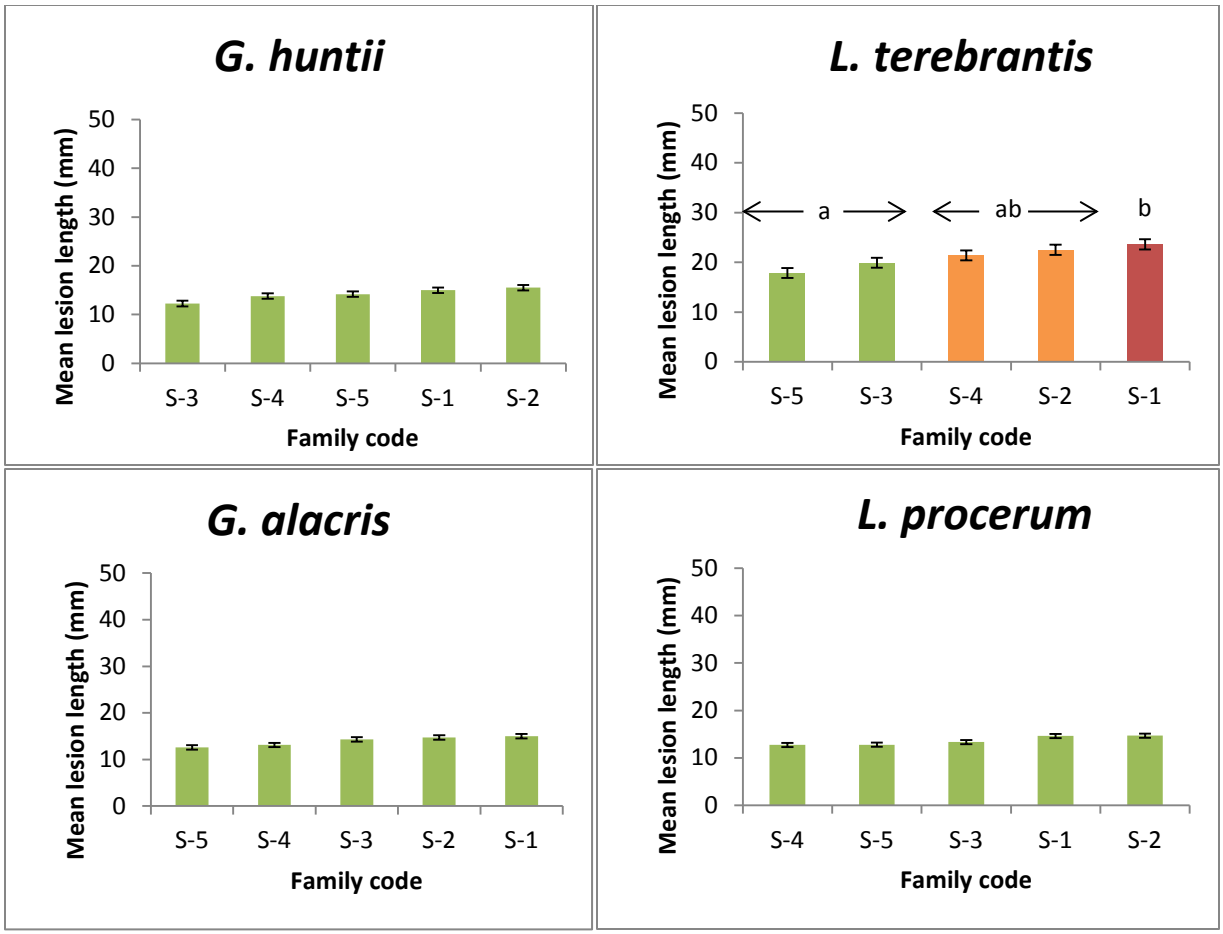

Figure 2.9. Average lesion length for slash pine families following inoculations with four ophiostomatoid fungal species from the year one family screening.

Table 2.5. Average lesion length, occlusion length, seedling survival and fungal re-isolation frequency at the culmination of the year one study for loblolly pine families.

Family	Lesion length (mm)	Occlusion length (mm)	Survival (%)	Re-isolation (%)
L-5	18(8.8)	20(9.7)	91	91
L-14	18(10.4)	23(11.0)	80	99
L-13	19(7.1)	22(10.1)	92	93
L-20	19(8.4)	22(10.4)	99	85
L-8	20(8.9)	23(11.4)	95	91
L-18	20(5.6)	25(9.2)	91	93
L-22	20(11.9)	23(13.5)	86	95
L-12	20(9.1)	23(9.9)	93	92
L-7	22(13.1)	24(13.4)	93	98
L-16	22(12.8)	26(14.4)	93	91
L-19	22(10.8)	26(12.5)	99	90
L-15	23(11.9)	26(13.2)	99	86
L-21	23(14.2)	26(15.4)	97	88
L-11	23(15.1)	27(15.1)	91	84
L-6	23(12.5)	26(13.3)	100	90
L-9	24(12.3)	27(14.5)	98	90
L-23	24(17.5)	27(18.0)	94	92
L-10	24(14.8)	29(16.8)	93	96
L-2	27(14.3)	31(14.5)	98	86
L-3	27(13.2)	31(14.3)	93	80
L-4	28(16.9)	32(17.3)	97	91
L-1	31(17.1)	34(17.7)	99	83

Note: Means and standard deviations (parentheses).

Table 2.6. Average lesion length, occlusion length, seedling survival and fungal re-isolation frequency at the culmination of the year one study for slash pine families.

Family	Lesion length (mm)	Occlusion length (mm)	Survival (%)	Re-isolation (%)
S-5	14(4.2)	17(6.5)	90	87
S-3	15(6.0)	18(8.2)	85	89
S-4	15(6.7	17(8.5)	87	92
S-2	17(7.5)	21(9.9)	86	89
S-1	17(7.4)	20(9.2)	92	89

Note: Means and standard deviations (parentheses).

Lesions did not extend beyond the inoculation site in either of the wound or wound+media controls and were not significantly different from each other (F = 0.06, P = 7990). These results were supported by the mixed model results from pairwise comparison of the treatments (Table 2.7). Further analysis was completed with four fungal treatments. Estimation of covariance parameters as presented in Table 2.8 indicated that the variation among the families was significantly different from zero for lesion length, and occlusion length. However, lesion width and lesion depth was not significantly different from zero for the loblolly pine families tested (Table 2.8). Type 3 fixed effects suggested that the fixed effects including blocks and treatments were significantly different for lesion length, occlusion length, lesion width and lesion depth (Table 2.9). Family x treatment interaction was found to be significant and the families were ranked overall and for each fungal species using lesion length on the basis of the estimate (i.e. mean family performance) (Table 2.10). Family ranking differed overall and for each treatment. As an example, family L-20 ranked first overall, second for *L. terebrantis*, fourth for *G. alacris* and twenty second for *L. procerum* (Table 2.10).

Table 2.7. Pairwise comparison of treatments for lesion and occlusion length for loblolly pine families (mixed model).

Treatment	Lesion length	Occlusion length
G. huntii vs. *L. procerum*	<0.0001	0.0066
G. huntii vs. *G. alacris*	0.0517	0.0875
G. huntii vs. *L. terebrantis*	<0.0001	<0.0001
L. procerum vs. *G. alacris*	0.0407	0.2999
L. procerum vs. *L. terebrantis*	<0.0001	<0.0001
G. alacris vs. *L. terebrantis*	<0.0001	<0.0001
Wound vs. Wound-Media	<0.7990	<0.7864
Wound vs. *G. huntii*	<0.0001	<0.0001
Wound vs. *L. procerum*	<0.0001	<0.0001
Wound vs. *G. alacris*	<0.0001	<0.0001
Wound vs. *L. terebrantis*	<0.0001	<0.0001
Wound-Media vs. *G. huntii*	<0.0001	<0.0001
Wound-Media vs. *L. procerum*	<0.0001	<0.0001
Wound-Media vs. *G. alacris*	<0.0001	<0.0001
Wound-Media vs. *L. terebrantis*	<0.0001	<0.0001

Note: t-value <0.05 shows significant differences at alpha=0.05.

Table 2.8. Covariance parameter estimates are presented. Variation among families is significantly different from zero for lesion length, and occlusion length. Family by treatment interaction is also significant; families can be ranked overall and for each fungus individually.

Cov parm	Lesion length	Lesion width	Lesion depth	Occlusion length
Family (F)	0.0261	0.1219	0.0514	0.0352
Trt (T)xF	0.0011	0.1729	--	0.0009
Block (B)xF	0.0022	0.0130	0.1424	0.0050
Residual	<0.0001	<0.0001	<0.0001	<0.0001

Note: Z-value <0.05 shows significant differences at alpha=0.05.

Table 2.9. Type 3 tests of fixed effects (F tests) suggest that all the effects are significant for lesion length, lesion width, lesion depth and occlusion length.

Effect	DF	Lesion length	Lesion width	Lesion depth	Occlusion length
Block (B)	2	<0.0001	<0.0001	<0.0001	<0.0001
Trt (T)	1	<0.0001	<0.0001	<0.0001	<0.0001
BxT	15	<0.0001	<0.0001	0.0008	<0.0001

Note: P- value <0.05 shows significant differences at alpha=0.05.

Table 2.10. Family ranking for lesion length (estimate) overall and across all four treatments.

Family	Estimate	Overall rank	Estimate	Rank GH	Estimate	Rank LT	Estimate	Rank GA	Estimate	Rank LP
L-20	-2.934	1	0.0223	14	-2.867	2	-0.628	4	1.6457	22
L-13	-2.215	2	0.6403	17	-1.415	6	-0.846	2	0.2414	13
L-8	-1.981	3	-0.342	9	-0.8	11	0.1202	14	-0.212	10
L-18	-1.865	4	-0.477	7	-1.714	4	0.397	18	0.6327	15
L-11	-1.547	5	0.36	15	-0.386	14	0.0744	13	-1.012	5
L-5	-1.376	6	1.4366	22	-3.462	1	0.2601	16	0.9082	19
L-16	-1.281	7	-0.481	6	-1.534	5	-0.189	10	1.4068	21
L-14	-1.046	8	-0.682	5	0.0474	15	0.1405	15	-0.157	11
L-19	-0.812	9	-0.227	10	-1.397	7	0.344	17	0.7741	17
L-6	-0.621	10	-0.895	3	-1.841	3	1.387	21	0.9623	20
L-15	-0.421	11	-0.791	4	0.2479	16	-0.508	5	0.7892	18
L-7	-0.338	12	1.3217	21	-0.868	9	-0.409	7	-0.255	8
L-10	-0.334	13	0.0066	13	-0.615	12	0.7361	20	-0.335	7
L-9	-0.013	14	1.1918	20	-0.816	10	-0.137	11	-0.246	9
L-21	0.1309	15	-1.009	2	0.3034	17	1.8111	22	-1.024	4
L-12	0.21	16	0.9363	18	-1.067	8	-0.127	12	0.3889	14
L-22	0.3104	17	0.9648	19	-0.461	13	-0.419	6	0.1085	12
L-23	0.4748	18	-1.759	1	3.3046	20	-0.28	9	-0.97	6
L-2	3.1606	19	-0.034	12	2.5937	19	0.4372	19	-1.029	3
L-3	3.6794	20	-0.447	8	2.3807	18	-0.331	8	0.6889	16
L-4	4.2569	21	-0.204	11	4.9687	21	-0.772	3	-1.342	2
L-1	4.5598	22	0.4685	16	5.3972	22	-1.06	1	-1.967	1

GH - *Grosmannia huntii*, LT - *Leptographium terebrantis*, GA – *Grosmannia alacris*,
LP - *Leptographium procerum*

2.4.2. Year Two

Mortality was observed after the fungal inoculation treatments were imposed on the seedlings. At the end of the experiment, 13% of the seedlings were dead. Analysis of maximum likelihood parameter estimates indicated that *L. terebrantis* significantly affected seedling survival, but *G. huntii*, wound and wound+media did not affect the survival significantly (Table 2.11). Seedling survival was found to be significantly different among the families and between the fungal treatments. However, the family x treatment interaction was not significant for seedling survival (Table 2.12). Lesion presence was not significantly different among the fungal treatments, or families suggesting that lesions occurred in almost all the seedlings inoculated with fungi (Table 2.12). In response to inoculations with *G. huntii* and *L. terebrantis*, dark brown sunken lesions were observed along the seedlings stems (Figure 2.7). Lesions extended vertically along both sides of the inoculation site. Radial movement was observed occasionally on the stems from the two fungal species. Lesions did not occur on the control treatments and lesion length was found to be significantly longer for both fungal species than for the controls (Table 2.13). Further analysis was conducted with fungal treatments separated.

Figure 2.10. Brown lesions reported on seedling stems following inoculations with *L. terebrantis*, *G. huntii* and Wound+media control.

Table 2.11. Analysis of maximum likelihood parameter estimates indicated that *Leptographium terebrantis* affected the survival significantly.

Treatment	DF	Estimate	Pr>ChiSq
G. huntii	1	-0.723	0.5626
L. terebrantis	1	-3.483	0.0010
Wound	1	21.456	0.9991
Wound-Media	0	0	--

Note: P- value <0.05 shows a significant difference at alpha=0.05.

Table 2.12. Results from logistic ANOVA indicate that survival but not lesion presence was significantly different among the families. Treatments also affected survival significantly. Treatment x family interaction was not found to be significant for survival and lesion presence.

Source	*DF	Pr>ChiSq	+DF	Pr>ChiSq
	Survival		Lesion presence	
Block (B)	2	<0.0001	2	0.0012
Trt (T)	3	<0.0001	1	0.3759
Family (F)	26	<0.0001	26	0.0826
TxF	78	0.6803	26	0.6648

*Control treatments were included in the analysis.
+Control treatments were excluded from the analysis.
Note: P- value <0.05 shows a significant difference at alpha=0.05.

Table 2.13. Pairwise comparison of treatments for lesion and occlusion length for loblolly pine families.

Treatment	Lesion length	Occlusion length
G. huntii vs. *L. terebrantis*	<0.0001	<0.0001
Wound vs. Wound-Media	0.1403	0.1128
Wound vs. *G. huntii*	<0.0001	<0.0001
Wound vs. *L. terebrantis*	<0.0001	<0.0001
Wound-Media vs. *G. huntii*	<0.0001	<0.0001
Wound-Media vs. *L. terebrantis*	<0.0001	<0.0001

Note: t- value <0.05 shows a significant difference at alpha=0.05.

Lesion length was considered a primary response variable and was used to rank the families. Average lesion length was found to be significantly different among the families ($F = 3.97$, $P < 0.0001$) with fungal treatments also affecting the lesion length significantly ($F = 532$, $P < 0.0001$) (Table 2.14). Occlusion length, lesion width and lesion depth were the other continuous variables measured and these differed significantly among the families and treatments (Table 2.14). Family x fungal interaction was not significant for the variables (lesion length, occlusion length, lesion width, and lesion depth) indicating that the treatments did not vary within a particular family (Table 2.14). Average lesion length was plotted for each family for the fungal treatments separately and overall by pooling the treatments together, as the family x fungal treatment interaction was not significant. When the seedlings were challenged with *G. huntii*, families were classified into two separate groups, significantly different from each other. Family L-49 could not be assigned to any of the groups and did not differ significantly from either group. Family L-42 had the shortest average lesion length among the families tested for *G. huntii* (Figure 2.8). Similar to *G. huntii*, average lesion length for each family when plotted for *L. terebrantis*, two groups of families were formed; the family groups were significantly different from each other. However, in this case as well, the family L-49 did not differ from both the groups (Figure 2.9). Family L-42 had smallest lesion length among the families tested for *L. terebrantis* also. When the two fungal treatments were pooled to plot the average lesion length, families were sorted into three groups (Figure 2.10). Families L-42 and L-41 had the shortest lesion length and differed significantly from the other two family groups (Figure 2.10). Along with other continuous variables, RCD and height were found to be significantly different among the loblolly pine families tested. However, no significant differences were found in final RCD and height in response to the fungal treatments (Table 2.15). Root to shoot ratio as calculated

from the final family morphological variables did not differ significantly between the two fungal treatments but was found to be significantly different from the wound control (Table 2.16). Significant differences in root to shoot ratio among the families were noticed (Table 2.17) but root to shoot ratio was found to be positively correlated to lesion length for family L-37 (F = 12.34, P = 0.026, R^2 = 0.7551). No relationship between initial root to shoot ratio, lesion length and final root to shoot ratio was observed by the correlation coefficients (Table 2.18).

Table 2.14. Probability of a greater F-value for lesion length, occlusion length, lesion width and lesion depth of loblolly pine families.

Effect	DF	Lesion length	Occlusion length	Lesion width	Lesion depth
Block (B)	2	<0.0001	<0.0001	<0.0001	<0.0001
Family (F)	26	<0.0001	0.0002	0.0103	0.0002
Trt (T)	1	<0.0001	<0.0001	<0.0001	<0.0001
TxF	78	0.5523	0.6372	0.6804	0.5808

Note: P- value <0.05 shows a significant difference at alpha=0.05.

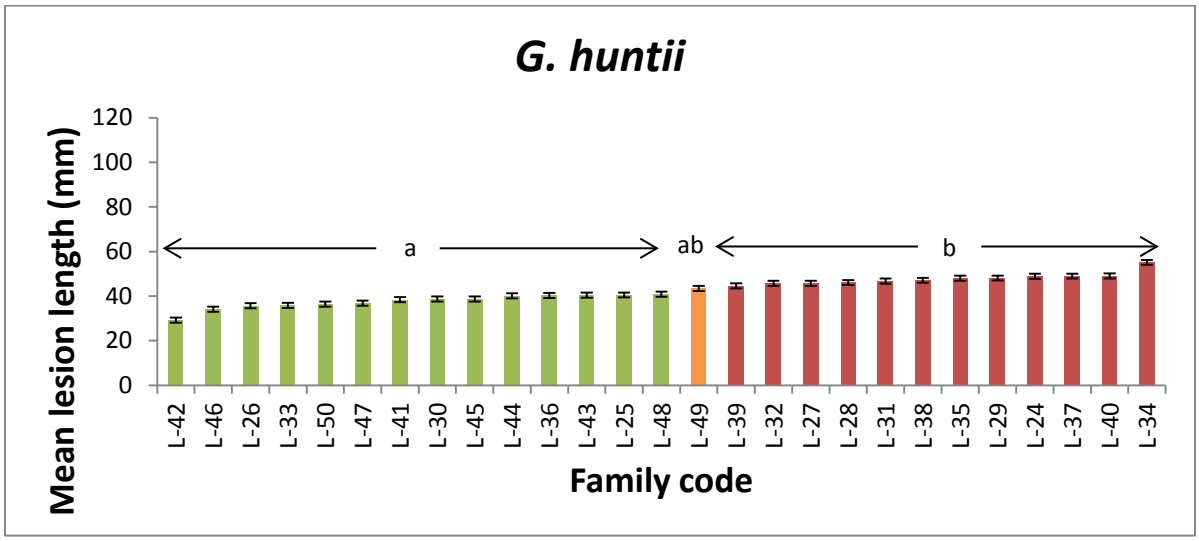

Figure 2.11. Average lesion length for loblolly pine families following inoculations with *Grosmannia huntti* from the year two family screening.

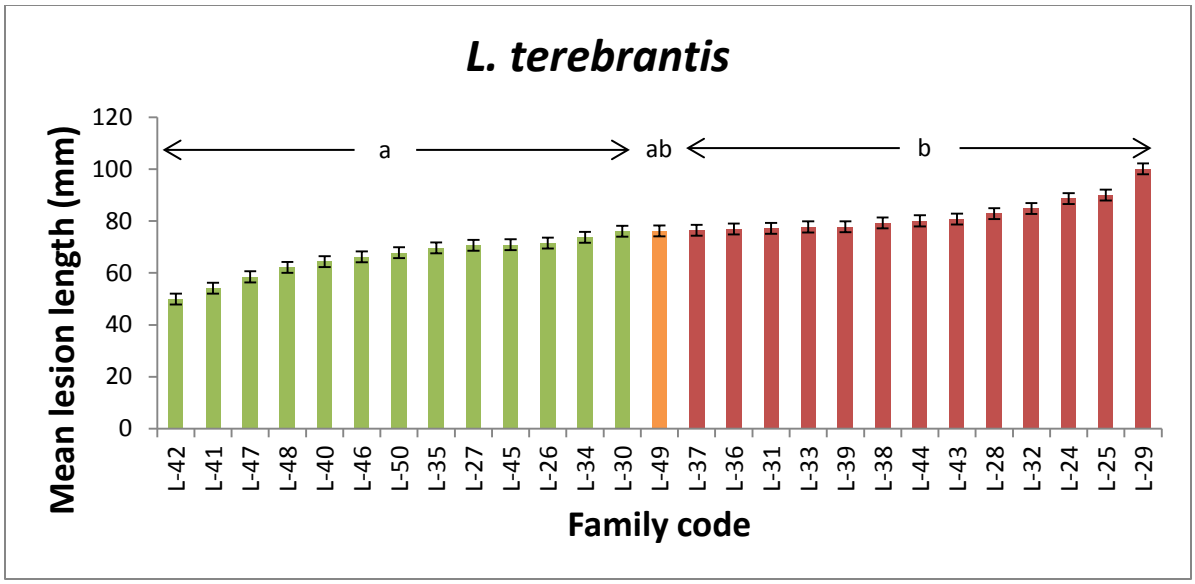

Figure 2.12. Average lesion length for loblolly pine families following inoculations with *Leptographium terebrantis* from the year two family screening.

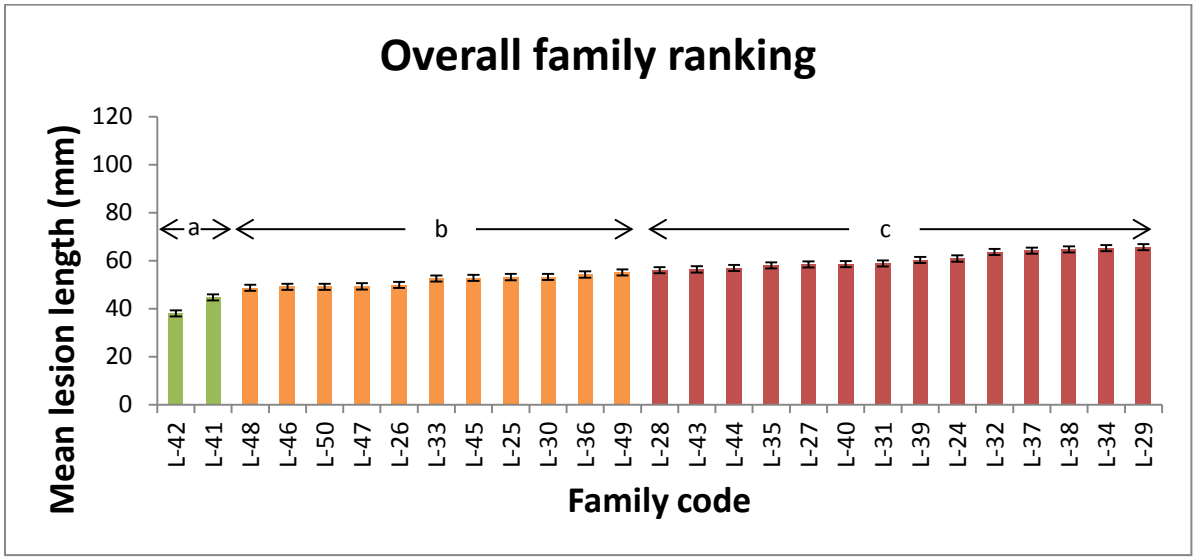

Figure 2.13. Average lesion length for loblolly pine families following inoculations with *Grosmannia huntti* and *Leptographium terebrantis* from the year two family screening.

Table 2.15. Probability of a greater F-value for final RCD and height of loblolly pine families.

Effect	DF	RCD	Total height
Block (B)	2	<0.0001	0.0728
Family (F)	26	<0.0001	<0.0001
Trt (T)	3	0.0737	0.1069
TxF	78	0.9836	0.9303

Note: P- value <0.05 shows a significant difference at alpha=0.05.

Table 2.16. Pairwise comparison of treatments for root to shoot ratio.

Treatment	Root to shoot ratio
G. huntii vs. *L. terebrantis*	0.6524
Wound vs. *G. huntii*	0.0014
Wound vs. *L. terebrantis*	0.0003

Note: P- value <0.05 shows significant differences at alpha=0.05.

Table 2.17. Probability of a greater F-value for root to shoot ratio.

Effect	DF	Root to shoot ratio
Block (B)	2	<0.0001
Family (F)	26	<0.0001
Trt (T)	2	0.0004
TxF	26	0.7542

Note: P- value <0.05 shows a significant difference at alpha=0.05.

Table 2.18. Pearson correlation coefficients between initial root to shoot ratio, lesion length and final root to shoot ratio.

	Initial R:S	Lesion length	Final R:S
Initial R:S	1.00000		
Lesion length	-0.07201 0.7211	1.00000	
Final R:S	-0.07461 0.7115	-0.03532 0.8611	1.00000

Note: P- value <0.05 shows a significant difference at alpha=0.05.

Pair-wise treatment comparison from the mixed model indicated that the wound and wound+media control treatments differed significantly from the two fungal treatments but not from each other (Table 2.19). Covariance parameters indicated that the variation among the families was significantly different from zero for lesion length, occlusion length, and lesion depth. However no significant variation from zero among the families was observed for lesion width (Table 2.20). Family x fungal treatment interaction was not found to be significant for lesion or occlusion length (Table 2.20) indicating that family ranking is not affected by fungal treatment. Treatments, as indicated from the Type 3 fixed effects, differed significantly for lesion length, lesion width, lesion depth and occlusion length (Table 2.21). The families were ranked overall and for each fungal species on the basis of mean family performance for lesion length (Table 2.22). Family L-42 had the smallest lesion length overall and for *L. terebrantis*. Average lesion length, occlusion length, survival and re-isolation for loblolly pine and slash pine families are shown in Table 2.23.

Table 2.19. Pair-wise comparison of treatments for lesion and occlusion length for loblolly pine families.

Treatment	Lesion length	Occlusion length
G. huntii vs. *L. terebrantis*	<0.0001	<0.0001
Wound vs. Wound-Media	0.1923	0.1726
Wound vs. *G. huntii*	<0.0001	<0.0001
Wound vs. *L. terebrantis*	<0.0001	<0.0001
Wound-Media vs. *G. huntii*	<0.0001	<0.0001
Wound-Media vs. *L. terebrantis*	<0.0001	<0.0001

Note: t- value <0.05 shows a significant difference at alpha=0.05.

Table 2.20. Covariance parameter estimates are presented. Variation among families is significantly different from zero for lesion length, and occlusion length. Familyxtreatment interaction is non-significant; families can be ranked overall.

Cov parm	Lesion length	Lesion width	Lesion depth	Occlusion length
Family (F)	0.0031	0.0611	0.0136	0.0026
Trt (T)xF	0.4272	0.3423	--	0.4888
Residual	<0.0001	<0.0001	<0.0001	<0.0001

Note: Z- value <0.05 shows a significant difference at alpha=0.05.

Table 2.21. Type 3 tests of fixed effects (F tests) suggest significant and non-significant effects for lesion length, lesion width, lesion depth and occlusion length.

Effect	DF	Lesion length	Lesion width	Lesion depth	Occlusion length
Block (B)	2	<0.0001	<0.1082	<0.0035	<0.0001
Trt (T)	1	<0.0001	<0.0001	<0.0001	<0.0001
BxT	15	<0.0304	<0.2847	<0.1589	<0.0059

Note: P- value <0.05 shows a significant difference at alpha=0.05.

Table 2.22. Family ranking for lesion length (estimate) overall and across all the four treatments.

Family	Estimate	Rank	Estimate	Rank GH	Estimate	Rank LT
L-42	-1.0018	1	0.0035	15	-0.035	1
L-41	-0.5208	2	0.0124	24	-0.029	2
L-46	-0.4108	3	-0.003	11	-0.01	7
L-47	-0.3834	4	0.0006	14	-0.013	5
L-50	-0.3334	5	-0.01	8	-9E-04	11
L-30	-0.2916	6	-0.011	6	0.0022	13
L-45	-0.2191	7	-0.006	10	-0.001	10
L-48	-0.2121	8	0.0055	19	-0.012	6
L-36	-0.1507	9	-0.011	7	0.0058	18
L-26	-0.1203	10	-0.013	3	0.009	21
L-33	-0.0835	11	-0.016	2	0.0129	22
L-49	0.0094	12	0.0058	20	-0.006	8
L-35	0.0394	13	0.0178	26	-0.017	4
L-44	0.0526	14	-0.012	5	0.0132	23
L-43	0.0947	15	-0.021	1	0.0238	27
L-25	0.1331	16	-0.012	4	0.016	25
L-40	0.1485	17	0.0222	27	-0.017	3
L-31	0.1496	18	0.0038	16	0.001	12
L-32	0.262	19	0.005	18	0.0033	16
L-24	0.2717	20	0.0044	17	0.0042	17
L-39	0.2851	21	0.0002	13	0.0089	20
L-29	0.3079	22	-0.006	9	0.016	26
L-27	0.3091	23	0.007	21	0.0028	14
L-34	0.3703	24	0.0148	25	-0.003	9
L-38	0.3787	25	-0.003	12	0.015	24
L-28	0.4361	26	0.0073	22	0.0066	19
L-37	0.4793	27	0.012	23	0.0032	15

Table 2.23. Average lesion length, occlusion length, seedling survival and fungal re-isolation frequency at the culmination of the year two study for loblolly pine families.

Family	Lesion length (mm)	Occlusion length (mm)	Survival (%)	Re-isolation (%)
L-42	38(23.4)	47(26.5)	95	95
L-41	45(27.7)	51(29.0)	91	96
L-48	49(25.3)	57(26.9)	89	85
L-46	49(31.6)	57(32.6)	90	94
L-50	49(26.8)	55(30.0)	89	92
L-47	49(30.2)	56(32.3)	93	100
L-26	50(27.5)	59(29.7)	89	99
L-33	53(37.2)	58(37.4)	88	90
L-45	53(39.8)	61(39.1)	91	97
L-25	53(39.3)	60(39.9)	79	95
L-30	53(32.3)	60(32.6)	91	90
L-36	54(34.8)	61(36.3)	91	92
L-49	55(28.3)	61(28.7)	90	94
L-28	56(30.0)	64(33.6)	79	90
L-43	56(39.9)	62(40.5)	86	99
L-44	57(35.2)	62(35.6)	87	96
L-35	58(39.4)	65(39.8)	91	96
L-27	58(34.2)	66(35.3)	82	86
L-40	59(36.6)	66(36.8)	81	97
L-31	59 (31.7)	67(33.3)	92	93
L-39	60(35.9)	67(37.2)	91	92
L-24	61(42.00)	69(40.6)	80	95
L-32	64(42.2)	71(43.3)	91	94
L-37	64(41.5)	71(43.3)	82	92
L-38	65(45.4)	71(47.0)	86	97
L-34	65(45.1)	72(45.3)	87	92
L-29	66(42.1)	76(46.5)	88	92
S-6	52(24.5)	58(25.5)	75	87
S-7	56(33.4)	64 (33.0)	78	88

Note: Means and standard deviations (parentheses).

2.5. Discussion

In the year one seedling family screening experiment, inoculation with the four different fungal species was able to identify loblolly and slash pine families that would be considered either more or less resistant to the interactions of the root-feeding bark beetles and the introduction of their associated fungi into these widely planted genotypes (Figures 2.8 & 2.9). All four fungal species successfully infected pine seedlings as lesions formed on all families of loblolly and slash pine. However, lesions did not extend the length of inoculation wound in several cases. While the virulence of ophiostomatoid fungi has been tested in previous studies (Nevill et al. 1995; Eckhardt et al. 2004a; Matusick and Eckhardt 2010a), these inoculation trials were the first to examine the within species variability of pine families for resistance against *Leptographium* and *Grosmannia* species. Differences in the relative virulence of fungal pathogens was seen in this study with *L. terebrantis* showing high variability in lesion and occlusion length among the seedling families tested as compared to the other three fungal species. Among the 23 loblolly pine families examined, the fungal species tested showed significant variability in symptomatology expression. But within each family, all four fungal species had similar effects without significant lesion differences as indicated by non-significant interaction of the fungi and families. As far as seedling host response to wounding, two controls were used; a wound control and a wound+media control. Neither of these treatments was detrimental to the seedlings and did not add to the lesion length. Wounding the stem of pine seedlings does not produce lesions; instead wounding forms a callus tissue that encloses the wound (Eckhardt et al. 2004a; Klepzig et al. 1995; Matusick and Eckhardt 2010a). Therefore, creating a wound in the seedling stem does not add to mortality. Rather than forming resin soaked lesions, the wounded area in the control treatments was found to have a healed

appearance with a visible wound mark on the seedlings stem. Control wounds were found to have significantly smaller lesions and did not produce occluded tissue when compared to the fungal inoculations.

In the second year inoculations, families were challenged only with *G. huntii* and *L. terebrantis*. This was decided on the basis of results from the first year as *G. alacris* and *L. procerum* showed low variation as compared to *G. huntii* and *L. terebrantis*. Both the fungal species, *G. huntii* and *L. terebrantis,* successfully infected the families included in the trials and showed a stronger response (larger lesions) as compared to the first year trials. However, the results were not statistically compared between the two years as all the seedlings in the second year were containerized as opposed to bare root seedlings in the first year. Two fungal species varied significantly in their virulence from each other among the loblolly pine families tested. However, likewise first year, no significant family x fungal interaction was seen, indicating that within a particular family, fungal treatments did not have a significant effect. Wound and wound+media treatments were included as controls and these did not produce lesions.

Dark brown, raised, and sunken lesions were observed on seedling stems following inoculations with fungi. In cross sections, deformation of seedling stems was observed showing only small portions of living tissue remaining. Girdling of stems leading to mortality was found in an earlier study following inoculations with *L. terebrantis* (Wingfield 1983). Lesions produced due to fungal inoculations extended beyond the wounded area on both sides on the stem of the seedlings. In certain cases the lesions failed to extend beyond the wounded area, especially in the seedlings inoculated with *L. procerum.* Lesions extended vertically in most of

the cases with some evidence of radial movement. Occlusion of vascular tissues is considered one of many host responses to fungal infection and has been used as a measure of seedling response to inoculation with blue stain fungi (Nevill et al. 1995). Generally, occlusion lengths are similar to lesion length and further support the virulence testing of ophiostomatoid fungi (Eckhardt et al. 2004a). Occlusion of the xylem was observed (as unstained tissue) after removing the bark tissue where the occlusion was observed extending over the entire lesion length. In many cases occlusion length did not extend across the lesion length. Similar lesion and occlusion responses were noticed in both the year one and year two studies.

Symptom development after inoculation of seedlings was localized in the form of lesions and occlusions. A small fraction of the seedlings died in the Year One experiment (7%). However, it is possible that mortality would have been more prevalent if the experiment continued for a longer time frame. Mortality associated with fungi was found in earlier experiments when the seedlings were kept for a longer period of time after imposing the inoculations (Harington and Cobb 1983; Wingfield 1983). Families L-14 and L-22 had lower survival percentages as compare to other loblolly pine families tested in the first year. Nevertheless the mortality was not significant in the Year One screening; *L. terebrantis* affected the seedling survival significantly in the year two study which is, in turn, consistent with the findings of Harington and Cobb (1983) and Wingfield (1983).

The consistent re-isolation of *Leptographium* species from the inoculated seedlings indicates their ability to infect pine seedlings. Similar results have been reported in a previous study but with lower re-isolations of fungal species from longleaf pine seedlings (Matusick et al.

2010). RCD and height were not affected by the treatments though RCD and height were found to be significantly different among the loblolly pine families. This difference was attributed to the differences in RCD and height of the seedling stock used. Further, it indicates that despite the development of localized symptoms in the form of lesions, occlusions and tissue deformation, fungal infection did not produce whole tree symptomatology. The lack of symptomology is consistent with previous trials conducted on southern pine seedlings (Matusick et al. 2010) and this was observed consistently in two studies conducted separately for two years. Significantly smaller final root to shoot ratio among the seedlings inoculated with fungal treatments compared to those serving as the wound control treatment indicate a decreased carbon allocation to the roots due to fungal infection. However, the root to shoot ratio could not be correlated to lesion length.

Of all the seedling genotypes tested during the Year One trials, families L-5, L-13, L-20 and L-8 had consistently smaller lesions overall and against the individual fungal species. Their host response is a good indication of being comparatively resistant to the *Leptographium* species tested in these trials. Families L-1, L-2, L-3 and L-4 had consistently larger lesions in all cases which would indicate more susceptibility to these particular fungal species. Deployment of these families should take into account the relative risk of the site if there is some concern about pine decline. In the case of *L. procerum*, a known weak pathogen, failed to produce significant lesion lengths. However, the pine family ranking was found to behave like other fungal species included in the study. Similar to other inoculations trials that used slash pine, (Matusick et al. 2010), the 5 slash pine families tested were found to be generally more resistant to the fungi used than the loblolly pine families. Average lesion and occlusion length were less in the slash pine

families. A small set of five slash pine families included in the study showed less variability without differences in lesion length and occlusion length. All the genotypes included in the second year screening trials developed lesions in response to fungal inoculations. Significant differences were reported among the families for lesion length, occlusion length, lesion width and lesion depth and some resistance to *Leptographium* fungal growth was observed in families L-42 and L-41. However, these families did not show complete resistance to the fungi as indicated from their average lesion and occlusion length (Table 2.23). All other families developed significantly larger lesions in response to *L. terebrantis* and *G. huntii*. The containerized seedlings used in the year two study performed better than the bare root seedlings used in the yeas one trial in terms of planting survival and growth which is consistent with previous studies in which out planting performance was studied between containerized and bare root seedlings (Barnett and McGilvray 1993). However, development of larger lesions in containerized seedlings during the Year Two warrants further study to compare the containerized and bare root seedlings response to *Leptographium* fungi. Larger lesion development during the Year Two trial could also be attributed to different genotypes used in the two studies and to genotype-environment interactions.

Generalized linear mixed model was fit to the data to test the breeding value of each family, conceptually similar to the mean family performance keeping the family effect random and treatments as fixed effect (Robinson 1991; Piepho et al. 2003). The general combining ability (GCA) estimates i.e. the best linear unbiased prediction (BLUP) estimates were used to rank the families on the basis of lesion length. When the families were kept random, interaction of family and fungal treatment was found to be significant in the Year One which is inconsistent

with the results when the families and treatments were kept fixed. However in the Year Two, results were consistent using both fixed and mixed effects models. While ranking the families on the basis of lesion length overall, it was noticed that in the Year One trial families L-20, L-13, L-8, L-18, L-11 and L-5 ranked lower while families L-1, L-2, L-3 and L-4 ranked highest among the families tested. These results are consistent with the family grouping from the fixed model analysis further supporting the indication of some seedling families being comparatively resistant to *Leptographium* as compared to others. However, the family ranking was not consistent for each fungal treatment which is due to the significant effects of the fungal treatments. Among the families tested in the Year Two trial, L-42 and L-41 ranked lowest overall and for *L. terebrantis*, consistent to the results when the families were kept as fixed effects.

Further studies should be done with some of the genotypes identified in the two year screening trials with a focus on identifying and characterizing the source of resistance to *Leptographium.* Also, studying the correlations between seedling family performance and mature trees concerning *Leptographium* species and pine decline should be considered.

Chapter Three

Identification of a seedling indicator of defense capacity by assessing nitrogen nutrition and family effects on *Grosmannia huntii* infection of loblolly pine

3.1. Abstract

The southeastern United States is experiencing isolated cases of tree mortality associated with a pine decline disease complex. Biotic and abiotic factors such as the activity of root-feeding bark beetles, root disease caused by *Leptographium* and *Grosmannia* fungal species, topography, and silvicultural practices have been studied as causal factors, but the role of physiological processes that sustain tree growth under pine decline is still being evaluated. Genotypes of loblolly pine vary in their carbon allocation to various plant parts. Pine families that have carbon allocation patterns favoring root system growth and defense chemicals may perform better against infection by pathogens such as *Leptographium* and *Grosmannia*. If so, an indicator variable that reflects these genetically controlled carbon allocation patterns could be identified to aid land managers in identifying the best loblolly pine genotype to plant where the risk of pine decline is high. This study was conducted to assess the effect of nitrogen nutrition on *Grosmannia huntii* infection, patterns of carbon allocation to above and below ground plant parts, and stem total phenolic concentration of 15 loblolly pine families currently utilized by the timber industry. Relationships between pattern of carbon allocation, concentration of total phenolics in stem tissue, and infection by *G. huntii* were evaluated and the resilience of these relationships under added nitrogen was assessed. Results indicated that *G. huntii* successfully

produced lesions on inoculated seedlings but that nitrogen concentration had no effect on lesion length, lesion width or stem total phenol concentration and lesion dimensions did not vary notably by family. Pathogen virulence under the study conditions may have interfered with lesion response to the treatments. However, family differences in stem total phenolic concentration indicate that there may be an opportunity to select genotypes with superior preformed defense capacity. Also, while carbon allocation pattern did not prove to be an effective predictor of disease resistance, total seedling size and foliage mass relative to the mass of other tissues were positively related to stem total phenolic concentration for several families. With continued research, therefore, these morphological variables show promise as indicators of preformed defense potential.

3.2. Introduction

A large area of the southeastern United States is managed for timberland with loblolly pine (*Pinus taeda* L.) and shortleaf pine (*P. echinata* Mill.) as the dominate tree species on 55 million acres; approximately one-fourth of all southern forests (Smith et al. 2007). While shortleaf pine is more widely distributed than loblolly pine, loblolly pine is the backbone of the southern pine industry, covering 80% of commercial forest area in the South (Smith et al. 2007) and having a geographical range extending across 15 states in the south and mid-Atlantic region (Baker and Langdon 1990). Mortality following poor growth rate, the appearance of an unhealthy crown, and an overall decline in tree vigor has been seen in loblolly pine over a 50 year period particularly in saw timber sized trees (Brown and McDowell 1986; Hess et al. 2002). Field observations have shown that ophiostomatoid fungi, with root feeding bark beetles as their vectors, are an important component of pine decline (Otrosina et al. 1999; Eckhardt et al. 2007).

Declining loblolly pine exhibits symptoms such as sparse and chlorotic foliage, fine and lateral root deterioration, large cone crops, and poor radial growth (Brown and McDowell 1968; Eckhardt et al. 2007). Premature mortality of loblolly pine has been well documented as a decline disease syndrome that involves interactions of biotic and abiotic factors (Eckhardt et al. 2004a). Root feeding bark beetles are attracted to forest stands due to stress induced by abiotic factors such as topography, increased slope and southwest facing aspects (Eckhardt et al. 2007; Eckhardt and Menard 2009). The beetle attack introduces the fungus to the trees and helps in transporting the fungus from infected trees to healthy trees (Paine et al. 1994). The fungus, in turn, aids to enhance the beetle brood by providing food for the developing larvae and by creating favorable conditions such as decreased host resistance to the beetles due to changes in host chemistry or formation of nutritive metabolic products for the beetles (Eckhardt et al. 2004b). This insect-fungus interaction increases the expansion of the decline disease complex and is understood as a mutualism in which the fungi and the beetles benefit each other (Paine et al. 1997). However, in certain conditions the fungus alone can contribute to decline by blocking the vascular tissues and hence preventing translocation of water and nutrients (Eckhardt et al. 2004a).

Resource deficiencies within a tree may accelerate pine decline by interfering with natural stress avoidance mechanisms (Eckhardt et al. 2010). Trees respond to, and sustain the resource stress by a variety of changes in physiology such as dynamic changes in crown leaf area which help trees maintain foliar physiology under low soil fertility and water availability (Pallardy et al. 1995). A subtle decline in leaf area that concentrates limiting mineral nutrients in the existing foliage may be favorable, for example, to achieve critical levels of the essential

elements required by photosynthesis. At the same time, the loss of some leaf area may also aid photosynthesis by increasing water availability within the crown. If the carbon allocation pattern favors the root system, resource stress is also reduced as demonstrated for loblolly pine in different soil types and moisture regimes in North Carolina (Hacke et al. 2000) and longleaf pine in Georgia (Addington et al. 2006). The ratio of root surface area to leaf area was greater on the sites with low soil water availability. This pattern of carbon allocation led to more water acquisition from the soil which helped to maintain stomatal conductance and evade water stress on the drier sites.

Trees are able to tolerate resource stress as long as whole-tree carbon demand is met by the leaf area, thus, providing sufficient energy to continue cellular processes. Severe stress, that causes a drop in leaf area below critical levels, however, may jeopardize tree performance (Eckhardt et al. 2010). In addition to leaf area adjustment and a carbon allocation pattern that favors the root system, a deep root system may help to meet the demand for water by accessing deep water that is either immediately translocated to the crown or is hydraulically redistributed to the surface soil horizons (Warren et al. 2007). In addition, any action that helps sustain the vascular transport of mineral nutrients and other solutes aids tree response to resource stress. Because many essential mineral nutrients (e.g., nitrogen, potassium, phosphorus) are dynamic and can be remobilized within the plant (Marschner 2012), new tissues rely on this source of some essential elements. Among the pines, nutrient remobilization from naturally senescing foliage is an important means of nutrient supply to new tissues in spring regardless soil fertility (Dalla-Tea and Jokela 1994). Nutrients can also be mobilized from older foliage before natural senescence to meet the nutritional demands of younger foliage (Nambiar and Fife 1991). In

doing so, the nutrient-demanding physiological processes of older and younger foliage persist during periods of nutritional resource stress as long as foliar nutrient concentrations are maintained at or above critical levels.

Nitrogen availability affects the seedling and tree growth rate, biomass partitioning to plant parts, defense chemicals synthesis, and susceptibility to the pathogens (Holopainen et al. 1995; Samuelson 2000; Rühmann et al. 2002; King et al. 2008). In a study done on Scots pine seedlings, for example, elevated nitrogen concentration resulted in increased biomass of above ground plant parts and decreased root biomass allocation (Holopainen et al. 1995). Specifically, a 36 to 60% increase in foliar nitrogen concentration above 1.4% caused decreases in starch, total monoterpene, and total phenolic concentrations of shoot and root tissues. At the same time, this increase in nitrogen concentration caused increases in tissue concentrations of eight amino acids. Increases in oviposition and growth of *Lygus rugulipennis* Popp. accompanied these responses suggesting that elevated nitrogen nutrition decreases resistance to herbivory (Holopainen et al. 1995). Others have also reported that conifer seedlings and trees have a lower concentration of defense chemicals under an elevated nitrogen environment indicating that nitrogen fertilization may play a role in plant pathogen interactions (Muzika 1993; Gebauer et al. 1998; Booker and Maier 2001). For example, Gebauer et al. (1993) reported lower concentrations of condensed tannins and total phenolics in the needles, stem, and lateral roots of loblolly pine seedlings as nitrogen availability increased.

The effects of genotype on loblolly pine carbon allocation patterns have been observed in previous studies (Bongarten and Teskey 1987; Li et al. 1991; Stovall and Seiler 2012). For

example, Li et al. (1991) reported that biomass allocation among the stem, needles, and roots of loblolly pine differed among 23 families. Stovall and Seiler (2012) found that divergence in carbon allocation between two loblolly pine clones occurred in the taproot and foliage. A carbon allocation pattern that supports the energy needs of a vigorous root system helps to maintain tree vigor by evading water and mineral nutrient stress when the availability of water and nutrients is low. Also, genotypes that allocate more carbon to defense chemicals in response to insect infestation may counteract insect vectored pathogens. Genotypes exhibiting small, rather than large nitrogen-induced decreases in the synthesis of defense chemicals could further benefit insect and disease resistance. It is hypothesized that under normal levels of nitrogen, genetically controlled carbon allocation patterns are indicative of pathogenic infection and defense chemical production, and under elevated levels of nitrogen nutrition, these relationships are negated for most families. The objectives of this study are first to identify the family morphological traits of loblolly pine that correlate to *G. huntii* infection after artificial inoculation, and the concentration of defense chemicals near the inoculation site. The second objective of the study is to evaluate the effect of nitrogen nutrition on relationships between loblolly pine family morphology, *G. huntii* infection, and defense chemical concentration. Results will be used to identify family morphological traits indicative of resistance to *G. huntii* and defense chemical production, and identify families exhibiting only a minimum effect of nitrogen nutrition on these relationships.

3.3. Material and Methods

Twenty seedlings per family from 15 loblolly pine families (300 seedlings) were planted in trade gallon pots filled with ProMix BX® (Premier Tech, Quebec, and Canada) peat based potting mix and placed into a greenhouse. One-half of the seedlings received 100 ml of either

normal (NN) or high nitrogen (HN) nutrient solution twice a week. A target foliar nitrogen (N) concentration of 1.0% was established for the NN treatment level because this is only slightly below the minimum foliar N concentration of 1.2% recommended for the normal physiological function of loblolly pine (Albaugh et al. 2010), and would likely accompany a carbon allocation shift toward the production of secondary metabolites such as defense compounds (Gebauer et al. 1998). The target concentration of foliar N for the HN treatment level was set at 1.5-2.5% because this represents the optimum foliar N concentration for planted loblolly pine seedlings (Dumroese 2003), and also would likely result in reduced rates of defense chemical production (Gebauer et al. 1998).

Information from three sources was synthesized to determine appropriate NH_4NO_3 application rates to achieve these target concentrations of foliar N. Specifically, Gebauer et al. (1998) quantified defense chemical concentrations of loblolly pine seedlings potted in sand and receiving daily applications of ammonium nitrate (NH_4NO_3) for 17 weeks. Distinct decreases in total phenolics and condensed tannins were observed by the daily application of 500 ml of 3.5 mmol/l (i.e., 280 mg/l) NH_4NO_3. Using sand culture, Dewald et al. (1992) achieved a slash pine foliar N concentration of 2 to 2.5% by the application of between 114 mg/l (40 mg/l N) and 857 mg/l (300 mg/l N) NH_4NO_3 two to three times per week. For loblolly pine seedlings potted in a mixture of sand-perlite-vermiculite (5:4:1), Samuelson (2000) obtained a foliar N concentration of 1.8% by the weekly application of 754 mg/l NH_4NO_3 (264 mg/l N) over a 21-week period. Thus, NN and HN levels were defined as the application of 100 ml of 280 mg/l (98 mg/l N) and 784 mg/l of NH_4NO_3 (274 mg/l N), respectively, to each potted seedling two times per week.

The NN and HN nutrient solutions provided equal amount of all other plant-essential mineral nutrients besides N (Table 3.1). Potted seedlings were watered to the point of saturation at least once per week as needed. Foliar nutrition was evaluated 14 weeks after the start of the study by pooling one fully elongated fascicle that grew during the study period per seedling among four families. Foliage was oven-dried (70°C) to equilibrium, ground to pass a 0.85 mm^2 screen, and evaluated for macro- and micronutrient concentrations (Waters Agricultural Laboratories, Inc., Camilla, GA). Results from this analysis indicated that a difference in foliar N concentration between the NN (n=4, mean ± standard deviation (SD): 1.2 ± 0.1%) and HN (n=4, mean ± standard deviation (SD): 1.3 ± 0.1%) levels had not been achieved. Therefore, the HN level was amended by increasing its NH_4NO_3 concentration from 784mg/l to 994 mg/l. At this time, foliar concentrations of phosphorus, potassium, calcium, and magnesium (i.e., (n=8, mean ± standard deviation) P: 0.21±0.02; K: 1.7±0.18; Ca: 0.90±0.84; Mg: 0.20±0.01%), were 75, 325, 500, 150% higher, respectively, than those recommended for loblolly pine (Albaugh et al. 2010). As a result, calcium and magnesium sources were removed from the nutrient solutions and phosphorus and potassium additions were reduced. Amounts of chemicals in the amended nutrient solutions are shown in Table 3.2.

Table 3.1. Concentration of chemicals in NN and HN nutrient solutions and amount of plant-essential elements applied per seedling during the first 14 weeks of the study.

Chemical name	Chemical formula	Concentration (mg/l)		Total amount of compound added in 35 applications (mg)		Element
		NN	HN	NN	HN	
Ammonium nitrate	NH_4NO_3	280	784	343.0	960.4	N
Potassium phosphate	KH_2PO_4	132	132	105.3	105.3	P
				132.6	132.6	K
Potassium chloride	KCl	71	71	130.2	130.2	K
Calcium sulphate	$CaSO_4.2H2O$	155	155	126.4	126.4	Ca
				100.9	100.9	S
Magnesium sulphate	$MgSO_4.7H2O$	182	182	82.8	82.8	Mg
				63.1	63.1	S
Iron chelate	Ferric EDTA[1]	26	26	13.8	13.8	Fe
				6.92	6.92	N
Boric acid	H_3BO_3	3.0	3.0	1.84	1.84	B
Zinc sulphate	$ZnSO_4.7H_2O$	1.4	1.4	1.11	1.11	Zn
				0.54	0.54	S
Copper chloride	$CuCl_2.2H_2O$	0.40	0.40	0.52	0.52	Cu
Sodium molybdate	$MoNa_2O_4.2H_2O$	0.05	0.05	0.07	0.07	Mo
Manganese sulphate	$MnSO_4.H_2O$	1.5	1.5	1.71	1.71	Mn
				1.00	1.00	S

[1] ethylenediaminetetraacetic acid

Before potting, 10 seedlings were randomly sub-sampled per family (i.e. 150 seedlings) to measure family morphological variables before the start of the study. For each seedling, root collar diameter (RCD) and total height (TH) were measured and seedlings were separated into fine roots (i.e. ≤ 2 mm diameter), coarse roots (i.e., > 2 mm diameter), stem, and foliage. Tissues were oven dried at 70°C to equilibrium. Root to shoot ratio (R:S) was calculated from the dry weight measurements. Seedlings were stem-inoculated with *G. huntii*, 12 weeks after potting. Two weeks before inoculations, the fungus was cultured on 2% malt extract agar (MEA) from single spore isolates of the fungus maintained in the laboratory. The wound inoculation method was followed which required making a vertical cut with a sterile razor blade in the lower stem of

the seedling about 2 cm from the surface of potting mix (Nevill et al. 1995; Eckhardt et al. 2004a). After placing a 3 mm diameter plug of medium colonized by mycelium in the cut, the wound was wrapped with sterile moist cotton, and sealed with Parafilm®.

Seedlings were placed on greenhouse benches in a randomized complete block (RCB), split plot design with 10 blocks (Steel and Torrie 1980). In the design, the two levels of nitrogen (NN and HN) were randomly assigned to two whole plots in each block. Subplots were one potted loblolly pine seedling from 15 families randomly placed in each whole plot.

Twenty-seven weeks after potting, one fascicle was pooled by family and nutrition treatment (i.e. 30 samples). Foliage was oven-dried (70°C) to equilibrium, ground to pass a 0.85 mm^2 screen, and evaluated for macro-and micronutrient concentrations (Waters Agricultural Laboratories, Inc., Camilla, GA). Twenty-eight weeks after potting, seedlings were harvested by gently shaking potting mix from root systems, washing root systems with tap water, and storing seedlings in plastic bags in the refrigerator. The presence or absence of a lesion, and lesion length, width, and depth, were measured by seedling. Lesion length was the length of dark brown tissue that occurred along, or extended from the vertical cut that was made during inoculation. After all lesion measurements were completed, shoots were separated from root systems, foliage was separated from the stem, and fine roots were separated from coarse roots. The foliage, fine roots, and coarse roots were placed in separate bags and oven-dried at 70°C to equilibrium.

From each stem, a 2 cm section approximately 0.5 cm above the point of inoculation was cut, freeze-dried, weighed, and ground to pass a 0.50 mm^2 screen. Ground stem tissues were analyzed for total phenolic concentration at the U.S. Forest Service Southern Research Station laboratory in Pineville, LA by a modification of the Folin-Ciocalteu method originally described by Singleton and Rossi (1965) and modified by Booker and Maier (2001) for loblolly pine. To confirm infection, re-isolation of associated fungi was conducted by cutting a 1 cm length of stem that was 0.5 cm above and below the lesion, and culturing it on CSMA (malt extract agar with 800 mg/l of cycloheximide and 200 mg/l of streptomycin sulfate). Cultures were incubated at room temperature for two weeks, and re-isolation success was determined visually. Oven-dry weights (70°C to equilibrium) of stem sections used for re-isolations were measured. Dry weights of the foliage, stem, fine roots, coarse roots, and total seedling were quantified, fractions of total seedling dry weight as foliage, stem, fine roots, and coarse roots, and R:S values were calculated.

Ten seedling response variables (lesion length and width, final TH, final RCD, total seedling dry weight, fraction of total seedling dry weight as foliage, stem, fine roots, and coarse roots, and stem total phenolic concentration) were analyzed in SAS statistical software (SAS Institute, 9.2 ed., Cary, NC) using the generalized linear model (GLM) procedure. In the RCB split plot design, nutrition treatments levels (NN and HN) were assigned to the whole plots and fifteen loblolly pine families were assigned to the subplots within each whole plot, and there were 10 blocks. Means by nutrition treatment and among families were evaluated by the Tukey pairwise comparison test. Multiple regression analyses were conducted with SAS statistical software (SAS Institute, 9.2 ed., Cary, NC) using the generalized linear model (GLM)

procedure. Relationship between lesion length and width and three independent variables (foliar nitrogen concentration, total stem phenolics, R:S) were assessed. Regressions were also conducted to determine the relationship between the variables representing virulence/defense (i.e. lesion length, lesion width, stem total phenolic concentration) and independent variables representing seedling growth (i.e. total seedling dry weight) and carbon allocation among plant components (i.e. R:S, and fraction of dry weight as foliage, stem, fine roots and coarse roots).

Table 3.2. Amended nutrient solutions applied as normal (NN) and high (HN) nitrogen treatments.

Chemical name	**Chemical formula**	**Concentration (mg/l)**		**Total amount of compound added in 19 applications (g)**		**Element**
		NN	HN	NN	HN	
Ammonium nitrate	NH_4NO_3	280	994	186.2	661.01	N
Potassium phosphate	KH_2PO_4	79	79	34.2	34.2	P
				43.1	43.1	K
Iron chelate	Ferric EDTA[1]	26	26	7.51	7.51	Fe
				3.75	3.75	N
Boric acid	H_3BO_3	3.0	3.0	1.00	1.00	B
Zinc sulphate	$ZnSO_4.7H_2O$	1.4	1.4	0.60	0.60	Zn
				0.30	0.30	S
Copper chloride	$CuCl_2.2H_2O$	0.40	0.40	0.28	0.28	Cu
Sodium molybdate	$MoNa_2O_4.2H_2O$	0.05	0.05	0.04	0.04	Mo
Manganese sulphate	$MnSO_4.H_2O$	1.5	1.5	0.93	0.93	Mn
				0.54	0.54	S

[1] ethylenediaminetetraacetic acid

3.4. Results

Pre-pot analyses indicated that the mean RCD of all families except L-4 met the minimum RCD (5 mm), and had TH (15-25 cm) values greater than the minimum recommended for planted loblolly pine (Johnson and Cline 1991). Mean R:S values of 13 families were less than 0.4 suggesting that root system size was sub-optimal at the time of potting for all but two

families (Johnson and Cline 1991). Means and standard deviations of TH, RCD, foliage, stem, fine root, and coarse root dry weights, and R:S for all the families are presented (Table 3.3). One week before the end of the study, the NN and HN seedlings had achieved target N levels (NN~1.2%; HN ~1.6%) and foliar concentrations of other plant-essential macro- and micronutrients were sufficient for loblolly pine according to Albaugh et al. (2010) (Table 3.4).

Table 3.3. Means followed by standard deviations (SD) of seedling total height, root collar diameter (RCD), foliage dry weight, stem dry weight, fine root dry weight, coarse root dry weight, and root to shoot ratio (R:S) from pre-pot measurements.

Family	RCD		Total height		Foliage dry weight		Stem dry weight		Fine root dry weight		Coarse root dry weight		R:S	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
L-1	6.19	0.96	38	3.0	4.3	1.3	2.8	0.9	0.5	0.3	0.8	0.3	0.2	0.1
L-2	6.59	0.83	33	4.3	4.3	1.8	2.6	1.6	0.6	0.3	1.1	0.5	0.2	0.1
L-5	7.69	1.34	38	4.0	6.9	2.3	3.8	1.4	0.7	0.3	2.0	1.2	0.2	0.1
L-6	4.22	0.64	31	1.3	2.2	1.2	1.3	0.5	0.3	0.2	0.3	0.2	0.2	0.0
L-7	5.30	0.77	33	2.3	2.8	0.9	1.4	0.4	0.6	0.3	0.6	0.6	0.3	0.1
L-8	5.91	1.51	36	4.9	4.1	1.6	2.0	1.1	0.5	0.3	0.8	0.6	0.2	0.1
L-9	7.61	1.00	35	2.5	7.2	1.5	3.2	0.7	1.3	0.5	2.0	0.5	0.3	0.1
L-10	5.62	1.79	33	4.1	4.1	2.3	1.8	1.1	1.0	0.9	1.5	1.7	0.4	0.1
L-11	7.97	1.33	31	3.5	5.8	1.8	2.7	1.0	1.5	0.6	2.5	1.1	0.5	0.2
L-12	7.43	0.49	36	4.7	5.1	0.9	2.9	0.5	0.8	0.4	1.2	0.5	0.3	0.1
L-13	7.63	1.16	34	3.4	5.9	1.2	3.2	0.9	1.0	0.5	1.4	0.7	0.3	0.1
L-16	9.02	1.32	35	3.8	7.9	2.3	4.9	2.0	1.1	0.8	3.3	1.3	0.4	0.1
L-18	6.80	1.49	38	5.0	5.2	2.7	3.3	1.5	0.8	0.4	1.0	0.6	0.2	0.1
L-22	6.36	1.75	33	4.8	4.5	2.4	2.3	1.5	1.3	2.1	1.2	1.0	0.3	0.2
L-23	6.54	0.93	35	5.5	4.9	1.3	2.6	0.8	1.4	1.5	1.6	1.0	0.4	0.3

Table 3.4. Concentrations of plant-essential macro- and micronutrients in loblolly pine foliage one week before the end of the study in response to normal (NN) and high (HN) levels of nitrogen nutrition.

Family	Nitrogen nutrition treatment level	N[1]	P	K	Ca	Mg	S	B	Zn	Mn	Fe[2]	Cu
		(%)						(ppm)				
L-1	HN	1.47	0.17	1.62	0.44	0.17	0.11	42	52	62	19	2
L-2	HN	1.53	0.19	1.71	0.46	0.19	0.13	39	51	60	20	3
L-5	HN	1.76	0.26	2.25	0.47	0.17	0.14	43	50	51	20	3
L-6	HN	1.67	0.20	1.61	0.39	0.16	0.13	38	52	66	26	3
L-7	HN	1.61	0.22	1.61	0.38	0.16	0.12	38	53	56	24	3
L-8	HN	1.58	0.19	1.75	0.48	0.18	0.13	39	46	69	23	3
L-9	HN	1.77	0.22	1.76	0.48	0.19	0.15	43	60	80	49	5
L-10	HN	1.54	0.20	1.67	0.44	0.16	0.12	44	47	69	22	2
L-11	HN	1.95	0.23	1.82	0.47	0.18	0.15	45	52	75	24	4
L-12	HN	1.71	0.23	1.76	0.31	0.14	0.13	45	50	62	22	4
L-13	HN	1.69	0.18	1.46	0.38	0.16	0.13	34	45	55	24	2
L-16	HN	1.61	0.19	1.63	0.34	0.15	0.12	36	43	51	26	2
L-18	HN	1.79	0.24	1.98	0.54	0.19	0.14	52	54	90	23	3
L-22	HN	1.86	0.25	2.05	0.45	0.18	0.14	51	57	55	22	3
L-23	HN	1.55	0.20	1.61	0.44	0.17	0.11	37	42	71	23	2
L-1	LN	1.28	0.20	2.16	0.59	0.26	0.11	56	53	52	22	3
L-2	LN	1.03	0.15	1.87	0.68	0.26	0.11	49	51	67	28	1
L-5	LN	1.00	0.14	1.66	0.67	0.26	0.11	54	46	68	32	2
L-6	LN	1.05	0.19	2.04	0.63	0.26	0.11	45	51	64	31	2
L-7	LN	1.23	0.19	1.99	0.6	0.24	0.12	43	51	67	28	2
L-8	LN	1.16	0.19	1.96	0.49	0.22	0.11	33	41	58	24	1
L-9	LN	1.05	0.21	2.15	0.53	0.23	0.13	34	55	55	29	3
L-10	LN	1.23	0.22	1.99	0.40	0.19	0.12	39	48	78	27	2
L-11	LN	1.29	0.23	2.23	0.55	0.25	0.14	38	56	72	27	3
L-12	LN	1.55	0.24	2.44	0.43	0.23	0.15	46	50	60	26	4
L-13	LN	1.12	0.19	1.73	0.41	0.21	0.12	29	49	48	27	3
L-16	LN	1.05	0.18	2.03	0.45	0.22	0.12	36	47	55	26	3
L-18	LN	1.29	0.20	2.08	0.60	0.23	0.14	54	54	92	28	3
L-22	LN	1.17	0.19	1.86	0.49	0.22	0.11	38	44	47	28	2
L-23	LN	0.98	0.18	1.73	0.52	0.20	0.10	33	46	64	29	2

[1] Foliar concentrations indicating sufficiency for loblolly pine according to Albaugh et al. (2010) and references cited therein: N: 1.2%, P: 0.12%, K: 0.35-0.40%, Ca: 0.15%, Mg: 0.08%, S: 0.10-0.12%, B: 4-8 ppm, Zn: 10-20 ppm, Mn: 20-40 ppm, Cu: 2-3 ppm.

[2] The foliar concentration of iron indicating sufficiency is 72 ppm for plants in general according to Marscher (2012).

Inoculations with *G. huntii* did not cause significant seedling mortality in the inoculated seedlings. Fourteen weeks after the seedlings were inoculated, brown colored lesions were observed on the seedlings stems. Although lesions were present on the inoculated seedlings, there were no differences in lesion length ($F = 0.10$, $P = 0.7569$) or lesion width ($F = 0.15$, $P = 0.7040$) between NN and HN treatments levels (Table 3.5). In addition, while family had no effect on lesion length ($F=0.61$, $P = 0.8591$), differences in lesion width were observed among the 15 families tested ($F = 3.11$, $P = 0.0002$). The T x F interaction was significant for lesion length ($F = 1.90$, $P = 0.0272$). Further analysis compared treatment means among the families within each nitrogen treatment and between nitrogen treatments within each family. In doing so, families L-1 and L-6 differed in lesion length between the NN and HN treatments levels. The T x F interactions for lesion width and stem total phenolic concentrations were non-significant and hence the means were compared only among the families. Family L-1 had a significantly different mean lesion width from families L-2 and L-13. Similarly, mean total phenol concentration was found to be significant between several family pairs (Table 3.6). Minor differences in mean lesion length and lesion width for each family under NN and HN treatments levels were seen with no significant differences such as family L-2 had similar mean lesion length and lesion width under both the treatment levels (Table 3.7 and 3.8). Means and standard deviations of stem total phenolic concentration among the families are presented in Figure 3.1. The fungus was consistently re-isolated from the seedlings with 95% or greater re-isolation from all the families except L-1 and L-2 (85% and 65% respectively).

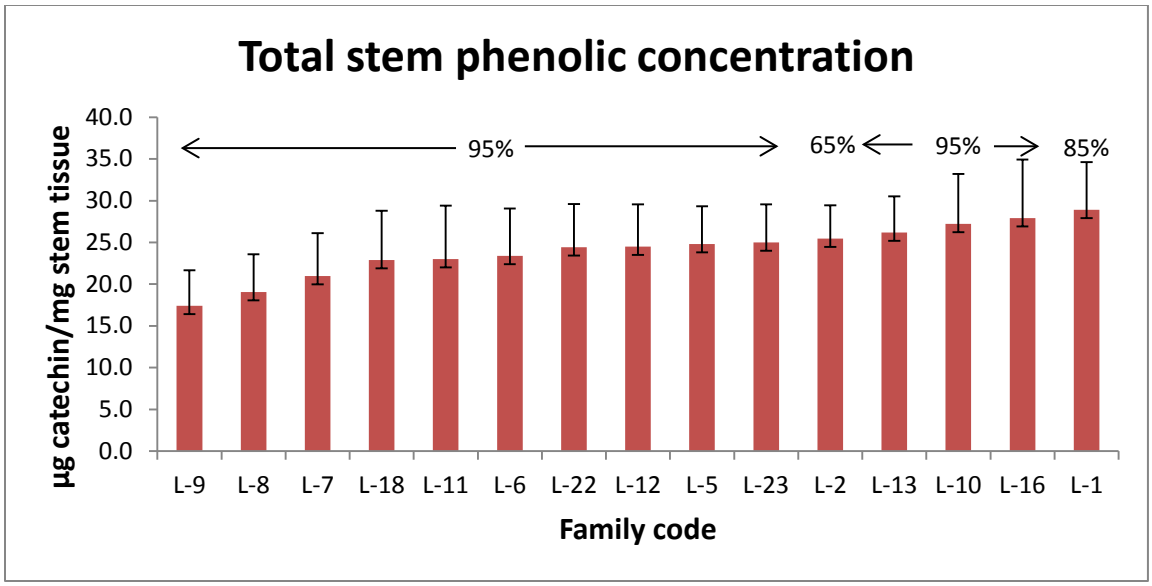

Figure 3.1. Means and standard deviations of stem total phenolic concentration among 15 loblolly pine families.

Table 3.5. Probability of a greater F-value for lesion length, lesion width and stem total phenolic concentration in response to normal (NN) and high (HN) nitrogen nutrition treatment levels for 15 loblolly pine families.

Source of variation	DF[1]	Lesion length	Lesion width	Stem total phenolic concentrations
Block (B)	9	0.0534	0.6736	0.0298
Treatment (T)	1	0.7569	0.7040	0.5065
BxT	9	0.2832	0.0534	0.7785
Family (F)	14	0.8708	0.0002	<0.0001
TxF	14	0.0272	0.1268	0.3641

[1]DF: degrees of freedom

Table 3.6. Differences between means for stem total phenolic concentration and lesion length among 15 loblolly pine families tested.

Stem total phenolic concentrations		**Lesion width**	
Family comparison	Mean differences	Family comparison	Mean differences
L-1- L-7	7.942	L-1 - L-13	2.0500
L-1 - L-8	9.850	L-1 - L-2	2.0500
L-1 - L-9	11.492		
L-1 - L-11	5.908		
L-1 - L-18	6.035		
L-2 - L-8	6.386		
L-2 - L-9	8.028		
L-5 - L-8	5.745		
L-5 - L-9	7.387		
L-6 - L-9	5.970		
L-7 - L-10	-6.233		
L-7 - L-16	-6.946		
L-8 - L-10	-8.141		
L-8 - L-13	-7.119		
L-8 - L-16	-8.855		
L-8 - L-23	-5.929		
L-9 - L-10	-9.783		
L-9 - L-12	-7.061		
L-9 - L-13	-8.761		
L-9 - L-16	-10.496		
L-9 - L-22	-6.996		
L-9 - L-23	-7.571		

Note: Only significant differences are presented.

Table 3.7. Means and standard deviations (SD) of lesion length for each loblolly pine family under normal (NN) and high (HN) nitrogen treatment levels.

Family	Trt	Lesion length		Family	Trt	Lesion length	
		Mean	SD			Mean	SD
L-1	HN	14	1.6	L-1	LN	16	3.3
L-2	HN	15	1.7	L-2	LN	15	2.1
L-5	HN	16	2.1	L-5	LN	16	2.2
L-6	HN	17	3.1	L-6	LN	14	1.7
L-7	HN	15	3.6	L-7	LN	15	2.4
L-8	HN	15	1.9	L-8	LN	15	2.2
L-9	HN	15	3.1	L-9	LN	15	2.1
L-10	HN	14	2.4	L-10	LN	16	2.1
L-11	HN	15	1.3	L-11	LN	15	1.9
L-12	HN	16	1.8	L-12	LN	16	2.2
L-13	HN	17	3.4	L-13	LN	15	2.7
L-16	HN	16	2.5	L-16	LN	17	2.9
L-18	HN	16	2.6	L-18	LN	15	1.9
L-22	HN	15	2.5	L-22	LN	15	3.5
L-23	HN	15	3.8	L-23	LN	17	3.7

Table 3.8. Means and standard deviations (SD) of lesion width for each loblolly pine family under normal (NN) and high (HN) nitrogen treatment levels.

Family	Trt	Lesion width		Family	Trt	Lesion width	
		Mean	SD			Mean	SD
L-1	HN	9	1.5	L-1	LN	9	1.2
L-2	HN	11	1.8	L-2	LN	11	1.9
L-5	HN	9	1.2	L-5	LN	10	1.2
L-6	HN	11	1.2	L-6	LN	11	2.0
L-7	HN	11	3.4	L-7	LN	10	1.9
L-8	HN	11	3.3	L-8	LN	10	1.2
L-9	HN	10	1.6	L-9	LN	11	1.9
L-10	HN	10	1.3	L-10	LN	10	1.5
L-11	HN	10	1.8	L-11	LN	11	1.9
L-12	HN	10	2.1	L-12	LN	9	1.9
L-13	HN	12	1.3	L-13	LN	11	1.6
L-16	HN	10	1.7	L-16	LN	11	1.3
L-18	HN	10	1.5	L-18	LN	10	2.0
L-22	HN	9	1.9	L-22	LN	10	1.9
L-23	HN	10	2.2	L-23	LN	11	0.9

Total height did not differ between NN and HN treatment levels ($F = 0.60$, $P = 0.4568$). However, TH was significantly different among the families ($F = 3.26$, $P < 0.0001$). Values of RCD differed significantly between the NN and HN treatment levels ($F = 10.71$, $P < 0.0096$) and it was also found to be significantly different among the families ($F = 4.36$, $P < 0.0001$). Nitrogen nutrition treatments affected total seedling dry weight significantly ($F = 8.39$, $P = 0.0177$), and total seedling dry weight differed significantly among the families as well ($F = 3.16$, $P = 0.0001$).

Fractions of total dry weight as foliage and fine roots were significantly affected by nitrogen nutrition treatment, while stem and coarse roots dry weight fractions did not differ between NN and HN treatment levels (Table 3.9). By the Tukey pairwise comparison test, however, only mean differences between the NN and HN treatment levels for fine root dry weight were significant. Seedling dry weight allocation to the foliage, stem, fine roots, and coarse roots differed significantly among the families (Table 3.9). Further analyses of TH, RCD, total seedling dry weight, and fraction of total seedling dry weight as foliage, stem, fine roots, and coarse roots was done by comparing the means among the families. Significant differences among family means for each of these variables were seen such as family L-13 had a significantly different mean total seedling dry weight from families L-1, L-5, L-6 and L-12 (Table 3.10 and 3.11). Mean values of seedling morphological variables at harvest are presented (Table 3.12).

Table 3.9. Probabilities of a greater F-value for total height, RCD, total seedling dry weight, and fraction of total dry weight as foliage, stem, fine roots, and coarse roots in response to normal (NN) and high (HN) nitrogen treatment levels for 15 loblolly pine families.

Source of variation	DF[1]	Total height	RCD	Total dry weight	Foliage	Stem	Fine root	Coarse root
					-------fraction of total dry weight-----			
Block (B)	9	0.0557	0.2619	0.0866	0.0772	0.1102	0.0175	0.4274
Trt (T)	1	0.4568	0.0096	0.0177	0.0014	0.5165	<0.0001	0.7388
BxT	9	0.7841	0.2009	0.7432	0.8823	0.7753	0.9151	0.3874
Family (F)	14	<0.0001	<0.0001	0.0001	0.0338	0.0168	<0.0001	0.0002
TxF	14	0.0575	0.5452	0.0508	0.4008	0.2594	0.0565	0.0819

[1]DF: degrees of freedom.

Table 3.10. Differences among the means of root collar diameter (RCD), total height, and total seedling dry weight for 15 loblolly pine families.

RCD		Total height		Total seedling Dry weight	
Family comparison	Mean differences	Family comparison	Mean differences	Family comparison	Mean differences
L-1 - L-2	-1.9631	L-1 - L-7	-16.682	L-1 - L-13	-10.878
L-1 - L-13	-2.3520	L-1 - L-16	-15.400	L-5 - L-13	-11.024
L-1 - L-16	-3.1580	L-6 - L-11	15.350	L-6 - L-13	-11.563
L-2 - L-5	2.1459	L-7 - L-10	18.707	L-12 - L-13	-12.002
L-5 - L-13	-2.5348	L-7 - L-11	20.132		
L-5 - L-16	-3.3408	L-7 - L-23	14.879		
L-6 - L-13	-1.9630	L-10 - L-16	-17.425		
L-6 - L-16	-2.7690	L-11 - L-16	-18.850		
L-7 - L-16	-2.2179				
L-8 - L-13	-1.9520				
L-8 - L-16	-2.7580				
L-9 - L-16	-2.2215				
L-10 - L-16	-1.7014				
L-11 - L-16	-1.8245				
L-12 - L-13	-1.6035				
L-12 - L-16	-2.4095				
L-13 - L-22	1.9826				
L-16 - L-18	2.3165				
L-16 - L-22	2.7887				
L-16 - L-23	2.1046				

Note: Only significant differences are presented.

Table 3.11. Differences among mean fraction of total seedling dry weight as fine roots, woody roots, and stem for 15 loblolly pine families.

Fine roots dry weight		**Coarse roots dry weight**		**Stem dry weight**	
Family comparison	Mean differences	Family comparison	Mean differences	Family comparison	Mean differences
L-2 - L-5	0.07339	L-1 - L-2	-0.04531	L-2 - L-5	-0.13465
L-2 - L-9	0.07664	L-1 - L-16	-0.04905		
L-2 - L-22	0.05828				
L-5 - L-7	-0.05434				
L-5 - L-8	-0.06657				
L-5 - L-18	-0.05577				
L-7 - L-9	0.05758				
L-8 - L-9	0.06982				
L-9 - L-11	-0.05626				
L-9 - L-13	-0.05518				
L-9 - L-18	-0.05902				

Note: Only significant differences are presented.

Table 3.12. Means followed by standard deviations (SD) of seedling total height, root collar diameter (RCD), total seedling dry weight, and fractions of dry weight as foliage, stem, and fine and coarse roots.

Family	Total height		RCD		Total dry weight		Foliage		Stem		Fine root		Coarse root	
							----------fraction of total dry weight----------							
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
L-1	74	14.7	9.90	1.62	33.4	12.2	0.5	0.1	0.3	0.1	0.1	0.0	0.1	0.0
L-2	82	12.0	11.86	1.48	41.1	7.2	0.5	0.1	0.3	0.1	0.2	0.0	0.1	0.0
L-5	81	15.2	9.72	1.20	33.2	9.8	0.4	0.1	0.4	0.1	0.1	0.0	0.1	0.0
L-6	86	17.1	10.29	1.51	32.7	10.0	0.5	0.1	0.3	0.1	0.1	0.0	0.1	0.0
L-7	91	22.5	10.84	1.85	37.8	7.7	0.4	0.1	0.3	0.1	0.1	0.0	0.1	0.0
L-8	82	19.9	10.30	1.80	41.2	5.6	0.4	0.0	0.3	0.0	0.2	0.0	0.1	0.0
L-9	77	11.5	10.84	1.57	39.2	8.7	0.5	0.1	0.4	0.1	0.1	0.0	0.1	0.0
L-10	72	10.7	11.36	1.55	36.5	8.1	0.4	0.1	0.4	0.1	0.1	0.1	0.1	0.0
L-11	71	10.5	11.23	1.87	35.4	9.1	0.5	0.1	0.3	0.1	0.1	0.0	0.1	0.0
L-12	81	16.7	10.65	2.03	32.2	12.3	0.5	0.1	0.3	0.1	0.1	0.0	0.1	0.0
L-13	85	21.4	12.25	1.40	44.2	8.9	0.5	0.1	0.3	0.1	0.1	0.0	0.1	0.0
L-16	90	15.1	13.06	4.69	41.5	12.7	0.5	0.1	0.3	0.1	0.1	0.0	0.1	0.0
L-18	80	16.8	10.74	2.28	34.6	14.1	0.4	0.1	0.3	0.1	0.2	0.1	0.1	0.0
L-22	80	13.1	10.27	1.05	33.8	7.9	0.4	0.1	0.4	0.1	0.1	0.0	0.1	0.0
L-23	76	13.1	10.95	1.70	39.4	11.9	0.5	0.1	0.3	0.1	0.1	0.0	0.1	0.0

Multiple regressions between lesion dimensions and independent variables among the families including foliar nitrogen concentration, R:S and total stem phenolic concentration did not indicate significant correlation (lesion length: $P = 0.9263$, $R^2 = 0.0174$; lesion width: $P = 0.0698$, $R^2 = 0.2342$). Because nitrogen nutrition treatment did not significantly affect lesion dimensions or stem total phenolic concentrations, and foliar nitrogen concentration was not a significant predictor of lesion dimensions, data associated with the NN and HN treatment levels were combined. With the combined data, regressions were conducted for each family between the dependent variables of lesion length, lesion width, and stem total phenolic concentration, and the independent variables of seedling morphological traits to determine the role of seedling growth and carbon allocation pattern in defense against *G. huntii*. Regressions between lesion dimensions and the fraction of total seedling dry weight as stem, foliage, fine roots, and coarse roots indicated that families L-1, L-23, L-6, L-7 and L-9 had a significant, negative relationship between lesion width and the fraction of total seedling dry weight allocated to the stem. No consistent, significant relationship was found between lesion length and any family morphological trait. However, family L-12 had a significant, negative relationship between lesion length and the fraction of total seedling dry weight allocated to the stem ($P = 0.0211$, $R^2 = 0.2752$), family L-13 had a significant, positive relationship between lesion length and the fraction of total seedling dry weight allocated to the coarse roots ($P = 0.3015$, $R^2 = 0.3015$), and family L-18 had a significant negative relationship between lesion length and the fraction of total seedling dry weight allocated to the stem ($P = 0.0274$, $R^2 = 0.2424$). For families L-1, L-12, L-13, L-18, L-22, L-5, lesion width was significantly and positively correlated to total seedling dry weight (Table 3.13). Families L-10 and L-9 showed significant positive correlation between lesion width and R:S (L-10: $P = 0.0327$, $R^2 = 0.2863$; L-9: $P = 0.0032$, $R^2 = 0.3910$). Lesion

width was also significantly and positively correlated to the fraction of total seedling dry weight allocated to coarse roots for family L-13 ($P = 0.0089$, $R^2 = 0.3085$) but was significantly and negatively correlated to the fraction of total seedling dry weight allocated to coarse roots for family L-22 ($P = 0.0034$, $R^2 = 0.4248$).

Stem total phenolic concentration was significantly and positively related to the fraction of total seedling dry weight allocated to foliage for families L-1, L-10, and L-9 and was significantly and positively correlated to fraction of coarse roots for family L-11 ($P = 0.0184$, $R^2 = 0.2718$). Stem total phenolic concentration was significantly and negatively correlated to fraction of dry weight as stem for family L-18 ($P = 0.0421$, $R^2 = 0.2337$). Stem total phenolic concentration was significantly and positively correlated to total seedling dry weight for families L-10, L-12, L-13, L-16, L-18 (Table 3.13) and to fraction of coarse root dry weight for family L-11 ($P = 0.0271$, $R^2 = 0.2432$).

Table 3.13. Probabilities of a greater F-value and regression coefficients for families having significant relationships between lesion width and independent variables (total seedling dry weight, and fractions of dry weight as foliage and stem).

Dependent variable	Family	Morphological variable	
		Stem dry weight	
Lesion width	L-1	0.0288	0.2388
	L-6	0.0123	0.3006
	L-7	0.0049	0.3331
	L-9	0.0028	0.4001
	L-23	0.0208	0.2506
		Total seedling dry weight	
Lesion width	L-1	0.0054	0.3564
	L-5	0.0012	0.4323
	L-12	0.0048	0.3823
	L-13	0.0252	0.2371
	L-18	<0.0001	0.7303
	L-22	0.0057	0.3885
		Total seedling dry weight	
Stem total phenolics	L-10	0.0309	0.2915
	L-12	<0.0001	0.6438
	L-13	0.0069	0.3567
	L-16	<0.0001	0.7389
	L-18	0.0099	0.3488
		Foliage dry weight	
Stem total phenolics	L-1	0.0130	0.3339
	L-10	0.0080	0.4056
	L-9	0.0326	0.2294

3.5. Discussion

The overall results from this study showed that the nitrogen nutrition did not affect susceptibility to *G. huntii* as indicated by the non-significant differences in lesion parameters between the two treatment levels (NN and HN). This is inconsistent with previous studies done with other pathogens such as when a nitrogen rich environment increased the susceptibility of Scots pine to tarnished plant bug *Lygus rugulipennis* Popp. (Holopainen et al. 1994). Also, longer lesions and excessive sporulation in apple trees developed by *Venturia inaequalis* (Cooke)

G. Wint. under high nitrogen cultures as compared to low nitrogen cultures (Rühmann et al. 2001).

Grosmannia huntii, relatively less known compared to other *Leptographium* species involved in southern pine decline, was selected for the inoculations in this experiment based on virulence trials where it caused significantly more damage to pine seedlings compared to that caused by the other *Leptographium* species tested (Matusick and Eckhardt 2010a). *Grosmannia huntii* successfully infected all the loblolly pine seedlings in the study with dark brown and sunken lesions seen on the seedling stems 14 weeks following inoculations. Previous studies have shown that in response to inoculations, dark brown lesions are seen on the seedling stems which normally extend vertically on either side of the length of the wound (Eckhardt et al. 2004a; Matusick and Eckhardt 2010a). However, the lesions associated with *G. huntii* in this study commonly showed minimal extension above and below the point of inoculation with poor radial movement. Radial movement is associated with highly virulent ophiostomatoid fungi such as *L. wageneri* (Cobb 1988). Not only was lesion extension minimal, but lesion length was not significantly different among 15 families. Consistent re-isolation from the stem tissue confirms infection and rejects the contention that inoculation was unsuccessful. Furthermore, a simultaneous study with *Leptographium* and *Grosmannia* fungi including *G. huntii* artificial stem inoculation of loblolly pine families resulted in a high degree of virulence (Chapter 2).

While increased virulence of *G. huntii* was anticipated with added nitrogen (Huber and Thompson 2007), absence of a distinct lesion dimension response to nitrogen nutrition treatment also suggests that the spread of *G. huntii* was limited by unknown factors. The seedlings in the

present study were housed in a greenhouse, and those exhibiting a high degree of *G. huntii* virulence were housed outdoors. It is hypothesized, therefore, that greenhouse environmental conditions played a negative role in *G. huntii* virulence and lesion development. An understanding of the environmental factors that control *G. huntii* infection is warranted before additional greenhouse experiments containing a stem inoculation component are attempted.

A negative relationship between lesion width and the fraction of dry weight allocated to seedling stems for five families (L-1, L-23, L-6, L-7, L-9), and a positive relationship between lesion width and total seedling dry weight for seven families (L-1, L-12, L-13, L-13, L-18, L-22, L-5) illustrates the difficulty of using lesion width to assess infection. The horizontal length of the wound needed for inoculation was a function of initial seedling size, and stem diameter growth essentially diluted the effect of the inoculum on vascular tissues that grow radially. Therefore, it was not possible to assess virulence by lesion width without first removing the influence of seedling size and stem growth rate. Nitrogen nutrition affected several seedling morphological traits. Root collar diameter which is the most valuable indicator of seedling quality and field performance (Johnson and Cline 1991), was greater with an increase in nitrogen availability from a sufficient to a high level. Total height was not significantly different between the NN and HN treatment levels. However, this is consistent with the finding of Dewald et al. (1992) who reported that among slash pine seedlings receiving optimal but not low, and high amounts of nitrogen, final height was not affected by high nitrogen concentration. Alternatively, comparison between low and high nitrogen levels, demonstrated distinct benefits of excess nitrogen to loblolly and slash pine seedling TH and RCD (Samuelson 2000).

Total dry weight was also significantly different between the NN and HN treatment levels with elevated nitrogen leading to larger seedlings. Carbon allocation to foliage was greater, while that to fine roots was lower, under the HN treatment level; whereas, carbon allocation to fine roots was greater, and that to foliage was lower, under the NN treatment level. These results support the assertion that leaf area and fine root biomass are dynamic in response to environmental conditions (Gower et al. 1995; Stovall et al. 2012). While others have found significant responses of loblolly pine seedling carbon allocation to coarse root and stem biomass in response to added nitrogen (Samuelson 2000; Stovall et al. 2012), the fraction of dry weight as coarse roots and stem did not differ significantly in this study. Absence of a nitrogen effect on woody tissues may be attributed to an inability to resolve these effects because the potted seedlings were variable in size at the start of the study (Table 3.3), the NN treatment level represented N-sufficiency rather than N-deficiency, and the nitrogen nutrition treatment was applied for only 27 weeks.

Simple and complex plant phenolic compounds serve as preformed (i.e., constitutive) and inducible means of resistance to insect attack and disease (Lattanzio et al. 2006). Past research has shown that elevated nitrogen levels depress the production of simple (e.g., caffeic acid) and complex (e.g., tannin) plant phenolic compounds (Ibrahim et al. 2011; Kováčik and Bačkor 2007; Muzika 1993). For example, Gebauer et al. (1998) found that total phenolic compound and condensed tannin concentrations in the foliage and lateral roots of loblolly pine seedlings were reduced as nitrogen availability increased. Booker and Maier (2001) reported a similar trend for loblolly pine receiving two levels each of nitrogen and carbon dioxide treatment. Elevated carbon dioxide not only boosted phenolic synthesis but it also increased whole-plant

carbon fixation which indirectly decreased tissue nitrogen concentration. It was suggested that in accordance with the growth differentiation balance hypothesis (Herms and Mattson 1992), the negative relationship between plant phenolic concentration and nitrogen addition was secondary in nature, arising in response to the ratio between carbohydrate production and utilization (i.e., source:sink ratio). Because carbohydrate utilization in the form of cell division and growth is nitrogen demanding, nitrogen amendments are diluted (i.e., the dilution effect) in plant tissues by accelerated growth (Jarrell and Beverly 1981). Booker and Maier (2001) suggested that phenolic compound synthesis serves as a mechanism to conserve fixed carbon that is not utilized for growth when nitrogen is limiting.

In the present study, sufficient nitrogen (i.e., NN) and excess nitrogen (i.e., HN) did not affect stem total phenolic concentrations. Based on the information presented by Booker and Maier (2001), lack of a nitrogen nutrition effect on total phenolic concentration may be explained by absence of different source:sink ratios between the NN and HN seedlings. While the HN seedlings exhibited more foliage and less fine root growth than the NN seedlings, stem and coarse root growth were unaffected by nitrogen nutrition treatment, and total seedling dry weight was only increased 8 percent by the HN treatment level. Perhaps the NN and HN treatment levels did not create a wide enough difference in carbon gain and seedling growth to diverge the source:sink ratios of the two treatment levels and as a result, change their stem total phenolic concentrations.

Naturally occurring phenolic compounds represent a source of preformed or constitutive defense (Lattanzio et al. 2006), and family differences in stem total phenolic concentration may

provide guidance regarding a genotype's potential for resistance before insect attack or pathogenic infection. Family L-1 produced the highest, and families L-8 and L-9 produced the lowest concentrations of stem phenolic compounds in this study. Seedlings from family L-1 were 15 percent smaller in total dry weight than those from family L-9. Thus, families L-1 and L-9 may have had different amounts of carbohydrate that could be shunted to defense compound synthesis accounting for differences in stem total phenolic concentration. However, families L-1 and L-8 were similar in total seedling dry weight, differing by only 3 percent. Similar seedling size indicates that these families may have been similar in their amounts of carbohydrate that could be shunted to defense compound synthesis. Therefore, dissimilar stem total phenolic concentration between families L-1 and L-8 point toward the possibility that genetic sources of loblolly pine with an elevated defense capacity could be selected for planting where the risk of pathogenic attack is high. However, a great deal of additional research is needed before this concept can be effectively transferred to the field.

Regression analyses between stem total phenolic concentrations and total seedling dry weights were significant for 33 percent of the families tested (L-10, L-12, L-13, L-16, L-18). If stem total phenolic concentration is representative of constitutive defense potential, then contrary to the growth differentiation balance hypothesis (Herms and Mattson 1992), growth and total phenolic concentration may be positively correlated as observed by Aspinwall et al. (2011). Thus it is possible that families with strong positive relationships between growth and total phenolic concentration are more effective at constitutive defense than families with a poor relationship between these variables.

Among fractions of dry weight allocated to the foliage, stem, fine roots, and coarse roots, the fraction of dry weight allocated to foliage was the strongest independent predictor of stem total phenolic concentration. Specifically, three families showed significant regressions between stem total phenolic concentration and fraction of dry weight allocated to foliage. This information supports the notion that for some loblolly pine families, there is a positive relationship between growth and constitutive defense potential (Aspinwall et al. 2011) because foliage production is closely tied to the growth of loblolly pine (Vose and Allen 1988; Luxmoore et al. 1995).

The hierarchy of plant carbon allocation prioritizes foliage and stem growth over root system growth and the production of nonstructural carbon containing compounds (e.g., phenolics, sugars, etc.) (Dickson 1991). This concept led to the hypothesis in the present study that a carbon allocation pattern favoring root system growth rather than foliage and stem growth would benefit defense chemical synthesis. If this were true, therefore, genetic control of R:S could parallel genetic control of natural levels of phenolic compound synthesis and constitutive defense potential. Correlation between stem total phenolic concentration and R:S was only significant for one family. Although R:S was not an acceptable predictor of constitutive defense potential, the results of this study indicate that there may be other seedling morphological variables that reflect defense capacity. Specifically, once loblolly pine seedlings meet established values of TH, RCD, and R:S that indicate superior quality, seedling size and/or foliage mass may gauge constitutive defense potential.

Chapter Four

Variation in root lesions of loblolly pine (*Pinus taeda* L.) families in response to *Leptographium* and *Grosmannia* root infecting fungi

4.1. Abstract

Pine decline has been observed as an emerging forest health problem in the southeastern United States. Complex interactions of biotic and abiotic factors are involved which together predispose, continuously stress and contribute to the decline. Presence of root-feeding beetles and their fungal associates such as *Leptographium* and *Grosmannia* have been consistently reported from the symptomatic stands. A study was conducted on fourteen year old loblolly pine stands at two locations with different open pollinated half sib families to determine their relative resistance to *Leptographium* and *Grosmannia* fungal genera. Inoculations were administered on the roots following an artificial inoculation method. Host responses to the inoculations were recorded as lesion length, lesion width, lesion depth, lesion area and occlusion length eight weeks after inoculations. Roots exhibited dark brown resin filled lesions and occluded tissues. Results indicated that the treatments had variable effects with *L. terebrantis* and *G. huntii* causing significantly larger lesions. However, no significant differences were observed in lesion parameters among the fourteen loblolly pine families included in the study.

4.2. Introduction

The southeastern United States is the leading timber producing region of the United States with loblolly pine grown as a commercially timber species (Schultz 1999). Loblolly pine is native to the southeastern United States and it is found throughout its range consisting of 14 states from New Jersey to central Florida and west to Texas (Schultz 1999). Loblolly pine has a fast growth rate and provides versatile marketable products. It is ecologically important as habitat to a diversity of wildlife and supports the region by economically providing 110, 000 jobs and $30 billion to the economy of the southeastern United States (Schultz 1999). Loblolly pine is vulnerable to a variety of abiotic and biotic agents such as wind throw, extreme temperatures, lightening damage, drought, flooding, insects and diseases. Reduced growth, tree decline and mortality have been associated with loblolly pine for the past 50 years and have been studied and well documented (Brown and McDowell 1968; Roth and Peacher 1971; Hess et al. 1999). The potential for serious future ecological and economic implications as a result of decline in loblolly pine cannot be neglected.

Decline in loblolly pine was first observed in the Talladega National Forest in the Oakmulgee Ranger District in central Alabama. Symptoms included thinning crowns, reduced radial growth and root deterioration in 40 to 50 year old stands (Brown and McDowell 1968). Studies indicated the involvement of unusual physiological and environmental conditions (Roth and Peacher 1971) and other pathogens such as Heterobasidion root rot, *Pythium* species and *Phytophthora cinnamomi* Rands. which is the causal organism of littleleaf disease (Campbell and Copeland, 1954; Brown and McDowell 1968). However, in a 3-year study led by Eckhardt et al. (2007), observations of affected stands showed damage to the lateral roots with the recovery of

root-feeding bark beetles (*Hylastes* species and *Hylobius pales* (Herbst.) and *Pachylobius picivorous* Germ.) and weevil species, as well as resin production. The recovery of *Leptographium* species including *L. procerm* (W.B. Kendr.) M.J. Wingf., *G. alacris* (formely *L. serpens*) T.A. Duong, Z.W. de Beer & M.J. Wingf. sp. nov., and *L. terebrantis* S.J. Barras and T.J. Perry were also reported from the root systems of the infected trees. Recently, *Grosmannia huntii* (R.C. Rob. Jeffr.) Zipfel, Z.W. de Beer & M.J. Wingf (*L. huntii* M.J. Wingfield) was recovered from the symptomatic loblolly pine roots and the insect vectors involved (Zanzot et al. 2010).

Presently, premature mortality in loblolly pine has been described as a decline disease syndrome that involves complex interactions of abiotic and biotic agents leading to tree mortality. Predisposing factors such as increased slopes, southwest facing aspects, genetics of the trees subjected to continuous stress, bark beetles and hence, ophiostomatoid fungi, through their mutualistic role with the bark beetles, contribute to tree decline (Eckhardt et al. 2004a; Eckhardt et al.2004b; Eckhardt and Menard 2008). Several other declines have been reported for confers in the United States with some prominent examples such as red pine (*P. resinosa* Aiton) decline in Wisconsin (Klepzig et al. 1991), ponderosa pine (*P. ponderosa* Laws.) root disease/decline in New Mexico (Livingstone et al. 1983), pole blight of western white pine (*P. monticola* Dougl. Ex D. Don) (Leaphart and Copeland 1957), and vascular tissue disruption caused by Procerum root disease in western white pine (Butnor et al. 2000). In all pine syndromes, involvement of ophiostomatoid fungi was reported either as a causal or contributing factor.

Artificial inoculations have been conducted on similar sized trees to determine the virulence of ophiostomatoid fungi (Långström et al. 2001; Rice et al. 2007). Fungal inoculations of above-ground parts such as tree stems is appropriate to mimic the bark beetles that naturally attack above ground plant parts and inoculate fungus into the tree stem (Schowalter and Filip 1993). However, in the case of root feeding bark beetles that introduce the fungus into the roots, mature tree root inoculations have been performed and proved appropriate (Wingfield andKnox-Davies 1980; Otrosina et al. 2002; Matusick et al. 2012). Following inoculations, host response is seen in the form of necrotic lesions with darkened phloem tissue and excess resin production at the point of inoculation (Parmeter et al. 1989). Invasion of the fungus is seen as wedge shaped sapwood discoloration with lesions extending longitudinally and laterally from the origin of infection (Bleiker and Uzunovic 2004). Restriction in hydraulic conductance through the xylem has been observed in ponderosa pine in response to *Leptographium wageneri* Kendrick. and *Heterobasidion irregulare* nom. nov. Garbelotto & Otrosina (Joseph et al. 1998). Furthermore, diffused symptomatology as decreased hydraulic conductivity, thin crowns, chlorotic foliage and decline was noticed in Scots pine (*Pinus sylvestris* L.) when inoculated with *Ophiostoma ips* (Rumb.) Nannf. (Fernandez et al. 2004).

Previous studies have been focused on studying the relative virulence of ophiostomatoid fungi without considering the variability in host genotype resistance. In a study done with two loblolly and two slash pine families, relative virulence of four ophiostomatoid fungi and *H. irregulare* was studied with *G. huntii* causing larger lesions. Although, the families were not compared, but the genetic differences in one family were held responsible for larger lesions in the wound+media control than the wound only control (Matusick 2010). This study was

conducted to determine the variability in symptomatology of loblolly pine genotypes commonly deployed by the timber companies in response to mature tree root inoculations with *Leptographium* and *Grosmannia* root infecting fungi.

4.3. Methods and Materials

The study was carried out in 14 year old stands at two locations provided by forest industry in Georgia and Florida. Two sites were selected from the lower gulf elite population trials planted across the range from Georgia to Mississippi and Texas. Each site consisted of trees from 14 families planted as single tree plots with 15 replications. The families were scattered throughout the trial and each site consisted of 210 trees.

Fungal cultures consisting of single spore isolates maintained in the Forest Health Dynamics Laboratory were used for inoculations. Two weeks before the inoculations the fungi were cultured on 2% malt extract agar (MEA). Four treatments were applied which consisted of two fungal treatments: *G. huntii*, *L. terebrantis* and two controls: wound and wound+media. For each treatment, two primary lateral roots were carefully excavated on each tree and the fungal species were applied to each root along with a control (Figure 4.1). About 30 cm (1 ft) of distance was maintained between the fungal and control treatments. The method given by Wright (1933) was used for root inoculation which consisted of creating a 13 mm wound on the root with a cork borer, peeling through the bark to the cambium. After removing the bark plug, a 10 mm plug of mycelium was placed in the wound followed by replacing the bark plug and sealing the wound with duct tape. In the case of the wound+media control, sterile media was placed in the wound and in the case of the wound control, wound was created in the same way

but nothing was inserted into the wound. The inoculation points were marked with pin flags and the roots were covered (Figure 4.2).

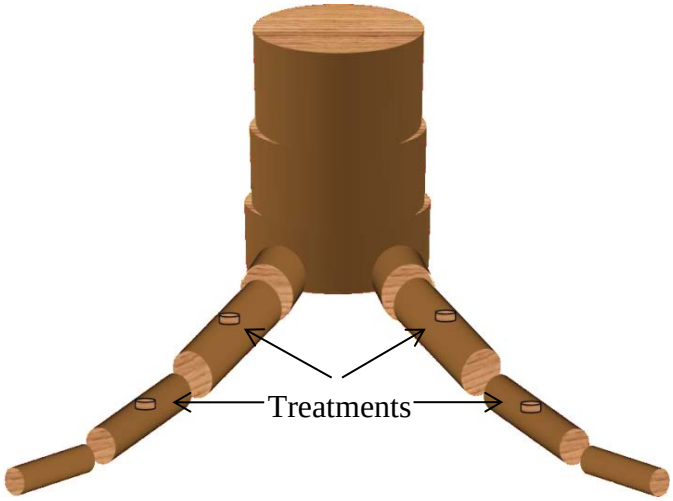

Figure 4.1. Diagram showing treatments as administered on the tree roots (Matusick 2010).

Figure 4.2. Roots covered with soil following inoculations with pin flags showing points of inoculation.

Eight weeks following inoculations, the roots were re-excavated, removed from the tree and returned to the laboratory at Auburn University for further processing and measurements.

Each root sample was inspected for insect activity and host responses to the inoculations were quantified by measuring the length, width and depth of the infected dark and discolored root tissue. The lesion depth was determined by cutting the root at the inoculation point and measuring the radial discolored sapwood. For determining the occluded root tissue, the root tissue was stained with a solution of FastGreen stain (FastGreen FCF; Sigma Chemical Co.) and water (0.25g/L of water) and the unstained tissue was recorded as occlusion length. Each root lesion margin was traced on transparency sheets and the lesion area determined using a Lasico® Planimeter (Lasico®, Los Angeles, CA). Small pieces of stem tissue from the distal and proximal portions of the inoculation site were plated on malt extract agar media amended with cycloheximide streptomycin (CSMA) to confirm infection.

The field design was set up as a randomized complete block design with single tree plots. Each site had 15 blocks with each family having one tree per block. The response variables to the inoculations were analyzed using SAS statistical software (SAS Institute, 9.2 ed., Cary, NC). A linear mixed model was fit to the data to compare the treatments and determine the family differences. In the model, the families were tested as a random sample of a breeding population with family x treatment interaction kept as a random effect. Site, block and treatment were kept fixed with the block effect nested within the sites. Covariance parameter estimates for each response variable (lesion length, occlusion length, lesion width, lesion depth, and lesion area) were used to determine the variation among the families and the families were ranked on the basis of general combining ability (GCA) estimates for lesion length. Fungal and control treatments were compared by type 3 fixed effects.

4.4. Results

Inoculation with *L. terebrantis* and *G. huntii* caused resin soaked brown lesions in the loblolly pine roots with distal and proximal extensions from the inoculation wound (Figure 4.3). Of the fungi tested on loblolly pine families, *L. terebrantis* caused longer lesion length, occlusion length, lesion width, lesion depth, and lesion area followed by *G. huntii* (Table 4.1). Pairwise comparison between the treatments indicated that lesions produced from *L. terebrantis* were significantly longer, wider and deeper than those of *G. huntii* (Table 4.2). Both fungal species caused significantly larger lesions than either the wound or wound+media control treatments. However, lesion length, width, depth and area, due to the presence of the media differed significantly from the wound only control (Table 4.2).

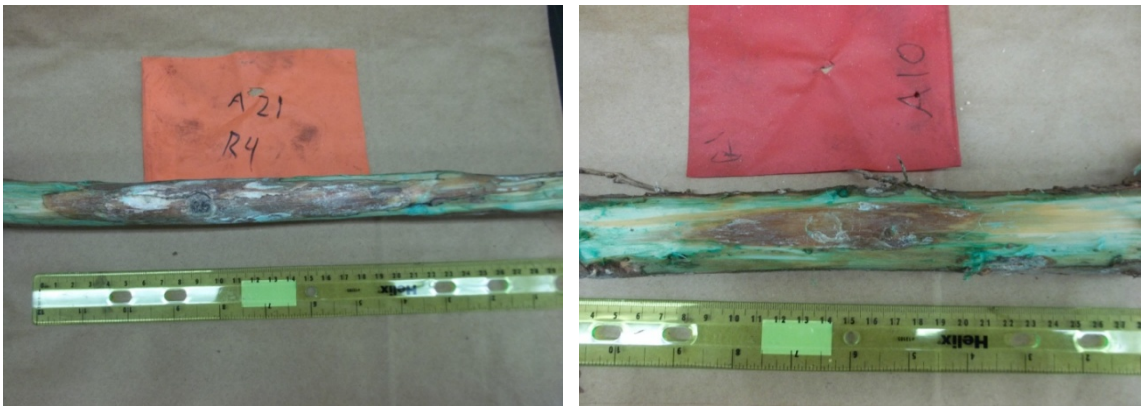

Figure 4.3. Lesions on the roots following inoculations with *Leptographium terebrantis* (Left) and *Grosmannia huntii* (Right).

Table 4.1. Least square means of treatments for lesion length, occlusion length, lesion width and lesion depth for loblolly pine families.

Variable	Treatment	Estimate	Std. error	DF	t Value	Pr > |t|
Lesion length	LT	13.5583	0.1934	1339	70.1	<.0001
	GH	11.1729	0.192	1339	58.2	<.0001
	WM	7.1301	0.1943	1339	36.69	<.0001
	W	5.2018	0.1942	1339	26.79	<.0001
Occlusion length	LT	13.7053	0.1836	1324	74.67	<.0001
	GH	11.389	0.1807	1324	63.01	<.0001
	WM	7.1749	0.1826	1324	39.28	<.0001
	W	5.2025	0.1823	1324	28.54	<.0001
Lesion width	LT	4.8947	0.03855	1337	126.98	<.0001
	GH	4.4909	0.03813	1337	117.78	<.0001
	WM	3.9262	0.03885	1337	101.07	<.0001
	W	3.6599	0.03879	1337	94.34	<.0001
Lesion depth	LT	3.0315	0.04612	1337	65.74	<.0001
	GH	2.6205	0.04573	1337	57.3	<.0001
	WM	1.9101	0.04634	1337	41.22	<.0001
	W	1.4531	0.04626	1337	31.41	<.0001
Lesion area	LT	3.5278	0.05133	1275	68.73	<.0001
	GH	2.8795	0.05080	1275	56.69	<.0001
	WM	1.7660	0.05213	1275	33.88	<.0001
	W	1.1516	0.05135	1275	22.43	<.0001

Note: P- value <0.05 shows a significant difference at alpha=0.05
GH- *Grosmannia huntii*, LT- *Leptographium terebrantis*, W- Wound,
WM- Wound+Media

Table 4.2. Differences in least square means of treatments for lesion length, occlusion length, lesion width and lesion depth for loblolly pine families.

Variable	Trt	Trt	Estimate	Std. error	DF	t Value	Pr > |t|
Lesion length	GH	LT	-2.3854	0.2702	1339	-8.83	<.0001
	GH	W	5.9712	0.2724	1339	21.92	<.0001
	GH	WM	4.0428	0.2712	1339	14.91	<.0001
	LT	W	8.3566	0.2729	1339	30.62	<.0001
	LT	WM	6.4282	0.2722	1339	23.61	<.0001
	W	WM	-1.9283	0.2732	1339	-7.06	<.0001
Occlusion length	GH	LT	-2.3163	0.2560	1324	-9.05	<.0001
	GH	W	6.1865	0.2566	1324	24.11	<.0001
	GH	WM	4.2141	0.2556	1324	16.49	<.0001
	LT	W	8.5028	0.2581	1324	32.94	<.0001
	LT	WM	6.5304	0.2577	1324	25.35	<.0001
	W	WM	-1.9724	0.2571	1324	-7.67	<.0001
Lesion width	GH	LT	-0.4037	0.05273	1337	-7.66	<.0001
	GH	W	0.8310	0.05339	1337	15.56	<.0001
	GH	WM	0.5647	0.05304	1337	10.65	<.0001
	LT	W	1.2347	0.05354	1337	23.06	<.0001
	LT	WM	0.9684	0.05335	1337	18.15	<.0001
	W	WM	-0.2663	0.05364	1337	-4.96	<.0001
Lesion depth	GH	LT	-0.4110	0.06313	1337	-6.51	<.0001
	GH	W	1.1674	0.06364	1337	18.34	<.0001
	GH	WM	0.7104	0.06337	1337	11.21	<.0001
	LT	W	1.5784	0.06380	1337	24.74	<.0001
	LT	WM	1.1214	0.06366	1337	17.61	<.0001
	W	WM	-0.4570	0.06386	1337	-7.16	<.0001
Lesion area	GH	LT	-0.6483	0.06808	1275	-9.52	<.0001
	GH	W	1.7279	0.06848	1275	25.23	<.0001
	GH	WM	1.1135	0.06874	1275	16.20	<.0001
	LT	W	2.3762	0.06874	1275	34.57	<.0001
	LT	WM	1.7618	0.06916	1275	25.47	<.0001
	W	WM	-0.6144	0.06927	1275	-8.87	<.0001

Note: P- value <0.05 shows a significant difference at alpha=0.05
GH- *Grosmannia huntii*, LT- *Leptographium terebrantis*, W- Wound,
WM- Wound+Media

Estimation of covariance parameters indicated that the variation among the families was not significantly different from zero for lesion length (Z = 0.09, P = 0.4653), occlusion length (Z = 0.06, P = 0.4775), lesion width (Z = 0.42, P = 0.3384), lesion depth (Z = 0.40, P = 0.3448) and lesion area (Z = 0.76, P = 0.2248) (Table 4.3). The family x treatment interaction was not found

to be significant for lesion length, width, depth, and occlusion length or lesion area (Table 4.3). Solutions for Type 3 fixed effects suggested that lesion length, occlusion length, lesion width and lesion area differed significantly between the two sites and among the treatments. Blocks were not found to be significantly different for lesion length, occlusion length and lesion area but were found to be significantly different for lesion width and lesion depth (Table 4.4). Although the variation among the families was not significantly different, the general combining ability (GCA) estimates were used to rank the families on the basis of lesion length overall and for each fungal species (Table 4.5). Average lesion length, occlusion length, lesion width and lesion depth for each treatment and for each family are presented in Table 4.6 and Table 4.7. Re-isolation percentage of *L. terebrantis* from inoculated loblolly pine roots was 92% while that of *G. huntii* was 58%.

Table 4.3. Covariance parameter estimates are presented. Variation among families is not significantly different from zero for lesion length, and occlusion length. Treatment x family interaction is non-significant.

Variable	Cov pram	Estimate	Std. error	Z value	Pr>Z
Lesion length	Family	0.005374	0.06168	0.09	0.4653
	Trt x Family	0.1644	0.1216	1.35	0.0882
	Residual	8.5194	0.3361	25.34	<.0001
Occlusion length	Family	0.002917	0.05171	0.06	0.4775
	Trt x Family	0.1425	0.1060	1.34	0.0894
	Residual	7.6081	0.3017	25.22	<.0001
Lesion width	Family	0.000920	0.002207	0.42	0.3384
	Trt x Family	0	--	--	--
	Residual	0.4807	0.01867	25.74	<.0001
Lesion depth	Family	0.001428	0.003575	0.40	0.3448
	Trt x Family	0.008169	0.006340	1.29	0.0988
	Residual	0.4843	0.01909	25.36	<.0001
Lesion area	Family	0.003830	0.005066	0.76	0.2248
	Trt x Family	0.008199	0.007531	1.09	0.1381
	Residual	0.5710	0.02308	24.73	<.0001

Note: P- value <0.05 shows a significant difference at alpha=0.05

Table 4.4. Type 3 tests of fixed effects (F tests) suggest significant and non-significant effects for lesion length, lesion width, lesion depth and occlusion length.

Variable	Effect	Num DF	Den DF	F value	Pr>F
Lesion length	Diameter	1	1339	22.04	<.0001
	Site	1	1339	28.87	<.0001
	Trt	3	1339	385.74	<.0001
	Block(Site)	28	1339	1.19	0.2268
Occlusion length	Diameter	1	1324	27.95	<.0001
	Site	1	1324	35.25	<.0001
	Trt	3	1324	450.96	<.0001
	Block(Site)	28	1324	1.23	0.1913
Lesion width	Diameter	1	1337	22.04	<.0001
	Site	1	1337	16.81	<.0001
	Trt	3	1337	215.30	<.0001
	Block(Site)	28	1337	1.98	0.0018
Lesion depth	Diameter	1	1337	34.46	<.0001
	Site	1	1337	0.02	0.9015
	Trt	3	1337	245.10	<.0001
	Block(Site)	28	1337	1.55	0.0343
Lesion area	Diameter	1	1275	14.38	0.0002
	Site	1	1275	19.05	<.0001
	Trt	3	1275	484.19	<.0001
	Block(Site)	28	1275	1.21	0.2051

Note: P- value <0.05 shows a significant difference at alpha=0.05

Table 4.5. Family ranking for lesion length (estimate) overall and across all the four treatments.

Family	Estimate	Rank	Estimate	Rank GH	Estimate	Rank LT
L-7	-0.022	1	-0.3354	1	-0.273	2
L-30	-0.022	2	-0.2874	2	-0.436	1
L-53	-0.014	3	-0.07579	7	-0.231	3
L-23	-0.014	4	0.06251	11	-0.206	4
L-18	-0.005	5	-0.1846	5	-0.029	8
L-37	-0.002	6	-0.1855	4	-0.074	6
L-1	-0.001	7	-0.03086	8	0.2711	13
L-6	-0.00095	8	-0.01837	9	0.0264	9
L-16	0.0087	9	-0.159	6	0.2066	11
L-13	0.0092	10	0.5948	14	-0.097	5
L-51	0.0095	11	0.03192	10	0.2165	12
L-40	0.012	12	0.4601	13	-0.034	7
L-8	0.013	13	-0.2248	3	0.4833	14
L-52	0.0287	14	0.3524	12	0.1755	10

Note: P- value <0.05 shows a significant difference at alpha=0.05

Table 4.6. Summary statistics for lesion length, occlusion length, lesion width and lesion depth.

Variable	Trt	N	Mean	SD	Minimum	Maximum
Lesion length	LT	342	195	103.7	13	590
(cm)	GH	351	133	79.6	16	510
	WM	336	59	55.2	12	315
	W	344	31	25	9	235
Occlusion length	LT	342	207	105.1	13	590
(cm)	GH	351	139	78.8	16	510
	WM	335	60	56.9	12	315
	W	344	31	25.5	9	235
Lesion width	LT	342	25	9.3	10	57
(cm)	GH	351	21	8.6	9	85
	WM	335	16	5.2	4	60
	W	343	14	3.6	5	38
Lesion depth	LT	341	10	3.9	0	25
(cm)	GH	351	7	2.9	0	30
	WM	335	4	3.2	0	23
	W	344	3	2.2	0	12
Lesion area	LT	327	45	37.2	2.6	331.1
	GH	340	23	20.3	1.1	160.6
	WM	314	9	11.3	0.6	77.8
	W	335	4	5.0	0.7	55.5

Table 4.7. Average lesion length, occlusion length, lesion width and lesion depth for each family.

Family	Lesion length	Lesion width	Occlusion length	Lesion depth
L-1	172(100.7)	23(9.4)	182(100.9)	8(3.5)
L-6	162(98.5)	22(8.0)	172(99.4)	9(3.7)
L-7	145(104.0)	22(7.7)	154(106.2)	8(3.0)
L-8	175(99.5)	23(8.5)	183(109.1)	9(4.1)
L-13	186(95.6)	23(9.7)	193(94.2)	8(3.4)
L-16	167(101.5)	23(10.3)	174(104.0)	9(3.7)
L-18	156(98.0)	21(6.1)	168(100.7)	8(4.0)
L-23	162(113.9)	23(11.6)	168(112.6)	9(4.6)
L-30	131(75.5)	23(10.1)	159(86.1)	8(4.2)
L-37	153(86.7)	25(9.4)	156(84.4)	9(4.2)
L-40	176(93.8)	24(8.9)	184(95.3)	9(3.3)
L-51	168(101.6)	24(8.8)	172(102.9)	8(3.1)
L-52	182(92.0)	23(7.2)	194(88.8)	9(2.5)
L-53	156(90.2)	24(12.0)	164(91.0)	8(4.0)

Note: Means followed by standard deviations in parenthesis.

4.5. Discussion

Root lesions following inoculations were consistently observed across all treatments including controls on the families tested in this experiment. Dark, discolored resin filled lesions have been observed in previous studies following inoculations with ophiostomatoid species in mature trees (Wingfield 1986; Matusick et al. 2012) (Figure 4.4). It was apparent that wound and wound+media controls also produced lesions but the lesions due to wound only were considerably small than those due to wound+media and the fungal treatments. However, lesion morphology due to wound+media control was similar to that of fungal species with occasionally greater lesion length than for the fungal treatments. Lesions due to control only have been observed in previous studies however, with lighter color due to wound control and pitch filled darker lesions from wound+media (Rice et al. 2007; Matusick et al. 2012).

Figure 4.4. Resin filled lesions on the roots following inoculations with *G. huntii* (Left) and *L. terebrantis* (Right).

Host reaction in the form of dark resinous lesions following fungal inoculations confirmed the virulence of these species to loblolly pine. Both *G. huntii* and *L. terebrantis* were pathogenic to the pine host which is consistent with previous studies that showlarge lesions when inoculated with *Leptographium* and *Grosmannia* species (Matusick et al. 2012). As observed in this study, *L. terebrantis* has been shown to be a moderate to virulent pathogen in previous studies when stem inoculations were performed on mature trees (Raffa and Smalley 1988; Wingfield 1986). *Grosmannia huntii* has been included in virulence trials on loblolly and slash pines recently where it was found to cause larger root lesions than other *Leptographium* species including *L. terebrantis* (Matusick et al. 2012). However, *L. terebrantis* was shown to be most virulent to young longleaf pine trees when stem inoculations were performed (Matusick and Eckhardt 2010b). Taken together these trials indicate that *L. terebrantis* is a moderate to severe pathogen of pines.

Damage to pine roots as a result of *Grosmannia* and *Leptographium* infection may potentially affect the activity and reproductive behavior of their root-feeding beetles. Root-

feeding beetle and weevil species such as *Hylastes* spp., *H. pales* and *P. picivorous* have been recovered from the symptomatic stands and are considered as potential vectors of these fungal species (Eckhardt et al. 2004b). Considerable damage to the roots in the form of galleries and frass was noticed in this study with no recovery of beetles which indicates that the damage potential of *Grosmannia* and *Leptographium* species is controlled by the beetle vectors (Figure 4.5).

Figure 4.5. Root-feeding beetle galleries and resin seen on the roots.

Pine hosts vary in their response to *Grosmannia* and *Leptographium* species with longleaf pine generally considered as tolerant to these fungal species as compared to loblolly and slash pines (Matusick and Eckhardt 2010a). Moreover, significant variation in lesion length was noticed among the loblolly pine families in a seedling inoculation study (Chapter 2). However, inconsistent with the seedling inoculations, no significant differences in lesion parameters were seen among the families when mature tree root inoculations were performed. It is probable that family differences in root inoculation trials could be observed in studies with longer durations than the eight weeks tested here. Although not significant, GCA estimates indicated the differences in lesion length among the families included in the study from a breeding population

of open pollinated families (Table 4.5). Further studies are needed to better understand the interactions of *Grosmannia* and *Leptographium* species with mature trees of loblolly pine genotypes and hence discover the role of genetics in *Leptographium* and *Grosmannia* resistance that could be used in seedling deployment strategies to minimize the effects of pine decline.

Chapter Five

Summary and Conclusions

5.1. Pine Decline in Southeastern United States

Natural and man-made disturbances have played a great role in determining the present status of southern pine dominated forests (Wear and Greis 2002). Forest succession has been continuously set back and moved forward due to natural disturbances such as flooding, forest wildfires and man-made changes such as logging, fuel wood cutting, and agriculture abandonment (Lorimer 2001). Along with these disturbances, southeastern forests are subject to numerous biotic insects and fungal pests. Substantial amount of tree and stand damage has occurred from insects especially southern pine beetle (*Dendroctonus frontalis* Zimmermann). This insect is responsible for damaging millions of acres of state, federal , industrial and private forests in several outbreaks from 1999 to 2003 (Thatcher and Barry 1982). Other important bark beetle species include the pine engraver beetles (*Ips* spp.), the regeneration weevils (*Hylobius* spp. and *Pachylobius* spp.) and the *Hylastes* spp. Among the major diseases, fusiform rust (*Cronartium quercum* f.sp. *fusiforme* Hedg. & Hunt) has resulted in large scale mortality of southern pines (Phelps and Czabator 1978) along with heterobasidion root rot (*Heterobasidion irregulare* nom. nov. Garbelotto & Otrosina) which cause root decay and wind throw

(Robbins 1984). Pitch canker has also caused significant mortality (*Fusarium circinatum* Nirenberg and O' Donnell) in some areas throughout the southern United States (Barnard and Blakeslee 1987).

Recenty, pine decline has been implicated as a serious root disease of southern pines. The working hypothesis is that blue-stain ophiostomatoid root inhibiting fungi and their insect vectors damage tree roots along with abiotic stress factors that result in premature tree mortality (Eckhardt et al. 2007). Insects and fungi have been shown to have a mutually beneficial relationship in which the oleoresin proportions are altered due to feeding activity of the beetles and this makes the tree environment favorable for fungal growth (Paine et al. 1994). The fungus, in turn, acts as a food source for the larvae and makes conditions favorable for insect activity thus helping in beetle brood enhancement (Eckhardt et al. 2004b). The ability of ophiostomatoid root fungi to infect southern pine species and the relative virulence of these fungi to southern pines have been shown to vary in their virulence to the three main southern pine species (Matusick and Eckhardt 2010a). The objective of this research was to inoculate some of the common loblolly pine genotypes deployed by the timber industry in the southeastern region to identify those genotypes that are resistant/tolerant to *Leptographium* and *Grosmannia* fungal species associated with the *Hylastes* beetle species.

5.2. Virulence of *Leptographium* and *Grosmannia* species

Virulence of ophiostomatoid fungi has been determined on the basis of host responses such as mortality, lesion dimensions and amount of tissue occlusions (Klepzig et al. 1996; Eckhardt et al. 2004a; Matusick and Eckhardt 2010a; Matusick and Eckhardt 2010b). The

virulence of *Leptographium* and *Grosmannia* species on studies established in 2011 indicated that *L. terebrantis, L. procerum, G. alacris* and *G. huntii* vary in their virulence in loblolly and slash pine species (Chapter 2). Of the fungi tested, *Leptographium terebrantis* appeared to be the most virulent with larger lesions and occlusions following inoculations. *Grosmannia huntii* was found to be more virulent than *G. alacris* and *L. procerum* and hence in 2012 only *L. terebrantis* and *G. huntii* were included in screening trials. *Grosmannia alacris* resulted in longer lesions on seedlings than *L. procerum* (Chapter 2). Following inoculations of mature loblolly pine tree roots, *L. terebrantis* produced larger lesions than *G. huntii* (Chapter 4).

When compared to other *Leptographium* species, less is known about *G. huntii*, thus this species was included in recent inoculation trials. Virulence rankings conducted on southern pines seedlings confirmed that this fungus is the most virulent pathogen among the fungal species tested (Matusick and Eckhardt 2010a; Matusick et al. 2012). However, when young longleaf trees were inoculated, *L. terebrantis* produced the largest sapwood discoloration (Matusick and Eckhardt 2010b). In other inoculation trials *L. terebrantis* is considered a moderate to severe pathogen. In several virulence testing studies this fungus has produced has produced diverse symptoms ranging from seedling mortality as seen by Wingfield (1986), losses in conduction noticed on Japanese black pine (*P. thunbergiana* Franco) and Scots pine (*P. sylvestris* L.) (Rane and Tattar 1987), and sapwood discoloration and discolored lesions (Eckhardt et al 2004a; Matusick and Eckhardt 2010a). Likewise, *L. procerum* has been reported as mild to weak pathogen in several previous studies (Wingfield 1986; Eckhardt et al. 2004a; Matusick and Eckhardt 2010a). While, Dochinger (1967) reported *L. procerum* as the causal organism of Procerum root disease on eastern white pine (*P. strobus* L.), generally *L. procerum*

is regarded as a weak pathogen. The insect associated *G. alacris* has been included in few seedling inoculation studies and its virulence compared to *L. terebrantis* and *L. procerum* (Eckhardt et al. 2004a; Zhou et al. 2002; Matusick and Eckhardt 2010a). In the virulence trials conducted on *Pinus* species in South Africa, *G. alacris* was considered a weak pathogen along with *Ophiostoma ips* and *Leptographium lundbergi* (Zhou et al. 2002). However, when inoculated into southern pine seedlings, *G. alacris* produced longer lesions than either *L. terebrantis* or *L. procerum* (Matusick and Eckhardt 2010a). Overall the results from the present inoculation trials strongly indicate that *L. terebrantis* and *G.huntii* can cause damage to pines while, *G. alacris* and *L. procerum* are relatively less virulent (Chapter 2).

5.3. Loblolly Pine Genetics and Resistance to *Leptographium* and *Grosmannia* species

Timber production in the southeastern United States has doubled since 1953 and the region contributes 58 % of total United States production and 16% of the world's share. Projections indicate that the southeastern region will contribute to about one-third of the United States production between 1995 and 2040 (Wear and Greis 2002). One of the reasons for increase in timber production and forest productivity is the combination of genetics and silviculture. The selection of superior trees for disease resistance, rapid growth and desirable wood quality has played an integral part in increasing forest productivity (Schultz 1997). One of the more troublesome limits on forest productivity was fusiform rust and pitch canker. Resistance to these two fungal diseases is genetically controlled and has been incorporated into tree breeding efforts (Schultz 1997). Unfortunately, genetically improved planting stock commonly deployed by the timber companies in the southeastern region have not been tested for resistance/tolerance to the root colonizing fungal species such as *Leptographium*. Thus, one of

the primary objectives was to determine if any of the commonly out planted genotypes are resistant/tolerant to either *Leptographium* and or *Grosmannia* fungi. Not surprisingly, seedlings screening in 2011 and 2012 identified some families that offered tolerance to *Leptographium* growth and had consistently smaller lesions as compared to other families included in the trials (Chapter 2). Unfortunately, a subset of these seedling same families were used in mature root screenings and did not respond as they had when tested as seedlings to *Leptographium* (Chapter 4). Some families showed similarity in ranking at both seedling and mature tree stages such as family L-30, while others such as family L-1, did not show similar trends in ranking as seedling and mature trees (Table 5.1). Family differences may be discernible at mature tree stages in longer term studies.

Table 5.1. Comparison of loblolly pine family ranking at seedling and mature tree stages.

	Family	Seedlings			Mature tree		
		Overall Rank	Rank GH	Rank LT	Overall Rank	Rank GH	Rank LT
Year One	L-1	22	16	22	7	8	13
	L-6	10	3	3	8	9	9
	L-7	12	21	9	1	1	2
	L-8	3	9	11	13	7	3
	L-13	2	17	6	10	14	5
	L-16	7	6	5	9	6	11
	L-18	4	7	4	5	5	8
	L-23	18	1	20	4	11	4
Year Two	L-30	6	6	13	2	2	1
	L-37	27	23	15	6	4	6
	L-40	17	27	3	12	13	7
Year Three	L-51	*	*	*	11	10	12
	L-52	*	*	*	14	12	10
	L-53	*	*	*	3	7	3

Note: Twenty two, twenty seven and fourteen loblolly pine families were included in year one, year two and mature tree trials respectively.

*Families to be screened in third year seedling trials.

5.4. Final Conclusions and Future Research

Ophiostomatoid fungi are virulent to southeastern pine species and consistently produced symptoms consisting of localized lesions and occlusions of the inoculated host tissues in both seedling and mature tree root inoculations. The symptoms are consistent such that the probability of causing severe root damage and producing diffused symptomology in above ground portions of the tree would be greater in long-term studies. Not surprisingly, the four ophiostomatoid fungal species vary in their virulence to southern pines tested with *L. terebrantis* the most virulent followed by *G. huntii*. *Grosmannia alacris* and *L. procerum* were found to be less virulent. These findings support the previous research regarding the variation in virulence

among these fungal species (Matusick and Eckhardt 2010a; Matusick and Eckhardt 2010b; Matusick et al. 2012).

Some seedling families had consistently smaller lesions and are a good indication of being tolerant to *Leptographium* infection. However, deployment of these families in the field should take into account the relative risk of the site and root-feeding beetle attacks. Since pine decline is gradual and involves complex interactions of abiotic and biotic factors, these findings merely provide basis for resistance to *Leptographium*. Variability in symptom expression was not enough to discern the differences among mature trees of the families included in this study. These differences may be more discernible if the study was carried out for a longer period of time. Future studies should be focused on understanding the genetic basis of resistance to *Leptographium* and identifying the role of genetics in governing tolerance/resistance to *Leptographium*.

References

Addington, R.N., Donovan, L.A., Mitchell, R.A., Vose, J.M., Pecot, S.D., Jack, S.B., Hacke, U.G., Sperry, J.S., and Oren, R. 2006. Adjustments in hydraulic architecture of *Pinus palustris* maintain similar stomatal conductance in xeric and mesic habitats. Plant Cell Environ. 29: 535-545.

Albaugh, T.J., Allen, H.L., Dougherty, P. M., Kress, L.W., and King, J.S. 1997. Leaf area and above and belowground growth responses of loblolly pine to nutrient and water additions. Forest Sci. 44(2): 317-328.

Albaugh, J.M., Blevins, L. Allen, H.L., Albaugh, T.J. Fox, T.R., Staple, J.L., and Rubilar, R.A. 2010. Characterization of foliar macro- and micronutrient concentrations and ratios in loblolly pine plantations in the southeastern United States. South. J. Appl. For. 34(2): 53-64.

Allen, H.L. 1987. Forest fertilizers: nutrient amendment, stand productivity, and environmental impact. J. Forest. 85: 37-46.

Aspinwall, M.J., King, J.S., Booker, F.L., and McKeand, S.E. 2011. Genetic effects on total phenolics, condensed tannins and nonstructural carbohydrates in loblolly pine (*Pinus taeda* L.) needles. Tree Physiol. 31: 831-842.

Baker, W. L. 1972. Eastern forest insects. U.S. Department of Agriculture, Miscellaneous Publication 1175. Washington, DC. pp. 642.

Baker, J.B., and Langdon, O.G. 1990. *Pinus taeda* L., Loblolly Pine. *In* Silvics of North America, Vol. 1: Conifers. *Edited by* R.M. Burns, and B.H. Honkala. U.S. Dep. Agric. Agric. Handb. 654, Washington, D.C. pp. 497-512.

Barnard, E.L., and Blakeslee, G.M. 1987. Pitch canker of southern pines. Plant pathology circular No. 302.Fla. Dept. of Agric. & Consumer Serv.

Barnard, E.L., Gilly, S.P., and Dixon, W.N. 1991. Incidence of *Heterobasidion annosum* and other root-infecting fungi in residual stumps and roots in thinned slash pine plantations in Florida. Plant Dis. 75: 823-828.

Barnett, J.P., and McGilvray, J.M. 1993. Performance of container and bareroot loblolly pine seedlings on bottomlands in South Carolina. South. J. Appl. For. 17: 80-83.

Barras, S.J., and Perry, T., 1971. *Leptographium terebrantis* sp. nov. associated with *Dendroctonus terebrans* in loblolly pine. Mycopathologia 43: 1-10.

Barron, G.L. 1968. The genera of hyphomycetes from soil. Williams & Wilkins, Baltimore, Maryland, USA. pp. 364.

Bechtold, W.A., Ruark, G.A., and Lloyd, F.T. 1991. Changing stand structure and regional growth reductions in Georgia's natural pine stands. Forest Sci. 37(2): 703-717.

Berryman, A.A., and Ferrel, G.T. 1988. The Fir Engraver Beetle in Western States. *In* Dynamics of forest insect populations: patterns, causes, implications, *Edited by* A.A. Berryman. New York: Plenum Press.

Bertagnole, C.L., Woo., J.Y., and Partridge, A.D. 1983. Pathogenicity of five *Verticicladiella* species to lodgepole pine. Can. J. Bot. 61: 1861-1867.

Bleiker, K.P., and Uzunovic, A. 2004. Fast- and slow-growing subalpine fir produce lesions of different sizes in response to inoculation with a blue-stain fungus associated with *Dryocoetes confuses* (Coleoptera: Scolytidae). Can. J. Bot. 82: 735-741.

Bongarten, B.C., and Teskey, R.O. 1987. Dry weight partitioning and its relationship to productivity in loblolly pine seedlings from seven sources. Forest Sci. 33(2): 255-267.

Booker, F.L., and Maier, C.A. 2001. Atmospheric carbon dioxide, irrigation, and fertilization effects on phenolic and nitrogen concentrations in loblolly pine (*Pinus taeda*) needles. Tree Physiol. 21: 609-616.

Brown, H.D., and McDowell, W.E. 1968. Status of loblolly pine die-off on the Oakmulgee District, Talladega National Forest, Alabama - 1968. U.S. Dep. Agric. For. Serv. Rept. No. 69-2-28.

Bryant, J.P., Chapin III, F.S., and Klein, D.R. 1983. Carbon nutrient balance in of boreal plants in relation to vertebrate herbivory. Oikos 40: 357-368.

Butnor, J. R., Seiler, J. R., and Gray, J. A. 2000. Influence of procerum root disease on the water relations of eastern white pine (*Pinus strobus* L.). J. Sustain. For. 10 (1/2): 95-105.

Campbell, W.A., and Copeland, O.L. 1954. Littleleaf disease of shortleaf and loblolly pines. U.S. Dep. Agric. For. Serv. Circular No. 940.

Campbell, R.K., and Rediske, J.H. 1966. Genetic variability of photosynthetic efficiency and dry-matter accumulation in seedling Douglas-fir. Silvae Genet. 15: 65-72.

Carles, S., Lamhamedi, M.S., Beaulieu, J., Stowe, D.C., and Margolis, H.A. 2012. Genetic parameters of morphological and physiological characteristics of containerized white spruce (*Picea glauca* [Moench.] Voss) seedlings. Tree Genet. Genomes 8: 39-51.

Castello, J.D., Silverborg, S.B. and Manion, P.D. 1985. Intensification of ash decline in New York State from 1962 through 1980. Plant Dis. 69: 243-246.

Cobb, F.W. 1988. *Leptographium wageneri*, cause of black-stain root disease: a review of its discovery, occurrence and biology with emphasis on pinyon and ponderosa pine. *In* *Leptographium* root diseases on conifers. *Edited by* T.C. Harrington and F.W. Cobb. American Phytopathological Society, St. Paul, MN.

Coley, P.D., Bryant, J.P., and chapin III F.S. 1985. Resource availability and plant antiherbivore defense. Science 230: 895-899.

Cook, S. P., and Hain, F.P. 1985. Qualitative examination of the hypersensitive response of loblolly pine, *Pinus taeda* L., inoculated with two fungal associates of the southern pine beetle, *Dendroctonus frontalis* Zimmermann (Coleoptera: Scolytidae). Environ. Entmol. 14(4): 396-400.

Copeland, O.L., and McAlpine, R.G. 1955. The interrelations of littleleaf, site index, soil, and ground cover in piedmont shortleaf pine stands. Ecology 36 (4): 635-641.

Dalla-tea, F., and Jokela, E.J. 1994. Needlefall returns and resorption rates of nutrients in young intensively managed slash and loblolly pine stands. Forest Sci. 40(4): 650-662.

Dewald, L., White, T.L., and Duryea M.L. 1992. Growth and phenology of seedlings of four contrasting slash pine families in ten nitrogen regimes. Tree Physiol. 11: 255-269.

Dickson, R.E. 1991. Assimilate distribution and storage. *In* Physiology of Trees. *Edited by* A.S. Raghavendra. John Wiley & Sons, New York. pp. 51-85.

Diamandis, S., Epstein, L., Cobb, F.W., Popenuck, T., and Hecht-Poinar, E. 1997. Development of *Leptographium wageneri* on root surfaces and other substrata. Eur. J. For. Path. 27: 381-390.

Dochinger, L.S. 1967. *Leptographium* root decline of eastern white pine. Phytopathology 57: 809.

Dumroese,R.K. 2003. Hardening fertilization and nutrient loading of conifer seedlings. *In* National Proceedings: Forest and Conservation Nursery Associations—2002. *Edited by* L.E. Riley, R.K. Dumroese, and T.D. Landis. RMRS-P 28. Forest Service Rocky Mountain Research Station, Ogden, UT. pp. 31-36.

Duong, T.A., De Beer, Z.W., Wingfield, B.D., and Wingfield, M.J. 2012. Phylogeny and taxonomy of species in the *Grosmannia serpens* complex. Mycologia 104(3): 715-732.

Eckhardt, L.G., and Menard, R.D. 2008. Topographic features associated with loblolly pine decline in central Alabama. Forest Ecol. Manag. 255: 1735-1739.

Eckhardt, L.G., and Menard, R.D. 2009**.** Declining loblolly pine stands: symptoms, causes and management options. AL Treasured Forest Magazine Volume XXV111, No. 2, pp. 10-12.

Eckhardt, L G., Jones, J.P., and Klepzig, K.D. 2004a. Pathogenicity of *Leptographium* species associated with loblolly pine decline. Plant Dis. 88: 1174-1178.

Eckhardt, L.G., Sword-Sayer, M.A., and Imm, D. 2010. State of pine decline in the southeastern United States. South J. Appl. For. 34: 138-141.

Eckhardt, L.G., Goyer, R.A., Klepzig, K.D., and Jones, J.P. 2004b. Interaction of *Hylastes* species (Coleoptera: Scolytidae) with *Leptographium* species associated with loblolly pine decline. J. Econ. Entomol. 97(2): 468-474.

Eckhardt, L.G., Weber, A.M., Menard, R.D., Jones, J.P., and Hess, N.J. 2007. Insect-fungal complex associated with loblolly pine decline in central Alabama. Forest Sci. 53(1): 84-92.

Edmonds, R.L., Agee, J.K., and Gara, R.I. (*Editors*) 2000. Forest Declines. *In* Forest health and protection. Waveland press, Inc. pp. 364-381

Feeny, P. 1976. Plant apparency and chemical defenses. Recent Adv. Phytochem. 10: 1-40.

Fernandez, M.M. F., Garcia, A.E., and Lieutier, F. 2004. Effects of various densities of *Ophiostoma ips* inoculations on *Pinus sylvestris* in north-western Spain. For. Path. 34: 213-223.

Gebauer, R.L.E., Strain, B.R., and Reynolds, J.F. 1998. The effect of elevated CO2 and N availability on tissue concentrations and whole plant pools of carbon-based secondary compounds in loblolly pine (*Pinus taeda*). Oecologia 113: 29-36.

Gadbury, G.L., Williams M.S., and Schreuder H.T. 2004. Revisiting the southern pine growth decline: Where we are 10 years later? US For. Serv. Gen. Tech. Rep. RMRS-GTR-124. pp. 10.

Gibbs, J.N., and Inman, A. 1991. The pine shoot beetle *Tomicus piniperda* as a vector of blue-stain fungi to windblown pine. Forestry 64(3): 239-249.

Gower, S.T., Isebrands, J.G., and Sheriff, D.W. 1995. Carbon allocation and accumulation in conifers. *In* Resource Physiology of Conifers, Acquisition, Allocation, and Utilization. *Editied by* W.K. Smith and T.M. Hinckley. Academic Press, New York. pp. 217-254.

Hacke, U.G., Sperry, J.S., Ewers, B.E., Ellsworth, D.S., Schafer, K.V.R., and Oren, R. 2000. Influence of soil porosity on water use in *Pinus taeda*. Oecologia. 124: 495-505.

Hansen, E.M., Goheen, D.J., Hessberg, P.F., Witcosky, J.J., and Schowalter, T.D. 1988. Biology and management of black-stain root disease in Douglas-fir. *In Leptographium* root diseases on conifers. *Edited by* T.C. Harrington and F.W. Cobb. American Phytopathological Society, St. Paul, MN.

Harrington, T.C. 1981. Cycloheximide sensitivity as a taxonomic character in *Ceratocystis*. Mycologia 73: 1123-1129.

Harrington, T.C. 1987. New combinations in *Ophiostoma* of *Ceratocystis* species with *Leptographium* anamorphs. Mycotaxon 28: 39-43.

Harrington, T.C., and Cobb F.W. 1983. Pathogenicity of *Leptographium* and *Verticicladiella* spp. isolated from roots of western North American conifers. Phytopathology 73: 596-599.

Hausner G., Reid J., and Klassen G.R. 1992. Do galeate-ascospore members of the *Cephaloascaceae*, *Endomycetaceae* and *Ophiostomataceae* share a common phylogeny? Mycologia 84: 870–881.

Haynes, R. W., Adams, D. M., Alig, R. J., Ince, P. J., Mills, J. R., and Zhou, X. 2007. The 2005 RPA timber assessment update. Gen. Tech. Rep. PNW-GTR-699. Portland, OR: U.S. Department of Agriculture, Forest Service, Pacific Northwest Research Station. pp. 212.

Haywood, J.D. 1994. Early growth reductions in short rotation loblolly and slash pine in central Louisiana. Southern J. Appl. For. 18: 35-39.

Herms, D.A., and Mattson, W.J. 1992. The dilemma of plants: to grow of defend. Q. Rev. Biol. 67: 283-335.

Hess, N.J., Otrosina, W.J., Carter, E.A., Steinman, J.R., Jones, J.P., Eckhardt, L.G., Weber,A.M., and Walkinshaw, C.H. 2002. Assessment of loblolly pine decline in central Alabama. Proceedings of the eleventh biennial southern silvicultural research conference. Gen. Tech. Rep. SRS–48.: U.S. Department of Agriculture, Forest Service, Southern Research Station, Asheville, NC. pp. 622.

Hessburg, P.F., and Hansen, E.M. 2000. Infection of Douglas-fir by *Leptographium wageneri*. Can. J. Bot. 78: 1254-161.

Hibben, C.R., and Silverborg, S.B. 1978. Severity and causes of ash dieback. J. Arboric. 4: 151-156.

Hicks, B.R., Cobb, F.W., Jr., and Gersper, P.L. 1980. Isolation of *Ceratocystis wageneri* from forest soil with a selective medium. Phytopathology 70: 880-883.

Hinkanen, T., and Haukioja, E. 1998. Intra- plant regulation of growth and plant- herbivore interactions. Ecoscience 5: 470-479.

Holopainen, K.J., Rikala, R., Kainulainen, P., and Oksanen, J. 1995. Resource partitioning to growth, storage and defense in nitrogen – fertilized Scots pine and susceptibility of the seedlings to the tarnished plant bug *Lygus rugulipennis.* New Phytol. 131: 521- 532.

Houston, D.R. 1981. Stress triggered tree diseases. The diebacks and declines. U.S. For. Serv. NS-INF-41-81. pp. 36.

Huber, D.M., and Thompson, I.A. 2007. Nitrogen and plant disease. *In* Mineral Nutrition and Plant Disease. *Edited by* L.E. Datnoff, W.H. Elmer, and D.M. Huber. The American Phytopathological Society Press, St. Paul, MN. pp. 31-44.

Hughes, S.J. 1953. Conidiophores, conidia, and classification. Can. J. Bot. 31: 577-659.

Ibrahim, M.H., Jaafar, H.Z.E., Rahmat, A., and Rahman, Z.A. 2011. Effects of nitrogen fertilization on synthesis of primary and secondary metabolites in three varieties of kacip Fatimah (*Labisia pumila* Blume). Int. J. Mol. Sci. 12: 5238-5254.

Jarrell, W.M., and Beverly, R.B. 1981. The dilution effect in plant nutrition studies. Adv. Agron. 34: 197-224.

Johnson, J.J., and Cline, M.L. 1991. Seedling quality of southern pines. *In* Forest Regeneration Manual, *Edited by* M.L. Duryea, and P.M. Dougherty. Klewer Academic Publishers, The Netherlands. pp. 143-159.

Joseph, G., Kelsey, R.G., and Thies, W.G. 1998. Hydraulic conductivity in roots of ponderosa pine infected with black-stain (*Leptographium wageneri*) or annosus (*Heterobasidion annosum*) root disease. Tree Physiol. 18: 333-339.

Karkan, R., and Baldwin, I.T., 1997. Induced responses to herbivory. Chicago: the University of Chicago Press.

Kendrick, B.W. 1962. The *Leptographium* complex. Can. J. Bot. 40 6: 771-797.

King, N.T., Seiler, J.R., Fox, T.R., and Johnsen K.H. 2008. Post-fertilization physiology and growth performance of loblolly pine clones. Tree Physiol. 28: 703-711.

Klepzig, K.D., Raffa, K.F., and Smalley, E.B. 1991. Association of an insect-fungal complex with red pine decline in Wisconsin. Forest Sci. 37(4): 1119-1139.

Klepzig, K.D., Smalley, E.B., and Raffa, K.F. 1996. Interactions of ecologically similar saprogenic fungi with healthy and abiotically stressed conifers. For. Ecol. Manag. 86: 163-169.

Klos, R.J., Wang G.G., Bauerle, W.L., and Rieck, J.R. 2009. Drought impact on forest growth and mortality in the southeast USA: an analysis using Forest Health and Monitoring data. Ecol. Appl. 19(3): 699-708.

Kováčik, J., and Bačkor, M. 2007. Changes of phenolic metabolism and oxidative status in nitrogen-deficient *Matricaria chamomilla* plants. Plant Soil 297: 255-265.

Kozlowski, T.T. 1969. Tree physiology and forest pests. J. Forestry 67(2): 118 - 123.

Kraus, J.F., and Hunt, D.L. 1971. Inherent variation of resistance to hypodermalethale in slash and loblolly pines. Forest Sci. 17(1):143-144.

Lackner, A.L., and Alexander, S.A. 1982. Occurrence and pathogenicity of *Verticicladiella procera* in Christmas tree plantations in Virginia. Plant Dis. 66: 211-212.

Långström, B., Solheim, H., Hellqvist, C., and Krokene, P. 2001. Host resistance in defoliated pine: effects of single and mass inoculations using bark beetle-associated blue-stain fungi. Agric. For. Entomol. 3: 211-216.

Lattanzio, V., Lattanzio, V.M.T., and Cardinali, A. 2006. Role of phenolics in the resistance mechanisms of plants against fungal pathogens and insects. *In* Phytochemistry: Advances in Research. Research Signpost, Kerala, India. pp. 23-67.

Leaphart, C.D., and Copeland, O.L. 1957. Root and soil relationships associated with the pole blight disease of western white pine. Soil Science Society Proceedings. pp. 551-554.

Ledig, T., and Perry, T.O. 1969. Net assimilation rate and growth in loblolly pine seedlings. Forest Sci. 15(4): 431-438.

Lewinsohn, E., Gijzen, M., and Croteau, R. 1991. Defence mechanisms of Conifers, Relationship of monoterpene cyclase activity to anatomical specialization and oleoresin monoterpene content. Plant Physiol. 96: 38-43.

Li, B., McKeand, S.E., and Allen, H.L. 1991. Genetic variation in nitrogen use efficiency of loblolly pine seedlings. Forest Sci. 37(2): 613-626.

Lieutier, F., 2002. Mechanisms of resistance in conifers and bark beetle attack strategies. *In* Mechanisms and deployment of resistance in trees to insects. *Edited by* M.R. Wagner et al. Kluwer Academic Publishers. Printed in the Netherlands. pp. 31-77.

Lieutier, F., Garcia, J., Romary, P., Yart, A., Jactel, H., and Sauvard, D. 1993. Inter-tree variability in the induced defense reaction of Scots pine to single inoculations by *Ophiostoma brunneo-ciliatum*, a bark-beetle-associated fungus. For. Ecol. Manag. 59: 257-270.

Litton, C.M., Raich, J.W., and Ryan, M.G. 2007. Carbon allocation in forest ecosystems. Glob. Change Biol. 13: 2089-2109.

Livingston, W.H., Mangini, A.C., and Mielke M.E. 1983. Association of root diseases and bark beetles with *Pinus Ponderosa* in New Mexico. Plant Dis. 67: 674-676.

Loomis, W.E. 1932. Growth-differentiation balance vs carbohydrate-nitrogen ratio. Proceedings American Society of Horticultural Science 29: 240-245.

Lorimer, C.G. 2001. Historical and ecological roles of disturbances in Eastern North American forests: 9000 years of change. Wildlife Soc. B. 29(2) 425-439.

Lorio, P.L. Jr. 1986. Growth-differentiation balance: a basis for understanding southern pine beetle-tree interactions. Forest Ecol. Manag. 14: 259-273.

Lorio, P.L., and Hodges, J.D. 1968. Microsite effects on oleoresin exudation pressure of large loblolly pines. Ecology 49(6): 1207-1210.

Luxmoore, R.J., Oren, R, Sheriff, D.W., and Thomas, R.B. 1995. Source-sink-storage relationships of conifers. *In* Resource Physiology of Conifers, Acquisition, Allocation, and Utilization. *Edited by* W.K. Smith and T.M. Hinckley. Academic Press, New York. pp. 179-216.

Manion, P. D. (*Editor*). 1981. Tree disease concepts. Prentice-Hall, Inc. NJ.

Marschner, P. 2012. Marschner's Mineral Nutrition of Higher Plants, third edition. Academic Press, New York. pp. 651.

Matusick, G. 2010. Pathogenicity and virulence of root-inhabiting ophiostomatoid fungi on *Pinus* species of the southeastern United States. Ph.D. Dissertation, School of Forestry and Wildlife Sciences, Auburn University, Auburn, AL.

Matusick, G., and Eckhardt, L.G. 2010a. Variation in virulence among four root-inhabiting ophiostomatoid fungi on *Pinus taeda* L., *P. palustris* Mill., and *P. elliottii* Englem. seedlings. Can. J. Plant Path. 32: 361-367.

Matusick, G., and Eckhardt, L.G. 2010b. The pathogenicity and virulence of four Ophiostomatoid fungi on young Longleaf pine trees. Can. J. Plant Path. 32(2): 172-176.

Matusick, G., Eckhardt, L.G., and Somers, G. 2010. The pathogenicity of four *Leptographium* species to mature longleaf pine roots. For. Ecol. Mgmt. 260: 2189-2195.

Matusick, G., Somers, G., and Eckhardt, L.G. 2012. Root lesions in large loblolly pine (*Pinus taeda* L.) following inoculation with four root-inhabiting ophiostomatoid fungi. Forest Pathol. 42: 37-43.

McCrady, R.L., and Jokela, E.J. 1997. Canopy dynamics, light interception,and radiation use efficiency of selected loblolly pine families. Forest Sci. 44(1): 64-72.

McKeand S., Mullin T., Byram T., and White T. 2003. Deployment of Genetically Improved Loblolly and Slash Pines in the South. J. Forest. 101(3): 32-37.

McKeand, S., Isik, F., Cumbie, P., and Whetten, R., 2009. Loblolly pine breeding in the Southern United States. *In* A joint Noveltree & Treebreedex event *abstract.*

Millers, I., Shriner, D.S., and Rizzo, D. 1989. History of hardwood decline in the eastern United States. U.S. Dep. Agric. For. Serv. Gen. Tech. Rep. NE-126.

Mueller-Dombois, D. 1983. Canopy dieback and successional processes in Pacific forests. Pacific Sci. 37:317-325.

Muzika, R.M. 1993. Terpenes and phenolics in response to nitrogen fertilization: a test of the carbon/nutrient balance hypothesis. Chemoecology 4(1): 3-7.

Nambiar, E.K., and Fife, D.N. 1991. Nutrient retranslocation in temperate conifers. Tree Physiol. 9: 185-207.

Nebeker, T.E., Hodges, J.D., and Blanche, C.A. 1993. Host response to bark beetle and pathogen colonization. *In* Beetle-Pathogen Interactions in Conifer Forests. *Edited by* T.D. Schowalter and G.M. Filip. Academic Press. San Diego, CA. pp. 157-169.

Neely, D. 1976. Iron deficiency chlorosis of shade trees. J. Arboric. 2: 128-130.

Nevill, R.J., Kelley, W.D., Hess, N.J., and Perry, T.J. 1995. Pathogenicity to loblolly pines of fungi recovered from trees attacked by southern pine beetles. South. J. Appl. For. 19(2): 78-83.

Nicholson, R.L., and Hammerschmidt, R. 1992. Phenolic compounds and their role in disease resistance. Ann. Rev. Phytopathology 30: 369-389.

Otrosina, W. J., Hess, N. J., Zarnoch, S. J., Perry, T. J., and Jones, J. P. 1997. Blue-stain fungi associated with roots of southern pine trees attacked by the southern pine beetle, *Dendroctonus frontalis*. Plant Dis. 81: 942-945.

Otrosina, W.J., Bannwart, D., and Roncadori, R.W. 1999. Root-infecting fungi associated with a decline of longleaf pine in the southeastern United States. Plant Soil 217: 145-150.

Otrosina, W.J., Walkinshaw, C.H., Zarnoch, S.J., Sung, S., and Sullivan, B.T. 2002. Root disease, longleaf pine mortality, and prescribed burning. *In* Proceedings of the eleventh biennial southern silvicultural research conference. U.S. Dep. Agric. For. Serv. Gen. Tech. Rep. SRS-48. Asheville, NC.

Owen, D.R., Lindahl, K.Q., Wood., D.L., and Parmeter Jr., J.R. 1987. Pathogenicity of fungi isolated from *Dendroctonus valens, D. brevicomis,* and *D. ponderosae* to ponderosa pine seedlings. Phytopathology 77: 631-636.

Paine, T.D., and Hanlon, C.C. 1994. Influence of oleoresin constituents from *Pinus ponderosa* and *Pinus jeffreyi* on the growth of the mycangial fungi from *Dendroctonus ponderosae* and *Dendroctonus brevicomis.* J. Chem. Ecol. 20: 2551-63.

Paine, T. D., Raffa, K. F., and Harrington, T. C. 1997. Interactions among scolytid bark beetles, their associated fungi, and live host conifers. Annu. Rev. Entomol. 42: 179-206.

Pallardy, S.G., Cermak, J., Ewers, F.W., Kaufmann, M.R., Parker, W.C., and Sperry, J.S. 1995. Water transport dynamics in trees and stands. *In* Resource physiology of conifers: Acquisition, allocation, and utilization. *Edited by* W.K. Smith, and T.M. Hinckley. Academic Press, Inc., New York. pp. 301–389.

Parmeter Jr., J.R. Slaughter, G.W., Chen, Mo-Mei., Wood, D.L., and Stubbs, H.A. 1989. Single and mixed inoculations of Ponderosa pine with fungal associated of *Dendroctonus* spp. Phytopathology 79: 768-772.

Pecknold, P.C. 1978. "Dutch Elm Disease" Historical Documents of the Purdue Cooperative Extension Service. Paper 401.

Phelps, W.R., and Czabator, F.L. 1978. Fusiform rust of southern pines. U.S. Dep. Agric., For. Serv., Forest Insect and Disease Leaflet 26.

Piepho, H.P., Büchse, A., and Emrich K. 2003. A hitchhiker's guide to mixed models for randomized experiments. J. Agron. Crop Sci. 189: 310-322.

Raffa, K.F. 1991. Induced defense reactions in conifer bark beetle systems. *Edited by* D.W. Tallamy and M.J. Raupp. Phytochemical Induction by Herbivores Acad Press NY. pp 245-276.

Raffa, K.F., and Smalley, E.B. 1988. Host resistance to invasion by lower stem and root infesting insects of pine: response to controlled inoculations with the fungal associate *Leptographium terebrantis*. Can. J. For. Res. 18: 675-681.

Rane, K.K., and Tattar, T.A. 1987. Pathogenicity of blue-stain fungi associated with *Dendroctonus terebrans*. Plant Dis. 71: 879-883.

Reid, R.W., Whitney, H.S., and Watson, J.A. 1967. Reactions of lodgepole pine to attack by *Dendroctonus ponderosae* Hopkins and blue stain fungi. Can. J. Bot. 45: 115- 126.

Retzlaff, W.A., Handest, J.A., O'Malley, D.M., McKeand, S.E., and Topa, M.A. 2001. Whole-tree biomass and carbon allocation of juvenile trees of loblolly pine (*Pinus taeda*): influence of genetics and fertilization. Can. J. Forest Res. 31: 960-970.

Rice, A.V., Thormann, M.N., and Langor, D.W. 2007. Virulence of, and interactions among, mountain pine beetle associated blue-stain fungi on two pine species and their hybrids in Alberta. Can. J. Bot. 85: 316-323.

Richardson, S.D. 1960. The role of physiology in forest tree improvement. *In* proceedings of the Fifth World Forestry Congress. Seattle, WA. pp. 733-741.

Robbins, K. 1984. Annosus root rot in eastern conifers. U.S. Dep. Agric., For. Serv., Forest Insect and Disease Leaflet 76.

Robinson, G.K., 1991. That BLUP is a good thing: The estimation of random effects. Stat. Sci. 6: 15-51.

Robinson-Jeffrey, R.C., and Grinchenko, A.H.H. 1964. A new fungus in the genus *Ceratocystis* occurring on blue-stained lodgepole pine attacked by bark beetles. Can. J. Bot. 42: 527-532.

Rossiter, M., Schultz, J.C., and Baldwin, I.T. 1998. Relationships among defoliation, red oak phenolics, and gypsy moth growth and reproduction. Ecology 69: 267-277.

Roth, E.R. 1954. Spread and intensification of the littleleaf disease of pine. J. Forest. 52: 592-596.

Roth, E.R., and Preacher, P.H. 1971. Alabama loblolly pine die-off evaluation. Report No. 72-2-9. U.S. Dep. Agric. For. Serv. Southeastern area, state and private forestry forest pest management group. Pineville, LA. 8p.

Ruel, J.J., Ayres, M.P., and Lorio, P.L. Jr. 1998. Loblolly pine responds to wounding with increased resin flow. Can. J. Forest Res. 28: 596-602.

Rühmnn, S. Leser, C., Bannert, M., and Treutter, D. 2002. Relationship between growth, secondary metabolism and resistance of apple. Plant Biol. 4: 137-143.

Samuelson, E.J. 2000. Effects of nitrogen on leaf physiology and growth of different families of loblolly and slash pine. New Forest. 19**:** 95-107.

Sandnes, A., and Solheim, H. 2002. Variation in tree size and resistance to *Ceratocystis polonica* in a monoclonal stand of *Picea abies*. Scand. J. For. Res. 17: 522-528.

Saxe, H. 1993. Triggering and predisposing factors in the "Red" decline syndrome of Norway spruce (*Pinus abies*). Trees 8: 39-48.

Saxon, M.E., Davis, M.A., Pritchard, S.G., Runion, G.B., Prior, S.A., Stelzer, H.E., Rogers, H.H., and Dute, R.R. 2004. Influence of elevated CO2, nitrogen, and *Pinus elliottii* genotypes on performance of the redheaded pine sawfly, *Neodiprion lecontei.* Can. J. Forest Res. 34: 1007-1017.

Schmidtling, R.C., and Amburgey, T.L. 1982. Genetic varratron in decay susceptibility and its relationship to growth and specific gravity in loblolly pine. Holzforschung 36.159-161.

Schowalter, T.D., and Filip, G.M. 1993. Bark beetle-pathogen-conifer interactions: an overview. In Beetle-Pathogen Interactions in Conifer Forests. Edited by T.D. Schowalter and G.M. Filip. Academic Press Inc. San Diego, CA. pp. 4.

Schutt, P., and Cowling, E.B. 1985. Waldsterben, a general decline of forests in central Europe: symptoms, development and possible causes. Plant Dis. 69: 548-558.

Scortichini, M. 2002. Bacterial canker and decline of European Hazelnut. Plant Dis. 86 (7) 704-709.

Seifert, K.A. 1993. Sapstain of commercial lumber by species of *Ophiostoma* and *Ceratocystis*. Chap. 16. *In Ceratocystis* and *Ophiostoma*, taxonomy, ecology and pathogenicity. *Edited by* J.M. Wingfield, K.A. Seifert, and J.F. Webber. APS Press, St. Paul, Minn. pp. 141–151.

Shultz, R.P. 1997. Loblolly pine, the ecology and culture of loblolly pine (*Pinus taeda* L.). U.S. Dep. Agric., Forest Service, Agric. Handb. 713. Washington, D.C. pp. 1-16.

Sinclair, W.A. 1967. Decline of hardwoods: possible causes. *In* Proceedings of the International Shade Tree Conference. pp. 17-32

Sinclair, W.A., and Hudler, G.W. 1988. Tree declines: Four concepts of causality. J. Arboric. 14(2): 29-35.

Singleton, V.L., and Rossi, Jr., J.A. 1965. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. Am. J. Enol. Viticult. 16: 144-158.

Smith, W.B., Patrik, D.M., Charles, H.P., and Scott, A.P. 2007. Forest Resources of the United States. A technical Document Supporting the Forest Service 2010 RPA Assessment.

Spatafora, J.W., and Blackwell, M. 1994. The polyphyletic origins of ophiostomatoid fungi. Mycol. Res. 98: 1-9.

Spencer, J.F.T., and Gorin, P.A.J. 1974. Systematics of the genera *Ceratocystis* and *Graphium.* Proton magnetic resonance spectra of the mannosecontaining polysaccharides as an aid in classification. Mycologia 63: 387-402.

Staley, J.M. 1965. Decline and mortality of red and scarlet oaks. Forest Sci. 11: 2-17.

Starkey, D.A., Oliveria, F., Mangini, A., and Mielke, M. 2004. Oak decline and red oak borer in the interior highlands of Arkansas and Missouri: Natural phenomena, severe occurrences. *In* Upland oak symposium: history, current conditions, and sustainability. U.S. Dep. Agric. For. Serv. Southern Research Station. Gen. Tech. Rep. SRS-73. Asheville, NC. pp. 311.

Steele, R.G.D., and Torrie, J.H. 1980. Principles and Procedures of Statistics, A Biometrical Approach, second edition. McGraw-Hill Book Company, New York. pp. 633.

Stovall, J.P., Fox, T.R., and Seiler, J.R. 2012. Short-term changes in biomass partitioning of two full-sib clones of *Pinus taeda* L. under differing fertilizer regimes over 4 months. Trees-Struct. Funct. 26(3) 951:961.

Sword, M.A., Tiarks, A.E., and Haywood, J.D. 1998. Establishment treatments affect the relationships among productivity and competing vegetation of loblolly pine on a Gulf Coastal Plain site. Forest Ecol. Manag. 105: 175-188.

Thatcher, R.C., and Barry, P.J. 1982. Southern Pine Beetle. Forest Insect and Disease Leaflet 49. USDA, Forest Service.

Trani, M.K., Brooks, R.T., Schmidt,T.L., Rudis, V.A., and Gabbard, C.M. 2001. Patterns and trends of early successional forests in the Eastern United States. Wildlife Soc. B. 29(2): 413-424.

Tyree, M.C., Seiler, J.R., Mayer, C.A., and Johnsen, K.H. 2009. *Pinus taeda* clones and soil nutrient availability: effects of soil organic matter incorporation and fertilization on biomass partitioning and leaf physiology. Tree Physiol. 29: 1117-1131.

US Forest Service. 1988. *The South's fourth forest.* Forest Resource, US For. Serv. Rep. No. 24. US For. Serv., Washington, DC. pp. 512.

Uzunovic, A., Yang, D-Q., Gagne, P., Breuil, C., Bernier, L., Byrne, A., Gignac, M., and Kim, S.H. 1999. Fungi causes sapstain in Canadian softwoods. Can. J. Microbiol. 45: 914-922.

Vose, J.M., and Allen, H.L. 1988. Leaf area, stemwood, and nutrition relationships in loblolly pine. Forest Sci. 34: 547-563.

Warren, J.M., Meinzer, F.C., Brooks, J.R., Domec, J.C., and Coulombe, R. 2007. Hydraulic redistribution of soil water in two old-growth coniferous forests: Quantifying patterns and controls. New Phytol. 173: 753-765.

Wear, D.N., and Greis, J.G. 2002. Southern forest resource assessment: summary of findings. J. Forest. 100: 6-14.

Wermelinger, B., Rigling, A., Mathis, D.S., and Dobbertin, M. 2008. Assessing the role of bark and wood boring insects in the decline of Scots pine (*Pinus sylvestris*) in the Swiss Rhone valley. Ecol. Entmol. 33: 239-249.

Westing, A.H. 1969. Plants and salt in the roadside environment. Phytopathology 59: 1174-1179.

Wingfield, M.J. 1983. Association of *Verticicladiella procera* and *Leptographium terebrantis* with insects in the lake states. Can. J. For. Res. 13: 1238-1245.

Wingfield, M.J. 1985. Reclassification of *Verticicadiella* based on conidial development. Trans. Br. Mycol. Soc. 85: 81-93.

Wingfield, M.J. 1986. Pathogenicity of *Leptographium procerum* and *Leptographium terebrantis* on *Pinus strobus* seedlings and established trees. Eur. J. For. Path. 16: 299-308.

Wingfield, M.J., and Knox-Davies, P.S. 1980. Root disease, associated with *Verticicladiella alacris*, of pines in South Africa. Plant Dis. 64: 569-571.

Wingfield, M.J., and Gibbs, J.N. 1991. *Leptographium* and *Graphium* species associated with pine infesting bark beetles in England. Mycol. Res. 95(11): 1257-1260.

Wingfield, M.J., Capretti, P., and Mackenzie, M. 1988. *Leptographium* spp. as root pathogens of conifers. An international perspective. *In Leptographium* root diseases on conifers. *Edited by* T.C. Harrington and F.W. Cobb. American Phytopathological Society, St. Paul, MN.

Wright, E. 1933. A cork-borer method for inoculating trees. Phytopathology 23: 487-488.

Zanzot, J.W. 2009. Biology and ecology of root-feeding beetles and ophiostomatoid fungi in sandhills longleaf pine stands. Ph.D. Dissertation, School of Forestry and Wildlife Sciences, Auburn University, Auburn, AL.

Zanzot, J.W., Matusick, G., and Eckhardt, L.G. 2010. Ecology of root-feeding beetles and their associated fungi on longleaf pine in Georgia. Eviron. Entomol. 39(2): 415-423.

Zipfel, R.D., de Beer, W., Jacobs, K., Wingfield, B.D., and Wingfield, M.J. 2006. Multi-gene phylogenies define *Ceratocystiopsis* and *Grosmannia* distinct *Ophiostoma*. Stud. Mycol. 55: 75-97.

Zhou, X.D., De Beer, Z.W. Wingfield, B.D., and Wingfield, M.J. 2002. Infection sequence and pathogenicity of *Ophiostoma ips*, *Leptographium serpens* and *L. lundbergii* to pines in South Africa. Fungal Divers. 10: 229-240.