ETIOLOGY AND MANAGEMENT OF A DIPLODIA TIP BLIGHT OUTBREAK ON SLASH PINE (*PINUS ELLIOTTII*) IN FLORIDA

By

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To Antonia and Ignacio, my kids
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ETIOLOGY AND MANAGEMENT OF A DIPLODIA TIP BLIGHT OUTBREAK ON SLASH PINE (PINUS ELLIOTTII) IN FLORIDA

By
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Chair: Jason A. Smith
Major: Forest Resources and Conservation

Extensive dieback and mortality of slash pine (Pinus elliottii) was reported in 2012 in central Florida. To determine the causal agent and disease biology, field monitoring and greenhouse experiments were carried out. Based on sequencing of the ITS-rDNA, Diplodia sapinea, Diplodia scrobiculata, Lasiodiplodia theobromae and Lasiodiplodia pseudotheobromae were isolated from resinous cankers from trees in the field. These “Diplodia tip blight pathogens” (D. sapinea and D. scrobiculata) were consistently recovered from low, medium and high incidence plots. A foliar nutrient analysis revealed mean nitrogen levels were significantly higher (p-value<0.01) from samples from trees in high disease severity plots versus healthy to medium disease severity plots. Significant differences in lesion lengths were observed when Pinus elliottii var. densa and Pinus elliottii var. elliottii were treated with high (3XN) and low levels (1XN) of nitrogen and inoculated with Diplodia sapinea. Disease progression plots displaying different incidence levels (low, intermediate and high) illustrated that intermediate incidence level plots had a significantly (p-value<0.05) more rapid disease progression compared with low and high incidence level plots. Sanitation (diseased trees removed and taken to landfill) reduced disease levels in plots with high disease
incidence. Random thinning significantly reduced disease severity in both disease incidence plots, when compared to the control. These results suggest that in Florida, care should be taken not to over-fertilize or plant slash pines too densely. The research reported here is being used to manage the disease and improve silviculture practices of slash pine plantings in urban areas of Florida.
CHAPTER 1
INTRODUCTION

The sudden occurrence of a plant disease (outbreaks) in a certain community can have devastating ecological, social and economic impacts (Allard et al., 2003). Chestnut blight, Dutch elm disease and mountain pine beetle are examples of tree diseases and pests that have created important ecological, economic and social impacts in the United States (Jones, 1981; Taylor et al., 2006; Freinkel, 2007). Outbreaks in pine plantations specifically, can be devastating to wood products industries that depend on them. For example, outbreaks of pitch canker caused by the fungus *Fusarium circinatum* in South Africa and *Phytophthora* in Chile, have had significant consequences for the timber industries in these regions (Coutinho et al., 2007; Durán et al., 2008).

Major disease outbreaks in trees are caused primarily by fungi, followed by Oomycetes and other organisms (Boyd et al., 2013). Outbreaks are generally associated with abiotic stress agents such as drought, tree wounding, nutritional imbalances and/or improper management practices. These factors can predispose trees to be weakened - allowing for infection by pathogens (Ayres and Lombardero, 2000). In South Africa, a long-term study showed that hail wounding events preceded more than 30 outbreaks of Diplodia tip blight in different pine species (Zwolinski et al., 1990). Its rapid appearance after wounding may be due to its pre-existing residency within the plant as an endophyte (Flowers et al., 2001). When environmental conditions become favorable, the fungi that cause Diplodia tip blight (*Diplodia sapinea* and *Diplodia scrobiculata*) shift to a pathogenic lifestyle and move into and parasitize living tissues of the tree (Flowers et al., 2001; Smith et al., 2002).
Nutrient availability imbalances and excessive fertilizer applications have been implicated as a major predisposing factor in certain tree disease outbreaks. Excessive nitrogen availability can induce abundant succulent shoot and foliar growth, potentially leading to more frequent wounds from insects and weather events (Hesterberg and Jurgensen, 1972). Likewise, numerous phytopathogenic fungi can become more aggressive when nitrogen levels are elevated in a host (Stanosz et al., 2004). These factors may favor disease outbreaks. For example, high levels of foliar and soil nitrogen are known to enhance disease severity in pitch canker (Lopez-Zamora et al., 2007). Likewise, excessive foliar nitrogen was associated with a Diplodia tip blight outbreak in the Netherlands (Van Dijk et al., 1992).

The first report of Diplodia tip blight was made on Pinus radiata D. Don and Pinus patula Schiede ex Schltdl. et Cham. in 1909 and 1930, respectively, in South Africa (Lundquist, 1987). By 1960, the disease was considered one of the most devastating in pine plantations (Swart et al. 1985 and Zwolinski et al., 1990).

Diplodia tip blight is caused by the fungal pathogens Diplodia sapinea and Diplodia scrobiculata, which can also cause tree decline and cankers on main stems (Blodgett et al., 1997). Diplodia sapinea was described initially as having two different morphotypes: A & B. Differences in the morphology and growth rate in morphotypes A and B were reported in the north central United States (Michigan, Minnesota and Wisconsin) (Palmer et al., 1987). Type A differed from type B in spore morphology (cultivated on Agar media), in virulence, and colony morphology, with type A having fluffy white to green mycelia and type B dark with grey mycelia appressed to the agar surface (Palmer et al., 1987). Type B has subsequently been recognized as a distinct
species, *Diplodia scrobiculata* (Burgess et al., 2003; Smith and Stanosz, 2006). *Diplodia scrobiculata* in previous studies was shown to be less virulent than *Diplodia sapinea* on pine species (De Wet et al., 2000).

Management of Diplodia tip blight with fungicides has been successful in nurseries, but the cost and practicality of application limits the use in pine plantations (Palmer et al., 1986). Thinning and sanitation/cultural methods are recommended in plantations and nurseries due to the reduction of stress by competition for nutrients and water and removing infected trees, reducing the amount of inoculum (Swart and Wingfield, 1991)

Other fungi that belong to the same family as *Diplodia* spp. (Botryosphaeriaceae) are associated with symptoms such as tip blight, stem cankers, fruit rots and dieback (von Arx, 1987) in numerous hosts worldwide, such as cacao, mango and other tropical and subtropical species (Ismail et al., 2012). Two members of this family, *Lasiodiplodia pseudotheobromae* and *Lasiodiplodia theobromae* were originally considered the same species, but due to the different characteristics of the spores and growth of mycelium, were separated into two species (Alvez, 2008).

Studies have previously shown that *Lasiodiplodia pseudotheobromae*, *Lasiodiplodia theobromae, Diplodia sapinea* and *Diplodia scrobiculata* can remain latent as endophytes until the host is stressed, allowing them to opportunistically infect their hosts (Peterson, 1977; Blodgett et al., 1997; Flowers et al., 2001; Smith et al., 2002). *Lasiodiplodia pseudotheobromae* is known to affect tropical trees (Ismail et al., 2012). However, *Lasiodiplodia theobromae* has been associated with *Diplodia sapinea* and
Diplodia scrobiculata in the southeastern United States in healthy and unhealthy pine stands (House, 2007).

The most important commercial pine species in the southeastern United States are slash pine and loblolly pine (Wear et al. 2007). These species are native to this area and have long been important for the wood products industry (Nelson, 2010). Two varieties are known for slash pine (Pinus elliottii Engelm.): typical slash pine (P. elliottii Engelm var. elliottii), and south Florida slash pine (P. elliottii Engelm. var. densa), the former is the most predominant, while the later grows naturally only in the southern half of Florida and in the keys. Both varieties are frequently used for landscaping along highways (Nelson, 2010). Loblolly pine (Pinus taeda L.) occurs on approximately 13 million hectares in the southeastern Unites States, and is one of the most robust and rapidly growing of the southern pines. It is well-suited for forest plantations and management, and contributes between $7,807-8,247 per ha\textsuperscript{1} to the regional economy (Susaeta et al., 2016). Slash and loblolly pines provide numerous ecological benefits such as forest cover, wildlife habitat and watershed protection (Schultz, 1997).

Historically, Diplodia tip blight has not been a common problem on slash pine and loblolly pine in Florida (Dr. Ed Barnard, Florida Div. of Forestry personal communication), which is confirmed by the lack of literature for these diseases in the Southeast US. However, a recent outbreak of tip and branch death of slash pine was detected along the highways of central Florida. This discovery could point to a larger potential threat to the pine resources of the state. Thus, the goal of this study was to determine the etiology and evaluate management strategies for this outbreak. The
specific objectives of this thesis are to: i) determine the etiology of Diplodia tip blight on slash pine in central Florida (chapter 2), ii) examine the role of nitrogen, sanitation and stand density management on the development of a Diplodia tip blight outbreak in central Florida (chapter 3).
CHAPTER 2
ETIOLOGY OF DIPLODIA TIP BLIGHT ON SLASH PINE IN CENTRAL FLORIDA

Background

*Pinus elliottii* Engelm. and *Pinus taeda* L. are the most important commercial pine species in the southeastern United States (Wear et al. 2007). *Pinus taeda* L. alone is present in more than 13 million hectares in this area, and contributes between $7,807-8,247 per ha$ to the regional economy (Susaeta et al., 2016). As these pines are native to this area, they are frequently challenged by numerous native diseases and pests. Among them, the most economically important natural enemies are: pitch canker (*Fusarium circinatum* Nirenberg & O'Donnell), fusiform rust (*Cronartium quercuum* Miyabe ex Shirai f. sp *fusiforme* (Cumm. Burds. & Snow), and the southern pine beetle (*Dendroctonus frontalis* Zimm.).

Pitch canker, is characterized by resin-soaked cankers on the trunk and on the terminal shoots. It has been a problem in southern and western United States where it has contributed to large plantation establishment failures and nursery losses (Dwinell and Phelps, 1977; Dwinell et al., 1985). Fusiform rust is characterized by “spindle-shaped” galls on stems and branches. These galls reduce growth, deform stems and cause mortality on disease prone sites. It has been a problem in the southeastern United States where it was a major obstacle to plantation forestry until resistant seed sources were identified (Cubbage et al., 2000). Southern pine beetle, the most destructive insect pest of pines in the southern United States, is characterized by pitch tubes on the bark of trunks of pines and galleries inside the bark that can girdle a tree and cause mortality (sometimes in very large outbreaks) (Lorio, 1986).
An outbreak of any of these diseases and pests can have important ecological, social and economic consequences (Allard et al., 2003). Generally, large outbreaks are associated with large numbers of susceptible hosts and abiotic stresses such as drought, tree wounding, nutritional imbalances and/or improper management practices. These factors can predispose trees to be weakened therefore allowing the establishment and infection by pathogens (Ayres and Lombardero, 2000).

To understand patterns or causes of outbreaks of tree diseases, several epidemiological models have been developed. Area under the disease progress curve (AUDPC) is a nonlinear growth function in epidemic analysis that can be used to assess disease development rate over time (Jeger, 2004). The AUDPC uses the trapezoid method and expresses the dynamic of an outbreak (Madden et al. 2007) standardizing variation seen in disease progression. It can be used to compare different patterns of disease progression and design management strategies (Jeger, 2004).

A recent outbreak of dieback on slash pines along highway buffer plantings in central Florida was investigated. Symptoms of branch death and resinous cankers and signs of pycnidia on the surface of cones and in some dead needles were observed. Although pitch canker is common in this region, these signs and symptoms do not match that disease. Instead, they are characteristic of Diplodia tip blight (von Arx, 1987; Phillips, et al., 2013).

Diplodia tip blight is caused by the fungal pathogens *Diplodia sapinea* and *Diplodia scrobiculata* (Botryosphaeriaceae), which can also cause tree decline and cankers on main stems (Blodgett et al., 1997). Historically, Diplodia tip blight has not been a common problem on pines in Florida (Dr. Ed Barnard, Florida Div. of Forestry...
personal communication), which is confirmed by the lack of literature for these diseases in the southeast US.

In this chapter, I provide a description of research studies that were conducted to examine the etiology of the disease outbreak in central Florida. A fungal survey was conducted on symptomatic trees planted by the Central Florida Expressway Authority (CFX) in Orlando, FL. Additionally, I tested the pathogenicity of the four most commonly isolated fungi and documented the progression of the disease.

**Materials and Methods**

**Field Study Sites**

The field study areas are located in slash pine buffer plantings established by the Central Florida Expressway Authority (CFX) on 3 sites along highways in Orange County, FL. (28°32'11.3"N 81°33'30.1"W). The average annual precipitation of the area is 1360 mm. The average annual temperature is 23.7°C, with average temperatures in summer of 28.3°C and in winter of 19.7°C, respectively (NOAA 1981-2010).

**Pathogen Field Sampling and Identification**

Detailed stratified sampling was carried out with 90 trees from plots displaying three different disease incidence levels (30 trees from a stand with <30% of trees with symptoms), 30 trees in a stand with intermediate symptomatic (30-50% of trees with symptoms) and 30 trees from a stand with an advanced symptomatic stage (>50% of trees with symptoms). By using these three categories we assessed the presence and activity of pathogens during various stages of development. Frequent association through all three stages would indicate a pathogen is contributing to the epidemic.

Branches from symptomatic trees were sampled in each stand using a pole pruner and transported in a cooler to the laboratory. Small sapwood pieces from the
margins of lesions from symptomatic branches with resinous cankers were excised, surface sterilized in 2.5% sodium hypochlorite solution and plated out on Acidified potato dextrose agar, (APDA; Difco Laboratories, Detroit, MI). The cultures were incubated in the dark for one week at 22°C and colonies with typical Diplodia and Lasiodiplodia morphology were sub-cultured and subsequently identified using molecular identification.

A Chi-square and Fisher exact test where the significance was evaluated at p-level <0.05, using a contingency table, were used to determine the association of the stand incidence level and the presence of fungi (Diplodia sapinea, D. scrobiculata, Lasiodiplodia theobromae, L. pseudotheobromae, and saprophytes). Analyses were carried out with the software R (R Core Team 2014).

Procedures for genomic DNA extraction from fungal cultures similar to described by Hulcr (2012) were used, using 20 µl of Ex-N-Amp® extraction solution (Sigma Aldrich), into which 20 µl of the fungal colony (mycelia and spores) were added and then macerated with a pestle and lysed by incubation at 96°C for 30 minutes. The deactivation of the sample was achieved by adding 20 µl of 3% BSA (bovine serum albumin), followed by vortexing and centrifugation at 13,000 rpm. The upper 20 µl of the supernatant was collected by pipette and was used as the final DNA template.

Amplification of the internal transcribed spacer (ITS) region of the nuclear ribosomal DNA was performed with the primers ITS1 (5’TCC GTA GGT GAA CCT GCG GG) and ITS4 (5’GCT GCG TTC TTC ATC GAT GC).

Polymerase chain reactions (PCRs) contained 2.5 µl of 10x ImmoBuffer (Manufacturer), 1.5 µl of 50 mM MgCl2, 2.5 µl of 2 mM dNTPs, 0.125 µl of Immolase
(Manufacturer), 15.375µl of distilled deionized water (Bioline), 1 µl of each primer (ITS1 and ITS4) and 1 µl of the DNA template with a final volume of 25 µl. The PCR reactions were conducted in a thermocycler (MJ Research PTC-200) with cycling parameters as follows: preheat at 94 °C for 5 min; 35 cycles of (i) denaturation at 94 °C for 60 secs, (ii) annealing at optimal temperature 52 °C for 60 secs, (iii) elongation at 72 °C for 60 secs, and a final elongation step of 5 min. The PCR amplicon was stained with SYBR Green I (Lonza, Rockland, ME, USA) separated by electrophoresis on 1% agarose gel in sodium boric acid buffer and photographed under UV light. Amplicon were purified with 2 µl of Exosap (Affymetrix) following manufacturer’s instructions. The purified amplicons were sent to the University of Florida’s Interdisciplinary Center for Biotechnology Research (ICBR) for Sanger sequencing.

Edited sequences were aligned using Geneious 5.6.6 (Biomatters Ltd., Auckland, New Zealand). BLASTn searches were then performed with default parameters (National Center for Biotechnology information (NCBI) website).

Pathogenicity Experiment

The pathogenicity of the fungi Diplodia sapinea, D. scrobiculata, Lasiodiplodia theobromae and L. pseudotheobromae was tested in a greenhouse experiment. Three-year-old trees of Pinus taeda L. (Loblolly pine), Pinus elliottii Engelm. var. elliottii (Typical Slash pine) and Pinus elliottii Engelm. var. densa (South Florida slash pine) were inoculated using a randomized complete block design with three replicates and two repetitions in the summers of 2015 and 2016 for each tree species.

Seedling stems were wounded using a 6-mm sterile cork borer at approximately 25 cm below the terminal shoot tip, and inoculated with 6 mm colonized agar plugs from the leading edge of an actively growing fungal colony, facing the cambial tissue of the
stem and wrapped with Parafilm® M (Pechiney Plastic). For controls, the inoculation was made with a non-colonized agar plug and wrapped with Parafilm. The trees were maintained in the greenhouse and watered as necessary. Parafilm was not removed until the end of the study.

Lesion lengths and external symptoms were evaluated after 11 weeks post inoculation. To complete Koch’s Postulates, one replicate from each of the different fungi was selected for pathogen re-isolation. This was accomplished by removal of sapwood (1 cm) from the margin between the live and dead tissue of the lesion, which was surface-sterilized in 2.5% sodium hypochlorite. This was followed by air-drying for 1 minute before being plated on acidified Potato Dextrose Agar (APDA). DNA was extracted and ITS regions were sequenced as described previously.

The mean lesion length produced by each fungus on the different pine species was measured following de-barking and was analyzed with One-Way ANOVA, significance was evaluated at p-level <0.05. A multiple means comparison was performed with Tukey’s test (post-hoc separation) with fungi as independent variable and lesion length as the dependent variable. All the analyses were performed in R statistical software (R Core Team 2014).

**Disease Progression Monitoring**

To monitor the disease progression along Central Florida Expressway slash pine plantings in Orange County, FL., nine linear 809.4 m² monitoring plots were established in buffer strips displaying three stages of disease incidence and severity:

1. Low (less than 30% of trees with symptoms)
2. Intermediate (30 to 50% of the trees showing symptoms)
3. High (> 50% of trees).
The plots were measured, mapped (Figure 2-1), flagged and GPS located. Photography was used to document symptom progression every three to five months for 15 months, beginning in April, 2014. The disease incidence was calculated as the percentage of trees with symptoms and ranged between 0 and 100%. Incidence was assessed for each stand at each time point (five total) (Table 2-1). To evaluate disease progression, and because the plots had different incidence levels at the beginning of the measurements, each plot was standardized with its own average incidence level of the starting point (table 2-2). Area Under the Disease Progress Curve (AUDPC), using the trapezoid method, was calculated using the disease incidence \((y_i)\) collected at various time points \((t_i)\) (Madden et al. 2007). The AUDPC was calculated using the following equation:

\[
AUDPC = \sum_{i=1}^{N-1} \left( \frac{y_i + y_{i+1}}{2} \right) (t_{i+1} - t_i)
\]

where \(y_i\) is the disease incidence rating at the time \(i\) (where \(i=1, 2, 3, 4, 5\)), and \(t_i\) is the time of the rating (Table 2-3).

The calculated AUDPC data were analyzed using ANOVA and Tukey’s multiple comparison was used for means separations \((\alpha=0.05)\). The analysis was performed using R statistics software (R Core Team 2014).

**Results**

**Pathogen Field Sampling and Identification**

To determine what pathogen was causing the disease outbreak in central Florida, we sampled from slash pine stands displaying different incidence levels (low, intermediate, and high). The Diplodia tip blight pathogens (Diplodia sapinea and D. sapinea).
scrobiculata) were consistently recovered from all three disease stages. There was no significant difference (p-value = 0.2) between presence of Diplodia spp. and incidence level of the plots sampled. However, lower levels of Diplodia tip blight pathogens were recovered in the high disease severity plots (Figure 2-2). A significant association (p-value = 0.02) between incidence plots and Lasiodiplodia spp. (Lasiodiplodia theobromae and L. pseudotheobromae) was found (Figure 2-3). Lower levels of Lasiodiplodia spp. were found in the low incidence, followed by high incidence plots and high levels were found in the intermediate incidence plots. Saprophyte recovery increased significantly (p-value = 0.009) with the level of incidence in the plot. These commonly recovered fungi were not considered pathogens and, thus, not important in the disease epidemiology at these sites.

Diplodia sapinea recovery was not significant in the different incidence levels (p-value=0.48) as shown in Figure 2-3. A significant relationship between the plot incidence level and the fungi Diplodia scrobiculata (p-value = 0.023), Lasiodiplodia theobromae (p-value = 3.347e-05), and Lasiodiplodia pseudotheobromae (p-value = 0.022) was found. Lower levels of Diplodia scrobiculata were found in high incidence levels, followed by intermediate incidence and the highest levels were found in the low incidence plots. Lower levels of Lasidiplodia theobromae and L. pseudotheobromae were found in low incidence plots and in low and high incidence plots, respectively. The highest presence of both fungi was found in intermediate incidence plots.

Pathogenicity Experiment

In the pathogenicity experiment, Pinus taeda (Figure 2-4) and Pinus elliottii var. elliottii showed no significant differences in the interaction of the treatments with the replication of the experiment (p-value>0.05). For Pinus taeda, Diplodia sapinea was the
fungus that caused the largest mean lesion length 11 weeks after inoculation (2.4 cm), followed by *Lasiodiplodia theobromae* (1.7 cm), *Diplodia scrobiculata* (1.5 cm) and *Lasiodiplodia pseudotheobromae* (1.5 cm). *Diplodia sapinea* caused significantly larger lesions than *Lasiodiplodia pseudotheobromae* and *Diplodia scrobiculata* (p-value<0.05), but not *Lasiodiplodia theobromae*. All fungal treatments were significantly different from the control (p-value<0.05).

None of the fungi caused symptoms on *Pinus taeda* L. after 11 weeks. Seedlings of *Pinus elliotti* var. *elliottii*, trees became symptomatic when inoculated with *D. sapinea* and *D. scrobiculata*, showing wilting of new shoots and resinosis on the lower part of the stem (Figure 2-6). *D. sapinea* produced significantly larger lesions than any other fungi (Figure 2-6). Average lesion length was 5.3 cm followed by *Lasiodiplodia theobromae* (3.4 cm), *Lasiodiplodia pseudotheobromae* (2.6 cm) and *Diplodia scrobiculata* with (2.4 cm). All fungal treatments were significantly different from the control (p-value<0.05). As expected, the negative control did not develop lesion or develop symptoms.

In *Pinus elliotti* var. *densa*, the interaction of the treatments with the experiment replication was statistically significant (p-value<0.05). Thus, the treatments were analyzed independently by experiment repetition. In the first experiment (Experiment A, Figure 2-5), *Diplodia sapinea, Lasiodiplodia theobromae* and *L. pseudotheobromae* showed similar lesion lengths (~1.9 cm) followed by *Diplodia scrobiculata* (1.3 cm). All fungi were significantly different than the control (p-value<0.05), but not significantly different among each other. In the second run of the experiment (Experiment B, Figure 2-5), the average lesion length caused by *D. sapinea* was significantly larger than any other fungi (3.9 cm). *Diplodia scrobiculata* (1.8 cm), *Lasiodiplodia pseudotheobromae*
(1.8 cm) were significantly different than the control, but not from *Lasiodiplodia theobromae* (0.9 cm). *Lasiodiplodia theobromae* was not significantly different from the control. In neither of the two experiments (A and B) did *Pinus elliottii* var. *densa* did not develop external symptoms in either experiments.

**Disease Progression Monitoring**

The number of symptomatic trees in the low incidence plots increased slightly from the beginning (April, 2014, 0%) to the end of the monitoring period (June, 2015, 13.3%) (Figure 2-7). The mean AUDPC of the low incidence plots was 115, being the lowest value compared with the other two levels of incidence plots (Table 2-3). Incidence was significantly higher for intermediate incidence level plots between the beginning and the end of monitoring. Intermediate plot incidence increased from 0% to 60% (Figure 2-7), with an AUDPC of 435, which was significantly higher (p-value<0.05) than the high (267) and low incidence plots (Table 2-3).

**Discussion**

Our pathogen field sampling recovered the pathogens causing the Diplodia tip blight disease in all incidence plot levels (Figure 2-2). *Diplodia sapinea* and *D. scrobiculata* were the primary pathogens causing Diplodia tip blight disease, producing cankers and shoot dieback in conifers (Luchi, et al., 2005). The recovery of these pathogens was possible in 56, 56 and 36% of the trees sampled in the low, intermediate and high incidence level plots, respectively. However, the presence of the Diplodia tip blight pathogens was not significantly associated (p-value > 0.05) with the incidence level of the stand sampled. When studying each of the fungi causing the Diplodia tip blight separately we found that only *D. scrobiculata* was significantly associated (p-value=0.02 with the stand incidence level, while *D. sapinea* was consistently observed
(20-30% of trees sampled) in all incidence levels (Figure 2-3). *Diplodia sapinea* is considered more virulent than *D. scrobiculata* or other fungi associated with Diplodia tip blight disease (Bihon et al., 2011); however, either of these species can cause Diplodia tip blight (Luchi, et al., 2005). This was also observed in our pathogenicity experiment, where *D. sapinea* was significantly more virulent than *D. scrobiculata* when inoculated on *P. elliottii* var. *elliottii* (Figure 2-6) and also in the second experimental repetition for *P. elliottii* var. *densa*. However, no differences were observed when inoculating these *Diplodia* spp. into *P. taeda* (Figure 2-4) and in the first repetition (experiment A) with *P. elliottii* var. *densa* (Figure 2-5). These different results in the two repetitions (A and B) with *P. elliottii* var. *densa* may be explained by the source (provenance) of the plants used in the experiments or by variable environmental conditions that may have affected the response of the trees (Smith et al., 2002). Additionally, *Pinus elliottii* var. *elliottii* was more susceptible to the Diplodia tip blight fungi than *Pinus taeda* and *Pinus elliottii* var. *densa*. *Diplodia* spp. can be opportunistic on stressed hosts (Peterson, 1977, Blodgett et al., 1997, Flowers et al., 2001) or may be primary pathogens if the strain has become more virulent (Blodgett and Bonello, 2003; Peterson, 1977), or a particularly susceptible seed source was used for planting (Smith et al., 2002).

*Lasiodiplodia* spp. frequency of recovery had a significant relationship with incidence plot level (p-value<0.01). The presence of both *L. theobromae* and *L. pseudotheobromae* followed the same pattern, both increased from the low to intermediate incidence level, and then decreased in the high incidence level; 20%-77%-37%, and 7%-30%-7%, respectively. The high presence of *L. theobromae* could be related to the outbreak. However, in the pathogenicity experiment with this fungus,
cankers developed under the bark but no visual external symptoms were observed on pines in this study at 11 weeks post inoculation (Figure 2-4, 2-5 and 2-6). Additionally, this fungus has been found in healthy and unhealthy pines in south Georgia, apparently living as an endophyte (House, 2007). Therefore, it is uncertain as to whether L. theobromae contributed significantly to this outbreak.

Lasiodiplodia pseudotheobromae has not been reported previously to cause mortality or dieback in any pine species. However, it is known to be at least a weak pathogen in other hosts such as Eucalyptus in Uruguay (Pérez et al., 2010), mango in Egypt (Ismail et al., 2012) and in other tropical and subtropical trees in China (JiaPing et al., 2010). In the greenhouse experiment, this fungus caused small lesions on the stem (under the bark), but no external symptoms in any of the pine tree species used in the study (Figure 2-4, 2-5 and 2-6). The results of the pathogenicity experiment indicate that Pinus elliottii var. elliottii was more susceptible to the four fungi, developing longer lesion lengths and more extensive symptoms than Pinus taeda and Pinus elliottii var. densa.

The presence of both Diplodia spp. and Lasiodiplodia spp. decreased in the high incidence level plots. This could be due to the increased presence of saprophytic fungal organisms (Figure 2-2), that compete with the main pathogen and thus decreasing its presence. Saprophytic fungi, for example, species of Pestalotiopsis, Penicillium among others establish on dead vs. diseased tissues (Sinclair and Lyon, 2005).

Diplodia sapinea is a well-known opportunistic pathogenic fungus distributed worldwide that affects conifers, including over 150 species of Abies, Araucaria, Cupressus, Picea, Pseudotsuga and Thuja (Sinclair and Lyon, 2005). However, this
fungus is best known as the causal agent of the disease commonly known as “Diplodia tip blight”, which primarily affects pines (Ostry and Juzwik., 2008, Farr and Rossman, 2016). Prior to this study the only host reported in Florida, previous to this study, was slash pine, *Pinus elliottii*, on which the disease was described as “dieback” (House, 2007). However, the current outbreak was considered unusual and mortality from this disease in Florida has been extremely limited in the past. Our field sampling and pathogenicity experiment results indicated that *Diplodia* spp. were the main cause of the outbreak in central Florida. However, trees in the pathogenicity experiment were maintained for 11 weeks, which might not be enough time to determine whether *Lasiodiplodia* spp. could cause blight and mortality in the pines used in the study. Thus, it would be advisable to increase the period of time to accurately determine if *Lasiodiplodia* spp. can also produce dieback.

The disease progression plots showed that the intermediate incidence level plots had a significantly (p-value<0.05) more rapid progression in percentage of trees infected compared with the low and high incidence level plots (slope=16.2) (Figure 2-7). The high incidence plots showed the second highest disease progression (slope=8.5), while the lowest progression was observed in the low incidence plots (slope=3.2). The AUDPC analysis, supported the findings of the progression curves, indicating that the increases in the intermediate incidence level plots were significantly larger compared with low and high incidence plots AUDPC (p-value<0.05).

The behavior of the progression of the disease in the different incidence levels was expected and could be explained based on the number of infected trees and the level of inoculum present in the stands. In the intermediate plots, the level of inoculum
was high, and the number of trees to be infected is high, while although inoculum was high, the number of plants to be infected was lower in the high incidence plots. This explains the faster progression in the intermediate plots. In the case of the low incidence levels, the number of infected trees was also high, but the level of inoculum was low. This likely explains its slower progression (Storer et al. 2002).

It took 14 months for the low level plots to reach the initial level observed in the intermediate plots (~20%) (Table 2-1). While the intermediate plots (initial levels 20-50%) reached 76% of trees infected during the same timeframe. In the case of the high incidence plots, almost all trees (~96%) were symptomatic by the end of the observation period.

The observations of this study are a key component when crafting a strategy to control the disease. There is a longer opportunity for reaction and management in low incidence level stands, but once they reach the intermediate levels (~20-50%), the reaction to control should be faster.
Figure 2-1. Map of locations for monitoring plots. H = High, I = Intermediate and, L = Low incidence levels.
Figure 2-2. Frequency of recovery (Number of isolates) by species group (*Diplodia* spp. and *Lasiodiplodia* spp.) from plots with different levels of disease incidence.

Figure 2-3. Presence of the species *Diplodia* sapinea, *D. scrobiculata*, *Lasiodiplodia theobromae* and *L. pseudotheobromae* from plots with different levels of disease incidence.
Figure 2-4. Lesion length (cm) produced by two Diplodia and two Lasiodiplodia species on Pinus taeda. Different letters indicate significantly different values using Tukey's multiple comparison test at p-value < 0.05.

Figure 2-5. Lesion length (cm) produced by two Diplodia and two Lasiodiplodia species on Pinus elliottii var. densa. A) Experiment 1, and B) Experiment 2. Different letters indicate significantly different values using Tukey's multiple comparison test at p-value < 0.05.
Figure 2-6. Lesion length (cm) produced by two *Diplodia* and two *Lasiodiplodia* species on *Pinus elliottii* var. elliottii. Different letters indicate significantly different values using Tukey’s multiple comparison test at p-value<0.05.

Figure 2-7. Standardized disease incidence progression of plots starting at different incidence levels during five time points. Disease incidence was rated using a 0 to 100% scale related to number of trees with symptoms.
Table 2-1. Average incidence at different time points in the three monitoring incidence level plots.

<table>
<thead>
<tr>
<th></th>
<th></th>
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<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td>Low</td>
<td>10</td>
<td>15</td>
<td>20</td>
<td>20</td>
<td>23.3</td>
</tr>
<tr>
<td>Intermediate</td>
<td>16.7</td>
<td>25</td>
<td>46.7</td>
<td>66.7</td>
<td>76.7</td>
</tr>
<tr>
<td>High</td>
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<td>71.7</td>
<td>86.7</td>
<td>93.3</td>
<td>96.7</td>
</tr>
</tbody>
</table>

Table 2-2. Standardized average incidence levels at the starting point of monitoring.

<table>
<thead>
<tr>
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</tr>
</thead>
<tbody>
<tr>
<td>Low</td>
<td>0</td>
<td>5.0</td>
<td>10</td>
<td>10.0</td>
<td>13.3</td>
</tr>
<tr>
<td>Intermediate</td>
<td>0</td>
<td>8.3</td>
<td>30.0</td>
<td>50.0</td>
<td>60.7</td>
</tr>
<tr>
<td>High</td>
<td>0</td>
<td>6.7</td>
<td>21.7</td>
<td>28.3</td>
<td>31.7</td>
</tr>
</tbody>
</table>

Table 2-3. Areas Under Disease Progress Curve (AUDPC) in different initial disease incidence level plots.

<table>
<thead>
<tr>
<th>Initial Disease Incidence</th>
<th>AUDPC*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low</td>
<td>115b</td>
</tr>
<tr>
<td>Intermediate</td>
<td>435a</td>
</tr>
<tr>
<td>High</td>
<td>267.5b</td>
</tr>
</tbody>
</table>

*Different letters indicate significantly different values (ANOVA; P<0.05)
CHAPTER 3
ROLE OF NITROGEN, SANITATION AND STAND DENSITY MANAGEMENT IN DEVELOPMENT OF A DIPLODIA TIP BLIGHT OUTBREAK IN CENTRAL FLORIDA

Background

Abiotic stressors such as drought, wounding, excessive or deficient nutritional levels and/or improper management practices are usually the most frequent predisposing factors in pathogen and pest outbreaks. These factors stress trees, thereby enabling the entrance and damage by pathogens (Ayres and Lombardero, 2000). Of all plant nutrients, excessive nitrogen is most commonly associated with elevated disease incidence and severity (Lopez-Zamora et al., 2007). Different studies have demonstrated the predisposition of trees to outbreaks following elevated nitrogen fertilization rates (Van Dijk et al., 1992). An excess of nitrogen induces succulent shoot and foliar growth, facilitating wounding from insects, hail or wind (Hesterberg and Jurgensen, 1972). For example, high levels of foliar and soil nitrogen are known to enhance disease severity of pitch canker (*Fusarium circinatum* Nirenberg & O'Donnell) in pines (Van Dijk et al., 1992; Lopez-Zamora et al., 2007), as well as fire blight (*Erwinia amylovora*) in pear and rust (*Puccinia graminis* f. sp. *tritici*) on wheat (Agrios, 2005). Likewise, trees severely affected by *Diplodia sapinea* showed approximately 60% more nitrogen than healthy trees (Van Dijk et al., 1992).

*Diplodia sapinea*, fungal causal agent of the disease Diplodia tip blight, is considered one of the most devastating pathogens in pine plantations (Swart et al. 1985 and Zwolinski et al., 1990). Diplodia tip blight is distributed worldwide and causes a variety of symptoms: shoot blight, branch and stem canker, blue stain and root rot and tree mortality (Desprez-Loustau, et al., 2006). The first outbreaks, associated with hail
damage, was reported in South Africa on *Pinus radiata* D. Don and *Pinus patula* Schiede ex Schltdl. et Cham. in 1909 and 1930, respectively (Lundquist, 1987).

Disease management for Diplodia tip blight and other diseases relies on a timely reaction with the correct silvicultural or chemical management strategy. Some of these treatments include thinning, pruning, prescribed fire and fungicide application (Powers et al., 1981; Swart and Wingfield, 1991; Castello, et al., 1995; Shindler and Reed 1996; Blakeslee, et al., 1999).

The management strategies for Diplodia tip blight include thinning, sanitation methods and fungicide applications (Swart and Wingfield, 1991). The first two methods are recommended for plantations and nurseries because they reduce the stress of competing for nutrients, water, and removing the infected trees also reduces the amount of inoculum in the stand (Swart and Wingfield, 1991). Fungicide application costs and logistics limit fungicide use in pine plantations and it is restricted to nurseries (Palmer et al., 1986).

In this chapter, research studies were conducted to examine the effect of nutrients in an ongoing Diplodia tip blight outbreak in Orlando, FL. In addition, the role of nitrogen in the disease severity of the Diplodia blight on inoculated plants was investigated. Finally, the role of sanitation and stand density on disease development were examined. In this study, I hypothesize that nitrogen increase the virulence of Diplodia tip blight and management practice will decrease level of disease in Orlando, FL.
Materials and Methods

Field Site for Nutrient Analysis

The field areas of study were located in slash pine (Pinus elliottii Engelm. var. elliottii) buffer plantings established by the Central Florida Expressway Authority (CFX) on three sites along highways in Orange County, FL (28°22'13.57"N 81°25'46.62"W, 28°22'28.48"N 81°25'27.00"W, and 28°32'59.39"N 81°27'47.70"W). The average annual precipitation of the area is 1288 mm. The average annual temperature is 23°C with average temperatures in summer of 28.2°C and in winter of 17°C, respectively (NOAA 1981-2010).

Field Foliar Nutrient Analysis

Prior to sampling, the three Pinus elliottii stands found along the CFX were categorized into healthy (<10% of trees with symptoms), intermediate (25-50%), and high (>50%) disease incidence levels of Diplodia tip blight. Foliar samples were taken with pole pruners from the upper 1/3rd of the canopy (Figure 3-1) from seven representative trees at each of the three sampling locations. Pine needle samples were kept in a cooler with ice while in the field. Each sample was dried to a constant weight in a conventional oven at 55 degrees Celsius for two days. Dry samples were sent to the Micro Macro International Laboratory (MMI, Atlanta, GA) for nutrient analysis.

Levels of macro and micro nutrients were analyzed with One-Way ANOVA, considering the original incidence level of the sampled tree as the independent variable. Multiple means comparison was performed with Tukey’s HSD where the significance was evaluated at p-level <0.05. All the analyses were performed in the statistical software R (R Core Team 2014).
Effect of Nitrogen On Disease Severity

One greenhouse study, under controlled conditions, was performed to evaluate the effect of different levels of nitrogen fertilization on disease severity caused by four fungal pathogens on three pine species. The study was repeated twice in the summers of 2015 and 2016 for each tree species (Table 3-1). Each experiment was designed as a randomized complete block, with three blocks. The treatments were arranged in a factorial design with fungal species (4 species and one control) and nitrogen fertilization rate (3 levels), for a total of 15 treatments. The fungal species tested were Diplodia sapinea (Fr.) Fuckel, Diplodia scrobiculata J. de Wet, Slippers & M.J. Wingf., Lasiodiplodia theobromae (Pat.) Griffon & Maubl., and Lasiodiplodia pseudotheobromae A.J.L. Phillips, A. Alves & Crous, plus a non-treated control. The fertilization treatments were preceded by a base application of Nutricote (18:6:8) (Florikan Inc.) (Figure 3-3) at the recommended dose per tree pot. The fertilization treatments were then imposed after a month and were applied as: 1.) no added nitrogen (equivalent to the recommended nitrogen for containerized pines (56.55 kg ha⁻¹)) (1XN). 2.) twice the recommended nitrogen dose (112.1 kg ha⁻¹, 2XN), and 3.) three times the recommended nitrogen dose (168.1 kg/ ha⁻¹) (3XN). The nitrogen source was urea (46:0:0) (Figure 3-3) and the dose was estimated for the pot size (11.4 liters) used in the study. Three-year old trees of Pinus taeda L. (Loblolly pine), Pinus elliottii Engelm. var. elliottii (Slash pine) and Pinus elliottii Engelm. var. densa (South Florida slash pine) were used in this study (Figure 3-2).

Three weeks after the nitrogen fertilization treatments were applied, the sapling stems were wounded and inoculated using a 6-mm sterile cork borer, at approximately 25 cm below the terminal shoot tip (Figure 3-4). A colonized six-mm agar plug (from the
marginal growth of each *in vitro* fungal culture) was inserted in the wounded area facing the cambial tissue of the stem and wrapped with Parafilm® M (Pechiney Plastic). For controls, the inoculation was made with a non-colonized agar plug wrapped with Parafilm® M. The trees were maintained in the greenhouse and watered as needed. The Parafilm was not removed until the end of the study.

Lesion lengths (Figure 3-5) and external symptoms were evaluated 11 weeks after inoculation. Following de-barking, lesion length was evaluated based on a scale of 0-7: 0 = 0-0.9 cm; 1 = 1-1.9 cm; 2 = 2-2.9 cm; 3 = 3-3.9 cm; 4 = 4-4.9 cm; 5 = 5-5.9 cm; 6 = 6-6.9 cm; 7 = 7 cm-tip dead. To complete Koch’s Postulates, one replicate from each of the different fungi was selected for pathogen re-isolation. This was accomplished by removal of sapwood (1 cm) from the margin between the live and dead tissue of the lesion. Excised tissue was surface-sterilized in 2.5% sodium hypochlorite. This was followed by air-drying for 1 minute before being plated on acidified Potato Dextrose Agar (APDA). DNA was extracted and ITS regions were sequenced as described in chapter 2.

**Field Site for Management Studies**

The two field studies for evaluating sanitation strategies for disease management were located in *Pinus elliottii* buffer plantings established by the Central Florida Expressway Authority (CFX) in Orange County, FL. The approximate locations were 28°22'28.48"N 81°25'27.00"W and 28°32'59.39"N 81°27'47.70"W. The average annual precipitation of the area is 1288 mm. The average annual temperature is 23° C with average temperatures in summer of 28.2° C and in winter of 17°C, respectively (NOAA 1981-2010). Two studies were established; one under low initial incidence of the
disease (Low, with <30% of the trees with symptoms) and the second under intermediate-high initial level of incidence (High, with >50% of trees with symptoms).

**Disease Management Study**

Four management treatments were applied in each study (Figure 3-6): 1) no treatment (control), 2) removal of all symptomatic trees (chipped and left on plot as mulch), 3) all symptomatic trees and debris removed and taken to landfill and 4) sanitation thinning of every other tree in the stand to reduce stand density and with the debris taken to the landfill. Each treatment was applied to a plot of 809.4 m² in size. In treatments 2, 3 and 4, the CFX only authorized removal of up to 30% of trees in a given study plot. Diameter at breast height (DBH) and disease severity of all trees in each plot were measured and scored, respectively in June 2014 and 2016. DBH was measured with a diameter measuring tape (Lufkin) at 1.3 m. Disease severity was estimated using a scale of 0 to 10, where 0=0% of tree with symptoms (healthy tree) and 10=100% of the tree with symptoms (Appendix B).

To evaluate the effect of the management strategies on plant growth, the percentage Basal area (BA) growth was calculated as proposed by Blodgett et al., (1997) using the Basal area in 2014 (BA2014) and 2016 (BA2016):

\[
\text{Basal Area} = \frac{\pi \times (DBH)^2}{40000}
\]

\[
\% \text{BA growth} = \frac{\text{BA2016} - \text{BA2014}}{\text{BA2014}} \times 100
\]

To evaluate the effect of the management strategies on the severity of the disease, the severity difference between the final and initial rating was calculated and analyzed. In both cases (BA and Severity), the analyses were carried out using R software (R Core Team 2014) with a two-way ANOVA including the initial disease
incidence and the management strategy. Multiple means comparison was performed with Tukey’s HSD test, where the significance was evaluated at p-level <0.05.
Results

Field Foliar Nutrient Analysis

The only nutrients that showed significant differences (p-value<0.001) with respect to disease severity level were nitrogen and molybdenum. There was a significant, and almost linear, association between the level of nitrogen and the increase in tree disease severity (Figure 3-7A). The nitrogen content (1.6%) of the trees with intermediate severity level were not significantly higher than the healthy trees (1.2%), whereas trees with high severity had a significantly higher nitrogen content (2.2%) than the other two levels. Molybdenum (Mo) followed a pattern opposite to nitrogen (Figure3-7B). The high severity trees had significantly lower Mo levels (0.1ppm) than both the intermediate (1.0ppm) and the healthy (1.4ppm) severity trees. The intermediate severity was not significantly higher in Mo than the healthy trees.

Effect of Nitrogen On Disease Severity

For Pinus elliottii var. elliottii and Pinus taeda, a model including both experiment repetitions showed no interaction effect of the experiment repetition with the treatments (p-value>0.05), so the repetitions were analyzed as one experiment, however for Pinus elliottii var. densa the interaction effect was significant.

For Pinus taeda, no significant difference was found for the factors nitrogen and nitrogen-by-pathogen interaction (p-value>0.05). However, the pathogen factor was significantly different (p-value<0.05). The fungus Diplodia sapinea caused a significantly longer lesion than the other three fungi (Figure 3-8). However, no differences were found among the three other fungi (Diplodia scrobiculata, Lasiodiplodia theobromae and Lasiodiplodia pseudotheobromae). All four fungi caused a significantly longer lesion
than the control (Figure 3-8). None of the treatments caused wilting symptoms in the crown of *Pinus taeda* seedlings.

In *Pinus elliottii var. elliottii* no significant difference was found for the nitrogen factor (p-value>0.05), but a significant difference was found for the factors pathogen and nitrogen-by-pathogen interaction (p-value<0.05). As expected, no lesion or symptoms developed in the seedlings from the control treatment (Figure 3-9). Thus, all fungi and nitrogen levels showed significantly larger lesions than the control (p-value<0.05). There was a nitrogen effect for *D. sapinea* only, where lesions in the 3XN treatment were significantly (p-value<0.05) longer than the 1XN treatment, although wilting symptoms were observed in all nitrogen treatments but more severe with 3XN. (Figure 3-9). Similar to *Pinus taeda*, there was no significant difference (p-value>0.05) in the lesion lengths created by *D. scrobiculata*, *Lasiodiplodia theobromae* and *L. pseudotheobromae*. Although lesion lengths were not significantly different (p-value>0.05) in seedlings inoculated with *D. scrobiculata*, wilting symptoms were observed at the 2XN treatment.

In *Pinus elliottii var. densa* the interaction of the treatments with the experiment replication was statistically significant (p-value<0.05). Thus, the treatments were analyzed by experimental replicate. In the first experimental replicate (Figure 3-10A), *Diplodia sapinea* was the only fungus that had a significant difference in lesion length among the levels of nitrogen. The 3XN produced significantly longer lesions than 1XN but not from 2XN whereas the 2XN and 1XN treatments were not significantly different (Figure 3-10A). For *D. sapinea* 3XN, and 2XN, along with *L. pseudotheobromae* 3XN showed a significantly larger lesion length than the control. No other pathogen or
nitrogen treatment was significantly different from the control treatment. In the second repetition of the experiment (Figure 3-10B), Diplodia sapinea showed the same pattern as the first run with 3XN lesions significantly larger than 1XN, but not different from 2XN treatment. D. sapinea for all levels of nitrogen, L. theobromae for 3XN and D. scrobiculata with 2XN treatment resulted in lesions significantly larger than the control (p-value<0.05). External symptoms were observed only for Diplodia sapinea for the treatment 3XN. Nitrogen also caused a significant effect on the L. theobromae lesion length. The lesion length for 3XN was larger (2.7) than the 2XN (1.0) and 1XN (1.0).

**Disease Management Study**

Diameter at breast height (DBH) and disease severity were determined pre- and post-treatments. A model including both incidence plots and their interactions with treatments was fitted for both traits. The DBH values were used to calculate the percentage of basal area (BA) growth. The analysis for percentage BA growth showed no significant (p-value=0.18) interaction of incidence plots with treatment (Figure 3-13). However, the treatment effect (p-value<0.001) and the sampling field effect were significantly different (p-value<0.001). Trees growing in low incidence plots grew almost twice (41%) as much as trees in high incidence plots (21%). The treatments removal/taken to landfill and sanitation thinning had the greatest BA growth among all the treatments (39% and 34%, respectively). However, removal/taken to landfill was the only sanitation treatment that improved BA growth (p-value<0.001), when compared to the control treatment. On the contrary, the treatments removal/left as mulch and sanitation thinning increased and decreased BA growth, respectively, when compared to the control, but not significantly (p-value=0.9 and 0.5).
Severity values were used to calculate the difference between the pre- and post-treatment (2014-2016). The analyses of the severity difference showed significant interactions (p-value<0.001) between the incidence plots and treatment. In the low disease incidence plots (Figure 3-14A), the control treatment showed a decrease in the severity level. However, the treatment sanitation thinning showed a significantly reduced severity (p-value<0.001) compared to all other treatments, including the control. The treatments removal/left as mulch, and removal/taken to landfill, did not vary in their severity from the beginning of the experiment and were not significantly different from zero (Figure 3-14A). In the high incidence plots (Figure 3-14B), the control treatment increased in severity, and was significantly higher than all other treatments (p-value<0.001). The treatment random/taken to landfill and random thinning were the only treatments that significantly decreased (p-value<0.001) the severity level observed in the trees. The treatment removal/left as mulch did not vary in severity from the beginning of the experiment and was not significantly different from zero (Figure 3-14B); similar to that observed in the low incidence plot.

Discussion

A set of field samplings and controlled experiments were carried out to determine contributing factors of the slash pine dieback outbreak in central Florida. The foliar analysis of samples from trees with different severity levels indicate that among 16 nutrients, only nitrogen and molybdenum were significantly different across the different severity levels (Figure 3-7). Mean nitrogen levels were significantly higher in trees exhibiting high disease severity versus healthy trees and intermediate disease severity (Figure 3-7A). The level of foliar nitrogen in the high disease severity trees (~2.3%) is considered high when compared to published values for healthy slash pine trees.
(Jokela, 2004). This was expected, as the nitrogen applied in these plots, 55-142 kg ha\(^{-1}\) (based on 1568-3913 trees ha\(^{-1}\)) every year for four years, was much higher than what is recommended in commercial forestry plantations using the same species (45-56 kg ha\(^{-1}\) of nitrogen at time of planting only) (Jokela, 2004). Furthermore, the planting densities observed in these stands were also higher, 1568-3913 trees ha\(^{-1}\), than the recommended planting density for this species (1250-1500 trees ha\(^{-1}\)). Molybdenum followed an opposite pattern than nitrogen, higher levels were observed in healthy trees compared to high disease severity trees. Molybdenum levels in high disease severity trees were also lower than the recommended level for this species (Figure 3-7B).

Molybdenum is required by plants in low concentration (<1 ppm) and is involved in the nitrate-reducing enzyme system. Also, nitrate reduction is an important step in N metabolism (Pallardy, 2008).

When studying the effect of nitrogen on disease severity under controlled conditions, higher nitrogen levels (3XN) significantly increased disease effects in *Pinus elliottii* var. *densa* and *Pinus elliottii* var. *elliottii* when inoculated with *Diplodia sapinea* (Figure 3-12). The same effect was observed only in the second experiment repetition for *Pinus elliottii* var. *densa* when inoculated with *L. theobromae*. However, the nitrogen did not cause a difference in the severity of the four pathogens when inoculated into *Pinus taeda* (Figure 3-8).

The two experimental repetitions showed no interaction with the treatments, with the exception for *Pinus elliottii* var. *densa*. For this species, the severity pattern was similar for *D. sapinea* and *D. scrobiculata*, but the magnitude of the lesions was higher for the second repetition. In contrast, for *L. pseudotheobromae* and *L. theobromae* the
pattern and lesions changed between repetitions; on average the lesion length was shorter in the second repetition, but for *L. theobromae* only at the 3XN rate. This difference could be explained by the genetic origin of the plants used; trees from same nursery were obtained, but no records of the family were available.

It is well known that stress to pine trees can be caused by numerous factors including drought, too much competition and over-fertilization (Bachi and Peterson, 1985; Nichols and Ostry, 1990; Blodgett, et al., 1997; Johnson, et al., 1997; Paoletti, et al., 2001). Each of these stresses can facilitate the establishment of pest and pathogens (Ayres and Lombardero, 2000). When environmental conditions become unfavorable for pines, *D. sapinea*, for example, turns pathogenic and moves into living internal tissues (Flowers et al., 2001; Smith et al., 2002). Also, previous research has shown that nitrogen increases susceptibility of pines to *D. sapinea* infection (Blodgett et al., 2005). Overall in this study, *Pinus elliottii* var. *elliottii* and *Pinus elliottii* var. *densa* were more susceptible to *D. sapinea* than other fungi, regardless of nitrogen levels.

A timely reaction with the correct silviculture or chemical management strategy is the key to contain an outbreak. In this study, one management (thinning) and two sanitation strategies (removal to landfill and mulching) were tested in field conditions. Overall, the treatment that removed diseased trees from the stand (*i.e.* removal/taken to landfill) produced the best response with respect to this disease. That is, increased growth of the trees and decreased disease incidence was observed in high incidence plots (Figure 3-13 and 3-14B). The same treatments did not change the severity level for low incidence plots. The treatment random thinning, while it did not produce a significant change in growth increment compared to the control, did significantly
decrease the severity of the disease in both low and high disease incidence plots, when compared to the control. Finally, the treatment that left the diseased trees as mulch in the stand did not change the severity of the disease in any of the plots. It has been recorded that treatments that remove trees from the stand or that decrease the inoculum load, tend to be better for disease control in general and particularly for Diplodia tip blight (Powers et al., 1981; Swart and Wingfield, 1991; Castello, et al., 1995; Shindler and Reed 1996; Blakeslee, et al., 1999). It is important to note that under high incidence of disease, the control treatment increased its severity, probably due to the high levels of inoculum in the stand. However, the treatments either decreased or maintained the same severity level. The restriction in number of trees allowed to be removed (30% of trees) and number of the experiments repetitions imposed by the CFX could have impacted the effect of the three treatments that were originally planned for removal of all diseased trees (removal/left as mulch, removal/taken to landfill and sanitation thinning) and the analysis, since pseudo-replication was part of the problem when it was analyzed. Thus, more consistent results could have been expected if restrictions by CFX were not applied.
Figure 3-1. Foliar sampling design of pine trees for nutrient analysis. Photo courtesy of author.

Figure 3-2. Greenhouse experiment (repetition 2) with three pine species. Photo courtesy of author.
Figure 3-3. Nutricote (left) and nitrogen (Urea) application (right). Photo courtesy of author.

Figure 3-4. Inoculation with agar plug (left) and wrapped with Parafilm (right). Photo courtesy of author.
Figure 3-5. Measurement of lesion length. Photo courtesy of author.
Figure 3-6. Maps showing locations of treatment plots. Low incidence plots (top); high incidence plots (bottom). 1 = No treatment; 2 = Removal of all symptomatic trees (chipped and left on site as mulch for remaining trees); 3 = Removal of all symptomatic trees and debris and taken to landfill; and 4 = Random thinning and taken to the landfill.
Figure 3-7. Percentage of nitrogen (A) and molybdenum (B) with respect to the disease severity level of sampled trees.
Figure 3-8. Lesion length produced by two Diplodia and two Lasiodiplodia species on Pinus taeda. Different letters indicate significantly different values using Tukey's multiple means comparison test at p-value<0.05.
Figure 3-9. Lesion length produced by two Diplodia and two Lasiodiplodia species on Pinus elliottii var. elliottii fertilized with different nitrogen levels. Different letters indicate significantly different values using Tukey's multiple means comparison test at p-value<0.05.
Figure 3-10. Lesion length produced by two Diplodia and two Lasiodiplodia species on Pinus elliottii var. densa at fertilized with different nitrogen levels. A) Experiment 1, and B) Experiment 2. Different letters indicate significantly different values using Tukey’s multiple means comparison test at p-value<0.05.
Figure 3-11. Wilting in *Pinus elliottii* var. *elliottii* with 3X nitrogen application after 11 weeks, inoculated with *D sapinea*. Photo courtesy of author.

Figure 3-12. *Pinus taeda* (left), *Pinus elliottii* var. *densa* (middle) and *Pinus elliottii* var. *elliottii* (right). *D. sapinea* inoculation with 3X nitrogen level, after five weeks. Photo courtesy of author.
Figure 3-13. Average percentage of Basal area growth (2014-2016) among sanitation and stand density treatments. Different letters indicate significantly different means (at p-value<0.05).
Figure 3-14. Severity difference between 2016 and 2014 (post-treatment). A) Low incidence plots, and B) High incidence plots. Different letters indicate significantly different mean severity differences (at p-value<0.05).
Table 3-1. Treatment arrangement of fungi and nitrogen levels for the greenhouse experiment with three different pine species.

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<thead>
<tr>
<th>Fungi treatment</th>
<th>Nitrogen Treatment</th>
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<td></td>
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<tr>
<td>D. sapinea</td>
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</tr>
<tr>
<td>D. scrobiculata</td>
<td>T2</td>
</tr>
<tr>
<td>L. theobromae</td>
<td>T3</td>
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<tr>
<td>L. pseudotheobromae</td>
<td>T4</td>
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</table>

T = Treatment. XN = Basis recommended fertilization
CHAPTER 4
CONCLUSION

Extensive dieback and mortality in slash pine (*Pinus elliottii*) plantings was reported in 2012 in Orlando, FL. To understand and clarify the causal agent, disease biology and its relationship with abiotic factors, a series of field studies and controlled experiments were carried out. In the field, a survey of diseased trees in different disease incidence level plots (low, intermediate and high) was also completed. Four different fungi were consistently recovered; *Diplodia sapinea, D. scrobiculata, Lasiodiplodia theobromae* and *L. pseudotheobromae*. Because these fungi are considered endophytes, a greenhouse study was performed to determinate the causal pathogen. *Diplodia sapinea* and *D. scrobiculata* were the most virulent fungi, causing similar symptoms as seen in the field. *Lasiodiplodia theobromae* and *L. pseudotheobromae* presented caused lesions in the pine species, but external symptoms as observed in the field were not produced.

A set of plots with different initial incidence levels were established and monitored for 15 months. Plots with an intermediate incidence level displayed the fastest progression of disease. This could be explained based on the number of asymptomatic trees to be infected and the level of inoculum present in the stand. Determining the pattern of disease progression is a key component of successful management. Based on the data from this study, a management priority should be given to plots with intermediate disease incidence. Low incidence plots have a slower progression and there is more time to act, while in the high incidence plots most trees are already infected and the effects of intervention are expected to be minimum.
Diplodia sapinea, D. scrobiculata, Lasiodiplodia theobromae and L. pseudotheobromae are fungi that can be present in trees without causing any disease expression (as endophytes), but are capable of transitioning to a pathogenic lifestyle, and produce an outbreak when trees are stressed due to abiotic factors. The outbreak in this study occurred in stands with higher planting density and fertilization than typically recommended for Pinus elliottii. A foliar nutrient sampling from trees growing in stands with different disease incidence levels was carried out. Nitrogen and molybdenum were the only nutrients associated with different disease levels (N was associated with higher disease levels and Mo was associated with lower disease levels). Excessive nitrogen has been known to increase host disease susceptibility and virulence of the pathogens of trees. This relationship was confirmed in a greenhouse experiment with different nitrogen levels applied in inoculated trees. Nitrogen increased the disease severity in Pinus elliottii var. densa and Pinus elliottii var. elliottii when inoculated with Diplodia sapinea.

A set of field plots were established in Orlando, FL along expressways with two different disease incidence levels (low and high). Within these plots, three silvicultural and a control treatment were applied. The treatment removal/taken to landfill significantly increased BA growth of the trees and decreased the severity of the disease in the high incidence level plots after two years following treatment. However, sanitation thinning is the best treatment for decreasing the severity of the disease in both low and high incidence plots. The treatment removal/left as a mulch did not improve the severity of the disease or BA growth in any of the plots.
Taken together, these studies indicate that the cause of pine dieback in Orlando, Florida was Diplodia tip blight disease caused by *Diplodia sapinea* and *D. scrobiculata*. This outbreak most likely was triggered by both high levels of nitrogen fertilization and planting density. By avoiding these predisposing factors and applying proper silvicultural management strategies, outbreaks by this disease could be avoided and/or managed in the region in the future.
APPENDIX A
MACRO AND MICRO NUTRIENTS OF SLASH PINE FOLIAR ANALYSIS AT DIFFERENT DISEASE LEVELS
Table A-1. Macro and micro nutrients of slash pine foliar analysis at different disease levels.

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<th>Mg</th>
<th>S</th>
<th>Fe</th>
<th>Mn</th>
<th>B</th>
<th>Cu</th>
<th>Zn</th>
<th>Mo</th>
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<td>2.4</td>
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Table B-1. Disease severity scale for management study.

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BIOGRAPHICAL SKETCH

Claudia A. Páez was born and lived in Santiago, Chile for 17 years. She attended high school at Siglo XXI to later moved to the South of Chile where she received her bachelor’s degree in forestry engineering from the Universidad Catolica de Temuco. After her graduation, she moved to Gainesville FL, where she learned English and worked as a technician in the forest pathology and forest genomic labs at the University of Florida. This experience influenced her to make the decision to obtain her master’s degree in forest resources and conservation from the University of Florida.