AUBURN UNIVERSITY
FOREST HEALTH
COOPERATIVE

RESEARCH REPORT 2014-03

VIRULENCE OF *LEPTOGRAPHIUM SERPENS* ON LONGLEAF PINE SEEDLING UNDER VARYING SOIL MOISTURE REGIMES

by
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ABSTRACT
Recently, *Leptographium serpens* has been recovered from the roots of declining and dead longleaf pine (*Pinus palustris*) in stands associated with various abiotic stresses. Although most data suggests L. serpens is pathogenic to various *Pinus* species, there is little known of its virulence on longleaf pine or its relationship with abiotic stress in causing disease. These trials examined the effects of *L. serpens* infection coupled with drought stress. Trials began with wound inoculations of bareroot longleaf pine seedlings in spring 2006 and 2007 at the seedling stress facility at Auburn University. Soon after inoculation, seedlings were also subjected to adequate moisture, moderate drought, or severe drought. Sixteen weeks after inoculation, longleaf pine survival, *L. serpens* virulence, and seedling growth characteristics were measured. Longleaf pine seedlings inoculated with *L. serpens* had 33% mortality (138/420) which was significantly greater than non-wounded control seedlings (22%, 47/211). Survival and lesion size on longleaf pine suggests that *L. serpens* is moderately pathogenic to longleaf pine seedlings. Separately, moisture stress associated with low soil moisture also contributed to seedling mortality. Results suggest *L. serpens* infection and moisture stress commonly experienced by southern pines act independently on longleaf pine.

INTRODUCTION
Longleaf pine (*Pinus palustris* Mill) was once the main southern pine species found throughout the southeastern United States, encompassing approximately 38 million hectares (Frost 1993). After nearly complete destruction of the longleaf pine ecosystem, restoration efforts in recent years have increased the planting of longleaf pine on many state and federal lands (Kush et al. 2004). Many factors have contributed to this renewed interest, including the requirement of longleaf pine forest for endangered and sensitive species, specifically the red-cockaded woodpecker (*Picoides borealis* Vieillot) (Alavalapati et al. 2002). Unfortunately, decline and premature tree mortality has recently been observed in longleaf pine stands (Otrosina et al. 1999; Otrosina et al. 2002). The current rate of longleaf pine mortality may affect future restoration efforts.

Studies concerning decline and premature tree mortality in loblolly pine (*P. taeda* L.) have identified several possible contributing factors, including a group of root- inhabiting stain fungi in the genus *Leptographium* (Eckhardt et al. 2007). Despite the common theory that longleaf pine is "resistant" to native insect and diseases (Johnson 1999; Pritchard et al. 1997), it is hypothesized that *Leptographium* species may be contributing to this decline and mortality of longleaf pine (Otrosina et al. 1999; Otrosina et al. 2002).

Leptographium serpens (Goidanich) Siemaszko has been associated with various pine diseases throughout the world (Wingfield et al. 1988), including root disease of two *Pinus* species in South Africa (Wingfield and Knox-Davies 1980). In inoculation tests, *L. serpens* (formerly *Verticicladiella alacris* [Wingfield and Marasas 1981]) was found to cause 20 cm lesions on pine roots after six months (Wingfield and Knox-Davies 1980). In contrast, Zhou et al. (2002) found *L. serpens* to be nonpathogenic to *Pinus* branches in South Africa using a similar inoculation technique. Within the United States, the fungus has been identified as a contributor to loblolly pine decline (Eckhardt et al. 2007). In loblolly pine seedling inoculations, *L. serpens* was more pathogenic than both *L. terebrantis* and *L. procerum* (Eckhardt et al. 2004a), causing 30 mm sapwood lesions after four months.

Many *Leptographium* species have been found associated with pine decline and mortality, usually with one or more contributing site or stand factors (Eckhardt et al. 2007; Klepzig et al. 1991). These syndromes are most often referred to as decline diseases, characterized by many factors contributing to the prolonged death of the tree (Manion 1981). It is possible that multiple inciting biotic and abiotic factors may be acting in conjunction with *Leptographium* to cause the observed longleaf pine mortality.

Inadequate soil moisture is often experienced by southern pines and imposes a significant stress on individual pine trees. Successive, extreme and devastating droughts in recent years (2000 to 2007) along with the pathogen's presence may contribute to the observed tree mortality. These studies seek to determine the virulence of *L. serpens* to longleaf pine seedlings grown in three soil moisture regimes.

MATERIALS AND METHODS

In all, 840 bareroot longleaf pine seedlings were used in inoculation trials with *L. serpens*. In January 2006 and 2007, pine seedlings were planted into 12 and 9 raised outdoor planting boxes (Fig. 3.1), respectively (40 per box). Planting boxes containing pure sand were housed under an open outdoor pavilion, which provide ambient sunlight, yet restricting natural precipitation. Seedlings were allowed to acclimate to their soil conditions with regular watering for two weeks prior to inoculation. Stem inoculations were administered with one isolate of *L. serpens* (LOB-R-00-309 / MYA-3315 Westervelt Company Land, AL) grown on 2% malt extract agar (MEA) plates.

Within each planting box, twenty seedlings were wound inoculated with a 3-mm- diameter colonized plug of MEA (Fig. 3.2) (adapted from Eckhardt et al. [2004a]). Also within each box, ten seedlings were wounded and ten seedlings were unwounded.

Within each replication (3 boxes), one of three moisture treatments were randomly assigned to

each box. The three watering treatments included adequate moisture (26 m^3 m^{-3}), moderate drought (18 m^3 m^{-3}) and severe drought (6 m^3 m^{-3}). Volumetric moisture levels were estimated (adapted from Turtola et al. [2003]), adjusted slightly for field capacity of pure sand and pine species. Soil moisture was regulated using moisture probe sensors, buried 10 cm below the soil line within each box and continuously measuring the dielectric constant. Solenoid valves, attached to each box, controlled the flow of water. Six irrigation nozzles were placed evenly around the perimeter of each planting box in order to ensure equal water distribution. Sixteen weeks after inoculation, seedlings were removed from the soil boxes and lesion length was measured on seedlings with identifiable lesions. To measure tissue occlusion length, the bark was removed from stem and root sections above and below the inoculation point. Seedlings were placed in a solution containing FastGreen FCF
(Sigma-Aldrich) stain in water (0.25 g/liter) for several days (Nevill et al. 1995) (Fig. 3.3) and the length of the unstained tissue portion represented the occlusion length.

Re-isolation was attempted on all seedlings with identifiable lesions by removing small portions of tissue surrounding the lesion and plating on MEA containing 800 mg/l of cycloheximide and 200 mg/l streptomycin sulfate. Seedling survival by treatment, dry-weight biomass of the fine roots, stem and taproot, root collar diameter and bud break was also measured.

Statistical analysis was conducted using SAS statistical software (SAS Institute, 9th ed., Cary, NC). Continuous variables were analyzed using a linear model in the General Linear Models procedure. Inoculation, moisture level, and trial year, including interactions between each factor, were included in the model. The analysis was run separately for each year (2006 and 2007) for those responses that differed between years. Comparisons between inoculation or soil moisture levels were made by the Tukey- Kramer multiple comparison test. All binary variables (mortality and bud break) were transformed to percentages prior to analysis. All percentages were transformed using the square root of the arcsine function to ensure a normal distribution. Transformed binary variables were subsequently used in the linear model for comparison.

RESULTS
Sixteen weeks after wounding and inoculation with *L. serpens*, longleaf pine mortality was 33% (Table 3.1). A significant difference in mortality was detected between the three inoculation treatments (F=6.61, P=0.0038). Mortality was greater within the wound-inoculated treatment when compared to both the wound (F=7.49, P=0.0098) and non-wounded (F=11.85, P=0.0015) controls. However, no difference was found between the wounded and non-wounded treatments (F=0.50, P=0.4860).

Seedlings within the adequate moisture treatment had significantly less mortality than either the severe (F=180.64, P=0.0473) or moderate drought (F=164.82, P=0.0495) treatments (Table 3.2). Mortality differences were not observed between the severe drought and moderate drought treatments (F=0.36, P=0.6552). No significant interaction was detected between inoculation and moisture factors for any response variable measured, including seedling mortality (F=1.63, P=0.1888).

Seedling health characteristics, including RCW, stem biomass, and bud break were not affected by either *L. serpens* inoculation (Table 3.1) or soil moisture level (Table 3.2). Stem and taproot biomass was significantly greater in seedlings not inoculated by *L. serpens* (*F*=3.18, *P*=0.0422). Also, biomass weights and root collar diameter were slightly less in seedlings under the low soil moisture treatment, though not significant.

Longleaf pine seedlings inoculated with *L. serpens* had dark-brown to black, sunken or slightly raised lesions surrounding the inoculation site. Resin was deposited in response to wounding and inoculation. Overall, 94% of living seedlings inoculated with *L. serpens* had lesions, with an average length of 9.3 mm. Lesion length on longleaf seedlings inoculated in 2006 (11.3 mm) was significantly greater (*F*=9.06, *P*=0.0088) than lesions measured in 2007 (8.0 mm)(Table 3.3). Average lesion lengths tended to increase as soil moisture decreased; however treatment means were not statistically significant (*F*=0.51, *P*=0.6112)(Table 3.4). Seedlings produced callus surrounding the lesion.

Occluded stem tissue was detected above and below the inoculation site in advance of all lesions. Similar to the lesion lengths, soil moisture had no affect on levels of tissue occlusion observed in longleaf pine seedlings (*F*=1.78, *P*=0.2017). Occlusion length did vary between the two study years, with lengths shorter overall in 2007 (*F*=3.18, *P*=0.0422).

DISCUSSION

These results suggest both *L. serpens* infection and low soil moisture negatively affect longleaf pine seedlings survival independently. Inoculation of longleaf pine seedlings with *L. serpens* and exposure to high levels of moisture stress contributed separately to mortality in longleaf pine seedlings. Previous pine seedling inoculation experiments with *Leptographium* species have resulted in conflicting results with respect to host mortality. Although some studies have reported that *Leptographium* species infection does not result in seedling mortality (Eckhardt et al. 2004a; Nevill et al. 1992a; Nevill et al. 1995), other inoculation trials have reported significant mortality (Harrington and Cobb 1983; Rane and Tattar 1987; Wingfield 1986). In this study, mortality of longleaf pine seedlings was significantly greater in those seedlings that were wounded and then inoculated with *L. serpens*. In addition to inoculation, the amount of soil moisture also contributed to seedling mortality. Seedlings experiencing low soil moisture were more susceptible to mortality compared to seedlings raised in adequate soil moisture conditions. The presence of *Leptographium* species in other *Pinus* species roots has been linked to abiotic stressors (Eckhardt et al. 2007; Klepzig et al. 1991; Otrosina et al. 2002) including, increasing slope (Eckhardt and Menard 2008) and limited moisture (Goheen et al. 1978). However, these trials indicate that soil moisture does not influence the severity of *Leptographium* species infection. Mortality in pine seedlings inoculated with *L. serpens* was not influenced by soil moisture.

The presence of a defined, darkened lesion surrounding the point of inoculation with *L. serpens* is consistent with other seedling inoculation studies (Eckhardt et al. 2004a; Harrington and Cobb 1983; Wingfield 1986). The presence of resin is similar to findings by Zhou et al. (2002) and callus tissue has been observed in inoculations with *L. procerum* (Wingfield 1983). Although *L. serpens* successfully colonized longleaf pine, growth within the seedling tissue was restricted to the wounded area. In most instances, fungal colonization of the seedling was minimal, particularly in seedlings with adequate moisture. Despite successful colonization by *L. serpens*, average lesion length differed between trial year (2006 versus 2007). Smaller lesion lengths and re-isolation success in 2007 could indicate a reduction in virulence that has been reported with another blue-stain fungus, *Ceratocystis polonica* (Krokene and Solheim 2001). Wounding and mortality of longleaf pine seedlings confirm that *L. serpens* is capable of infecting and killing longleaf pine. However, the amount of mortality and average lesion length on adequately watered seedlings suggests *L. serpens* is a mild to moderate pathogen to healthy longleaf pine seedlings.

Tissue occlusion surrounding the inoculation site was associated with *L. serpens* infection. Occlusion length did not differ among the soil moisture treatments. In contrast, Croisé and Lieutier (1993) found a reduction in host response as water stress increases. Occluded tissues are formed by resin movement into the sapwood surrounding the point of infection. Generally this pathogen-induced defense strategy is considered an advantage; however evidence suggests blockage of the xylem tissues may significantly alter water transport (Horner and Alexander 1985; Joseph et al. 1998). In these trials, mortality was not affected by soil moistures in inoculated seedlings, suggesting xylem blockage was not severe enough to contribute to mortality. The occlusion length difference between study years illustrates a weaker seedling response in 2007, supporting the hypothesis that the *L. serpens* isolate lost virulence between study years.

Infection by *L. serpens* affected the production of new stem and taproot biomass compared to control seedlings. In field observations of pine affected by *Leptographium* species, crown growth often appears stagnant with thin, sparse crowns (Eckhardt et al. 2007; Klepzig et al. 1991; Menard 2007). In loblolly pine, reduced radial growth and crown density has been positively correlated with *Leptographium* infection and root deterioration (Eckhardt et al. 2007). It is thought that *Leptographium* infection significantly alters water and nutrient movement (Horner and Alexander 1985), which leads to the observed reduction in growth.

These inoculation and moisture stress studies indicate that both *L. serpens* infection and low soil moisture contribute to longleaf pine mortality. However, these data show no interaction between inoculation and soil moisture factors. Although both factors negatively affect the survival of longleaf pine seedlings, their affect was independent. On the forest scale, longleaf pine experiencing low soil moisture or *L. serpens* infection are under more stress, with an increased susceptibility to premature mortality. Future studies related to multiple stresses and host reactions are required to better understand the premature pine mortality observed in the southeastern United States.

Table 3.1 Seedling mortality, root collar diameter (RCD), stem biomass, and bud break following inoculation with *L. serpens* and controls.

Treatment	Mortality (%)	RCD (mm)	Stem Biomass (g)	Bud Break (%)
Control (n=211)	6 (9)a	11.0 (2.4)a	11.64 (6.87)a	73 (20)a
Wound (n=209)	40 (30)b	10.2 (2.5)a	10.99 (5.71)a	58 (33)a
Inoculated (n=420)	41 (26)b	9.8 (2.5) a	10.47 (6.51)a	66 (24)a

Note: Means (followed by standard deviation in parentheses) followed by the same letter within a column are not different at alpha= 0.05.

Table 3.2 Seedling mortality, root collar diameter (RCD), stem biomass, and bud break in seedlings experiencing three soil moisture treatments.

Treatment	Mortality (%)	RCD (mm)	Stem Biomass (g)	Bud Break (%)
Adequate (n=280)	6 (9)a	11.0 (2.4)a	11.64 (6.87)a	73 (20)a
Moderate Drought (n=280)	40 (30)b	10.2 (2.5)a	10.99 (5.71)a	58 (33)a
Severe Drought (n=280)	41 (26)b	9.8 (2.5) a	10.47 (6.51)a	66 (24)a

Note: Means (followed by standard deviation in parentheses) followed by the same letter within a column are not different at alpha= 0.05.

Table 3.3 Effects of inoculation of *Leptographium serpens* on lesion development, length, host response, and infection.

Treatment	Lesion Presence (%)	Lesion Length^y (mm)		Occlusion Length (mm)		Re-Isolation (%)	
		2006	2007	2006	2007	2006	2007
Control (n=211)	0.0	0.00	0.00	0.00	0.00	0	0
Wound (n=209)	3.9 (19.5)b	1.00 (0)b	6.20 (2.28)b	3.00 (0)b	8.80 (8.23)b	0	0
Inoculated (n=420)	94.0 (23.8)a	11.29 (5.96)a	7.95 (3.05)a	18.99 (8.42)a	13.03 (6.18)a	100 (0)	85.6 (16.44)

Note: Numbers followed by the same letter within a column are not significantly different at *P*=0.05, based on Tukey-Kramer

Note: Variables with both years shown were found to have significant differences by experiment year and analyzed separately

Table 3.4 Lesion presence, length, length of occluded tissues, and *L. serpens* re-isolation following inoculations of seedlings experiencing three soil moisture treatments.

Soil Moisture	Lesion (%)	Lesion Length (mm)	Occlusion Length (mm)	Re-isolation (%)
Adequate (n=280)	95 (5)a	9.0 (4.81)a	17.3 (8.6)a	95.7 (5.0)a
Moderate Drought (n=280)	96 (5)a	9.9 (4.3)a	16.3 (7.0)a	91.8 (10.9)a
Severe Drought (n=280)	94 (7)a	10.0 (6.4)a	13.8 (7.7)a	90.8 (10.4)a

Note: Means (followed by standard deviation in parentheses) followed by the same letter within in a column are not different at *P*=0.05, based on the Duncan grouping multiple comparison test.

Fig. 3.1 Longleaf pine seedlings planted in raised boxes experiencing three separate soil moisture regimes.

Fig. 3.2 Wound inoculation with *L. serpens* in the upper stem.

Fig. 3.3 Seedlings placed in FastGreen FCF stain solution.