Virulence of *Leptographium serpens* on Longleaf Pine Seedlings Under Varying Soil Moisture Regimes

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ABSTRACT

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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 suggest that *L. serpens* is pathogenic to various *Pinus* spp., 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 nonwounded 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 that *L. serpens* infection and moisture stress commonly experienced by southern pines act independently to stress longleaf pine.

Additional keywords: pine decline, root disease, stain fungi

Longleaf pine (*Pinus palustris* Mill) was once the main southern pine species found throughout the southeastern United States, encompassing approximately 38 million hectares (6). After nearly complete destruction of the longleaf pine ecosystem, restoration efforts in recent years have caused resurgence in the planting of longleaf pine on many state and federal lands (14). Many factors have significantly contributed to this renewed interest, including the requirement of longleaf pine forest for several endangered and sensitive species, specifically the red-cockaded woodpecker (*Picoides borealis* Vieillot) (1). Unfortunately, decline and premature tree mortality has recently been observed in longleaf pine stands (19,20). The current rate of mature longleaf pine mortality may affect future restoration efforts.

Studies concerning a similar decline and premature tree mortality in loblolly pine (*Pinus taeda* L.) have identified several possible contributing factors, including a group of root-inhabiting stain fungi in the genus *Leptographium* (3,5). Despite the common theory that longleaf pine is "resistant" to native insect and diseases (10,22), it is hypothesized that *Leptographium* spp. may be contributing to this decline and mortality of longleaf pine (19,20).

Leptographium serpens (Goid.) Siemaszko has been associated with various pine diseases throughout the world (27), including root disease of two *Pinus* spp. in South Africa (28). In inoculation tests, *L. serpens* (formerly *Verticiladiella alacris*; 29) was found to cause 20-cm lesions on pine roots after 6 months (28). In contrast, Zhou et al. (30) found *L. serpens* to be nonpathogenic to *Pinus* branches in South Africa using a similar inoculation technique. Within the United States, it has been identified as a contributor to loblolly pine decline (5). In loblolly pine seedling inoculations, *L. serpens* was more pathogenic than both *L. terebrantis* and *L. procerum* (3), causing 30-mm sapwood lesions after 4 months.

Many *Leptographium* spp. have been found associated with pine decline and mortality, usually with one or more contributing site or stand factors (5,12). These syndromes are most often referred to as decline diseases, characterized by many factors contributing to the prolonged death of the tree (15). It is possible that multiple inciting biotic and abiotic factors may be acting in conjunction with *Leptographium* spp. 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.

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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, respectively (40 per box). Planting boxes containing pure sand were housed under an open outdoor pavilion, which provided ambient sunlight yet restricted natural precipitation. Seedlings were allowed to acclimate to their soil conditions with regular watering for 2 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, 20 seedlings were wound inoculated with a 3-mm-diameter colonized plug of MEA (adapted from Eckhardt et al.) (3). Also within each box, 10 seedlings were wounded and 10 seedlings remained nonwounded. After imposing the inoculation treatments, seedlings were then subjected to one of three soil moisture regimes.

Within each replication (three boxes), one of three moisture treatments was 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.; 22), 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 measurements were made. Lesion length was measured on seedlings with identifiable lesions. In order to measure tissue occlusion length, the bark was removed from stem and root sections above and below the inoculation point. The seedlings were then placed in a solution containing FastGreen FCF (Sigma-Aldrich) stain in water (0.25 g/liter) for several days (18,21). The length of the unstained tissue portion represented the length of occlusion. Reisolation attempts were made on all seedlings with identifiable lesions by surface sterilizing small portions of tissue surrounding the lesion and plating on MEA containing cycloheximide at 800 mg/liter and streptomycin sulfate at 200 mg/liter. Seedling survival by treatment, dry-weight biomass of the fine roots, stem, and taproot, root collar diameter, and bud break were also measured and recorded.

Statistical analysis was conducted using SAS statistical software (9th ed.; SAS Institute, 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 (status 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 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 with both the wounded (F = 7.49, P = 0.0098) and nonwounded (F = 11.85, P = 0.0015) controls. However, no difference was found between the wounded and nonwounded 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 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).

Inoculation of longleaf pine seedlings with *L. serpens* caused dark-brown to black sunken or slightly raised lesions surrounding the inoculation point. Pitch was deposited in response to inoculation and wounding. Seedlings often produced callous surrounding the lesion. 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). Average lesion lengths tended to increase as soil moisture decreased; however, treatment means were not statistically significant (F = 0.51, P = 0.6112).

Occluded stem tissue was detected above and below the inoculation point in advance of all lesions. Like that of the lesion length, soil moisture treatments had no significant 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).

Generally, seedling health characteristics were not affected by either *L. serpens* infection (Table 1) or soil moisture level (Table 2). Stem and taproot biomass was significantly greater in seedlings not affected by *L. serpens* (F = 3.18, P = 0.0422). Also, biomass weights and root collar diameter were slightly decreased in seedlings experiencing low soil moisture, though not significant.

DISCUSSION

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* spp. have resulted in conflicting results with respect to host mortality. Although some studies have reported that *Leptographium* spp. infection does not result in seedling mortality (3,17,18), other inoculation trials have reported observing significant mortality (8,23,25). 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 with seedlings raised in adequate soil moisture conditions. The presence of *Leptographium* spp. in other *Pinus* spp. roots has been linked to abiotic stressors (5,12,20), including increasing slope (4) and limited moisture (7). However, these trials indicate that soil moisture does not influence the severity of *Leptographium* spp. infection. Mortality in pine seedlings inoculated with *L. serpens* was not influenced by soil moisture. These results suggest that both *L. serpens* infection and low soil moisture negatively affect longleaf pine seedling survival independently.

Table 1. Effects of inoculation of *Leptographium serpens* on survival, seedling growth, and host response of longleaf pine seedlings[x]

Treatment	Mortality (%)	Stem biomass (g)	RCD[z] (mm)	Bud break (%)	Lesion presence (%)	Lesion length (mm)[y]		Occlusion length (mm)		Reisolation (%)	
						2006	2007	2006	2007	2006	2007
Control (n = 211)	22 b	11.56 a	10.3 a	69.6 a	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Wound (n = 209)	27 b	11.40 a	10.2 a	67.9 a	3.9 b	1.0 b	6.2 b	3.0 b	8.8 b	0 b	0 b
Inoculated (n = 420)	33 a	10.14 b	10.4 a	59.3 a	94.0 a	11.3 a	8.0 a	19.0 a	13.0 a	100 a	85.6 a

[x] Numbers followed by the same letter within a column are not significantly different at P = 0.05, based on Tukey-Kramer test. N/A = not available.
[y] Variables with both years shown were significantly different between study years based on Tukey-Kramer test.
[z] Root collar diameter.

Table 2. Effects of soil moisture on survival, seedling health, and host response to *Leptographium serpens* on longleaf pine seedlings[y]

Soil moisture	Mortality (%)	Bud break (%)	Lesion length (mm)	Occlusion length (mm)	Reisolation (%)	RCD[z] (mm)	Stem biomass (g)	Fine root biomass (g)
Adequate (n = 280)	6 b	72.8 a	9.0 a	17.3 a	95.7 a	11.0 a	11.64 a	1.88 a
Moderate drought (n = 280)	40 a	57.8 a	9.9 a	16.3 a	91.8 a	10.2 a	10.99 a	1.56 a
Severe drought (n = 280)	41 a	66.2 a	10.0 a	13.8 a	90.8 a	9.8 a	10.47 a	1.32 a

[y] Numbers followed by the same letter within each column are not significantly different at the P = 0.05 level, based on Tukey Kramer test.
[z] Root collar diameter.

The presence of a defined, darkened lesion surrounding the point of inoculation with *L. serpens* is consistent with other seedling inoculation studies (5,8,26). The pitch response is similar to findings by Zhou et al. (30). Callous tissue surrounding the darkened lesion was also evident, which has also been observed in inoculations with *L. procerum* (25). Although *L. serpens* successfully colonized longleaf pine tissue, growth within the seedling tissue was generally restricted to the wounded area. In most instances, fungal colonization of the seedling was minimal, particularly in seedlings with adequate moisture. Despite consistent successful colonization by *L. serpens*, average lesion length differed between trial years (2006 versus 2007). Decreased lesion length and reisolation in 2007 suggests a reduction in virulence which has been reported with another blue-stain fungus, *Ceratocystis polonica* (13). Wounding and subsequent 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 suggest that *L. serpens* is a mild to moderate pathogen to healthy longleaf pine seedlings.

Tissue occlusion surrounding the inoculation site was consistently associated with *L. serpens* infection. Occlusion length did not differ significantly among the soil moisture treatments. In contrast, Croisé and Lieutier (2) found a reduction in host response as water stress increased. Occluded tissues are formed by resin flow into the sapwood surrounding the point of infection. Generally, this pathogen-induced defense strategy is considered an advantage; however, evidence suggests that blockage of the xylem tissues may significantly alter water transport (9,11). In these trials, mortality was not affected by soil moisture level in inoculated seedlings, suggesting that xylem blockage was not sufficiently severe to contribute. 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* limited the production of stem and taproot biomass compared with control seedlings. In field observations of pines affected by *Leptographium* spp., growth often appears stagnant, with thin, sparse crowns (5,12,16). In loblolly pine, reduced radial growth and crown density has been positively correlated with *Leptographium* spp. infection and root deterioration (5). It is presumed that *Leptographium* spp. infection significantly alters water and nutrient movement (9), which leads to the observed reduction in growth.

These studies indicate that both *L. serpens* infection and low soil moisture contribute to longleaf pine stress and 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 activities are independent. Future studies related to multiple-stress host reactions are required in order to better understand the observed premature mortality. 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.

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