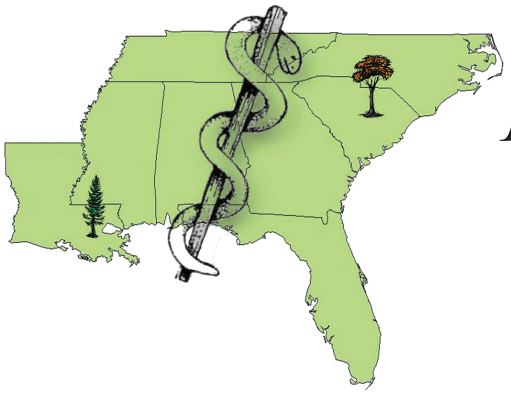

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EFFECTS OF TROPOSPHERIC OZONE ON LOBLOLLY PINE SEEDLINGS INOCULATED WITH ROOT INFECTING OPHIOSTOMATOID FUNGI

by
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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.

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.

MATERIALS AND METHODS

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.

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.

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 19th and ended August 14th 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).

Inoculations

Stem inoculations were conducted as described by Nevill et al. (1995) from May 26 – 29th 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.

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 $Volume = diameter^2 \times height$ 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.

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.

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.

RESULTS

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.

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).

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).

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).

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).

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).

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).

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.

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).

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). 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) 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). 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).

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.

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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**

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

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.

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**

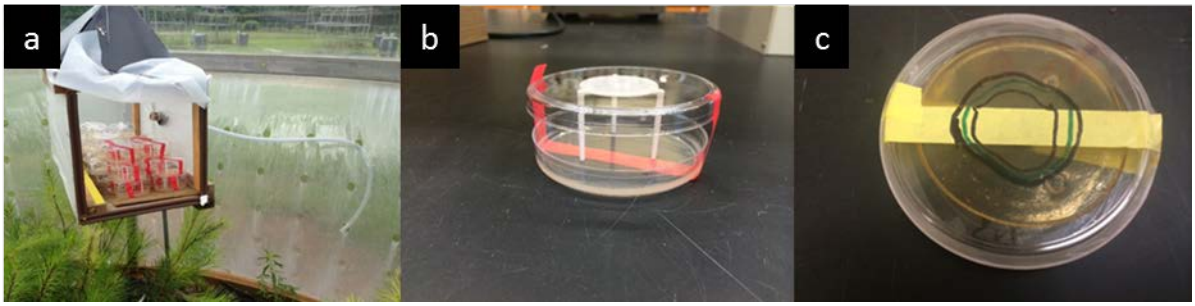

Figure 2.1. (a) Wood frame box with hose attachment placed in the OTC for air exposure. (b) Plastic tripod/Pizza Stacker® used to prevent media desiccation and allow air flow over the fungus. (c) Growth area marked on the underside of the plate.

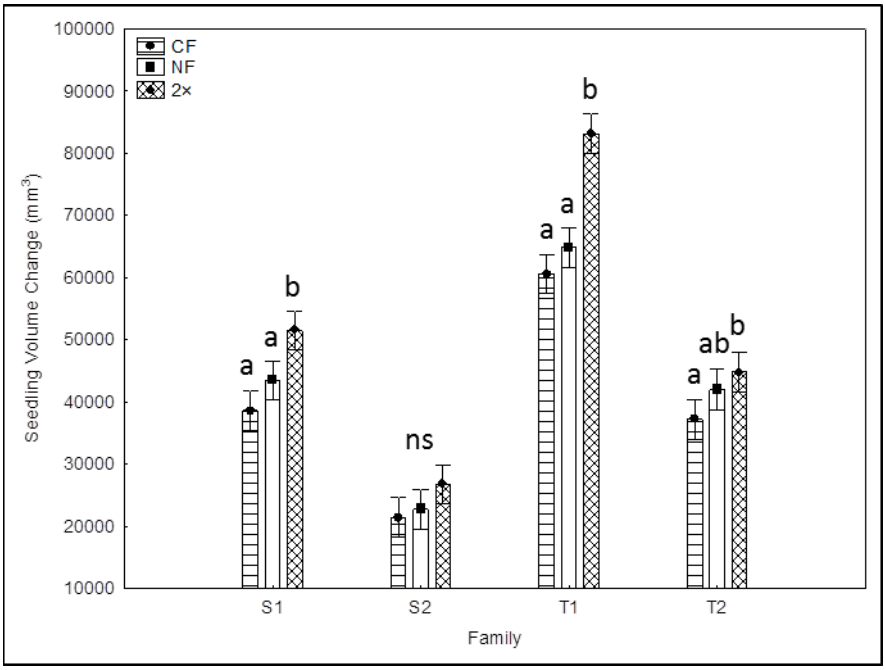

Figure 2.2. Seedling volume change from January to August 2013 by family and ozone treatment. Letters are from Tukey pair-wise comparisons (specific to each family). ns = no significant difference for each family between ozone treatments. CF = charcoal filtered, NF = non-filtered, 2× = twice NF. S1 and 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.

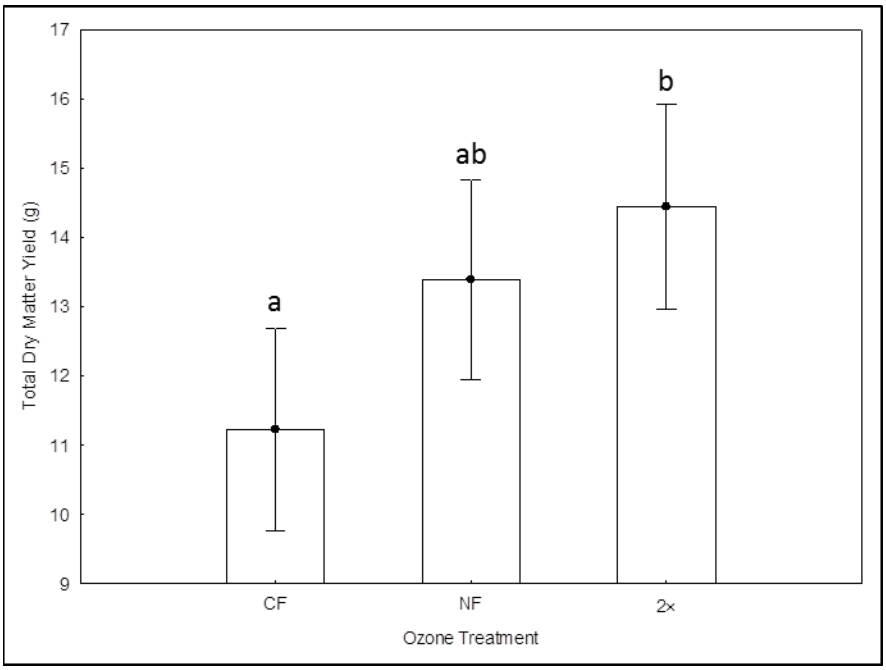

Figure 2.3. Total dry matter yield of seedlings by ozone treatment. Letters are from Tukey pair-wise comparisons. CF = charcoal filtered, NF = non-filtered, 2× = twice NF. Bars denote 95% confidence intervals.

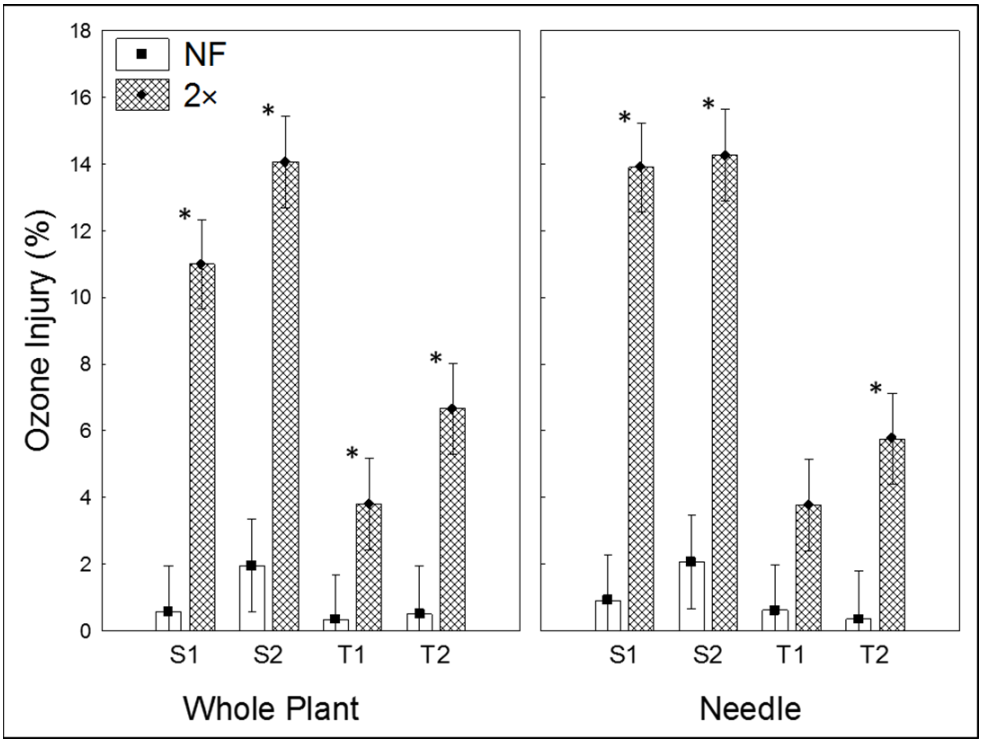

Figure 2.4. Percent injured by family by ozone treatment from August 2013. A = ambient, 2× = twice NF. S1 and S2 denote families chosen for susceptibility to root infecting ophiostomatoid fungi. T1 and T2 denote families chosen for tolerance. Asterisks denote a significant difference within each family.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

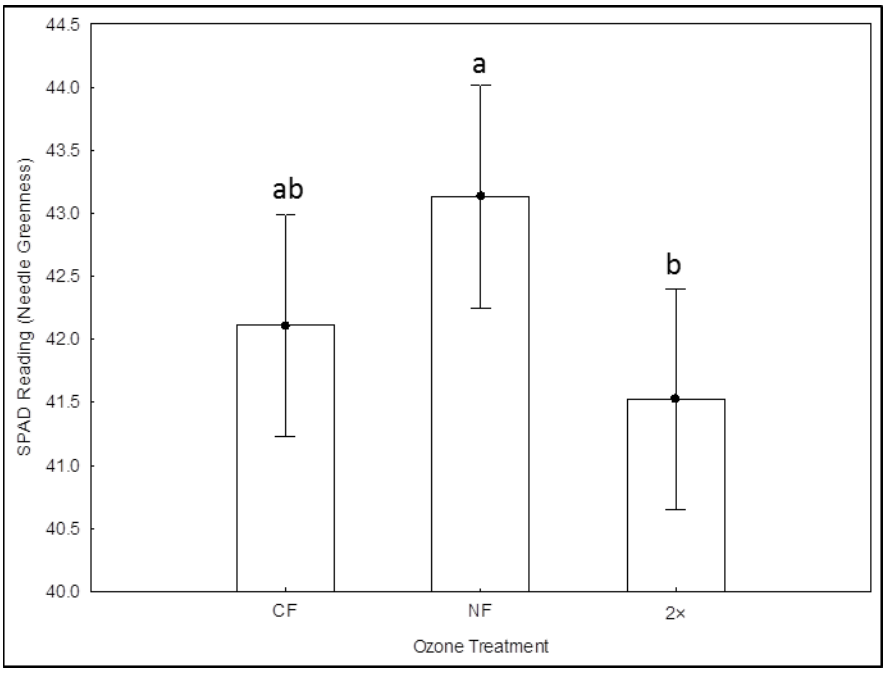

Figure 2.5. Needle greenness by ozone treatment. Letters are from Tukey pair-wise comparisons. CF = charcoal filtered, NF = non-filtered, 2× = twice NF. Bars denote 95% confidence intervals.

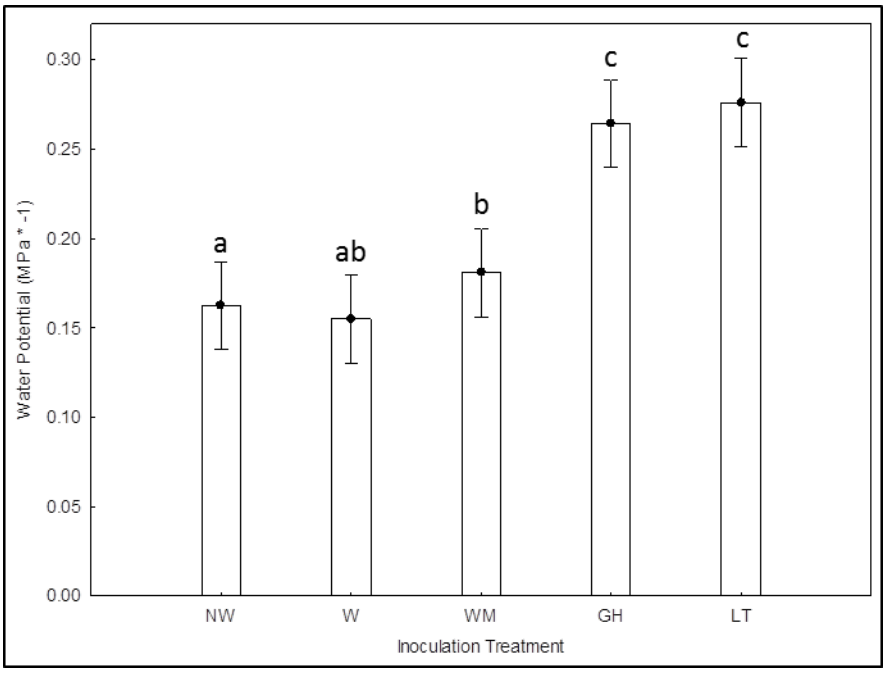

Figure 2.6. Midday water potentials (megapascals*-1) of seedlings by inoculation treatment. Letters are from Tukey pair-wise comparisons. NW = no wound, W = wound only, WM = wound+media, GH = *G. huntii*, LT = *L. terebrantis*. Bars denote 95% confidence intervals.

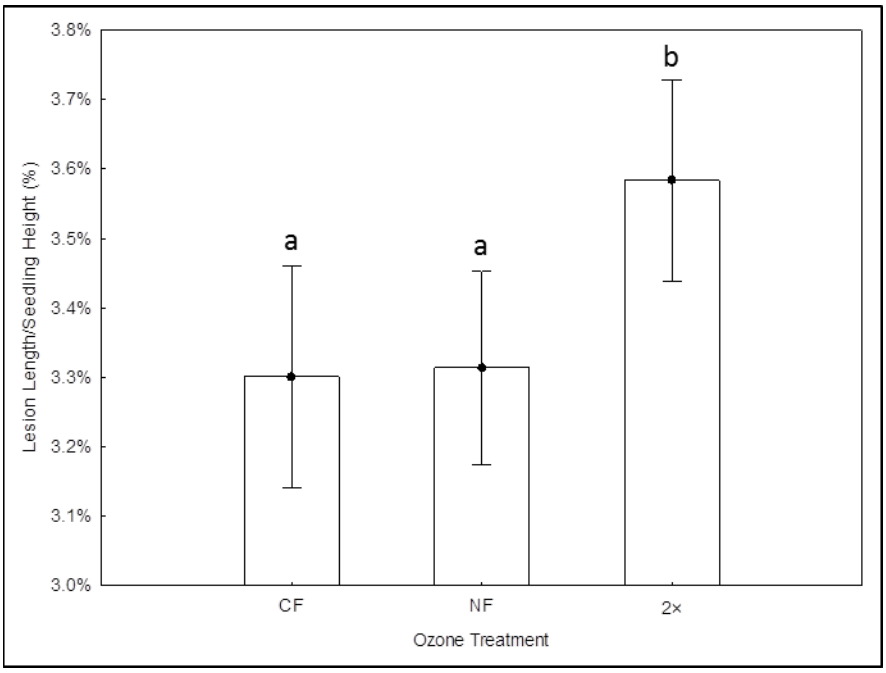

Figure 2.7. Lesion lengths relative to seedling heights. Letters are from Tukey pair-wise comparisons. CF = charcoal filtered, NF = non-filtered, 2× = twice NF. Bars denote 95% confidence intervals.

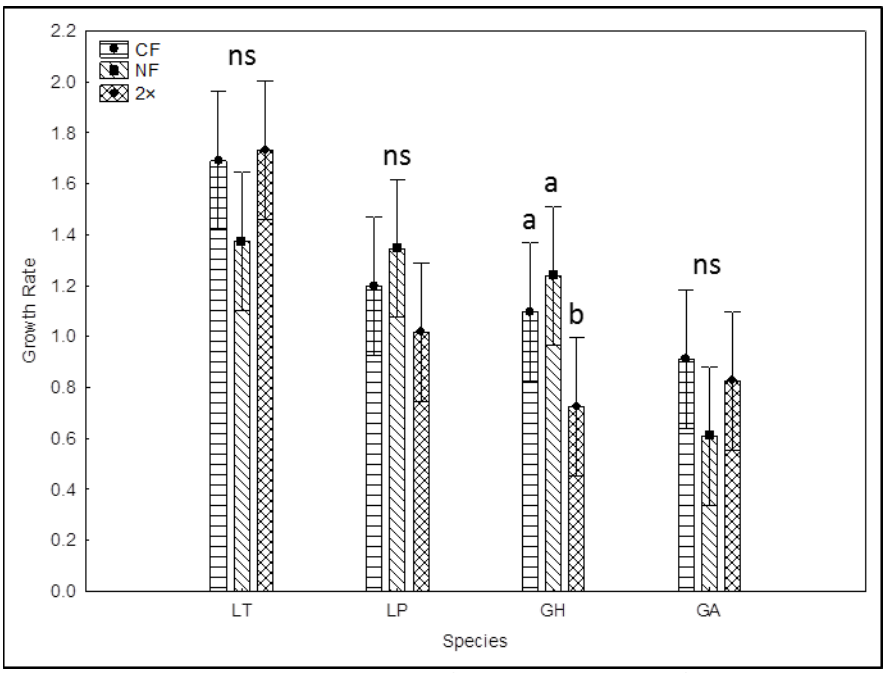

Figure 2.8. Growth (final area cm^2 – initial area cm^2 / final area cm^2) for each fungus by ozone treatment. Letters are from Tukey pair-wise comparisons. ns = no significant different for each fungus between ozone treatments. CF = charcoal filtered, NF = non-filtered, 2× = twice NF. LT = *L. terebrantis*,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