Response of *Pinus taeda* L. Families to Root-Inhabiting Ophiostomatoid Fungi

by

Pratima Devkota

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Approved by

Lori Eckhardt, Professor of Forest Entomology and Pathology
Scott Enebak, Professor of Forest Pathology
Zhaofei Fan, Associate Professor of Forestry
Joseph Kloepper, Professor of Plant Pathology
Kathy Lawrence, Professor of Plant Pathology

Abstract

Pinus taeda (loblolly pine), a most widely cultivated timber species in the southern U.S., creates 110,000 job opportunities and contributes 30 billion dollars per year to the economy. However, insect-vectored root-infecting ophiostomatoid fungi, *Leptographium terebrantis,* and *Grosmannia huntii* are potential threats to sustainable *P. taeda* forest management in the southern U.S. Understanding the intra-species response of *P. taeda* to these fungi is critical to mitigate the potential problem due to these fungi. Thus, the objectives of my research are: (i) to determine the intraspecific tolerance/susceptibility of *P. taeda* to *L. terebrantis* and *G. huntii*, (ii) to understand whether intraspecific tolerance of *P. taeda* to *L. terebrantis* and *G. huntii* remain same regardless of the tree growth stage (iii) to understand the interaction of the vascular-inhabiting fungi and *P. taeda* under drought conditions, (iv) to determine the antibiosis potential of these fungi by plant growth-promoting rhizobacteria (PGPR), (v) to understand whether PGPR can induce resistance of *P. taeda* families to these fungi, (vi) to determine the intraspecific variation in virulence of *L. terebrantis,* (vii) to determine the growth potential of most virulent *L. terebrantis* at different inoculum densities in *P. taeda* wood segments, and (viii) to determine the growth potential of various blue-stain fungi on *P. taeda* stem segments.

In study 1, seedlings from 94 *P. taeda* families were artificially inoculated at the stem with *L. terebrantis* and *G. huntii* and family responses were studied. In study 2, the roots of the mature *P. taeda* trees from 4 families were inoculated with these two fungi to

understand the intraspecific response of mature trees. In study 3, *P. taeda* families were exposed to drought and simultaneously inoculated with vascular-inhabiting fungi and impacts were studied. In study 4, PGPR strains and ophiostomatoid fungi were plated together in a dual agar plate and the antibiosis potential of PGPR strains to fungi was studied. In addition, induced systemic resistance of *P. taeda* to *L. terebrantis* and *G. huntii* were studied by inoculating PGPR in soil and fungi in stems of *P. taeda* seedlings. In study 5, most virulent *L. terebrantis* was inoculated to *P. taeda* stem segments at different inoculum densities. In study 6, various blue-staining ophiostomatoid fungi were cultured in *P. taeda* stem segments to study their growth potential.

The results suggest *P. taeda* families vary in tolerance to ophiostomatoid fungi with potential for selection of relatively tolerant families. Moreover, this intra-species variation in tolerance is an inherent character of *P. taeda*, regardless of the tree growth stage. The growth and productivity of *P. taeda* seedlings decrease and fungal pathogenicity increase under severe drought. Specific strains of PGPR have the ability to inhibit the growth of blue-staining fungi *in vitro*. Specific PGPR strains have the capacity to induce systemic resistance of *P. taeda* during fungal infection. Fungal growth and blue-staining potential are high when fungal inoculation points are closer. Growth and staining potential of *L. terebrantis* in *P. taeda* stem segment is higher compared to *G. huntii* and *G. alacris*. This study provides meaningful insights into *P. taeda* and ophiostomatoid fungal interaction.

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CHAPTER I

Introduction and Review of Literature

1.1 Forestry in the southern United States

The southern United States (U.S.) comprises 108.1 million hectares of forestland of which approximately 13% of the total volume of the timber in the U.S. is produced in the south. This region incorporates almost 50% of world's entire industrial forest plantations used for commercial timber production (Prestemon and Abt, 2002). *Pinus taeda* L. (loblolly pine) and *P. echinata* Mill. (shortleaf pine) are the major timber species Oswalt et al. (2012) which together account for 71% of softwood volume in the southern U.S. as shown in Table 1.1.

Table 1.1 Volume of timberland by softwood species in the southern U.S. (Oswalt et al., 2012)

Softwood Species	Total volume
Pinus taeda and *P. echinata*	71%
Pinus palustris Mill. (longleaf pine) and *P. elliottii* Englem. (slash pine)	13%
Cypress	6%
Pinus strobus L. (white pine) and *P. resinosa* Aiton (red pine)	2%
Yellow pines	5%
Tsuga canadensis (L.) Carr. (Eastern hemlock)	1%
Picea spp. (Spruce) and *Abies balsamea* (L.) Mill. (Balsam fir)	<1%
Other softwoods	2%

1.2 *Pinus taeda*

Pinus taeda (Figure 1.1), the most important commercial timber species in the southern U.S. is grown on 11.7 million hectares (Baker and Balmer, 1983a; Rauscher,

2004) which is 80% of the commercial forest area in the south (Smith et al., 1990). The geographical range of this species extends across 15 states in the south and mid-Atlantic region (Baker and Langdon, 1990). Approximately, one billion *P. taeda* seedling are planted each year (McNabb and Enebak, 2008). *Pinus taeda* grows in a variety of conditions producing a high wood volume yield per acre. It provides all-purpose marketable forest products, ecosystem services and place for recreational activities making it the most economical pine species in the southern U.S (Poudel, 2014; Schultz, 1997).

Figure 1.1 *Pinus taeda* stands in Cordele, Georgia, USA.

1.2.1 History of *Pinus taeda*

Pinus taeda was only a minor component (2.2 million hectares) of hardwood dominated Piedmont forest before European settlement in the U.S. (before the 1800's). These forests were converted into agricultural lands (especially cotton farming) after

European settlement. However, the introduction of the boll weevil (*Anthonomus grandis grandis* Boheman) impacted the growing cotton industry, and piedmont and coastal plains in the south remained unused. Abandoned land with eroded soil created a favorable condition for the spread of the light-seeded *P. taeda* trees resulting in the widespread growth of pure *P. taeda* stand (Schultz, 1997). Fire control programs conducted in the early 1900s further favored those stands. Furthermore, *P. taeda* stands expanded to cover the natural range of the species from eastern Texas to central Florida to southern New Jersey (Schultz, 1997).

1.2.2 Indicators of health and vigor of *P. taeda*

Crown morphology is one of the indicators of *P. taeda* tree health. Trees with larger crowns (high crown density) are stronger and have higher energy available for the stem growth. The live crown ratio indicates the health and growth potential of individual trees. Healthy *P. taeda* trees in the southern U.S have a live crown ratio of 0.40, or greater (Zhao et al., 2012).

Healthy roots are another indicator of the good health of *P. taeda*. The root system of a *P. taeda* consists of a taproot, lateral roots, and fine roots. The tap root is strong and extends downward. Lateral roots are smaller than taproot and grow in all directions covering larger areas. Ten or more lateral roots usually originate from the tap root of a tree at depths of 15-to-100 cm depending on the soil texture. Whereas, 3-to-10 lateral roots grow within 15 cm below the ground surface. These roots live for the entire life of a tree. Lateral and fine roots are the primary supporting roots, and soil water and nutrient

absorbing roots respectively (Schultz, 1997). Healthy roots are vital to *P. taeda* health and productivity.

1.2.3 Biotic and abiotic factors affecting growth of *P. taeda*

Various biotic and abiotic factors put *P. taeda* health at risk. Factors that influence *P. taeda* growth are temperature, soil type, fertilization, daylight length, moisture, genetics, wind and flooding, high and freezing temperature. Also, vegetative competition may affect the growth (Schultz, 1997). The soil moisture availability is critical for the growth of *P. taeda*. Moisture stress causes various physiological changes in *P. taeda* tree. Prolonged moisture stress can hinder or interrupt tree growth by affecting stomatal opening (Schultz, 1997).

Soil moisture conditions influence the stomatal opening of *P. taeda.* The needles of *P. taeda* seedlings growing under warm and moist condition have 70 % of the stomata open 14 hours after watering. Under similar soil moisture condition, 62 hours after watering, stomatal opening drops to 30% (Newton et al., 1987). However, individual trees may respond differently to moisture stress due to genetic differences. Drought resistant trees can resume the normal functioning of all the tissues after severe dehydration (Newton et al., 1987).

Various plant growth characteristics are affected by soil moisture. Such as annual rings, relative amounts of early wood and latewood production, needle growth and development, and root growth. In drier years, the radial growth of mature *P. taeda* trees reduces by 20% to 30% (Hiller and Brown, 1967). Moisture stress may negatively impact root growth and rate of water absorption by roots. The root-to-shoot ratio is larger in

seedlings growing under dry soil conditions than that compared to seedling growing under the moist soil. Needles grow 1 mm/day until they reach 70% of their maturity if the soil moisture is near field capacity. However, in drying soil, growth is only 0.4 to 0.6 mm/day until they reach maximum length (Kaufmann, 1968). *Pinus taeda* is tolerant to low levels of soil O_2 caused by excess moisture associated with flooding (Williston, 1962). Abiotic factors play a role in predisposing the trees to organisms like insects and fungi (Baker, 1972).

Pinus taeda is a host to many insects among which some cause significant damage. For example, *Dendroctonus frontalis* Zimmermann (southern pine beetle) among others is the most destructive pest in the southern U.S and is distinguished as a persistent threat of pine trees. Several outbreaks of this insect from 1999 to 2003 have occurred causing damage to millions of acres of state, federal, industrial and private forests (Thatcher and Barry, 1982). *Pinus taeda* stands on poor site, adverse weather, overstocking or over maturity are likely to be more stressed and are susceptible to infestation by *D. frontalis*. Even well-managed stands adjacent to stand with stressed trees are also at risk when the beetle populations are high (Baker and Balmer, 1983b).

Several other insects negatively impact *P. taeda* health. For example, pine engraver beetles (*Ips* spp.) are another important pest group of the southern pine forests. They consist of three species *I. calligraphus* (Germar), *I. grandicollis* (Eichoff) and *I. avulsus* (Eichoff) which have six, five and four spines respectively on their bodies' surfaces. These insects attack dying, unhealthy and recently felled trees (Connor and Wilkinson, 1983). Pine tip moths (*Rhyacionia* spp.) often infest the tips of the young

seedlings and saplings which are under 5 years of age (Fettig et al., 2000). Regeneration weevils such as *Hylobius pales* (Herbst) and *Pachylobius picivorus* (Germar) with their ophiostomatoid fungal associates are associated with *Pinus strobus* L. (white pine) and *Pinus resinosa* Sol. ex Aiton (red pine), and *P. taeda* decline in the northeastern and the southern U.S. (Erbilgin and Raffa, 2000; Eckhardt et al., 2007) respectively. Similarly, *Hylastes* spp. feed on the roots of pre-stressed conifers (Wood, 1982; Jacobs and Wingfield, 2001). *Hylastes salebrosus* Eichoff and *H. tenuis* Eichoff have been associated with pine decline (Klepzig et al., 1995; Jacobs and Wingfield, 2001; Eckhardt et al., 2007). However, accurate economic assessment of the damages caused by these pests in the southern U.S. forests is limited in recent literature.

Pinus taeda is a suitable host to fusiform rust caused by *Cronartium quercuum* f. sp. *fusiforme* (*Fusarium* spp. and *Macrophomia* spp.), root rot (*Heterobasidion irregulare* Fr.) and heart rot (*Phellinus pini* Tho. Ex. Fr.) (Baker and Balmer, 1983b) (Table 1.2). *Leptographium* Lagerb. and Melin and *Ophiostoma* H. and P. Sydow, commonly known as ophiostomatoid fungi infect lateral roots of pine trees, and, have been associated with pine decline (Harrington and Cobb, 1988; Eckhardt et al., 2004a; Eckhardt et al., 2007).

Table 1.2 Common fungal diseases of pine forests of the southern U.S.

Pathogen	Diseases	Host	References
Cronartium quercuum fusiforme	Fusiform rust	*P. elliottii*	Lamb and Sleeth (1940)
		P. taeda	Burdsall and Snow (1977)
			Lamb and Sleeth (1940)
Heterobasidion irregulare	Annosus root-rot	*P. elliottii*	Powers and Boyce (1961)
		P. taeda	Lightle (1960)
		P. echinata	Berry and Dooling (1962)
Armillaria spp.	Armallaria root- rot	Conifers	Hepting and Downs (1944)
Phytopthora cinnamomic	Littleleaf disease	Shortleaf pine	Hepting and Jemison (1950)
		Radiata pine	Newhook 1959
Hypoderma spp.	Needle cast disease	Loblolly pine	Boyce (1958);
		Longleaf pine	Hepting and Jemison (1950)
		Shortleaf pine	Boyce (1958)
Lophodermium pinastri		Slash pine	Boyce (1958)
Bifusella linearis		Virginia pine	Boyce (1958)
		Mountain pine	Boyce (1958)
		Pitch pine	Boyce (1958)
		All pines	Boyce (1958)
		Eastern white pine	Boyce (1958)
Fusarium circinatum	Pitch canker	Loblolly pine	Nirenberg and O'Donnell (1988)
Mycosphaerella dearnessii	Brown spot needle blight	Longleaf pine	Siggers (1944)
Leptographium procerum	Procerum root disease	Loblolly pine	Eckhardt et al.(2004a); Eckhardt et al. (2007)
Leptographium procerum	Southern Pine Decline (SPD)	Loblolly pine	Eckhardt (2004b);
Leptographium terebrantis		Longleaf pine	Zanzot et al. (2010); Matusick et al. (2010)
Grosmannia huntii			
Grosmannia alacris			

1.3 Concepts of forest decline

Forest decline can occur at a stand level or landscape level. Trees of the same species showing out-of-season leaf discoloration, diameter increment reduction and loss of crown vigor indicate a decline. Dieback often comes together with the decline which is the further concatenation of decline. Loss of leaves in the crown of the top limbs of the tree indicates the dieback. Tree death often follows the decline (Manion, 1981).

Forest decline is multifactorial in origin, and thus, often difficult to determine the exact factors causing decline (Manion, 1981). However, a common interpretation of the literature is that forest decline is a disorder involving abiotic stresses that modifies the physiology and predisposes trees to dieback and damage caused by biotic agents. There are several hypotheses regarding factors in forest decline.

The host, stress, and pathogen hypothesis describes decline as a disease. This assumption suggests that some form of environmental stress alters the host tissues, making it vulnerable to pathogen invasion. Invaded pathogen weakens the plant tissues, and further leads to tree decline (Ciesla and Donaubauer, 1994). The synchronous cohort senescence hypothesis considers decline as a part of forest dynamics involving various interacting factors. This concept suggests that certain sudden eliciting environmental factors such as drought and flood predispose the trees to pest and pathogen attacks (Ciesla and Donaubauer, 1994).

The most widely accepted hypothesis of forest decline is the chain reaction theory given by Sinclair (1965). According to this hypothesis, when predisposing, inciting and contributing factors come into play together, they cause forest decline (Sinclair, 1965). Manion (1981) assimilated this theory in his death spiral theory which helps to clarify the concept of decline and describes different biotic and abiotic agents as factors causing

decline. Moreover, this model gives emphasis on pathology which supports the idea that forest decline is a form of a disease. The predisposing factors are long-term factors such as the genetic potential of the tree, age, climate, soil factors, and air pollution. The inciting factors are short term factors such as frost, drought, salt, and mechanical injury. The contributing factors are bark beetles, canker fungi, and root decay fungi. Thus, the decline can be a slow process in which pathogens and insects always appear towards the end (Manion and Lachance, 1992).

1.4 Pine Decline (PD)

Pine Decline (PD) was first reported in 1959 in the Talladega National Forest in the Okmulgee and Tuscaloosa Ranger districts in Alabama (Brown and McDowell, 1968). This decline was termed as "loblolly pine die-off." Further incidences of mortality were reported during the early 1970s, which included the symptoms such as chlorotic crowns, thinned crowns, deteriorated fine and lateral roots and reduced growth (Roth and Preacher, 1971).

Phytophthora cinnamomi Rands was initially suggested as the causal agent of "loblolly pine die-off" (Hess et al., 1999). In the early 2000s, there were more reports of loblolly pine decline. Hess et al. (2002) further examined the conditions and factors associated with this early tree mortality and recovered many fungi from the soil root zone including, *P. cinnamomi* and *Leptographium* spp. Furthermore, Eckhardt et al. (2007) conducted a three-year study to investigate the roles of fungi and root-feeding bark beetles in pine decline. Root-feeding bark beetles such as *H. salebrosus* Eichoff, *H. tenuis*, *P. picivorus* and *H. pales* were recovered from areas with symptomatic trees showing symptoms of decline (Eckhardt et al., 2007). Eckhardt et al. (2004a) had

previously found that *Hylastes* species could vector the fungi *Leptographium* spp. Further studies have provided sufficient supporting evidence to prove the hypothesis that root-feeding bark beetles and their fungal associates are biotic factors associated with PD.

Recently, premature mortality in *P. taeda* has been described as a disease decline disorder which involves a complex interaction of biotic and abiotic factors (Eckhardt et al., 2007). The predisposing factors are abiotic factors including topography, drought, increased slope, and southwest facing aspects that predispose the pines to biotic factors such as bark beetles. The abiotic factors induce stress on the trees, and the stressed trees will then attract root and lower stem feeding bark beetles (Eckhardt et al., 2007). These beetles carry the fungus from an infected tree to a stressed tree by feeding activity (Paine and Hanlon, 1994). There is a mutualistic relationship between the ophiostomatoid fungi and the root feeding bark beetles that lead to an expansion of the decline disease complex (Paine et al., 1997). Figure 1.2 shows an advanced hypothesis of PD termed as diseae circle, in which various biotic and abiotic factors are adapted from the literature. Various predisposing factors such as tree genetics, age and topographical location of the tree, climatic and soil factors act as predisposing factors. Stand density, stand species composition, natural disturbance, anthropogenic factors and soil moisture stress act as inciting factors which stress the trees. Then the stressed trees attract insects and the associated fungi that act as contributing factors in the PD.

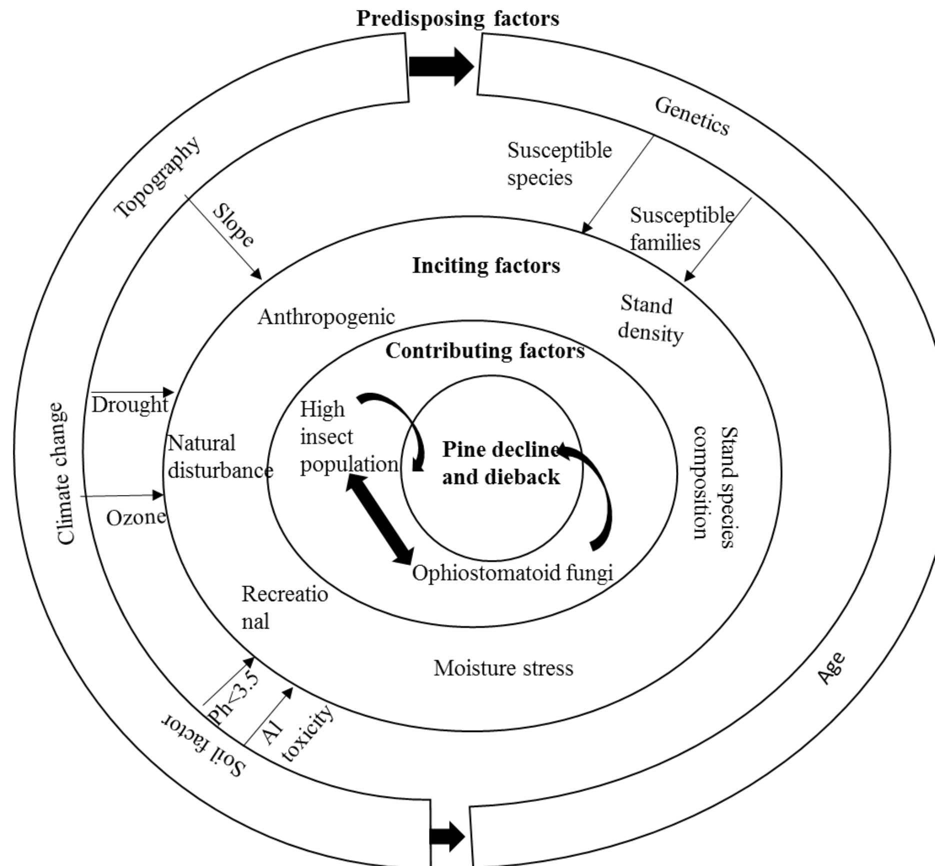

Figure 1.2 Model of southern pine decline (disease circle). Factors are adapted from many literatures.

1.5 Ophiostomatoid fungi

Ophiostomatoid fungi contains diverse anamorphs and teleomorphs with different patterns of conidium ontogeny, ascomatal centrum development, different biochemical composition and symbiotic association with the insect vector (Upadhyay, 1993). Some genera in this group are *Ophiostoma* Syd. and P. Syd., *Ceratocystis* Ellis and Halst., *Ceratocystiopsis* H.P Upadhyay and W.B. Kendr., *Grosmannia* Goid. as well as related asexual genera *Leptographium*, *Pesotum* J.L. Crane and Schokn., *Sporothrix* Hektoen and C.F. Perkins, *Hyalorhinocladiella* H.P. Upadhyay and W.B. Kendr., *Thielaviopsis* Went, *Chalara* Rabenh., and *Graphium* Cord. (Repe and Jurc, 2010).

Many of ophiostomatoid fungi are conifer pathogens and blue-staining fungi (Repe and Jurc 2010). They have darkly pigmented hyphae which cause blue staining of the plant tissue (Jacobs and Wingfield, 2001. They pose mucilaginous ascospores and conidia. The slimy masses of the spores are produced at the top of the long conidiophores which helps surface attachment to vectors (Wingfield et al.,1993). The fungi lead to superficial discoloration on the wood surface by sporulation. When they penetrate the wood, they cause blue coloration which reduces the economic value of the timber (Uzunovic et al., 1999).

1.5.1 *Leptographium* species (Lagerb and Melin.)

Leptographium, the anamorph of *Ophiostoma,* is distinguished by having dark mononematous conidiophores that give rise to a series of branching metulae. These conidiophores have conidiogenous cells at the terminal end which produce single-celled pigmented hyaline conidia. The conidia are produced through enteroblastic ontogeny and

holoblastic proliferation (Jacobs and Wingfield, 2001). Conidia are ideally suited for dispersal by insects as they accumulate in a slimy mass at the top of the conidiophore (Figure 1.3).

Leptographium spp. have cellulose, rhamnose, and chitin in their cell walls and are tolerant to high concentrations of cycloheximide (Jacobs and Wingfield, 2001). *Ceratocystis* could be associated with *Leptographium* species. The characteristics of *Ophiostoma* and the *Ceratocystis* are similar which led to doubt regarding the validity of the genera. However, currently, these two genera are accepted as being phylogenetically unrelated (Hausner et al., 1993; Spatafora and Blackwell, 1994).

Leptographium spp. have been recovered throughout the world. In the Northern Hemisphere, the fungus has been recorded from U.S., Canada, Europe, Germany, Italy, Japan, Vietnam, Taiwan, and China. Whereas, in the Southern Hemisphere, the fungus has been reported from New Zealand, South Africa, Central Africa and Australia (Jacobs and Wingfield, 2001). In most cases, *Leptographium* species have been reported from conifers (Kendrick, 1962; Harrington, 1988) and, only a few cases have been reported from deciduous trees (Kendrick,1962). This fungus has been found to be one of the most pathogenic fungus isolated from declining *P. taeda* in the southern U.S. (Hess et al., 1999). *Leptographium terebrantis* Barras and. Perry is vectored by *Dendroctonus terebrans* (Oliver) infecting *P. taeda* (Barras and Perry, 1971). This fungus also was isolated from the root samples and insects captured from several *P. taeda* plantations in Alabama (Eckhardt et al., 2007). The continuous re-isolation of *L. terebrantis* from southern forests indicates that the fungus has the potential to become a devastating pathogen in the future.

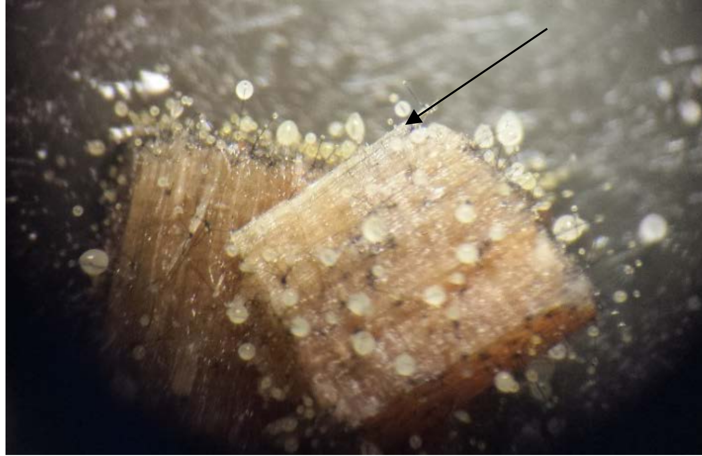

Figure 1.3 Conidia of *Leptographium terebrantis* in *Pinus taeda* root section.

Leptographium procerum (W.B. Kendr.) M. J. Wingf., previously known as *Verticicladiella procera* Kendrick (Kendrick, 1962; Wingfield, 1983) was first identified from *P. banksiana* Lamb. (jack pine) (Kendrick, 1962) and has been recovered from other conifer species including *P. palustris* (longleaf pine) (Otrosina et al., 1999), *P. taeda* (Eckhardt et al., 2004a) and *P. elliottii* (slash pine) by Barnard et al. (1991). This fungus has been isolated from declining white pine from the U.S. (Houston, 1969; Lackner and Alexander, 1982; Wingfield and Marasas, 1983), Yugoslavia (Halambek, 1976) and New Zealand (Shaw and Dick, 1980; Wingfield and Marasas, 1983).

The insect vectors associated with *L. procerum* are *D. frontalis* Zimmermann (Otrosina et al., 1999), *Hylastes* spp. (Wingfield and Gibbs, 1991; Eckhardt et al., 2007) and *H. pales* by (Klepzig et al., 1991). Though *L. procerum* have repeatedly been isolated from various pine decline site, pathogenicity tests have shown that it is a weak pathogen compared to *L.* terebrantis, *Grosmannia huntii* (R.C. Rob. Jeffr.) Zipfel, Z.W. de Beer and M.J. Wingf. and *G. alacris* T.A. Duong, Z.W. de Beer and M.J. Wingf. sp. nov (Wingfield, 1986; Matusick et al., 2012).

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

Grosmannia huntii was formerly known as *Ophiostoma huntii* (Zipfel et al., 2006). The teleomorphic form *Grosmannia huntii* and anamorphic form *Leptographium huntii* were first described occurring on *Pinus contorta* Dougl. var. *latifolia* Engelm. (lodgepole pine) infested by *D. ponderosae* Hopkins (mountain pine beetle) in British Columbia, Canada (Robionson-Jeffrey and Grinchenko, 1964). *Grosmannia huntii* have distinct serpentine hyphae as shown in Figure 1.4. The colonies initially grow hyaline, and the olivaceous pigmentation appears with time. It produces sparse conidiophores in culture. If present, each conidiophore has two primary branches. The conidiophore form on the aerial mycelium gives rise to ovoid conidia (Jacobs and Wingfield, 2001).

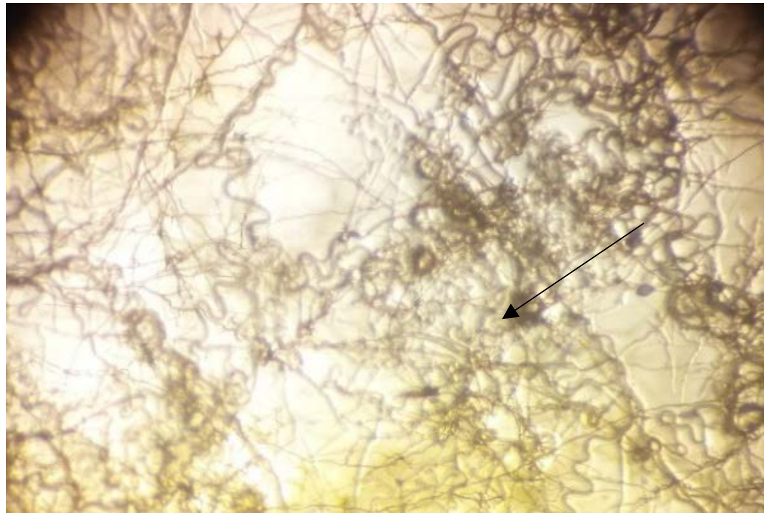

Figure 1.4 Serpentine hyphae of *Grosmannia huntii.*

Grosmannia huntii has been isolated from New Zealand from *Hylastes ater* (Payk) stumps, and seedlings of pine trees infested with *Hylastes ater* (Reay et al., 2005). It also occurs in Australia, associated with *H. ater, Tomicus piniperda* (Linnaeus) in Europe and *H. macer* LeConte and *Ips pini* (Say) in the U.S. (Harrington, 1988; Wingfield and Gibbs, 1991; Zhou et al., 2004). In the U.S., it has been isolated from

root-feeding bark beetles in California (Kim et al., 2011), Georgia (Zanzot et al., 2010), Alabama (Matusick et al., 2013). *Grosmannia huntii* has recently been described as relatively virulent fungi among other *Leptographium* spp. and *Grosmannia* spp. in the southern U.S. (Zanzot et al., 2010; Matusick et al., 2012).

1.5.3 *Grosmannia alacris T. A. Duong, Z. W. de Beer and M. J. Wingf.*

Grosmannia alacris T. A. Duong, Z. W. de Beer and M. J. Wingf. renamed from *L. serpens* (Goid.) (Duong et al., 2012) was previously described as *Verticicladiella alacris* (Wingfield and Marasas, 1981). *Grosmannia alacris* has been isolated from diseased roots of *P. pinea* L. in Italy and *P. radiata* D. Don and *P. pinaster* Aiton in South Africa (Wingfield et al., 1988). In culture medium, the fungus forms black mycelium and has relatively slow growth rate compared to either *L. terebrantis* or *L. procerum*. Serpentine shaped hyphae are the most important characteristic of this fungus. *Grosmannia alacris* has been successfully isolated from *H. tenuis* and *H. salebrosus* (Eckhardt et al., 2007) and has been reported to produce longer lesions on mature loblolly pine tree roots when compared to lesions produced by either *L. terebrantis* or *L. procerum* (Matusick et al., 2012).

1.6 Concept of virulence and pathogenicity

The terms pathogenicity and virulence are commonly used in the literature dealing with fungal pathogens. However, the usage of these terms is often confusing. Pathogenicity is the ability of a disease-causing agent to cause disease which may be either natural or experimental. It considers only the qualitative factors associated with the level of pathogenicity. Whereas, virulence is the capability of a disease-causing agent to cause disease. Virulence considers both qualitative and quantitative factors related to the

degree of pathogenicity (Watson and Brandly, 1949). Virulence is used to compare the disease evoking capacity of an isolate compared to another. Also, it is used to refer to the process of the disease establishment and to indicate the relative amount of damage to the host (Shaner et al., 1992). Various inoculation techniques can assess the relative virulence and the pathogenicity of pathogens.

1.7 Methods of fungal inoculation

Inoculation experiments satisfy the Koch's Postulates and confirm the causal relationship between disease and the disease-causing agent. Experiments with artificial inoculation of fungi is a simplified method to determine the pathogenicity and relative virulence of ophiostomatoid fungi (Wingfield and Marasas, 1983, Matusick and Eckhardt, 2010). Various stages of the tree from seedling to sapling to mature trees have been used for inoculations (Matusick and Eckhardt, 2010).

Wright (1933) described the method for an artificial inoculation study to mimic natural inoculation of fungi by bark beetles in the tree. In this approach, the bark around the point of inoculation is surface sterilized with 70% ethyl alcohol and bark disc is removed with a sterilized 6 mm cork borer to create an artificial wound. A disc of the fungal mycelium from the culture plate is taken and inserted into the wound in the tree using a sterile plunger. The inoculation site is then covered with sterile moistened cotton balls and then finally covered with a wrapper (Wright, 1933).

Artificial inoculation experiments have been conducted to determine the virulence and the pathogenicity of the ophiostomatoid fungi in conifers (Yamaoka et al., 2000; Molnar, 1965; Lieutier et al., 2009; Jankowiak, 2006; Lee et al., 2006; Matusick et al., 2012). Hepting and Roth (1946) artificially inoculated mature *Pinus virginiana* Mill.

(Virginia pine), *P. echinata* and *P. rigida* Mill. (Pitch pine) trees with *Fusarium* species. The fungal inoculation resulted in an infection and death of those trees (Hepting and Roth, 1946). Molnar (1965) used a similar method and inoculated, *Abies lasiocarpa* (Hook.) Nutt. (Alpine fir) trees (15.24-38.1 cm in diameter) with *Ceratocystis dryocetidis* Kendrick and Molnar. Sterile agar was inoculated as a control (Molnar, 1965). Paine and Stephen (1987) used the similar inoculation technique to inoculate an 18-year-old *P. taeda* (19 - 19.5 cm in diameter at 1.5 m height). The choice of inoculation method depends on the age of the tree.

1.7.1 Mature tree root inoculation

Mature tree root inoculation studies in conifers have been performed to understand the pathogenicity of root-feeding beetle vectored fungi and their role in pine decline. Declining longleaf pine roots with evidence of insect attacks, resinosis and necrosis have been excavated and sampled for the presence of root-infecting fungi (Otrosina et al., 1999). To determine the effect caused by these fungi, root inoculation experiments seem a more accurate portrayal of disease etiology. Healthy primary lateral roots have been inoculated on longleaf pine to determine the extent the root damage caused by ophiostomatoid fungi (Matusick et al., 2010). This inoculation technique seems more promising as it mimics the natural inoculation of ophiostomatoid fungi by root-feeding bark beetles.

1.7.2 Mature tree stem inoculation

Wound inoculations of ophiostomatoid fungi in the cambium of mature trees bark is the most commonly used technique to assess the fungal pathogenicity (Wright, 1933; Matusick et al., 2016). Stem inoculations of the root pathogen *L. terebrantis* has been

repeated to give similar results as root inoculation in *P. taeda* and *P. palustris*. Also, stem inoculation techniques can act as the surrogate to understand the relative pathogenicity of root fungal pathogens; *G. huntii*, *G. alacris*, *H. irregulare* and *L. procerum* in the same pine species (Matusick et al., 2016).

Inoculations of fungus at a single point in each tree has been performed with *P. resinosa* Sol. ex Aiton (red pine) (Raffa and Smalley, 1988), *P. ponderosa* Douglas ex C. Lawson (ponderosa pine) (Parmeter et al., 1989), *P. taeda* (Eckhardt, 2004b), and *P. palustris* (Matusick and Eckhardt, 2010). Multiple inoculations (multiple dosages) like 200, 400 or 800 inoculation sites/m^2 of the surface of the bark has been also performed in Douglas-fir (Ross and Solheim, 1997), 400 inoculation sites/m^2 *Picea abies* (L.) H. Karst. (Norway spruce) (Krokene and Solheim, 1998). These artificial inoculations have resulted in lesions occurring in the inoculated hosts.

Measurement of the necrotic lesion area formed around the point of inoculation has been useful to evaluate fungal virulence (Matusick and Eckhardt, 2010; Raffa and Smalley, 1988). The fungi in multiple mass inoculation experiments extended well beyond the necrotic region, colonizing the phloem and sapwood, finally killing the tree (Christiansen et al., 1987). These mature tree, mass inoculations experiments, mimic the natural scenario of an introduction of the fungi by bark beetles and are a reliable estimate of the fungal-virulence (Solheim 1993; Solheim and Krokene, 1998).

The inoculum size and the number of inoculation points are the primary factors to be considered while performing inoculation experiments. Wound size created for inoculations varies between 5 - 19 mm between studies which might lead to a difference in results between studies. Inoculation techniques using multiple inoculation points m^{-2}

would be more practical in determining the actual impact of the beetle- vectored fungi. The studies on the impact of multiple point inoculations on the physiology of southern pine species are limited.

1.7.3 Seedling inoculation

Numerous seedling inoculation experiments have been conducted to determine the pathogenicity of ophiostomatoid fungi on pine trees and to assess the relative virulence of different ophiostomatoid fungi (Harrington, 1993; Krokene and Solheim, 1998; Parmeter et al., 1989; Wingfield, 1986; Yamaoka et al., 1995). Inoculation in a seedling stem under aseptic conditions is performed by inoculating colonized mycelium in slanting cut made in the seedling stem (Singh et al., 2014; Matusick and Eckhardt, 2010; Nevill et al., 1995). Various host response characteristics and symptoms can then be observed to determine successful fungal colonization.

Various host response characteristics such as mortality (Wingfield, 1983), dark resinous lesion (Matusick and Eckhardt, 2010; Nevill et al., 1995), chlorotic needles, inner bark necrosis and reduction in water potential (Rane and Tattar, 1987) are observed following inoculation of ophiostomatid fungi in *P. taeda*. In addition, other responses like occlusion of vascular tissues (Singh et al., 2014; Matusick and Eckhardt, 2010) as well as a change in monoterpene levels and carbohydrate composition have been observed (Cook and Hain, 1985). The defense system of small seedlings may not be similar to a mature tree and therefore the use of seedlings to estimate virulence in the mature trees may affect the accuracy. Thus, careful experimentation and elucidation of the results are necessary.

Krokene and Solheim (1998) studied the relevance of seedling inoculation study in determining the pathogenicity of *Capperia polonica* Adamczewski and *Ophiostoma*

spp. by comparing it with inoculation in 40-year-old *P. abies* trees. They repeated that the fungal isolate showed similar virulence in seedlings when compared to mass inoculation study using 40–year–old trees. Some studies have suggested that fungi appear to be more pathogenic to seedlings than to mature trees (Basham, 1970). However, seedling inoculation experiments allow to control over the soil temperature and moisture to assess the pathogenicity of fungi. Lower inoculum load should be used in seedling inoculations compared to mature trees to get proportionally accurate equivalent lesion.

1.7.4 Lesion measurement

The fungi associated with PD when inoculated in pine trees, cause the necrotic lesions. The lesion extends radially from the point of inoculation. The relationship between vertical lesion length and depth have not gained proper attention (Parmeter et al., 1992). Many authors focused only on vertical lesion length (Cook and Hain 1985; Paine et al., 1988; Raffa and Berryman, 1982; Wingfield, 1986). Since the sapwood depth is limiting, it is therefore suggested that the vertical extension of the lesion from the point of inoculation is the better measure of pathogenicity (Parmeter et al., 1992). Placing the infected stem in a dye solution and measuring the area through which the dye could not pass has been used as a measure of the sapwood occlusion (Matusick et al., 2008; Parmeter et al., 1992; Singh et al., 2014).

The time period between fungal inoculation and lesion measurement is also an important factor to consider. Cook and Hain (1985), repeated there is a significant increase in lesion length produced by *O. minus* (Hedge.) H. et P. Syd. on *P. taeda* when measured at 2 weeks and 4 weeks. Similarly, Raffa and Smalley (1988) found a continuous increase in lesion length produced by *Ceratocystis nigrocarpa* (Davids.) and

C. ips (Rumb) on either *P. resinosa* or *P. banksiana* from 1 to 5 weeks after inoculation. Parmeter et al. (1992), with the purpose of finding a time frame of the radial development and extension of sapwood occlusion, inoculated 36-37-year-old trees of *P. ponderosa* and measured the lesion and occlusion length weekly. In the study, lesion and occlusion sizes were not different between 9 and 17 weeks post fungal inoculation. They reported that the lesion and occlusion reached maximum size (near endpoint) 8-10 weeks post inoculation. The lesion length in loblolly pine seedlings has been measured at 8 (Singh et al., 2014; Chieppa et al., 2015) and 16 weeks (Eckhardt, 2004b; Matusick et al., 2008) following inoculation.

The lesion caused by the blue stain fungus can be an evidence of both fungal growth and the resistance response of the host tree (Solheim, 1992). Lesion length has been used as a strong indicator of the fungal pathogenicity (Molnar, 1965; Paine et al., 1997; Matusick et al., 2012; Singh et al., 2014; Chieppa et al., 2015). Smaller lesion lengths may indicate that the tree can produce the effective defensive mechanism towards the fungi whereas longer lesion as the response produced by less resistant trees (Horntvedt, 1988; Solheim, 1988; Singh et al., 2014). Also, the most pathogenic fungus grows for a longer period under oxygen-deficient condition than the less pathogenic one. The ability of the fungus to tolerate the oxygen-deficient condition may be related to the volume of the occluded sapwood (Solheim, 1992).

1.8 Host defense

Active defense mechanisms in conifers helps them to protect from insect and pathogens. These defense mechanisms involve the production of terpenoids and phenolics that are toxic to bark beetles. Oleoresin, a major chemical constituent of

conifers, is a complex mixture of terpenes. Its secretion plays a significant role in the defensive mechanism of conifers against beetles, fungi, and microbes (Turtola et al., 2003) and conifers have unique anatomical structures that store and transport these chemicals.

Recently, researchers have been focused on inter-species and intra-species variation in tolerance of pine trees (Matusick et al., 2010; Singh et al., 2014). The response of the host tree to inoculation of beetle-vectored-fungi is the reliable means of identifying the relative host resistance (Raffa and Smalley, 1988). Fungal metabolism, but not the structural components have been found to responsible for eliciting the host defense mechanism (Raffa and Smalley, 1988). So, the inter-species and intra-species variance of the pine can be studied regarding their response towards the pathogenic fungi (Singh et al., 2014). Thus, studying the response of the tree to fungi would be a promising step in selecting tolerant pine varieties to mitigate the pine decline problem.

1.9 Tree-to-tree variation

Pinus taeda trees within a stand may have significant genetic variation. For example, as the quality and quantity of chemicals are under strong genetic control different levels of monoterpenes can be present in individual trees (Rockwood, 1972). Also, there is significant genetic variation among the trees regarding survival, growth, development, resistance to insects and disease-causing agents. These genetic variations allow selection of specific traits and thus help to generate families having desirable properties (Schultz, 1997).

1.10 Climate change in host-pathogen interactions

Abiotic stress factors like adverse climatic condition have an influence on the susceptibility of host conifers to attacks by pathogens and insects (Lindberg and Johansson, 1992). Climate change issues like increasing levels of ozone at of rate of 0.3% to 2% per year (Balsing, 2009), fluctuating mean annual precipitation and increasing temperature are prevalent (Paoletti et al., 2009). The fungal pathogens may behave in a different manner under future climate change conditions (Chieppa et al., 2015; Klepzig et al., 2001). For example, the forests of the southeastern U.S. have experienced severe droughts in recent years, and these drought events significantly affected tree growth and mortality (Klos et al., 2009; Wang et al., 2010). Drought-stressed *P. palustris* seedlings inoculated with *G. huntii* have resulted in an increased infection and high mortality in longleaf pine (Matusick et al., 2008). Seedlings treated with ozone were more susceptible to ophiostomatoid fungi. *Pinus taeda* families which were susceptible to pine decline associated fungi were found to be more sensitive to ozone treatment (Chieppa et al., 2015). Therefore, it is important to understand the pathogen virulence and host defense system under other predicted climate change scenarios.

1.11 Possible solutions to root infecting ophiostomatoid fungi

Ophiostomatoid fungi have the potential to become a more virulent pathogen in a changed environment. Global trade of logs and wood products has enhanced the introduction of pathogens into new environments. One of the examples is Dutch Elm disease caused by *O. ulmi* (Buisman) Melin and Nannf. associated with *Scolytus* spp. (Brasier, 2001). This disease is a plant vascular wilting disease that caused massive tree mortality in Europe and North America in the 20th century. Similarly, ophiostomatoid

fungi were found to be problematic in *Pinus* species and *Pieca abies* (L.) H. Karst (Norway spruce) (Friedl, 2004). Various studies have suggested, though the present damage is at low levels, the predicted environmental changes in the future may bring new threats at a larger level.

The first strategy to mitigate this problem is to investigate the potential pathogen that might negatively influence the forest health (Repe and Jurc, 2010). Prognostic and early diagnostic procedures must be adopted (Jurc et al., 2006). Identification of the pathogen by microscopy and culture may be time taking. In addition, some of the microbes cannot be cultured. Use of recent molecular techniques such as loop-mediated isothermal amplification (LAMP) and clustered regularly interspaced short palindromic repeats (CRISPER) might be promising approach to detect pathogen.

Various fungicides have been used for treatment where the damage is severe. However, the use of fungicides has been found to be problematic. Fungicides have a broad spectrum of the mode of action and may kill other useful microorganisms. Thus, the application of the fungicide is not recommended (Jmones and Mangels, 2002).

Another good approach to mitigate potential problem due to PD in industrial plantations may be breeding trees for resistance to ophiostomatoid fungi. This can be done by phenotypic selection of tree or genetic improvement of the trees. This method seems suitable, but often it is dubious that resistance may change from young to adult. Screening studies should be conducted both on mature and the young trees (Repe and Jurc, 2010).

Fungal virulence may also change over time (Dwinell, 2001). So, the regular monitoring and diagnosis of the tree are very crucial. Other possible solutions would be

to study the biocontrol potential of the beneficial microbes in the rhizosphere and phyllosphere of the tree. For example, albino strains of *Ophiostoma* spp. do not synthesize the melanin-like pigment (Farrell et al., 1993; Schroeder et al., 2002) and have been found to be effective in stopping the pathogenic fungi. The chemical method of treatment and kiln drying is done to treat wood vulnerable to attack by blue-stain fungi. Fungal biology and the disease pathogenesis should be properly studied to fight against the pathogen and disease.

1.12 Conclusion

The purpose of this review is to view the impact of the insect-vectored vascular-inhabiting ophiostomatoid fungi to the *Pinus* species in the southern U.S. There have been some research conducted to understand the pathogenicity and the virulence of these fungal species on *Pinus* species. Also, some studies have been carried out to understand the inter-species variability in the tolerance of *Pinus* species. Further research and testing are required to better understand the intra-species and inter-stock type variability in tolerance of *P. taeda* to ophiostomatoid fungi. Furthermore, it is necessary to develop an efficient artificial fungal inoculation technique to mimic the natural inoculation of the fungi by bark beetles to understand the impact caused by these fungi.

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CHAPTER II

Intraspecific and Inter-Stocktype Response of *Pinus taeda* L. to *Grosmannia huntii* and *Leptographium terebrantis*

2.1 Abstract

Vascular-inhabiting ophiostomatoid fungi are among the contributing factors of premature decline and mortality of *Pinus taeda* (loblolly pine). The aim of this experiment was to examine intraspecific variation in tolerance of *P. taeda* to ophiostomatoid fungi. Containerized and bare-root seedlings for 94 and 4 families respectively, were artificially inoculated at the stem with *L. terebrantis* and *G. huntii*. Eight weeks post inoculation, lesion and occlusion were measured on each seedling to determine variation of host responses. *Pinus taeda* showed wide intraspecific variation in tolerance/susceptibility to both *L. terebrantis* and *G. huntii*. The two interspecies stocktypes (bare-root and container) of *P. taeda* had similar tolerance to fungi. Results suggest both seedling stocktypes can be used in virulence screening studies. *Pinus taeda* families more tolerant to ophiostomatoid fungi can be separated from less tolerant. These results will help land managers in making decisions to plant most appropriate *P. taeda* families to minimize the potential impact of ophiostomatoid fungi.

2.2 Introduction

Pinus taeda L. (loblolly pine) is the leading pine species which comprises 50% of the total softwood volume grown in the south (Schultz, 1997; Oswalt et al., 2014). The number of *P. taeda* seedlings planted in the southern U.S. each year reaches a billion (McNabb and Enebak, 2008). *Pinus taeda* plantations provide marketable forest products, provides habitat for wildlife, and place for recreational activities and thus contribute a considerable portion of the southern U.S. economy (Poudel et al., 2017; Schultz, 1997). Over the past 40 years, however, there have been reports of Pine Decline.

Pine Decline (PD), a decline disease syndrome, first reported by Brown and Mc Dowell (1968) at Talladega National Forest, Oakmulgee Ranger District in 1959. The decline was indicated by short chlorotic needles, sparse crowns, reduced radial growth and premature mortality. Subsequent reports of decline urged scientists to conduct further studies that revealed the association of beetle-vectored ophiostomatoid fungi with PD (Hess et al., 1999; Hess et al., 2002). Consistent isolation of ophiostomatoid fungi: *Leptographium terebrantis* S.J. Barras and T. J Perry, *Grosmannia huntii* R.C. Rob. Jeffr, *L. procerum* Kendrick M.J. Wingfield and *Grosmannia alacris* T.A. Duong, Z.W. de Beer and M.J Wingfield, from the roots of declining trees (Eckhardt et al., 2007) emphasizes the role of fungi in decline process, thus warranting further controlled experimental studies incorporating *P. taeda* and fungi.

Leptographium terebrantis and *G. huntii*, are distributed worldwide as pathogens of conifers (Jacobs and Wingfield, 2001). Whereas, in North America, the former pathogen is relatively more problematic (Wingfield et al., 1988; Matusick and Eckhardt, 2010). However, mature root inoculations in *P. taeda*, *P. palustris* (longleaf pine) and *P. elliottii* (slash pine) in the southern U.S. revealed relatively higher virulence of *G. huntii*

than *L. terebrantis* (Matusick and Eckhardt, 2010). One of the immediate effects of *L. terebrantis* and *G. huntii* in *Pinus* hosts include resin-soaking, sapwood discoloration and lesions in the phloem (Wingfield 1986; Matusick and Eckhardt 2010; Chieppa et al., 2017).

Ophiostomatoid fungi affect pine species differently with *P. taeda* being relatively more susceptible than *P. palustris* and *P. elliotti* (Matusick et al., 2010). Singh et al. (2014) studied intra-species variation in tolerance of *P. taeda* to *L. terebrantis* and *G. huntii* and found *P. taeda* families widely varied in tolerance/susceptibility to both *Leptographium* and *Grosmannia* species. Various *Pinus* species have shown intra-species variation in tolerance to other tree pathogens and prompted the launch of tree breeding initiatives. For instance, open-pollinated families of *Pinus thunbergii* Parl., and *P. densiflora* Sieb. et Zucc., inoculated with a pine wood nematode, *Bursaphelenchus xylophilus* (Steiner et Buhrer) Nickle in Japan (Akiba et al., 2012) exhibited intra-species variation. Use of tolerant families in the breeding program has resulted in 92 clones of *P. densiflora* and 16 clones of *P. thunbergi*. Similarly, *Pinus sylvestris* L. (Scots pine) had intraspecific variation in susceptibility to dothistroma needle blight caused by fungi *Dothistroma septosporum* (Dorog.) Morlet. with implications for more tolerant families in breeding programs (Fraser et al., 2015).

Bare-root and containerized seedlings are the two stock types used in previous screening studies (Singh et al., 2014; Chieppa et al., 2017). Bare-root seedlings are grown in soil beds in an open field with the removal of soil during harvest. Containerized seedlings are grown in containers containing artificial media under a shelter or controlled greenhouse environment with root and soil media maintained together from harvest to re-

planting (Grossnickle et al., 2016). The lifting of bare-root seedlings in nurseries in the southeastern U.S. involves a number of operational procedures which might affect root viability (Starkey and Enebak, 2013). In contrast, root damage is minimal in containerized seedlings. Containerized seedlings usually have higher survival success rates than bare-root after replanting (Sloan et al., 1987; South et al., 2005). However, the use of a greenhouse, growth medium, proper irrigation and fertilization may make containerized seedlings more susceptible to biotic diseases (Grossnickle et al., 2016). Singh et al. (2014) studied intraspecific variation in tolerance to ophiostomatoid fungi in either containerized or bare-root seedlings respectively in two separate years. The containerized seedlings had both higher field survival and longer lesions compared to bare-root seedlings. However, the results were inconclusive as interfamily-stocktype differences were not studied. Thus, the study of the response of two stocktypes (container-grown and bare-root seedlings) of the same families of *P. taeda* is necessary.

The variation in susceptibility to pathogen observed in few families cannot be generalized to the whole population. However, despite this knowledge, and the enormous threat that the ophiostomatoid fungi pose to *P. taeda*, the question of whether intraspecific variation in tolerance/susceptibility to ophiostomatoid fungi remains unexplored in many *P. taeda* families. The hypotheses of this study are: (i) *Pinus taeda* families out-planted in the southern U.S. have intra-specific variation in tolerance to two major fungi associated with PD, (ii) *Pinus taeda* stocktypes vary in tolerance to ophiostomatoid fungi, and (iii) The connector families will show a similar response to the fungal inoculation in all the three years.

2.3 Materials and methods

2.3.1 Experimental design

Container-grown seedlings from 33, 38 and 23 different *P. taeda* families were studied in 2013, 2014 and 2016 respectively (Table 2.1, Table 2.2 and Table 2.3). In 2014, bare-root seedlings from 4 families same as container grown families also were studied. The genetic distinction among groups is based on female parent, so the term "family" is utilized. Families L05, L09, L16, L38, L49, and L50 were included each year and served as connector families. The genetic distinction between these families is unknown but families L49 and L50 represent the wildtype families. Families used belong to the most commonly out-planted half-sib (open-pollinated), or full-sib (controlled-pollinated) *P. taeda* families in the southern U.S. These families were derived from the tree genetic improvement programs conducted by North Carolina Tree Improvement Cooperative. Seeds from all families were grown within a single forest company nursery each year to minimize environmental variability. Nine-month-old, containerized seedlings and bare-root seedlings were extracted from individual containers and common nursery beds and used in the experiment. Twelve seedlings per family were randomly selected and separated into the stem, needles, coarse root and fine root and dried at oven (75 °C for 72 h) for initial biomass assessment.

The study site is an outdoor research facility of the School of Forestry and Wildlife Sciences, Auburn University, located in Auburn, Alabama. A randomized complete block design with six blocks (3 replications) (Figure 2.2) was established with the random assignment of families and treatments within each block. Seedlings were planted in one-gallon pots filled with ProMIx BX® (Premier Tech, Quebec, and Canada)

peat-based potting media. The seedlings with a mean height of 30 cm and root collar diameter of 4.5 mm (Figure 2.1) were chosen for planting to reduce individual seedling variability. The planted seedlings were allowed to acclimatize in the ambient climate condition at the experimental site for 2 months prior commencement of stem inoculations. Seedlings were irrigated when required.

Figure 2.1 Container-grown *Pinus taeda* seedlings from a single family before re-planting.

Table 2.1 *Pinus taeda* families for the year 2013.

Family ID	Company
L05	Rayonier
L09	Rayonier
L16	Rayonier
L38	Arborgen
L49	Arborgen
L50	Arborgen
L51	Rayonier
L52	Arborgen
L53	Westervelt
L54	Rayonier
L55	Rayonier
L56	Rayonier
L57	Rayonier
L58	Weyerhaeuser
L59	Weyerhaeuser
L60	Weyerhaeuser
L61	Weyerhaeuser
L62	Plum Creek
L63	Plum Creek
L64	Plum Creek
L65	Plum Creek
L66	Arborgen
L67	Arborgen
L68	Arborgen
L69	Hancock
L70	Hancock
L71	Hancock
L72	Hancock
L73	Hancock
L74	Westervelt
L75	Westervelt
L76	Westervelt
L77	Westervelt

Notes: L05, L09, L16, L38, L49 and L50 replicated each year, L49: FM2 and L50: SEF-Mix are wildtype families

Table 2.2 *Pinus taeda* families for the year 2014.

Family code	Company
L78	Arborgen
L79	Weyehaeuser
L80	Rayonier
L81*	Arborgen
L16	Rayonier
L82	Arborgen
L83	Weyehaeuser
L84	Westervelt
L85	Arborgen
L86	Plum Creek
L05	Rayonier
L87	Arborgen
L88	Plum Creek
L38*	Arborgen
L89	Plum Creek
L90	Westervelt
L91	Plum Creek
L92	Arborgen
L93	Arborgen
L94	Westervelt
L95	Westervelt
L09*	Rayonier
L96	Hancock
L97	Hancock
L98	Arborgen
L99	Rayonier
L100	Rayonier
L101	Rayonier
L102	Weyehaeuser
L103	Westervelt
L104	Arborgen
L105	Arborgen
L106	Weyehaeuser
L107	Hancock
L49	Arborgen
L108	Weyehaeuser
L109*	Plum Creek
L50	Arborgen

Notes: L05, L09, L16, L38, L49 and L50 replicated each year, L49: FM2, L50: SEF-Mix are wildtype families, * represents families with both bare-root and container-grown seedlings.

Table 2.3 *Pinus taeda* families for the year 2016.

Family code	Company
L114	Rayonier
L118	Rayonier
L05	Rayonier
L16	Rayonier
L123	Rayonier
L117	Rayonier
L124	Rayonier
L116	Plum Creek
L122	Plum Creek
L115	Plum Creek
L111	Westervelt
L33	Westervelt
L09	Weyehauser
L126	Weyehauser
L127	Weyehauser
L128	Weyehauser
L129	Weyehauser
L130	Weyehauser
L112	Plum Creek
L113	Plum Creek
L38	Arborgen
L49	Arborgen
L50	Arborgen

Notes: L05, L09, L16, L38, L49 and L50 replicated each year, L49: FM2, L50: SEF-Mix are wildtype families.

Figure 2.2 *Pinus taeda* seedlings planted in six blocks (RCBD) in the outdoor planting space**.**

2.3.2 Inoculation of fungi

Single spore isolates of *Leptographium terebrantis* (ATCC accession no. MYA-3316) and *Grosmannia huntii* (ATCC accession no. MYA-3311) maintained at 4 °C in Forest Health Dynamics Laboratory at Auburn University, AL, U.S. were used for the stem inoculations. These isolates were sub-cultured in Malt Extract Agar, 2 weeks before the start of the stem inoculation. The *L. terebrantis* isolate was isolated from the lateral root of *P. taeda* in the Talladega National Forest, Oakmulgee Ranger District, AL, U.S. The *G. huntii* isolate was isolated from the lateral root of *P. taeda* from Fort Benning Military Reservation, GA, U.S. Those trees exhibited symptoms of PD such as thin crowns and lateral roots with a damaged localized root tissue as described by Eckhardt et al. (2007).

Seven seedlings (in 2013 and 2014) and 10 seedlings (in 2016) from each family received 1 of 4 inoculation treatments. The inoculation treatments were: (i) wound (control), (ii) wound + sterile media (control), (iii) wound + media with *L. terebrantis,* and (iv) wound + media with *G. huntii*. To perform inoculation, an 11-mm vertical wound (< 2 mm deep) in root-collar-area 2 cm above the soil line was created with a sterile razor blade (Figure 2.3). Wound control received a sterile cut only. Wound + media control received a sterile agar plug in the wound. Media with fungus treatment received a 3-mm agar plug with actively growing fungal mycelium from the edge of the agar plate, inoculated (fungus-side-down) in the wound. Inoculation points were covered with sterile moist cotton balls to prevent desiccation of the fungal media. Furthermore, the inoculation area was wrapped with Parafilm® to prevent further contamination.

Figure 2.3 Artificial inoculation of fungi in the stem of *Pinus taeda*.

2.3.3 Measurements

Seedling height and Root-Collar-Diameter (RCD) were measured on individual seedlings prior to stem inoculations and at harvest. During harvest, 7 seedlings/family/block were clipped at the soil level and placed in a tub that contained solution of Fast-Green stain (FastGreen FCF; Sigma Chemical Co., St. Louis, MO, USA) and distilled water mixed in a ratio of 0.25 g L^{-1}. Seedlings were exposed to the solution for 72 h to allow the capillary movement of dye through the stem. Three seedlings/family/block (in 2016) were gently pulled from the pot with roots and used for seedling dry matter biomass measurements as described earlier.

Figure 2.4 *Pinus taeda* seedlings dipped in FastGreen stain after clipping.

After removal from the Fast-Green solution, the lesion-length-width-depth, occlusion- length-width, and depth were measured on each seedling. The dark brown dead tissue section around the inoculation site was considered lesion length. Stem tissue

lacking capillary action to allow Fast-Green dye to pass through it was recorded as occlusion.

To verify Koch's Postulates of disease diagnosis, one centimeter of stem surrounding the lesion was removed from the stem and plated in MEA amended with 800 mg L^{-1} of cycloheximide and 200 mg L^{-1} of streptomycin sulphate. Plates were incubated at room temperature for 14 days and fungal recovery from each stem piece was identified and scored.

2.3.4 Statistical analyses

Mixed-models were used to analyze the lesion and occlusion data with family and treatment as fixed effects and the block as a random effect. PROC MIXED statement was used in SAS 9.4. The data were checked for normality and homogeneity of variance and log transformations were performed if there was a violation of these assumptions. Estimate statements were used to evaluate effects of each treatment. Multiple comparison tests were performed using Tukey-Kramer test at a 5% significance level. Graphs were created in STATISTICA 10.

The data were analyzed using mixed model. This model has both fixed and random effects. The statistical model used was

$$Y_{ijk} = \mu + Cov + T_i + B_j + F_k + FT + E_{ijk} \quad \ldots\ldots\ldots (1)$$

Where,

Y_{ijk} = Response variable (for example : lesion length, occlusion length)

μ = Mean of parameter

T_{ij} = Fixed effect of treatments in block j (i = 1(GH).., 2(LT).., 3(WM)..,4(W))

Cov = Initial root collar diameter of seedling as a covariate

B_j = Random Effect associated with block (j = 1..6)

F_k = Fixed Effects of family (k = 1..n)

FT_{ki} = Interaction effect of loblolly pine family and treatments (GH, LT, W and WM)

E_{ijk} = Residual with mean zero and constant variance (Random error)

Working hypotheses:

Family and inoculation treatments are under fixed effects. We want to compare whether there is significant differences in lesion length among the families.

We were mainly interested in testing family effects:

Ho: $\mu_1 = \mu_2 = \cdots \mu_k$ (Mean lesion length of the families are not significantly different)

HA: $\mu_1 \neq \mu_k$ (Mean lesion length is different in at least between two families)

Also, when inoculation treatment effects are considered:

Ho: $T_1 = T_2 = \cdots T_i = 0$ (There is no effect of the treatment on lesion development)

Ha: $T_i \neq 0$ for at least one fungal treatment

2.4 Results

2.4.1 Year 2013

Both the fungi used in the inoculation trials led to dark brown lesions in seedlings form families tested (Figure 2.5). The fungal re-isolation from seedling stems was 96 to 98%. Seedling survival was significantly different among the families tested (Chi-sq = 68.36, P <0.0001) and among the inoculation treatments (Chi-sq = 1419.86, P <0.0001). However, the seedling survival was not different between the seedlings receiving different inoculations within a family.

The lesion length produced by the wound and wound + media was significantly smaller than that caused by the fungal treatments. So, the effect of the controls were removed from the model. The average lesion length caused by both fungal treatments on different *P. taeda* families is shown in Figure 2.6. *Leptographium terebrantis* caused longer lesions that those by *G. huntii* (P <0.0001) as given in Table 2.1. Family L73 had the shortest lesions and families L68 and L66 had the longest lesions when treated with *L. terebrantis*. Whereas families L51 and L73 had the shortest lesions and L55, L66 and L67 had the longer lesions when treated with *G. huntii* (Table S1). Occlusion observed was moving both vertically and radially. The occlusion length produced as a result of *L. terebrantis* inoculation was significantly higher than that produced by *G. huntii* (P <0.0001).

Figure 2.5 The dark necrotic lesions observed in *Pinus taeda* seedlings 8 weeks following inoculations.

Covariance parameter estimates showed that lesion length for families tested were significantly different from zero (Z = 0.02). The average overall lesion length and those

caused by *G. huntii* and *L. terebrantis* is shown in Figure 2.6, 2.7, and 2.8, respectively. Lesion width was not significantly different from zero (Z = 0.19). Similarly, occlusion length (Z = 0.35) and occlusion width (Z = 0.47) was not significantly different from zero (Table 2.4).

The length, width and depth of lesions were found to be affected by fungal treatments as shown by type three fixed effects (Table 2.5). A family x treatment interaction was not found to be significant (P = 0.07) which indicates within each family two fungi did not cause differing sizes of lesion. Thus, an overall ranking of families (in terms of lesion length) can be done as shown in Table S2.

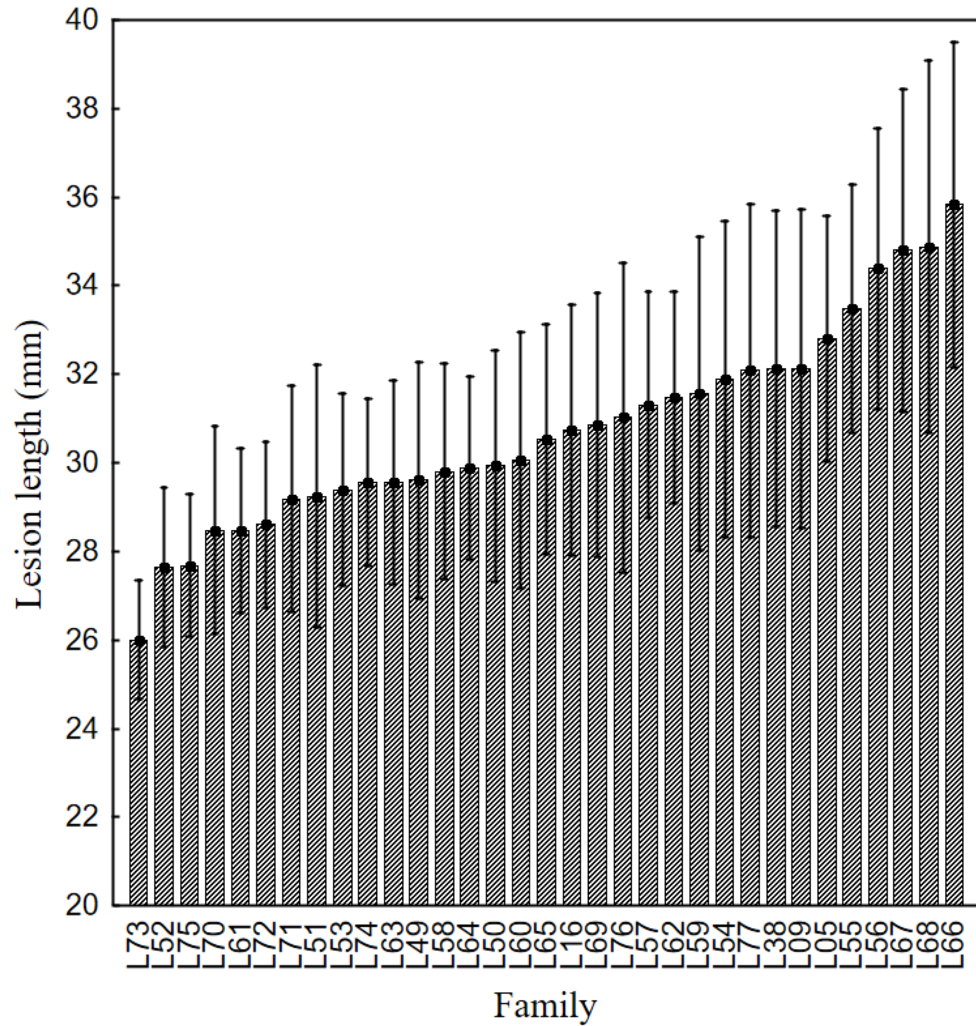

Figure 2.6 Mean lesion length caused by the both fungal treatments in *Pinus taeda* families (year: 2013). Error bars indicate 95% confidence interval.

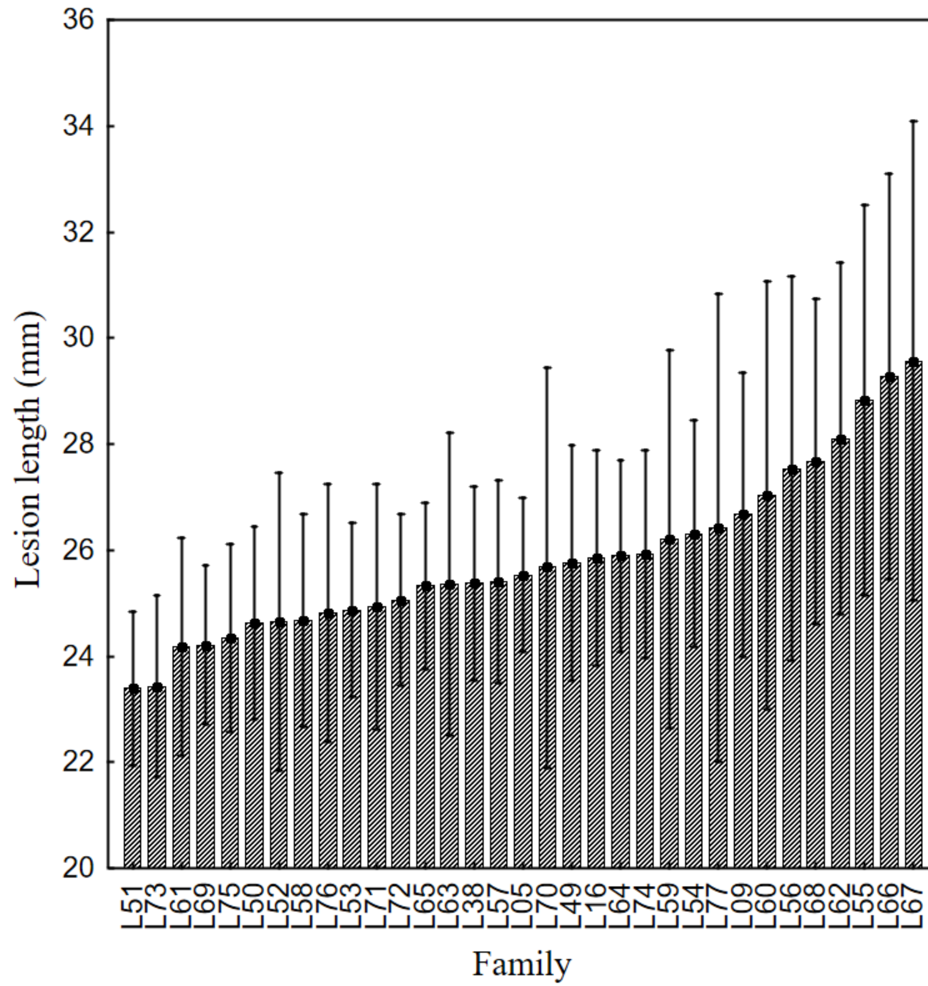

Figure 2.7 Mean lesion length caused by *Grosmannia huntii* in different *Pinus taeda* families (year: 2013). Error bars indicate 95% confidence interval.

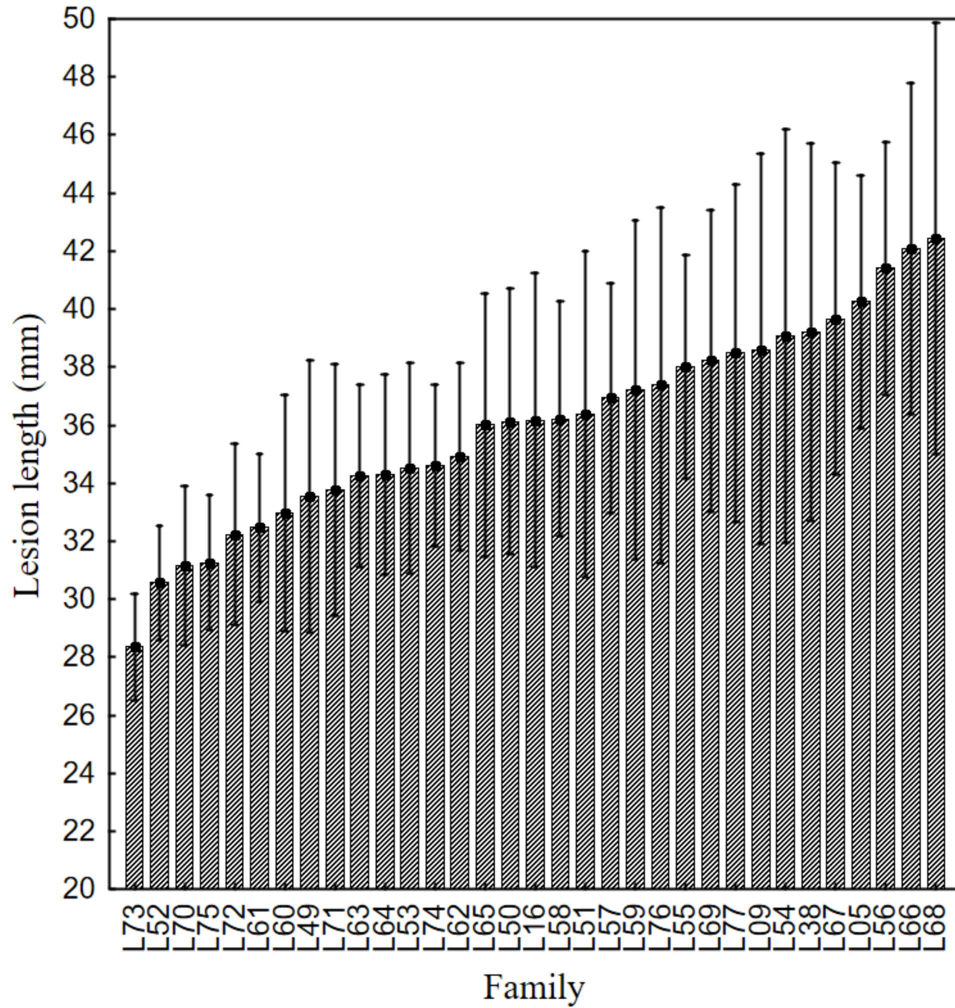

Figure 2.8 Mean lesion length caused by *Leptographium terebrantis* in different *Pinus taeda* families (year: 2013). Error bars indicate 95% confidence interval.

Table 2.4 Covariance parameter estimates form mixed-model (Year 2013).

Variable	Cov Parm	Estimate	SE	Z Value	Pr > Z
Lesion length	Family	2.88	1.42	2.03	0.0267
	Family*Trt	1.74	1.16	1.50	0.0710
	Residual	116.24	3.26	35.70	<0.0001
Lesion width	Family	0.03	0.03	0.88	0.1901
	Family*Trt	0.01	0.04	0.31	0.3248
	Residual	6.53	0.18	35.69	<0.0001
Occlusion length	Family	5.80	15.94	0.36	0.3546
	Family*Trt	28.33	20.41	1.39	0.0832
	Residual	915.17	39.02	23.45	<0.0001
Occlusion width	Family	0.01	0.16	0.08	0.4761
	Family*Trt	0.14	0.21	0.65	0.2652
	Residual	10.33	0.46	22.37	<0.0001

Note: Trt: Fungal treatment.

Table 2.5 Type three fixed effects from mixed-model. Initial root collar diameter (RCD) was used as covariate (year 2013).

Variable	Effect	DF	F Value	Pr>F
Lesion length	RCD	1	1.68	0.2000
	Block	5	44.74	<0.0001
	Trt	1	369.20	<0.0001
	Block*Trt	5	33.74	<0.0001
Lesion width	RCD	1	29.10	<0.0001
	Block	5	22.08	<0.0001
	Trt	1	323.42	<0.0001
	Block*Trt	5	17.57	<0.0001
Lesion depth	RCD	1	9.53	<0.0020
	Block	5	4.43	<0.0005
	Trt	1	17.83	<0.0001
	Block*Trt	5	0.31	<0.9000
Occlusion length	RCD	1	0.35	<0.5558
	Block	5	20.91	<0.0001
	Trt	1	16.99	<0.0001
	Block*Trt	5	9.40	<0.0001
Occlusion width	RCD	1	3.04	0.0800
	Block	5	10.71	<0.0001
	Trt	1	4.75	0.0300
	Block*Trt	5	2.58	0.0200
Occlusion depth	RCD	1	67.86	<0.0005
	Block	5	4.99	0.0030
	Trt	1	62.25	<0.0001
	Block*Trt	5	1.85	0.1300

Note: RCD: Root-collar diameter and Trt: Fungal treatment.

2.4.2 Year 2014

In 2014, the survival of inoculated seedlings was significantly different among the families (Chi-sq = 188.32, P <0.0001) but was not due to inoculation treatments (Chi-sq = 4.29, P = 0.2321). Inoculation of the two fungi resulted in dark brown necrotic lesions around inoculation zone. Re-isolation of the fungi from the inoculated seedlings ranged from 62% to 82% (Table S6). Consistent re-solation of the fungi proved the success of the inoculation.

Lesion length caused by both fungal treatments were significantly longer than those resulting from the control treatments as shown by pairwise comparisons test (Table 2.6). The effects of both controls were removed, and only the effects of the fungal treatments were included in the model. *Grosmannia huntii* produced significantly longer lesion length than *L. terebrantis* (P <0.0001). Similarly, occlusion length caused by *G. huntii* was significantly longer than that caused by *L. terebrantis* (P <0.0001) (Table 2.7).

Table 2.6 Pairwise comparisons between all inoculation treatments for lesion length.

Treatment	Estimate (mm)	Standard error	Adj P
GH vs LT	7.10	0.50	<0.0001
GH vs W	14.89	0.50	<0.0001
GH vs WM	14.92	0.50	<0.0001
LT vs W	7.78	0.50	<0.0001
LT vs WM	7.82	0.50	<0.0001
W vs WM	0.04	0.50	0.9999

Note: GH: *Grosmannia huntii,* LT: *Leptographium terebrantis*, W: Wound, WM: Wound + media.

Table 2.7 Pairwise comparisons between all inoculation treatments for occlusion length.

Treatment	Estimate (mm)	Standard error	Adj P
GH vs LT	13.26	0.67	<0.0001
GH vs W	29.19	0.67	<0.0001
GH vs WM	28.02	0.67	<0.0001
LT vs W	15.94	0.67	<0.0001
LT vs WM	14.76	0.67	<0.0001
W vs WM	-1.18	0.67	0.2933

Note: GH: *Grosmannia huntii,* LT: *Leptographium terebrantis*, W: Wound, WM: Wound + media.

Lesion and occlusion length was significantly different among the families as indicated by covariance parameter estimates (Table 2.8). However, family and treatment interaction was not statistically significant for both lesion length and occlusion length. Lesion and occlusion length, depth and width were found to be affected by fungal treatments (P <0.0001) (Table 2.9). Families L108 and L99 had shorter lesions and L81 and L91 had longest lesions when treated with *L. terebrantis*. Whereas, families L86 and L108 had the shortest lesions and families L88 and L91 had the longest lesions when treated by *G. huntii*.

There was no significant difference in lesion length between the two stocktypes used in the trial when challenged with *L. terebrantis* and *G. huntii* (Figure 2.9 and 2.10). Although differences were observed among different families, none of the same two families had significantly different lesion lengths (Figure 2.9 and 2.10).

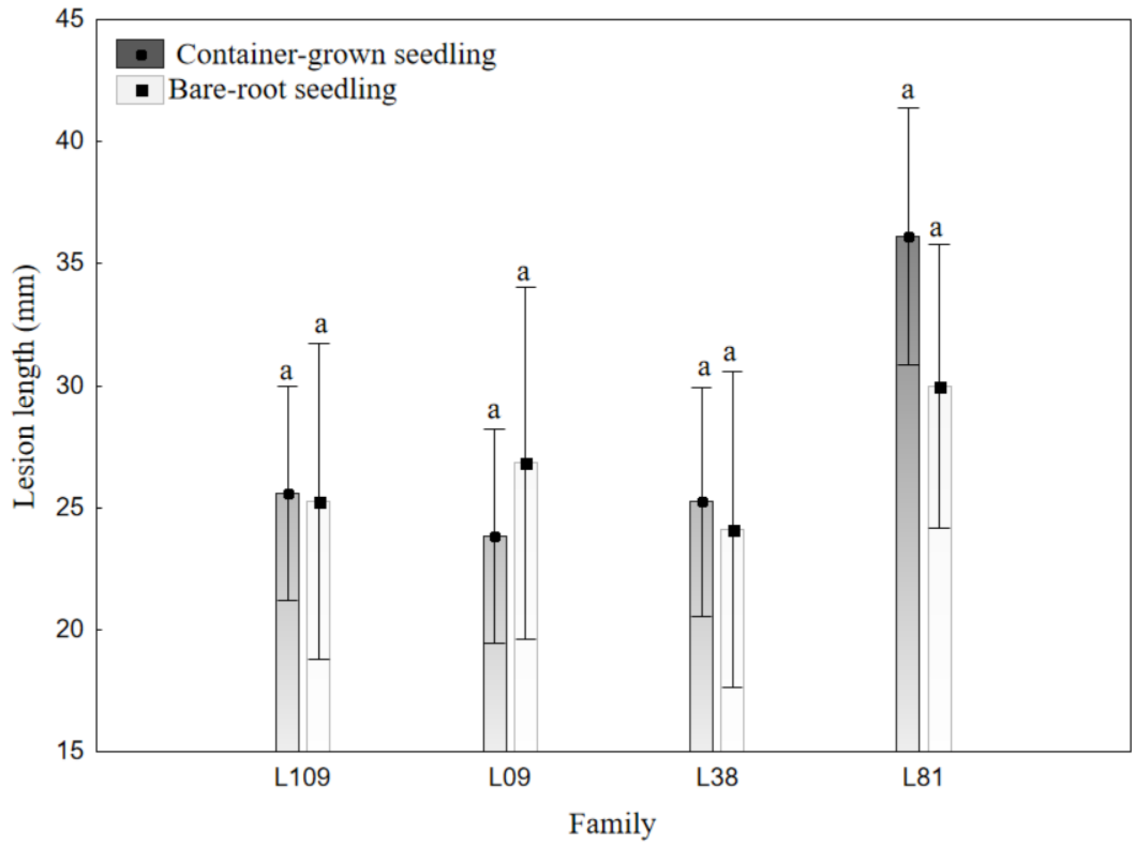

Figure 2.9 Mean lesion length on bare-root and container-grown families *P. taeda* families inoculated with *Leptographium terebrantis* (year: 2014). Error bars indicate 95% confidence interval. Different letters indicate significant differences between same bare-root and container-grown seedlings within same family.

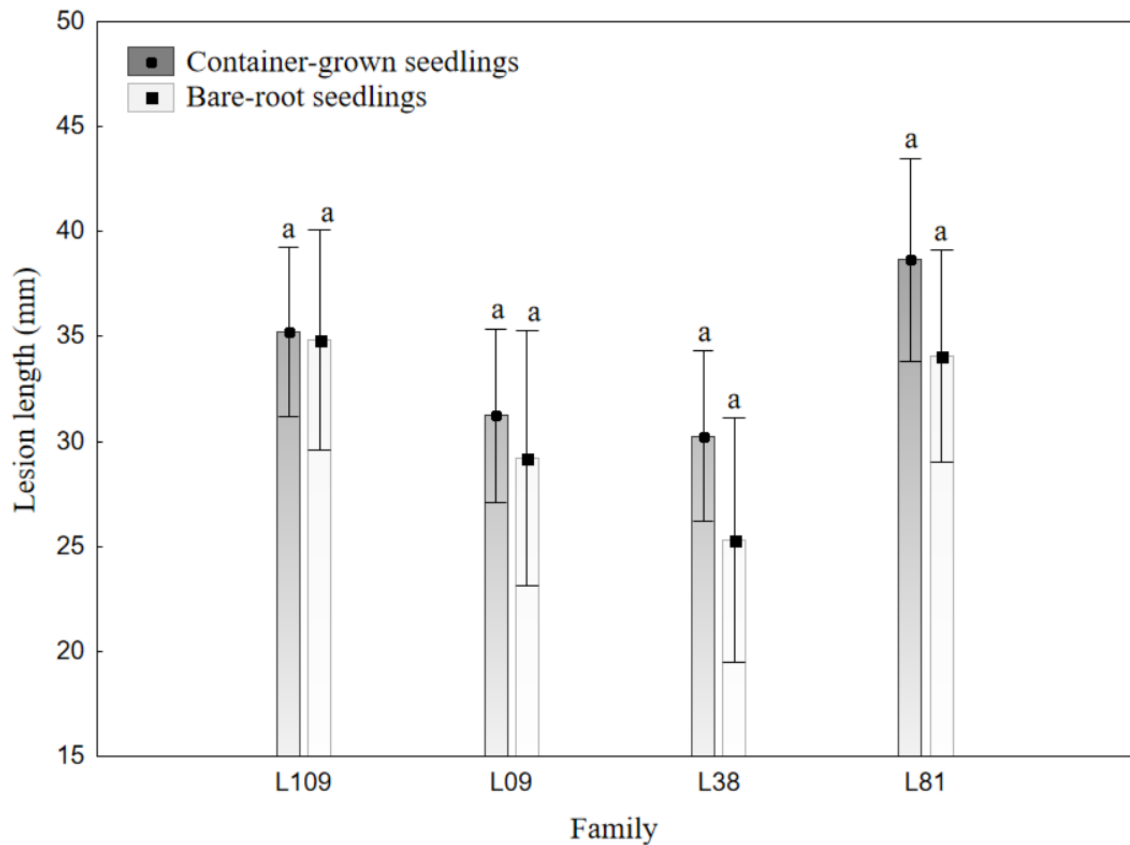

Figure 2.10 Mean lesion length on bare-root and container-grown *Pinus taeda* families inoculated with *Leptographium terebrantis* (year: 2014). Error bars indicate 95% confidence interval. Different letters indicate significant differences between bare-root and container-grown seedlings within same family.

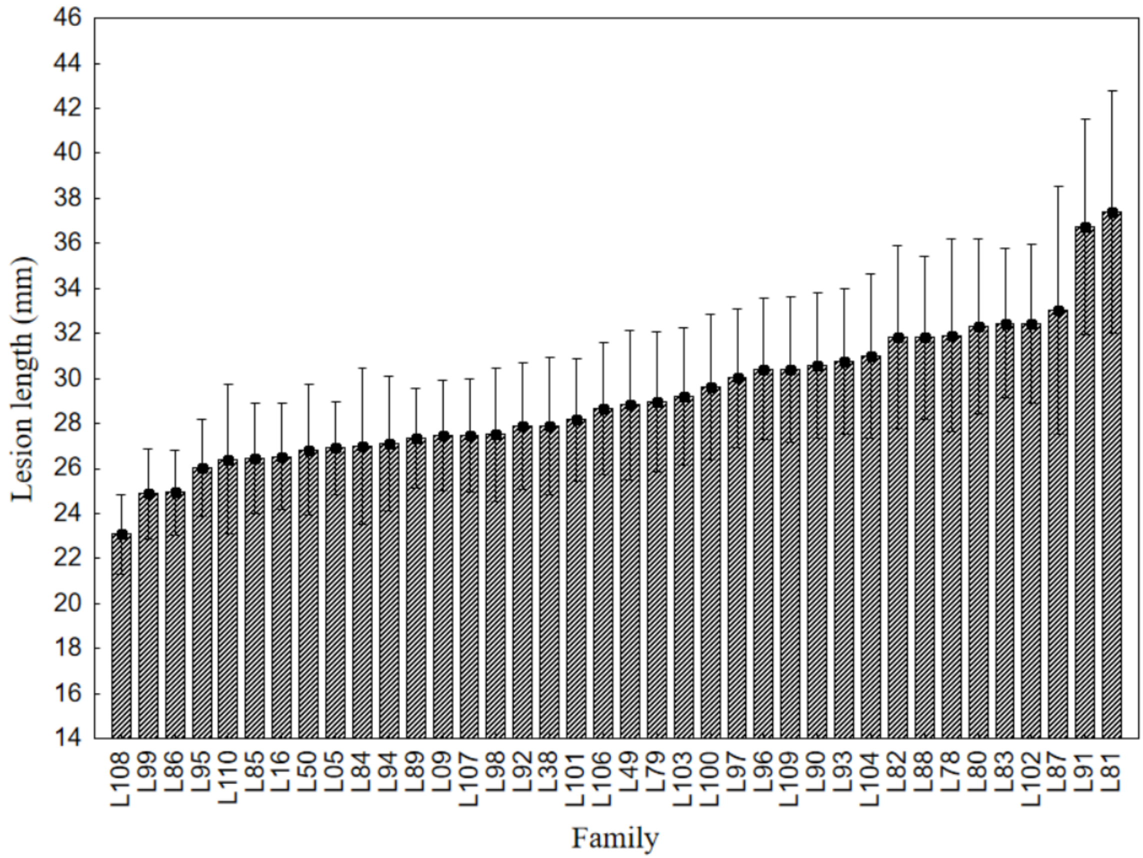

Figure 2.11 Mean lesion length caused by both the fungal treatments on *Pinus taeda* families (year: 2014). Error bars indicate 95% confidence interval.

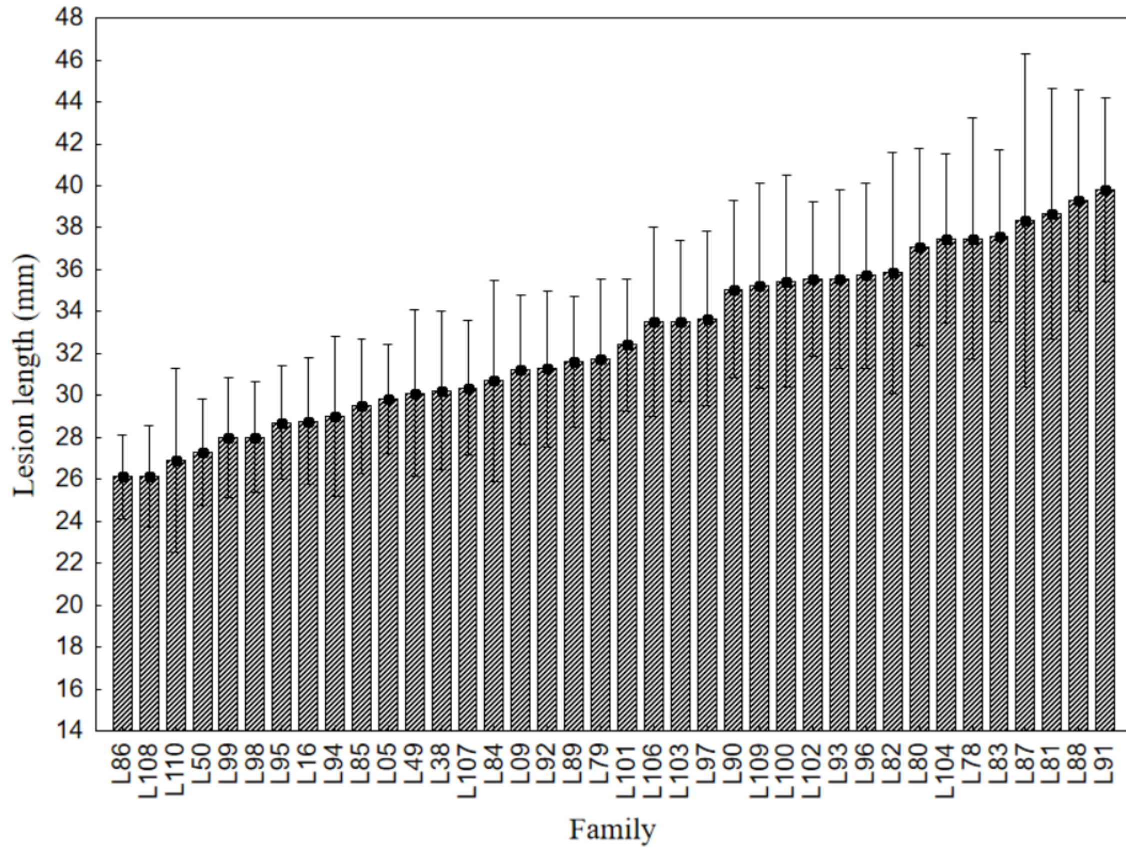

Figure 2.12 Mean lesion length caused by *Grosmannia huntii* on different *Pinus taeda* families (year: 2014). Error bars indicate 95% confidence interval.

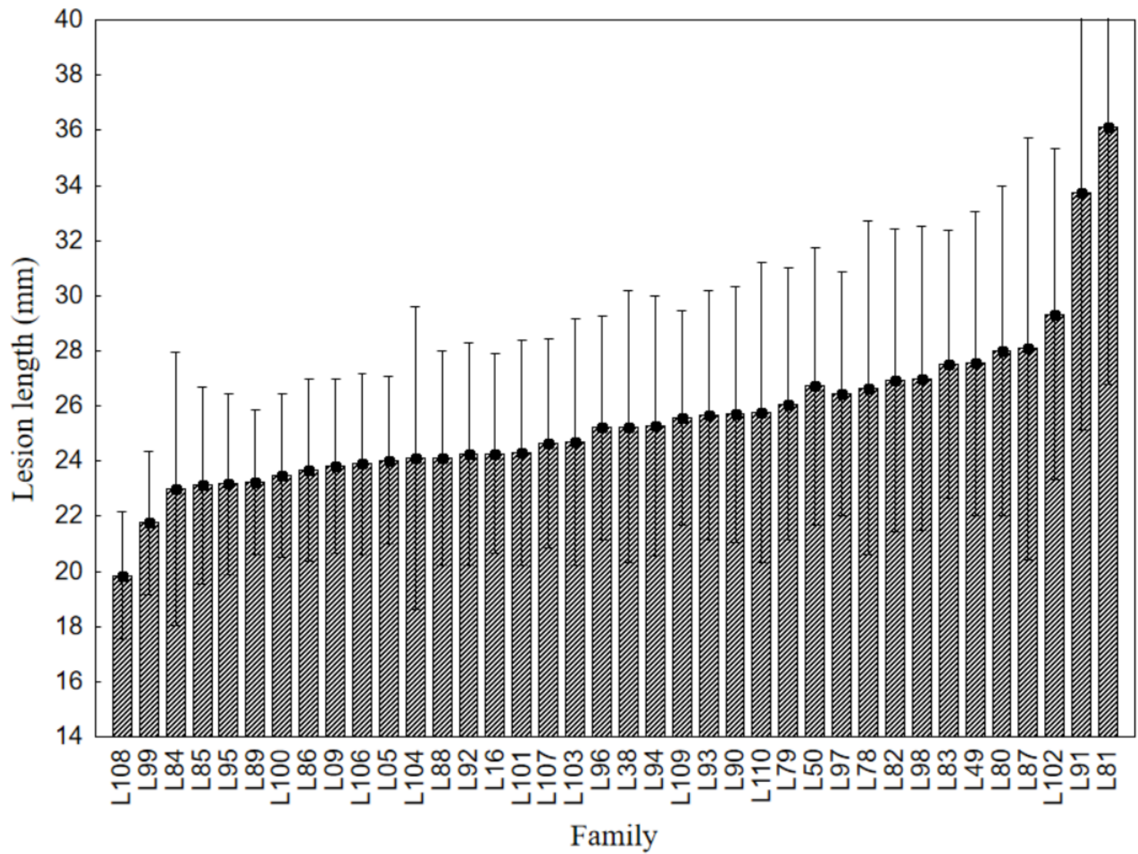

Figure 2.13 Mean lesion length caused by *Leptographium terebrantis* on different *Pinus taeda* families (year: 2014). Error bars indicate 95% confidence interval.

Table 2.8 Covariance parameter estimates from the mixed model (Year 2014).

Parameter	Covariance Parameter	Estimate	Standard Error	Z values	Pr>Z
Lesion length	Family	6.16	2.16	2.85	0.0022
	Family*treatment	1.46	1.26	1.16	0.1230
	Residual	149.07	3.94	37.85	<0.0001
Occlusion length	Family	7.34	3.49	2.13	0.0166
	Family*treatment	4.60	2.89	1.59	0.0620
	Residual	289.50	7.69	37.65	<0.0001

Table 2.9 Type three fixed effects from the mixed model (Year 2014).

Variables	Effect	Num DF	Den DF	F value	Pr>F
Lesion length	RCD	1	2864	6.98	0.0083
	Block	5	2864	117.11	<0.0001
	Trt	1	2864	179.62	<0.0001
	Block*Trt	5	2864	115.03	<0.0001
Lesion width	RCD	1	2864	137.60	<0.0001
	Block	5	2864	74.62	<0.0001
	Trt	1	2864	75.75	<0.0001
	Block*Trt	5	2864	10.29	<0.0001
Lesion depth	RCD	1	2864	68.03	<0.2426
	Block	5	2864	16.98	<0.0001
	Trt	1	2864	35.98	<0.0001
	Block*Trt	5	2864	53.15	<0.0001
Occlusion length	RCD	1	2833	13.02	0.0003
	Block	5	2833	187.50	<0.0001
	Trt	3	2833	295.73	<0.0001
	Block*Trt	15	2833	245.55	<0.0001
Occlusion width	RCD	1	2833	266.42	<0.0001
	Block	5	2833	234.15	<0.0001
	Trt	3	2833	574.56	<0.0001
	Block*Trt	15	2833	174.19	<0.0001
Occlusion depth	RCD	1	2832	177.16	<0.0001
	Block	5	2832	147.25	<0.0001
	Trt	3	2832	427.68	<0.0001
	Block*Trt	15	2832	161.18	<0.0001

Note: Trt: Treatment, RCD: Root-collar diameter.

2.4.3 Year 2016

The seedling survival was not significantly different among the family. All families had 100% seedling survival except families L50, L114 and L127 which had 97% survival rate. Neither *G. huntii* (F = 9.94, χ^2 = 0.99) nor *L. terebrantis* (F = 3.21, χ^2 = 0.67) affected seedling survival. The re-isolation success of *G. huntii* and *L. terebrantis* was 96% and 93% respectively from the inoculated seedlings.

Inoculation of *L. terebrantis* and *G. huntii* in *P. taeda* seedlings resulted in dark brown lesions around the inoculation points. The lesions caused by both fungal treatments were significantly longer than those lesions resulting from the control treatments (Table 2.10). Lesion length, width and depth and occlusion length, width and depth differed significantly between two fungal treatments (P = <0.0001) and families (P = <0.0001). The fungal treatment and family interaction was significant for lesion length (P = 0.002), occlusion length (P = <0.0001) and occlusion depth (P = 0.0171). However, the interaction was not significant for lesion width (P = 0.3784), lesion depth (P = 0.3049) and occlusion width (P = 0.0505) (Table 2.11). Families, L126, L130 and L129 had the longest, and L118 and L09 had the shortest lesion length when treated with *G. huntii*. Families, L126 and L129 had the longest average lesion length, and L33 and L111 had the shortest lesion length when challenged with *L. terebrantis*.

Inoculations did not alter seedling biomass, however, biomass varied by family ($F_{(22, 1420)}$ = 13.40, P = <0.001). Fungal treatment interaction was not significant ($F_{(66, 1420)}$ = 0.93, P = 0.6369) indicating that biomass did not change in seedlings within families receiving different inoculation treatments.

Table 2.10 Pairwise comparison of log lesion between the treatments.

Treatment	Estimate	Standard error	DF	t Value	Pr > |t|
GH vs LT	-0.01	0.01	3943	-10.39	<0.0001
GH vs W	0.40	0.01	3943	40.60	<0.0001
GH vs WM	0.31	0.01	3943	32.56	<0.0001
LT vs W	0.50	0.01	3943	51.74	<0.0001
LT vs WM	0.41	0.01	3943	43.75	<0.0001
W vs WM	-0.09	0.01	3943	-8.99	<0.0001

Note: GH: *Grosmannia huntii,* LT: *Leptographium terebrantis*, W: Wound, and WM: Wound + media.

Table 2.11 Treatment and family fixed effects from mixed model.

Variable	Effect	Num DF	Den DF	F Value	Pr > F
Lesion length	Block	5	1971	178.45	<0.0001
	IRCD	1	1971	8.88	0.0029
	Family	22	1971	4.48	<0.0001
	Trt	1	1971	137.51	<0.0001
	Trt*Family	22	1971	2.10	0.002
Lesion width	Block	5	1963	26.06	<0.0001
	IRCD	1	1963	13.23	0.0003
	Family	22	1963	2.54	0.0001
	Trt	1	1963	206.43	<0.0001
	Trt*Family	22	1963	1.07	0.3784
Lesion depth	Block	5	1971	69.23	<0.0001
	IRCD	1	1971	39.99	<0.0001
	Family	22	1971	2.15	0.0015
	Trt	1	1971	130.79	<0.0001
	Trt*Family	22	1971	1.13	0.3049
Occlusion length	Block	5	1952	223.98	<0.0001
	IRCD	1	1952	5.49	0.0193
	Family	22	1952	5.16	<0.0001
	Trt	1	1952	552.47	<0.0001
	Trt*Family	22	1952	2.65	<0.0001
Occlusion depth	Block	5	1952	36.32	<0.0001
	IRCD	1	1952	37.41	<0.0001
	Family	22	1952	2.51	0.0001
	Trt	1	1952	587.09	<0.0001
	Trt*Family	22	1952	1.75	0.0171
Occlusion width	Block	5	1952	112.38	<0.0001
	IRCD	1	1952	29.71	<0.0001
	Family	22	1952	2.93	<0.0001
	Trt	1	1952	546.88	<0.0001
	Trt*Family	22	1952	1.55	0.0505

Note: RCD: Initial Root-collar-diameter, Trt: Treatment.

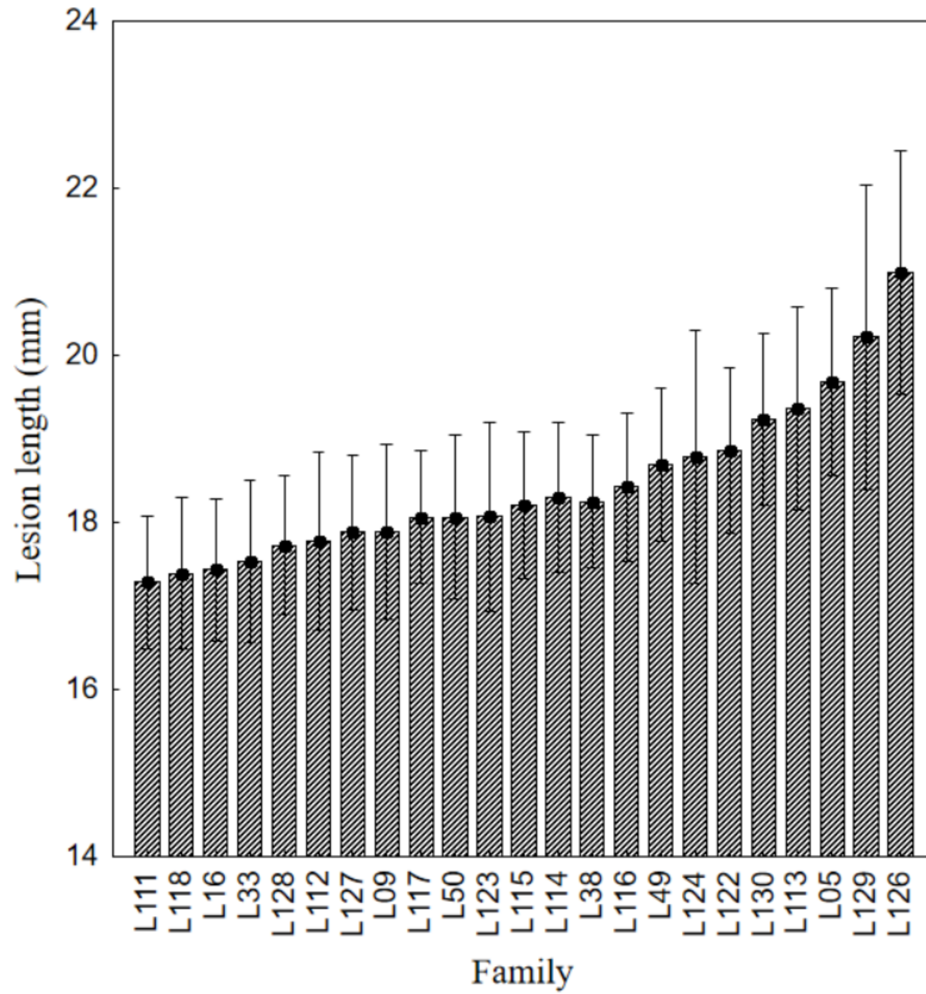

Figure 2.14 The overall mean lesion lengths caused by both the fungal treatments on *Pinus taeda* families (year: 2016). 95% confidence intervals are indicated by error bars.

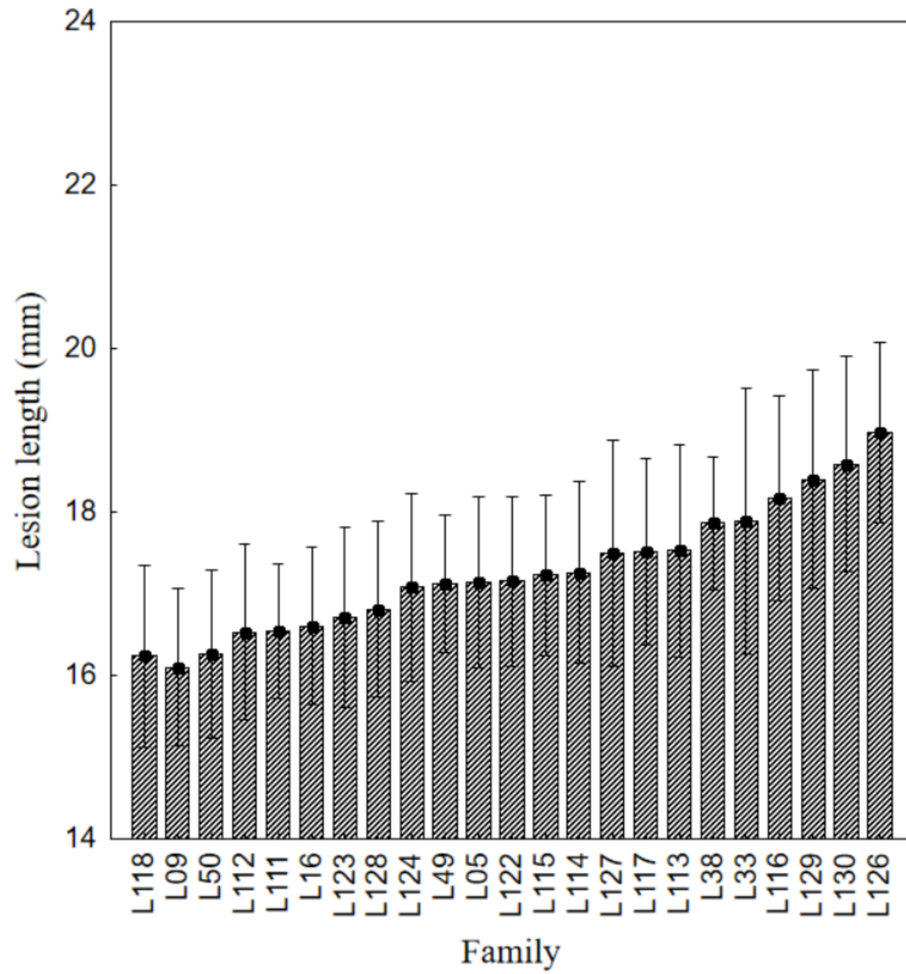

Figure 2.15 Mean lesion length caused by *Grosmannia huntii* on *Pinus taeda* families (year: 2016). Error bars indicate 95% confidence interval.

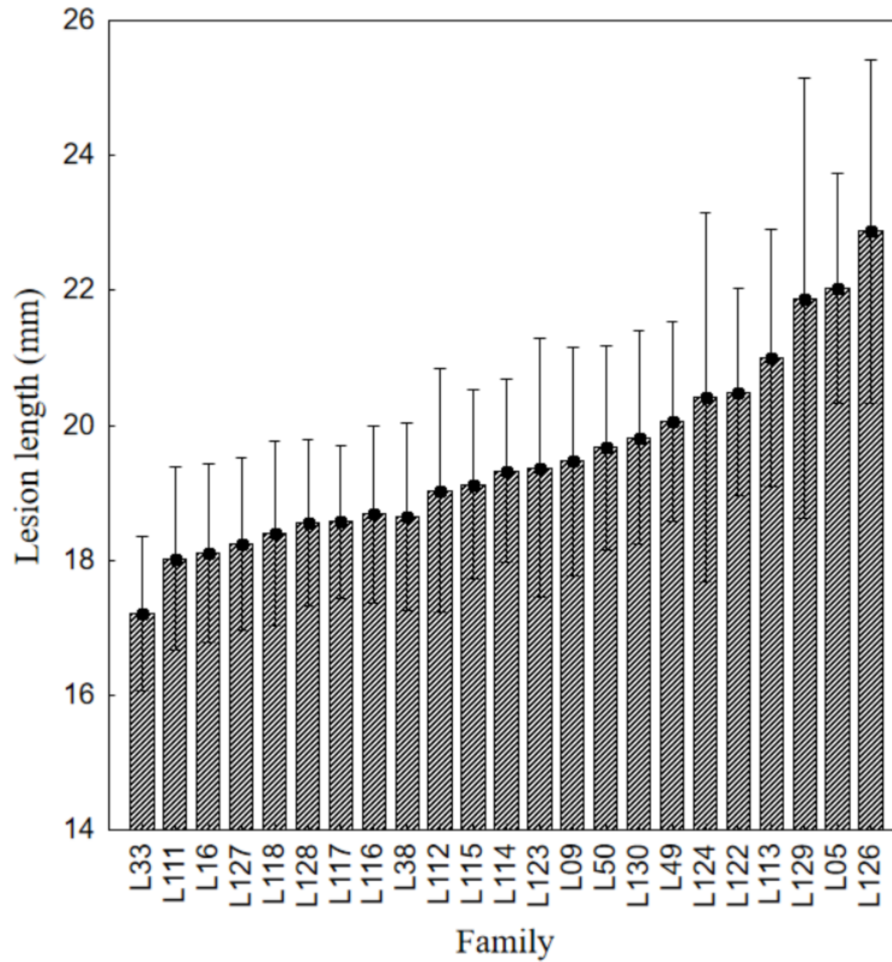

Figure 2.16 Mean lesion length caused by *Leptographium terebrantis* on *Pinus taeda* families (year: 2016). Error bars indicate the 95% confidence interval of the mean.

2.4.4 Response of connector families in all years

The 6 connector families which were inoculated every year did not show any family x year interaction (in terms of lesion length). However, the lesion formation varied by year of inoculation. The lesion length was smallest in the year 2016 and greatest in 2014. These families did not respond differently to the fungal inoculation (in terms of lesion length) within each year. The six families responded similarly to the fungal inoculation (in terms of lesion length) in within each experimental year (Figure 2.17).

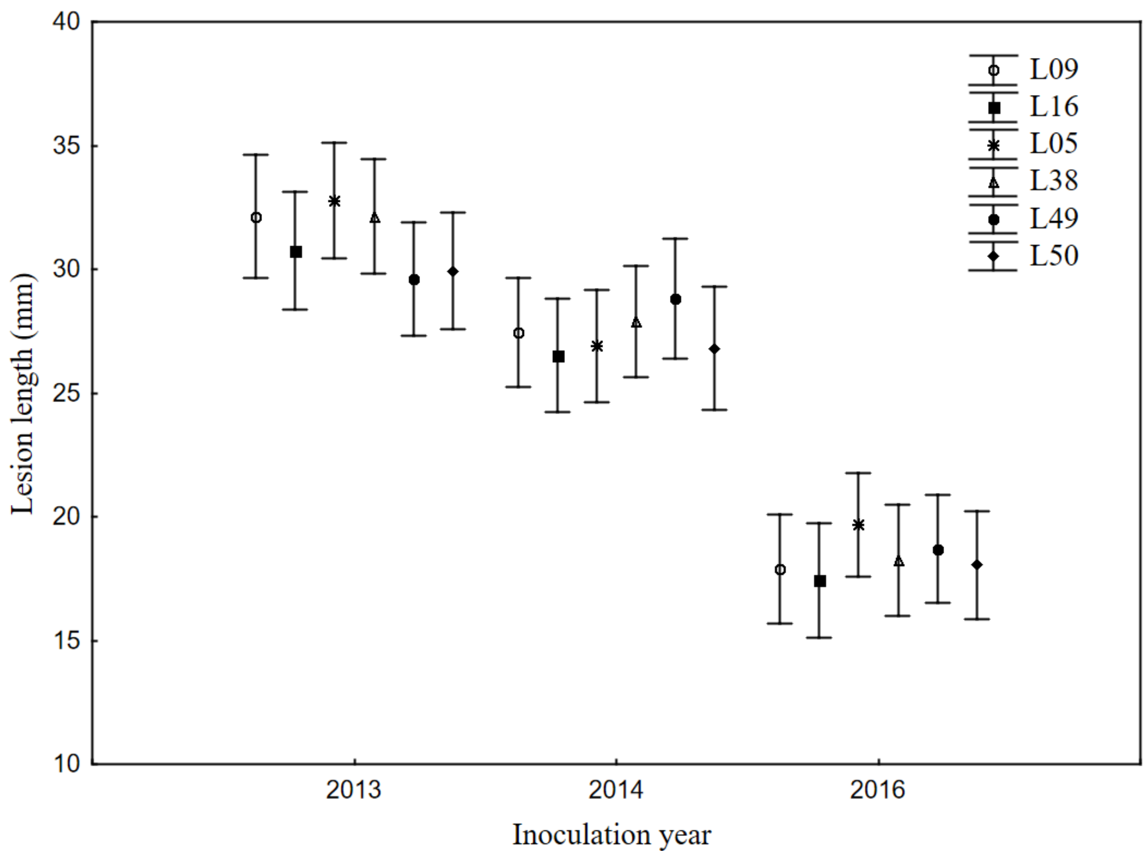

Figure 2.17 Mean lesion length of connector *Pinus taeda* families on each inoculation year. Error bars represent 95% confidence interval.

2.5 Discussion

There was intra-species variation in tolerance/susceptibility (regarding lesion length and occlusion length) of *P. taeda* to *L. terebrantis* and *G. huntii*. Similar variation

was observed in the study of different *Pinus* families in response to *Fusarium circinatum* Roux et al. (2007), and clones of *Ulmus americans* L. (American elm) to *Ophiostoma ulmi* (Buism). Tchernoff (1965), reported similar response of *Quercus robur* L. (pedunculate oak) families to *Phytophthora cambivora* (Petri) Buisman. Jankowiak et al. (2013). Current screening trials show that there is significant potential for selecting PD tolerant *P. taeda* from current southeastern U.S. planting stock. These families have the potential for use as parents in breeding programs to maximize the disease tolerance in *Pinus taeda* and thus to ensure that losses due to fungi associated with PD can be minimized in the future.

Intraspecific variation in tolerance of the *P. taeda* to ophiostomatoid fungi is independent of the seedling growth conditions. Bare-root and container-grown seedlings demonstrated no inter-stocktype differences in response to ophiostomatoid fungi (Figure 2.17). Both stocktypes of a single family responded similarly. Containerized seedlings had higher survival rate than the bare-root, making former stocktype more favorable to use in the experimental design studies.

Lesions observed in host seedling after fungal inoculation serves as a reliable estimator of the fungal virulence as well host response (Matusick and Eckhardt, 2010, Matusick et al., 2010). While other host responses such as lesion depth, lesion width, occlusion length, and occlusion depth and occlusion width also were measured, these responses did not provide sufficient evidence to draw any conclusions regarding relative tolerance as the depth of sapwood in limited. Occlusion length acted as supporting response variable.

Pinus taeda families with shorter lesion were considered relatively tolerant to the fungi than the families with longer lesions. In an ecological scale, the bark-beetle and the associated fungi (i) must overcome the tree defense, and (ii) obtain food from the tree. The utilization of the tree's resources such as sapwood resources and non-structural carbohydrates (NSC) by the trees in defense may lead to depletion of the resources. The relatively tolerant families can defend against the fungi by utilizing fewer resources. In the susceptible family, the successfully colonized ophiostomatoid fungi use the tree's resources, and the resources decline over time impacting the growth and development of the tree. The trees with relatively larger lesions as a response to fungal inoculation have greater resource reduction (Lahr and Krokene, 2013).

As indicated by the relatively longer lesion and occlusion length, *L. terebrantis* and *G. huntii* were found to be relatively more pathogenic in 2013 than 2014 or 2016. Our results are similar to those of Singh et al. 2014 where they reported that the pathogenicity of the fungi varied among years. Singh et al. (2014) gave two possible explanations for this variation: (i) use of different seedling stocktypes in different years or (ii) genotype x environment interaction. The former reason can be excluded as our results suggest that inter-stocktype response to ophiostomatoid fungi do not exist. Thus, genotype x environment interaction might have resulted in differences in fungal pathogenicity among years. In this regard, in January 2013 (when seedlings were potted), lowest and monthly average temperature were -1 ° and 12 ºC respectively (according to weather underground[1]). In contrast, in January 2014, the lowest and the average temperature was -12 ºC and 3 ºC respectively which was lower compared to 2013. In

[1] http://www.wunderground.com

addition, seedlings were subjected to a winter storm on January 28. Thus, the observed differences in fungal pathogenicity between years could be due to family x environment interaction. This can be further supported by Petäistö et al (1993) who suggested that trees experiencing cold and frost also are more susceptible to infection by *Gremmeniella abietina*.

The ophiostomatoid fungi varied in virulence among each year of inoculation. In 2013 and 2016, *L. terebrantis* was found to be more virulent than *G. huntii*. Whereas, in 2014 (when the seedling growing condition was cold), *G. huntii* was relatively more virulent (in terms of lesion length) than *L. terebrantis*. Our finding that *G. huntii* is relatively virulent than *L. terebrantis* has been supported by Matusick and Eckhardt, 2010 where they reported *G. huntii* to be more virulent than *L. terebrantis* in *Pinus* species in the southern U.S. Although we lack experiments with controlled temperature and fungal virulence, results suggest the disease-causing ability of the pathogen is associated with either how stressed the hosts are due to adverse environmental condition or how conducive is the condition for the growth of pathogen (Stenlid and Oliva, 2016). Our results underline the need to include the role of the environment while predicting the impact of the invasive pathogens (Dukes et al., 2009). Tolerance to the *L. terebrantis* and *G. huntii* varies between the families, and the findings are consistent with the previous years.

Fungi had no effect on seedling growth regarding seedling biomass. Some short-term studies have found that the growth ability of *Pinus taeda* seedling may vary between the family independent of the fungal inoculation (Chieppa et al. 2015; Chieppa et al., 2017). Some other conifer and ophiostomatoid fungal interactions studies have not

considered the impact of fungi on seedling growth (Matusick and Eckhardt, 2010; Singh et al., 2014). Oliva et al., (2014) suggests that vascular-inhabiting fungi obtain sugar from degraded cells around the lesion and xylem sap in order to thrive. In addition, allocation of the more carbon in defense of vascular-inhabiting fungi cause resource starvation which decreases plant productivity (Oliva et al., 2014). In a natural scenario, bark beetles inoculate the fungi on previously stressed trees. So, it is possible that the fungi negatively impact the growth and biomass of the trees in the field at a higher level. We, therefore, suggest that long-term studies should be conducted to understand the impact of these fungi on seedling biomass.

Families utilized in the present study have the desired attributes (undisclosed) depending on the objective of the forest companies. These families responded differently to the *L. terebrantis* and *G. huntii*. Whereas, the wild-type families had the intermediate levels of virulence. This suggests that different families chosen for desired attributes differ in their tolerance towards the ophiostomatoid fungi, but wildtype families do not differ. Wild-type families may be relatively less susceptible to fungi than some of the susceptible families but use of these families may not meet the objective of the timber companies in the southern U.S. Thus a particular attribute such as growth phenology, wood density, wood volume, etc. may or may not benefit the plant against the attack by the studied fungi. Thus, the selection of families tolerant to these fungi provides an opportunity to make a decision regarding the planting of the suitable *P. taeda* families.

In conclusion, *P. taeda* families show wide variation in response to ophiostomatoid fungi associated with the pine decline thus indicating family genetics play an important role in the variation in response to the fungi. Containerized and bare-

root seedling stocktypes of the same family are equally susceptible or tolerant to *L. terebrantis* and *G. huntii* suggesting both types of the seedlings can be used in further screening studies. Pathogenicity of the two fungi varies even within a particular family so relative tolerance of *P. taeda* families to *L. terebrantis* and *G. huntii* should be considered separately. Since the tolerance of the families is tested at the premature stage future, studies should be performed on mature *P. taeda* families. Also, future studies should be focused on transcriptional changes in the tolerant and susceptible families following fungal inoculation to improve our understanding of mechanisms of disease tolerance.

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CHAPTER III

Intra-species Variation in Response of Mature *Pinus taeda* Families to Root-infecting Ophiostomatoid Fungi

3.1 Abstract

Seedling screening studies have shown intra-species variation in susceptibility of *Pinus taeda* (loblolly pine) to *Leptographium terebrantis* and *Grosmannia huntii,* the causal agents of *Pinus* species root-infection. Roots of mature *P. taeda* families determined as susceptible and tolerant to *L. terebrantis* and *G. huntii* by previous seedling screening trials were artificially inoculated with the same fungal isolates. Dark necrotic lesion and the vascular occlusion were recorded 8 weeks later. Families previously considered as susceptible had longer lesions and occlusions when compared to the tolerant families. The variation in susceptibility/tolerance pattern remained similar as exhibited by families at seedling trial. The studies indicate that intra-species variation in relative susceptibility of *P. taeda* to *L. terebrantis* and *G. huntii* remain the same regardless of the tree age.

3.2 Introduction

Pinus taeda L., an important timber species, is commercially planted on approximately 11.7 million hectares of land in the southern U.S. (Baker and Langdon, 1990; Rauscher, 2004). This species, when fertilized, can produce high per hectare wood volume yields (Fox et al., 2004) providing an all-purpose forest product such as furniture,

pulpwood, composite boards, crates, boxes and pallets (Schultz, 1997). In addition, *P. taeda* stands provide habitat for wildlife including endangered species such as red-cockaded woodpecker (Jones and Hunt, 1996), and also place for wildlife watching and hunting (Poudel et al., 2016). This pine species directly or indirectly contributes $30 billion to the economy of the southern U.S. (Schultz, 1999).

A factor impacting the growth and optimal productivity of this important pine species is root-infecting pathogenic fungi, among which some are associated with Pine Decline (PD) (Otrosina et al., 1999; Eckhardt et al., 2004a). Pine Decline, a decline disease syndrome, was first observed by Brown and Mc Dowell (1968) on the Talladega National Forest in Oakmulgee Ranger District located in central Alabama, U.S. Brown and Mc Dowell (1968) reported 40-to-50-year-old *P. taeda* stands with symptoms of decline that included thinning crowns, reduced radial growth, and root deteriorations. This decline syndrome has subsequently been reported across the southeastern U.S. (Hess et al., 2002; Eckhardt et al., 2007).

The decline-disease spiral, the widely accepted model of forest decline, given by Manion (1981) involves interactions of 3 factors as follows: (i) factors bringing trees under constant stress (predisposing factors), (ii) short-term factors increasing the severity of stress (inciting factors), and (iii) factors a playing role at the end (contributing factors). In the context of PD, tree genetics and increased slopes are the predisposing, drought and ozone are the inciting, and root-feeding bark beetles and their associated ophiostomatoid fungi are the contributing factors (Eckhardt et al., 2004a; Eckhardt et al., 2004b; Eckhardt et al., 2007; Eckhardt and Menard, 2008). Root-feeding bark beetles such as *Hylobius pales* Herbst., *Hylastes* spp. and *Pachylobius picivorus* (Germar) act as vectors in

introducing their ophiostomatoid fungal associates, namely *Leptographium terebrantis* S.J. Barras and T.J. Perry, *Grosmannia huntii* R.C. Rob. Jeffr, and *Leptographium procerum* (W.B. Kendr.) M.J. Wingf., into roots of *P. taeda* trees (Eckhardt et al., 2004 a; Eckhardt et al., 2004b). *Leptographium terebrantis* and *G. huntii* were found to be more virulent to southern *Pinus* species (Matusick et al., 2010; Singh et al., 2014) thus warranting further need to study these fungi. Also, the inter-species and intra-species susceptibility of the host (a major predisposing factor) to fungi contributing to PD are still poorly known.

Ophiostomatoid fungi cause lesions in the phloem and occlusion in the xylem of artificially inoculated seedling stems (Eckhardt et al., 2004a), stems (Matusick et al., 2016), and roots (Matusick et al., 2010) of mature *P. taeda* trees. Furthermore, these fungi use sugars, defense compounds and sugars leaking from degraded cell walls to surive and proliferate inside xylem conduits (Hammerbacher et al., 2013). Concomitantly, trees synthesize defensive carbon compounds and form tyloses and structures that can compartmentalize fungal spread and infection (Yadeta and Thoma, 2013). Fungal spread and tyloses formation both disturb plant water transport (Joseph et al., 1988) resulting in tree decline and death. Moreover, investment by the tree in defense reaction occurs at the expense of radial growth (Krokene et al., 2008).

Artificial inoculation of *Grosmannia* and *Leptographium* species into roots of *P. taeda*, *P. palustris* Mill. (longleaf pine) and *P. elliotti* Englem. (slash pine) by Matusick et al. (2010) showed inter-species variation in susceptibility/tolerance. *Pinus taeda* was the most susceptible among 3 *Pinus* species. Furthermore, Singh et al. (2014) conducted a seedling screening study to examine intra-species variability of *P. taeda* tolerance to *G.*

huntii and *L. terebrantis*. Results indicate that *P. taeda* families have varying levels of susceptibility/tolerance to those fungi. However, given that these fungi affect mature trees in the ecological scenario, the reliability of results obtained from families at a seedling stage is yet to be answered. To address this important question, we further sought to determine the intra-species variation in tolerance/susceptibility of mature *P. taeda* trees to *L. terebrantis* and *G. huntii* based on the seedling studies performed by Singh et al. (2014). We hypothesize that intra-species variation in tolerance/susceptibility of *P. taeda* to ophiostomatoid fungi is an inherent character of a family regardless of the tree's age.

3.3 Materials and method

3.3.1 Experimental design

Pinus taeda stands from 4 families in Alabama and Georgia (~ 31°53'N and 85°8'W, 81.16 m above the sea level) were chosen. Among 4 families, T1 and T2 represent the families relatively tolerant to ophiostomatoid fungi based on seedling screening trials conducted by Singh et al. (2014). Conversely, S1 and S2, represent susceptible families. The study was conducted twice; once in summer 2015 (June 15 - August 15) and again in spring 2016 (March 15 - May 15). A total of 25 healthy mature *P. taeda* trees, 17-year-old (for 2015) and 18-year-old (for 2016) with no visible signs or symptoms of aboveground disease were selected per family. Selected trees had a mean diameter at breast height and height of 19.5 cm (± 2.8 cm), and 13 m (± 2 m) respectively.

3.3.2 Inoculation experiment

Roots were artificially inoculated with fungal cultures consisting of single spore isolates of *L. terebrantis* (ATCC accession no. MYA-3316) and *G. huntii* (ATCC accession no. MYA-3311) maintained at 4 ºC in Malt Extract Agar (MEA) in the Forest Health Dynamics Laboratory at Auburn University. These fungal isolates were cultured on 2% MEA, two weeks prior to root inoculations. The *L. terebrantis* and *G. huntii* isolates used in the study were respectively isolated from the roots of *P. taeda* from the Talladega National Forest, Oakmulgee Ranger District, AL, USA and of *P. palustris* from the Fort Benning Military Reservation, GA, USA exhibiting symptoms of decline such as localized tissue damage and defoliating crowns as described by Eckhardt et al. (2007). Several previous artificial stem and root inoculation studies have used these isolates (Matusick et al., 2010; Singh et al., 2014; Chieppa et al., 2017).

In the field, two primary lateral roots were excavated with hand tools that had been sterilized using 70% ethanol from each *P. taeda* tree without damaging roots (Figure 3.1). On each excavated root, two wounds to the cambium layer and 30 cm horizontally apart from each other, were created by hitting a rubber mallet on a 13-mm diameter sterile steel arch punch. The root inoculation included removing the bark plug and placing the 10-mm agar plug (fungus-side-down) with actively growing fungi in the wound (Figure 3.2). There were 2 fungal treatments: *L. terebrantis, G. huntii* and 2 control treatments: wound with sterile media and wound without media. One of each fungal treatment and a control treatment were randomly paired together in each of the roots per tree. Following inoculation, the bark was replaced, and the wound sealed with duct tape to minimize further contamination. The inoculation points of the roots were marked with labeled pin flags and covered with soil.

Figure 3.1 Two primary lateral roots of *Pinus taeda* excavated for the fungal inoculation.

Figure 3.2 Inoculation of the agar plug with fungi in the lateral root of *Pinus taeda.*

3.3.3 Laboratory measurements

Eight weeks post-inoculation, the inoculated roots were re-excavated and removed from the tree (Figure 3.3). The exposed ends of the roots were painted with Drylok Latex Masonry Waterproofer (Scranton, PA, USA) and were transported to the lab at Auburn University for further processing (Figure 3.4). To observe the necrotized and occluded tissues, bark around the inoculation area was removed and painted with a solution of FastGreen stain (FastGreen FCF; Sigma Chemical Co.) (0.25 g/L of water). The necrotic tissue area on each inoculation site was traced on a clear transparent sheet, and lesion area was determined by using a Lasico Planimeter (Lasico®, Los Angeles, CA) as described by Matusick et al. (2012). The amount of unstained tissue around the fungal inoculation point determined the occlusion length. The root samples were cut transversely at the point of the inoculation and the discolored sapwood was measured as occlusion depth. Small pieces of stem tissue from the distal and proximal portions of the inoculation site were plated on MEA amended with cycloheximide and streptomycin to confirm fungal infection by re-isolation of the inoculated fungi.

Figure 3.3 Re-excavation of the fungi inoculated lateral roots of *Pinus taeda* for further measurements.

Figure 3.4 *Pinus taeda* root samples ready for measurement in the laboratory.

3.3.4 Statistical analysis

Data were analyzed using multivariate analysis of variance (MANOVA) in SAS 9.4 (SAS Institute, Inc., Cary, NC, USA) using the PROC GLM statement. Family, fungal treatments and study period were kept as fixed effects. Possible interactions also were tested in the model. Root-diameter was used as a covariate. The data met assumptions such as the normal distribution of residuals and homogeneity of variance, and did not warrant further transformation. Lesion length and area were used as the strongest response variables (Matusick et al., 2010; Singh et al., 2014). As the response variables (lesion and occlusion) differed by study period (replication), data was analyzed separately for each study period.

The general linear model

$$y_{ik} = \beta_0 + T_i + F_k + TF_{ik} + coV + \mathcal{E}_{ik} \quad (1)$$

was used, where y_{ik} is response variable such as lesion length, occlusion length, and depth of ith fungal treatment in k^{th} family, coefficients β_0 is the intercept, T_i express the effect of fungal treatment, F_k express the effect of *P. taeda* family, TF_{ik} express the treatment and family interaction, root diameter was used as covariate (coV), and $\mathcal{E}_{ik}$ express the random error for i^{th} treatment in k^{th} family. Estimate statements were used to estimate the differences between the families. Graphs were created on STATISTICA 10 (Statsoft, Inc., Tulsa, OK, USA).

3.4 Results

The inoculation of *L. terebrantis* and *G. huntii* into the roots of mature *P. taeda* led to necrosis and resin soaking around the initial point of inoculation (Figure 3.5 and Figure 3.7)). The inoculation of the wound and wound with sterile agar resulted in

significantly smaller lesions than those caused by fungal inoculation (Figure 3.6). Families responded differently to the fungal inoculation at each study periods in terms of lesion area ($F_{(3,374)} = 10.48$, $p = <0.0001$), lesion length ($F_{(3,374)} = 7.69$, $p = <0.0001$) and occlusion length ($F_{(3,374)} = 3.68$, $p = 0.01$) (Figure 3.8). Hence, the data from summer and spring root inoculation studies were analyzed separately. The inoculation of the fungi into roots resulted in more tissue necrosis (in terms of lesion area) in the spring 2016 inoculation compared to the summer 2015 inoculation (Figure 3.9).

Figure 3.5 Resinosis and necrosis in the root 8 weeks following inoculation with *Leptographium terebrantis* in family S2.

Figure 3.6 Wound control treated root section with small necrotic area.

Figure 3.7 Pie-shaped occlusion observed at the cross-section of inoculated root.

3.4.2 Summer 2015 inoculation

Families differed in their overall response to the fungal inoculation during summer 2015 ($F_{(21, 543)} = 3.01$, $p = <0.0001$) (Table 3.1). However, within a single family, the pathogenicity of the two fungi were not different ($F_{(21, 543)} = 1.07$, $p = 0.38$). *Leptographium terebrantis* and *G. huntii* did not differ in their virulence (in terms of occlusion length) ($F_{(7,179)} = 1.41$, $p = 0.21$) (Figure 3.10).

Four *P. taeda* families differed in their response to inoculated fungi in terms of the area ($p = <0.0001$) and length of the lesion ($p = 0.01$), and length ($p = 0.03$) and depth ($p = <0.0001$) of the vascular occlusion (Table 3.1). The lesion area did not differ significantly between two families (T1 and T2) tolerant to ophiostomatoid fungi ($p = 0.07$) and between two families (S1 and S2) susceptible to ophiostomatoid fungi ($p = 0.30$) (Table 3.2 and 3.3). The lesion area observed in tolerant family T2 was substantially smaller than susceptible families S1 and S2 (Table 3.2 and 3.3). Families showed a similar trend for lesion length. Susceptible family (S1) had a significantly longer occlusion length compared to tolerant families (T1 and T2) (Table 3.2 and 3.3). However, the depth and width of occlusion at the cross section underneath the fungal inoculation point were significantly shorter in susceptible families S1 and S2 compared to tolerant family T1 (Table 3.3).

The success of re-isolation of *G. huntii* from the inoculated root was 92%, 80%, 92%, and 92% for family T1, T2, S1, and S2 respectively. Similarly, for *L. terebrantis* the re-isolation success was 100%, 96%, 88%, and 100% for family T1, T2, S1, and S2 respectively. The higher success of re-isolation of the fungi inoculated in root proved the success of fungal inoculation and root-infection.

3.4.3 Spring 2016 inoculation

Pinus taeda families responded differently to the fungal inoculation in spring 2016 ($F_{(21, 552)} = 4.63$, $p = <0.0001$). However, family and fungal treatment interaction were significant ($F_{(21,552)} = 2.27$, $p = <0.0001$), suggesting an overall variation in the pathogenicity of *L. terebrantis* and *G. huntii* within each family. The families responded differently to *G. huntii* and *L. terebrantis* in terms of lesion area ($p = 0.03$) and occlusion length ($p = 0.01$) (Table 3.1). *Leptographium terebrantis* caused significantly longer occlusion length than *G. huntii* in all of the families (Figure 3.10).

Area of the lesions caused by both fungi was significantly smaller in roots of tolerant families (T1 and T2) compared to the susceptible families (S1 and S2) (Table 3.4 and 3.5). The length of occlusion caused by *G. huntii* was significantly longer in the susceptible family S2 compared to all other families. However, occlusion length caused by *L. terebrantis* did not differ in between the four *P. taeda* families (Table 3.4 and 3.5). Both fungi caused lesions with a larger area and longer and wider length in susceptible family S2 followed by susceptible S1 (Table 3.5).

The success of re-isolation of *L. terebrantis* from inoculated roots was 100%, 100%, 92%, and 100% from family T1, T2, S1, and S2 respectively. Similarly, the re-isolation success of *G. huntii* was 84%, 76%, 64%, and 88%, from family T1, T2, S1, and S2 respectively. Consistent re-isolation of the fungi previously inoculated in root proved the success of fungal inoculation and infection.

Table 3.1 P-values for fixed effect and possible interactions for summer 2015 and spring 2016 inoculation.

Time	Variable	DF	Lesion Area	Lesion Length	Lesion Width	Occlusion Length	Lesion Depth	Occlusion Width	Occlusion Depth
2015	RD	1	0.01	0.01	0.01	0.08	0.65	<0.0001	0.02
	Fam	3	<0.0001	0.31	0.31	0.03	0.12	0.10	<0.0001
	Trt	1	0.08	0.10	0.10	0.10	0.58	0.34	0.02
	Fam*trt	3	0.85	0.46	0.46	0.86	0.38	0.94	0.51
2016	RD	1	0.03	0.86	<0.0001	0.99	<0.0001	<0.0001	<0.0001
	Fam	3	<0.0001	<0.0001	<0.0001	0.03	0.02	0.43	0.12
	Trt	1	0.002	<0.0001	0.62	0.31	0.31	0.10	<0.0001
	Fam*Trt	3	0.03	0.32	0.54	0.01	0.37	0.73	0.29

(Note: RD: Root-diameter, Fam: Family, Trt: Fungal treatment). P-values were estimated at $\alpha = 0.05$.

Table 3.2 Familywise mean and standard deviation of all the response variables for 2015 summer inoculation.

Family	N	Lesion Area (mm^2)	Lesion Length (mm)	Lesion Width (mm)	Occlusion Length (mm)	Lesion Depth (mm)	Occlusion Width (mm)	Occlusion Depth (mm)
T1	50	1734.44^{ab}	58.50^{a}	38.46^{a}	85.34^{a}	3.64^{a}	13.49^{a}	6.81^{a}
		(1010.62)	(18.62)	(15.51)	(24.90)	(1.43)	(3.00)	(3.02)
T2	49	1411.59^{b}	57.02^{ab}	37.86^{a}	85.54^{a}	3.96^{a}	14.22^{a}	7.11^{a}
		(635.08)	(12.46)	(12.33)	(29.08)	(1.54)	(2.45)	(3.08)
S1	46	2400.76^{c}	76.41^{c}	43.13^{a}	102.09^{b}	3.35^{a}	13.37^{a}	5.54^{b}
		(1553.76)	(28.38)	(12.22)	(38.42)	(0.96)	(3.68)	(2.67)
S2	49	2125.22^{ac}	67.23^{ac}	38.38^{a}	86.96^{a}	5.84^{b}	12.74^{a}	5.42^{b}
		(1255.88)	(22.66)	(16.04)	(25.65)	(10.98)	(4.08)	(2.86)

T1 and T2: Families considered tolerant to root-infecting ophiostomatoid fungi at seedling stage, and S1 and S2: Families considered susceptible root-infecting ophiostomatoid fungi at seedling stage. Means followed by the standard deviation in parenthesis. Numbers followed by same letters within each column are not significantly different at $\alpha = 0.05$.

Table 3.3 Parameter estimates of the response variables between the families in summer 2015 inoculation.

	Lesion Area (mm²)		Lesion Length (mm)		Occlusion Length (mm)		Occlusion Depth (mm)	
Family	Estimate	p-value	Estimate	p-value	Estimate	p-value	Estimate	p-value
T1 vs T2	419.78	0.07	2.67	0.53	1.49	0.80	-0.08	0.89
	(230.30)		(4.27)		(6.10)		(0.58)	
T1 vs S1	-545.09	0.02	-16.41	0.0002	-14.63	0.01	1.54	0.01
	(235.31)		(4.37)		(6.16)		(0.59)	
T1 vs S2	-305.25	0.18	-7.67	0.07	-0.13	0.98	1.59	0.007
	(229.76)		(4.26)		(6.01)		(0.58)	
T2 vs S1	-964.87	<0.0001	-19.09	<0.0001	-16.12	0.008	1.62	0.006
	(232.88)		(4.32)		(5.99)		(0.57)	
T2 vs S2	-725.02	0.002	-10.34	0.02	-1.62	0.78	1.66	0.004
	(229.04)		(4.25)		(6.10)		(0.58)	
S1 vs S2	239.84	0.30	8.74	0.04	-14.50	0.01	0.04	0.94
	(233.05)		(4.33)		(6.09)		(0.58)	

T1 and T2: Families considered tolerant to root-infecting ophiostomatoid fungi at seedling stage, and S1 and S2: Families considered susceptible to root-infecting ophiostomatoid fungi at seedling stage. Estimates followed by the standard error in parenthesis. P-values show significant differences at $\alpha = 0.05$.

Table 3.4 Means of response variables in four families inoculated with *L. terebrantis* and *G. huntii* in spring 2016.

Fungi	Family	N	Lesion Area (mm^2)	Lesion Length (mm)	Lesion Width (mm)	Occlusion Length (mm)	Occlusion Depth (mm)
LT	T1	25	2330.88^{a}	95.44^{a}	33.24^{a}	256.04^{a}	12.23^{a}
			(729.69)	(31.40)	(5.02)	(81.64)	(5.22)
	T2	25	2608.20^{a}	103.26a^{b}	37.86^{a}	206.20^{a}	16.35^{a}
			(847.55)	(26.31)	(13.53)	(83.11)	(8.31)
	S1	25	4764.84^{b}	115.31a^{b}	50.41^{a}	251.84^{a}	14.96^{a}
			(2126.54)	(41.78)	(17.83)	(104.67)	(5.93)
	S2	25	6435.40^{c}	164.520^{c}	53.79^{a}	255.54^{a}	14.11^{a}
			(4610.77)	(96.08)	(22.2)	(95.32)	(5.71)
GH	T1	25	2063.64^{a}	81.59^{a}	34.97^{a}	180.95^{a}	10.04^{a}
			(791.27)	(21.57)	(12.71)	(90.84)	(3.89)
	T2	25	2429.40^{a}	83.01^{a}	38.07^{b}	248.07^{b}	9.70^{a}
			(1443.48)	(34.94)	(10.40)	(106.15)	(3.47)
	S1	22	3529.73^{b}	92.71^{a}	45.85^{b}	179.96^{ab}	13.83^{b}
			(2151.41)	(37.39)	(19.89)	(101.89)	(9.15)
	S2	25	3886.36^{b}	115.75^{c}	50.52^{c}	295.69^{c}	10.13^{ab}
			(2524.02)	(62.44)	(13.28)	(190.79)	(3.89)

Note: LT: *Leptographium terebrantis*, and GH: *Grosmannia huntii*. T1 and T2: Families considered tolerant to root-infecting ophiostomatoid fungi at seedling stage, and S1 and S2: Families considered susceptible to root-infecting ophiostomatoid fungi at seedling stage. Means followed by the standard deviation in parenthesis. Numbers followed by same letters within each column within each fungus are not significantly different at $\alpha = 0.05$.

Table 3.5 Estimate of difference in response variables between families inoculated with *L. terebrantis* and *G. huntii* in spring 2016.

		Lesion Area (mm^2)		Lesion Length (mm)		Lesion Width (mm)		Occlusion Length (mm)		Occlusion Depth (mm)	
Fungi	Family	Estimate	p-value	Estimate	p-value	Estimate	p-value	Estimate	p-value	Estimate	p-value
LT	T1 vs T2	-129.41	0.86	-10.27	0.53	-1.30	0.77	39.73	0.14	-0.73	0.53
		(758.05)		(16.43)		(4.44)		(26.47)		(1.16)	
	T1 vs S1	-25359.26	0.002	-21.10	0.19	-15.50	0.0006	-0.91	0.97	-1.03	0.37
		(742.07)		(16.08)		(4.35)		(25.90)		(1.13)	
	T1 vs S2	-4095.43	<0.0001	-69.23	<0.0001	-20.34	<0.0001	-0.11	0.99	-1.69	0.14
		(736.60)		(15.96)		(4.32)		(25.71)		(1.13)	
	T2 vs S1	-2229.84	0.003	-10.82	0.50	-14.20	0.0015	-40.63	0.12	-0.30	0.79
		(741.85)		(16.06)		(4.35)		(25.90)		(1.13)	
	T2 vs S2	-3966.01	<0.0001	-58.95	0.0005	-19.04	<0.0001	-39.84	0.13	-0.96	0.41
		(755.51)		(16.37)		(4.43)		(26.38)		(1.15)	
	S1 vs S2	-1736.17	0.02	-48.12	0.004	-4.85	0.27	0.79	0.98	-0.66	0.56
		(740.80)		(16.05)		(4.34)		(25.86)		(1.13)	
GH	T1 vs T2	-333.94	0.51	-1.07	0.93	-2.70	0.46	-66.07	0.07	0.44	0.76
		(501.45)		(11.81)		(3.61)		(36.49)		(1.47)	
	T1 vs S1	-1344.11	0.01	-9.81	0.43	-9.37	0.01	5.12	0.90	-3.39	0.03
		(519.85)		(12.25)		(3.74)		(37.83)		(1.53)	
	T1 vs S2	-1952.93	0.0002	-35.55	0.004	-17.17	<0.0001	-119.15	0.001	-0.51	0.73
		(503.35)		(11.86)		(3.63)		(36.63)		(1.48)	
	T2 vs S1	-1011.17	0.05	-8.75	0.48	-6.68	0.07	-71.12	0.06	-3.84	0.01
		(519.50)		(12.23)		(3.74)		(36.77)		(1.53)	
	T2 vs S2	-1619.99	0.002	-34.50	0.005	-14.76	0.0001	-53.13	0.15	-0.96	0.52
		(504.50)		(11.88)		(3.64)		(36.71)		(1.48)	
	S1 vs S2	-608.82	0.25	-25.74	0.04	-7.80	0.04	-124.26	0.002	2.88	0.07
		(525.46)		(12.40)		(3.79)		(38.23)		(1.54)	

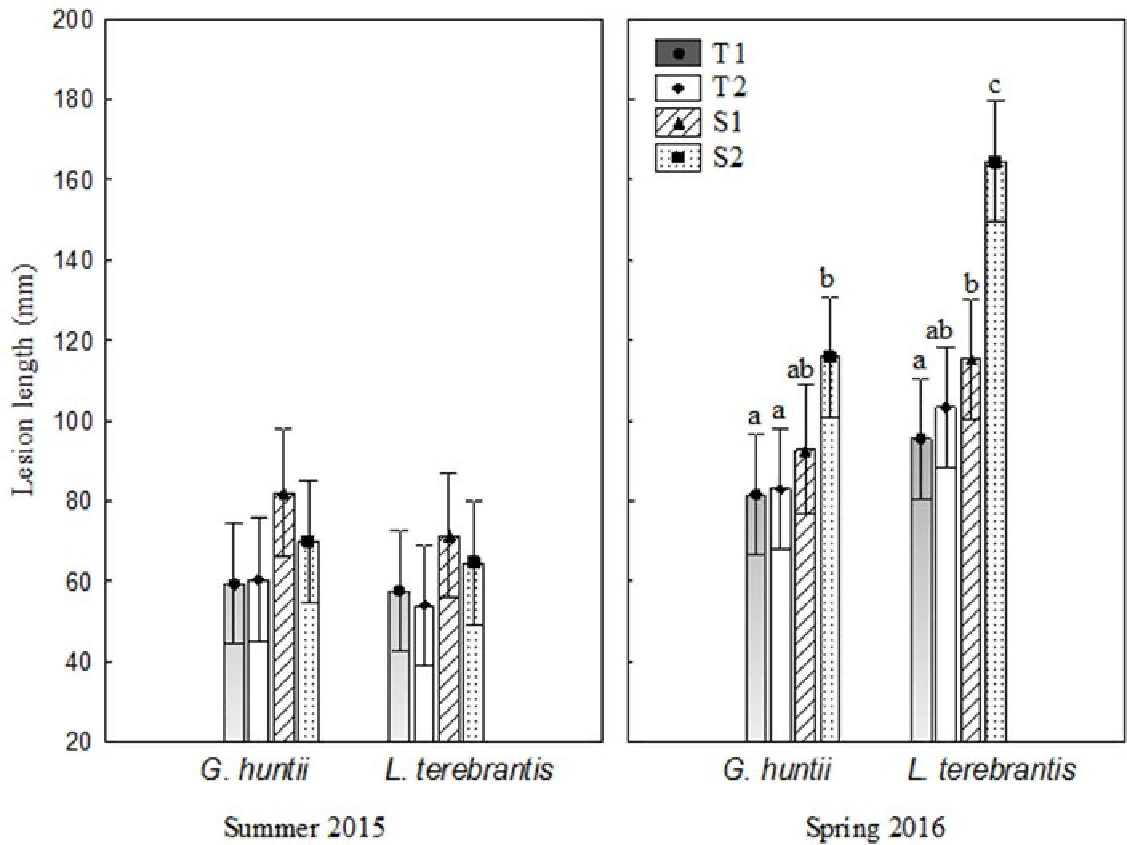

Figure 3.8 Lesion length caused by the two different fungi in summer 2015 and spring 2016 inoculations. T1 and T2: Families tolerant to root-infecting ophiostomatoid fungi at seedling stage. S1 and S2: Families susceptible to root-infecting fungi and seedling stage. 95% confidence intervals are indicated by error bars.

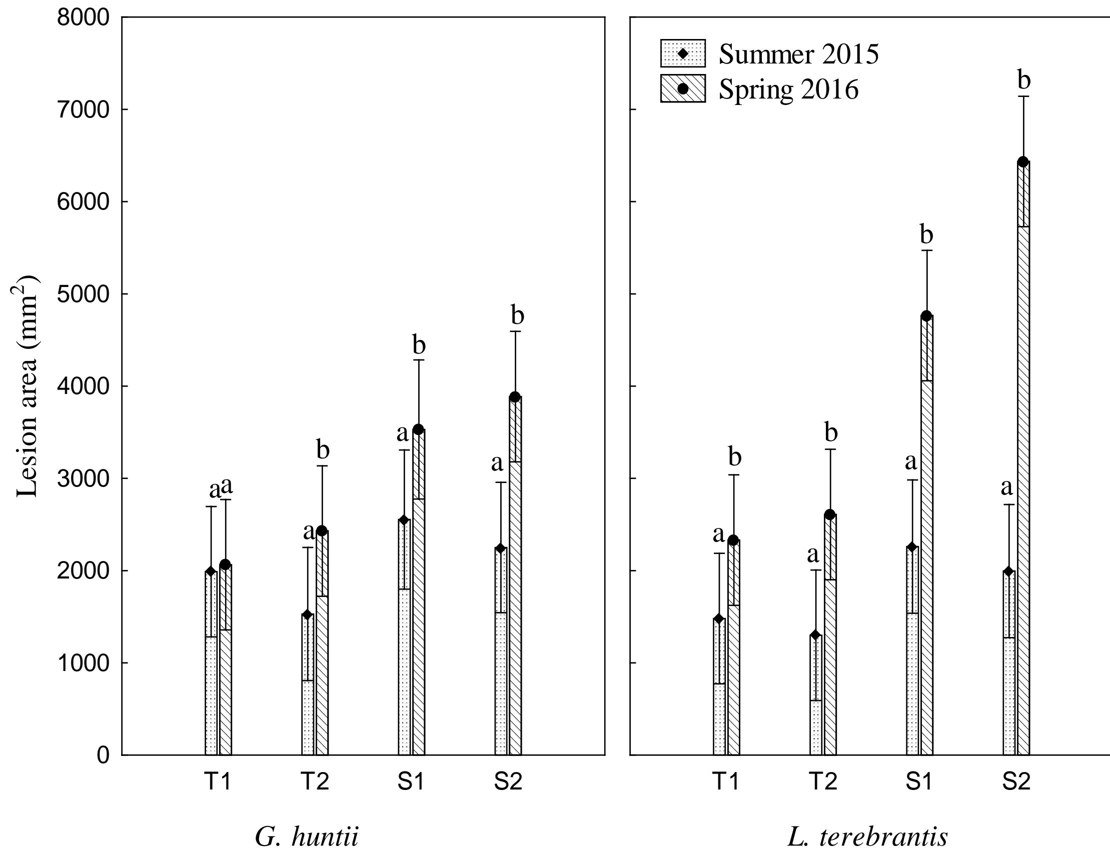

Figure 3.9 Lesion area caused by the two different fungi in summer 2015 and spring 2016 inoculations. T1 and T2: Families tolerant to root-infecting ophiostomatoid fungi at seedling stage. S1 and S2: Families susceptible to root-infecting ophiostomatoid fungi at seedling stage. 95% confidence intervals are indicated by error bars.

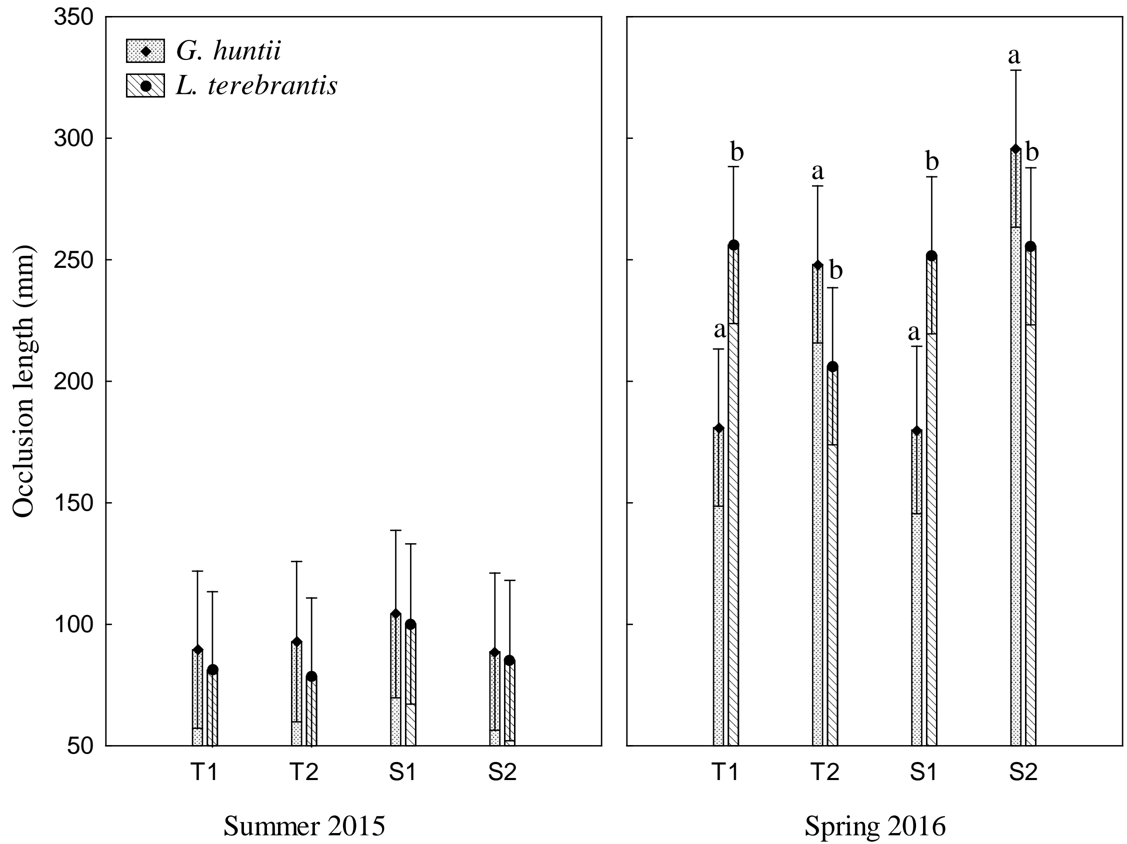

Figure 3.10 The length of occluded tissue produced by the two different fungi in summer 2015 and spring 2016 inoculations. T1 and T2: Families tolerant to root-infecting fungi. S1 and S2: Families susceptible to root-infecting ophiostomatoid fungi at seedling stage. 95% confidence intervals are indicated by error bars.

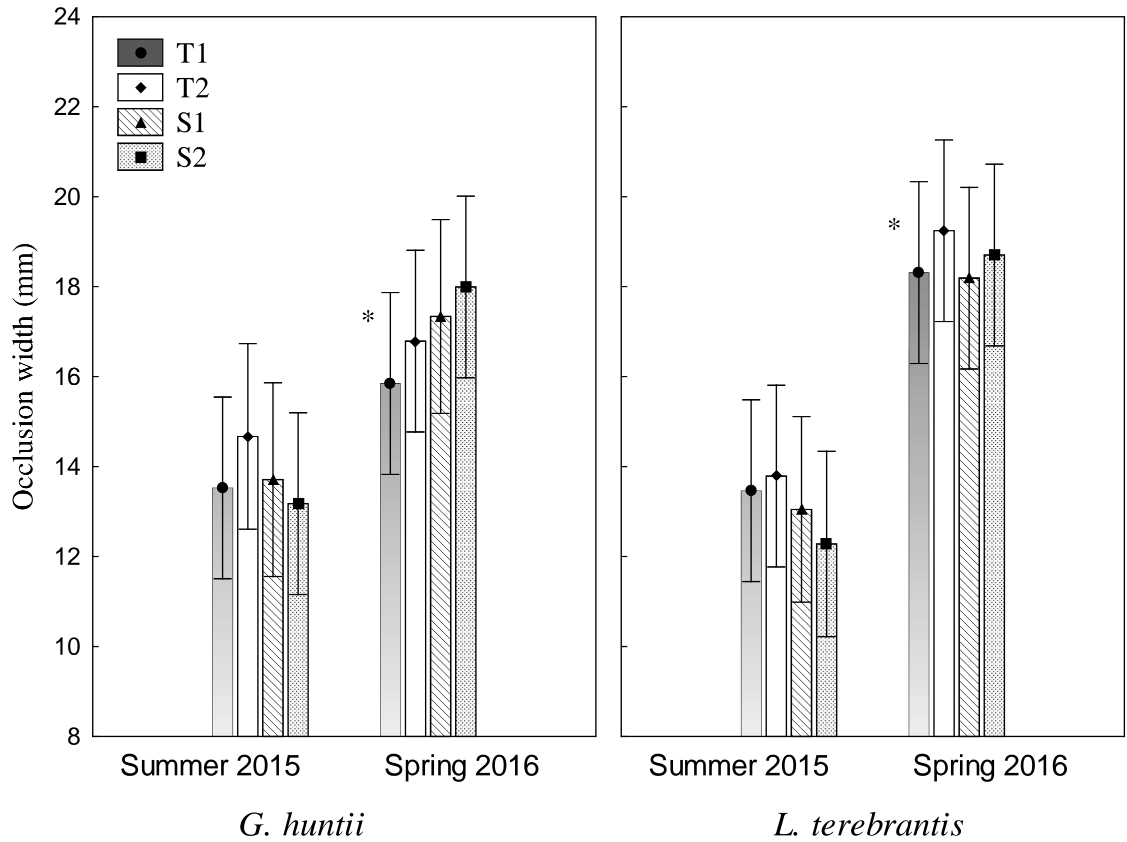

Figure 3.11 The width of occluded tissue produced by the two different fungi in summer 2015 and spring 2016 inoculations. T1 and T2: Families tolerant to root-infecting fungi. S1 and S2: Families susceptible to root-infecting ophiostomatoid fungi and seedling stage. 95% confidence intervals are indicated by error bars.

3.5 Discussion

This is the first study to show within species variation in susceptibility of mature *P. taeda* to ophiostomatoid fungi. Families S1 and S2 were more susceptible to root-infection caused by ophiostomatoid compared to family T1 and T2 (Figure 3.8) confirming the result of Singh et al. (2014) that found four *P. taeda* families used in the present study plus others have different levels of tolerance to root-infecting ophiostomatoid fungi. Intraspecific variation in susceptibility of mature *Pinus* species to ophiostomatoid fungi have been observed in some other conifer hosts (Rice et al., 2007a; Rice et al., 2007b). Several other researchers have reported considerable variation in susceptibility to pitch canker within *P. taeda* (Dwinell et al. 1977; Gordon et al., 1998; Schultz et al. 1990; Kelley and Williams, 1982) and *P. radiata* (Correll et al., 1991; Roux et al., 2007). The present study shows that there is potential for selecting ophiostomatoid fungi tolerant *P. taeda* families from the current southern U.S. planting stock reducing the potential losses due to PD.

The use of seedlings to screen tolerance/susceptibility of *P. taeda* families to *L. terebrantis* and *G. huntii* was questioned by Coyle et al. (2015). However, these results confirmed that the use of seedlings in screening studies is consistent and reliable. While, differences in the actual lesion size to define family susceptibility levels varies between the present study when compared to that of Singh et al. (2014) families performed similarly in both studies. Variation in susceptibility exists within and between *Pinus* spp., and this variation must be quantified before host susceptibility/tolerance can be generalized across the entire range of this host.

In the spring, larger lesions and occlusions caused by *L. terebrantis* indicated a higher virulence level of *L. terebrantis* over *G. huntii*. Similar results were reported by

Singh et al. (2014). Our results are not in agreement with the findings of Matusick et al. (2010) where they found *Grosmannia huntii* to be relatively more virulent than *L. terebrantis*. These discrepancies about fungal virulence can be attributed to the consideration of intra-species variability in *P. taeda* in our study which was not considered in the previous study. This implies that virulence of pathogens can be underestimated if the intra-species response is not integrated into the selection of disease tolerant hosts.

Lesions and occlusions were observed in all of tree roots. Similar hypersensitive response was observed in *P. taeda* following fungal inoculation in various previous studies (Matusick et al., 2008; Matusick et al., 2010; Matusick et al., 2012; Otrosina et al., 2000). The host response observed in control treated roots were significantly lower than those produced by the roots that were inoculated with fungal treatments. Similar observations were made by Singh et al. (2014) in their seedling inoculation experiment.

Lesion size determines the susceptibility of the host (Stephen and Paine, 1985; Matusick and Eckhardt, 2010; Singh et al., 2014). The introduction of ophiostomatoid fungi in *P. taeda* induces ethylene production which further regulates monoterpene production and guides the lesion formation (Popp et al., 1995). Paine et al. (1997) further concluded that higher monoterpene accumulation denotes elevated plant defense to invading insect vectors and pathogens. We, however, suggest that a smaller lesion length indicates that with a shorter response the host plant can suppress the effect of the fungal pathogen. The family that can block the fungal movement with less resin response is more tolerant to root-infecting fungi. The higher carbon investment of the tree in defense can result in the reduced radial growth (Krokene et al., 2008), alteration in conductive

tissue (Joseph et al., 1998), xylem disruption and immediate mortality (Tyree and Zimmermann, 2002). We, thus suggest that the tolerant families T1 and T2 inhibit the fungi at the expense of less carbon and are thus less prone to infection and further reduction in productivity by ophiostomatoid fungi.

A number of the secondary metabolites produced by the host highly influence the ability of root pathogens to spread in the host (Eckhardt et al., 2009). Trees with fewer resin ducts are more susceptible to fungal infection and prone to attack by insect vectors compared to those with more ducts (Ferrenberg et al., 2014). It is, however, unclear whether variarion in either the resin constituents or the resin duct length and area among the families results in inhibition of the fungal growth. The amount of production, the rate of flow and the concentration of chemical content are heritable traits that guide the resin defense in pine trees (Chhatre et al., 2013; Westbrook et al., 2013). The inherent factors that direct the susceptibility and tolerance of *P. taeda* families to ophiostomatoid fungi are still unknown.

A relatively severe hypersensitive response was observed in spring inoculation compared to summer inoculation. Several studies suggest the timing of fungal inoculation influence the response of trees (Paine, 1984; Stephen and Paine, 1985; Matusick et al., 2010) and the results greatly support our findings. Resin production is highest in the root cells formed immediately following the meristem differentiation and differ throughout the year (Berryman, 1972). Tree defense is highest in May and continues to decrease until December (Blanche et al., 1992). Thus, lesions are larger during the growing season that the dormant season (Stephen and Paine, 1985; Matusick et al., 2010). The larger lesion response requires higher carbon at the inoculation site (Guérard et al., 2007.) There is a

trade-off between investment of tree in defense and decrease of the radial growth and loss of conductive tissue (Oliva et al., 2014).

Recent research has made great strides towards understanding intra-species variation in disease tolerance, but their implication in mature trees in an ecological scenario remains understudied. This paper presents the first study to show intra-species variation in disease tolerance exists in mature *P. taeda* trees. Our results suggest that the susceptibility and tolerance to *L. terebrantis* and *G. huntii* is an inherent property of families, regardless of the life stage of *P. taeda*. The family genetics, a significant role player in susceptibility and tolerance to pathogens, should be considered in future studies. It is suggested that future studies of the molecular, anatomical and chemical mechanism of defense strategies will improve our understanding of these findings.

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CHAPTER IV

The Role of *Leptographium terebrantis* and *Grosmannia huntii* Invasion in Driving Drought-Related Decline in *Pinus taeda* Families

4.1 Abstract

The complex interaction of various biotic and abiotic factors may put the overall stand health of *Pinus* spp. at risk. A study was designed to determine the combined impact of drought and vascular-inhabiting fungi (*Leptographium terebrantis* and *Grosmannia huntii*) in *Pinus taeda* (loblolly pine). Seedlings from two *P. taeda* families were planted and watering treatments: i. normal moisture, ii. medium drought, and iii. severe drought were applied. One month following initiation of watering treatments, seedling stems were artificially inoculated with *L. terebrantis* and *G. huntii*. Drought and fungal interaction significantly affected lesion and occlusion length, and seedling fine root dry matter biomass yield and needle-to-fine-root dry biomass ratio. *Leptographium terebrantis* was more virulent in severe drought conditions. The tolerant family was also more tolerant to drought, indicating tolerance to of *P. taeda* to drought may be improved through appropriate family selection. Drought and vascular-inhabiting fungi may negatively impact *P. taeda* stand health.

4.2 Introduction

Adverse climatic conditions like drought have been shown to be responsible for a number of forest declines throughout the world (Adams et al., 2013; Cailleret et al.,

2014). Recent incidents of tree decline and mortality have been related to increased mean annual temperatures and decreased mean annual rainfall in European forests (Carnicer et al., 2011) and increased droughts in the southwestern (van Mantgem et al., 2009) and southeastern U.S. (Klos et al., 2009; Wang et al., 2010). Drought events are expected to become more common in future (IPCC, 2013) resulting in drought-induced forest mortality (Peng et al., 2011). As forests play a key role in the global water and carbon cycles, a feedback loop exists between climate change and forest function (Bonan, 2008). Despite the effects of drought on forest function, mechanisms underlying forest decline and mortality are still weakly understood (Mc Dowell et al., 2011).

According to Turtola et al. (2003), low soil moisture influences the production of specific chemicals in conifers rendering the trees more susceptible to both pathogen and insect attack. For example, bark beetle infestation in drought-weakened *Pinus* forests may occur many years after the end of the climatological drought (Raffa et al., 2008). Beetle-vectored, vascular-inhabiting pathogens can have a devastating effect on drought stressed trees (Oliva et al., 2014). Vascular wilt pathogens such as *Ceratocystis* Ellis and Halst., *Leptographium,* and *Grosmannia* Goid. species thrive in the xylem of *Pinus* spp. (Yadeta and Thomma, 2013; Singh et al., 2014). *Pinus* spp. defend against these fungi by producing resins that clog the plant vascular conducting tissues (Matusick and Eckhardt, 2010). Clogging of plant conduits disturb plant water transport, resulting in hydraulic failure that leads to tree mortality (Oliva et al., 2014). Thus, adverse climatic conditions easily influence susceptibility of conifer hosts to pathogen and insect attack (Lindberg and Johansson, 1992).

Models of forest decline incorporate predisposing factors, inciting factor and contributing factors (Sinclair 1966; Manion, 1981). In this context, *P. taeda* L. decline in the southern U.S. has been associated with (i) predisposing factors like tree genetics, age, ozone and adverse soil conditions, (ii) inciting factors such as drought and increased ozone and (iii) contributing factors like root-feeding bark beetles and beetle-vectored vascular-inhabiting fungi (Eckhardt et al., 2004; Eckhardt and Menard, 2008; Eckhardt et al., 2010). The vascular inhabiting fungal pathogens are considered to be the major driving factors in the final phase of drought-induced tree and stand mortality (Oliva et al., 2014).

Pathologists many have been suffering from false dichotomy of drought vs biotic attack (Mc Dowell et al., 2013). Many studies have focused primarily on individual factors: (i) drought and its subsequent effect on plant physiology (Noormets et al., 2010; Maggard et al., 2016) or (ii) biotic agents and its subsequent impact on tree health (Matusick and Eckhardt, 2010; Singh et al., 2014). However, the evidence for the mechanisms suggested by these individual factors is inconclusive and a more integrated approach focusing on interrelations between drought and the biotic agents on tree growth and functioning are needed.

Recently, a limited number of studies have concentrated on the interaction of drought and vascular-inhabiting fungi (Matusick et al., 2008; Chieppa et al., 2017). However, these studies were conducted for shorter time periods and deployed both drought and fungal treatment at the same time (Matusick et al., 2008; Chieppa et al., 2017), despite the fact that these vascular-inhabiting fungi come into play only after the predisposition of trees to a drought event. Thus, a closer examination of the impact of *L.*

terebrantis and *G. huntii* on *P. taeda* trees predisposed to drought is needed. In this concern, we address the following questions: (i) Does the virulence of *L. terebrantis* and *G. huntii* in *P. taeda* increase under increasing drought?, (ii) Does drought stress increase the susceptibility of *P. taeda* families to these fungi?, and (iii) Is infection by vascular-inhabiting fungi under drought likely to enhance tree decline directly through increased investment in occlusion and indirectly through a reduction of plant growth?

4.3 Methodology

4.3.1 Experimental set-up

The experiment was conducted in the research facility of the Southern Forest Nursery Management Cooperative Auburn, AL, USA. The facility contained an open outdoor pavilion with 12 raised wooden boxes (120 cm long and 100 cm wide) filled with pure sand. Plastic transparent roof covered the pavilion to exclude ambient rainfall. A system was used for automatic irrigation prior to the commencement of the study.

Figure 4.1 *Pinus taeda* seedlings planted in sand filled boxes experiencing different watering treatments**.**

4.3.2 Seedling planting

One-year-old, bare-root seedlings from two commercially grown *P. taeda* families were used. Seeds were sown in March 2014 and seedlings were lifted from the nursery in January 2015. Based on previous findings by Singh et al., (2014), one family was considered “susceptible” (S) and one “tolerant” (T) to root-infecting ophiostomatoid fungi. In February 2015, 630 seedlings (35 per family in each box) were planted in 9 wooden boxes and watered to field capacity for 4 weeks until watering treatments were initiated.

4.3.3 Moisture treatment

Three watering treatments: i. normal moisture, ii. moderate drought, and iii. severe drought were deployed to 3 boxes (3 replicates/treatment) in March 2015. The watering treatments were determined based on the volumetric water content of the pure sand and loblolly pine. The well-watered soil sample was taken with soil core of known volume. The wet weight and dry weight (72 h at 105 ℃) of the soil were determined and the volumetric water content of the soil sample at field capacity (FC) was determined by using the following formula:

$$V = \frac{Wmass - Dmass}{Pw\,Vs}$$

Where *Wmass* is the mass before drying and *Dmass* is the mass after drying, *Pw* is the

density of

water (1000 kg m^{-3}) and *Vs* is the total volume of the soil sample (sum of air, water, and soil). The volumetric water content for the FC was 32 $m^3\,m^{-3}$. The watering treatments

were as follows: i. 75 % of FC (normal water i.e 28 $m^3 m^{-3}$), ii. 50 % of FC (medium drought i.e. 18 $m^3 m^{-3}$), and iii. 25 % of FC (severe drought i.e. 11 $m^3 m^{-3}$). Soil water content was constantly monitored in each box using an external moisture probe (Figure 4.2) and irrigation was programmed to meet volumetric water content of each box.

Figure 4.2 Soil moisture being monitored with a soil moisture meter.

4.3.4 Inoculation treatment

One month into the three watering treatments (April 2015), artificial stem inoculations were conducted using the method described by Nevill et al. (1995), Singh et al. (2014), and Chieppa et al. (2017) using wound + inoculum method. Five inoculation treatments applied were as follows: *L. terebrantis* (LOB-R-00-805/ATCC accession no. MYA-3316), *G. huntii* (LLP-R-02/ATCC accession no. MYA-3311), wound, wound + media and no wound. Seven seedlings per family within a box received each inoculation treatment. The *L. terebrantis* isolate used was isolated from a root of *P. taeda* exhibiting local tissue damage and deteriorating crowns from the Talladega National Forest,

Oakmulgee Ranger District, AL, USA (Eckhardt et al., 2007). The *G. huntii* isolate used was isolated from a root of *P. palustris* Mill. showing similar characteristics of decline from the Fort Benning Military Reservation, GA, USA. These isolates have been used in previous artificial inoculation studies (Matusick and Eckhardt, 2010; Singh et al., 2014; Chieppa et al., 2017). The fungal isolates were maintained at 4 ºC in Malt Extract Agar (MEA) before use and were plated on 2 % MEA plate, 14 days prior to the inoculation experiment.

To perform the inoculation, 13 mm (<2 mm depth) of seedling bark at the stem, ~ 3 cm above soil line was cut vertically with a sterile razor blade. The single pre-punched plug of agar (3 mm) with actively growing fungal mycelium was placed (fungus-side-towards wound) in the wound in each seedling. Sterile agar was inoculated in the wound in case of wound + media inoculation. A sterile cut was made for wound control. No wound was made in seedling receiving no wound treatment. Wounds on the stems were then wrapped with sterile cotton balls moistened with deionized water to prevent desiccation of MEA and wrapped with Parafilm® to avoid contamination.

Figure 4.3 Inoculation of agar plug in *Pinus taeda* stem.

4.3.5 Pre-harvesting measurements

4.3.5.1 Growth and size measurement

Height and root-collar diameter (RCD) measurements were collected from each seedling prior to watering treatment (March 2015), stem inoculation (April 2015) and seedling harvesting (September 2015). Before seedling planting (February 2015), 12 extra seedlings from each family were randomly selected for height, RCD, and biomass measurements and the averages are given in Table 4.1. The number of new buds developed were also counted on individual seedlings before watering (March 2015) and inoculation treatments (April 2015) and prior to seedling harvesting (September 2015).

Table 4.1 Initial family height, RCD, dry wet matter of seedling averages and standard deviation (n = 12 per family).

F	H (cm)	RCD (mm)	FR (g)	CR (g)	N(g)	S (g)
S	23.5 (2.2)	4.29 (0.68)	0.45 (0.17)	0.39 (0.21)	1.84 (0.60)	1.10 (0.26)
T	27.5 (3.5)	5.34 (1.02)	0.89 (0.64)	0.38 (0.38)	1.48 (0.47)	1.43 (0.52)

F: Family, S and T denote loblolly pine family selected for its susceptibility and tolerance to ophiostomatoid fungi, RCD: Root-collar diameter, FR: Fine Root, CR: Coarse root, N: Needle, and S: Stem. Means followed my standard deviation in parenthesis.

4.3.5.2 Needle greenness and chlorophyll content

Needle greenness was measured non-destructively on 5-7 needles that reached physiological maturity using Soil-Plant Analysis Development-502 (SPAD) chlorophyll meter (Spectrum Tech. Inc., Plainfield, IL, USA) prior to inoculation treatment and at seedling harvest. Three measurements were taken from each seedling and then averaged.

Needle chlorophyll content was measured in two needles for all treatments. Samples were collected by extracting 0.25 g of the needle material in 6.25 ml 95 % ethanol. The sample was kept in the dark in a water bath for 24 h and then allowed to cool to room temperature and vortexed at slow speed for 1 minute. Samples were centrifuged for 5 minutes at 13500 g, and the extract was collected. The extract absorbance was measured at 665, 645 and 470 nm using a UV-Vis spectrophotometer (Thermo ScientificTM, USA). Chlorophyll content Chlorophyll-a (Chla) and Chlorophyll-b (Chlb) was calculated by using the following formula (Guo et al., 2010):

$$Chla = 13.95A665 - 6.88A649$$

and

$$Chlb = 24.96A649 - 7.32A665$$

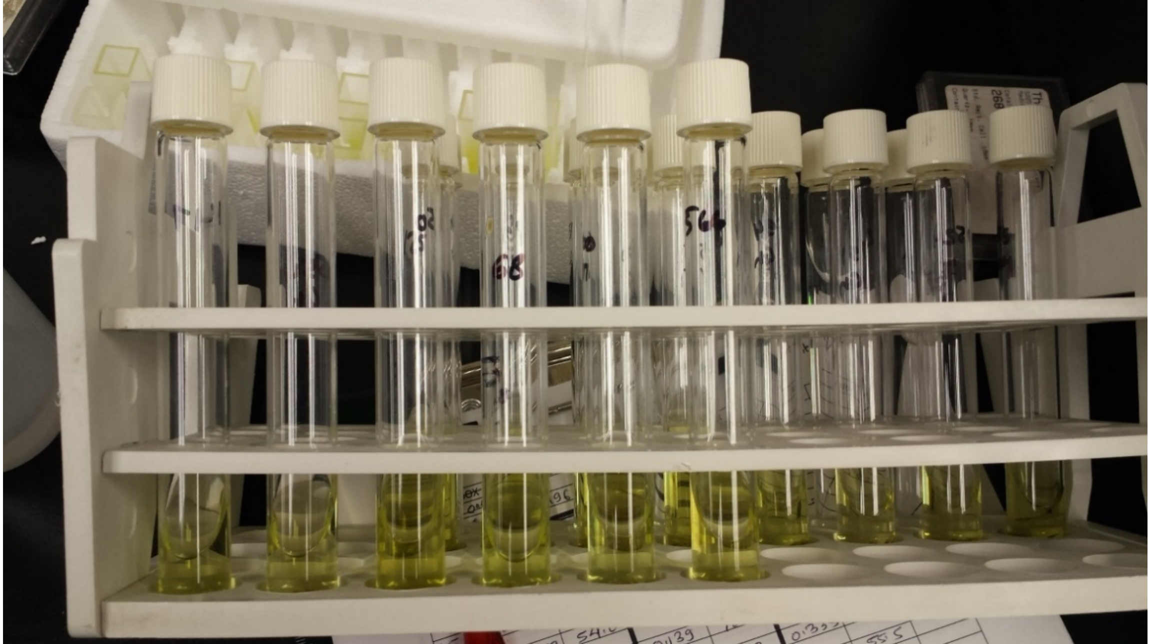

Figure 4.4 Samples containing chlorophyll extracted from *Pinus taeda* needles.

4.3.5.3 Predawn water potential

Two seedlings from each treatment, replicate, were destructively sampled (end of August 2015) for pre-dawn needle water potential (*Ψpd*) measurements. Three mature fascicles from each seedling were harvested between 2:30 AM and 5:00 AM, placed in an air-tight plastic bag and kept in a dark box until measured. A Scholander pressure bomb (PMS Instrument Company, Albany, OR, USA) was used to measure *Ψpd*, within 5 hours of collection.

4.3.5.4 Relative leaf water content

The relative leaf water content (RWC) was measured on needle fascicles used for the water potential measurement. The fresh weight of the fascicle was determined then soaked in sterile deionized water for 24 h. The fascicle was oven dried at 75 ºC for 48 h, re-weighed and RCW was calculated by the following formula:

$$RCW = \frac{W - DW}{TW - DW} \, X \, 100 \, \%$$

Where W = Sample fresh weight, TW = Turgid weight of needle, DW = Dry weight of the sample.

4.3.6 Post-harvest measurement

4.3.6.1 Inoculation response

In September 2015, four seedlings from each treatment within each box were cut at the stem above the soil level (September 2015). Seedling stems were placed in mixture of stain (FastGreen FCF; Sigma Chemical Co., St. Louis, MO, USA) and water in a ratio of 0.25 g L^{-1} for 72 h. The bark near the inoculation point was carefully scraped away to the xylem and the lesion and occlusion length and width were measured. The phloem colonized by the fungi was measured as the lesion. The portion of the xylem that did not allow the stain to pass through it was considered an occlusion. Two pieces (~ 3 mm) of stem tissue surrounding the lesion were cut and plated on MEA with cycloheximide at 800 mg L^{-1} and streptomycin sulfate at 200 mg L^{-1} to confirm fungal re-isolation. Stem sections of control seedlings also were also plated to confirm no contamination.

4.3.6.2 Seedling biomass

Three remaining seedlings from each treatment combination per box were used for dry biomass measurements. Each seedling was separated into needles (N), stem (S), coarse root (CR) and fine root (FR) and allowed to dry at 75 ºC for 72 h then weighed.

Figure 4.5 *Pinus taeda* seedling separated into coarse root (CR), fine root (FR), needles (N) and stem (S) for biomass measurement.

4.3.7 Statistical analysis

The experimental design was a randomized complete block (RCBD) design with replicates at all levels. The data for the seedling survival were analyzed by logistic regression using R 3.3.1.

Similarly, the general linear model was used to analyze the other response variables. The general linear model used was:

$$y = \beta o + \beta 1 + \beta 2 + \beta 3 + \beta 4 + \beta 5 + \beta 6 + \beta 7 + \mathcal{E}$$

Where, y is the response variable (lesion and occlusion length, depth and width, and needle, stem, coarse root, fine root). $\beta 0$ is the intercept, $\beta 1$ is the family effect, $\beta 2$ is the fungal effect, $\beta 3$ is the moisture effect, $\beta 4$ is the family x fungal interaction, $\beta 5$ is the interaction of fungal treatment x moisture treatment and $\beta 6$ is the interaction of family x moisture treatment and $\beta 7$ is the random effect of the box and $\mathcal{E}$ is the residual error. Multiple comparisons tests were performed by using post hoc Tukey (Honest Significant Difference) procedures. All the assumptions of normality and homogeneity of the

variance were inspected. All the statistical analysis were conducted using SAS (Version 9.4, SAS Institute, Inc., Cary, NC, USA), R Core 3.3.1 and STATISTICA (Statsoft, Inc., Tulsa, OK, USA).

4.4 Results

4.4.1 Seedling survival

No specific pattern regarding the seedling mortality was observed during the study period. Seedling survival was not affected by family of *P. taeda*, watering treatment, and fungal inoculation. An overall result of the effects of different treatments in the response variables is presented in Table 4.2.

Table 4.2 An overview of the effect of different treatments on seedlings response variables.

		Treatments/Combinations							
Measurement	Transformation	*n*	Mos	Fam	Ino	Mos x Fam	Fam x Ino	Mos x Ino	Fam x Mos x Ino
LL	Log10	429	***	***	***	NS	**	***	NS
LL/Ht	Log10	420	***	***	***	NS	NS	**	NS
LD	Log10	425	**	NS	***	NS	NS	NS	*
LW	Log10	419	*	NS	***	NS	NS	*	NS
OL	Log10	419	NS	*	***	NS	*	**	NS
OL/Ht	Log10	410	**	***	***	NS	NS	*	NS
OD	Log10	417	**	*	***	NS	NS	NS	NS
OW	Log10	417	**	NS	***	NS	NS	NS	NS
Ny	Log10	450	**	***	*	NS	NS	NS	NS
Sy	Log10	430	***	***	**	NS	NS	NS	NS
Cry	Log10	428	***	***	NS	NS	NS	NS	*
Fry	Raw	428	***	***	**	NS	NS	**	NS
S/R	Log10	399	***	***	NS	NS	NS	NS	NS
Fry/Cry	Log10	385	NS	*	*	NS	NS	NS	NS
Ny/Fry	Log10	375	**	NS	NS	NS	NS	**	NS
Hti	Raw	609	***	***	**	NS	NS	NS	NS
NG	Log10	315	NS	NS	***	NS	NS	NS	NS
SVC	Square root	597	***	***	**	NS	*	NS	NS
Ψpd	Log10	149	NS	NS	**	NS	NS	NS	NS
RCW	Log10	150	**	NS	NS	NS	NS	NS	NS
BP	Log10	463	*	NS	**	NS	NS	*	NS

*: $P<0.05$, **: $P<0.01$, ***: $P<0.0001$, Mos: watering treatment, Ino: Inoculation treatment, Fam: Family, LL: Lesion length, LL/Ht: Lesion length Height^{-1}, OL/Ht: Occlusion length Height^{-1}, LD: Lesion depth, OL: Occlusion length, Ny: Needle dry matter yield (DMY), Sy: Stem DMY, Cry: Crude root DMY, Fry: Fine root DMY, Hti: Height increase, NG: Needle greenness, SVC: Seedling volume change, *Ψpd*: Predawn water potential, RCW: Relative water content, BP: Bud production.

4.4.2 Lesion and occlusion

Dark brown necrotic tissues were observed at the inoculation point in all the inoculated seedlings. The re-isolation of *G. huntii* and *L. terebrantis* was 89% and 92%, respectively indicating successful fungal inoculation. Seedling lesions with the control inoculations were significantly smaller than fungal inoculations, indicating fungi, but not the wound caused lesion. The lesions in the wound and wound + media did not extend beyond the inoculation zone.

Lesion length/seedling height (LL/Ht) was significantly affected by family and the interaction between inoculation and watering conditions (Table 4.2). Family S (susceptible family) had the greatest lesion and lesion length/height ratio. *L. terebrantis* resulted in the highest lesion length/height ratio in severe drought condition (Figure 4.6).

Lesion length was significantly affected by watering treatment, family, inoculation, family x inoculation and watering treatment x inoculation. *Leptographium terebrantis* resulted in significantly longer lesion than *G. huntii* within both tolerant (T) and susceptible (S) family. The seedlings under severe drought challenged with *L. terebrantis* had the longest lesions (Table 4.3). Under the medium drought condition, there was greater variation in lesion length caused by both fungi. The lesion caused by *L. terebrantis* was significantly longer in the susceptible family than the tolerant family.

Lesion depth was significantly affected by the watering condition, inoculation and family x watering x inoculation (Table 4.2). *Leptographium terebrantis* caused deeper lesions than *G. huntii* in all treatment and treatment combinations (Table 4.5). Occlusion length was significantly affected by family, inoculation, family x inoculation, watering treatment x inoculation interaction (Table 4.2). The occlusion length caused by the wound and wound + media inoculation was not different among the various watering treatments and smaller than the

caused by fungal inoculation. *Leptographium terebrantis* caused the longest length of occlusion in seedlings under severe drought (Table 4.3).

Occlusion length/seedling height was affected by watering treatment, family, inoculation and watering treatment x inoculation interaction (Table 4.2). Within each watering treatment, *L. terebrantis* caused significantly higher occlusion length/seedling height than *G. huntii* (Table 4.3). Occlusion length was also affected by family, inoculation, family x inoculation, and watering treatment x inoculation (Table 4.2). *Leptographium terebrantis* caused longer occlusion lengths than *G. huntii* in both families (Table 4.4). Seedlings under moderate drought had greatest occlusion length as a response to *L. terebrantis* inoculation. However, the occlusion length/seedling height ratio was not significantly different between the seedlings receiving different watering treatments. This ratio was significantly higher in seedlings inoculated with *L. terebrantis* than *G. huntii* (Table 4.3), indicating the higher virulence of *L. terebrantis*.

Occlusion depth produced in the seedlings was significantly affected by watering treatment, family type, and inoculation. *Grosmannia huntii* and *L. terebrantis* caused significantly deeper occlusions than wound and wound + media inoculated seedlings, suggesting successful fungal inoculation. *Leptographium terebrantis* caused deeper occlusion than *G. huntii* under all watering conditions.

Figure 4.6 Necrotic tissue (lesion) on the stem of *P. taeda* seedling under severe drought inoculated with *L. terebrantis.*

Figure 4.7 Dark necrotic tissue in stem cross-section of *Pinus taeda* following fungal inoculation.

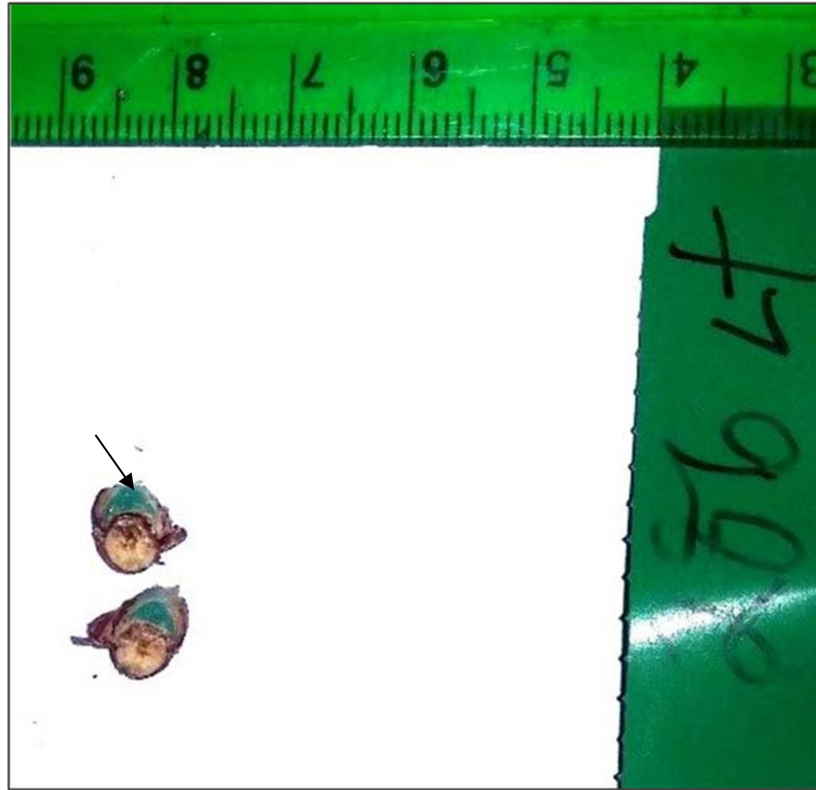

Figure 4.8 Cross-section of the *Pinus taeda* vascular tissue occluded as a result of *Leptographium terebrantis* inoculation. Arrow indicates the growth of new tissue on the opposite site of inoculation.

Table 4.3 Effects of inoculation and watering treatments on lesion and occlusion.

Inoculation	Moisture	LL (mm)	LL/Ht	LD (mm)	OL (mm)	OL/Ht	OD (mm)
GH	N	21.73 (2.48) d	0.50 (0.12) b	1.51 (0.57) b	27.27 (4.98) c	0.63 (0.19) c	0.46 (0.75) b
	MD	24.06 (6.59) d	0.56 (0.19) b	2.31 (0.55) b	28.71 (12.31) c	0.67 (0.22) bc	0.61 (0.79) b
	SD	22.47 (6.50) d	0.73 (0.78) b	0.40 (0.51) b	27.64 (12.16) c	0.63 (0.94) bc	0.59 (0.93) b
LT	N	27.97 (5.65) c	0.65 (0.21) b	2.44 (0.56) a	39.37 (9.22) b	0.93 (0.37) ab	0.82 (1.85) a
	MD	24.06 (10.64) a	0.56 (0.44) a	1.58 (0.60) a	54.52 (7.32) a	1.32 (0.47) a	0.67 (1.85) a
	SD	38.27 (11.60) b	1.04 (0.46) a	2.14 (0.47) a	44.39 (12.65) b	1.20 (0.48) a	2.57 (0.54) d
WM	N	12.73 (1.52) e	0.28 (0.09) c	0.64 (0.34) c	12.75 (3.43) d	0.28 (0.13) d	0.40 (0.01) c
	MD	12.67 (3.57) e	0.26 (0.09) c	0.72 (0.33) cd	12.34 (4.83) d	0.25 (0.12) d	0.54 (0.01) c
	SD	12.73 (3.30) e	0.43 (0.67) c	0.78 (0.46) cd	13.04 (4.23) d	0.44 (0.70) d	0.56 (0.01) c
W	N	12.73 (3.54) e	0.28 (0.10) c	0.64 (0.31) cd	12.75 (4.31) d	0.28 (0.12) d	0.54 (0.01) c
	MD	12.52 (3.73) e	0.27 (0.10) c	0.62 (0.35) d	11.82 (4.70) d	0.25 (0.11) d	0.43 (0.01) c
	SD	12.42 (3.52) e	0.46 (0.70) c	0.60 (0.26) d	11.12 (5.00) d	0.42 (0.69) d	0.42 (0.01) c

GH: *Grosmannia huntii*, LT: *Leptographium terebrantis*, WM: Wound + media, W: Wound, N: Normal watering, MD: Medium drought, SD: Severe drought, LL: Lesion length, LL/Ht: Lesion length Seedling height^{-1}, LD: Lesion depth, OL: Occlusion length, OL/Ht: Occlusion length Seedling height^{-1}, OD: Occlusion depth. Means followed by the standard deviation in parenthesis. Numbers followed by different letters within a column indicate significant differences at $\alpha = 0.05$

Table 4.4 Effects of family and inoculation on the lesion and occlusion length.

Family	Inoculation	Lesion length (mm)		Occlusion length (mm)	
		n		*n*	
Tolerant	*G. huntii*	58	21.45 (3.65) c	58	25.78 (5.94) b
	L. terebrantis	50	35.22(10.24) b	50	43.62 (12.91) a
	Wound	58	12.74 (3.51) d	58	11.93(4.79) c
	Wound + media	55	12.76 (2.99) d	55	12.65 (4.17) c
Susceptible	*G. huntii*	58	24.03 (6.84) c	58	29.95 (10.61) b
	L. terebrantis	55	40.41 (12.99) a	55	49.45 (12.87) a
	Wound	54	12.34 (3.65) d	54	11.78 (4.64) c
	Wound + media	51	13.0 (2.92)d	51	4.16 (4.21)c

Means followed by the standard deviation in parenthesis. Numbers followed by different letters within a column indicate significant differences at $\alpha = 0.05$.

Table 4.5 Effect on family x watering treatment x inoculation on lesion depth.

Family	Watering treatment	Inoculation	*n*	Lesion depth (mm)
Tolerant	Normal	*G. huntii*	18	1.41 (0.70) bcd
		L. terebrantis	14	2.55 (0.59) a
		Wound	20	0.62 (0.38) ef
		Wound + media	19	0.89 (0.38) cde
	Moderate drought	*G. huntii*	19	1.65 (0.44) ab
		L. terebrantis	19	2.29 (0.70) a
		Wound	19	0.50 (0.23) f
		Wound + media	16	0.80 (0.29) ef
	Severe drought	*G. huntii*	21	1.34 (0.51) bcd
		L. terebrantis	17	2.16 (0.49) a
		Wound	19	0.66 (0.27) ef
		Wound + media	20	0.69 (0.36) ef
Susceptible	Normal	*G. huntii*	19	1.60 (0.42) ab
		L. terebrantis	15	2.35 (0.54) a
		Wound	15	0.66 (0.19) ef
		Wound + media	14	0.85 (0.30) def
	Moderate drought	*G. huntii*	18	1.51 (0.65) bc
		L. terebrantis	19	2.33 (0.50) a
		Wound	18	0.75 (0.42) ef
		Wound + media	16	0.65 (0.35) ef
	Severe drought	*G. huntii*	21	1.45 (0.51) bc
		L. terebrantis	21	2.13 (0.46) a
		Wound	21	0.55 (0.24) ef
		Wound + media	21	0.87 (0.53) def

Means followed by the standard deviation in parenthesis. Numbers followed by different letters within a column indicate significant differences at $\alpha = 0.05$.

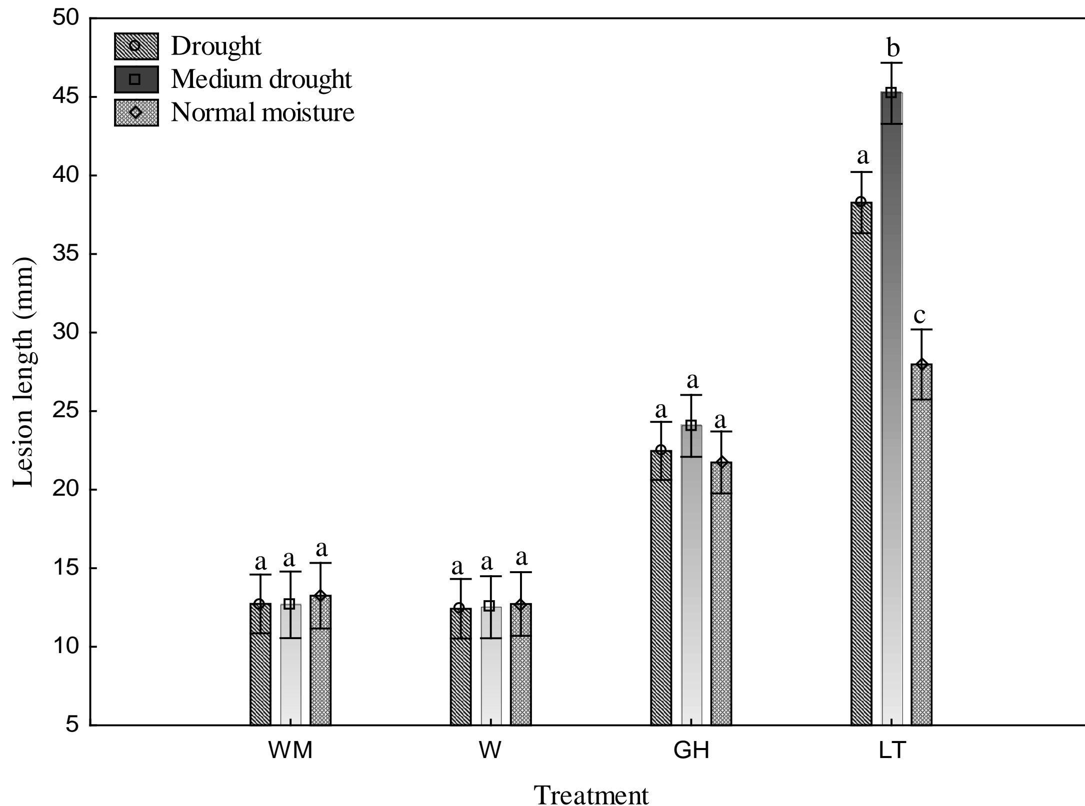

Figure 4.9 Mean lesion length produced in seedlings under different watering treatments. Different letters denote significant differences between watering treatments within each inoculation treatment at α = 0.05.

4.4.3 Seedling volume increment

Watering treatment, family, inoculation, and family x inoculation interaction significantly affected seedling growth during the experiment (Table 4.2). The susceptible family (S) grew less than tolerant family (T). This reduction in growth rate cannot be attributed to the response of different family to fungal inoculation as the growth was not significantly different between the fungi inoculated and control seedlings. Thus, the tolerant family has more capability to grow compared to susceptible family. Volume increment of seedlings under drought stress was low.

Table 4.6 Effect of family and inoculation on seedling volume change (SVC).

Family	Inoculation	*n*	SVC (mm^3)
Tolerant family	*G. huntii*	62	11.86 (8.51) ab
	L. terebrantis	56	10.93 (6.28) abc
	No wound	57	16.97 (10.59) a
	Wound	61	16.88 (10.76) a
	Wound + media	63	14.47 (8.66) a
Susceptible family	*G. huntii*	61	7.67 (5.52) dc
	L. terebrantis	59	8.25 (5.84) bcd
	No wound	59	8.32 (7.45) d
	Wound	60	8.18 (6.13) dc
	Wound + media	59	8.50 (5.34) bcd

Means followed by the standard deviation in parenthesis. Numbers followed by different letters within a column indicate significant differences at $\alpha = 0.05$.

4.4.4 Bud-outbreak number increment

Bud-outbreak was significantly affected by inoculation x watering treatment. The bud production was not affected by family. The control (NW) had the greatest number of the buds with no differences among medium drought and normal watering. Seedlings challenged with *G. huntii* had significantly fewer buds in the seedling under the severe drought than that under the high and medium drought. However, the increment in the number of bud-outbreak was not different in the seedlings inoculated with *L. terebrantis* under different watering treatments.

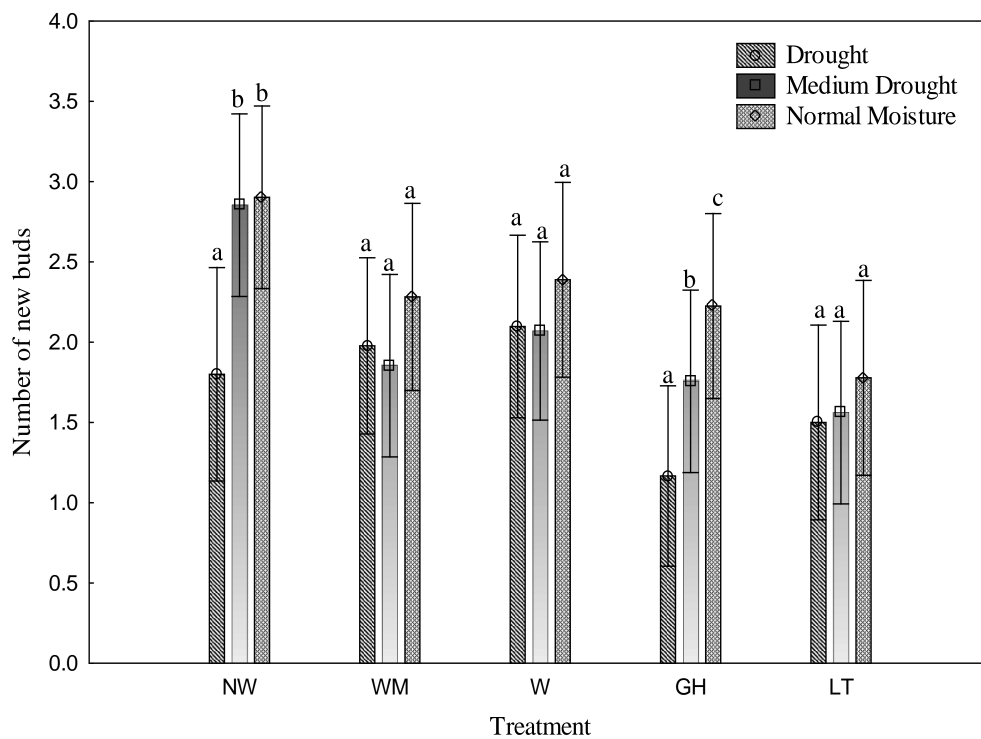

Figure 4.10 Mean numbers of bud break in seedlings under different fungal and watering treatments.

Table 4.7 Effect of watering treatment and inoculation on increment in number of bud outbreak (BP).

Watering	Inoculation	*n*	BP
Normal water	*G. huntii*	40	2.23 (2.15) ab
	L. terebrantis	36	1.78 (1.51) abc
	No wound	41	2.90 (1.89) ab
	Wound	36	2.39 (2.05) ab
	Wound + media	39	2.28 (1.59) abc
Severe drought	*G. huntii*	42	1.17 (1.10) c
	L. terebrantis	36	1.50 (1.42) abc
	No wound	30	1.80 (2.41) abc
	Wound	41	1.95 (2.51) abc
	Wound + media	44	1.98 (1.64) abc
Medium drought	*G. huntii*	41	1.61 (1.84) abc
	L. terebrantis	41	1.56 (1.14) bc
	No wound	41	2.85 (2.57) a
	Wound	43	2.07 (2.16) abc
	Wound + media	41	1.85 (1.35) abc

Means followed by the standard deviation in parenthesis. Numbers followed by different letters within a column indicate significant differences at $\alpha = 0.05$.

4.4.5 Seedling height increment

Overall seedling growth was affected by the watering treatment, family type, and inoculation. Tolerant family (T) had more growth than the susceptible family (S) in all 3 of the watering conditions (Table 4.10). The growth increase was significantly less in seedlings inoculated with *G. huntii* than control seedlings (Table 4.8). However, *L. terebrantis* did not affect seedling growth.

4.4.6 Seedling dry matter yield

Needle dry matter yield (Ny) was affected by watering treatments, family and inoculation (Table 4.2). Stem dry matter (Sy) was also significantly affected by watering treatments, family and inoculation (Table 4.2).

Watering treatments, family, and family x moisture x watering treatment significantly affected coarse root dry matter yield (Cry) (Table 4.2). *Pinus taeda* seedlings

from susceptible family (S) under severe drought inoculated by *L. terebrantis* had significantly lower dry matter yield when compared to both normal watering and moderate drought. *Leptographium terebrantis* inoculation resulted in less Cry of the susceptible family under the normal watering condition. However, under both the moderate and severe drought, Cry of seedlings inoculated with *L. terebrantis* did not differ.

Fine root dry matter yield (Fry) was significantly affected by watering treatment, family, inoculation and watering treatment x inoculation. Drought treatments did not result in a significant reduction of Fry in the wound, wound + media and no wound inoculated seedlings. Under normal watering and moderate drought, *L. trerebrantis* did not reduce Fry when compared to control seedlings. However, Fry of seedlings inoculated with *L. terebrantis* was significantly less than seedlings under severe drought as compared those under the moderate drought and normal watering condition. Fine root dry matter yield was not different between the seedlings inoculated with *L. terebrantis* and *G. huntii* under any of the watering conditions (Table 4.11).

The shoot-to-root dry matter yield ratio (Sy/Ry) was significantly different between the seedlings from two families and three moisture treatments (Table 4.2). The family considered tolerant to ophiostomatoid fungi had lower Sy/Ry ratio than family considered susceptible (Table 4.14). Seedlings under severe and moderate drought had higher Sy/Ry ratio than seedlings receiving normal watering treatment (Table 4.15). Control and fungal inoculation treatments did not affect Sy/Ry.

Similarly, the coarse-root-to fine root dry matter yield ratio (Cry/Fry) was also significantly different between seedlings from two families. The family considered tolerant to ophiostomatoid fungi had significantly higher Cry/Fry ratio than susceptible

family. The *L. terebrantis* inoculated seedlings had higher Cry/Fry ratio than wound inoculated seedlings (Table 4.14).

Needle-to-fine-root dry matter yield ratio (Ny/Fry) was significantly affected by watering treatment and fungal inoculation. Seedlings inoculated with *G. huntii* and *L. terebrantis* under severe drought had significantly higher Ny/Fry ratio than that under normal watering (Table 4.16).

Figure 4.11 Roots of *Pinus taeda* seedling following harvest; left: Severe drought, right: Medium drought.

Figure 4.12 Seedling parts of two different *Pinus taeda* seedlings grown under different watering treatment. Left: seedling under severe drought and right: seedling under normal watering**.**

Table 4.8 Inoculation x family interaction on seedling height, stem dry matter and needle dry matter yield.

Inoculation	Family	Height growth (Ht)		Stem dry matter yield (Sy)		Needle dry matter yield (Ny)	
		n	cm	*n*	(g)	*n*	(g)
G. huntii	T	63	20.7 (10.5) abc	43	2.89 (1.68) c	51	3.47 (2.52) abc
	S	63	16.0 (10.6) d	43	2.15 (1.27) c	41	3.13 (2.10) abc
L. terebrantis	T	56	20.3 (8.1) abcd	46	2.98 (1.73) bc	48	3.16 (1.76) bc
	S	60	15.9 (10.7) d	46	2.52 (1.39) c	55	2.72 (2.41) c
Wound	T	63	25.2 (10.6) a	41	3.96 (1.24) ab	40	4.46 (2.98) ab
	S	60	18.3 (8.9) cd	40	2.60 (1.41) c	39	3.23 (2.44) abc
Wound + media	T	64	23.8 (9.8) abc	43	0.46 (2.03) abc	43	4.60 (2.30) ab
	S	62	17.9 (12.6) bcd	42	2.30 (1.09) c	44	3.15 (1.71) abc
No wound	T	58	26.6 (10.1) ab	42	4.35 (2.35) a	44	4.99 (3.99) a
	S	60	18.2 (10.6) d	44	2.30 (1.09) c	45	3.07 (2.79) bc

Means followed by the standard deviation in parenthesis. Numbers followed by different letters within a column indicate significant differences at $\alpha = 0.05$.

Table 4.9 Inoculation x family interaction on fine and coarse root dry matter yield.

		Fine root dry matter yield (Fry)		Coarse root dry matter yield (Cry)	
Inoculation	Family	*n*	(g)	*n*	(g)
G. huntii	Tolerant	43	0.58 (0.61) ab	43	0.88 (0.65) abcd
	Susceptible	41	0.33 (0.41) b	41	0.51 (0.42) d
L. terebrantis	Tolerant	44	0.49 (0.44) b	44	1.20 (1.03) a
	Susceptible	47	0.35 (0.45) b	47	0.53 (0.38) d
Wound	Tolerant	41	0.87 (0.95) a	41	1.06 (0.67) ab
	Susceptible	41	0.52 (0.52) ab	41	0.57 (0.50) cd
Wound + media	Tolerant	43	0.60 (0.47) ab	43	0.98 (0.69) abcd
	Susceptible	41	0.40 (0.26) ab	41	0.61 (1.11) bcd
No wound	Tolerant	45	0.88 (0.92) a	45	1.04 (0.72) abc
	Susceptible	44	0.54 (0.68) ab	45	0.59 (0.58) cd

Means followed by the standard deviation in parenthesis. Numbers followed by different letters within a column indicate significant differences at $\alpha = 0.05$.

Table 4.10 The effect of watering treatment x family interaction on seedling growth and biomass yields.

Drought	Family	*n*	Hty (cm)	*n*	Sy (g)	*n*	Ny (g)	*n*	Fry (g)	*n*	Cry (g)
N	T	101	25.6 (9.8) a	68	4.33 (2.21) a	77	4.36 (3.39) a	69	1.02 (0.87) a	69	1.27 (0.86) a
	S	97	18.3 (10.0) bc	67	2.87 (1.38) bc	71	3.45 (2.85) ab	66	0.53 (0.43) b	66	0.80 (0.99) bc
MD	T	106	25.4 (8.9) a	76	3.53 (1.84) ab	79	4.43 (2.94) a	75	0.59 (0.70) ab	75	1.13 (0.80) ab
	S	106	18.5 (10.4) bc	75	2.62 (1.41) c	78	3.33 (2.21) ab	77	0.51 (0.61) b	77	0.56 (0.39) cd
SD	T	97	18.6 (10.1) b	72	2.74 (1.29) bc	70	3.40 (1.85) ab	72	0.46 (0.43) bc	72	0.70 (0.49) c
	S	102	15.0 (11.5) c	73	1.86 (1.18) d	75	2.35 (1.63) b	71	0.25 (0.32) c	72	0.34 (0.29) d

N: Normal watering, MD: Medium watering, SD: Severe drought, Hty: Height growth, Sy: Stem dry yield increase, Ny: Needle dry yield increase, Fry: Fine root dry yield increase, Cry: Coarse root dry yield increase. Means followed by the standard deviation in parenthesis. Numbers followed by different letters within a column indicate significant differences at $\alpha = 0.05$.

Table 4.11 The effect of watering treatment x inoculation interaction on fine root dry matter yield (Fry).

Watering	Inoculation	*n*	Fine root dry yield (g)
Normal water	*G. huntii*	26	0.80 (0.56) a
	L. terebrantis	27	0.60 (0.49) ab
	No wound	32	0.78 (0.81) ab
	Wound	24	1.17 (1.09) a
	Wound + media	26	0.60 (0.39) ab
Severe drought	*G. huntii*	30	0.24 (0.32) bc
	L. terebrantis	32	0.17 (0.33) c
	No wound	23	0.52 (0.43) ab
	Wound	30	0.49 (0.47) ab
	Wound + media	28	0.40 (0.30) ab
Medium drought	*G. huntii*	28	0.38 (0.55) ab
	L. terebrantis	32	0.52 (0.42) ab
	No wound	34	0.78 (1.02) a
	Wound	28	0.51 (0.55) ab
	Wound + media	30	0.51 (0.45) ab

Means followed by the standard deviation in parenthesis. Numbers followed by different letters within a column indicate significant differences at $\alpha = 0.05$.

Table 4.12 The effect of family x watering treatment x inoculation interaction on coarse root dry matter yield (Cry).

Family	Watering	Inoculation	*n*	Coarse root yield (g)
Tolerant	Normal	*G. huntii*	14	1.23 (0.72) abc
		L. terebrantis	13	1.80 (1.32) a
		No wound	18	1.09 (0.66) abc
		Wound	11	1.33 (0.70) abc
		Wound + media	13	1.00 (0.63) abc
	Severe drought	*G. huntii*	15	0.68 (0.55) bc
		L. terebrantis	15	0.59 (0.25) bc
		No wound	12	0.55 (0.42) bc
		Wound	15	0.77 (0.40) bc
		Wound + media	15	0.87 (0.71) abc
	Moderate drought	*G. huntii*	14	0.73 (0.58) bc
		L. terebrantis	16	1.28 (0.95) abc
		No wound	15	1.37 (0.81) ab
		Wound	15	1.16 (0.78) abc
		Wound + media	15	1.07 (0.76) abc
Susceptible	Normal	*G. huntii*	12	0.84 (0.46) abc
		L. terebrantis	14	0.45 (0.35) bc
		No wound	14	0.74 (0.75) bc
		Wound	13	0.97 (0.64) abc
		Wound + media	13	1.05 (1.92) abc
	Severe drought	*G. huntii*	15	0.33 (0.28) c
		L. terebrantis	17	0.42 (0.38) c
		No wound	12	0.31 (0.29) c
		Wound	15	0.33 (0.26) c
		Wound + media	13	0.29 (0.19) c
	Moderate drought	*G. huntii*	14	0.42 (0.37) c
		L. terebrantis	16	0.71 (0.35) bc
		No wound	19	0.65 (0.55) bc
		Wound	13	0.44 (0.28) bc
		Wound + media	15	0.50 (0.20) bc

Means followed by the standard deviation in parenthesis. Numbers followed by different letters within a column indicate significant differences at $\alpha = 0.05$.

Table 4.13 Shoot-to-root and coarse-to-fine-root dry matter yield ratio (Sy/Ry) of different families.

Family	*n*	Sy/Ry	*n*	Cry/Fry (log)
Tolerant	202	1.56 (0.62) a	202	0.49 (1.09) a
Susceptible	198	1.87 (0.73) b	184	0.26 (0.96) b

Means followed by the standard deviation in parenthesis. Numbers followed by different letters within a column indicate significant differences at $\alpha = 0.05$.

Table 4.14 Coarse-to-fine-root dry matter yield ratio (Cry/Fry) of different inoculation treatment.

Inoculation	*n*	Cry/Fry (log)
G. huntii	70	0.33 (1.07) ab
L. terebrantis	83	0.68 (1.10) a
No wound	81	0.33 (1.02) ab
Wound	77	0.24 (0.98) b
Wound + media	75	0.27 (0.94) ab

Means followed by the standard deviation in parenthesis. Numbers followed by different letters within a column indicate significant differences at $\alpha = 0.05$.

Table 4.15 Effect of different watering treatment on shoot-to-root dry matter yield ratio (S/R).

Watering treatment	*n*	S/R (log)
Normal watering	130	1.51 (0.69) b
Medium drought	141	1.72 (0.70) a
Severe drought	129	1.89 (0.63) a

Means followed by the standard deviation in parenthesis. Numbers followed by different letters within a column indicate significant differences at $\alpha = 0.05$.

Table 4.16 Effect of watering treatment x inoculation on needle-to-fine-root dry matter yield ratio (Ny/Fry).

Watering treatment	Inoculation	*n*	Ny/Fry (log)
Normal watering	*G. huntii*	24	1.43 (1.21) c
	L. terebrantis	26	1.61 (0.96) c
	No wound	29	1.83 (1.35) bc
	Wound	23	1.69 (0.92) bc
	Wound + media	25	2.00 (1.07) abc
Severe drought	*G. huntii*	22	2.52 (1.38) ab
	L. terebrantis	25	2.69 (1.01) a
	No wound	20	1.96 (0.58) abc
	Wound	26	1.84 (0.88) abc
	Wound + media	24	1.98 (0.76) abc
Medium drought	*G. huntii*	21	2.16 (0.56) abc
	L. terebrantis	31	1.92 (0.83) abc
	No wound	32	1.87 (1.14) abc
	Wound	21	2.34 (0.87) abc
	Wound + media	27	2.10 (0.77) abc

Means followed by the standard deviation in parenthesis. Numbers followed by different letters within a column indicate significant differences at $\alpha = 0.05$.

4.4.7 Needle greenness and needle chlorophyll content

The needle greenness was significantly different between the inoculation treatments (P = <0.0001). Seedlings treated with *L. terebrantis* and *G. huntii* had significantly lower needle greenness (Table 4.17). However, the watering treatment did not significantly impact needle greenness (Table 4.2). The needle chlorophyll content was not significantly affected by fungi (P = 0.52671), watering treatment, or watering treatment x inoculation interaction ($F_{(1, 35)} = 0.97$, P = 0.43770).

Table 4.17 The effect of inoculation treatment on needle greenness.

Inoculation treatment	*n*	Needle greenness
G. huntii	64	42.21 (10.62) b
L. terebrantis	63	41.48 (1.96) b
No wound	62	50.76 (8.27) a
Wound	61	51.71 (10.04) a
Wound + media	65	52.19 (10.10) a

Means followed by the standard deviation in parenthesis. Numbers followed by different letters within a column indicate significant differences at $\alpha = 0.05$.

4.4.8 Pre-dawn water potential measurement

Pre-dawn needle water potential (Ψpd) was significantly affected by inoculation, ($P = 0.00218$) (Table 4.2). Seedlings inoculated with *L. terebrantis* and *G. huntii* had significantly more negative Ψpd than the no wound seedlings (Table 4.18). However, fungal inoculation did not cause significantly negative water potential than wound and wound + media controls.

Table 4.18 The effect of inoculation treatment interaction on water potential (Ψpd).

Inoculation treatment	*n*	Ψpd (-Mpa)
G. huntii	19	7.53 (2.37) a
L. terebrantis	18	7.52 (1.95) a
No wound	21	5.12 (1.49) b
Wound	15	5.92 (2.32) ab
Wound + media	19	6.29 (1.53) ab

Means followed by the standard deviation in parenthesis. Numbers followed by different letters within a column indicate significant differences at $\alpha = 0.05$.

4.4.9 Relative water content

The relative water content (RWC) was significantly affected by watering treatments (Table 4.2). However, it was not significantly affected by the inoculation, family type and any of the interaction. Seedling under moderate drought had significantly low needle relative water content than the normal watering condition and severe drought (Table 4.19).

Table 4.19 The effect of watering treatments on relative water content.

Watering treatment	*n*	Relative water content (RWC)
Normal water	47	84.18 (10.63) a
Severe drought	51	82.48 (8.19) a
Moderate drought	52	76.79 (9.86) b

Means followed by the standard deviation in parenthesis. Numbers followed by different letters within a column indicate significant differences at $\alpha = 0.05$.

4.5 Discussion

The relative susceptibility of *P. taeda* families to vascular-inhabiting ophiostomatoid fungi is not influenced by different watering conditions. The tolerant family grew better than the susceptible family selected for susceptibility. *Leptographium terebrantis* was consistently more virulent as determined by lesion length to *P. taeda* than *G. huntii.* There was an interaction between watering and inoculation treatment in case of lesion length and occlusion length. Previous studies by Matusick et al. (2008) and Chieppa et al. (2017) did not find any evidence of soil moisture and fungal interaction.

The virulence of *L. terebrantis* when inoculated into seedlings (in terms of LL/Ht, LD, and OD) increased under severe drought condition. Virulence of *G. huntii* (in terms of lesion and occlusion), however, remained same under different watering conditions. Salle et al. (2008) reported longer length by *L. yunnanense* in moderately water-stressed *P. yunnanenesis* (Franch.). We observed similar results only in terms of occlusion length. In contrast, Christiansen and Glosli (1996) reported that phloem damage and blue staining due to *Ceratocystis polonica* was greater in the well-watered trees than in the water-stressed trees. This difference in result between their study and the present study might be due to the variation in the species-specific threshold.

Virulence of *G. huntii* was increased regarding bud outbreak and Fry. Bud outbreak and Fry as a measure of seedling productivity was significantly lower in

seedlings inoculated with *G. huntii* under severe drought, nevertheless, LL/Ht and OL/Ht ratio caused by the fungi did not differ between three watering conditions. This suggests that virulence of *G. huntii* increases to some extend under severe drought. Matusick et al. (2008) found no evidence in bud-break in *P. palustris* Mill. inoculated with *G. alacris* (formerly *G. serpens*) under varying soil moisture regimes at 16 weeks. This difference in result between our study and their study may be due to species-specific response.

The infection by vascular-inhabiting fungi *L. terebrantis* was likely to affect seedling health under severe drought through increased investment of seedling in LL/Ht and reduction in fine root dry yield (in both families) and coarse root dry matter yield (in the susceptible family). However, *G. huntii* inoculated seedlings under drought did not show increased defense responses to *inoculated* fungi than compared to the seedlings under the normal watering and moderate drought conditions. Also, the seedlings growth parameters were not different between the control inoculated and the fungal inoculated seedling. Thus, this may suggest that fungus *L. terebrantis* may utilize more resources of the plant under drought conditions (Oliva et al. 2014) and fungus *G. huntii* may affect the plant health independent of the drought (Chieppa et al., 2017).

Localized damage to the vascular conducting tissue was observed in inoculated *P. taeda* seedlings. The spread of the fungal mycelium into the sapwood might have caused damage to the tracheid walls. Such damage can result in cavitation and embolism (Zimmermann, 1983). The xylem blockage can be irreversible due to resin deposition and tyloses formation. Complete xylem blockage in some of the seedlings under severe drought treatment was observed. In such seedlings, surprisingly, the development of the new tissues (Figure 4.7) on the opposite side of the fungal inoculation which would have

helped in the survival of the plant. However, the growth of the tissues around the fungi inoculated side was completely halted. It could be an adaptive trait of *P. taeda* that would allow the plant to be decoupled from drought as well as pathogen stress. Moreover, the growth of such seedlings was halted suggesting a potential tradeoff between this adaptive trait and plant growth.

Both the present study and few additional studies indicate that in general, the family chosen for tolerance to ophiostomatoid fungi should have more growth potential in terms of seedling volume change and height increment e.g., Chieppa et al. (2015) and Chieppa et al. (2017). *Pinus taeda* families used in the studies above and in the current study were the same. Therefore, it is not surprising that the results between studies were similar. Inoculated and non-inoculated controls did not show any difference in growth potential suggesting wound and media do not have any effect on seedling growth. Taken together, the previous studies and present together show some support for the higher growth potential of tolerant families. Other indicators of plant growth (increment in the number of new bud-break) was not different between the two families.

The tolerant family tended to yield more stem and needle dry matter than the susceptible family. Stem and needle dry weight yield was less under severe drought conditions when compared to the normal water. To our knowledge, this is the first study to show a two-way interaction of watering x inoculation (in terms of fine root dry matter yield) and three-way interaction of watering treatment x family x inoculation (in terms of coarse root dry matter yield) in *P. taeda* under different watering treatments. Here, *L. terebrantis* caused less coarse root biomass yield in severe drought conditions. The results of our study have been supported by that of Croisé et al. (2001). Unlike, Croisé et

al. (2001) we only performed single point inoculation. Massive inoculation of the fungi might lead to more detrimental effects on the *P. taeda* seedlings.

The seedlings inoculated with *L. terebrantis* under severe drought allocated significantly little biomass to roots as compared to other seedlings within same or other watering treatments. The allocation of biomass to shoot increases above and root decreases under adverse environmental conditions. Survival of plant decreases as this aboveground and belowground biomass ratio reaches a certain threshold. Above that threshold, evaporative surface (needles) increases as compared to the absorbing root surface (Cregg, 1994). Adversely, high root-to-shoot-ratio implies that the plant has the capability to cope with the drought. As the number or the length of the root increases larger soil volume can be accessed to extract more available water (Niu et al., 2008).

Inoculation of *L. terebrantis* and *G. huntii* result in a decrease in the needle greenness as compared to the control seedlings. Our results are in contrast with the previous studies performed by Chieppa et al. (2017) where they did not report any decrease in needle greenness. This discrepancy between the present study and previous study might be due to the longer study duration deployed in our study.

Inoculated *P. taeda* had lower needle water potential than the control treatments. While some seedlings inoculated with *L. terebrantis* and *G. huntii* in severe drought had water potential values lower than -1.4 MPa, no strong overall pattern was observed. The vulnerability of seedling to physiological damage may depends on individual seedling vigor. For example, it has been postulated that xylem embolism and cavitation occurs at low soil moisture levels (Croisé et al., 2001). For instance, the xylem embolism and loss of hydraulic conductivity begin at -2 MPa xylem water potential in *Pinus sylvestris* L.

(Cochard, 1992). Croisé et al. (2001) reported a drop in hydraulic conductivity and needle water potential in water stressed *Pinus sylvestris* following inoculation with *L. wingfieldii*. A similar trend was not observed in the present study.

Future studies should be focused on longer-term monitoring of the fungal inoculated *P. taeda* seedlings under projected climate change scenario such as drought, increase in temperature and CO_2. The damage on an ecological scale might be higher than what we observed in our controlled study as we know that the mass attack of the beetles occurs in trees pre-stressed with drought in the natural scenario. Thus, mass inoculation of the fungi in the stressed mature *P. taeda* trees can provide a better understanding of host-microbe and environment interactions.

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CHAPTER V

Towards Biocontrol of Blue-Stain Fungi by Plant Growth-Promoting Rhizobacteria

5.1 Abstract

*Leptographium terebranti*s, *Grosmannia huntii* and *G. alacris* cause blue-staining of wood and also are associated with the decline of *Pinus* species in the southeastern U.S. Twenty-nine plant growth-promoting rhizobacteria (PGPR) were tested for antibiosis against these ophiostomatoid fungi *in vitro*. Three PGPR strains were tested for their capacity to induce resistance of *P. taeda* L. to these fungi by co-treating seedling with PGPR and fungus in the greenhouse. The dark necrotic tissue around fungal inoculation sites and seedling biomass were measured after 8 weeks. In antibiosis test, all PGPR strains inhibited the growth of the three fungal pathogens. In greenhouse assay, PGPR treated *P. taeda* resulted in higher plant biomass and smaller necrotic tissue. In conclusion, tested PGPR strains have potential as biocontrol agents.

5.2 Introduction

Plant growth-promoting rhizobacteria (PGPR) consist of symbiotic rhizospheric bacteria that with the capacity to colonize plant roots, stimulate plant growth (Kloepper and Schroth, 1978; Chanway et al., 1991; Orhan et al., 2006). They often inhibit pathogenic microorganisms (Beneduzi et al., 2012; Liu et al., 2016a) or induce systemic resistance (ISR) of the plant to disease (Liu et al., 2016b; Kumar et al., 2016) and abiotic

stresses (Bresson et al., 2013; Egamberdieva and Lugtenberg, 2014). Specific strains of PGPR induce systemic resistance of plants against fungal (Liu et al., 2016b; Enebak and Carey, 2000), bacterial (Liu et al., 1995) and viral (Raupach et al., 1996) diseases as well as insects (Zehnder et al., 1997) and nematodes (Kokalis-Burelle et al., 2003). PGPR have the ability to compete with other microorganisms for nutrients and space (Liu et al., 2017). PGPR inhibit other microbes by producing antibiotics such as pyrrolnitrin and pyocyanine, and as well as siderophores which limit the iron required for the growth of pathogens (Ramamoorthy et al., 2001). More specifically, PGPR strains can act against fungal pathogens by the producing lytic enzymes such as chitinases and B-1, 3-glucanases which degrade chitin and glucan present in the fungal cell wall by specific PGPR isolates (Ramamoorthy et al., 2001).

The use of microbes, including PGPR (Plant Growth Promoting Rhizobacteria), as a biological control method and, is an environmentally friendly approach (Lugtenberg and Kamilova, 2009) to disease control, and their use is steadily increasing globally (Lugtenberg, 2014). Dunham (2016) has projected the market of biocontrol to reach 5 billion dollars by 2020. Adoption of PGPR as a biocontrol in agriculture offers an attractive way to replace chemical fertilizers, pesticides and other supplements (Ashrafuzzaman et al., 2009). Also, PGPR strains with a broad spectrum of activity protect plant through multiple mechanisms (Liu et al., 2017) which are unachievable by other chemical and management treatments (Beneduzi et al., 2012; Iturritxa et al., 2017). Recently, application of PGPR is gaining attention in agroforestry (Chanway, 1997; Enebak and Carey, 2000; Enebak, 2005; Iturritxa et al., 2017). Specific isolates of PGPR

have shown effectiveness against sap-stain fungi and wood decaying fungi (Seifert et al., 1987).

Leptographium terebrantis S.J. Barras and T.J. Perry*, L. procerum* Kendrick M.J. Wingfield and *Grosmannia huntii* R. C. Rob. Jeffr is among the ophiostomatoid fungi associated with the decline of *Pinus* species in the southern U.S. (Eckhardt et al., 2007). These ophiostomatoid fungi infect *Pinus* root, cause the lesion, thrive in vascular tissues, and further disturb plant hydraulic conductance (Wingfield, 1988; Matusick et al., 2010; Hammerbacher et al., 2013; Oliva et al., 2014). Furthermore, they cause blue-stain which reduce the aesthetic value of wood leading to a significant economic loss in the timber industries (Bruce et al., 2003). Taking into account, high risk of these fungal pathogens, it is essential to advance rapidly in understanding the efficient control measures.

The understanding of the activity of the PGPR towards blue-staining ophiostomatoid fungi could be beneficial to overcome the root disease and pine decline problems. Thus, the overall aim of this study was to understand the possibility of using PGPR to control the harmful effects of blue-staining fungi on *P. taeda.* The specific objectives of the study were (i) to assess the antibiosis potential of PGPR strains *in vitro* against *L. terebrantis*, *G. huntii* and *G. alacris* by PGPR strains and (ii) to evaluate the capacity of PGPR strains, *Bacillus pumilus* (INR7), *B. pumilus* (SE-34) and *Serratia marcescens* (90 -166) to induce systemic resistance of *P. taeda* to *L. terebrantis* and *G. huntii*.

5.3 Methodology

5.3.1 *In vitro* screening of antagonistic activity

Twenty-eight PGPR strains of *B. velezensis* and a single strain of *Paenibacillus peoriae* and *Bacillus altitudinis* maintained at PGPR lab culture collection at Department of Entomology and Plant Pathology at Auburn University, Auburn, Alabama, U.S. (Table 1) were studied. All strains were previously identified using 16S rDNA sequencing with the comparison to sequences of type strains. The bacteria isolates were stored at -80 °C in Tryptic Soy Broth (TSB) (Difco Laboratories, Detroit, MI, USA) amended with 30 % glycerol. Each strain was streaked onto Tryptic Soy Agar (TSA) three days before the start of the experiment. After incubation at 28 °C for 48 h, purity of the culture was checked. A single colony (10 µl) of PGPR was inoculated in 25 ml TSB and incubated at 28 °C for 48 h with continuous shaking (150 rpm). Pellets obtained by centrifuging bacterial cultures at 3500 rpm for 15 min were re-suspended in sterile distilled water at a concentration of 10^8 CFU ml^{-1} (Liu et al., 2016a).

Table 5.1 Twenty-nine PGPR strains used in the in vitro antagonism screening.

Strains	Identification
AP52	*Bacillus velezensis*
AP69	*Bacillus altitudinis*
AP136	*Bacillus velezensis*
AP188	*Bacillus velezensis*
AP194	*Bacillus velezensis*
AP195	*Bacillus velezensis*
AP196	*Bacillus velezensis*
AP197	*Bacillus velezensis*
AP198	*Bacillus velezensis*
AP199	*Bacillus velezensis*
AP200	*Bacillus velezensis*
AP201	*Bacillus velezensis*
AP203	*Bacillus velezensis*
AP208	*Bacillus velezensis*
AP210	*Bacillus velezensis*
AP211	*Bacillus velezensis*
AP212	*Bacillus velezensis*
AP213	*Bacillus velezensis*
AP214	*Bacillus velezensis*
AP218	*Bacillus velezensis*
AP241	*Bacillus velezensis*
AP294	*Paenibacillus peoriae*
AP295	*Bacillus velezensis*
AP296	*Bacillus velezensis*
AP297	*Bacillus velezensis*
AP298	*Bacillus velezensis*
AP301	*Bacillus velezensis*
AP305	*Bacillus velezensis*
H57	*Bacillus velezensis*

5.3.1.1 Preparation of fungal cultures

Three fungi, *L. terebrantis* (ATCC MYA-3316), *G. huntii* (ATCC MYA-3311) and *L. procerum* (ATCC MYA-3313), maintained at 2 % MEA at 4 °C in the culture collection of Forest Health Dynamics Laboratory, School of Forestry and Wildlife Sciences at Auburn University were included in the study. These fungal isolates were obtained from roots of symptomatic *P. taeda* and *P. palustris* Mill. as the method described by Eckhardt et al. (2007). A description of fungal isolates are provided in Table 5.2. Fourteen days before the experiment these isolates were cultured on 2 % Malt Extract Agar (MEA) and incubated at room temperature.

Table 5.2 Isolation site and source of fungal isolates used in the study.

Fungi	Isolate number (ATCC accession no.)	Isolation site	Host Species
Grosmannia huntii	LLP-R-02-100 (MYA-3311)	Fort Benning Military Reservation, GA	*Pinus palustris*
Grosmannia alacris	LOB-R-00-309 (MYA-3315)	Westervelt Company Land, AL	*Pinus taeda*
Leptographium terebrantis	LOB-R-00-805 (MYA-3316)	Talladega National Forest, Oakmulgee Ranger District, AL	*Pinus taeda*

5.3.1.2 Antibiosis test

A dual agar plate system was designed to support the growth of PGPR strains and the challenged fungal pathogen in the same Petri plate (Figure 5.1). These plates had four holes (13-mm diameter) filled with melted TSA at three equidistant edges in a Potato Dextrose Agar (PDA) plate. Ten µl of the PGPR suspensions prepared earlier were inoculated in the TSA following solidification. In the control side of the plate, 10 µl of

the sterile water without PGPR suspension were inoculated. Plates were incubated at 28 °C for 48 h. Plates were exposed to ultraviolet (1000 x 100 µJ cm^{-2}) for 2 minutes to prevent bacterial growth outside the TSA-holes. Seven-mm of agar plug with actively growing fungal hyphae was placed in the center of antibiosis testing plate and incubated at room temperature. The study was conducted in triplicate.

Figure 5.1 Fungal disc inoculated at the center of the agar plate.

5.3.1.3 Measurements

Inhibition zones were measured from the edge of the PGPR strain to the fungal pathogen with the help of a digital caliper once the growth of control reached to the edge of the plate (Figure 5.2). The inhibition index was calculated using the formula given by Vincent (1947):

$$I = \frac{C - T}{C} \times 100$$

Where, I = inhibition index, C = growth in control, T = growth in treatment

The inhibition index was calculated for all strains of PGPR. Then the inhibition indices were categorized based on inhibition zones. It was categorized as strong (+++),

medium (++) and weak (+) when $I > 20\%$, $10\% < I < 20\%$ and $I < 10\%$ respectively (Wahyudi and Astuti, 2011).

Figure 5.2 Fungal growth inhibited at the three sides.

5.3.2 Induced systemic resistance study

5.3.2.1 Study design

One-year-old *P. taeda* seedlings were received from Rayonier in January 2016. Seedlings represented either susceptible (S) or tolerant (T) family to ophiostomatoid fungi as determined from a study by Singh et al. (2014). Dry biomass of 12 seedlings of each family was measured by separating needles, stem, coarse root and fine root and allowing them to dry in the oven at 75 ºC for 72 h. Then 400 seedlings from each family were planted in one-gallon plastic pots. The potting medium was ProMix BX® (Premier Tech, Quebec, and Canada) peat-based potting media. The seedlings were kept in the greenhouse and allowed to acclimatize for 3 months (Figure 5.3). The study was designed as a completely randomized block design with 3 PGPR and 2 fungal treatment combinations.

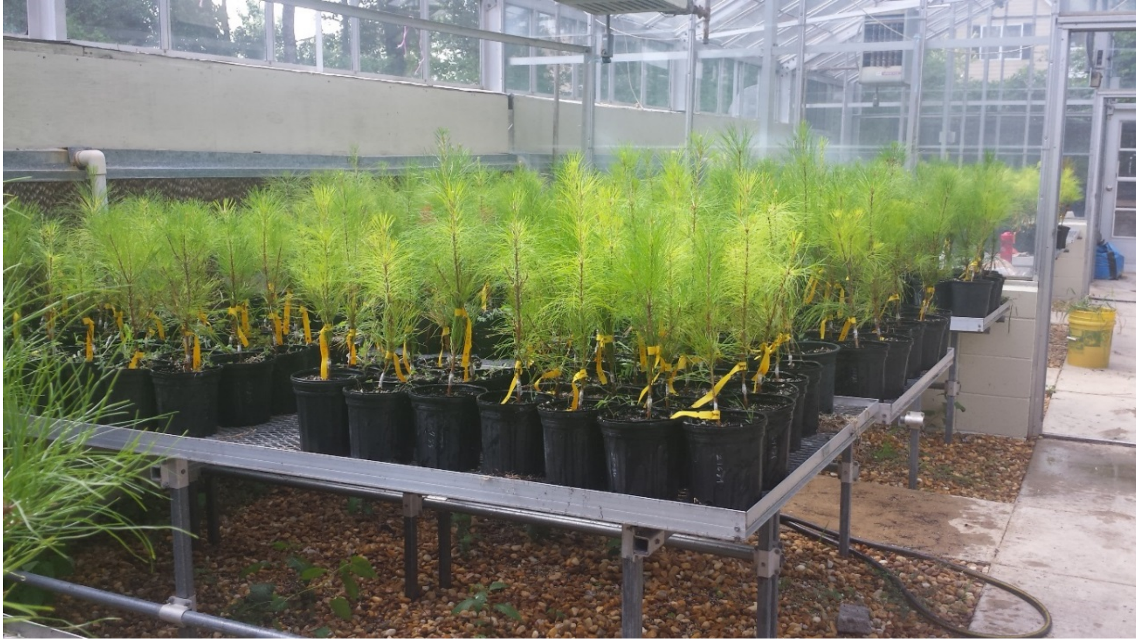

Figure 5.3 Loblolly pine seedlings after application of the PGPR and the fungal treatment.

5.3.2.2 PGPR selection

Three PGPR strains: *B. pumilus* INR7, *B. pumilus* SE-34 and *S. marcescens* (90 - 166), previously shown to induce systemic resistance of conifers to fusiform rust (Enebak and Carey 2000), were included in the study. These strains were obtained from the culture collection of the PGPR lab maintained at the Department of Entomology and Plant Pathology at Auburn University, Alabama, U.S. These strains were stored in Tryptic Soy Broth (TSB) with 30 % glycerol at -80 ºC until used. Pure cultures of PGPR strains were streaked on TSA plates, three days before inoculations. Suspensions of 10^8 colony forming unit ml^{-1} of each rhizobacterial suspension were prepared as described by Enebak et al. (1998). Seedlings were carefully pulled out and were washed in running tap

water. Sixty seedlings of each family were randomly selected, and the root balls were dipped in each PGPR solution for one hour. Root balls of control seedlings were immersed in sterile distilled water. Seedlings were carefully replanted following inoculation.

5.3.2.3 Fungal treatment

Single spore cultures of *L. terebrantis* (ATCC accession no. MYA-3316) and *G. huntii* (ATCC accession no. MYA-3316) were cultured on MEA 14 days before the inoculation experiment. Two weeks following PGPR inoculation, 30 seedlings of each family treated with each PGPR were artificially inoculated with each fungus. To inoculate seedlings, an 8-mm vertical cut (< 2 mm deep) was made in the stem 2 cm above the soil line. Then a 3-mm plug of agar with actively growing fungal hyphae was inserted (fungus-side-down) into the cut. The inoculation point was gently wrapped with a moistened sterile cotton ball and covered with Parafilm® respectively to prevent sudden desiccation of agar media and prevent any external contamination. The inoculation experiment was carried out in two replications: one in April 2016 and another in May 2016.

5.3.2.4 Measurements

Eight weeks following fungal inoculation, seedlings were carefully harvested by clipping each seedling above the soil line. Stems of harvested seedlings were placed in a solution of stain (FastGreen FCF; Sigma Chemical Co, St. Louis, MO, USA) and water in a ratio of 0.25 g L^{-1} for 48 h. Staining allowed easier visualization of infected tissue. The bark tissue around the inoculation point was scraped, and the length of the necrotic tissue

was measured. Biological control efficacy of 3 PGPR strains was calculated using the following formula:

Biological control efficacy (BCE) $= \frac{C-T}{C} \times 100$

Where, C = average lesion in control, T = average lesion in treatment group

Ten seedlings from each fungal treatment within each family and PGPR treatment were selected for biomass yield measurements. The seedlings were separated into needles, stem, coarse root and fine root and dried at 75 °C for 72 h**.** Whole plant biomass was calculated by adding the dry weight of all seedling components. Family mean biomass determined before the start of the experiment was subtracted from each final seedling biomass to estimate biomass gain by seedlings during the experiment (dry matter yield). The biological efficacy of 3 PGPR strains for biomass yield was calculated.

5.3.3 Statistical analysis

Data were analyzed by using Proc GLM statement on SAS 9.4 software (SAS Institute, Cary, NC, USA). Data for lesion and biomass were log transformed. Two-way analysis of variance was performed on the response variables like lesion and the seedling biomass. Treatment means were separated by Tukey's Honest Significant Difference at $\alpha = 0.05$.

5.4 Results

5.4.1 *In vitro* antagonistic activity screening

Growth of the fungal isolates adjacent to PGPR strains were inhibited. The fungi grew well to the edge of the plate in the control sides. Near the fungal inhibition zone, fungal sporulation was halted. Different strains of PGPR produced significantly different inhibition zones to *L. procerum* ($F_{(28, 232)} = 87.08$, $P = <0.0001$), indicating that the

ability to inhibit the growth of the fungi varies among strains. Strains AP188 (76.30 %) and AP195 (76.30 %) had the highest index of inhibition (Table 5.3). Whereas, strain AP211 (39.26 %) had the lowest inhibition index for *L. procerum*. Different PGPR strains showed different antagonistic behavior to *L. terebrantis* ($F_{(28, 232)} = 47.77$, $P = <0.0001$). Strain AP214 (67.16 %) had the highest inhibition activity, and strain AP69 (9.52 %) had the least inhibition index. The inhibition index for *L. terebrantis* was lower than that of the *L. procerum*. Similarly, the inhibition zones produced for *G. huntii* also were significantly different among the PGPR strains ($F_{(28, 232)} = 44.35$, $P = <0.0001$). Strains, AP295 (73.58 %) and AP301 (73.58 %) had the highest inhibition index and AP69 had the lowest. The inhibition index of all the strains was strong (Table 5.3).

Table 5.3 Mean inhibition zone (mm) and inhibition index by 30 PGPR strains to 3 fungi.

Strain	N	*L. procerum*		*L. terebrantis*		*G. huntii*	
		Inhibition	Index	Inhibition	Index	Inhibition	Index
AP136	9	12.33	+++	4.67	++	11.78	+++
AP188	9	21.33	+++	12.44	+++	16.00	+++
AP194	9	18.44	+++	7.11	+++	17.22	+++
AP195	9	21.33	+++	10.11	+++	13.33	+++
AP196	9	19.44	+++	12.78	+++	14.78	+++
AP197	9	15.11	+++	12.11	+++	17.11	+++
AP198	9	9.00	+++	11.00	+++	12.78	+++
AP199	9	13.22	+++	3.44	++	18.44	+++
AP200	9	18.89	+++	7.89	+++	13.22	+++
AP201	9	9.56	+++	4.33	+++	14.89	+++
AP203	9	11.33	+++	8.33	+++	16.67	+++
AP208	9	8.44	+++	8.00	+++	14.56	+++
AP210	9	9.89	+++	4.44	++	7.67	+++
AP211	9	4.67	+++	13.44	+++	12.67	+++
AP212	9	20.56	+++	11.89	+++	11.78	+++
AP213	9	20.89	+++	8.78	+++	18.11	+++
AP214	9	17.11	+++	17.22	+++	16.67	+++
AP218	9	25.22	+++	11.11	+++	15.00	+++
AP241	9	13.00	+++	8.11	+++	18.89	+++
AP294	9	12.44	+++	9.33	+++	19.67	+++
AP295	9	11.89	+++	13.78	+++	20.11	+++
AP296	9	20.67	+++	10.22	++	16.11	+++
AP297	9	12.89	+++	5.89	+++	14.89	+++
AP298	9	9.33	+++	5.33	+++	14.67	+++
AP301	9	18.44	+++	7.56	+++	20.11	+++
AP305	9	16.56	+++	17.00	+++	16.67	+++
AP52	9	13.56	+++	15.33	+++	8.78	+++
AP69	9	5.78	+++	1.44	+	0.56	+
H57	9	8.56	+++	4.33	++	15.33	+++

Index= Inhibition Index (I) [(+++) = strong when I ≥ 20 %, (++) = medium when I < 10 % and I > 20% and (+) = weak when I <10 %)].

5.4.2 Resistance Study

5.4.2.1 Infection reduction

The pathogenic fungi caused a dark necrotic lesion in all of the inoculated seedlings. The lesion length produced by the fungal treatment was significantly different between the two study periods ($F_{(1, 471)} = 15.72$, $P = <0.0007$). Thus, the data were analyzed individually for each of the replications. Two different *P. taeda* families did not produce significantly different ($F_{(1,141)} = 2.65$, $P = 0.1040$) lesion length overall. So, the data were pooled together for further analysis.

In the first replication of the study, *L. terebrantis* and *G. huntii* fungi did not produce significantly different lesion lengths ($P = 0.9286$) (Table 5.4). Thus, the data from both of the fungi were pooled together and further analysis was performed for overall fungi. All the PGPR strains caused significant reductions in the lesion length than the control. *Bacillus pumilus* INR7 caused the highest reduction in lesion length (Table 5.5). The strains *S. marcescens* (90 -166), *B. pumilus* (INR7), and *B. pumilus* (SE-34) reduced the lesion production by 13.26 %, 21.44 %, and 14.73 %, respectively.

In the second replication of the study, lesion length was not different between the two *P. taeda* families ($F_{(1, 184)} = 1.86$, $P = 0.1739$) (Table 4). However, lesion length was different among seedlings treated with different PGPR ($F_{(3, 184)} = 4.66$, $P = 0.0036$), indicating that the effect of the PGPR differed between two families. In family S, the biological efficacy of *B. pumulis* (INR7) was 10.45 % in terms of lesion reduction. However, the other two PGPR strains did not cause significantly reduced lesion length than the control. In family T, the biological efficacy of *B. pumilus* (INR7) and *B. pumilus*

(SE-34) in terms of lesion reduction was 17.72 % and 11.89 % respectively. However, *S. marcescens* (90 -166) did not cause significantly smaller lesion than control (Table 5.5).

Table 5.4 The p-values showing the effect of the independent variables for lesion length.

Replication	Source	DF	F Value	Pr > F
Replication1	Family	1	1.15	0.2839
	Fungi	1	0.01	0.9286
	PGPR	3	40.15	<0.0001
	Fungi*PGPR	3	1.18	0.3173
	Family*PGR	3	0.70	0.5541
Replication 2	Family	1	1.86	0.1739
	Fungi	1	5.62	0.0188
	PGPR	3	19.45	<0.0001
	Fungi*PGPR	3	1.89	0.1328
	Family*PGPR	3	4.66	0.0036

Note: PGPR: Plant Growth-Promoting Rhizobacteria treatment, Fungi: Fungal treatment.

Table 5.5 Mean length of the lesion produced by fungi in seedlings inoculated with different PGPR.

	Replication 1		Replication 2			
	Overall Family		Family S		Family T	
PGPR	N	LL (mm)	N	LL (mm)	N	LL (mm)
90 -166	79	18.25 (2.59)a	25	20.77(1.57)ad	25	18.94 (2.24)a
INR7	74	16.53 (2.84)b	26	17.83 (2.01)b	26	17.37 (3.20)b
SE-34	68	17.93 (2.28)a	24	19.20 (1.63)dc	25	18.60 (1.82)dc
Control	71	21.04 (2.34)c	23	19.91 (1.41)a	22	21.11 (1.71)a

Note: LL: Lesion length. Means followed by standard deviation in parenthesis. Different letters indicate that statistical significant differences at α = 0.05, Family S: Susceptible family, Family T: Tolerant family.

5.4.2.2 Biomass yield

The total biomass yield by the seedlings was significantly different between the replications of the study ($F_{(1, 226)} = 103.91$, $P = <0.0001$). Thus, the biomass yield was calculated separately for each of the replication of study. There was an overall significant effect of PGPR treatment on biomass yield ($F_{(3,226)} = 11.03$, $P = <0.0001$).

In the first replication of the study, the biomass gained by two families were different ($F_{(1,152)} = 4.61$, $P = 0.0334$) (Table 5.6). Also, the interaction between the family and PGPR was significant ($P = 0.0055$) (Table 5.6) referring that the activity of PGPR was significantly different among two families. In family S, the biomass of the seedlings inoculated with *B. pumilus* (SE-34) was significantly higher than the control. In family T, mean biomass gain in the seedlings inoculated with all three PGPR was significantly higher than the control (Table 5.7).

In the second replicate of the study the biomass yield was significantly different between the families. Family T had higher biomass than family S ($P = 0.0093$) (Table 5.7). The mean biomass yield of the seedlings treated with PGPR was higher than the control in both families, although non-significantly (Table 5.8).

Table 5.6 The p-values showing biomass yield for study replicates 1 and 2.

Replication	Source	DF	F value	Pr > F
Relication 1 (log)	Family	1	4.61	0.0334
	PGPR	3	8.85	<0.0001
	Fungi	1	0.27	0.6226
	Fungi*PGPR	3	0.23	0.8435
	Family*PGPR	3	4.15	0.0055
Replication 2 (log)	Family	1	6.62	0.0093
	PGPR	3	0.96	0.2187
	Fungi	1	0.62	0.6289
	Fungi*PGPR	3	0.31	0.7788
	Family*PGPR	3	0.6	0.6178

Note: PGPR: Plant Growth-Promoting Rhizobacteria treatment, Fungi: Fungal treatment.

Table 5.7 Mean biomass yield of *Pinus taeda* seedlings inoculated with different PGPR.

	Replication 1				Replication 2			
		Family S		Family T		Family S		Family T
PGPR	N	Wt (g)	N	Wt (g)	N	Wt (g)	N	Wt (g)
90 - 166	21	7.47 (2.83) ac	18	9.06 (2.59) abc	9	3.22 (0.76) a	10	3.71 (1.41) a
INR7	24	6.68 (4.44) ac	20	9.41 (2.56) b	9	2.54 (1.51) a	10	4.19 (2.22) a
SE-34	19	8.02 (3.37) ab	20	7.18 (3.14) c	10	3.20 (1.77) a	9	5.00 (2.22) a
Control	21	4.98 (1.39) c	21	5.01 (1.58) d	7	2.41 (1.21) a	11	3.28 (1.67) a

Note: LL: Lesion length. Means followed by the standard deviation in parenthesis. Different letters indicate that statistical significant differences at $\alpha = 0.05$, Family S: Susceptible family, Family T: Tolerant family.

5.5 Discussion

5.5.1 Antibiosis study

The results suggest that the studied PGPR strains have the ability to inhibit the growth of the ophiostomatoid fungal pathogens *in vitro*. All the 29 elite strains outcompeted all three ophiostomatoid fungi. Strains AP195 and AP198 were highly antagonistic to *L. procerum,* AP305 to *L. terebrantis* and AP295 to *G. huntii* respectively. These PGPRs were previously shown to have a broad spectrum of antagonistic activity against foliar pathogens, vascular pathogens and root pathogens (Liu et al., 2016a). Our results are in agreement with Zhang et al. (2014) who reported that *B. subtilis* strain B37 exhibited antagonistic activity towards the sap-staining fungi *Lasiodiplodia theobromae* and *B. subtilis* strain C186 towards *Ceratocystis* sp. and *Ophiostoma* sp. Similarly, Gradinger et al. (2009) reported that the filamentous fungi; *Trichoderma* sp. and *Phlebiopsis gigantica,* can inhibit the growth and staining of the fungi *Sphaeropsis sapinea* and *Ophiostoma minus* in both laboratory and field conditions, respectively.

Twenty-seven of the 29 PGPR strains studied in this study were *B. velezensis* which are known to contain many strains with potent biocontrol activity (Balhara et al., 2011; Liu et al., 2016a; Liu et al., 2016b; Liu et al., 2017). These PGPR strains pose a broad spectrum of activity against plant pathogens from 6 different genera (Liu et al., 2017). Furthermore, most of these strains were reported to produce five traits related to plant growth promotion such as nitrogen fixation, phosphate solubalization, indole-3-acetic acid (IAA production), siderophore production, and biofilm formation. Moreover, specific strains of *B. velezensis* have been reported to produce various bioactive compounds (Ruiz-Garcia, 2005; Yu et al., 2002; Argulles-Arias et al., 2009). It is,

therefore, not surprising that all of the tested PGPR strains used in the present study were antagonistic to the fungal pathogens. Production of bioactive compounds and or other anti-fungal metabolites might have contributed to the high level of antagonism that we observed in our study. The study and characterization of these bioactive compounds might lead to a discovery of novel compounds which can be used by forest product companies to control the growth of blue stain and other possible fungi that damage wood products.

This is the first study to show the *in vitro* antibiosis of blue-stain fungi by strains of *B. velezensis*. The dual agar plate study provides a very rapid assay to improve understanding of the antagonistic activity of the PGPR strains. However, there are cases where the *in vitro* and *plant*a studies have been correlated (Gupta et al., 2010; Mesanza et al., 2016) and have not been correlated (Ran et al., 2005). The PGPR studied in this study are bacilli. Use of these PGPR may be beneficial *in planta* under the adverse climatic condition like drought as bacilli can form dormant endospores which are tolerant to UV and desiccation (Nicholson, 2002). In the future, the antagonistic ability of rhizobacteria should be tested both *in vitro* and *in planta*.

5.5.2 Induced systemic resistance study

To our knowledge, this is the first study to show certain strains of PGPR can induce systemic resistance of conifer to *L. terebrantis* and *G. huntii*. Furthermore, studied PGPR strains also have the ability to promote *P. taeda* growth. Even though co-inoculation of fungi and PGPR did not induce full resistance against fungal infection in *P. taeda*, lesion length caused by fungi in PGPR treated seedlings were relatively longer than the control (PGPR untreated) seedlings in most cases. Thus, our results are in

agreement with similar studies reporting classical ISR against pine tree pathogens. For example, Enebak and Carey (2000) reported PGPR inoculation reduced that fusiform rust infection caused by the fungus (*Cronartium quercuum* f. sp. *fusiforme*) by 50 % in pine seedlings. Similarly, Iturritxa et al. (2017) and Mesanza et al. (2016) reported *Pseudomonas fluorescens*, *Bacillus simplex* and *Erwinia billingiae* isolated from healthy pine tree promoted the growth and survival of *P. radiata* against *Heterobasidion annosum* and *Armallaria malallea,* and *Fusarium circinatum* respectively.

PGPR inoculated *P. taeda* showed some support for intraspecific variation in plant growth in terms of biomass gain. The tolerant family (T) not only had the ability to tolerate the fungal infection but also had the capacity to grow more (in terms of biomass) than the susceptible family (S). Our results are following Enebak (2005), where he reported that different rhizobacterial strains had the differential efficacy to promote plant growth. Furthermore, he suggested that the effect of rhizobacterial inoculations may be species specific. Our results suggest the effect of rhizobacteria may be intra-specific. Regarding lesion length, PGPR strain *B. pumilus* (INR 7) had the highest efficacy induced plant resistance to the fungal infection. This relatively higher induction efficiency of a particular strain suggests that potential of PGPR for inducing systemic resistance against fungal pathogens is rhizobacterial strain-specific.

The biomass yield of the seedlings inoculated with PGPR was significantly higher in the first replicate of the study. Out results are similar to Shishidio et al. (1996) where they reported that the biomass of spruce seedlings was improved by 18 % when inoculated with rhizobacteria. In the second replicate of the study, the biomass yield of the PGPR inoculated seedlings was still higher than control seedlings even though non-

significant. *Pinus taeda* were in a less active growth phase during the second round of study than first (Stephen and Paine, 1985). The difference in plant development period might have caused the differences in seedling biomass gain between two replicates.

One major impediment we faced while performing the experiment was selecting the best way to measure and rate disease. The tested PGPR strains caused some tolerance but not total disease resistance. Hence, we measured lesion size following pathogen inoculation and compared between the PGPR-treated and the control seedlings. Total biomass gain by the seedling was used as a measure to determine seedling growth.

In a forest ecosystem, soil microorganisms play a vital role in nutrient cycling, plant growth regulation (Hart et al., 1994) and soil quality maintenance (Jeffries et al. 2003). Fungi and the bacteria are the major components of the rhizosphere microflora (Chanway, 1996). Rhizosphere soil contains 10-100 times higher bacterial population than that of the non-rhizosphere soil (Rouatt and Katznelson, 1961). Among those many microbes in the soil they may be positively impacting the health of the trees. Therefore, the study of the rhizobacterial community differences in symptomatic and asymptomatic *P. taeda* stands would be a promising method to understand and identify the rhizobacterial strains involved in promoting tree health. Also, the study of the plant growth promotion by mycorrhizal fungi can be equally exciting. Finally, rhizobacteria have the ability to promote *P. taeda* growth and reduce the infection of blue stain fungi. Thus, future studies should focus on identifying those antimicrobials and the secondary metabolites of PGPR which inhibited the fungal growth. Also, the study of the various molecular pathways involved in the induction of the resistance will provide the greater

insights in the understanding of the induced systemic resistance of *P. taeda* to ophiostomatoid fungi.

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CHAPTER VI

Intra-specific variation in virulence and effect of inoculum density of *Leptographium terebrantis* to *Pinus taeda* L.

6.1 Abstract

Leptographium terebrantis, a pathogenic, root-feeding bark-beetle vectored fungi, causes root infection and blue-staining in conifers. Intra-specific virulence and impact of different inoculum densities of *L. terebrantis* on *P. taeda* are unknown. This study determined the intraspecific variation in virulence of *L. terebrantis* to *P. taeda* and examined the growth ability of relatively virulent *L. terebrantis* at different inoculum densities. Forty-two, *L. terebrantis* isolates were used to inoculate *P. taeda* seedling stems, and the developed lesion was studied. Toothpicks cultured with relatively virulent *L. terebrantis* were inoculated into *P. taeda* stem segments at different densities, and blue-stain caused by fungi was measured after 8 weeks. The results suggest virulence of *L. terebrantis* varies among isolates. At higher inoculation density, *L. terebrantis* have higher growth and staining potential which may negatively impact *P. taeda*.

6.2 Introduction

Leptographium species are distributed worldwide with most species as inhabitants of conifers (Jacobs and Wingfield, 2001). These fungi are more common in the southern U.S. where conifer are the major species. *Leptographium procerum* (W.B. Kendr.) M.J.

Wingf and *L. terebrantis* S.J. Barras and T.J. Perry are among the root-feeding bark beetle vectored *Leptographium* species, frequently isolated from roots of declining *Pinus taeda* L. (loblolly pine) in the southern U.S. (Eckhardt et al., 2004). *Leptographium terebrantis* is relatively more pathogenic blue-staining and root-infecting fungi (Singh et al., 2014).

Leptographium terebrantis causes lesions in the phloem and resin flow in the xylem in wound-inoculated seedlings and mature trees of several conifers (Klepzig et al. 1996). Although the pathogenicity of *L. terebrantis* on loblolly pine is relatively well established in seedlings and young trees (Eckhardt et al., 2004; Matusick et al., 2016, Matusick and Eckhardt, 2010; Nevill et al., 1995; Singh et al., 2014), its ability to grow in and cause occlusion in *P. taeda* wood and trees at different inoculation densities is unknown.

Following invasion in wood, the blue-stain fungi initially grow in ray cells in the sapwood and subsequently start to proliferate in ray parenchyma cells. Fungal hyphae enter the tracheid by forming a penetration peg. Bordered pit cells facilitate the tracheid to tracheid movement, leading to the longitudinal movement of the fungus (Bergvinson and Borden, 1992). Movement of fungi in the tracheid blocks the fundamental water conducting system in the plant. Resin spillage from the resin duct epithelial cells destroyed by the fungus restricts the further movement of water (Ballard et al., 1982).

The potential damage by beetle vectored fungus depends upon the inoculation density and host tree susceptibility (Croise et al., 1998). The inoculum density (the number of the fungal inoculum per unit), plays a significant role in determining the

impact of the blue-stain fungi (Horntvedt et al., 1983; Solheim, 1992). Mass inoculation loads have been found to cause the sudden decline of tree health (Christiansen, 1985; Horntvedt et al., 1983; Solheim and Krokene., 1988). However, there have been no studies which can relate the radial inoculum density to the blockage of the vascular tissue.

The high frequency of *L. terebrantis* isolation from *Pinus* forests (Eckhardt et al., 2007) shows that the fungus will have the potential to become a devastating pathogen in the future. However, is it still unclear whether all the isolates have equal potential to impact the health of the tree? Also, in an ecological scenario, mass inoculation of fungi is more likely. Moreover, it is important to understand the growing ability of blue-stain fungus in the absence of the host-tree response before any conclusion can be drawn. Thus, to get better insights about virulence and pathogenicity of *L. terebrantis* we will try to answer following questions: (i) Is there an intra-specific variation in virulence of *L. terebrantis*? and (ii) How does *L. terebrantis* grow at different inoculum densities?

6.3 Material and methods

6.3.1 *Leptographium terebrantis* pathogenicity testing

An artificial stem inoculation study was performed in *P. taeda* seedlings from a single family. In January of 2015, a total of 820 one-year-old bare-root *P. taeda* seedlings were lifted from Rayonier and were re-planted in one-gallon pots with ProMix BX® (Premier Tech, Quebec, and Canada) peat-based potting media. The re-planted seedlings were placed in an outdoor research facility at Auburn University, allowed to grow under natural conditions (Figure 6.1), and watered when required. Seedling height and root-

collar diameter (RCD) were measured before the fungal inoculation and final measurements.

Figure 6.1 *Pinus taeda* seedlings in randomized controlled design in an outdoor space.

The experiment was designed as randomized complete block design with 19 seedlings randomly chosen for treatment with an isolate of *L. terebrantis* forty-two isolates were maintained at 4 °C in Forest Health Dynamics Laboratory, Auburn University, Auburn, AL, U.S. These isolates were isolated from the roots of declining *Pinus* stands throughout the southern U.S. (Table S9; Eckhardt et al., 2007). Fungal isolates were plated on Malt Extract Agar (MEA) plates and incubated at room temperature for 14 days before inoculation in seedlings.

A 1-cm vertical wound 2 cm above the root-collar area was created in each seedling, and 3 mm agar plug with actively growing fungi were placed (fungus-side-down). Control seedlings were inoculated with sterile agar. The inoculation point was covered with a moist cotton ball to prevent desiccation of agar media and gently wrapped with Parafilm® to prevent further contamination. After 8 weeks, seedlings were cut above the soil line and placed in stain (FastGreen FCF; Sigma Chemical Co., St. Louis, MO, USA) and distilled water mixed in a ratio of 0.25 g L^{-1} for 48 h to allow the dye to move through them. The bark around the inoculation point was then carefully scraped, and the dark necrotic tissue was measured as the lesion. The vertical portion of the seedling stem that did not allow the capillary movement of dye was recorded as occlusion length. The length and width of the lesion were also measured.

6.3.2 ***Leptographium terebrantis*** **inoculations in** ***P. taeda*** **stem sections (blots)**

6.3.2.1 Wood segment preparation

Seven, five-year-old, *P. taeda* saplings with no above-ground signs and symptoms of disease were selected from naturally regenerating forest in Auburn, Alabama, U.S.,

from which 50 segments of 15.24 cm length were cut. These wood segments were transported to the lab on the ice box, and both ends of segments were sealed by dipping in the melted wax within an hour to prevent the moisture loss and stored at 8º C until used.

6.3.2.2 Preparation of culture

Sterilized wooden toothpicks were used for inoculation. Approximately 900 toothpicks were soaked in Malt Extract Broth (MEB) overnight in a sterile environment and broth was drained the next day while other 900 were not soaked (Figure 6.2). Fifteen toothpicks either soaked or un-soaked in MEB were aligned in petri plate with MEA covering half of the plate (Figure 6.3) in such a way that half of each toothpick touched the media whereas the other half did not. Then a plug of the *L. terebrantis* was placed in the plate and incubated at 25 ºC in the dark for 14 days. The *L. terebrantis* isolate used in the study was the most virulent among the isolates screened in the seedling inoculation study described earlier.

Figure 6.2 Malt Extract Broth soaked and un-soaked sterile toothpicks.

Figure 6.3 Wooden toothpicks placed into the MEA plate.

6.3.2.3 Inoculation of *Pinus taeda* stem segments

A 0.15-mm diameter drill bit was used to drill a 5-mm deep hole in the wood segments. The holes in the segments were then inoculated with toothpicks colonized by fungi. The number and the distance between the holes determined the density of inoculation treatment. The inoculation points were (i) 1.27 cm apart sideways and parallel, (ii) 1.27 cm apart in a single line, (iii) 2.54 cm in a parallel line, and (iv) 2.54 cm apart in single line (Figure 6.4). There were 6 replicates (stem segments) of each treatment (2 replicates for control) for broth soaked and un-soaked toothpicks. Controls segments received sterile toothpicks without any fungus. The inoculated stem segments were then kept in bins with sterilized sand under the shade. (Figure 6.5). The top of the containers was covered with a moist sterile cheese cloth to prevent moisture loss.

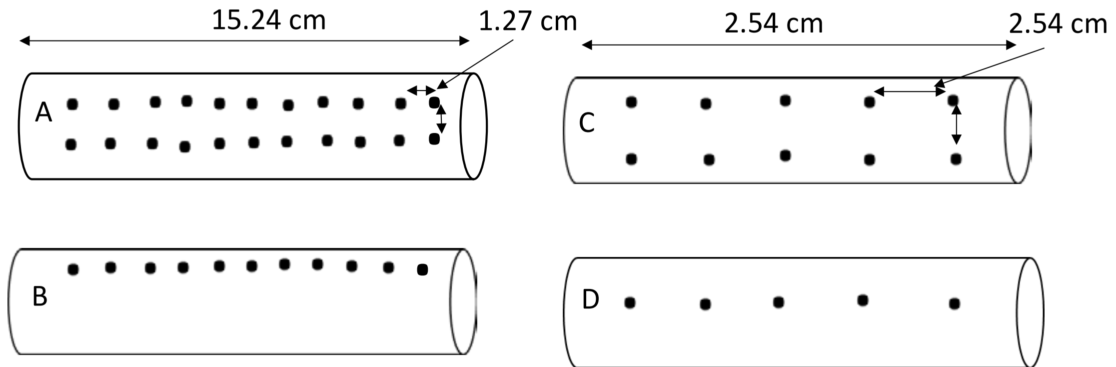

Figure 6.4 *Pinus taeda* stem segments showing different inoculation points for inoculation of *Leptographium tererantis.* A: 1.27 cm apart sideways and parallel, B: 1.27 cm apart in a single line, C: 2.54 cm in a double line and D: 2.54 cm in a single line.

Figure 6.5 Inoculated stem segments kept in a container with sterile sand.

6.3.2.4 Laboratory Measurement

After 8 weeks, smaller circular segments of wood (wooden cookies) were made by cutting at the center of each inoculation point with a band saw to observe fungal growth (Figure 6.6). The area of the fungal stains at a cross-section of each wooden cookie was traced on a transparent sheet and measured using a Lasico® Planimeter

(Lasico®, Los Angeles, CA, USA). The total volume of each wood segment was calculated using formula (Vtotal = $\pi r^2 h$). The volume of the entire stained area in each bole was calculated using the formula (Vstained = S x L where, S = average stained area of each cross section and L = total length of the bole). The total percentage stains in each bole was calculated using formula (%stain = $SL/\pi r^2 h$ x 100).

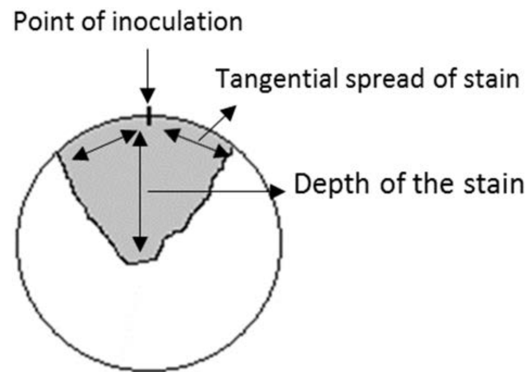

Figure 6.6 Cross-section of the stem segment showing point of inoculation, tangential spread, and depth of the stain.

6.3.3 Analysis

Data were analyzed using a general linear model (GLM) in SAS statistical software (SAS Institute, 9.4 versions, Cary, NC). Data were first checked for normality, and equal variance and transformations were done using reciprocal transformation for occlusion from stem segments. Bolt diameter was used as a covariate in the model. Pair-wise comparisons were undertaken using the Post Hoc Tukey's test on the four fungal treatments at $\alpha = 0.05$.

6.4 Results

6.4.1 *Leptographium terebrantis* pathogenicity

All *L. terebrantis* isolates caused lesions and occlusions in all of the inoculated seedlings. Different isolates of *L. terebrantis* caused significantly different size of lesion ($F_{(42, 773)} = 7.22$, $P = <0.0001$). The lesion depth was also different among the treatments ($F_{(42, 773)} = 2.80$, $P = <0.0001$). Occlusion length and depth was significantly different between the isolates ($F_{(42,773)} = 5.83$, $P = <0.0001$; $F_{(42,772)} = 3.99$, $P = <0.0001$).

Among the 42 isolates of *L. terebrantis,* 41 isolates had significantly longer lesion length than the control treatment. All the isolates had longer occlusion length than the control. Isolate MYA-3316 had the highest mean lesion length and occlusion length (Figure 6.7). The lesion caused by this isolate was 4.77 (± 1.14) mm longer than that resulting from the control ($P = <0.0001$) (Table S10). Fungal isolate R-00-44-ss6 resulted in 2.39 (± 1.14) mm longer lesion than the control ($P = <0.0001$).

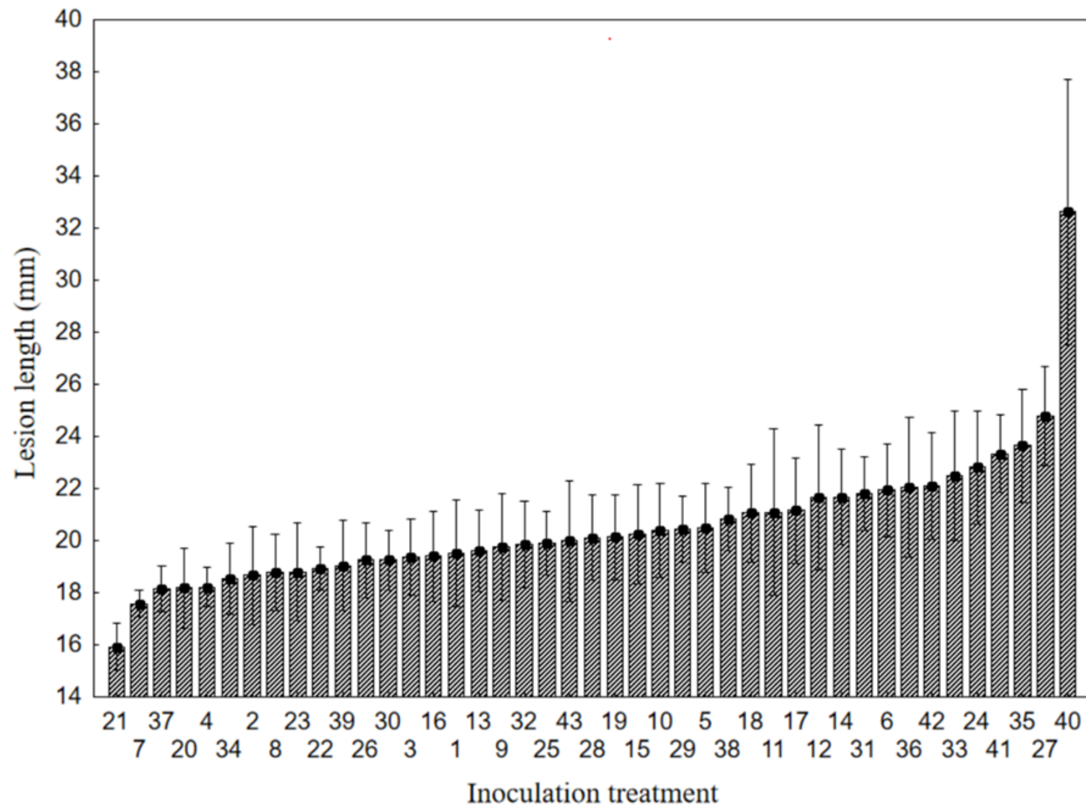

Figure 6.7 The mean lesion length produced in *Pinus taeda* seedlings following inoculation with various *Leptographium terebrantis* isolates. Error bars indicate 95% confidence intervals at $\alpha = 0.05$.

6.4.2 Growth of *L. terebrantis* in *P. taeda* segments

Dark blue pie-shaped staining was observed in stem segments, eight weeks following inoculation with *L. terebrantis* isolates. Any signs of damage and fungal staining were not observed in control stem segments inoculated with sterile toothpicks. The stem diameter had no significant effect on the stain development, but including it in the model increased our model performance (Table 6.1). The staining produced by the soaked and un-soaked toothpick was significantly different ($F_{(1, 39)} = 6.72$, $P = 0.0009$) as shown in Table 6.2 and Figure 6.8.

Figure 6.8 **A** Cutting of the bolts with the band saw **B** Development of the fungal stain in different treatments **C** Staining developed in soaked 2.54 cm treatment.

Table 6.1 The effects of different dependent variables for percentage of fungal staining (N = 48).

Source	DF	F value	Pr > F
Diameter	1	1.06	0.3103
Media	1	10.24	0.0027
Treatment	3	6.72	0.0009
Media*Treatment	3	0.24	0.8707

(Note: Treatment: different inoculum load; Media: soaked or un-soaked).

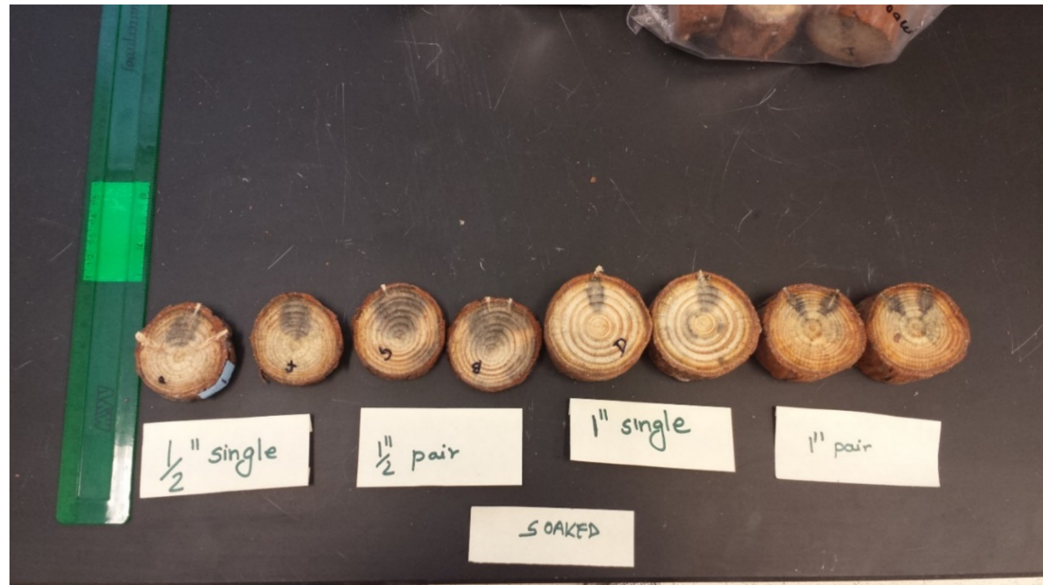

Figure 6.9 Cross-section of wood stained with *Leptographium terebrantis* at different inoculation treatments.

The toothpicks soaked in MEB caused 2.81% more staining than the un-soaked toothpick (P = 0.0027) (Figure 6.10). When the toothpicks were 1.27 cm apart in a double line, then they caused 4.73 % more staining than that caused by the lowest load of inoculation (i.e. 2.54 cm in a single line) (Figure 6.9). There was 100 % occlusion in some of the stem segments with 1.27 cm apart inoculation points in a double line.

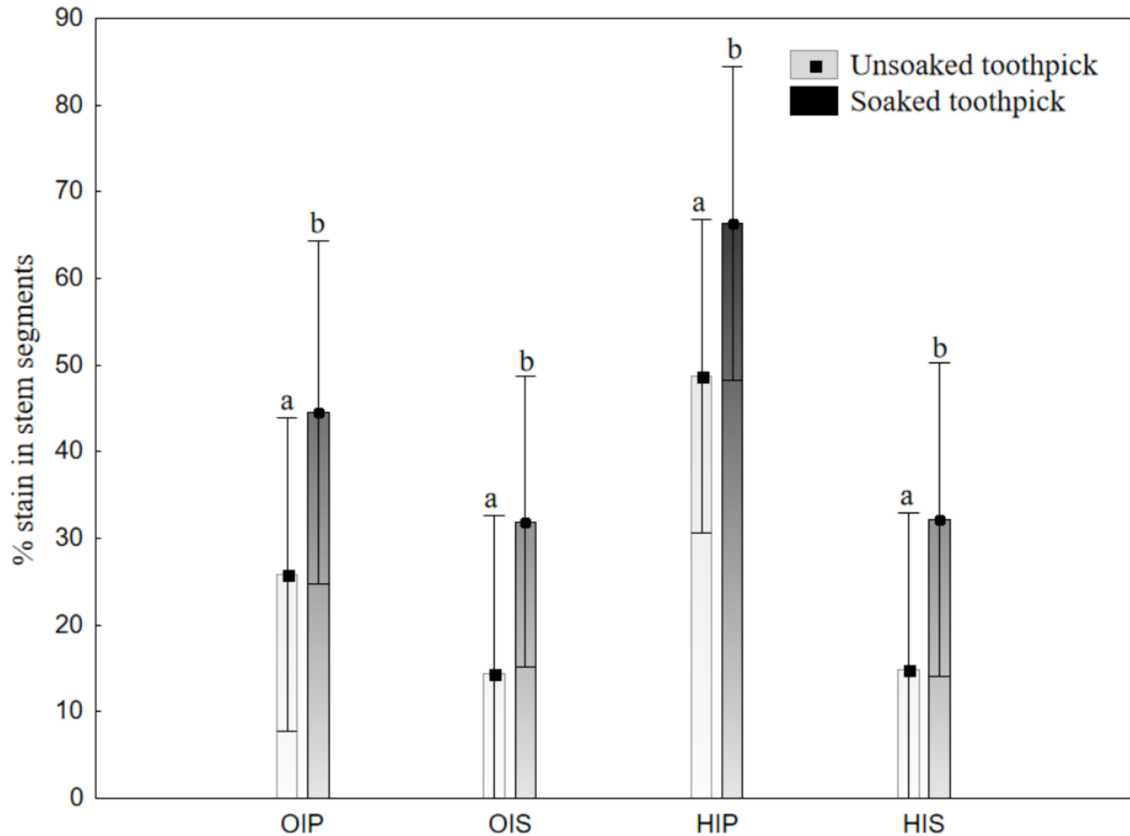

Figure 6.10 The percentage of the total tissue stained by *L. terebrantis* at different inoculum loads in *P. taeda* stem sections. Different letters indicate significant differences at α = 0.05. (OIP: 2.54 cm in double line, OIS: 2.54 in single line, HIP: 1.27 cm apart in double line, and HIS: 1.27 cm apart in single line).

Table 6.2 Estimation of the difference in staining caused by various inoculum load in *Pinus taeda* stem segments.

Parameter	Log (Estimate) stain %	Standard error	T-value	Pr > |t|
So vs Unso	-0.281	0.088	-3.2	0.0027
HIP vs HIS	-0.465	0.125	-3.71	0.0006
HIP vs OIP	-0.252	0.132	-1.91	0.0639
HIP vs OIS	-0.473	0.120	-3.95	0.0003
HIS vs OIP	0.212	0.126	1.68	0.1003
HIS vs OIS	-0.009	0.123	-0.07	0.9447
OIP vs OIS	-0.221	0.130	-1.7	0.0963

(Notes: So: Toothpicks soaked in broth, Unso: Toothpicks un-soaked in broth, HIP: 1.27 cm apart in a double line, HIS: 1.27 cm apart in a single line, OIP: 2.54 cm in double line, and OIS: 2.54 cm in a single line).

6.5 Discussion

Forty-two *Leptographium terebrantis* isolates showed intraspecific variation in virulence. Different isolates can act as relatively more or less virulent pathogens on a single family of *P. taeda*. Similar results were obtained by Parmeter et al. (1989) when they inoculated immature *Pinus ponderosa* Laws. with two different isolates of *L. terebrantis*. Our findings are also supported by Lieutier et al. (2004) where they found variation in the growth virulence of *L. wingfieldii* Morelet. in *P. sylvestris* L. (scots pine). In contrast, Wingfield (1986) did not find any significant differences in the virulence of different *L. procerum* isolates in *P. strobus* L. (white pine) seedlings. Also, Swedjemark et al. (1999) did not found any evidence of intraspecific variation in growth of *Heterobasidion annosum* (Fr.) Bref. in *Picea abies* (L.)H. Karst. (Norway spruce) and *P. sylvestris*. Thus, these previous studies and present study taken together suggests that intraspecific variation in virulence of the fungi pathogenic to forest trees may be isolate specific or host specific.

Mortality of *P. taeda* seedlings were not observed during this study as had been observed in other pathogenicity studies (Harrington and Cobb, 1983; Parmeter et al. 1989; Eckhardt et al., 2004; Singh et al., 2014). Pathogenicity and virulence of fungal inoculants to *Pinus* species are usually assessed based on lesion length and area caused by the fungus (Nevill et al., 1995; Parmeter et al., 1989). The isolate *L. terebrantis* (ATCC accession no MYA – 3316) caused the longest lesion and occlusion. The longer lesion denotes the higher virulence of some fungi as compared to the other fungal isolates. With the same level of defense by *P. taeda***,** relatively more virulent fungal

isolates were able to cause varying lengths of necrotic lesions and blockage of the water conducting vascular tissue.

The vertical length of the inoculation points did not cause a difference in fungal growth. There was no variation in the fungal spread when the inoculum load was 1.27 cm and 2.54 cm in single lines. This suggests that the frequency of inoculation points at the radial cross-section plays a significant role in the movement of fungi but not the frequency of the vertical inoculation. The blue-staining fungi move through the ray parenchyma cells and grow substantially within ray cells and move to the tracheid through the bordered pits (Ballard et al., 1982; Ballardt et al., 1984). Therefore, when the two inoculation points were near, they merged with each other and expanded the dark-blue-stain. Thus, inoculation points at least 1.27 cm vertically and longitudinally apart from each other is suggested for future artificial inoculation studies in *P. taeda* trees to understand the impact of a high inoculum load.

The results suggest that observed fungal movement might be associated with embolism formation in living conifer hosts and it might become more severe at higher loads of *L. terebrantis* inoculation. Tissue embolism decreases the hydraulic conductance and concomitantly disturbs the water balance in the plant (Mayr et al., 2014). For example, sap-fungi inoculation in an embolized drought stressed trees might cause complete hydraulic failure and plant mortality (Oliva et al., 2014). As bark beetles usually attack and inocualte fungi in previously stressed tree (Raffa et al., 1993), the results of the fungal inoculation on an ecological scale might be more detrimental to the host tree. Future studies should be focused on the consequences of high inoculum loads

of *L. terebrantis* on whole tree physiology to quantify the impact caused by the fungi in *P. taeda* health.

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CHAPTER VII

Growth and Staining Ability of Various Blue-Staining Fungi in *Pinus taeda* L. Stem Segments

7.1 Abstract

Blue-staining of conifer wood is caused by various ophiostomatoid fungi and is a major problem in the forest products industry. Toothpicks cultured with *Leptographium terebrantis*, *Grosmannia huntii,* and *G. alacris* were inoculated in loblolly pine stem segments. Growth potential (in terms of stain area) were determined eight weeks after inoculation. *Leptographium terebrantis* had relatively higher growth potential than *G. alacris* and *G. huntii*. Results indicate that wood staining by ophiostomatoid fungi is species dependent.

7.2 Introduction

Discoloration of conifer sapwood by the presence of certain fungal hyphae results in blue-stain (Seifert et al., 1993). The fungi of the genus *Ceratocystis*, *Ophiostoma*, *Leptographium,* and *Grosmannia* species are associated with the blue-staining of lumber (Kirisits, 2007). Staining and discoloration of the wood caused by the blue-staining fungi decrease the value and the aesthetic quality of the wood. Although the wood strength is little affected, the discoloration is of a significant problem in the paper and pulp industry as additional treatment is required for stained

wood (Behrendt et al., 1995). Apart from their staining ability some species of ophiostomatoid fungi can be pathogenic to the host (Matusick et al., 2010). Information regarding the staining abilities of these fungi on important *Pinus* species, *P. taeda* is limited in literature.

The blue stain is the result of melanin production, a secondary metabolite of fungal growth, is an important biological pigment (Zimmerman et al., 1995). This pigment aids in fungal penetration into the host by helping in appressoria formation and perithecia formation (Zimmermann et al., 1995). Melanization may also help in adaptation of the organism to changing climate such as increasing temperature and drought (Roulin, 2014). It may confer UV resistance and desiccation (Bell and Wheeler, 1986). Thus, melanization in blue stain fungi may provide it with adaptation mechanisms even in adverse climatic conditions.

Bark beetle associates vector blue-staining fungi into trees (Paine et al., 1997). Initial fungal growth occurs in the sapwood rays. The hyphae then penetrate to the cell wall and subsequently ray parenchyma cell where it grows rapidly. It can form an appressorium-like structure with a penetration peg which can rupture the cell wall and move to the adjacent cells. Finally, fungal hyphae enter the tracheid by penetrating the bordered pit cells. Infrequent branching and longitudinal growth of the hyphae causes further proliferation of the fungi (Ballard et al., 1984).

The hypersensitive responses such as lesion and occlusion following inoculation of *G. huntii*, *G. alacris*, *L. terebrantis* and *L. procerum* into roots and stems of living *Pinus* trees and seedlings has been studied (Matusick and Eckhardt, 2010, Singh et al.,

2014). The lesion and occlusion are the responses produced by the tree after the fungal inoculation which does not necessarily provide an idea about how far fungi can grow. Thus, the staining or the growth potential of the various blue-stain fungus has been a question of much speculation. A simple study was designed to mimic the artificial inoculation of the different blue-staining fungi by bark-beetles. This study seeks to determine the growth ability of the three different blue stain fungi: *L. terebrantis*, *G. huntii* and *G. alacris* in loblolly pine stem segments.

7.3 Materials and methods

7.3.1 Wood section preparation

Seven, five-year-old, *P. taeda* saplings with no visible signs and symptoms of disease were selected from a naturally regenerating forest near Auburn, Alabama, U.S. were cut into 15.24 cm long, 20 stem segments. Stem segments were kept on the ice and transported to the lab. Further, the exposed ends of the segments were sealed by dipping in the melted wax within an hour to prevent the moisture loss and stored in a cooler at 8º C until used.

7.3.2 Preparation of culture

Sterilized wooden toothpicks were soaked in Malt Extract Broth (MEB) overnight in a sterile environment and drained the next day. Petri plates with Malt Extract Agar (MEA) covering only half of the plate were prepared and a plug of agar with *L. terebrantis* (ATCC MYA-3316) was placed in the agar medium. The toothpicks were aligned so that half of each toothpick touched the media whereas the other half did

not. This allowed the growth and sporulation of the fungi on the exposed ends of toothpicks. The culture plates were incubated for 14 days at 25ºC in the dark.

7.3.3 Inoculation of *P. taeda* bolts

Four holes, 2.54 cm apart and 5-mm deep were drilled with 0.15-mm diameter drill bit. The holes in *P. taeda* bolts were then inoculated with fungal cultured toothpicks. The stem segments were kept in a bin containing sterile sand. The top of the bin was covered with a sterile cheesecloth to prevent the moisture loss.

Figure 7.1 *Pinus taeda* bolts with inoculation holes for inoculation of fungal cultured toothpicks.

7.3.4 Laboratory Measurement

Eight weeks after inoculation, wood segments were cut at each inoculation point with a band saw to observe the staining caused by fungi. Then the area of the fungal staining and cross-section of the wooden cookies were traced on a transparent sheet and measured using a Lasico® Planimeter (Lasico®, Los Angeles, CA, USA). The total

percentage of the area stained by the fungi at the cross-section at the point of toothpick inoculation was calculated. Also, the depth of the fungal staining below the inoculation points was calculated. The estimates of the effect caused by three different fungi were analyzed using the general linear model in R 3.3.1. Estimates of effects were analyzed at $\alpha = 0.05$.

7.4 Results

The inoculation of three fungi led to the inward spread of the blue-staining fungi. The staining was dark-blue and pie-shaped (Figure 7.2). The wood segments receiving the control toothpicks did not develop blue stain, so they were removed from the further analysis.

Figure 7.2 Blue-stain caused by various fungi in the stem segments.

The staining in the cross section varied significantly among the fungi ($F_{(2, 69)}$ = 5.06, *P* = 0.0089). The cross-section area stained by *L. terebrantis* was 1.69 cm^2 (±1.23,

95 % CI) higher than that by *G. alacris, P* = 0.00808, R^2 = 0.11. Similarly, the area stained caused by the *G. huntii* was 0.16 cm^2 (± 1.24, 95% CI) lesser than that of the *G. alacris*. However, it was not statistically significant (*P* = 0.80). Similarly, fungi *L. terebrantis* caused 1.85 (± 1.24, 95 % CI) cm^2 higher staining than that of *G. huntii*.

The depth of the staining in the cross section was also significantly different among the fungi ($F_{(2, 69)}$ = 2.33, *P* = 0.0375, R^2 = 0.11) (Figure 7.3). The depth of the stain caused by the fungi *L. terebrantis* was 3.73 (± 3.62, 95 % CI) cm^2 higher than that caused by *G. alacris* (*P* = 0.04386). The depth of the fungal staining caused by *G. huntii* was 1.60 (± 3.62, 95 % CI) cm^2 higher than that caused by *G. alacris* (*P* = 0.3796). Similarly, the depth of the fungal staining caused by *L. terebrantis* was 5.37 (± 3.62, 95 % CI) cm^2 higher than that caused by *G. huntii* (*P* = 0.00447).

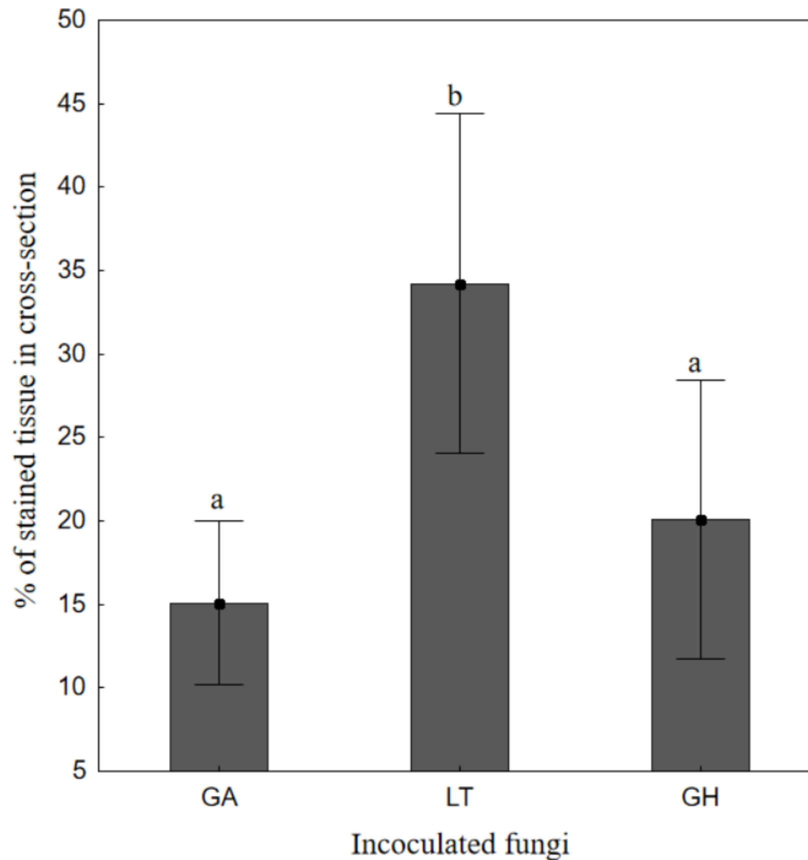

Figure 7.3 Percentage of the stain area in the cross-section caused by three fungi. (GA: *Grosmannia alacris*, LT: *Leptographium terebrantis*, GH: *Grosmannia huntii*). Different letters denote significant differences at α = 0.05.

With 1 cm^2 increase in the cross-sectional area the stain increased by 0.21 (± 0.14, 95 % CI), $P = 0.0047$, $R^2 = 0.27$ when treated with *G. alacris*. We observed that when cross sectional area of bolt increased by 1 cm^2 then the stain caused by *G. huntii* decreased by 0.15 (± 0.35, 95 % CI), $P = 0.3812$, $R^2 = 0.04$. We found that with 1 cm^2 increase in the cross-sectional area of the blot the stain caused by *L. terebrantis* decreased by -0.14 (± 0.44, 95 % CI), $P = 0.34$, $R^2 = 0.02$.

7.5 Discussion

There was variation in the staining ability of various blue-staining fungi. *Leptographium terebrantis* had the highest blue staining ability among the three fungi tested. Yang (2001) reported similar findings by evaluating the growth of 20 isolates of sap-staining fungi on *P. banksiana* Lamb. log sections. Similarly, Matusick and Eckhardt (2010) also found that *L. terebrantis* can cause relatively higher sapwood occlusions than *G. huntii*, *L. procerum* and *G. alacris* in roots of *P. palustris* Mill. These differences in staining ability might be due to the variation in virulence of the fungi.

Leptographium terebrantis had the ability to stain the wood surface up to the deeper levels. Singh et al. (2014) also found that *L. terebrantis* was relatively virulent than *G. huntii* when inoculated in the *P. taeda* seedlings. This also reflects that fungi can go deeper into the vascular tissues and stain the wood and block the vascular tissues thus affecting the whole plant physiology.

The differential ability of different blue-staining fungi to grow and stain wood tissue may depend on the fungal melanin production pathways (Wang and Breuil, 2002). Melanized appressorium is necessary for the cell to cell movement. The relatively higher level of blue-stain caused by *L. terebrantis* suggests that melanization occurs faster in this fungi. More rapid melanization may allow this fungus to quickly adapt to the changing climate (Roulin, 2014). However, much less is known about the role of the melanin in the pathogenesis of these blue-staining fungi. It is possible that the melanin biosynthesis pathways in the three fungi we studied are different. Further studies should focus on the mechanisms of melanin biosynthesis pathways and blue-

stain development. Also, the study of wood anatomy associated with the fungal invasion and the fungal spread in tracheids of *P. taeda* might give a better insight of the mechanism behind the different rates of movement of the three different fungi. Furthermore, studying the fungal growth phase in which fungal pigments are produced would be an important step towards preventing the stain caused by the blue stain fungi.

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CHAPTER VIII
General Conclusions

8.1 Ophiostomatoid fungi

Ophiostomatoid fungi, include several primary tree pathogens that cause blue-staining in wood (Repe and Jurc, 2010). They have been widely reported from around the world (Jacobs and Wingfield, 2001), but more specifically in the southern U.S., they are associated with pine decline (PD) (Eckhardt et al., 2007). The important fungal genera involved in the decline are *Leptographium*, and *Grosmannia* species (Eckhardt et al., 2007). They have sticky conidia at the top of the long conidiophore which is ideal for attachment and the dispersal by bark beetles (Jacobs and Wingfield, 2001).

8.2 The decline of pine in the southern U.S.

Pine Decline has been regarded as a threat to the sustainable forest production and management in the southern U.S. The decline was first noticed and documented in 1959 in *P. taeda* forest in Talladega National Forest in Alabama (Brown and McDowell, 1968). Then increased incidences of the decline occurred in the 1970s and 2000s. Initially, *Phytopthora cinnamomi* Rands. was thought to be involved in the decline. However, further researches reported that root-feeding bark beetles and the related ophiostomatoid fungi were found to be associated with the decline (Eckhardt et al., 2007).

The symptoms of the decline are short and chlorotic needles, reduction in radial growth, deterioration of the fine and the lateral roots and finally decline and the mortality of the trees (Brown and McDowell, 1968; Eckhardt et al., 2007). Manion (1981), gave the death spiral theory which can be used to understand the process of the decline. Contributing factors, predisposing factors, and the inciting factors are involved in this decline process. The predisposing factors are topography, drought, increased slope and southwest facing aspects and the inciting factors are high ozone, increased soil moisture, and mechanical damage, stand density, and the contributing factors are high insect population and the fungi carried by the insects. Thus, pathogens play major role at the end and cause the decline of the trees (Eckhardt et al., 2007).

8.3 Current understanding

Leptographium terbrantis and *G. huntii* are the most common pathogens of *Pinus species. Pinus taeda,* the most common timber species in the southern U.S. is susceptible to these fungi. These fungi have been associated with pine decline which is a threat to forest production and management in the southern U.S. Despite the threat, little has been done to understand the fungal pathogenicity and host response. Artificial inoculation studies have provided great insights to our understanding. Trees from various stages such as seedling, sapling, and mature trees have been used for inoculation (Matusick and Eckhardt, 2010; Singh et al., 2014). The inoculation of primary lateral roots of *P. taeda* with the ophiostomatoid fungi can be considered most promising as it mimics the natural inoculation of the ophiostomatoid fungi by root

feeding bark beetles. Measurement of the necrotic area following the fungal inoculation has been useful to evaluate the virulence and pathogenicity of the fungi.

8.4 Approaches to improve understanding

This dissertation focuses on improving the previous understanding of the fungi associated with pine decline. The central hypotheses of this dissertation are: (i) there is intra-specific variation in tolerance of *P. taeda* families to *L. terbrantis* and *G. huntii* with some family being more tolerant and some being more susceptible (ii) tolerance to those fungi is the inherent property of *P. taeda* family independent of the of the tree (iii) pathogenicity of the fungi changes under the soil moisture stress conditions and the (iv) Plant Growth-Promoting Rhizobacteria will inhibit the growth of the fungi and also induces systemic resistance to fungal infection in *P. taeda*, (v) Virulence of *L. terebrantis* varies among different isolates, and (vi) *Leptographium terebrantis* has more staining ability at higher inoculation loads.

Artificial inoculation studies were conducted (Chapter 2-7). In chapters 2, 4, 5 and 6, stems of *P. taeda* seedlings were inoculated with studied fungi. However, in chapter 3, the primary lateral roots of mature *P. taeda* were inoculated. More than 90 families of *P. taeda* were screened for their tolerance to *L. terebrantis* and *G. huntti.* Two families showing tolerant and two showing the susceptibility to the fungi in the seedling screening study were inoculated in the field (Chapter 3). In chapter 4, two loblolly pine families were grown under different moisture regimes and inoculated with *G. huntii* and *L. terebrantis*. In chapter 5, fungi and PGPR were inoculated in media plate, and antagonistic activity was measured *in vitro*. Also, in another study fungi were

inoculated in stems of *P. taeda* seedling following the inoculation of the PGPR. In chapter 6, most virulent *L. terebrantis* among 42 isolates of *L. terebrantis* isolated from roots of declining *Pinus* species by Eckhardt (2007) was determined. Most pathogenic *L. terebrantis* was inoculated into *P. taeda* bolts at different inoculum densities.

8.5 Research results

Pinus taeda families vary significantly in tolerance to *L. terebrantis* and *G. huntii*. The lesion length was considered the strongest response variable. Bare-root and container-grown seedlings of the same family behaved similarly with respect to virulence and host resistance. The tolerant families and susceptible families as determined from the seedling inoculation study showed a similar pattern of the response to the fungi indicating that the tolerance of *P. taeda* to *L. terebrantis* and *G. huntii* is a result of family genetics independent of tree age. Also, the production of large brown necrotic lesions in the lateral root following the fungal inoculation suggests that the fungi are root-infecting fungi. Even with a single point inoculation, extended damage was observed in eight weeks.

Pinus taeda pre-exposed to moderate drought were more likely to cause fungal infection than seedling pre-exposed to severe drought following the *L. terebrantis* inoculation. The interaction between fungi and soil moisture was weak. The seedling volume change, dry needle biomass, and dry stem biomass were lower in the seedlings receiving fungal treatments under the drought conditions. The family selected for susceptibility to *L. terebrantis* and *G. huntii* had a bigger infection and had less biomass gain.

In chapter 5, studied PGPR strains had the ability to inhibit the growth of the fungi *in vitro*. These results suggest that these PGPR strains produce some metabolites or antagonistic agents that can inhibit fungal growth. Also, specific strains of PGPR have the ability to induce systemic resistance of *P. taeda* to *L. terebrantis* and *G. huntii*. Studied PGPR strains also promoted the plant growth. Results suggest that specific PGPR strains can be used as biocontrol agents and growth promoters.

In chapter 6, different isolates of *L. terebrantis* had varying virulence. This suggests that in ecological scale more virulent strains might exist. *Leptographium terebrantis* can cause blue-stain in wood tissues following a week after the inoculation. The frequency and distance between the radial inoculation lead to the quicker spread of the fungi following inoculation. *Leptographium terebrantis* causes and extended blue staining of the wood tissues than *G. huntii*, *L. procerum,* and *G. alacris*. Results suggest that *L. terebrantis* can easily move through the bordered pits and may alter the hydraulic conductance of *P. taeda* trees in the field.

8.6 Potential future research

To better understand the host-pathogen interaction, molecular mechanisms involved in the interaction should be focused. Since our research is only focused on the phenotypic variations in the tolerance and susceptibility of the *P. taeda* seedlings, exploring the differential expression of genes following the fungal inoculation will give clear insights to our understanding. Continued and long-term monitoring of the seedlings should be performed to understand the effect of different moisture and the fungal treatments. Also, isolation of the rhizobacterial communities and studying their

anti-fungal activity and growth promoting activity would be an appropriate approach for site selection for *P. taeda* plantation. To better understand the impact of *L. terebrantis* on *P. taeda* health long term monitoring of the plant physiological response at different inoculum densities should be performed.

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Appendix

Table S1. Mean lesion length and occlusion length (overall and across each fungus) (2013) (N = 1978).

Fam	Lesion length			Occlusion length		
	Overall mean (mm)	Mean (GH) (mm)	Mean (LT) (mm)	Overall mean (mm)	Mean (GH) (mm)	Mean (LT) (mm)
L05	32.80(12.35)	25.53(4.52)	40.25(13.40)	52.91(26.39)	58.15(46.87)	51.33(17.19)
L09	32.13(15.10)	26.67(8.17)	38.61(18.65)	59.78(42.30)	58.43(48.59)	60.62(39.16)
L16	30.75(12.37)	25.86(6.35)	36.17(15.00)	59.86(38.16)	45.22(17.92)	62.79(40.65)
L38	32.13(16.09)	25.38(5.79)	39.22(20.05)	51.70(23.76)	37.319(15.65)	58.10(24.16)
L49	29.98(11.65)	26.41(5.78)	33.55(14.67)	51.32(26.11)	49.23(30.97)	51.86(25.34)
L50	29.93(11.59)	24.63(5.83)	36.12(13.50)	48.85(17.41)	41.21(10.32)	52.28(19.02)
L51	29.24(13.30)	23.39(4.77)	36.38(16.61)	50.21(32.01)	31.76(6.88)	61.28(36.02)
L52	27.64(8.30)	24.65(9.02)	30.57(6.37)	42.97(14.82)	34.00(11.06)	47.65(14.55)
L53	29.39(9.53)	24.88(5.22)	34.53(10.73)	55.69(36.72)	36.31(10.36)	58.67(38.49)
L54	31.90(15.30)	26.31(6.79)	39.07(19.74)	55.90(28.37)	48.12(28.50)	60.65(28.02)
L55	33.48(12.68)	28.83(11.54)	38.02(12.20)	52.18(30.62)	46.27(35.62)	55.94(26.88)
L56	34.39(14.32)	27.54(11.49)	41.41(13.62)	72.58(47.22)	60.53(34.70)	77.88(51.52)
L57	31.31(11.41)	25.40(5.91)	36.94(12.55)	57.31(33.35)	40.61(8.50)	64.73(37.54)
L58	29.81(10.96)	24.689(6.68)	36.21(11.94)	50.56(23.75)	38.02(13.62)	57.45(25.24)
L59	32.02(14.47)	26.22(10.53)	38.35(15.64)	51.41(20.37)	42.89(23.07)	55.46(18.15)
L60	30.45(12.58)	27.03(12.47)	33.78(11.92)	65.57(46.13)	57.76(32.47)	68.58(50.66)
L61	28.48(8.40)	24.18(6.32)	32.47(8.18)	53.23(29.63)	70.75(60.15)	48.69(13.26)
L62	31.47(10.92)	28.11(10.67)	34.91(10.02)	62.96(42.13)	55.00(31.45)	68.53(48.24)
L63	29.95(9.67)	25.99(8.17)	34.24(9.43)	43.72(18.50)	34.40(13.08)	46.32(19.15)
L64	29.89(9.42)	25.90(5.88)	34.29(10.64)	52.39(32.50)	40.49(21.80)	57.84(35.43)
L65	30.53(11.52)	25.33(4.89)	36.01(13.82)	46.80(16.79)	39.62(13.70)	50.11(17.28)
L66	35.84(16.73)	29.27(11.95)	42.09(18.30)	69.37(52.83)	42.31(23.32)	82.89(58.32)
L67	34.80(16.65)	29.57(14.14)	39.67(17.47)	67.83(36.84)	83.96(49.43)	61.15(28.65)
L68	34.88(18.88)	27.67(9.73)	42.45(22.94)	55.32(27.73)	39.02(16.25)	65.42(28.82)
L69	30.86(13.24)	24.22(4.72)	38.22(15.63)	56.27(41.30)	35.95(13.04)	65.65(46.48)
L70	28.48(10.67)	25.68(11.81)	31.14(8.80)	44.84(14.03)	35.07(5.82)	48.66(14.51)
L71	29.18(11.53)	24.93(7.41)	33.77(13.37)	47.69(36.76)	36.52(12.84)	52.61(42.66)
L72	28.61(8.39)	25.06(5.05)	32.24(9.57)	51.09(26.30)	41.53(15.19)	54.28(28.63)
L73	26.30(5.70)	24.00(4.10)	28.35(6.17)	40.30(18.21)	51.72(26.79)	34.59(8.32)
L74	29.96(7.39)	26.53(4.97)	34.61(7.65)	48.72(24.16)	41.03(13.57)	53.72(28.28)
L75	27.68(7.37)	24.35(5.77)	31.27(7.27)	48.76(29.95)	41.12(17.96)	52.58(34.14)
L76	31.02(15.77)	24.82(7.69)	37.38(19.16)	56.69(33.97)	32.68(12.13)	68.18(35.18)
L77	32.90(16.43)	27.71(13.41)	38.49(17.68)	55.98(36.27)	47.82(32.55)	60.40(38.06)

Note: Means followed by the standard deviation in parenthesis, Fam: Family, GH: *Grosmannia huntii*; LT: *Leptographium terebrantis*, W: Wound, and WM: Wound + media.

Table S2. Estimate and rank of lesion length (overall and among the fungus) (2013).

Family	Overall Estimate	Rank	Estimate (LT)	Rank	Estimate (GH)	Rank
L73	-2.93	1	-2.14	1	0.36	27
L52	-1.99	2	-1.47	2	0.27	26
L75	-1.77	3	-1.15	4	0.08	19
L61	-1.59	4	-0.90	6	-0.06	14
L70	-1.55	5	-1.36	3	0.42	29
L72	-1.30	6	-0.91	5	0.12	21
L71	-0.88	7	-0.55	11	0.02	18
L53	-0.65	8	-0.27	13	-0.12	13
L64	-0.60	9	-0.61	10	0.24	24
L49	-0.58	10	-0.76	8	0.41	28
L51	-0.51	11	0.37	21	-0.67	2
L60	-0.48	12	-0.78	7	0.49	30
L63	-0.42	13	-0.37	12	0.12	20
L58	-0.34	14	0.18	18	-0.39	7
L50	-0.32	15	0.20	19	-0.39	6
L65	-0.11	16	-0.02	15	-0.04	16
L57	-0.02	17	0.27	20	-0.28	10
L74	0.00	18	-0.23	14	0.23	23
L76	0.07	19	0.45	22	-0.40	5
L69	0.11	20	0.78	25	-0.71	1
L16	0.18	21	0.11	16	-0.01	17
L62	0.18	22	-0.66	9	0.77	33
L38	0.62	23	0.78	27	-0.41	4
L59	0.77	24	0.78	26	-0.31	9
L54	0.87	25	0.89	29	-0.36	8
L05	1.02	26	1.12	30	-0.50	3
L77	1.16	27	0.53	23	0.17	22
L09	1.24	28	0.81	28	-0.06	15
L55	1.29	29	0.17	17	0.61	32
L56	1.89	30	1.29	32	-0.14	12
L67	1.97	31	0.63	24	0.56	31
L68	2.17	32	1.59	33	-0.28	11
L66	2.48	33	1.25	31	0.26	25

Note: LT: *Leptographium terebrantis*; GH: *Grosmannia huntii*.

Table S3. Re-isolation percentage of various fungi from inoculated seedlings of different *Pinus ta*eda families (2013).

Family	Overall	LT	GH
L05	96	98	93
L09	96	97	92
L16	96	95	95
L38	98	98	95
L49	98	98	95
L50	97	97	95
L51	96	97	93
L52	95	98	91
L53	96	95	95
L54	95	97	93
L55	95	98	90
L56	98	98	95
L57	99	98	98
L58	99	97	98
L59	96	97	92
L60	99	98	98
L61	96	98	93
L62	96	95	95
L63	96	97	93
L64	90	95	84
L65	95	95	93
L66	96	95	95
L67	96	95	95
L68	95	93	95
L69	92	92	90
L70	98	95	98
L71	93	93	91
L72	96	95	95
L73	98	98	95
L74	96	91	98
L75	98	98	95
L76	96	98	93
L77	91	91	90

Note: LT: *Leptographium terebrantis*, GH: *Grosmannia huntii*.

Table S4. Mean lesion and occlusion length produced in the families (overall and among the fungal treatments) (2014).

Family	Lesion length (mm)			Occlusion length (mm)		
	Overall mean	Mean (GH)	Mean(LT)	Overall Mean	Mean (GH)	Mean (LT)
L05	26.91(9.46)	29.86(8.24)	24.03(9.78)	36.17(17.52)	43.85(13.21)	28.66(18.09)
L09	27.45(11.71)	31.24(11.73)	23.83(10.59)	36.63(18.48)	44.79(15.93)	29.01(17.56)
L100	29.62(14.05)	35.44(15.61)	23.49(8.86)	36.59(18.72)	45.27(16.37)	27.44(16.71)
L101	28.15(12.21)	32.41(9.66)	24.31(13.09)	39.66(20.21)	49.18(16.52)	31.07(19.53)
L102	32.44(16.19)	35.55(11.83)	29.33(19.25)	42.02(23.18)	49.55(15.01)	34.49(27.32)
L103	29.18(13.06)	33.55(11.46)	24.7(13.22)	38.14(20.16)	47.59(16.32)	28.42(19.25)
L104	30.99(14.96)	37.47(11.57)	24.11(15.23)	35.96(21.82)	45.36(16.66)	25.31(22.31)
L106	28.65(13.20)	33.5(14.12)	23.91(10.38)	35.20(17.66)	42.61(15.80)	27.97(16.49)
L107	27.47(11.56)	30.36(10.18)	24.64(12.22)	37.24(20.36)	44.54(13.57)	30.12(23.33)
L108	23.07(8.09)	26.14(7.78)	19.85(7.17)	28.51(14.58)	34.87(11.86)	21.83(14.29)
L109	30.41(15.55)	35.24(16.41)	25.58(13.11)	36.10(20.86)	42.61(19.68)	29.15(20.03)
L110	26.42(12.96)	26.89(12.75)	25.77(13.48)	30.66(16.68)	31.36(13.39)	29.69(20.69)
L16	26.53(10.72)	28.77(9.54)	24.28(11.46)	37.06(21.52)	40.08(12.35)	34.03(27.69)
L38	27.89(14.13)	30.25(12.57)	25.25(15.44)	33.24(20.28)	38.39(19.89)	27.44(19.34)
L49	28.83(14.33)	30.11(11.87)	27.55(16.50)	32.25(17.59)	34.19(12.83)	30.32(21.33)
L50	26.81(12.16)	27.29(7.18)	26.38(15.40)	30.20(16.95)	33.58(10.63)	27.18(20.75)
L78	31.92(18.42)	37.49(16.94)	26.65(18.42)	35.44(23.52)	44.03(19.16)	27.54(24.61)
L79	28.96(14.13)	31.71(12.35)	26.07(15.42)	36.97(20.94)	42.64(15.34)	31.02(24.34)
L80	32.31(17.55)	37.07(14.31)	28.02(19.20)	41.49(25.41)	51.14(18.99)	32.54(27.47)
L81	37.39(21.59)	38.67(16.66)	36.11(25.81)	43.78(27.02)	45.85(22.09)	41.55(31.72)
L82	31.81(16.28)	35.84(16.79)	26.95(14.47)	37.61(19.43)	44.29(15.74)	29.54(20.63)
L83	32.44(15.14)	37.61(12.88)	27.51(15.62)	40.68(22.60)	50.74(15.76)	31.11(24.09)
L84	26.98(13.27)	30.70(12.84)	22.99(12.76)	34.69(19.37)	42.08(14.56)	26.46(20.97)
L85	26.47(10.98)	29.49(10.34)	23.13(10.82)	38.11(19.21)	44.25(13.62)	31.31(22.18)
L86	24.93(8.61)	26.13(6.45)	23.67(10.34)	34.36(17.33)	36.94(11.64)	31.67(21.61)
L87	33.01(24.29)	38.34(23.88)	28.08(23.91)	41.87(28.72)	49.56(25.58)	34.94(29.92)
L88	31.81(16.46)	39.31(16.76)	24.12(12.16)	41.70(23.18)	52.11(20.58)	31.04(20.92)
L89	27.37(10.02)	31.60(9.74)	23.23(8.53)	37.03(18.21)	45.15(13.02)	28.92(19.16)
L90	30.56(14.46)	35.07(13.42)	25.70(14.11)	41.28(20.97)	47.73(18.52)	33.94(21.42)
L91	36.72(20.24)	39.80(12.82)	33.73(25.32)	47.79(32.23)	52.90(16.85)	42.83(41.85)
L92	27.87(11.73)	31.28(10.95)	24.26(11.59)	35.75(17.50)	42.75(11.50)	28.34(19.76)
L93	30.75(14.54)	35.57(13.52)	25.68(13.98)	37.22(20.62)	43.69(18.31)	30.59(20.97)
L94	27.13(13.43)	28.98(11.92)	25.27(14.70)	34.00(19.95)	38.92(15.19)	29.09(22.93)
L95	26.02(9.86)	28.72(8.77)	23.18(10.24)	36.13(18.52)	42.79(12.82)	29.13(21.02)
L96	30.40(14.22)	35.71(13.74)	25.22(12.82)	39.45(19.87)	48.97(16.74)	30.16(18.37)
L97	30.01(13.62)	33.67(12.70)	26.45(13.69)	38.67(20.67)	45.79(15.72)	31.56(22.69)
L98	27.52(13.20)	28.02(8.23)	27.00(16.96)	32.31(18.36)	33.14(10.82)	31.42(24.01)
L99	24.87(9.14)	27.97(9.08)	21.76(8.18)	33.75(17.11)	42.41(13.92)	25.08(15.66)

Note: Means followed by the standard deviation in parenthesis.

Table S5. Family estimate and ranking for lesion length (overall and across both the treatments) (year: 2014).

Family	Estimate	Overall rank	Estimate	GH rank	Estimate	LT rank
L108	-4.30	1	-0.66	5	-0.36	6
L86	-3.12	2	-1.00	2	0.27	30
L99	-3.09	3	-0.50	9	-0.23	10
L95	-2.35	4	-0.47	10	-0.08	21
L85	-2.14	5	-0.31	14	-0.20	12
L16	-1.92	6	-0.60	8	0.14	27
L110	-1.88	7	0.39	32	-0.84	4
L05	-1.58	8	-0.38	12	0.00	22
L92	-1.57	9	-0.21	15	-0.16	16
L94	-1.34	10	-0.62	6	0.30	31
L50	-1.28	11	-0.94	3	0.64	35
L89	-1.17	12	0.08	22	-0.36	5
L98	-1.14	13	-1.05	1	0.78	37
L107	-1.13	14	-0.31	13	0.05	24
L84	-0.88	15	-0.09	17	-0.12	20
L09	-0.84	16	-0.03	18	-0.17	13
L38	-0.76	17	-0.41	11	0.23	29
L101	-0.72	18	0.00	19	-0.17	14
L49	-0.38	19	-0.60	7	0.51	34
L79	-0.27	20	-0.21	16	0.14	28
L106	-0.15	21	0.33	26	-0.37	4
L100	0.01	22	0.67	33	-0.67	2
L103	0.40	23	0.25	24	-0.16	15
L97	0.49	24	0.04	21	0.08	25
L90	1.00	25	0.47	29	-0.24	9
L93	1.02	26	0.45	28	-0.21	11
L96	1.06	27	0.59	31	-0.34	7
L109	1.31	28	0.62	32	-0.31	8
L82	1.70	29	0.30	25	0.11	26
L88	1.76	30	1.36	38	-0.95	1
L104	1.87	31	0.96	37	-0.52	3
L80	2.26	32	0.49	30	0.04	23
L78	2.28	33	0.70	35	-0.16	17
L83	2.35	34	0.69	34	-0.13	19
L87	2.40	35	-0.14	18	-0.14	18
L102	2.42	36	0.13	23	0.44	33
L91	4.53	37	0.36	27	0.71	36
L81	5.13	38	0.01	20	1.21	38

Table S6. Re-isolation and survival percentage of various *Pinus taeda* families (2014).

Family	Total	LT	GH	Survival
L05	71	71	71	98
L09	69	68	70	95
L100	67	65	69	99
L101	64	60	68	98
L102	81	71	90	98
L103	74	72	76	99
L104	70	72	68	96
L106	63	73	53	98
L107	73	73	74	99
L108	67	66	69	99
L109	72	76	67	97
L110	74	73	74	89
L16	63	59	68	99
L38	65	60	69	94
L49	77	76	79	95
L50	68	68	67	88
L78	68	63	73	93
L79	68	65	76	99
L80	64	64	64	99
L81	77	85	69	97
L82	72	62	80	94
L83	72	79	66	99
L84	78	82	73	99
L85	68	68	67	99
L86	63	60	67	98
L87	84	85	84	98
L88	78	83	73	99
L89	74	76	73	99
L90	78	76	80	99
L91	69	69	69	99
L92	77	79	75	99
L93	73	74	71	99
L94	74	78	71	99
L95	78	75	81	99
L96	68	63	73	99
L97	75	74	76	99
L98	76	79	73	96
L99	72	71	73	99

Table S7. Least square means estimate of lesion caused by *Grosmannia huntii* (2016).

Family	Estimate	Standard error	DF	Lower CI	Upper CI
L05	0.66	0.05	934	0.57	0.76
L09	0.60	0.05	934	0.51	0.70
L111	0.60	0.05	934	0.51	0.70
L112	0.72	0.05	934	0.62	0.81
L113	0.75	0.05	934	0.65	0.85
L114	0.65	0.05	934	0.56	0.75
L115	0.78	0.05	934	0.69	0.88
L116	0.72	0.05	934	0.63	0.82
L117	0.67	0.05	934	0.58	0.77
L118	0.62	0.05	934	0.52	0.72
L122	0.65	0.05	934	0.55	0.75
L123	0.59	0.05	934	0.49	0.68
L124	0.72	0.05	934	0.62	0.81
L126	0.78	0.05	934	0.68	0.87
L127	0.65	0.05	934	0.55	0.75
L128	0.76	0.05	934	0.66	0.86
L129	0.73	0.05	934	0.64	0.83
L130	0.73	0.05	934	0.63	0.83
L16	0.78	0.05	934	0.67	0.88
L33	0.71	0.05	934	0.61	0.81
L38	0.78	0.05	934	0.68	0.88
L49	0.79	0.05	934	0.69	0.88
L50	0.61	0.05	934	0.52	0.71

Note: CI: Confidence interval.

Table S8. Least square mean estimates of lesion caused by *Leptographium terebrantis* (2016).

Family	Estimate	Standard Error	DF	Lower CI	Upper CI
L05	3.01	0.03	971	2.95	3.08
L09	2.94	0.03	971	2.88	3.00
L111	2.87	0.03	971	2.81	2.94
L112	2.94	0.03	971	2.88	3.01
L113	3.02	0.03	971	2.96	3.08
L114	2.95	0.03	971	2.89	3.01
L115	2.93	0.03	971	2.87	2.99
L116	2.91	0.03	971	2.84	2.97
L117	2.90	0.03	971	2.84	2.96
L118	2.90	0.03	971	2.84	2.96
L122	3.00	0.03	971	2.94	3.06
L123	2.93	0.03	971	2.87	2.99
L124	2.96	0.03	971	2.90	3.02
L126	3.06	0.03	971	3.00	3.12
L127	2.88	0.03	971	2.82	2.94
L128	2.92	0.03	971	2.86	2.98
L129	3.10	0.03	971	3.04	3.16
L130	2.96	0.03	971	2.90	3.02
L16	2.87	0.03	971	2.81	2.93
L33	2.83	0.03	971	2.77	2.89
L38	2.90	0.03	971	2.83	2.96
L49	2.98	0.03	971	2.93	3.04
L50	2.96	0.03	971	2.90	3.02

Note: CI: Confidence interval.

Table S9. Different isolates of *Leptographium terebrantis* used in the pathogenicity testing (Eckhardt et al., 2007).

No	Isolate no	Plot	Source	Insect	Location	County	Physioregion	Soil	Ownership
1	I-03-3150	C2	Insect	Hs	N 33 42.889 W 85 34.725	Clebrune	Ridge and Valley	Fruithurst loam	State
2	I-03-2040	P1	Insect	Hs	N 33 28.485 W 85 44.315	Clay	Piedmont	Madison sandy loam	Federal
3	I-03-2069	P19	Insect	Hs	N 32 55.911 W 87 23.147	Bibb	Coastal Plain	Suffolk loamy sand	Federal
4	I-03-3125	P17	Insect	Hs	N 32 57.850 W 87 22.933	Bibb	Coastal Plain	Maubila flaggy sandy loam, eroded	Federal
5	I-03-2072	C7	Insect	Hs	N 32 46.117 W 86 59.283	Chilton	Coastal Plain	Smithdale sandy loam	Federal
6	I-03-1848	P15	Insect	Pp	N 32 59.835 W 87 29.780	Hale	Coastal Plain	Smithdale sandy loam	Federal
7	I-03-2122	P1	Insect	Hp	N 33 28.485 W 85 44.315	Clay	Piedmont	Madison sandy loam	Federal
8	I-03-2077	P3	Insect	Hp	N 33 27.870 W 85 44.325	Clay	Piedmont	Madison sandy clay loam, eroded	Federal
9	I-03-3107	P14	Insect	Pp	N 32 57.928 W 87 24.715	Clay	Piedmont	Louisa sandy loam	Federal
10	I-03-2013	P2	Insect	Ht	N 33 28.194 W 85 44.276	Clay	Piedmont	Madison sandy clay loam, eroded	Federal
11	I-03-1770	P4	Insect	Hs	N 33 30.444 W 85 42.114	Cleburne	Piedmont	Madison sandy loam	Federal
12	I-03-2031	P21	Insect	Hs	N 32 55.818 W 87 25.595	Hale	Coastal Plain	Smithdale sandy loam	Federal
13	I-03-3196	P8	Insect	Hs	N 33 28.660 W 85 44.663	Bibb	Piedmont	Maubila flaggy sandy loam	Federal
14	I-03-1953	P9	Insect	BTB	N 33 28.722 W 85 45.094	Clay	Piedmont	Louisa sandy loam	Federal
15	I-03-1772	P32	Insect	BTB	N 32 47.302 W 87 01.456	Perry	Coastal Plain	Suffolk loamy sand	Federal
16	I-03-2123	P31	Insect	BTB	N 33 22.603 W W 87 26.853	Tuscaloosa	Cumberland Plateau	Smithdale sandy loam	Industry
17	I-03-2030	P18	Insect	Pp	N 32 57.767 W 87 22.805	Bibb	Coastal Plain	Maubila flaggy sandy loam	Federal
18	I-03-2098	P16	Insect	Ht	N 32 50.741 W 87 29.733	Hale	Coastal Plain	Riverview sandy loam	Federal
19	I-03-2099	P5	Insect	Hs	N 33 30.212 W 85 85 41.895	Cleburne	Piedmont	Madison sandy loam	Federal
20	I-03-3179	P21	Insect	Pp	N 32 55.818 W 87 25.595	Hale	Coastal Plain	Smithdale sandy loam	Federal
21	Control		-						
22	I-03-1973	P7	Insect	BTB	N 33 42.797 W 85 40.100	Calhoun	Ridge and Valley	Fine-loamy, mixed, semi-active	State
23	I-03-2163	P10	Insect	BTB	N 33 23.384 W 85 56.898	Talladega	Piedmont		Federal
24	I-03-2026	P31	Insect	Hs	N 33 22.603 W W 87 26.853	Tuscaloosa	Cumberland Plateau	Smithdale sandy loam	Industry

25	I-03-1911	P29	Insect	Ht	N 33 22.585 W 87 26.694	Tuscaloosa	Cumberland Plateau	Smithdale sandy loam	Industry
26	I-03-1995	P30	Insect	Hp	N 33 22.537 W 87 26.882	Tuscaloosa	Cumberland Plateau	Smithdale sandy loam	Industry
27	I-03-2133	P26	Insect	Ht	N 32 23.206 W 87 26.245	Tuscaloosa	Cumberland Plateau	Sweatman sandy loam	Industry
28	I-03-3123	P27	Insect	Hp	N 33 23.229 W 87 26.356	Tuscaloosa	Cumberland Plateau	Suffolk sandy loam	Industry
29	R-03-3122	P24	Root	LOB	N 33 24.697 W 87 26.900	Tuscaloosa	Cumberland Plateau	Sweatman sandy loam	Industry
30	R-03-2088	P23	Root	LOB	N 33 24.806 W 87 26.657	Tuscaloosa	Cumberland Plateau	Suffolk loamy sand	Industry
31	R-03-3134	P21	Root	LOB	N 32 55.818 W 87 25.595	Hale	Coastal Plain	Smithdale sandy loam	Federal
32	R-03-1687	P6	Root	LOB	N 33 42.826 W 85 40.920	Calhoun	Ridge and Valley	Decatur clay loam, eroded	State
33	R-03-3134	P22	Root	LOB	N 32 56.113 W 87 26.420	Hale	Hale	Maubila flaggy sandy loam	Federal
34	R-03-3111	P3	Root	LOB	N 33 27.870 W 85 44.325	Clay	Clay	Madison sandy clay loam, eroded	Federal
35	R-03-1593	P6	Root	LOB	N 33 42.826 W 85 40.920	Calhoun	Calhoun	Decatur clay loam, eroded	State
36	R-03-1622	P12	Root	LOB	N 32 58.802 W 87 27.123	Bibb	Bibb	Maubila flaggy sandy loam	Federal
37	R-00-407	P15	Root	LOB	N 32 59.835 W 87 29.780	Hale	Hale	Smithdale sandy loam	Federal
38	R-00-366	P19	Root	LOB	N 32 55.911 W 87 23.147	Bibb	Bibb	Suffolk loamy sand	Federal
39	R-00-87-ss205	P32	Root	LOB	N 32 47.302 W 87 01.456	Perry	Perry	Suffolk loamy sand	Federal
40	ATCC MYA-3316	P21	Root	LOB	N 32 55.818 W 87 25.595	Hale	Hale	Smithdale sandy loam	Federal
41	R-00-44-ss6	P3	Root	LOB	N 33 27.870 W 85 44.325	Clay	Clay	Madison sandy clay loam, eroded	Federal
42	I-03-1731	P4	Insect	LOB	N 33 30.444 W 85 42.114	Cleburne	Cleburne	Madison sandy loam	Federal
43	Clem	Unknown	Root	LOB					

Table S10. Estimates of lesion length (log) of 42 different *Leptographium terebrantis* isolates compared to control treatment.

Isolate name	Estimate	Standard Error	t value	Pr > |t|
Intercept	2.76	0.04	68.99	<0.0001
I-03-3150	0.18	0.06	3.13	0.0018
I-03-2040	0.15	0.06	2.60	0.0094
I-03-2069	0.19	0.06	3.38	0.0008
I-03-3125	0.14	0.06	2.44	0.0150
I-03-2072	0.24	0.06	4.31	<0.0001
I-03-1848	0.31	0.06	5.53	<0.0001
I-03-2122	0.1	0.06	1.83	0.0677
I-03-2077	0.16	0.06	2.83	0.0048
I-03-3107	0.2	0.06	3.57	0.0004
I-03-2013	0.24	0.06	4.22	<0.0001
I-03-1770	0.25	0.06	4.33	<0.0001
I-03-2031	0.28	0.06	5.01	<0.0001
I-03-3196	0.2	0.06	3.58	0.0004
I-03-1953	0.3	0.06	5.32	<0.0001
I-03-1772	0.23	0.06	4.05	<0.0001
I-03-2123	0.19	0.06	3.33	0.0009
I-03-2030	0.27	0.06	4.81	<0.0001
I-03-2098	0.27	0.06	4.77	<0.0001
I-03-2099	0.23	0.06	4.04	<0.0001
I-03-3179	0.14	0.06	2.48	0.0134
I-03-1973	0.18	0.06	3.07	0.0022
I-03-2163	0.16	0.06	2.76	0.0060
I-03-2026	0.35	0.06	6.16	<0.0001
I-03-1911	0.22	0.06	3.93	<0.0001
I-03-1995	0.19	0.06	3.27	0.0011
I-03-2133	0.44	0.06	7.72	<0.0001
I-03-3123	0.23	0.06	4.01	<0.0001
R-03-3122	0.25	0.06	4.38	<0.0001
R-03-2088	0.19	0.06	3.35	0.0009
R-03-3134	0.31	0.06	5.52	<0.0001
R-03-1687	0.21	0.06	3.77	0.0002
R-03-3134	0.33	0.06	5.80	<0.0001
R-03-3111	0.15	0.06	2.63	0.0088
R-03-1593	0.39	0.06	6.82	<0.0001
R-03-1622	0.31	0.06	5.42	<0.0001
R-00-407	0.13	0.06	2.34	0.0195
R-00-366	0.27	0.06	4.74	<0.0001
R-00-87-ss205	0.17	0.06	3.00	0.0028
*LOB-R-00-805	0.68	0.06	11.93	<0.0001

R-00-44-ss6	0.38	0.06	6.73	<0.0001
I-03-1731	0.32	0.06	5.65	<0.0001
Clem	0.21	0.06	3.71	0.0002

(* Singh et al., 2014, ATCC: MYA - 3316).