**Interaction of Future Climate Change Scenarios of Elevated Tropospheric Ozone and Altered Rainfall on Loblolly Pine Seedlings Inoculated with Ophiostomatoid Fungi**

by

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

Auburn, Alabama
May 10, 2015

Keywords: root disease, pine decline, tropospheric ozone,
drought, global climate change



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## Abstract

Southern Pine Decline is a cause of premature mortality of *Pinus* species in the Southeastern United States. While the pathogenicity of ophiostomatoid fungi have been observed both in the laboratory and the field, the driving mechanisms for success of fungal infection, as well as the bark-beetle vectors is less understood. The goal of this thesis is to provide insight into the role of future climatic conditions, specifically elevated tropospheric ozone and altered precipitation patterns, in the progression of the Southern Pine Decline on loblolly pine. Two scientific questions were address: (1) will predicted future concentrations of tropospheric ozone affect loblolly pine vigor and increase susceptibility to root infecting ophiostomatoid fungi?; and (2) will predicted future rainfall patterns affect loblolly pine vigor and increase susceptibility to root infecting ophiostomatoid fungi?

The first question was addressed in 2013, utilizing open-top chambers, three ozone concentrations and stem inoculations of four families of loblolly pine. Two of the families used were selected for tolerance to root infecting ophiostomatoid fungi, while the others were more susceptible. The second question was addressed in 2014, utilizing capped open-top chambers, simulated rainfall treatments and stem inoculations of four families of loblolly pine. Two of the families used were selected for tolerance to root infecting ophiostomatoid fungi, while the others were more susceptible.

Overall, changes in climatic conditions are anticipated to increase Southern Pine Decline severity and incidence. There was a strong link between tolerance to root infecting ophiostomatoid fungi and susceptibility to elevated ozone concentrations. There was no strong relationship between sensitivity to moisture stress and susceptibility to root infecting ophiostomatoid fungi. In the future, ozone and precipitation patterns may work in tandem, as well as with Southern Pine Decline, and therefore may play an even more important role in the productivity of loblolly pine.

Acknowledgments

"A journey of a thousand miles begins with a single step." – Lao Tzu

I am thankful to my major professors, Drs. Lori Eckhardt and Art Chappelka, for their guidance and support. Without them, I would have not been able to succeed. In such a short period of time we have accomplished so much and I am deeply grateful for all of the opportunities you have given me. I also would like to thank Dr. Scott Enebak. Because of my interactions with him, I am a better student and a better person. Dr. Ryan Nadel generously offered his knowledge of statistics, experimental design and methodology. Tessa Bauman made herself available night and day. Because of all of you, I am confident that we maximized the quality of research during my time at Auburn University. For that, I can never thank all of you enough.

I also would like to thank the Alabama Agricultural Experiment Station for providing the funding for my research. Special thanks goes to Rayonier and Arborgen for their support. The School of Forestry and Wildlife Sciences was an amazing place to attend. Dr. Graeme Lockaby, Patti Staudenmaier and Audrey Grindle are all wonderful people who always made time for me. They are superb examples of the quality of person I encountered at the SFWS. It has been a true pleasure getting to know the staff, faculty and students.

Auburn University allowed me to choose the path that I wanted to take. I am deeply grateful that I had the opportunity to cross paths with Nick Barnwell, Pratima Devkota and Andrea Cole. Adam Trautwig, a friend of many years, played an integral role in experience during my undergraduate studies and I am thankful we were able to collaborate once again.

Thank you to all my family and friends, especially Hannah Sherman who has always been thoughtful and encouraging. Finally, a thank you must go to Kate Fuller. Without her love and support, I would have never had the courage to embark on this journey.

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## Introduction and Literature Review

### 1.1 CLIMATE CHANGE

#### 1.1.1 Climate Change Defined

The hypothesis that human activities could influence the Earth's climate was first postulated more than a century ago (Arrhenius, 1896) and became more developed during the 20$^{th}$ century (MacCracken et al. 2000, IPCC 2013). The Intergovernmental Panel on Climate Change (IPCC) reported that previous climate change assessments have already come to pass through multiple lines of evidence that the climate is changing at an accelerated rate across our planet, largely as a result of human activities. The most compelling evidence of climate change originates from observations of the atmosphere, land, oceans and cryosphere (IPCC 2013). Both climate and climate change have various definitions. These can cause uncertainty when considering management and mitigation strategies (Parmesan and Yohe 2003, Sasaki and Putz 2009). To clarify, below are several useful definitions:

"The climate is described by such measures as the average temperature, precipitation and soil moisture as well as the magnitude and frequency of their variations, the likelihood of floods and droughts, the temperature of the oceans and the paths and intensities of the winds and ocean currents" – MacCracken et al. (2000).

"Climate change is a change in the state of the climate that can be identified by the changes in the mean and/or variability of its properties and that persist for extended period, typically decades or longer" – IPCC (2013).

"Climate change refers to any significant change in the measure of climate that lasts for an extended period of time. In other words, climate change includes major changes in temperature, precipitation and wind patterns, among others, that occur over several decades or

longer" – United States Environmental Protection Agency glossary; http://www.epa.gov/climatechange/glossary.html

### 1.1.2 Drivers of Climate Change

In 1750, carbon dioxide, methane and nitrous oxide concentrations were approximately 279 parts per million (ppm), 721 parts per billion (ppb) and 270 ppb, respectively. In 2011 these concentrations had increased to 391 ppm, 1803 ppb and 324 ppb respectively (Walsh et al. 2014). The atmospheric concentrations are currently higher than any levels over the last 800,000 years. Carbon dioxide is primarily caused from the burning of fossil fuels and the subsequent emissions and secondarily from land use change emissions (Walsh et al. 2014). The ocean has absorbed about 30% of the emitted anthropogenic carbon dioxide, causing ocean acidification and a lowering of the pH (Walsh et al. 2014). Changes in vegetation cover can affect the energy balances, change surface reflectivity, evapotranspiration rates, wind drag and the amount by which snow cover can increase surface reflectivity in winter (Pitman et al. 1999).

Evidence suggests that these variations have been driven primarily by changes in the seasonal and latitudinal distributions of solar radiation caused by cyclic variations in the Earth's orbit around the Sun, perhaps amplified by a number of factors. These factors include changes in glacial height and extent, in ocean circulations and in the atmospheric carbon dioxide and methane concentrations that were apparently driven by the initial temperature change (MacCracken et al. 2000, IPCC 2013).

### 1.1.3 Observations of Climate Change

The physical and chemical climate of the earth has changed rapidly over the last 100 years and is predicted to change in the future (Christensen et al. 2007, IPCC 2013). The maximum and minimum surface temperatures have globally increased since 1950 and 1983-2012 was the warmest 30 year period in the last 800 years recorded (Stocker et al. 2013). Climate reconstructions over the past thousand years using ice cores, tree rings, vegetation types and other proxy measures indicate that the warming of the 20$^{th}$ century is unprecedented when compared to other natural variations prior to this century that were presumably caused by solar, volcanic and other natural influences (Walsh et al. 2014). An ice-core record from Antarctica

analyzing the past 420,000 years indicates temperatures in that regions have been up to 10 °F (6 °C) colder than the last 40,000 years of that period (MacCracken et al. 2000).

### 1.1.4 Global Change and Global Circulation Models

The physical and chemical climate of the earth has changed rapidly over the last 100 years and is predicted to continue in the future (Christensen et al. 2007, IPPC 2013, Karl et al. 2009). The IPCC (2013) determined that many aspects of climate systems are showing evidence of climate change. Both anthropogenic and natural causes are attributed to increased greenhouse gas emissions and the ensuing temperature changes (IPPC 2013). Various models predict the global temperature to increase approximately 3 °C over the next 50 to 100 years (Wang and Schimel 2003, Easterling and Apps 2005, IPPC 2013). Two major global circulation models, the Hadley and Canadian models, predict temperature increases from 1.7-5.5 °C by 2030 and 1.0-2.3 °C by 2100 (MacCracken et al. 2000). The models differ in predicted rainfall patterns in the Southeastern United States into 2090: 20% greater in the Hadley model and 10% less in the Canadian model (MacCracken et al. 2000). An increase in precipitation may be negligible if predicted evapotranspiration rates continue to increase (Dale et al. 2001, Seager et al. 2009). Increased temperatures, evapotranspiration, and extreme weather events brought on by a continuous climate change will increase the frequency and severity of stress factors such as drought. Droughts can cause reductions in tree vigor which can results in predisposition to various stresses including fungal pathogens (Hepting 1963, Manion 1991, Hepting 1971) that lead to many forest declines (Sturrock et al. 2011).

### 1.1.5 Predicted Changes in Climate

Global climate is changing and this change is apparent across a wide range of observations of which the warming of the past 50 years is primarily due to human activity (Walsh et al. 2014). Various models predict the global temperature to increase approximately 3 °C over the next 50 to 100 years (Wang and Shimel 2003, Easterling and Apps 2005, IPCC 2013).

Climate change prediction models that have used both natural and anthropogenic factors of climate change have been more accurate, over the last 50 to 100 years, than those using natural factors alone (Walsh et al. 2014). These models predict that warmer climates will occur

in coming centuries. Prolonged snow-free period and increasing frequency and intensity of droughts are expected to elevate the frequency of forest fires in many regions (Kirilenko and Sedjo 2007). Droughts in the Southwestern U.S. and periods of extreme heat are projected to become more intense and periods of colder weather less intense worldwide (Walsh et al. 2014). There is high confidence that annual mean surface warming since the 20$^{th}$ century has reversed long-term cooling trends in the past 5000 years in the mid-to-high latitudes of the Northern Hemisphere (Stocker et al. 2013) while average precipitation in the U.S. has increased since 1900 (Walsh et al. 2014). Some areas have had increases in precipitation greater than the national average, and some areas have had decreases. More winter and spring precipitation is projected in the Northern U.S. and less for the Southwestern region of the U.S. over this century (Walsh et al. 2014)

The impacts of climate change may become less predictable in the future, and may have unforeseen consequences. For example, under one version of the Hadley Global Circulation Model, there is an expected increase of 18% in wood growth by 2030, which over time will gradually decrease. Economic impacts may increase or decrease the prices on lumber in an unpredictable way (Kirilenko and Sedjo 2007). Other changes in climate and uncertainties in predictions could have reverberating effects throughout ecological and socioeconomic systems.

### 1.1.6 Impacts of Climate Change on Forests and Forest Health

Combustion of fossil fuels and the release of greenhouse gases is the main driver of the ongoing climate change, global changes in temperature, shifts in precipitation, and an increase in frequency, extremity, and intensity of storms (Paoletti et al. 2009). It is likely that natural disturbances in forest ecosystems will be altered by climate change and there is evidence that warmer temperatures have already shifted the habitats and ranges of some forest species (Kirilenko and Sedjo 2007). Climate change-induced modifications of frequency and intensity of forest wildfires, outbreaks of insects and pathogens and extreme events such as high winds may be more important than the direct impact of higher temperatures and elevated carbon dioxide (Kirilenko and Sedjo 2007). The direct effects of climate change on individual plants and plant communities may occur in the absence of pathogens, but also may bring about changes in plants that will affect their interactions with pathogens (Garret et al. 2006).

Both drought and tropospheric ozone are issues of concern to the Southeastern U.S. ecosystems (Phillips et al. 2009, IPCC 2013, Wear and Greis 2002, Chameides et al. 1988). Climate change can have impacts on forest diseases and decline complexes (Manion 1991, La Porta et al. 2008, Edmonds et al. 2000). Potential concerns include increased abiotic stresses, increased pathogen distributions, reduced host distributions and host physiological changes conducive to disease (Sturrock et al. 2011). The future between climatic conditions and how they will affect diseases and declines is complex and variable and further research is needed in this area (Duke et al. 2008).

### 1.1.7 Climate Change Effects on Forestry

Changing temperature and precipitation patterns and increasing atmospheric carbon dioxide concentrations are likely to drive changes in natural and managed forests (Kirilenko and Sedjo 2007). While the understanding of how temperature and precipitation changes will occur is limited, there has been extensive research into the effects of elevated carbon dioxide on vegetation physiology (Curtis and Wang 1998) because of its role in plant growth and the implications for the forest product industry, natural resource managers, ecologists and atmospheric scientists.

A long-term study conducted by Duke University, in North Carolina, has monitored the effects of carbon dioxide enrichment on net primary production of loblolly pine (*Pinus* taeda L.) since 1994. DeLucia et al. (1999) found that after two years dominant pine trees exposed to elevated carbon dioxide had increased growth rates by 26% as well as a 25% increase in net primary production. Although this may seem positive for timber producers, the authors noted that limited rates of nitrogen mineralization over longer periods of time would likely offsetthe gains predicted in primary productivity. In another study from Duke University the authors note that elevated carbon dioxide increases production for non-production species as well (Ellsworth et al. 2012) which is turn can lead to undesirable forests.

Other studies on agronomic crops and forest trees have shown changes in photosynthesis, stomatal conductance, biomass allocation and water use can have both beneficial and detrimental effects when exposed to increased carbon dioxide levels (Rogers et al. 1983a, Rogers et al. 1983b, Curtis and Wang 1998, Poorter 1993).

Other than carbon dioxide, there are many other changes that can occur in forest ecosystems. Changes in soil chemistry can alter forestry and forest management practices (Vose et al. 2012). Major topics of interest would include variation in disturbance regimes, changes in precipitation and temperature patterns and soil chemistry alterations. These factors can all have an effect on management objectives.

Ectomycorrhizal colonization of root tips on loblolly seedlings exposed to ozone decreased with increasing ozone concentrations (Meier et al. 1990). In a study by Garrett et al. (1982), it was found that loblolly pine seedlings exposed to ozone and sulfur dioxide became more resistant to the deleterious effects when ectomycorrhizae were present. These studies confirm that mycorrhizal fungi are important for growth and production in both forest and agricultural settings. How these fungi and plant hosts are responding to climate change and its impact on site productivity, however, yet to be determined.

Carbon dioxide and ozone affect fungal productivity and community composition. The primary effects would be on dispersal, colonization and sporocarp-dependent food webs in aspen-maple communities (Andrew and Lilleskov 2009). Such impacts could result in a loss of productivity in both timber growth and atmospheric carbon fixation by trees. Sulfur dioxide and ozone are known to have adverse effects on both endo- and ectomycorrhizae in loblolly pine seedlings (Mahoney et al. 1985). In the nitrogen limited boreal forest, high C and low N conditions revealed that mycorrhizae actually limited the donation of N to host plants and invested in self growth (Näsholm et al. 2013).

## 1.2 TROPOSPHERIC OZONE

### 1.2.1 Historical Perspective

Increases in anthropogenic air pollutants have shown to adversely affect many plants (Manning 1975). Tropospheric ozone ($O_3$) is produced by photochemical reactions involving hydrocarbons and nitrogen oxides and has increased at a rate of 0.3%-2.0% per year, due to an increase in fossil fuel combustion (Blasing 2009, IPCC 2013, Thompson 1992, Vingarzan 2004). The ubiquitous nature of ozone and the fact that tree response is altered by many other factors (light, nutrition, moisture etc.), highlighted the difficulties to determine whether ambient ozone concentrations significantly affect tree growth and productivity in the field (Chappelka and Samuelson 1998). The effect originates with cellular injury that causes metabolic changes

and alterations in growth if the dose (amount present at a particular time) is sufficient and plant protective or repair mechanism are overcome (Lefohn 1992).

Even in non-urban locations, the presence and formation of ozone occurs in the absence of anthropogenic hydrocarbons and nitrogen oxides. These processes include transport of ozone from the stratosphere to the troposphere, photochemical formation and subsequent deposition in the planetary boundary layer and transport from urban to non-urban areas (Altshuller 1986). Regardless of land use in a given area, meteorological conditions play a large role in ozone formation (Chang et al. 2010). These conditions include temperature, wind speed, cloud cover, solar radiation and atmospheric mixing and in the Eastern U.S., high ozone concentrations are noted during high pressure systems which have warm temperatures, little wind and cloudless skies (U.S. EPA 2006, Seinfeld and Pandis 2012). Two uncertainties regarding ozone formation are emission scenarios of ozone precursors, and changes in weather and climatic conditions (Chang et al. 2010).

### 1.2.2 Ozone Formation

Summarized by Lehfon (1992), the mechanism for stratospheric ozone formation was developed by chemist Sydney Chapman. Chapman (1930) describes the photolytic mechanisms of stratospheric ozone and destruction as follows:

$$\text{(Eq 1) } O_2 + \text{sunlight } (\lambda \leq 320 \text{ nm}) \rightarrow O + O$$

$$\text{(Eq 2) } O + O_2 \rightarrow O_3$$

$$\text{(Eq 3) } O_3 + \text{sunlight } (\lambda \leq 320 \text{ nm}) \rightarrow O_2 + O$$

$$\text{(Eq 4) } O + O_2 \rightarrow O_3$$

Many others (Hampson 1964, Molina and Rowland 1974, Stolarski and Cicerone 1974) found that equations 3 and 4 did not occur quickly enough to deplete the generation of ozone from equations 1 and 2. Lefhon (1992) goes on to note that we still do not understand all mechanisms involved in the formation of ozone but subsequent studies to Chapman's (1930) showed that

oxidation of hydrocarbons (R) and carbon monoxide (CO) in the presence of nitrogen oxides (NOx) and sunlight play a large role. For example, in tropospheric ozone formation hydroxyl radicals (OH) are integral (Eq 4 and 5).

(Eq 4) $O_3$ + sunlight ($\lambda \leq 320$ nm) $\rightarrow$ O + $O_2$

(Eq 5) O + $H_2O \rightarrow 2OH$

In remote areas, with few nonmethane hydrocarbons, CO triggers ozone formation once OH radicals are produced (Eq 6-11).

(Eq 6) CO + OH $\rightarrow$ $CO_2$ + H

(Eq 7) H + $O_2 \rightarrow HO_2$

(Eq 8) $HO_2$ + NO $\rightarrow$ OH + $NO_2$

(Eq 9) $NO_2$ + sunlight ($\lambda \leq 420$ nm) $\rightarrow$ NO + O

(Eq 10) O + $O_2 \rightarrow O_3$

(Eq 11) Net: CO + $2O_2 \rightarrow CO_2 + O_3$

When methane is present, the reaction produces formaldehyde (HCHO), at which time the process is very similar to equations 8, 9 and 10 (Eq 12 and 13).

(Eq 12) Net: $CH_4 + 2O_2$ + 2 sunlight ($\lambda \leq 320$ nm) $\rightarrow$ HCHO + $H_2O + 2O_3$

(Eq 13) Net: RH + $4O_2$ + 2 sunlight ($\lambda \leq 320$ nm) $\rightarrow$ RCHO + $H_2O + 2O_3$

Based on these proposed ozone formation equations, Lehfon (1992) proposed four important generalizations:

1. Increases concentrations of $NO_X$, associated with anthropogenic emissions, increases the rate of photochemical ozone production.
2. Areas with low concentrations of $NO_X$ act in controlling ozone formation.
3. In pristine environments natural levels of $NO_X$ and hydrocarbons can only contribute to ozone formation and allow background ozone concentrations of 20 to 30 ppb.
4. In areas with high $NO_X$ levels, hydrocarbons can increase ozone formation. In these areas, when hydrocarbons are not present, $NO_X$ can hinder ozone formation.

### 1.2.3 Ozone Effects on Vegetation

Ozone causes injury to plants upon entering leaf stomata, dissolving in the aqueous layer lining the cell walls, diffusing through the cellular membranes and reacting with cellular components and metabolic processes (Samuelson and Kelley 2001). Vegetation exposed to ozone show symptoms of injury including chlorotic mottling, browning, and chlorotic leaves and stippling (Lehfon 1992, Krupa and Manning 1988, Thomas 1961, among many others).

Plants exposed to ozone not only exhibit symptoms but also show changes in normal physiological functions (Karnosky et al. 2007). Ozone stress can reduce carbon fixation, alter rates of leaf and root respiration, cause shifts in carbon allocation and disrupt nutrient allocation patterns (Chappelka and Samuelson 1998).

### 1.2.4 Ozone Effects on Ecosystems

The air pollutants of most concern to forests are nitrogen deposition and tropospheric ozone (Paoletti et al. 2009). Nitrogen deposition can affect ecosystems through increases in soil inorganic N which can have fertilization effects, while the acidic nature of N deposition can lead to acidification effects (Throop and Lerdau 2004). Fertilization effects can cause changes in forest productivity which effects carbon balance in ecosystems (Vose et al. 2012). Changes in acidification can change other soil nutrient and cation availability (Likens et al.1996, Schulze 1989, Shortle and Smith 1988).

Ozone can have effects on exposed individual plants' physiology (Karnosky et al. 2007). As the individuals change, whether singly or in concert, the structure and function of the community change (Chappelka and Samuelson 1998). An example is presented by Sun et al. (2012) who described how trees exposed to ozone lose the ability to regulate water through loss

of stomatal control. Overtime this can cause the community streams to lose water. This can have negative impacts on water quality and community resilience.

In general, air pollutants including ozone, can cause stress. Odum (1985) summarized how stressors can affect ecosystems and communities. In hardwood communities this can drastically alter the amount of sunlight penetrating the canopy through increased leaf senescence via ozone exposure. This may result in shifts in species composition in the understory which can have numerous large scale effects including altered fire regimes and decreased resiliency to invasive species.

Kim et al. (1998) reported that ozone may influence substrate quality and microbial activity which reduced litter decomposition rates. This is a prime example of how air pollution can affect structure and function of a community. Kim et al. (1998) also reported that under warmer temperatures litter decomposed faster. As mentioned before, ozone can also increase leaf senescence which can heat understory litter increasing decomposition rates. This exemplifies the complexity of the effects of air pollution on community structure and function.

Air pollutants can cause changes in structure and function of an ecosystem or community. Canopy gas-exchange, alterations in canopy cover and edaphic factors can all play a role in these changes in structure and function. Inherently, communities are comprised of many species. Antagonistic and synergistic relationships between air pollutants and a particular species present can have consequences for all species present in the system.

### 1.2.5 Ozone Effects on Plant Pathogens/Pests

Manning (1975) stated that obligate parasitism by fungi seems to be impaired by ozone exposure and ozone-injured host tissue. Also, facultative parasitism seems to be favored by host plants exposed to ozone (Sandermann Jr. 2000). While a unified body of work has yet to emerge on this topic (Heagle 1973, Garrett et al. 2006), there are several concepts within the literature that show how ozone can affect pathogen-host relationships. Based on several articles (Garrett et al. 2006, Sturrock et al. 2011) there are three common relationships found when analyzing climate-host-pathogen relationships:

1. Climate can have an effect on the pathogen's virulence, abundance, distribution and general biology/ecology;

2. Climate can have an effect on the host's defenses, abundance, distribution and general biology/ecology.
3. Climate can change the way the host and pathogen interact, through direct or indirect effects.

## 1.3 ALTERED PRECIPITATION AND DROUGHT

### 1.3.1 Historical Perspective

Interactions between changing precipitation regimes and other aspects of climate change are likely to affect terrestrial ecosystems (Weltzin et al. 2003). Weltzin et al. (2003) states that soil moisture rates are driven by precipitation effects and the rate of precipitation change will affect ecosystems in various ways. These affects will depend on the interactions between soil moisture and vegetation dynamics. A good example of these interactions is presented by Tschaplinski et al. (1995). The authors found that among three deciduous tree species, the rates of growth and gas exchange were independent of carbon dioxide and drought treatments. This provides a good model to discuss the effects of drought and carbon dioxide singly on trees, and how these may interact to affect terrestrial ecosystem health.

First, understanding the concepts of droughts is important as to understand their effects on social and biological systems. Droughts are extreme meteorological events that have long durations and are unpredictable (Mishra and Singh 2010). The distinction between heat waves and droughts is the temporal scale over which they occur; with heat waves typically lasting weeks while droughts last months to years (Chang and Wallace 1987). Droughts take on many forms and have resonant effects throughout the world. A review paper from Wilhite et al. (1985) discusses various perceptions of drought affect mitigation and preparedness strategies. Several definitions of drought were provided by Mishra and Singh (2010):

1. Meteorological drought is lack of precipitation over a region for a period of time.
2. Hydrological drought is related to a period with inadequate surface and subsurface water resources for establishing water uses of a given water resources management systems.
3. Agricultural drought refers to a period with declining soil moisture and subsequent crop failure without any reference to surface water resources.
4. Socio-economic drought is a failure of water resources systems to meet water demand and thus associating droughts with supply and demand for an economic good.

Drought can result in increased susceptibility to pathogens, decreased growth and altered disturbance regimes (wildfires). Parks et al. (2012) found that drought is a major driver of widespread wildfire in the southwestern U.S. Dale et al. (2001) stated that ecosystems in general that are exposed to drought due to increasing temperatures could increase evaporative demands triggering water stress. Any increase in stress can trigger numerous direct and indirect effects. For example, drought can reduce resistance to beetle attacks by changing resin production and resin content (Jones et al. 2004).

Increasing carbon dioxide concentrations in the atmosphere can have positive effects on growth for both hardwood and coniferous tree species (Saxe et al. 1998). Although increased carbon dioxide can reduce stomatal conductance this may not make trees more resistant to drought conditions. The literature shows that there is generally a species specific response to carbon dioxide and water regulation and this varies across sites (Saxe et al. 1998).

Beerling et al. (1996) found that stomatal conductance of three eastern hardwood species varied given elevated carbon dioxide treatments. While European beech (*Fagus sylvatica* L.) and English oak (*Quercus robur* L.) decreased conductance over two years, European white birch (*Betula pendula* Roth) increased. The response among species is just one example of the variable host responses of individual plants and communities to elevated carbon dioxide levels and drought. This variation also may be based upon specific site characteristics (Dale et al. 2001).

Both changes in drought and carbon dioxide levels will play major roles in terrestrial ecosystem changes. More often than not, managed lands will prove challenging to landowners when creating management plans to mitigate climate change. In order to better understand this topic, future research should focus on endeavors that examine widespread landscape species that occur in regions where physical climate change is most likely to occur. This future research should concentrate on keeping socially and economically important ecosystems healthy (e.g. pine plantations in the Southeast U.S., Douglas-fir forests in the Northwestern U.S.) and result in lands with value to become resilient to both chemical and physical climate change.

### 1.3.2 Precipitation Causes/Formation

The scientific community has made great strides in understanding North American droughts and improved monitoring and forecasting tools (Mariotti et al. 2013). In North America, major droughts over the last 100 years can be attributed to either heating or cooling of

sea surface temperatures in the tropical Pacific Ocean. This causes changes in radiating forces that affect the North American landmass causing atmospheric hydrological changes. It is important to note that droughts occurring anywhere globally have impacts and effects over other regions on Earth as well (Cook et al. 2007).

### 1.3.3 Precipitation Effects on Vegetation

The response of vegetation to water stress has been well documented (Hsiao 1973, Griffiths and Parry 2002). Of concern to forest managers are specific species utilized in forestry as well as several main parameters that indicate plant health. These include items such as growth rate, photosynthesis and disease tolerance. While plants respond differently to water stress, there are a few generalizations that can be made. Drought stress is defined as "a moderate loss of water, which leads to stomatal closure and limitation of gas exchange (Jaleel et al. 2009) and water potential (Tyree and Hammel 1972, Scholander et al. 1966) is widely accepted as the fundamental measure of plant water status (Hsiao 1973). Jaleel et al. (2009) describes several important factors of drought stress including diminished growth, loss of turgor, impaired mitosis and reduced cell elongation and enlargement. Hsiao (1973) discusses other factors involved in drought stress including wall synthesis, protein synthesis, chlorophyll synthesis and production, stomatal opening and carbon assimilation among many others. Typically, desirable tree functions either slow down or cease functioning (at the wilting point) in most vegetation.

### 1.3.4 Precipitation Effects on Pathogens/Pests

The effects of altered precipitation can have various effects on host-pathogen interactions. The interactions between drought stress and pathogens are similar to the section described above (See Tropospheric Ozone: Effects on Pathogens section above). Other factors to consider when dealing with moisture, rather than pollutants, are the increased effects of moisture on fungal pathogens. These interactions have been reviewed extensively (Desprez-Loustau et al. 2006, Sturrock et al. 2011, Chakraborty et al. 2000, Garrett et al. 2006, Scherm and Coakley 2003). In brief, pathogens such as rusts (*Cronartium* spp.) and watermolds (e.g. *Phytophthora* spp.) can increase in inoculum concentrations during periods of high moisture and precipitation (Cao et al. 1997, Smith et al. 2008, Van Arsdel 1972). The opposite also can be true for restricting species presence and distribution.

Of concern are pests that vector pathogenic fungi (e.g. ophiostomatoid fungi) (Kirisits 2004, Bentz et al. 2010). The influence of bark beetles, among other pests, is well established in the literature and typically, drought causes host plants to become stressed leading to greater infestations (Jones et al. 2004, Jactel et al. 2012, Klepzig et al. 2004, Koricheva et al. 1998). Host stress is one factor playing a part in this interaction, but insect physiology and ecology shifts can have drastic effects as well (Clarke and Fraser 2004, Gillooly et al. 2001).

## 1.4 Forest and Tree Decline Concepts

Forest declines are caused by the interaction of a number of interchangeable, specifically ordered abiotic and biotic factors to produce a gradual general deterioration, often ending in the death of a tree (Manion 1991, Hepting 1963). Manion (1991) theorizes declines are caused by predisposing factors, inciting factors and contributing factors. Predisposing factors occur over a long period of time and include genetic potential, site factors and age. Inciting factors occur over a short-period and can include air pollution exposure, seasonal to annual droughts and insect defoliation. Contributing factors can be long-term but are often attributed to opportunistic biotic agents (Manion 1991).

These factors are often hard to describe and their effects can be variable. Decline symptoms are difficult to diagnose but hosts often exhibit dieback, stunted growth and an overall deterioration of health. Reports of forest decline have increased in recent years, although they may have been occurring for a long time (Manion and Lachance 1992). By the end of the 21$^{st}$ century forest ecosystems in the U.S. will be different than today. Changes in forest structure and function are not only caused by chemical climate change but significant short-term effects via altered disturbance regimes (Vose et al. 2012).

## 1.5 FORESTRY

### 1.5.1 Forestry in the Southeastern United States

Colonization, farming, and intensive logging in the 1800s followed by fire control and extensive planting of loblolly pine in the 1900s, converted the southern pine from predominantly longleaf pine to predominantly loblolly pine in less than 100 years (Schultz 1997). In more recent years (1950-2000), Brown et al. (2005) found that in the Southeastern Plains ecoregion urban environments increased by 1.4% while forest cover declined 1.8%. Despite this trend,

forestry continues to remain an important aspect of the Southeastern economy. Currently, the Southeastern U.S. represents the main softwood producing area accounting for 64% of total timber harvest in the country (Smith et al. 2001).

### 1.5.2 Loblolly Pine

Loblolly pine is one of the most hardy and versatile of all southern pines and accounts for nearly half of all growing stock in the subsection *Australes* (North and Central America and Caribbean pines) of the genus *Pinus* (Schultz 1997). The species current range spans from central New Jersey, southwest to east Texas and east to central Florida but is absent in the Mississippi River Valley (Schultz 1997). The range also extends into the coastal plain throughout the Piedmont Plateau and into the Appalachian Highlands (Baker and Langdon 1990). Before European settlement in North America, loblolly pine forests existed on approximately 2 million hectares and currently this area has expanded to 13.4 million hectares (Schultz 1997). This expansion was due to the silvicultural habits of the tree. The species ability to regenerate rapidly on abandoned cultivated lands has given loblolly pine the nickname "old-field pine" (Schultz 1997). Loblolly historically grew on moist sites that were not subject to regular burning and was a minor part of upland and lowland ecosystems (Schultz 1997). Today loblolly pine is grown in pure and mixed stands (Schultz 1997) and can be considered off-site in many locations (Hess et al. 1999, Hess et. all 2002).

Loblolly pine reaches maturity at 80 to 100 years on average sites and grows to 27 to 34 m in height and 71 to 76 cm in diameter. Roots make up about 20% of the total tree biomass. Loblolly pine is characterized by pale blue-green to yellow-green needles 13 to 22 cm in length and 1.2 to 1.4 mm in dia in fascicles of three. Needles are slender, generally triangular in cross section, rigid, and slightly twisted and have basal sheaths 10 to 20 mm in length. Loblolly pine is monoecious. Strobili form in clusters of 3 to 10, and are 2.5 to 3.8 cm in length and 0.6 to 0.8 cm in width. Pollen contains two air sacs which facilitate long-distance dispersal by air currents. Female flowers are ovoid and are 1.0 to 1.5 cm in length and 0.5 to 0.7 cm in width. Mature cones are 5 to 15 cm in length, 4 cm in dia and light reddish brown in color. Cones are ovoid-cylindrical to narrowly conical and are armored. The bark is light to dark brown and turns reddish brown or gray with age (description from Schultz 1997).

Loblolly pine is a susceptible species to various biotic agents found throughout the

Southeastern U.S. In Alabama, several major pests are southern pine beetle (*Dendroctonus frontalis* Zimmerman), Ips engraver beetles (*Ips* spp. Eichoff and Germar), black turpentine beetle (*D. terebrans* Olivier), pitch canker (*Fusarium circinatum* Nirenberg and O'Donnell) and southern pine decline (*Leptographium spp.* Barras and Perry and others) (Barnard and Dixon 1983, Price 2008, Cordell 1989). Because pests and disease are an integral part of the loblolly pine ecosystem, their management must be integrated into management themes (Schultz 1997).

In 2010, Alabama sales of forest products and related sectors totaled $11.2 billion (Fields et al. 2011). Southeastern forest ecosystems are a critically important resource, providing forest products such as timber and pulp, ecosystem services such as water purification and flood mitigation, and habitat for endangered species (Duke et al. 2008). Unfortunately, the land base of the Southeastern U.S. has been impacted by previous agricultural practices. Southern pine forests, both planted and naturally seem to be experiencing local and regional decline in function and productivity. (Duke et al. 2008) An increase in the type or extent of forest health problems in the southern pine systems would be important as these forests are a dominant landscape feature (Prestemon and Abt 2002, Trani 2002). The loss of dominant organisms in the landscape can create complex challenges for forest owners and managers. Forest and tree health is critical when considering water quality, recreation, wildlife management and threatened and endangered species conservation.

### 1.5.3 Southern Pine Decline

Southern Pine Decline (SPD) is associated with premature death of southern *Pinus* species (Harrington and Cobb 1983, Ostrina et al. 1997, Eckhardt et al. 2004a). Southern Pine Decline (formerly pine decline or Loblolly Pine Decline) was first observed in the Talladega National Forest, Alabama in 1959 (Brown and McDowell 1968). Like littleleaf disease (*Phytophthora cinnamomi* Werres) of shortleaf pine (*Pinus echinata* Mill.) (Campbell and Copeland 1956), SPD is characterized by lateral root deterioration, loss of fine roots, reduced radially growth, thinning foliage and heavy cone crops just prior to mortality (Brown and McDowell 1968). Historically SPD has been found in loblolly pine trees 50+ years in age (Brown and McDowell 1968) but more recently has been reported in stands as young as 13 years old (personal communication with LG Eckhardt).

Southern Pine Decline is caused by a series of biotic and abiotic factors that include root

pathogenic fungi (*Leptographium* and *Grosmannia* spp.), their root-feeding bark beetle/root weevil vectors (*Hylastes salebrosus* Eichoff, *H. tenuis* Eichoff, *Hylobius pales* Herbst., and *Pachylobius picivorus* Germar), resource stress (nutrient deficiencies, moisture stress, edaphic factors) and management strategies (overstocking, mechanical injury and fire stress) (Eckhardt et al. 2010).

The fungus, *Leptographium terebrantis* Barras and Perry, causes lesions in the phloem and resin-soaking in the xylem of inoculated seedlings and mature trees of several conifers (Wingfield 1983, Eckhardt et al. 2004a, Matusick and Eckhardt 2010). *Grosmannia huntii* (Rob.-Jeffr.) Zipfel, de Beer and Wingfield is a related fungal pathogen that has been reported to be more virulent on young loblolly pine seedlings (Matusik and Eckhardt 2010). *Grosmannia* fungal species and their anamorphic (asexual) states, *Leptographium* spp., are grouped in the order Ophiostomales. Ophiostomatoid fungi consist of a suite of fungal pathogens of deciduous and coniferous trees.

Both *L. terebrantis* and *G. huntii* are closely associated with root-feeding bark beetle vectors (Wingfield 1983, Paine et al. 1997, Eckhardt et al. 2007). Scolytid bark beetles (Coleoptera: Scolytinae) are the primary group of vectors for ophiostomatoid fungi (Paine et al. 1997). Zanzot et al. (2010) reported that both *L. terebrantis* and *G. huntii* were most commonly found on *Hylastes tenuis* (sampling conducted 2006-2007 in Georgia), although many bark beetles and regeneration weevils (Curculionidae) act as vectors. Vectoring beetle species carry the fungi on their exoskeletons on special structures (Harrington 1988). The fungi are introduced into the tree by their insect vectors, colonize and grow within the larval feeding galleries and then spread within the tree or forest stand by emerging adults carrying the fungus (Barras and Perry 1971, Klepzig et al. 1995a, Klepzig et al. 1991). Evidence supports that the pathogenicity of *Leptographium* species increases as beetles begin to feed because of changes in oleoresin components (Eckhardt et al. 2009). Eckhardt et al. (2004b) reported a significant increase in *Hylastes* spp. emergence in roots inoculated with *L. terebrantis* when compared to sterile roots, indicating an enhancement in *Hylastes* reproduction. The symbiotic relationship proposed may be essential to the onset and progress of SPD.

Trees under stress due to injury release elevated levels of secondary compounds (Kimmerer and Kozlowski 1982, Kelsey and Joseph 2001). Klepzig et al. (1995b) found that inoculated red pine contained greater concentrations of terpenes than unwounded and wounded

controls. Ethanol, terpenes and mixtures of the two are successful attractants of bark beetles and regeneration weevils (Miller and Rabaglia 2009).

Southern Pine Decline management can be difficult given the various sites where loblolly pine is planted. Prevention and control measures include: (i) reducing rotation age, (ii) harvest stands greater than 40 years of age and replant with appropriate species, (iii) selectively thinning stands of 25-40 years of age, (iv) limit soil compaction and maintaining stand health via proper thinning and fertilization regimes in stands of 15-25 years of age. Currently loblolly pine is planted on 80% of all southern pine plantations in the Southern U.S. Best management strategies should include intensive monitoring programs to aid in early detection (Eckhardt and Menard 2009).

### 1.5.4 Ophiostomatoid Fungi: Pathogenicity and Characteristics

#### 1.5.4.1 *Leptographium terebrantis*

*Leptographium terebrantis* (Kendrick) Wingfield is commonly associated with dying or declining pine trees throughout the U.S. and Canada (Harrington 1988). It is common on many pine species (Klepzig et al. 1991, Harrington 1988), and in the Southeast U.S. is particularly of concern on loblolly pine (Eckhardt et al. 2007) and longleaf pine (Otrosina et al. 2002). The morphology is typical of most *Leptographium* species and lacks distinguishable characteristics and is often identified by hyphal growth characteristics (Jacobs and Wingfield 2001).

#### 1.5.4.2 *Grosmannia huntii*

*Grosmannia huntii* (formeraly *Ceratocystis huntii* Robinson-Jeffery and Grinchenko) is less commonly found in the U.S. and is readily distinguished from *Leptographium* anamorphs by the presence of perithecia with black bases and small hyphal hairs. Conidia (*L. huntii*) are often present and are similar to other *Leptographium* spp. *Grossmania huntii* can be distinguished by serpentine hyphae and an abundance of aerial hyphae (Jacobs and Wingfield 2001, Robinson-Jeffrey and Grinchenko 1964). Recently, *G. huntii* has been found to be non-native to North America (TA Duong personal communication).

#### 1.5.4.3 *Leptographium procerum*

*Leptographium procerum* is found throughout North America, South Africa and Europe. In the Eastern U.S. it is associated with Procerum Root Disease which infects eastern white pine (*Pinus strobus* L.) when root feeding bark beetles and weevils attack stressed trees (Nevill and Alexander 1992a, Nevill and Alexander 1992b). In the U.S., *L. procerum* is also associated with decline of red pine (*Pinus resinosa* Sol. ex. Aiton) in the Great Lakes region. *Leptographium procerum* is distinguished from other similar species by rhizoid-like structures at the base of conidiophores.

#### 1.5.4.4 *Grosmannia alacris*

*Grosmannia alacris*, formerly known as *Verticicladiella alacris* Wingifled and Marasas, is non-native to the Southeastern U.S. and is native to South Africa (Seifert et al. 2013, Duong et al. 2014),. It is found on *Pinus* species in both regions (Eckhardt et al. 2007, Wingfield and Knox-Davies 1980, Jacobs and Wingfield 2001). *Grosmannia alacris* can be distinguished from other fungal species by the presence of perithecia, serpentine hyphae and a lack of aerial hyphae in culture (Jacobs and Wingfield 2001).

## 1.6 OBJECTIVES

The major goal of this research is to describe the interactions between *L. terebrantis* and *G. huntii* associated with Southern Pine Decline in the presence of predicted climatic conditions expected in the next 50 to 100 years in the Southeastern U.S. To accomplish this, two objectives were addressed:

a) To examine the effects of seedlings inoculated with ophiostomatoid fungi in the presence of elevated ozone concentrations;
b) To examine the effects of seedlings inoculated with ophiostomatoid fungi in the presence of altered precipitation regimes.

# Chapter 2

## Effects of tropospheric ozone on loblolly pine seedlings inoculated with root infecting ophiostomatoid fungi

### 2.1 ABSTRACT

Seedlings from four families of loblolly pine (*Pinus taeda* L.) were exposed in open-top chambers to charcoal-filtered air, non-filtered air or air amended with ozone to 2 times ambient. Two of the families used were selected for their tolerance to root infecting ophiostomatoid fungi. The other two families were selected for their susceptibility to root infecting ophiostomatoid fungi. After 44 days of ozone exposure, seedlings were treated with five inoculation treatments: no wound, wound only, wound+media, *Grosmannia huntii* and *Leptographium terebrantis*. After 77 additional days of exposure, seedlings were harvested. Seedling volume, dry matter yield, relative chlorophyll content, water potential and lesion characteristics were measured by treatment and analyzed using ANOVA procedures. The results indicate that seedlings selected for their susceptibility to root infecting ophiostomatoid fungi were also more sensitive to ozone than seedlings tolerant to root infecting ophiostomatoid fungi. Overall lesion length was greater on seedlings exposed to elevated ozone concentrations. Inoculation with *L. terebrantis* and *G. huntii* also caused seedlings to be more water stressed during midday while ozone had no effect. Relative chlorophyll content was not affected by inoculation treatment but was lowest in seedlings exposed to elevated ozone concentrations. There was no evidence to support the hypothesis ozone and root infecting ophiostomatoid fungi work in tandem to decrease loblolly pine vigor, however, the results indicate that susceptibility to root infecting ophiostomatoid fungi and sensitivity to ozone may be linked.

**Keywords** – tropospheric ozone, loblolly pine, root infecting ophiostomatoid fungi, *Leptographium terebrantis*, *Grosmannia huntii*

## 2.2 INTRODUCTION

Combustion of fossil fuels is the main driver of the ongoing climate change. Consequently, changes in annual mean temperatures, shifts in precipitation and an increase in frequency, extremity, and intensity of storms are predicted under future climate scenarios (Paoletti et al. 2009). It is also likely that natural disturbances, such as fires and pest (insects and plant pathogens) outbreaks in forest ecosystems will be altered by climate change. There is evidence that warmer temperatures have already shifted the habitats and ranges of some forest species (Kirilenko and Sedjo 2007, Bentz et al. 2010). These climatic events may be more important than the direct impact of higher temperatures and elevated carbon dioxide levels (Kirilenko and Sedjo 2007). While direct effects of climate change on individual plants and vegetation communities may occur in the absence of plant pathogens, climate change will also affect their interactions with pathogenic organisms (Garret et al. 2006).

For example, obligate biotroph infections by fungi appear to be reduced by ozone exposure and ozone-injured host tissue, while necrotrophic pathogens seem to be favored by host plants exposed to elevated ozone (Manning 1975, Manning and von Tiedemann 1995, Sandermann 2000). While a consensus has yet to emerge on ozone and plant pathogen interactions (Heagle 1973, Garrett et al. 2006), there are several parameters within the literature that indicate ozone can affect pathogen-host relationships. Based on several studies (Garrett et al. 2006, Sturrock et al. 2011, Manning and von Tiedemann 1995) there are three common relationships to look for when analyzing climate-host-pathogen relationships: 1) climate can affect the pathogen's virulence, abundance, distribution and general biology/ecology; 2) climate can alter the host's defense, abundance, distribution and general biology/ecology; and 3) climate can change the way the host and pathogen interact, through direct and/or indirect effects.

Loblolly pine (*Pinus taeda* L.) is planted in 80% of all southern pine plantations in the Southeastern U.S. and is susceptible to various biotic agents. In Alabama, several major pests include southern pine beetle (*Dendroctonus frontalis*), Ips engraver beetles (*Ips* species) and black turpentine beetle (*D. terebrans*). Regarding plant pathogens, loblolly pine is susceptible to pitch canker fungus (*Fusarium circinatum*) and fusiform rust (*Cronartium fusiforme*). One insect and fungal association has resulted in SPD (*Leptographium spp.* and *Hylastes spp.*) (Barnard and Dixon 1983, Price 2008, Cordell 1989).

Southern Pine Decline is the term for decline of, in general, southern *Pinus* species and is associated with the premature mortality of loblolly pine (Harrington and Cobb 1983, Otrosina et al. 1997, Eckhardt et al. 2004a) and is the consequence of a series of biotic and abiotic factors. These include root pathogenic fungi (*Leptographium* and *Grosmannia* spp.), their root-feeding beetle vectors (*Hylastes salebrosus* Eichoff, *H. tenuis* Eichoff, *Hylobius pales* Herbst., and *Pachylobius picivorus* Germar), resource stress (nutrient deficiencies, other edaphic factors), management strategies such as overstocking, mechanical injury and fire stress (Eckhardt et al. 2010). When loblolly pine is inoculated with *Leptographium terebrantis*, the fungus causes lesions in the phloem and resin-soaking in the xylem of seedlings and mature trees of several conifers (Wingfield 1983, Eckhardt et al. 2004b, Matusick and Eckhardt 2010). *Grosmannia huntii* is a related pathogen reported as being more virulent on young pine seedlings than *L. terebrantis* (Matusick and Eckhardt et al. 2010).

Increases in anthropogenic air pollutants have shown to adversely affect numerous plant species (Manning 1975, Manning and von Tiedemann 1995). Tropospheric ozone is produced by photochemical reactions involving hydrocarbons and nitrogen oxides and has increased at a rate of 0.3%-2.0% per year due to an increase in fossil fuel combustion (Blasing 2009, IPCC 2013, Thompson 1992, Vingarzan 2004). The ubiquitous nature of this pollutant and the fact that tree response is altered by many factors (light, nutrition, moisture etc.), it is difficult to determine if the effects of ambient ozone concentrations significantly affect tree growth and productivity in the field (Chappelka and Samuelson 1998). The effect of ozone on plant growth begins with cellular injury that results in metabolic changes and alterations in growth if the dose is sufficient and plant repair mechanism are overcome (Lefohn 1992).

Plant response to pathogens has been shown to be altered by the exposure of ozone (Heagle 1973). Ozone can alter tree vigor and reduce defensive compounds which in turn predispose plants to infection and colonization by a pathogen (Sandermann et al. 1998). Working with loblolly pine, Carey and Kelley (1994) reported that ozone predisposed trees to the pitch canker fungus, *Fusarium circinatum*. Cankers caused by this fungus were smaller for resistant loblolly pine families compared with susceptible loblolly pine families. Elevated ozone concentrations resulted in larger cankers caused by the pathogen regardless of tree family sensitivity to the pathogen.

There are only a few studies on ozone interactions with tree root pathogens (James et al.

1980, Lackner and Alexander 1983, Fenn et al. 1990). Early research was conducted in Southern California with ponderosa (*Pinus ponderosa* Lawson) and Jeffrey pine (*Pinus jeffreyi* Balf.) and their relationship with the root-rot fungus *Heterobasidion irregular* Garbelotto and Otrosina, formerly *H. annosum* (Fr) Bref. (James et al. 1980). Fenn et al. (1990) investigated the effects of ozone exposure on black stain root disease; caused by *Leptographium wageneri var. ponderosum* Harrington and Cobb of ponderosa pine. In California they reported increases in foliar injury and decreases in stem growth for inoculated seedlings. Lesion length increased with increasing ozone concentrations. Their findings indicate an interaction among these stress agents in the trees' growth. Lackner and Alexander (1983) excavated roots from air pollution sensitive and tolerant trees in the Blue Ridge Parkway in Virginia. They recovered several ophiostomatoid fungi and *H. irregulare* from the roots of sensitive trees, but no fungi were recovered from tolerant trees.

Jones et al. (2001), in an assessment of the effect of potential future climate change scenarios for the Southeastern U.S., reported multiple factors such as ozone and changes in water availability are important. Water availability and ozone levels alter loblolly pine vigor and in unison with biotic organisms, such as *L. terebrantis* or *G. huntii*, may have the potential to exacerbate pine decline and reduce productivity. The overall objective of this study was to elucidate the interactions of *L. terebrantis* and *G. huntii* in the presence of predicted climatic conditions expected in the next 50 to 100 years in the Southeastern U.S. Specific hypotheses include: (1) loblolly pine seedlings will be more susceptible to *L. terebrantis* and *G. huntii* when exposed to elevated ozone concentrations; (2) loblolly pine seedlings susceptible to *L. terebrantis* and *G. huntii* will be also be sensitive to ozone injury; (3) hyphal growth of *L. terebrantis* and *G. huntii* are not affected by the presence of elevated ozone.

## 2.3 MATERIALS AND METHODS

### 2.3.1 Study Site and Open-Top Chambers

The research site (approximately 0.02 $km^2$) is located approximately 5 km north of the Auburn University Campus, Auburn, AL, U.S. The site contains 24 open-top chambers (OTCs), monitoring sheds and a small laboratory. The OTCs were 4.8 m height x 4.5 m diameter aluminum framed structures with fans (1.5 horse-power motors), chamber plastics and Teflon tubing (Gilliland et al. 2012). Before the initiation of the study (March 2013), vegetation from

each OTC was sprayed with glyphosate and removed. The bare soil was covered with landscape fabric.

### 2.3.2 Seedlings

Seedlings from four loblolly pine families were used in this study (lifted from the nursery November 2012) with two families considered tolerant to root infecting ophiostomatoid fungi (T1 and T2), while the other two were more susceptible (S1 and S2) (Singh et al. 2014). In January 2013, 2700 seedlings (750 per family) were planted in trade gallon pots with ProMix BX® peat-based potting mix (Premier Tech, Quebec, Canada). Seedlings were kept in a shade house and watered until mid-April when they were deployed into OTCs for acclimation before inoculations in late May 2013.

### 2.3.3 Ozone Treatments

Three ozone fumigation treatments were used (replicated 3 times): (1) CF = charcoal filtered (~0.5 x ambient air), representative of more pristine environments, (2) NF = non-filtered air, representative of ambient air in the Auburn, AL area and other rural areas in the Piedmont region of the U.S., and (3) 2× = (twice NF) representative of concentrations currently found around large urban areas such as either Atlanta, GA or Birmingham, AL (Chameides and Cowling 1995). The 2× is indicative of potential future ozone scenarios for rural Piedmont regions over the next 50 years (Thompson 1992, Vingarzan 2004).

Ozone was generated by passing pure oxygen ($O_2$) through a high-intensity electrical discharge source (Griffin Inc., Lodi, NJ) and added to the OTCs through Teflon tubing connected to the fan box for 12 hours/day (09:00-21:00) for 7 days/week. Fans were turned off from 23:00-05:00 to allow natural dew formation. Ozone concentrations were monitored using U.S. EPA approved Model 49 TECO Ozone analyzers (Thermo Environmental Instruments, Inc., Hopkinton, MA). Instruments were calibrated based on U.S. EPA quality assurance guidelines. Fumigation began on April 19$^{th}$ and ended August 14$^{th}$ 2013. Of the 118 days of fumigation, the first 41 days were utilized to acclimate seedlings to chamber conditions and ozone concentrations. Once inoculated, seedling fumigation continued for 77 more days (41 + 77 = 118 days).

### 2.3.5 Inoculations

Stem inoculations were conducted as described by Nevill et al. (1995) from May 26 – 29$^{th}$ 2013 using the wound+inoculum method. Five inoculation treatments were used: no wound (NW), wound only (W), wound+media (WM), *L. terebrantis* (LT) and *G. huntii* (GH). A sterile razor blade was used to cut a 5 cm vertical lesion into the bark 5 cm above the soil line to inoculate trees. Agar plugs of 2% MEA (3 mm) were placed into the wound. Media was either sterile or had *L. terebrantis* or *G. huntii* growing. All wound and inoculations were wrapped in cotton soaked with deionized water and each wound area stem region was wrapped in Parafilm® to retard desiccation.

### 2.3.6 Measurements and Harvest

Seedling root collar diameter (RCD) and shoot lengths were recorded January and August of 2013 for all seedlings. Seedling volume change ($Volume_{Final} - Volume_{Initial} = Volume_{Change}$) was used to determine overall growth for individual seedlings. The equation for volume *Volume = diameter$^2$ x height* has been used to reliably estimate volume in seedlings and mature trees (Ruehle et al. 1984).

During seedling potting in January 2013, 40 seedlings from each family were destructively sampled and separated into needles (NE), shoot (SH), coarse roots (CR) and fine roots (FR < 2.0 mm dia). Components were placed in drying-ovens for 72 hours at 70 °C and average dry matter recorded. At the end of the inoculation x exposure period, two seedlings from each treatment combination, from each chamber, were selected for final biomass determination. Initial family means for each component (needles, coarse roots etc.) were subtracted to estimate biomass growth over the experiment, referred to as dry matter yield.

Eleven seedlings from each treatment, in all nine OTCs, were nondestructively sampled for relative leaf chlorophyll using a SPAD-502 chlorophyll meter (Spectrum Tech. Inc., Plainfield, IL) during the final harvest (August 2013). Needles from the first 2013 flush were selected due to their physiological maturity (Sasek et al. 1991). These same seedlings were also evaluated for incidence (% of seedlings exhibiting symptoms) and severity of visible ozone injury using a modified Horsfall-Barratt rating scale (Horsfall and Barratt 1945, Chappelka et al. 2003). Whole plants were rated for severity of visible injury using the following categories: 0%,

1-6%, 7-25%, 26-50%, 51-75% and 76-100%. Needles displaying visible symptoms were then rated using the same scale.

The same 11 seedlings per treatment were taken to the laboratory and measured for lesion characteristics. Seedlings were cut at the soil line and placed in plastic bins filled with FastGreen stain (FastGreen FCF; Sigma Chemical Co., U.S.) as described by Singh et al. (2014). Lesion length, width and depth were measured along the stem and two pieces of stem tissue from each lesion were collected and plated on malt extract agar with cyclohexamide and streptomycin sulfate for re-isolation (Singh et al. 2014).

The remaining two seedlings from each treatment combination treatment per chamber, were examined for water potential using a Scholander pressure bomb (PMS Instrument Company, Albany, OR) during final harvest. Five cm of a lateral branch was cut off each seedling and sampled as described by Kaufmann (1968). Midday sampling occurred between 1300-1500 h. Predawn sampling occurred between 0200-0500 h, however due to significant variation between replicates the data were not analyzed and thus not included in the analysis.

### 2.3.7 Fungal Growth Study

The wound+inoculum method used in these trials could potentially expose the fungi to ambient air conditions within the OTCs. The fluctuating temperature, increased ozone exposure and heavy precipitation in OTCs can compromise the wound dress (Paramfilm®) on inoculated seedlings. Therefore, we also conducted a plate (petri dish, 100 mm x 15 mm) fungal growth study to determine if ozone had any positive or negative effects on the fungi used in the inoculation trial.

Nine wood-frame boxes (one per OTC) were constructed and wrapped in polyethylene film. A plastic hose was inserted into the box containing the petri dishes and the other end was placed into the perforated plastic of each open-top chamber that provides the various ozone concentrations (CF, NF, 2×) into the box. Boxes were suspended in the OTCs from aluminum bars at a height of ~1.5 meters above the ground (Figure 2.1a).

Two percent MEA, amended with cyclohexamide and streptomycin sulface (1600 and 400 mg/l respectively) and glycerol (32%) to retard desiccation in the boxes for at least 5 days, allowed fungi to reach the media margin. Ophiostomatoid fungi are tolerant to cyclohexamide and streptomycin sulfate, but show varying degrees of growth reduction on the selective media

(Jacobs and Wingfield 2001). Each petri dish was inoculated with a 3 mm plug of 2% MEA containing an active culture of each fungus. Inoculated petri dishes cultured on the laboratory bench for three days before deployment into the boxes. The fungi used were *L. terebrantis* (LT), *G. huntii* (GH), *L. procerum* (LP) and *G. alacris* (GA). *Leptographium procerum* and *G. alacris* were included as they also are root infecting ophiostomatoid fungi. At the time of deployment, petri dish lids were raised from the cups using surface sterilized (ethanol dipped, autoclaved and dried in a laminar flow hood under UV light) plastic tripods (Pizza Stackers®, Royal, Coatesville, PA) which allow air flow over the fungus as well as assist in evaporation prevention (Figure 2.1b).

To determine if temperature was affecting by utilizing the boxes, thermocouple wires were placed inside one chamber and within the box inside that chamber. Plates were deployed into the boxes after 3 days and were kept in boxes for 5 days. Fungal growth was measured on the day of deployment and Day 5 by marking the current growing margin of the fungus (Figure 2.1c). At 6 days, the media edge had begun to shrink indicating desiccation. Fungal area was measured using a LASICO planimeter (LASICO Co., Los Angeles, CA) and growth rate was calculated using the formula: final area – initial / initial x 100.

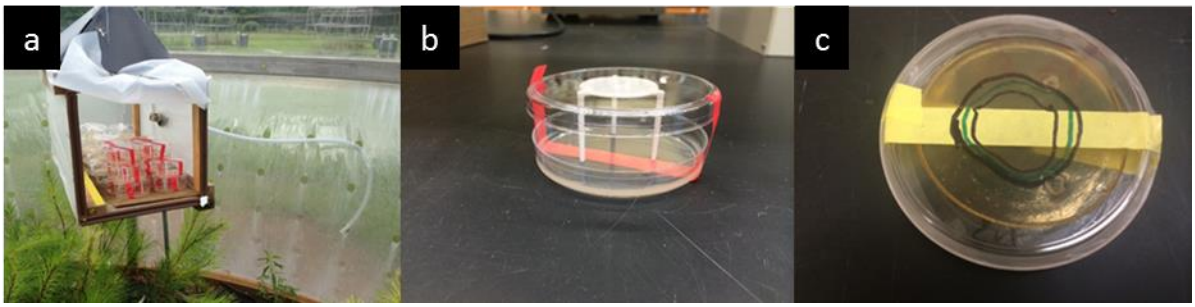


**Figure 2.1**. (a) Wood frame box with hose attachment placed in the OTC for air exposure. (b) Plastic tripod/Pizza Stacker® used to prevent media desiccation and allow air flow over the fungus. (c) Growth area marked on the underside of the plate.

### 2.3.8 Data Analysis

For the inoculation x family x ozone concentration study, the experimental design was a split-split-split plot with replicates at all levels. The three concentrations of ozone, four loblolly pine families and five inoculation treatments produced sixty treatment combinations. Each treatment combination was replicated fifteen times in each chamber at the beginning of the

study. Statistical analyses were conducted using SAS (SAS Institute, Inc. Cary, NC) ANOVA procedures (Glimmex procedures). Initial ANOVA tests were to ensure there was no significant variation between replicates. Post-hoc Tukey (Honest Significant Difference – HSD) procedures were conducted to further investigate treatment effects. Alpha was set at 0.05. Graphics were produced using STATISTICA (StatSoft, Inc. Tulsa, OK).

For the fungal growth study the experimental design was a split-split plot with replicates at all levels. Three ozone concentration and four species of fungi produced twelve treatment combinations. Each treatment combination was replicated five times in each chamber. Statistical procedures followed the same methodology as the main study described above.

## 2.4 RESULTS

### 2.4.1 Climatic Data and Ozone Exposures

Mean 12-h (0900-2100 h) ozone concentrations (Table 2.1) over the five month experiment were 14, 23 and 37 ppb for CF, NF and 2× respectively. The seasonal 12hr AOT40 values (ppm · $hr^{-1}$) for CF, NF and 2× were 0.027, 1.631 and 31.277 respectively. Seasonal W126 values (ppm · $hr^{-1}$) were 0.033, 0.423 and 21.913 for CF, NF and 2×, respectively).

Monthly air temperatures (24-hr avg) (Table 2.2) were similar to the 30-year averages throughout the experimental period; 22.5 ° and 22.9 °C, respectively for April-August 2013 and the 30-yr average. Total precipitation in the Auburn, AL area for April-August 2013 was 70.1 cm was 1.3 times greater than the 30-yr average of 54.9 cm.

**Table 2.1**. 12-h ozone concentration for each ozone treatment, 12-h W126 and 12-h AOT40

| Month | 12-h Ozone Conc. (ppb) | | | | | 12-h W126 | | | 12-h AOT40 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | CF | NF | 2× | Avg. 1-h Daily Max (2×) | 1-h Monthly Max (2×) | CF | NF | 2× | CF | NF | 2× |
| April | 17 | 26 | 41 | 90 | 97 | 0.000 | 0.000 | 0.010 | 0 | 38 | 666 |
| May | 19 | 29 | 48 | 99 | 154 | 0.000 | 0.001 | 0.019 | 27 | 1205 | 13116 |
| June | 15 | 23 | 39 | 88 | 140 | 0.023 | 0.310 | 13.980 | 0 | 337 | 9378 |
| July | 12 | 19 | 30 | 73 | 140 | 0.010 | 0.112 | 7.909 | 0 | 51 | 5606 |
| August | 9 | 16 | 28 | 79 | 102 | 0.000 | 0.000 | 0.004 | 0 | 0 | 2511 |
| **Average** | **14** | **23** | **37** | **86** | **127** | **0.007** | **0.085** | **4.384** | **5** | **326** | **6255** |

CF = charcoal-filtered, NF = non-filtered, 2× – twice non-filtered. Avg. 1-h Daily Max (2×) = the average of all daily ozone peaks/maximum values in the month in 2× ozone treatments. 1-h Monthly Max (2×) = the maximum ozone value for the entire month in the 2× ozone treatments. AOT40 (ppb · $hr^{-1}$) = accumulated ozone values over a threshold of 40 ppb. W126 (ppm · $hr^{-1}$) = cumulative weighting index (Lefohn 1992).

**Table 2.2**. Precipitation (cm) and temperature (°C) in Auburn, AL during the experimental period and the 30-yr (1971-2000) average for Auburn, AL (AWIS, Inc.)

| Month | 2013 Avg. Temperature (°C) | 30-yr Avg. Temperature (°C) | 2013 Rainfall (cm) | 30-yr Avg. Rainfall (cm) |
|---|---|---|---|---|
| April | 17.8 | 16.3 | 5.1 | 10.7 |
| May | 20.4 | 21.2 | 14.7 | 9.7 |
| June | 25.7 | 24.9 | 10.2 | 10.4 |
| July | 24.5 | 26.2 | 21.3 | 15.0 |
| August | 24.3 | 26.1 | 18.8 | 9.1 |
| **Average** | **22.5** | **22.9** | **14.0** | **11.0** |

### 2.4.2 Seedling Volume Change

Overall seedling growth increased ($P < 0.0001$) when exposed to elevated ozone concentrations compared to CF and NF seedlings (Table 2.3, 11.7% and 8.5% respectively) across all treatments. Inoculation had no effect on seedling growth ($P = 0.585$). Regarding family differences, T1 was found to have the greatest volume growth while S2 had the least (41.4% less than T1). Families S1 and T2 grew 18.8% and 20.9% less than T1. There was a significant ozone concentration × family effect (Table 2.3). S1 grew more ($P < 0.011$) when exposed to elevated ozone compared to NF and CF as shown in Figure 2.2 (13.7% and 8.0% respectively). S2 volume growth was not significantly different between ozone treatments ($P > 0.064$). T1 volume growth was greater ($P < 0.0001$) in 2× chambers than those T1 seedlings grown in NF and CF (10.8% and 13.6% respectively). T2 grew 8.0% ($P = 0.024$) more in 2× compared to T2 seedlings grown in CF but not significantly more than T2 seedlings in NF chambers ($P = 0.947$). There were no other significant interactions between treatments and treatment combinations (Table 2.3).

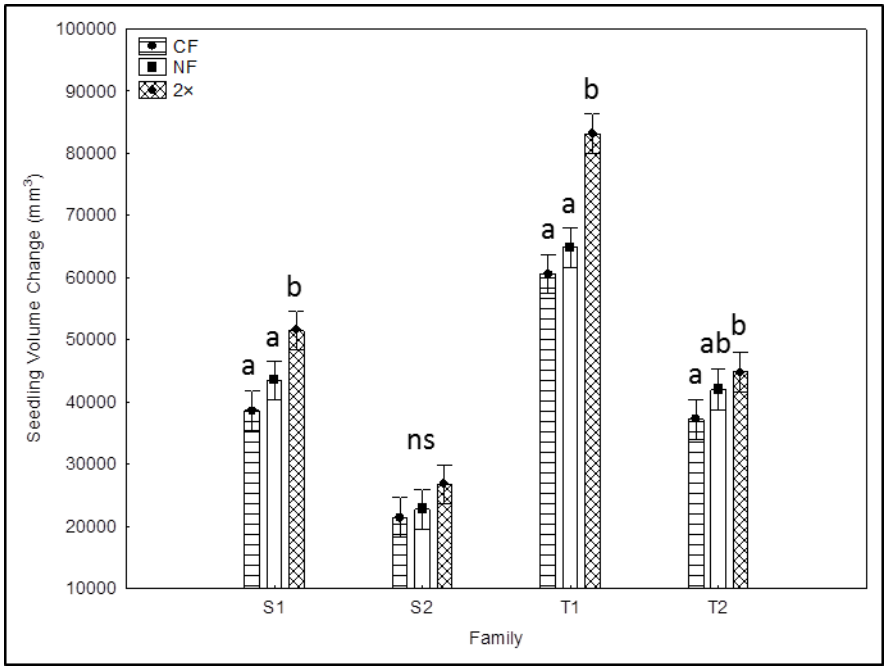


**Figure 2.2**. Seedling volume change from January to August 2013 by family and ozone treatment. Letters are from Tukey pair-wise comparisons (specific to each family). ns = no significant difference for each family between ozone treatments. CF = charcoal filtered, NF = non-filtered, 2× = twice NF. S1 and S2 denote families chosen for susceptibility to root infecting ophiostomatoid fungi while T1 and T2 denote families chosen for their tolerance to the fungi. Bars denote 95% confidence intervals.

**Table 2.3.** ANOVA *P*-values for each treatment combination by measurements

| Measurement | | Seedling Volume Change | Dry Matter Yield | | | | | Plant Ozone Injury | Leaf Ozone Injury | SPAD (needle greenness) | Predawn Water Potential | Midday Water Potential | Lesion Length | Lesion Length/ Seedling Height |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Needles | Shoots | Coarse Roots | Fine Roots | Total | | | | | | | |
| ANOVA F-Test *P*-Values by Treatment Combination | Family | < 0.0001*** | < 0.001** | < 0.001** | < 0.001** | < 0.001** | < 0.0001*** | < 0.0001*** | < 0.0001*** | < 0.0001*** | N/A | 0.001* | 0.026* | < 0.001** |
| | O3 | < 0.0001*** | < 0.001** | 0.046* | 0.682 | 0.913 | 0.008* | < 0.0001*** | < 0.0001*** | 0.037* | N/A | < 0.0001*** | 0.014* | 0.002* |
| | Inoculation | 0.585 | 0.523 | 0.499 | 0.531 | 0.884 | 0.360 | 0.151 | 0.091 | 0.942 | N/A | < 0.001** | < 0.001** | < 0.001** |
| | Family* O3 | < 0.001** | 0.669 | 0.370 | 0.272 | 0.458 | 0.446 | < 0.001** | < 0.001** | 0.248 | N/A | 0.958 | 0.880 | 0.517 |
| | Family* Inoculation | 0.041* | 0.568 | 0.022* | 0.189 | 0.864 | 0.265 | 0.597 | 0.268 | 0.182 | N/A | 0.421 | 0.211 | 0.324 |
| | O3* Inoculation | 0.379 | 0.054 | 0.712 | 0.203 | 0.577 | 0.159 | 0.849 | 0.108 | 0.565 | N/A | 0.038* | 0.304 | 0.183 |
| | Family*O3* Inoculation | 0.958 | 0.594 | 0.235 | 0.696 | 0.139 | 0.321 | 0.528 | 0.779 | 0.079 | N/A | 0.700 | 0.260 | 0.088 |

### 2.4.3 Dry Matter Yield

Needle dry matter yield (DMY) and shoot DMY were found to be similar among treatments so the results for aboveground DMY (needles + shoots) are reported. Coarse root DMY and fine root DMY were found to be similar so reported are the results for belowground DMY (coarse roots + fine roots).

Seedlings exposed to elevated ozone had greater (12.8%) aboveground DMY compared to CF seedlings (Table 2.3). Inoculation treatments had no effect on seedling aboveground DMY ($P = 0.499$). Regarding families, T1 had the greatest aboveground DMY and S2 had the least (36.8% less than T1, $P < 0.0001$). S1 and T2 had intermediate aboveground DMY but were not different (14.6% and 19.8% less than T1 respectively, $P = 0.383$). There were no other significant interactions between treatments or treatment combinations (Table 2.3).

Seedlings grown in CF chambers had less belowground DMY (14.9%) compared to trees grown in 2× chambers (Table 2.3). Inoculation had no effect on seedling belowground DMY ($P = 0.531$). Examining the family main effects (Table 2.3), T1 had the greatest belowground DMY and S2 had the least ($P < 0.0001$, 51.3% less than T1). S1 and T2 had belowground DMY between S2 and T1 but were not different ($P = 0.995$, 29.8% and 27.1% less than T1 respectively). There were no other significant interactions between treatments or treatment combinations (Table 2.3).

Seedlings exposed to elevated ozone had an increase in total DMY compared to seedlings grown in CF chambers (11.9%, $P = 0.003$, Figure 2.3). Inoculation had no effect on total seedling DMY ($P = 0.360$). T1 had the greatest total DMY and S2 had the least (40.7% less than T1, $P < 0.0001$). S1 and T2 had intermediate total DMY but were not different (17.7% and 21.3% less than T1 respectively, $P = 0.600$). There were no other significant interactions between treatments or treatment combinations (Table 2.3).

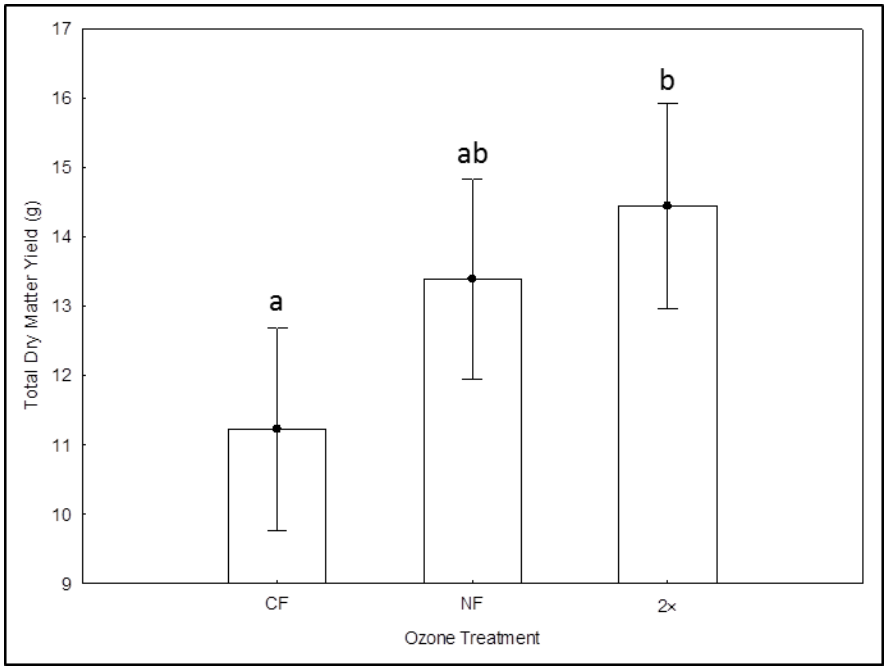


**Figure 2.3**. Total dry matter yield of seedlings by ozone treatment. Letters are from Tukey pair-wise comparisons. CF = charcoal filtered, NF = non-filtered, 2× = twice NF. Bars denote 95% confidence intervals.

### 2.4.4 Visible Injury

Seedlings grown in CF chambers had no visible ozone symptoms and are not included in this section. Incidence of ozone injury in families S1 and S2 were greater (52.9%) than those found in T1 and T2 (Table 2.4). The severity of ozone injury regarding whole plants and needles were similar (Figure 2.4). Seedlings exposed to elevated ozone exhibited 10.6 × more symptoms on whole plants ($P < 0.0001$, Figure 2.4). Ambient (NF) ozone exposures resulted in no significant difference in visible ozone injury between the four families tested ($P > 0.808$), however susceptible families (S1 and S2) exposed to elevated ozone (2×) had 2.4 × more injury than tolerant families ($P < 0.014$). Seedlings exposed to elevated ozone were found to have 9.9 × more ozone injury on needles ($P < 0.0001$, Figure 2.4). Susceptible families (to root infecting ophiostomatoid fungi) of loblolly pine were found to have 3 × more needle ozone injury compared to tolerant families ($P < 0.0001$). Injury levels on family T1, when exposed to elevated ozone, were not different than injury levels on S2 seedlings in NF chambers ($P = 0.481$).

Inoculation had no role in whole plant or leaf level ozone injury ($P > 0.091$). No other interactions were found to be significant (Table 2.3).

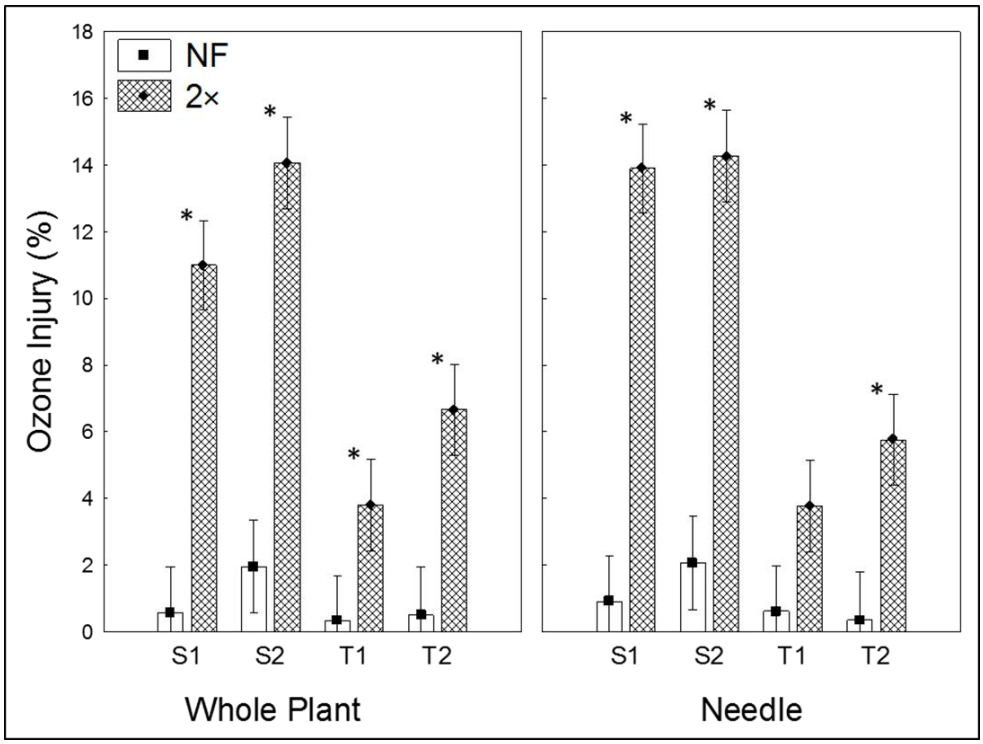


**Figure 2.4**. Percent injured by family by ozone treatment from August 2013. A = ambient, 2× = twice NF. S1 and S2 denote families chosen for susceptibility to root infecting ophiostomatoid fungi. T1 and T2 denote families chosen for tolerance. Asterisks denote a significant difference within each family. Bars indicate 95% confidence intervals.

**Table 2.4**. Incidence (%) of ozone injury by family at final harvest (August 2013)

| Incidence (%) of Ozone Injury | | | | |
|---|---|---|---|---|
| Pine Family | CF | NF | 2× | Total |
| S1 | 0% | 11% | 70% | 28% |
| S2 | 0% | 27% | 78% | 35% |
| T1 | 0% | 5% | 37% | 14% |
| T2 | 0% | 6% | 42% | 17% |
| Total | 0% | 12% | 57% | 23% |

CF = charcoal filtered, A = ambient, 2× = twice NF. S1 CF, n = 115; S1 NF, n = 118; S1 2×, n = 124; S2 CF, n = 113; S2 NF, n = 113; S2 2×, n = 116; T1 CF, n = 115; T1 NF, n = 120; T1 2×, n = 115; T2 CF, n = 112; T2 NF, n = 106; T2 2×, n = 118. S1 and S2 denote families chosen for susceptibility to root infecting ophiostomatoid fungi. T1 and T2 denote families selected for tolerance to root infecting ophiostomatoid fungi.

### 2.4.5 Needle Greenness

Seedlings grown in CF chambers were found to have no significant difference in needle greenness compared to other chamber treatments ($P > 0.224$), however 2× seedlings had lower needle greenness (13.7%) than those grown in NF chambers ($P = 0.021$, Figure 2.5). Loblolly pine family S2 was found to have the lowest needle greenness compared to other families (5.0 – 8.6%, $P < 0.0001$), however, other families had no difference between them ($P > 0.194$). No other treatments or treatment combinations had significant effects on needle greenness (Table 2.3).

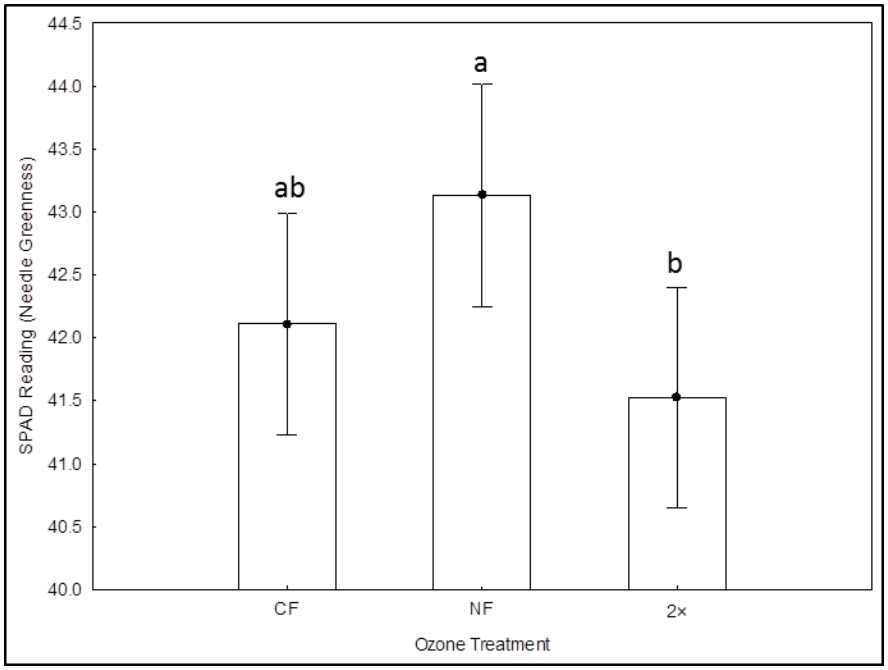


**Figure 2.5**. Needle greenness by ozone treatment. Letters are from Tukey pair-wise comparisons. CF = charcoal filtered, NF = non-filtered, 2× = twice NF. Bars denote 95% confidence intervals.

### 2.4.6 Midday Water Potential

Seedlings grown in NF and 2× chambers were not different for midday water potential (Table 2.3). Seedlings grown in CF chambers, however, were 12.1% and 9.6% more water stressed than those grown in NF and 2× treatments ($P < 0.0001$). Family T2 was found to be more water stressed than S1 and S2 seedlings (6.1% and 7.1% respectively, $P = 0.001$). Seedlings inoculated with GH and LT were more water stressed ($P < 0.0001$) than NW, W and WM controls (22.1% and 24.6% respectively, Figure 2.6). Families selected for susceptibility to root infecting ophiostomatoid fungi were more water stressed when inoculated with GH or LT than the controls (S1 – 16.8% and 23.8% more stressed than the control average, $P < 0.0001$; S2 – 21.9% and 22.6% more stressed than the control average, $P < 0.002$). There were no other significant interactions between treatments and treatment combinations relevant to the studies hypotheses (Table 2.3).

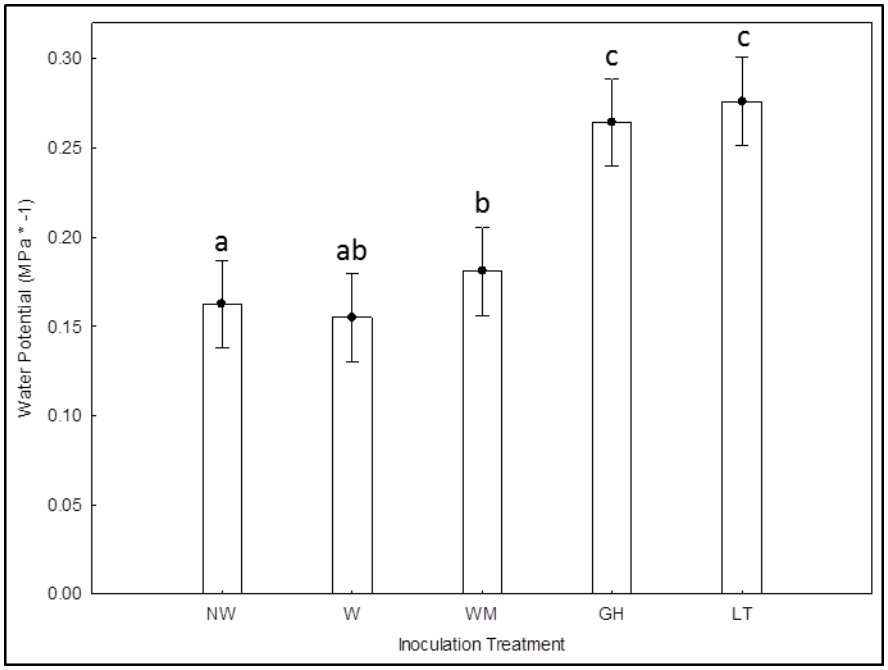


**Figure 2.6**. Midday water potentials (megapascals*-1) of seedlings by inoculation treatment. Letters are from Tukey pair-wise comparisons. NW = no wound, W = wound only, WM = wound+media, GH = *G. huntii*, LT = *L. terebrantis*. Bars denote 95% confidence intervals.

### 2.4.7 Lesion Measurements

Overall, lesion length and lesion length ratio were affected by ozone concentration (Table 2.3). Seedlings exposed to elevated ozone (2×) were found to have longer lesions ($P < 0.010$) compared to NF and CF grown seedlings; 2.4% and 2.8% smaller respectively. Lesion length ratio (lesion length/seedling height) was also greater ($P = 0.018$) in 2× chambers compared to NF and CF grown seedlings as shown in Figure 2.7; 7.5% and 7.8% smaller respectively. Seedlings grown in 2× chambers also had greater lesion length ratios. Families S1, S2 and T1 did not significantly differ in overall lesion length ($P > 0.262$); however T2 lesion length was found to be at least 2.4% greater than all other families ($P < 0.028$). However, relative to seedling height, lesion length (lesion length ratio) was found to greatest in S2 while T1 had the smallest lesion length ratio (25.1% smaller, $P < 0.0001$). T1 was found to have the second largest lesion ratio (7.8% smaller than S2, $P = 0.044$) while S1 was found to have the third largest lesion length ratio (16.3% smaller than S2, $P < 0.0001$).

Inoculated seedling W and WM controls did not differ in lesion length ($P = 0.416$) or lesion length ratio ($P = 0.744$). Seedlings inoculated with LT were found to have lesion lengths 13.7% greater than the control average (the average of the W and WM estimated means) and 8.6% greater than GH inoculated seedlings ($P < 0.0001$). Seedlings inoculated with LT had lesion length ratios 45.7% greater than the control average and 29.0% greater than GH inoculated seedlings ($P < 0.0001$). Seedlings inoculated with GH were found to have lesion lengths 15.9% greater than W inoculated seedlings ($P = 0.005$) but were not different than WM inoculated seedlings ($P = 0.060$).

S1 seedlings inoculated with LT had lesion lengths 17.9% greater than the control average and 8.8% greater than GH inoculated seedlings ($P < 0.001$). S1 seedlings inoculated with GH were found to have lesion lengths 8.3% greater than the control average ($P < 0.046$). S1 seedlings inoculated with LT were found to have lesion length ratios 68.0% greater than the control average and 34.5% greater than GH inoculated seedlings ($P < 0.0001$), however, GH inoculated seedlings were not different than the control average ($P > 0.225$). S2 seedlings inoculated with LT had lesion lengths 11.2% greater than the control average and 9.1% greater than GH inoculated seedlings ($P < 0.001$), however GH inoculated seedling were not different than the control average ($P = 0.999$). S2 seedlings inoculated with LT were found to have lesion length ratios 29.3% greater than the control average and GH inoculated seedlings ($P < 0.008$). T1 seedlings inoculated with LT were found to have lesion lengths 15.3% greater than the control average and 10.4% greater than GH inoculated seedlings ($P < 0.001$), however, GH inoculated seedlings were not different than the control average ($P > 0.291$). T1 seedlings inoculated with LT were found to have lesion length ratios 52.1% greater than the control average and 31.7% greater than GH inoculated seedlings ($P < 0.005$). T2 seedlings inoculated with LT had lesion lengths 10.8% greater than the control average and 6.4% greater than GH inoculated seedlings ($P < 0.043$), however, GH inoculated seedlings were not different than the control average ($P > 0.799$). T2 seedlings inoculated with LT were found to have lesion length ratios 40.5% greater than the control average and 25.4% greater than GH inoculated seedlings ($P < 0.005$), however, GH inoculated seedlings were not different than the control average ($P > 0.963$). There were no other significant interactions between treatments and treatment combinations (Table 2.3).

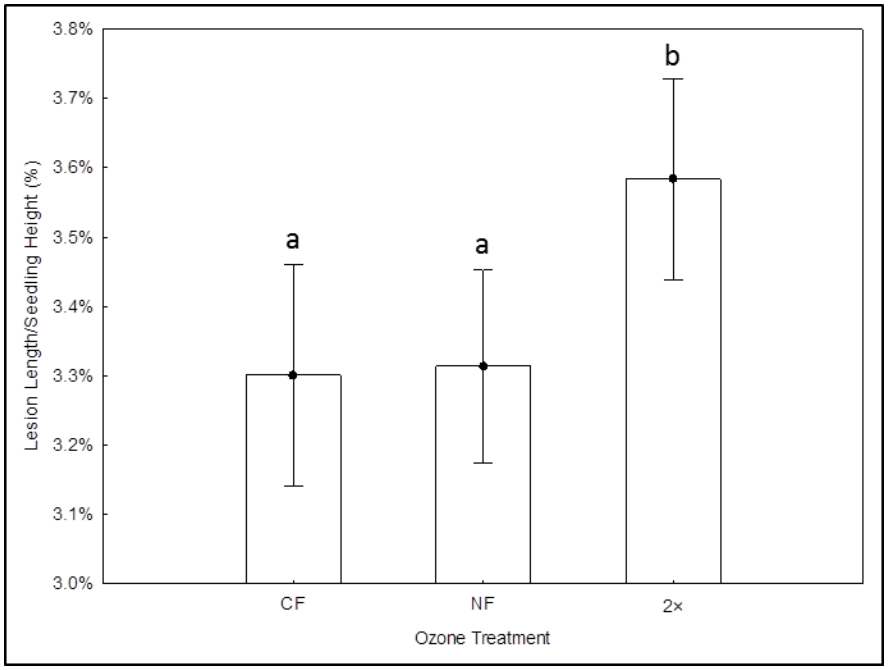


**Figure 2.7**. Lesion lengths relative to seedling heights. Letters are from Tukey pair-wise comparisons. CF = charcoal filtered, NF = non-filtered, 2× = twice NF. Bars denote 95% confidence intervals.

### 2.4.8 Fungal Growth Study

*Ozone Exposure and Temperature Data*

The highest 12-hour ambient ozone concentration (NF) was 37 ppb on the first and second day of the experimental period. The 5-day ozone average for each chamber was 15, 28 and 50 ppb for CF, NF and 2× treatments, respectively. The maximum ozone concentration (94 ppb) occurred on the third day of the experimental period in the 2× chamber treatment (Table 2.5). The maximum, average and minimum in the wood-box during the experimental period was 41, 27 and 21 °C. The OTC temperature maximum, average and minimum was 39, 27 and 20 °C. Based on these temperature readings we determined the difference between the OTC and wood box was negligible and unlikely to affect the fungal growth.

**Table 2.5**. 12-hour ozone concentration (ppb) for the 5 day experimental period

| Ozone concentration (12-hr ppb) by ozone treatment | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Date | Day | CF | | | NF | | | 2× | | |
| | | Min | Avg | Max | Min | Avg | Max | Min | Avg | Max |
| 3-Aug | 1 | 6 | 14 | 20 | 9 | 27 | 37 | 9 | 34 | 79 |
| 4-Aug | 2 | 9 | 16 | 22 | 14 | 30 | 37 | 14 | 42 | 82 |
| 5-Aug | 3 | 8 | 14 | 18 | 13 | 28 | 35 | 15 | 63 | 94 |
| 6-Aug | 4 | 5 | 12 | 20 | 10 | 25 | 36 | 8 | 52 | 89 |
| 7-Aug | 5 | 11 | 17 | 24 | 18 | 29 | 36 | 22 | 57 | 80 |
| **Average** | | **8** | **15** | **21** | **13** | **28** | **36** | **14** | **50** | **85** |

*Fungal Growth Analysis*

Overall, of the four root infecting ophiostomatoid fungi examined, *L. terebrantis* was found to have the greatest growth ($P < 0.003$). *Grosmannia alacris* had the slowest growth (51.1% lower than LT, $P < 0.005$). *Leptographium procerum* and *G. huntii* growth did not from each other ($P > 0.061$). Both *L. procerum* and *G. huntii* growth were 23.5% and 36.9% less than *L. terebrantis* ($P < 0.005$). *Leptographium terebrantis*, *L. procerum* and *G. alacris* had no difference in growth between ozone treatments ($P > 0.788$, $P > 0.622$ and $P > 0.071$ respectively). *Grosmannia huntii* was not significantly different in growth between NF and CF chambers ($P = 0.999$), however, *G. huntii* growth was reduced when exposed to elevated ozone by 41.7% and 35.8% respectively ($P < 0.003$, Figure 2.8).

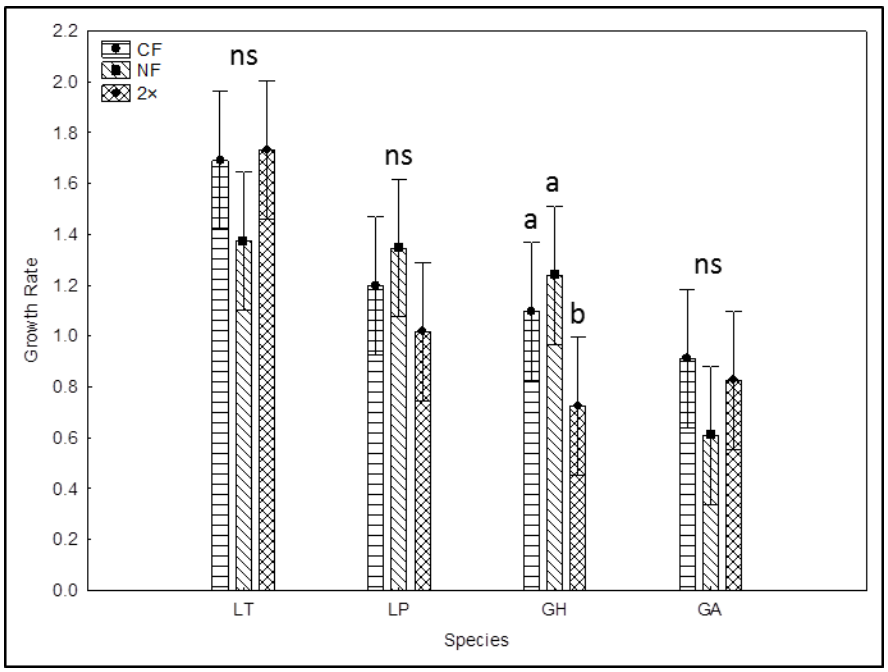


**Figure 2.8**. Growth (final area $cm^2$ – initial area $cm^2$ / final area $cm^2$) for each fungus by ozone treatment. Letters are from Tukey pair-wise comparisons. ns = no significant different for each fungus between ozone treatments. CF = charcoal filtered, NF = non-filtered, 2× = twice NF. LT = *L. terebrantis*, LP = *L. procerum*, GH = *G. huntii*, GA = *G. alacris*. Bars denote 95% confidence intervals.

## 2.5 DISCUSSION

Previous research has found links between air quality and increase susceptibility to various biotic agents (Lackner and Alexander 1983, Fenn et al. 1990, James et al. 1980). To address this question, we placed loblolly pine seedlings inoculated with root infecting ophiostomatoid fungi into OTCs. The late summer of 2013 was cooler and wetter than the 30-year average for Auburn, AL. These conditions are not ideal for ozone formation (U.S. EPA 2006, Seinfeld and Pandis 2012) and likely caused reductions in normal ambient ozone levels during July and August of 2013.

Although multiple exposures to ozone have resulted in reductions in growth of loblolly pine (Taylor 1994), there is evidence that shorter durations of ozone exposure can increase aboveground growth (Spence et al. 1990). Spence et al. (1990) hypothesized that ozone injury could affect phloem loading from needles resulting in a reduction in photosynthate transport to

roots. They found the lack of transport caused accumulation of photosynthates in stems and branches which in turn increased aboveground dry matter yield 50-60%. This mechanism may be important to aboveground growth during seasonal ozone fumigation periods observed in these OTC trials, but longer exposures may eventually overcome plant defense mechanisms resulting in growth decreases (Waring 1987, Lefohn 1992, Anderson 2003). The loblolly pine families selected for their tolerance to root infecting ophiostomatoid fungi were larger (volume and dry matter). Perhaps the resiliency of these families is based upon reserves of carbohydrates. Carey and Kelley (1994) suggested aboveground and belowground growth competed as energy sinks. We did not observe this interaction, rather drew conclusions similar to those reported by Spence et al. (1990) and Anderson et al. (1997), where short fumigation periods caused increase aboveground growth.

The exposure to the loblolly pine families to ozone reduced loblolly pine vigor, however, lesions produced from *L. terebrantis* and *G. huntii* were no greater given increasing ozone concentrations than those from NF and CF treatments. The increase in lesion length was observed when all inoculation treatments were averaged indicating loblolly pine became more susceptible to mechanical stress, and possibly, root infecting ophiostomatoid fungi. The susceptible loblolly pine families (S1 and S2) exhibited greater incidence and severity of ozone injury than the tolerant families (T1 and T2). This indicates that tolerance to root infecting ophiostomatoid fungi and ozone sensitivity may be linked in the loblolly pine families tested in this experiment. Based on the results of the fungal growth study, of the four fungal pathogens, only *G. huntii* had a reduced growth when exposed to elevated ozone concentrations.

Decreased photosynthetic rates have been observed under elevated ozone conditions in loblolly pine (Taylor 1994, Spence et al. 1990, Cooley and Manning 1987). Along with a decrease in chlorophyll content (needle greenness) (Richardson et al. 2002), seedlings exposed to ozone exhibited increased needle injury, typically expressed as chlorotic mottling (Grulke and Lee 1997). It should be noted that family T1 when exposed to elevated ozone displayed injury levels similar to those found on S2 seedlings grown in NF chambers. This indicates that there is variation in ozone sensitivity between loblolly pine families used in this study and that T1 was the least sensitive. The link between susceptibility to root infecting ophiostomatoid fungi seems to be linked to levels of ozone injury, indicating seedlings that are more susceptible to root infecting ophiostomatoid fungi also are more sensitive to ozone. Disease tolerance and ozone

sensitivity may be linked, but as mentioned above, tolerant families were, in general, larger and may be able to compensate for reductions in carbon uptake and decreased phloem loading.

Ozone had no significant interactions on plant midday water potential. Rather, inoculation treatment played a significant role in water regulation. The lesions caused by the fungi *L. terebrantis* and *G. huntii* likely inhibited water uptake as all seedlings were irrigated evenly throughout the duration of the study (Wingfield 1983, Paine 1984, Owen et al. 1987).

Although three of the four pine families used in the study had increased growth when exposed to tropospheric ozone, we also saw larger lesion lengths relative to the seedling size. This indicates that seedlings did become more susceptible when exposed to elevated ozone concentrations. Overall lesions caused by the fungi may be inhibited in host plants exposed to ozone via system acquired resistance (Sandermann et al. 1998). Carey and Kelley (1994) found similar results regarding lesion length but were able to determine the pathogen (*F. circinatum*) caused a larger lesion when exposed to elevated ozone concentrations. However, in our study, fungal growth may have been inhibited by the presence of ozone, as seen with *G. huntii* during the plate growth study. It is important to note that while *G. huntii* growth on media was retarded by ozone, these fungi are typically found within roots of mature trees and therefore would not be affected by ambient ozone concentrations.

Because seedlings were observed to increase in aboveground growth when exposed to elevated ozone, we felt it necessary to determine if the percentage of the tree colonized (lesion length ratio) was greater. We did find that overall lesion length ratio increased with elevated ozone concentrations but the relationship was not specific to any inoculation treatment. Whether or not ozone affected the ability of the fungi to colonize seedling stems in our study is debatable, however, seedlings were increasingly susceptible to mechanical stress. Mechanical stress can have negative impacts on plant water transport (Sperry 2011); however, we only saw this in seedlings inoculated with root infecting ophiostomatoid fungi.

Our results suggest that families susceptible to root infecting ophiostomatoid fungi also may be more sensitive to changes in climatic conditions, in this case elevated ozone concentrations. Incidence and severity of ozone injury was greater for seedlings that were selected for susceptibility than those selected for tolerance. Needles from the SPD susceptible seedlings did show symptoms of ozone injury, primarily chlorotic mottling characteristics of ozone injury (Grulke and Lee 1997).

## 2.6 CONCLUSIONS

While open-top chambers and the wound+inoculum method are useful tools in studying interactions between air quality and plant pathogens, there are many limitations (Lefohn 1992). Outlined by Lefohn (1992), limitations include problems drawing comparisons between ozone treatments, limited space, microclimate effects on soil moisture, and pest/pathogen incidence and increased plant growth at cooler ambient temperatures. Particularly with root diseases, fungal pathogens and air quality may interact directly and cause unexplainable anomalies within the data that would not occur in field conditions (Manning and von Tiedemann 1995). In the case of this study, the response of fungal growth to elevated ozone concentrations was examined but could not be controlled. It is important to improve on these methodologies in the future given expected changes in ecosystem processes (Paoletti et al. 2009, Kirilenko and Sedjo 2007).

To our knowledge, this is the first study to examine the interaction of loblolly pine seedlings inoculated with root infecting ophiostomatoid fungi and ozone. While North American emissions of ozone precursors have decreased in the last few decades, the role global emissions and transport of pollutants have become increasingly important (Cooper et al. 2012). In the Southeastern U.S., populations have increased in recent years and are expected to cause alterations to the landscape (U.S. Bureau of the Census 2009, Wear and Greis 2002, Milesi et al. 2003). This will likely cause increasing biogenic volatile organic compounds emissions, increased $NO_x$ from increased automobile use and increased temperatures associated with climate change causing ozone concentrations to increase in the Southeastern U.S. (Gonzalez-Abraham et al. 2014).

The role of pine production in the Southeastern U.S. is profound (Prestemon and Abt 2002). Climate change is expected to change the intensity and frequency of extreme weather events as well as insect/disease outbreaks (Paoletti et al. 2009, Kirilenko and Sedjo 2007, Bentz et al. 2010). It is important to acknowledge that the 'business-as-usual' philosophy on forest regeneration and management no longer applies in a global or regional context. The interactions between air pollutants and plant pathogens are likely to increase in occurrence and therefore it is important to gather information on these interactions to understand how to protect forests in the future.

Overall, in our study susceptible families did not perform worse than tolerant families in all facets, however, the chamber environment is likely to disrupt normal physiological processes

and change the biological behavior of each family uniquely (Lefohn 1992, Adams et al. 1988). Lefohn (1992) reported changes in microhabitat conditions and growth patterns of plant species grown in OTCs. Given the unknown physiological changes with the loblolly pine families used in OTCs, it is difficult to determine if the dichotomy of tolerant-susceptible remains valid.

In conclusion, regardless of loblolly pine family we tested, seedlings were affected on several physiological parameters by both ozone concentration and inoculation treatment. The main treatment factors affecting physiological variables were ozone concentration and pine family. There is no conclusive evidence to support that the relationship between ozone and SPD fungi is synergistic/antagonistic. We do know that susceptibility to fungi is increased under elevated ozone growing conditions but we would recommend a longer experimental duration as well as increasing the number of families used in future studies to strengthen our conclusions regarding links between susceptibility to root infecting ophiostomatoid fungi and ozone sensitivity.

# Chapter 3

## Interaction of simulated rainfall treatments on Loblolly pine seedlings inoculated with root infecting ophiostomatoid fungi

### 3.1 ABSTRACT

Seedlings from four families of loblolly pine (*Pinus taeda* L.) grow in capped open-top chambers were exposed to three different weekly moisture regimes. Moisture regimes varied in intensity and frequency of irrigation events, and were the same in the amount of irrigation received. Two of the families were selected for their tolerance to root infecting ophiostomatoid fungi, while the other two were selected for their susceptibility. Seedlings were inoculated with five inoculation treatments: no wound, wound only, wound+media, *Grosmannia huntii* and *Leptographium terebrantis*. After 13 weeks of moisture and inoculation treatments, seedlings were harvested. Seedling volume dry matter, yield, relative needle greenness, water potential and lesion characteristics were measured and analyzed for treatment effects using ANOVA procedures. Moisture stress increased susceptibility to inoculation in one loblolly pine family, however the increase was not specific to either root infecting ophiostomatoid fungi. In general, seedlings irrigated 7 days.week$^{-1}$ showed greater chlorophyll content and growth compared to seedlings irrigated 4 days.week$^{-1}$. Seedlings irrigated 3 days.week$^{-1}$ appeared to begin to reduce metabolic functions as confidence intervals were larger than other moisture treatments. In conclusion, moisture had no effect on the virulence of the zfungi.

**Keywords** – altered precipitation, loblolly pine, Southern Pine Decline, *Leptographium terebrantis*, *Grosmannia huntii*

### 3.2 INTRODUCTION

Southern Pine Decline (SPD) is the term attributed to the premature death of *Pinus* spp. in the Southern U.S. due to a series of biotic and abiotic factors (Harrington and Cobb 1983, Otrosina et al. 1997, Eckhardt et al. 2007). These factors include associated root pathogenic

fungi (*Leptographium* and *Grosmannia* spp.) and their root-feeding beetle vectors (*Hylastes salebrosus*, *H. tenuis*, *Hylobius pales*, and *Pachylobius picivorus*). Predisposing abiotic factors include resource stress (nutrient deficiencies, edaphic factors, and moisture stress), management strategies such as overstocking, mechanical injury and prescribed burning (Eckhardt et al. 2010). Studies have shown that when loblolly pine is inoculated with *Leptographium terebrantis*, the fungus can result in the development of lesions in the phloem and resin-soaking in the xylem (Wingfield 1983, Eckhardt et al.2004, Matusick and Eckhardt 2010). *Grosmannia huntii* is a related fungal pathogen and has been reported to be more virulent on young pine seedlings when compared to *L. terebrantis* (Matusik and Eckhardt 2010).

Future climate change scenarios may play a significant role in the predisposing factors associated with SPD. A comparison of two global circulation climate models indicate an increase in mean temperature globally (MacCracken et al. 2000), although the rate of increase is uncertain (MacCracken et al. 2000, IPCC 2013). Another uncertainty is how much precipitation will occur in the Southeastern U.S. in the next 50-100 years (MacCracken et al. 2000, IPCC 2013). Observed precipitation patterns over the past 60 years may indicate future rainfall for a region. For example, in 2007 the worst drought in 100 years occurred in the Southern U.S. and was followed by flooding in 2009 (Wang et al. 2010). While changes in the intensity and frequency of summer precipitation may continue in the Southeastern U.S., there is still debate as to its underlying cause (Wang et al. 2010, Li et al. 2011, Seager et al. 2009). Another trend in precipitation patterns has been the daily variation in precipitation events where storms are occurring less frequently but are characterized by more intense rainfall for longer durations in North America (Kunkel et al. 2013, Muschinski and Katz 2013, IPCC 2013).

A concern when considering future precipitation patterns is how forests will respond to altered drying and wetting periods (Hanson and Weltzin 2000, MacCracken et al. 2000). Trees may thrive during wetter periods and experience moisture stress if evaporative losses increase during warmer, drier periods (Neilson and Drapek 1998). Changes in precipitation patterns can have cascading effects throughout forest ecosystems. Droughts can reduce tree vigor and alter insect and pathogen physiology (Dale et al. 2001). The body of literature on this subject is extensive (Desprez-Loustau et al. 2006), however the effects of precipitation changes are anticipated to be unique based on the host's and pathogen's physiology (Rouault et al. 2006, Sturrock et al. 2011).

The impact that changes in precipitation will have on insect populations in the Southeastern U.S. needs to be understood, specifically for those insects that vector pathogenic fungi (e.g. ophiostomatoid fungi) (Kirisits 2004, Bentz et al. 2010). One example is the influence of drought stress and its impact on host trees and subsequent influence on bark beetle populations. It has been shown that an increase in drought stress of the host tree results in greater infestations of bark beetles (Jones et al. 2001, Jactel et al. 2012, Klepzig et al. 2004, Koricheva et al. 1998). Host stress is only a single factor in this interaction, as insect physiology and ecology shifts can have effects on the host-insect relationship as well (Clarke and Fraser 2004, Gillooly et al. 2001).

Numerous studies have examined the effects of precipitation changes on plants and fungal pathogen interactions. These studies usually compare a sufficiently watered control and a reduced water treatment (Seiler and Johnson 1988, Goheen et al. 1978, Croisé et al. 2001, Meier et al. 1990, Matusick et al. 2008). While these studies provide insight as to host plant responses to periods of reduced moisture availability, less is known as to the impact that fluctuating moisture availability will have on host-pathogen interactions. Some studies indicate that fluctuating moisture availability results in decreased productivity of loblolly pine (Tschaplinski et al. 199).

In an assessment of the effect of potential future climate change scenarios for the Southeastern U.S., Jones et al. (2001) stated that changes in water availability are important and require further investigation. Water availability can cause alterations in loblolly pine vigor resulting in biotic organisms, such as *L. terebrantis* or *G. huntii,* potentially exacerbating declines and reducing productivity.

The overall objective of this study was to elucidate the interactions of two root infecting ophiostomatoid fungi (*L. terebrantis* and *G. huntii*) in the presence of climatic conditions predicted in the next 50 to 100 years in the Southeastern U.S. More specifically, our main aim was to understand how changes in precipitation patterns may affect loblolly pine infected with the root infecting ophiostomatoid fungi. The hypotheses tested include: (1) loblolly pine will become more susceptible to *L. terebrantis* and *G. huntii* as the irrigation regime is altered in intensity and frequency; and (2) loblolly pine families selected for their tolerance to root infecting ophiostomatoid fungi would be more tolerant to changes in the intensity and frequency.

## 3.3 MATERIALS AND METHODS

### 3.3.1 Study Site and Capped Open-Top Chamber

The research site (approximately 0.02 $km^2$ in area) is located approximately 5 km North of Auburn University Campus, Auburn, AL, U.S. The site contained 24 open-top chambers (OTCs), monitoring sheds and a small laboratory. The OTCs were 4.8 m height x 4.5 m diameter aluminum framed structures with fans (1.5 horse-power motors) and chamber plastics (Gilliland et al. 2012). Plastic caps were attached to each OTC to exclude ambient rainfall and permitted adequate airflow (Heagle et al. 1989).

Prior to the commencement of the study (March 2014), the vegetation growing in each OTC was killed with a 3% solution of glyphosate. Once dead, the vegetation was removed prior to the ground being covered with landscape fabric to prevent further unwanted vegetation growth within each OTC.

### 3.3.2 Seedlings

Bareroot seedlings from four commercially grown loblolly pine families were used for this study (lifted/extracted from the nursery in November 2013). Based on previous studies, two of these loblolly pine families were considered "tolerant" (T1 and T2) and two "susceptible" (S1 and S2) to ophiostomatoid root infecting ophiostomatoid fungi (Singh et al. 2014). In January 2014, 2700 seedlings (750 per family) were planted in trade gallon pots with ProMix BX® peat-based potting mix (Premier Tech, Quebec, Canada). Seedlings were kept under a shade house and watered daily for 17 weeks until being deployed into the OTCs in May 2014.

### 3.3.3 Irrigation Treatments

To determine the longest duration the potting mix could go before difficulty rewetting, eight seedlings (two from each family) were placed in a greenhouse at approximately 90 °F (~32 °C). After 3 days of water being withheld, the potting mix became dried out and therefore, the longest period between irrigation events was set at 2 days.

Three simulated precipitation treatments were used (with each treatment having 3 replicates). The treatments were as follows: (1) irrigated 3 $days.week^{-1}$ (3D) during the experimental period, (2) irrigated 4 $days.week^{-1}$ (4D) during the experimental period, (3) irrigated daily (7D). Irrigation nozzles within each OTC were adjusted to ensure an even water

distribution and flow rates within and between the chambers. These were adusted to ensure 58 minutes of irrigation resulted in 1 inch or 25.4 mm of precipitation. While the days of irrigation varied between treatments, each chamber received the same amount of precipitation at the end of each week (Table 3.1). Weekly irrigation values were estimated based on the 30-yr (1971-2000) average precipitation for Auburn, AL. In June 2014 a 20% increase was given to all treatment amounts to compensate for higher temperatures and increased airflow in the chamber (Table 3.2). Monitoring throughout June indicated this adjustment approximately offset the increased loss of moisture in the chambers.

**Table 3.1**. Summary of irrigation treatments (minute quantities) by month

| Month | Week | Days of Rain | Minutes/Day | Minutes/Week | Amount/Week (cm) |
|---|---|---|---|---|---|
| May | 1 | 7D | 9 | 63 | 2.8 |
| | | 4D | 15 | 60 | 2.6 |
| | | 3D | 21 | 63 | 2.8 |
| | 2 | 7D | 9 | 63 | 2.8 |
| | | 4D | 15 | 60 | 2.6 |
| | | 3D | 21 | 63 | 2.8 |
| June | 3 | 7D | 9 | 63 | 2.8 |
| | | 4D | 15 | 60 | 2.6 |
| | | 3D | 21 | 63 | 2.8 |
| | 4 | 7D | 9 | 63 | 2.8 |
| | | 4D | 15 | 60 | 2.6 |
| | | 3D | 21 | 63 | 2.8 |
| | 5 | 7D | 9 | 63 | 2.8 |
| | | 4D | 15 | 60 | 2.6 |
| | | 3D | 21 | 63 | 2.8 |
| | 6 | 7D | 11 | 76 | 3.3 |
| | | 4D | 18 | 72 | 3.2 |
| | | 3D | 25 | 76 | 3.3 |
| July | 7 | 7D | 14 | 101 | 4.4 |
| | | 4D | 25 | 101 | 4.4 |
| | | 3D | 32 | 97 | 4.3 |
| | 8 | 7D | 14 | 101 | 4.4 |
| | | 4D | 25 | 101 | 4.4 |
| | | 3D | 32 | 97 | 4.3 |
| | 9 | 7D | 14 | 101 | 4.4 |
| | | 4D | 25 | 101 | 4.4 |
| | | 3D | 32 | 97 | 4.3 |
| | 10 | 7D | 14 | 101 | 4.4 |
| | | 4D | 25 | 101 | 4.4 |
| | | 3D | 32 | 97 | 4.3 |
| | 11 | 7D | 14 | 101 | 4.4 |
| | | 4D | 25 | 101 | 4.4 |
| | | 3D | 32 | 97 | 4.3 |
| August | 12 | 7D | 11 | 76 | 3.3 |
| | | 4D | 18 | 72 | 3.2 |
| | | 3D | 25 | 76 | 3.3 |
| | 13 | 7D | 11 | 76 | 3.3 |
| | | 4D | 18 | 72 | 3.2 |
| | | 3D | 25 | 76 | 3.3 |

7D = irrigated 7 days.week$^{-1}$. 4D = irrigated 4 days.week$^{-1}$. 3D = irrigated 3 days.week$^{-1}$

**Table 3.2**. Summary of irrigation treatments (rainfall quantity) compared to the average precipitation for Auburn, AL (1971-2000) (AWIS, Inc.).

| Month | Weeks | Days of Rain (chamber) | Total Irrigation (cm) | Monthly Average For Study (cm) | Monthly Average (1971-2000) | Monthly Average (1971-2000) + 20% |
|---|---|---|---|---|---|---|
| May | 2 | 7D | 5.5 | 11.0 | 9.7 | N/A |
| | | 4D | 5.2 | 10.4 | | |
| | | 3D | 5.5 | 11.0 | | |
| June* | 4 | 7D | 10.9 | 10.9 | 10.3 | 10.9 |
| | | 4D | 11.0 | 11.0 | | |
| | | 3D | 11.6 | 11.6 | | |
| July* | 5 | 7D | 22.1 | 17.7 | 14.9 | 17.9 |
| | | 4D | 22.1 | 17.7 | | |
| | | 3D | 21.3 | 17.0 | | |
| August* | 2 | 7D | 6.6 | 13.2 | 9.2 | 11.0 |
| | | 4D | 6.3 | 12.6 | | |
| | | 3D | 6.6 | 13.2 | | |

"Weeks" indicates how many weeks irrigation was applied for the month. 7D = irrigated 7 days.week$^{-1}$. 4D = irrigated 4 days.week$^{-1}$ (Sunday, Tuesday, Thursday, Saturday). 3D = irrigated 3 days.week$^{-1}$ (Monday, Wednesday, Friday). The a*sterisks* denote months where a 20% increase was applied to all irrigation times (in minutes) in order to compensate for increased evaporation rates as a result of the fans and increased temperatures inside the capped open-top chambers. N/A denotes 'not applicable' as a 20% increase in irrigation time was not applied to the month of May.

### 3.3.4 Inoculations

Stem inoculations were conducted as described by Nevill et al. (1995) in May 2014 using the wound+inoculum method. Five inoculation treatments were used in this study: no wound (NW), wound only (W), wound+media (WM), *L. terebrantis* (LT) and *G. huntii* (GH). To

inoculate seedlings, a sterile razor blade was used to cut a 5 cm vertical lesion into the bark 5 cm above the soil line. Plugs of 2% malt extract agar (MEA, 3 mm) were placed into the wound. Media was either sterile or had cultures of *L. terebrantis* (LT) or *G. huntii* (GH) growing on it. Seedling stem wounds were wrapped in cotton dampened with deionized water and then wrapped in Parafilm ® to prevent desiccation of the MEA and avoid contact with other biological contaminants.

### 3.3.5 Measurements and Harvest

Root collar diameter (RCD) and height measurements were recorded for all seedlings at both the study initiation (February 2014) and completion (August 2014). Seedling volume change was calculated ($Volume_{Final} - Volume_{Initial} = Volume_{Change}$) to determine overall growth for individual seedlings. The equation $Volume = RCD^2 \; x \; height$ was used to estimate seedling volume (Ruehle et al. 1984).

During planting in February, 40 seedlings from each family were destructively harvested into needles (NE), shoot (SH), coarse roots (CR) and fine roots (FR). These components were placed in drying-ovens for 70 °C at 72 hours. At the conclusion of the study (August 2014), two seedlings from each treatment combination and chamber were selected for final dry matter seedling biomass. Initial family averages for each component (needles, shoots etc.) were subtracted to estimate dry matter yield.

Eleven seedlings from each treatment combination, from all nine OTCs, were examined for relative leaf chlorophyll using a SPAD-502 chlorophyll meter (Spectrum Tech. Inc., Plainfield, IL) during final harvest (August 2014). Needles from the first 2013 flush were selected as they had reached physiological maturity (Sasek et al. 1991).

The remaining two seedlings from each combination treatment, from each chamber, were sampled for water potential measurements using a Scholander pressure bomb (PMS Instrument Company, Albany, OR). Five cm of a lateral branch was cut off each seedling and sampled as described by Kaufmann (1968). Midday sampling occurred between 1300-1500 h. Predawn water potential sampling occurred between 0300- 0500 h. Seedlings were sampled on days of irrigation and days where irrigation was withheld, however, because of significant variation between replicates the data for predawn water potential on non-irrigated days were not analyzed and thus are not included in the analysis.

Another sample of 11 seedlings per treatment was measured for lesion characteristics. These seedlings were cut at the soil line and placed in plastic bins filled with FastGreen stain (FastGreen FCF; Sigma Chemical Co., U.S.) as described by Singh et al. (2014). After 72 hours, stems were removed and the lesion length, width and depth were measured. Two pieces of stem tissue from each lesion were removed from the stem and plated on malt extract agar with cyclohexamide and streptomycin sulfate for fungal re-isolation (Singh et al. 2014).

### 3.3.6 Data Analysis

The experimental design was a split-split-split plot with replicates at all levels. Thee irrigation treatments, 4 loblolly pine families and 5 inoculation treatments produced 60 treatment combinations. Each treatment combination was replicated 15 times in each chamber at the beginning of the study. All statistical analyses were conducted using SAS (Version 9.3 SAS Institute, Inc. Cary, NC) and STATISTICA (Statsoft, Inc. Tulsa, OK). ANOVA procedures (Glimmex procedures), followed by post hoc Tukey (Honest Significant Difference – HSD) procedures were undertaken to further investigate treatment effects. Alpha was set at 0.05.

## 3.4 RESULTS

### 3.4.1 Needle Greenness

Seedlings in 3D and 4D chambers had no difference in needle greenness ($P = 0.418$). Seedlings in 7D treatments had 6.1% greener needles ($P < 0.0001$) compared to seedlings in 4D treatments, although 7D treatments were not different than 3D seedlings (Figure 3.1). Neither family nor inoculation treatments affected needling greenness ($P = 0.323$ and $P = 0.675$, respectively). Family S1 in 4D treatments had less needle greenness when compared to the same family in 7D treatments ($P = 0.033$), however, 3D seedlings were not different from 4D ($P = 0.883$) or 7D ($P = 0.991$) seedlings. There were no other significant interactions between treatments or treatment combinations (Table 3.3).

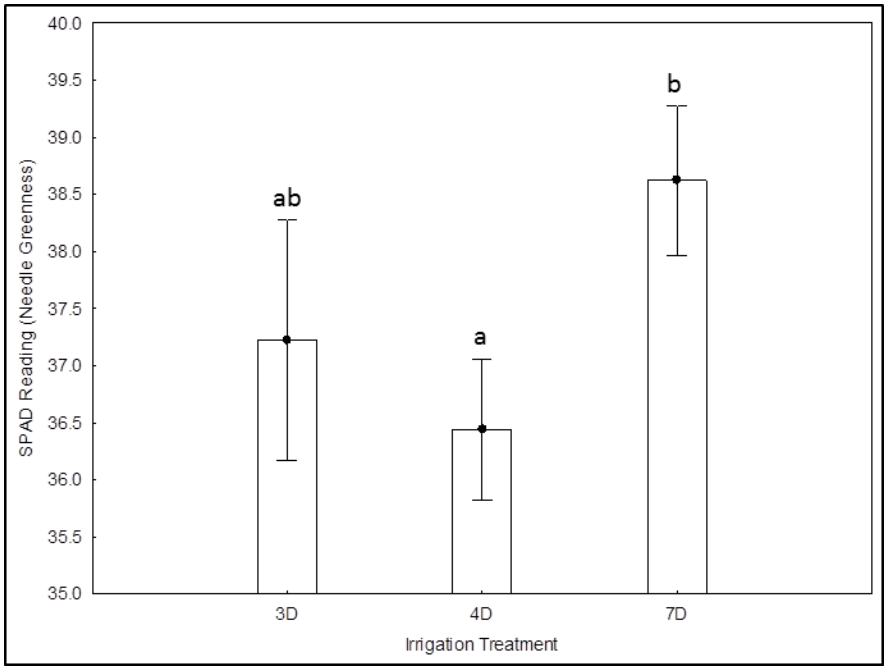


**Figure 3.1**. Needle greenness by irrigation treatment. Letters are from Tukey pair-wise comparisons. Bars denote 95% confidence intervals. 3D = 3 days.week$^{-1}$ irrigation treatment; 4D = 4 days.week$^{-1}$ irrigation treatment; 7D = 7 days.week$^{-1}$ irrigation treatment.

**Table 3.3a**. ANOVA F-Test Values and *P*-Values by treatment (Irrigation, Family)

| Measurement | n | ANOVA F-Test Values and *P*-Values by Treatment Combination | | | | | |
|---|---|---|---|---|---|---|---|
| | | Irrigation | | | Family | | |
| | | df | F-value | *P*-Value | df | F-value | *P*-Value |
| Seedling Volume Change | 2043 | 3 | 4.40 | 0.009* | 3 | 197.76 | < 0.0001*** |
| Dry Matter_Total | 294 | 3 | 4.74 | 0.08 | 3 | 96.04 | < 0.0001*** |
| Needles | 298 | 3 | 2.54 | 0.009* | 3 | 71.76 | < 0.0001*** |
| Shoots | 298 | 3 | 4.79 | 0.017* | 3 | 48.52 | < 0.0001*** |
| Aboveground (Ne+Sh) | 298 | 3 | 4.11 | 0.012* | 3 | 79.01 | < 0.0001*** |
| Coarse Roots | 298 | 3 | 4.49 | 0.002* | 3 | 124.83 | < 0.0001*** |
| Fine Roots | 298 | 3 | 6.27 | 0.008* | 3 | 43.12 | < 0.0001*** |
| Belowground (Cr+Fr) | 298 | 3 | 4.96 | < 0.0001*** | 3 | 122.14 | < 0.0001*** |
| SPAD | 1561 | 3 | 11.63 | 0.90 | 3 | 1.16 | 0.323 |
| Lesion Length | 1217 | 3 | 0.10 | 0.17 | 3 | 5.12 | 0.002* |
| Lesion Volume | 1215 | 3 | 1.76 | 0.31 | 3 | 10.98 | < 0.0001*** |
| Lesion Length/ Seedling Height | 1194 | 3 | 1.81 | 0.14 | 3 | 71.88 | < 0.0001*** |
| Lesion Volume/ Seedling Volume | 1192 | 3 | 1.93 | < 0.0001*** | 3 | 90.83 | < 0.0001*** |
| Mid-Day Wet Water Potential | 284 | 3 | 10.74 | < 0.0001*** | 3 | 0.17 | 0.918 |
| Mid-Day Dry Water Potential | 281 | 3 | 14.09 | < 0.0001*** | 3 | 1.07 | 0.361 |
| Pre-Dawn Wet Water Potential | 276 | 3 | 12.98 | n/a | 3 | 0.10 | 0.963 |
| Pre-Dawn Dry Water Potential | n/a | n/a | n/a | 0.00 | n/a | n/a | n/a |

* denotes $P < 0.05$; ** denotes $P < 0.001$; *** denotes $P < 0.0001$. F(df, n) denotes the degrees of freedom (df) and sample size (n) of the ANOVA F-Test. “n/a” denotes data were not analyzed.

**Table 3.3b**. ANOVA F-Test Values and *P*-Values by treatment (Inoculation, Family*Irrigation)

| Measurement | n | ANOVA F-Test Values and P-Values by Treatment Combination | | | | | |
|---|---|---|---|---|---|---|---|
| | | Inoculation | | | Family*Irrigation | | |
| | | df | F-value | *P*-Value | df | F-value | *P*-Value |
| Seedling Volume Change | 2043 | 4 | 0.92 | 0.453 | 6 | 6.71 | < 0.0001*** |
| Dry Matter_Total | 294 | 4 | 3.01 | 0.019* | 6 | 3.86 | < 0.001** |
| Needles | 298 | 4 | 4.40 | 0.002* | 6 | 4.61 | < 0.001** |
| Shoots | 298 | 4 | 0.46 | 0.767 | 6 | 2.26 | 0.037* |
| Aboveground (Ne+Sh) | 298 | 4 | 2.77 | 0.027* | 6 | 4.22 | < 0.001** |
| Coarse Roots | 298 | 4 | 1.46 | 0.215 | 6 | 2.58 | 0.019* |
| Fine Roots | 298 | 4 | 4.28 | 0.002* | 6 | 1.69 | 0.123 |
| Belowground (Cr+Fr) | 298 | 4 | 3.02 | 0.018* | 6 | 1.58 | 0.151 |
| SPAD | 1561 | 4 | 0.58 | 0.675 | 6 | 0.5 | 0.832 |
| Lesion Length | 1217 | 3 | 84.40 | < 0.0001*** | 6 | 0.84 | 0.538 |
| Lesion Volume | 1215 | 3 | 208.07 | < 0.0001*** | 6 | 1.24 | 0.283 |
| Lesion Length/ Seedling Height | 1194 | 3 | 58.37 | < 0.0001*** | 6 | 3.83 | < 0.001** |
| Lesion Volume/ Seedling Volume | 1192 | 3 | 137.93 | < 0.0001*** | 6 | 3.8 | < 0.001** |
| Mid-Day Wet Water Potential | 284 | 4 | 3.19 | 0.014* | 6 | 0.8 | 0.574 |
| Mid-Day Dry Water Potential | 281 | 4 | 0.99 | 0.412 | 6 | 1.58 | 0.152 |
| Pre-Dawn Wet Water Potential | 276 | 4 | 0.25 | 0.907 | 6 | 2.2 | 0.044* |
| Pre-Dawn Dry Water Potential | n/a | n/a | n/a | n/a | n/a | n/a | n/a |

* denotes $P < 0.05$; ** denotes $P < 0.001$; *** denotes $P < 0.0001$. F(df, n) denotes the degrees of freedom (df) and sample size (n) of the ANOVA F-Test. "n/a" denotes data were not analyzed.

**Table 3.3c**. ANOVA F-Test Values and *P*-Values by treatment (Family*Inoculation, Irrigation*Inoculation)

| Measurement | ANOVA F-Test Values and P-Values by Treatment Combination | | | | | |
|---|---|---|---|---|---|---|
| | Family*Inoculation | | | Irrigation*Inoculation | | |
| | df | F-value | *P*-Value | df | F-value | *P*-Value |
| Seedling Volume Change | 12 | 0.75 | 0.699 | 8 | 1.48 | 0.158 |
| Dry Matter_Total | 12 | 1.17 | 0.302 | 8 | 1.07 | 0.383 |
| Needles | 12 | 1.15 | 0.318 | 8 | 1.28 | 0.254 |
| Shoots | 12 | 0.98 | 0.465 | 8 | 0.98 | 0.453 |
| Aboveground (Ne+Sh) | 12 | 1.21 | 0.277 | 8 | 1.25 | 0.268 |
| Coarse Roots | 12 | 0.78 | 0.675 | 8 | 0.54 | 0.828 |
| Fine Roots | 12 | 0.82 | 0.631 | 8 | 1.25 | 0.268 |
| Belowground (Cr+Fr) | 12 | 0.77 | 0.685 | 8 | 0.84 | 0.568 |
| SPAD | 12 | 1.33 | 0.195 | 8 | 1.33 | 0.225 |
| Lesion Length | 9 | 4.48 | < 0.0001*** | 6 | 0.77 | 0.591 |
| Lesion Volume | 9 | 2.07 | 0.029* | 6 | 2.44 | 0.024* |
| Lesion Length/ Seedling Height | 9 | 2.57 | 0.006* | 6 | 0.27 | 0.950 |
| Lesion Volume/ Seedling Volume | 9 | 1.14 | 0.332 | 6 | 1.04 | 0.395 |
| Mid-Day Wet Water Potential | 12 | 1.43 | 0.154 | 8 | 2.68 | 0.007* |
| Mid-Day Dry Water Potential | 12 | 1.24 | 0.253 | 8 | 0.53 | 0.832 |
| Pre-Dawn Wet Water Potential | 12 | 0.5 | 0.913 | 8 | 1.01 | 0.428 |
| Pre-Dawn Dry Water Potential | n/a | n/a | n/a | n/a | n/a | n/a |

* denotes $P < 0.05$; ** denotes $P < 0.001$; *** denotes $P < 0.0001$. F(df, n) denotes the degrees of freedom (df) and sample size (n) of the ANOVA F-Test. “n/a” denotes data were not analyzed.

**Table 3.3d**. ANOVA F-Test Values and *P*-Values by treatment

| Measurement | ANOVA F-Test Values and P-Values by Treatment Combination | | |
|---|---|---|---|
| | Family*Irrigation* Inoculation | | |
| | df | F-value | *P*-Value |
| Seedling Volume Change | 24 | 0.69 | 0.865 |
| Dry Matter_Total | 24 | 1.41 | 0.097 |
| Needles | 24 | 1.29 | 0.171 |
| Shoots | 24 | 1.45 | 0.082 |
| Aboveground (Ne+Sh) | 24 | 1.44 | 0.087 |
| Coarse Roots | 24 | 1.28 | 0.179 |
| Fine Roots | 24 | 1.00 | 0.466 |
| Belowground (Cr+Fr) | 24 | 1.20 | 0.240 |
| SPAD | 24 | 1.41 | 0.090 |
| Lesion Length | 18 | 1.17 | 0.280 |
| Lesion Volume | 18 | 1.11 | 0.340 |
| Lesion Length/ Seedling Height | 18 | 1.07 | 0.378 |
| Lesion Volume/ Seedling Volume | 18 | 0.94 | 0.524 |
| Mid-Day Wet Water Potential | 24 | 0.95 | 0.527 |
| Mid-Day Dry Water Potential | 24 | 1.10 | 0.348 |
| Pre-Dawn Wet Water Potential | 24 | 1.17 | 0.269 |
| Pre-Dawn Dry Water Potential | n/a | n/a | n/a |

* denotes $P < 0.05$; ** denotes $P < 0.001$; *** denotes $P < 0.0001$. F(df, n) denotes the degrees of freedom (df) and sample size (n) of the ANOVA F-Test. “n/a” denotes data were not analyzed.

### 3.4.2 Seedling Volume Change

Seedlings grown in 7D treatments had a greater change in volume compared to 3D (0.7%, $P < 0.0001$) and 4D (1.2%, $P = 0.015$) treatments. Seedling volume changes in 3D and 4D treatments were not significantly different ($P = 0.060$) from each other. The T1 seedlings had the largest volume change ($P < 0.0001$) compared to S2 seedlings that had the least (11.7% less than T1, $P < 0.0001$). T2 was found to have the second largest change in seedling volume (1.5% less than T1, $P < 0.0001$), followed by S1 (8.6% less than T1, $P < 0.0001$). Inoculation had no effect on seedling volume changes ($P = 0.805$). S1 seedlings grew more in the 7D treatments compared to either the 3D (5.3%, $P < 0.0001$) or 4D (4.0%, $P < 0.001$) treatments. S2 was not significantly affected by irrigation treatment ($P > 0.999$). T1 seedlings grown in 3D treatments were found to have a greater volume change compared to seedlings in 4D (1.8%, $P < 0.001$) and 7D treatments (1.1%, $P < 0.001$). T1 seedlings in 4D and 7D changes were found to be not significantly different ($P = 0.999$, Figure 3.2). T2 seedlings grown in 4D treatments were not significantly different from those grown in 3D ($P = 0.510$) or 7D ($P = 0.262$) treatments, although T2 seedlings grown in 3D treatments were found to have greater volume changes compared to seedlings grown in 7D treatments (4.1%, $P = 0.023$). There were no other significant interactions between treatments or treatment combinations (Table 3.3).

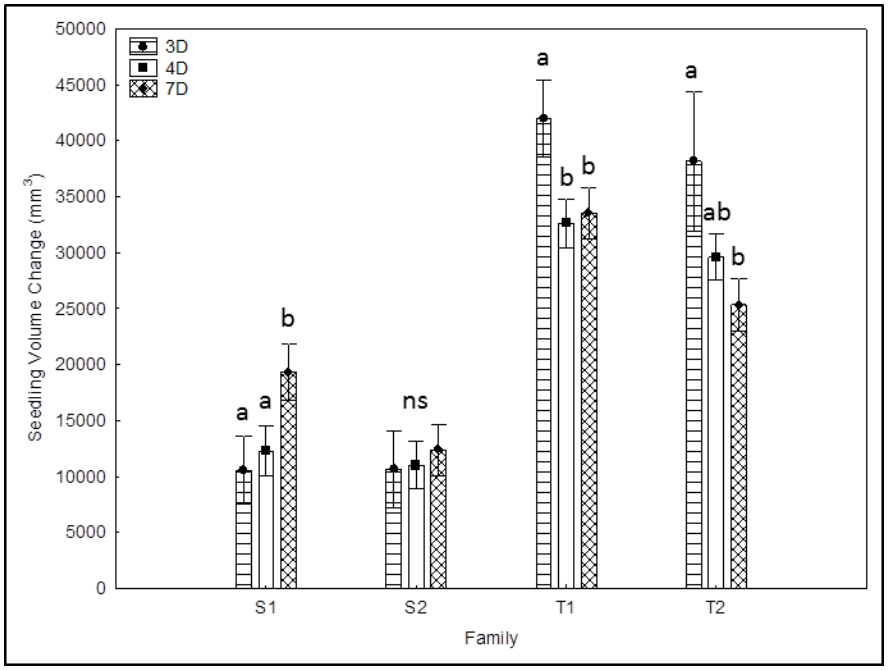


**Figure 3.2**. Seedling volume change by loblolly pine family by irrigation treatment. Letters are from Tukey pair-wise comparisons and are specific to each family. Bars denote 95% confidence. S1 and S2 denote families selected for susceptibility to root infecting ophiostomatoid fungi. T1 and T2 denote families selected for tolerance to root infecting ophiostomatoid fungi. "ns" denotes "no significance."

### 3.4.3 Dry Matter Yield

Needle dry matter yield (DMY) and shoot DMY were found to be similar so those response variables were combined for analyses and named aboveground DMY (needles + shoots). Coarse root DMY and fine root DMY were found to be similar so those were also combined for analysis and reported as belowground DMY (coarse roots + fine roots).

### 3.4.4 Aboveground DMY

Seedlings grown in 7D treatments had 7.1% greater ($P$ = 0.010) aboveground DMY compared to seedlings grown in 4D treatments (7.1%), however, there was no difference ($P$ = 0.188) between 4D and 3D aboveground DMY. The two tolerant families of loblolly pine had no difference in DMY ($P$ = 0.051) when compared. Likewise, S1 and S2 seedlings were not

significantly different in aboveground DMY ($P = 0.230$), however, both had significantly less ($P < 0.0001$) aboveground DMY compared to T1 and T2 seedlings (22.4% and 27.5% less than T1). Inoculation treatment had no significant effect on aboveground DMY ($P = 0.192$). Families S2, T1 and T2 DMY was not significantly affected by irrigation treatment ($P = 1.000$, $P > 0.659$, $P > 0.728$ respectively). S1 seedlings grown in 7D treatments had greater aboveground DMY compared to both 3D (20.8%) and 4D (22.9%) treatments ($P < 0.002$). There were no other significant interactions between treatments or treatment combinations (Table 3.3).

### 3.4.5 Belowground DMY

Seedlings grown in 3D treatments were found to have no significant difference in belowground DMY compared to seedlings grown in 4D ($P = 0.569$) and 7D ($P = 0.085$) treatments, however, seedlings grown in 7D treatments had 8.4% greater belowground DMY compared to seedlings grown in 4D treatments ($P = 0.005$). T1 and T2 seedlings had the greatest belowground DMY, although were not significantly different from each other ($P = 0.442$). S1 and S2 seedlings belowground DMY were not significantly different ($P = 0.229$), and both S1 and S2 seedlings had less belowground DMY compared to both T1 and T2 (32.9% and 38.5% compared to T1, $P < 0.0001$). Inoculation had no significant effect on belowground DMY ($P = 0.064$). There were no other significant interactions between treatments or treatment combinations (Table 3.3).

### 3.4.6 Total DMY

Seedlings grown in 3D treatments were found to have no difference in total DMY compared to seedlings grown in 4D ($P = 0.636$) and 7D ($P = 0.073$) treatments, however, seedlings grown in 7D treatment were found to have greater total DMY compared to seedlings grown in 4D treatments (7.4%, $P = 0.005$, Figure 3.3). T1 and T2 seedlings were found to have the greatest total belowground DMY, although were not different from each other ($P = 0.072$). Seedling families S1 and S2 total DMY were not different from each other ($P = 0.211$), and both S1 and S2 seedlings were found to have significantly less belowground DMY when compared to both “tolerant” families (24.3% and 29.5% less than T1 respectively, $P < 0.0001$). Inoculation had no effect on total DMY ($P > 0.120$). Families S2, T1 and T2 were not significantly affected for total DMY by treatments. S1 seedlings grown in 7D treatments had significantly greater total

DMY compared to seedlings grown in 3D (20.4%) and 4D (22.3%) treatments ($P < 0.003$), however, 3D and 4D seedlings were not different from each other ($P = 1.000$). There were no other significant interactions between treatments or treatment combinations (Table 3.3).

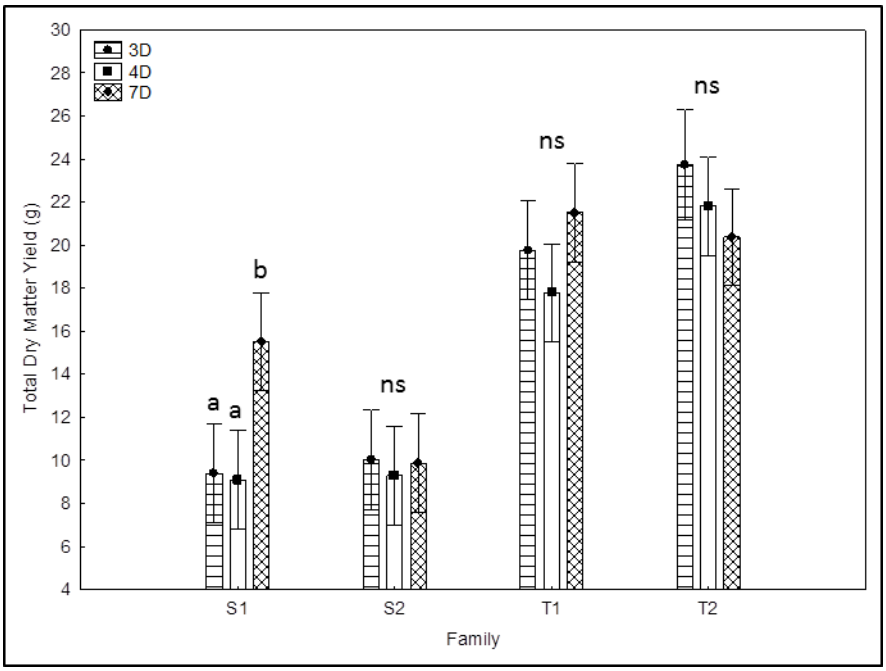


**Figure 3.3**. Whole plant (total) dry matter yield by loblolly pine family by irrigation treatment. Letters are from Tukey pair-wise comparisons. Bars denote 95% confidence intervals. S1 and S2 denote loblolly pine families selected for their susceptibility to root infecting ophiostomatoid fungi. T1 and T2 denote families selected for their tolerance to root infecting ophiostomatoid fungi.

### 3.4.7 Predawn Water Potential on Irrigated Days

Seedlings grown in 4D treatments had lower water potentials compared to seedling grown in 3D (15.7%) and 7D (20.8%) treatments ($P < 0.001$), however, there was no difference in predawn water potential between seedlings grown in 3D and 7D treatments ($P = 0.395$). Family and inoculation each had no effect on water potential ($P > 0.907$). Predawn water potential was the same for families S1, T1 and T2 and were not affected by irrigation treatment ($P > 0.143$). S2 seedlings grown in 3D treatments were not significantly affected compared to seedlings grown in 4D ($P = 0.956$) or 7D ($P = 0.089$) treatments, however, seedlings grown in

4D treatments were more water stressed compared to those grown in 7D treatments (35.9%, $P < 0.001$). There were no other significant interactions between treatments or treatment combinations (Table 3.3).

### 3.4.8 Midday Water Potential on Irrigated Days

Seedlings grown in 3D treatments were more water stressed compared to seedlings grown in 4D (10.7%) and 7D (9.4%) treatments ($P < 0.001$, Figure 3.4), however, there was no difference between the 4D and 7D treatments ($P = 0.702$). There were no significant difference ($P = 0.917$) in midday water potential values between seedling families. Seedlings inoculated with GH were more water stressed than the NW (9.2%) and WM (9.6%) controls ($P < 0.040$). The water potentials of the four seedling families were not affected by inoculation treatment ($P = 0.153$) or irrigation treatment ($P = 0.574$). Wound only seedlings, LT and GH had no difference in water potential between irrigation treatments ($P > 0.221$). Seedlings that were not inoculated (NW) grown in 7D treatments were not different from NW seedlings grown in 3D and 4D treatments ($P > 0.599$). Wound+media seedlings grown in 7D treatments were not different from seedlings grown in 3D and 4D treatments ($P > 0.149$), however, WM seedlings grown in 3D treatments had lower water potentials (18.6% ) compared to those grown in 4D treatments ($P = 0.048$). There were no other significant interactions between treatments or treatment combinations (Table 3.3).

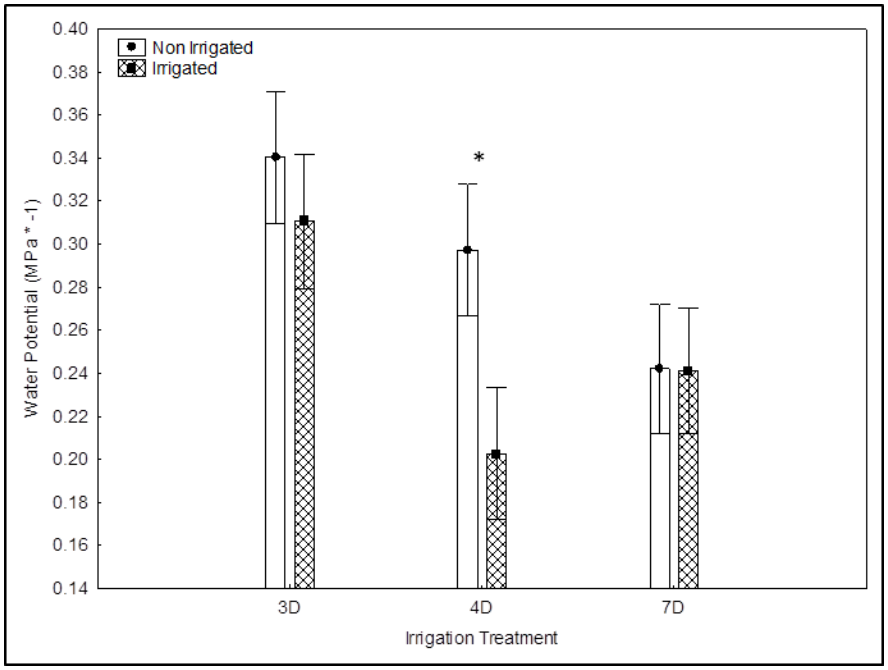


**Figure 3.4**. Midday water potential (-MPa) by irrigation treatment and day of irrigation. The asterisk denotes a significant difference in water potential. Bars indicated 95% confidence intervals. 3D = 3 days.week$^{-1}$ irrigation treatment; 4D = 4 days.week$^{-1}$ irrigation treatment; 7D = 7 days.week$^{-1}$ irrigation treatment.

### 3.4.9 Midday Water Potential on Non-Irrigated Days

Seedlings grown in 7D treatments were less water stressed than those grown in 3D or 4D treatments (19.1% and 12.8% respectively, $P < 0.003$), however, seedlings grown in 3D treatments and 4D treatments were not significantly different from each other ($P = 0.143$, Figure 3.4). There was no difference in water potentials between seedling families ($P = 0.361$) or inoculation treatments ($P = 0.412$). Families S1, T1 and T2 water potentials values were not affected by irrigation treatment ($P > 0.070$). S2 seedlings grown in 4D treatments were not different than seedlings grown in 3D and 7D treatments ($P > 0.424$), however, S2 seedlings grown in 3D treatments were more water stressed than those grown in 7D treatments (25.1%, $P = 0.009$). There were no other significant interactions to report between treatment or treatment combinations (Table 3.3).

### 3.4.10 Lesion Length and Lesion Length/Seedling Height

Lesion length was unaffected by irrigation treatment ($P = 0.905$). S1, T1 and T2 overall had no difference in lesion length ($P > 0.563$). Seedlings inoculated with control treatments (W and WM) had similar lesion lengths ($P = 0.827$). Similarly seedlings inoculated with LT and GH had similar lesion lengths ($P = 0.729$). Both GH and LT inoculated seedlings had significantly larger lesions compared to the controls (8.1% and 8.3% respectively, $P < 0.0001$).

All families inoculated with W and WM controls were found to have no differences in lesion length ($P > 0.983$). Putatively susceptible seedlings in family S1 that were inoculated with LT and GH had larger lesion lengths compared to the non-inoculated seedlings within that family (8.1% and 10.0% respectively, $P < 0.0001$). The family S2 seedlings inoculated with LT and GH had larger lesion lengths compared to the controls (12.9% and 9.6% respectively, $P < 0.0001$), however, lesions produced by the fungi were not significantly different from each other ($P = 0.097$). T1 seedlings with LT and GH had larger lesions when compared to the non-inoculated control (5.2% and 6.0% respectively, $P < 0.0001$). There was no difference in the fungi used in the trial as both LT and GH lesion lengths were similar on the T1 family ($P = 1.000$). Likewise the putatively tolerant family seedlings inoculated with the LT and GH treatments had larger lesions when compared to the non-inoculated controls (6.7% for both, $P < 0.0001$). There was no difference in virulence among the two fungi as both LT and GH lesion lengths were similar on family T2 ($P = 1.000$).

Lesion length ratio (lesion length.seedling height$^{-1}$) was greatest in the S2 family and lowest in the T1 family (9.2% less than S2, $P < 0.0001$). S1 lesion length ratio was smaller than the lesion length ratio on the S2 family seedlings (2.6%, $P < 0.001$). T2 lesion length ratio was lower than S2 seedlings (7.8%, $P < 0.0001$). Irrigation treatment had no effect on seedling lesion length ratios ($P = 0.307$). Lesion length ratio for W and WM seedlings were similar for both wounds ($P = 0.991$). Likewise, the two fungal inoculations (GH and LT) were similar to each other ($P = 0.808$). Seedlings inoculated with GH and LT had lesion length ratios 7.0% and 7.5% greater than the non-inoculated control average ($P < 0.0001$). S2, T1 and T2 seedling lesion length ratios were not affected by the various irrigation treatments ($P > 0.406$). S1 seedlings in 7D treatments had smaller lesion length ratios compared to S1 seedlings in 3D treatments (5.1%, $P = 0.026$) (Table 3.3).

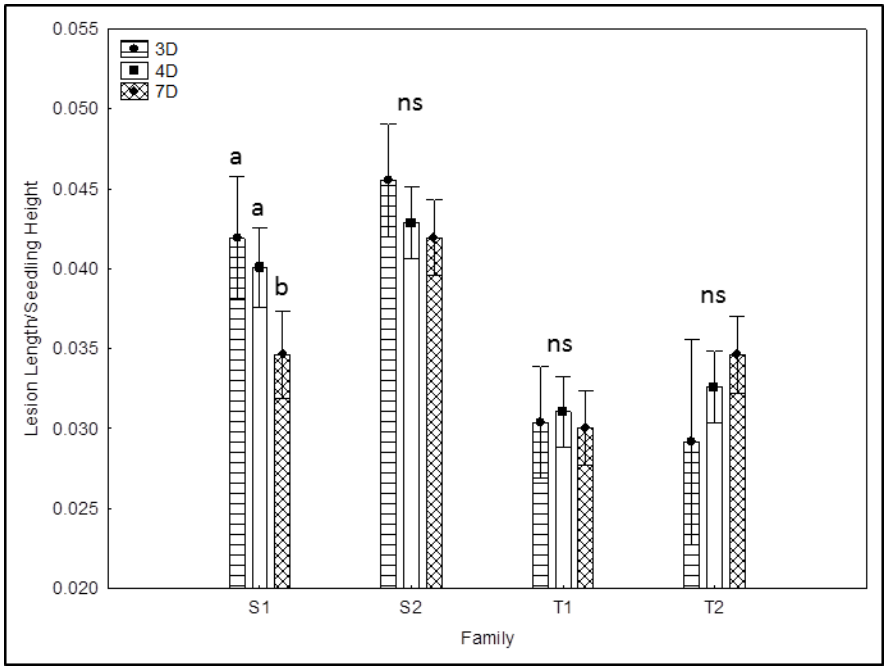


**Figure 3.5**. Lesion length/seedling height (lesion length ratio) by irrigation treatment and loblolly pine family. Letters indicate post hoc Tukey pair-wise comparisons. "ns" denotes "no significance." Bars denote 95% confidence intervals.

### 3.4.11 Lesion Volume and Lesion Volume/Seedling Volume

When examining the effect of the fungal inoculations on the four seedling families lesion volume was greatest in the tolerant T1 and T2 families ($P = 0.891$). For families S1 and S2 lesion volumes were similar to each other ($P = 0.637$) and smaller than the lesion volumes on the T1 and T2 seedling families (3.7% and 2.5% less than T1 respectively, $P < 0.005$).

The various irrigation treatments had no effect on lesion volume ($P = 0.110$). Wound only seedlings had the smallest lesion volumes in contrast to LT inoculated seedlings which had the largest lesion volumes (15.8% larger than W, $P < 0.0001$). Wound+media (WM) and GH seedlings were found to have the largest lesion volumes (4.9% and 14.4% respectively) compared to W seedlings ($P < 0.0001$).

Lesion volume ratio was not significantly different among the two families T1 and T2 ($P = 0.384$). S1 and S2 ratios were less than those of T1 and T2 (16.4% and 21.1% less than tolerant families' average, $P < 0.0001$). Irrigation treatments had no effect on lesion length ratio ($P = 0.145$). Seedlings inoculated with LT had the largest lesion volume ratios (28.2%, 23.2%

and 4.1% greater than W, WM and GH seedlings respectively, $P < 0.040$). Lesion volume ratios in GH seedlings were found to be greater than W and WM controls (25.1% and 19.8% greater than W and WM seedlings respectively, $P < 0.0001$), which were also different (WM were 6.6% greater than W seedlings, $P < 0.0001$). Family S2, T1 and T2 lesion volume ratios were not affected by the 3 different irrigation treatments ($P > 0.447$). S1 seedlings in 3D and 4D chambers were not different ($P = 0.865$), however, S1 seedlings in 3D chambers had greater lesion volume ratios than those grown in 7D chambers (12.8%, $P = 0.004$) (Table 3.3).

## 3.5 DISCUSSION

The alteration of irrigation patterns in the OTC to simulate precipitation changes due to climate change did not result in an increase in susceptibility of four commonly grown loblolly pine families to the root infecting ophiostomatoid fungi. One family did have an increase in injury from the wounding process but it was restricted to S1 and was not specific to either *L. terebrantis* or *G. huntii*. This indicates that some loblolly pine families do not necessarily become more susceptible to root infecting ophiostomatoid fungi, but may become more susceptible as a result of mechanical stress. Based on the results of Singh et al. (2014), it is difficult to say what host-pathogen interactions are creating the tolerance-susceptible spectrum in loblolly pine seedlings.

Water stress has been found to result in a decrease in net photosynthesis in loblolly pine (Samuelson et al. 2014) which is accompanied by a decrease in transpiration rate (Groninger et al. 1996). Seiler and Johnson (1988) found evidence that water stress conditioning allowed loblolly pine seedlings to photosynthesize at lower water potentials than usual that may explain why the 3D irrigation treatment was not different than the 4D and 7D treatments. In our study, 3D seedling responses had large 95% confidence intervals, which may indicate some seedlings had begun to reduce metabolic functions. This would likely causes some seedlings to decrease photosynthetic rates while others continue to actively photosynthesize, which in our study, caused an increase in uncertainty.

Loblolly pine has been shown to have reduced growth when exposed to moisture stress (Meier et al. 1990, Seiler and Johnson 1988, Tschaplinkski et al. 1993). The degree to which loblolly pine responds to moisture stress has been shown to be affected by the seed source location (Seiler and Johnson 1988). In these trials, only S1 had reduced growth given the water

stress treatments for both dry matter yield and volume growth. In both tolerant families, dry matter yield was not affected, however, seedlings had greater volume growth when watered 3 days.week$^{-1}$ compared to other treatments. Therefore, the response of loblolly pine to alterations in moisture availability will likely be family dependent. Given the results of this study, it is difficult to determine if there is a link between tolerances to root infecting ophiostomatoid fungi and drought.

In a previous study, it was observed that inoculation with root infecting ophiostomatoid fungi increased midday water stress when compared to non-inoculated control seedlings (Chapter 2). However, in this study, there was no moisture x inoculation interactions. Seedlings irrigated 3 days.week$^{-1}$ were not able to recover after irrigation an event, which strengthens the idea that some seedlings had begun to reduce metabolic processes. Seedlings irrigated 4 days.week$^{-1}$ were able to recover to water potentials similar to those in seedlings watered 4 days.week$^{-1}$.

## 3.6 CONCLUSIONS

The results of the study indicate that tolerance to root infecting ophiostomatoid fungi may be linked to moisture stress sensitivity. One of the two susceptible families used was also increasingly sensitive to moisture stress. The same family (S1) that was sensitive to changes in moisture also had a larger lesion or wound when irrigated less frequently. This was not specific to inoculation with the pathogenic fungi and therefore we reject the hypothesis that altered moisture availability increases loblolly pine susceptibility to root infecting ophiostomatoid fungi. We can conclude that the strategy to compensate for mechanical stress/wounding is compromised by moisture stress in some families of loblolly pine.

While most work concerning loblolly pine response to drought has not focused on seedlings (Graham et al. 2012, Murthy et al. 1996, Cregg et al. 1988), seedlings will be most likely to succumb to moisture stress (Allen et al. 2010) unless catastrophic drought occurs in the Southeastern U.S. While some studies have utilized seedlings (Seiler and Johnson 1988, Goheen et al. 1978, Croisé et al. 2001, Meier et al. 1990, Matusick et al. 2008), little is known how fluctuating water stress will affect host-pathogen interactions.

It is important to note that there are two overall precipitation patterns that are occurring in North America given the changing climate. The first is the year to decade variation in

precipitation patterns that occur during the summer months in the Southeastern U.S. (Wang et al. 2010, Li et al. 2011, Seager et al. 2009, IPCC 2013). The second is the minute to daily variation in precipitation extremes (high moisture to low moisture) that is occurring (Westra et al. 2014). While the long term trends may contribute to pest and disease outbreaks and factor into forest decline, the short term variations in moisture availability will likely affect regeneration and understory species in forest ecosystems. It is important to consider the differences between precipitation patterns when designing experiments and considering the results. Distinguishing between the effects of altered moisture availability becomes increasingly important as multiple stresses, in our case root infecting ophiostomatoid fungi, are added to the design.

Based on several studies (Garrett et al. 2006, Sturrock et al. 2011, Manning and von Tiedemann 1995) there are three common relationships to look for when analyzing climate-host-pathogen relationships: (1) Climate can affect the pathogen's virulence, abundance, distribution and general biology/ecology; (2) climate can alter the host's defense, abundance, distribution and general biology/ecology; and (3) climate can change the way the host and pathogen interact, through direct or indirect effects. When applying this framework to insect-fungal disease relationships, examining multiple species-species interactions can become complex. It is important to understand the underlying physiological mechanisms for these interactions. For instance, this study focuses on host-fungal pathogen relationships to changes in irrigation (precipitiation) patterns that may occur with climate change. While bark beetles have been shown to capitalize on trees with reduced vigor caused by moisture stress (Jones et al. 2004, Jactel et al. 2012, Klepzig et al. 2004, Koricheva et al. 1998), there is little evidence to support increased pathogen virulence in moisture stressed trees (Goheen et al. 1978, Matusick et al. 2008, Joseph et al. 1998, Croisé et al. 2001). Further studies should examine the insect vector-climate interactions when investigating insect-fungal disease relationships.

# Chapter 4

# Summary and Conclusions

## 4.1 Loblolly Pine and Southern Pine Decline

Loblolly pine is integral to the economic and ecological functions of the Southeastern U.S. (Fields et al. 2011, Duke et al. 2008, Prestemon and Abt 2002). The modified landscape creates challenges to pine production (Schultz et al. 1997) and will likely be exacerbated by changes in climate. Manion (1991) theorized three components that act as underlying causes of forest or tree decline. Predisposing factors are those that are long-term that stress trees. They can be site related or inherited traits (genetic potential). Decline in health and vigor, even mortality, can occur when short-term disturbances incite (inciting factors) host tree defense responses. Contributing factors are those that results from the interactions between predisposing and inciting factors. In the case of SPD, root infecting ophiostomatoid fungi contribute to the decline of Southern *Pinus* species. Loblolly pine, because of its utility to land owner, has considerable genetic diversity from coastal plains to the piedmont regions, therefore genetic potential may play a role in the success of ophiostomatoid fungi and their bark beetle vectors.

## 4.2 Climate Change in the Southeastern United States

Tropospheric ozone and drought are considered potential threats to forests in the Southeastern U.S. (Jones et al. 2001). While ozone concentrations have been moderated by air pollution legislation (Clean Air Acts of 1970 and 1990), the increasing temperatures and human population could increase ozone concentrations in the future (U.S. Bureau of the Census 2009, Wear and Greis 2002, Milesi et al. 2003, Gonzalez-Abraham et al. 2014). Changes in precipitation also have been observed and are expected to become more intense in the future (MacCracken et al. 2000, IPCC 2013, Wang et al. 2010, Seager et al. 2009). The Southeastern U.S. is already experiencing climatic changes which have had detrimental effects to both humans and natural ecosystems (Wang et al. 2010).

## 4.3 Interactions between Southern Pine Decline and Climate Change

Shifts in climate will change the way species interact with each other and individually (Manning and von Tiedemann 1995). In the Southeastern U.S., exposure to elevated concentrations of ozone over multiple seasons is predicted to decrease loblolly pine vigor and increase the tree's susceptibility to root infecting ophiostomatoid fungi. Drought and altered precipitation regimes will likely have negative impacts as well. Typically, the attributors of host-pathogen-environment interactions are easily categorized. When examining insect-fungal disease complexes, such as SPD, there is greater complexity to be considered. There is no evidence that loblolly pine genotypes will respond similarly to climate change. There is also no evidence that insects and root infecting ophiostomatoid fungi will respond to climate change. Therefore, the interactions of organisms (hosts, vectors, pathogens) cannot be easily understood. Individual plants and insects that are capable of rapid acclimation to climate change and variability will likely be successful. Through experimental trials, the knowledge gained from testing and monitoring ecosystems and climate change will prove integral to the success of ecological and economic sustainability.

## 4.4 Final Research Summary and Potential Research

Although, neither elevated ozone (Chapter 2) nor moisture stress (Chapter 3) resulted in increased virulence of *L. terebrantis* or *G. huntii* when inoculated into loblolly pine families, there is evidence to support the hypotheses outlined for the study. Tropospheric ozone induced a host response, even at low concentrations, and caused visible injury. Families selected for the susceptibility to root infecting ophiostomatoid fungi had significantly greater ozone injury, occurring on a higher percentage of the total plants. This indicates that families of loblolly pine that test more tolerant to root infecting ophiostomatoid fungi than others may withstand short-term exposure to elevated ozone concentrations. This relationship has been seen in a similar study (Carey and Kelley 1994). The relationship between family susceptibility and moisture stress is weak. Typically root pathogens and moisture stress act independently, as observed by others (Goheen et al. 1978, Matusick et al. 2008, Joseph et al. 1998, Croisé et al. 2001). Seedlings exposed to intense and infrequent irrigation events began to reduce metabolic functions towards the end of the experimental period. This strategy will likely cause seedlings to be outcompeted by other more tolerant vegetation as well as result in mortality. Family affected

the response of the seedlings to water stress treatments. One of the two susceptible families had less growth with infrequent moisture events, while both tolerant families had more growth with infrequent events. Seiler and Johnson (1988) found that seed source affects the response of loblolly pine to water stress. Our results agree, however, the response is not strongly linked to tolerance to root infecting ophiostomatoid fungi. To better understand the relationship between disease tolerance and moisture stress sensitivity, more loblolly pine families should be tested.

To better understand the relationship between disease tolerance and sensitivity to moisture stress, a more thorough approach would be recommended. Using either soil moisture probes in larger planting pots (as described by Matusick et al. 2008), or conducting a through-fall exclusion methodology experiment would be appropriate. Because our seedlings were potted and placed in OTCs on uneven ground, there are water runoff issues that can affect the relative humidity uniquely during different time periods of the day. The OTCs themselves can also have drying effects on warm days. This can alter the rate of evaporation from seedlings and cause a chamber effect, as seen with our predawn water potential measurements.

Future research should focus on the effects of elevated carbon dioxide and warming temperatures with SPD. Another component missing in the climate-SPD interaction is the role of the bark beetles vectoring the ophiostomatoid fungi. Current monitoring efforts should focus on changes in the chemical and physical climate during insect monitoring trials. Elevated carbon dioxide and warming will likely alter host vigor and productivity which may increase or decrease susceptibility of loblolly pine to biotic and abiotic agents.

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