Inoculation of Loblolly and Slash Pine Roots with Four *Leptographium* species and *Heterobasidion annosum*

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ABSTRACT

Root diseases of southeastern *Pinus* species contribute to significant tree mortality every year. Root wound inoculations were performed on two half-sib families of both loblolly and slash pines. Four hundred pine seedlings of each family were planted in a blocked pattern in 1997-1998. Fifty individuals were randomly chosen from each family in both 2007 and 2008 for inoculations. Four *Leptographium* species and *Heterobasidion annosum* were paired together, leaving ten possible treatment pairings, in order to directly test the virulence between the pathogens. Each treatment pairing was randomly assigned to five trees within each of the four pine families in 2007 and 2008. Approximately one meter from the root collar, a wound inoculation was performed with a 1 cm diameter disk of actively growing mycelium. Either a simple wound or sterile media control was imposed approximately 30 cm from the root collar on each root. After eight weeks, several response measurements were made on the darkened lesion, commonly formed following inoculation. *Leptographium huntii* caused the development of longer, wider and deeper lesions than all other species. *Leptographium procerum* and *H. annosum* affected root tissue similarly overall and caused the development of similar sized lesions in loblolly pine. Lesion surface area and total root volume affected by inoculation was significantly larger on average in slash pine hosts when compared to loblolly pines. Results suggest pine hosts react differently to pathogenic root fungi. *Leptographium huntii* appears to elicit the strongest host reaction following infection, ultimately leading to significant root damage. Slash pines react more strongly to invading root pathogens than loblolly pine overall.

INTRODUCTION

Root disease has historically played a critical role in contributing to mortality in southern pine species. Annosus root disease has been known to cause significant losses in recently thinned southern pine plantations since the late 1940's (Stambaugh, 1989). Considerable research has been conducted in Europe and throughout the United States to both better understand the biology and to develop practical solutions. Infection has been found to occur through several different pathways including through natural and wound openings in the cortical cells (Werner et al., 2005). Similarly, mortality associated with other root diseases have been observed since 1940 (Siggers and Doak, 1940). Littleleaf disease of shortleaf and loblolly pine (Campbell and Copeland, 1954) and "loblolly pine die-off" (Brown and McDowell, 1968), more recently called loblolly pine decline, cause deterioration of root tissue leading to a slow decline towards death. *Leptographium* and *Grosmannia* species, associated with loblolly pine decline, are thought to infect through wounds caused by bark beetle and weevil vectors (Eckhardt et al., 2007). Few pathogenicity and virulence tests have been conducted with *Leptographium* and *Grosmannia* species on their mature southern pine hosts. Also, despite nearly consistent observations of both *Heterobasidion annosum* and *Leptographium/ Grosmannia* infection since the 1940's, no direct comparison has been made concerning their relative virulence. These studies seek to compare the relative virulence of several *Leptographium/ Grosmannia* species and *H. annosum* to plantation grown ten year-old loblolly and slash pine roots. Few large experiments have examined infection of these common root pathogens to mature tree roots. These studies seek to better understand primary root damage associated with fungal introduction.

Fig. 1. Four Leptographium species growing in stored slants

Fig. 2. Agar plates containing cultures used in inoculation

Fig. 3. Wound created on lateral root prior to inoculation

Fig. 4. Duct tape covering the control and inoculation treatments prior to being buried

Fig. 5. Re-escavating inoculated roots eight weeks following the inoculation

Fig. 6. Pile of inoculated roots following removal from the ground

Fig. 7. Measurements being taken from root material inoculated

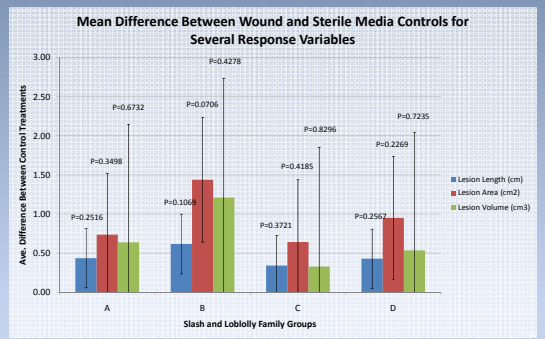

Fig. 8. Average difference between two types of controls for lesion length, lesion area, and lesion volume in each pine family group

Fig. 9. Typical result following a control treatment

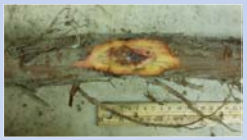

Fig. 10. Typical lesion resulting from *H. annosum* inoculation

Fig. 11. Typical lesion following inoculation with *L. terebrantis*

Fig. 12. Typical lesion formed following inoculation with L. huntii

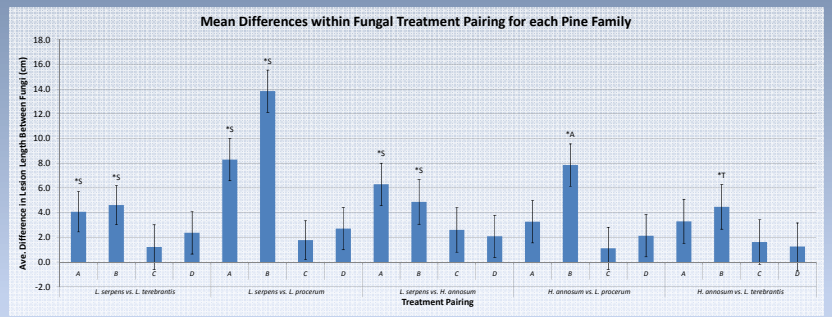

Fig. 13. Average lesion length difference between fungal species within a treatment pairing for each pine family group

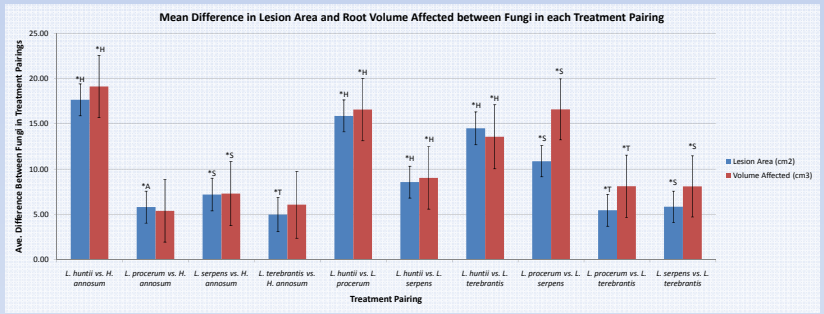

Fig. 14. Average lesion area and affected root volume differences between fungi within each treatment pairing

MATERIALS AND METHODS

Wound root inoculations were performed on two half-sib families of ten-year old plantation grown loblolly and slash pines in Covington County, Alabama. Four blue-stain fungi (Fig. 1), including *Leptographium huntii*, *L. serpens*, *L. terebrantis* and *L. procerum* and *H. annosum* were all paired on sample trees in order to make direct virulence comparisons. A total of ten pairings were used as treatments and randomly applied to five trees within each half-sib family in November of 2007 and 2008. All fungi were plated on 2% malt extract agar approximately fourteen days prior to inoculation (Fig. 2). Two primary lateral roots were excavated on each tree. Fungal species within a treatment pairing were randomly assigned a root and inoculated approximately 70 cm (2 feet) from the root collar. Either a wound or wound+sterile media control was applied to each root approximately 30.5 cm (1 foot) from the root collar. Wounds were created with a 1.3 cm diameter punch, exposing the cambium (Fig. 3). A 1 cm diameter disk of actively-growing mycelium was aseptically placed on the exposed cambium. The cork tissue removed from the wound was subsequently placed on the mycelial disk and the inoculation point was covered in duct tape. All inoculation points were marked with colored pin flags and buried (Fig. 4). After eight weeks all inoculated roots were removed from the ground for measurements (Fig. 5-6). The lesion length (measured on the surface of the phloem), lesion width, lesion depth, and root diameter were measured. Also, the lesion outline was traced on clear transparent sheets and later used to measure lesion area using a Lasico® planimeter. Lesion areas were multiplied by lesion depths to produce an estimate of lesion volume affected. Finally, small pieces of tissue were taken from each inoculation and control and plated on malt extract agar to confirm re-isolation of the inoculated fungi (Fig. 7).

All statistical analysis was conducted used SAS 9.13 statistical software. Control measurements were subtracted from all inoculation measurements on their respective roots prior to analysis. Next for all continuous response variables, values from one fungal treatment were subtracted from the second treatment on each tree, producing one value for each tree. All continuous values were tested using the GLM procedure and individual Estimate statements, testing whether the average difference between fungi were statistically different from zero. These analyses allowed for the most direct comparison between fungal species.

RESULTS/DISCUSSION

Both wound and wound + media controls were used in the inoculation tests. No statistical differences were observed between the two control types for measurements of lesion length, lesion area, and affected root volume (Fig. 8). In root inoculations of pines in South Africa, only a sterile media control was used and proved to be an accurate control measure (Wingfield and Knox-Davies, 1980).

Dark, resin impregnated tissue was observed surrounding the point of inoculation with all five fungi tested. Tissue surrounding control treatments were often lighter in color compared to fungal inoculations (Figs. 9-12). A significant statistical interaction existed between treatment pairing and tree family group. *Leptographium serpens* produced a significantly longer lesion than *L. terebrantis*, *L. procerum*, and *H. annosum* in both slash pine families, however was not significantly longer in loblolly pine families (Fig. 14). In loblolly pine seedling inoculations, *L. serpens* has been shown to cause a larger lesion than both *L. terebrantis* and *L. procerum* (Eckhardt et al., 2004). *Heterobasidion annosum* and *L. procerum* produced lesions of similar length, with the exception of slash family B, where *H. annosum* was larger. Similarly, lesions produced by *L. terebrantis* were not longer than those produced by *H. annosum*, except for slash family B.

Lesions, resulting from fungal infection often extended in both the longitudinal and radial directions within the root tissue. The total lesion area and approximate root volume affected by inoculation showed similar results (Fig. 15). *Leptographium huntii* was associated with significantly larger lesions than all other fungi. As a result, it affected more total root volume than any other treatment. *Leptographium serpens* was significantly greater in both measures compared to *L. terebrantis*, *L. procerum* and *H. annosum*. *Heterobasidion annosum* caused larger lesions than *L. procerum* but not *L. terebrantis*. Finally, *L. terebrantis* produced larger lesions and affected more root volume than *L. procerum*, which has been observed previously in pine stems (Eckhardt et al. 2004).

These studies illustrate the ability of all four root fungi to infect and cause resin-soaked lesions in slash and loblolly pine roots. *Leptographium huntii* appears to cause significantly more damage to root tissue, compared to all other fungi tested. *Leptographium serpens* and in some measures *L. terebrantis* also appear to cause larger lesions than *H. annosum*, a well-known primary pathogen of southern pines. These studies help to establish the pathogenicity and relative virulence of several prominent southern pine root pathogens.