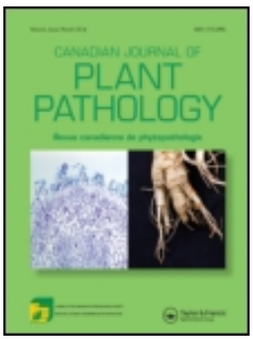

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# The pathogenicity and virulence of four Ophiostomatoid fungi on young Longleaf pine trees

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# The pathogenicity and virulence of four Ophiostomatoid fungi on young Longleaf pine trees

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**Abstract:** In southeastern USA, insect vectors transfer pathogenic ophiostomatoid fungi that cause disease in southern pines. During 2007 and 2008, potted longleaf pines (*P. palustris* Mill.), of similar ages ranging in height from 58 to 198 cm, were inoculated with the following fungi to assess their pathogenicity (and virulence): *Grosmannia huntii*, *Leptographium procerum*, *L. serpens* and *L. terebrantis*. Seventeen weeks after inoculation, *L. terebrantis*, *L. serpens* and *G. huntii* were found to cause significantly larger lesions and more sapwood discolouration than wounded uninoculated controls. *Leptographium terebrantis* caused significantly more sapwood discolouration than all other fungi. Despite significant sapwood occlusion after fungal inoculation, no reductions in needle water potentials were observed between treatments. All fungal species were successfully re-isolated from longleaf pine trees.

**Keywords:** *Grosmannia huntii, Leptographium serpens, Leptographium procerum, Leptographium terebrantis*, ophiostomatoid fungi, pathogenicity, *Pinus palustris*

**Résumé:** Dans le sud-est des États-Unis, des insectes agissent comme vecteurs de champignons ophiostomatoïdes pathogènes qui causent des maladies chez les pins du Sud. En 2007 et 2008, des pins des marais en pots (*Pinus palustris* Mill.), d'à peu près du même âge et mesurant de 58 à 198 cm de hauteur, ont été inoculés avec les champignons suivants afin d'évaluer leur pathogénicité (et leur virulence): *Grosmannia huntii*, *Leptographium procerum*, *L. serpens* et *L. terebrantis*. Dix-sept semaines après l'inoculation, *L. terebrantis*, *L. serpens* et *G. huntii* causaient des lésions significativement plus graves et provoquaient davantage de décoloration de l'aubier que les arbres témoins blessés, mais non inoculés. *Leptographium terebrantis* a causé davantage de décoloration que tous les autres champignons. Malgré une occlusion significative de l'aubier après inoculation fongique, aucune réduction du potentiel hydrique des aiguilles n'a été observée. Toutes les espèces fongiques qui se trouvaient dans les pins des marais ont été de nouveau isolées.

**Mots clés:** champignons ophiostomatoïdes, *Grosmannia huntii*, *Leptographium procerum*, *Leptographium serpens*, *Leptographium terebrantis*, pathogénicité, *Pinus palustris*

## Introduction

Root-colonizing ophiostomatoid fungi in the genus *Grosmannia* (previously *Ophiostoma* Zipfel *et al*. [2006]) and their anamorphs *Leptographium* (Lagerberg & Melin) cause diseases on conifers (Wingfield *et al*., 1988). *Leptographium wageneri* (W.B. Kendr.) M.J. Wingfield, the causal agent of black-stain root disease, extensively colonizes the outer xylem of infested trees and causes mortality in many western conifer species (Cobb, 1988). Procerum root disease (caused by *L. procerum* [Kendrick] M.J. Wingfield), may girdle and kill many conifer species (Alexander *et al*. 1988), particularly white pine (*Pinus strobus* L.) (Dochinger, 1967). *Leptographium* species have also been identified as contributors to decline diseases, specifically red pine (*P. resinosa* Ait)

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decline (contributed by *L. procerum* and *L. terebrantis* S.J. Barras & T.J. Perry; Klepzig *et al*., 1991) and loblolly pine (*P. taeda* L.) decline (contributed by *L. procerum*, *L. terebrantis* and *L. serpens* (Goidanich) Siemaszko; Eckhardt *et al*., 2007).

*Hylobius pales* Herbst. (pales weevil) and *Pachylobius picivorous* Germar. (pitch-eating weevil) commonly feed in the lower stem and near the root collar of sapling-sized pines (Edmonds *et al*., 2000). In young longleaf pine plantations, weevil damage in the lower stem and *Hylastes* damage on the lateral roots have been observed (G. Matusick, unpublished observations) and the wounds represent potential infection sites for fungi. Natural inoculation of young pines with ophiostomatoid fungi has been illustrated in other pine systems around the world. For example, in *Pinus radiata* D. Don seedlings, *Hylastes atar* (Paykull) has been shown to transmit ophiostomatoid species during feeding (Reay *et al*., 2002). Nevill & Alexander (1992) observed the transmission of *L. procerum* to five-year-old eastern white pine (*P. strobus* L.) by *Hylobius pales* adults. In addition, *L. procerum* has been isolated from various sapling-sized pines attacked by weevils (Wingfield, 1983).

Longleaf pine (*P. palustris* Mill.) once occupied approximately 370,000 km$^2$ in the southeastern USA, but is now a relatively minor component of the ecosystem, residing on just 2% of its original range (Frost, 1993). Based on trends in seedling production, however, planting of longleaf pine has been increasing (Hainds, 2002). This is in part due to the species' relative resistance to several insect pests and diseases (Snow *et al*., 1990) and the habitat it provides for endangered plant and animal species, such as the red-cockaded woodpecker (*Picoides borealis* Vieillot) (Brockway *et al*., 2005). Decline and premature mortality have recently been observed in 30–45-year-old longleaf pine stands. *Leptographium procerum* and *L. terebrantis* have been associated with increasing crown symptoms (Otrosina *et al*., 1999), similar to loblolly pine decline (Eckhardt *et al*., 2007). More recently, *L. serpens* and *G. huntii* (R.C. Rob. Jeffr.) Zipfel, Z.W. de Beer & M.J. Wingfield were isolated from roots of symptomatic longleaf pines. *Leptographium* species are commonly isolated from Curculionid beetles in longleaf pine stands, including *Hylastes* species, *Hylobius pales* and *Pachylobius picivorus* (Zanzot, 2009).

There are a few reported inoculation studies with ophiostomatoid fungi on longleaf pine. *Leptographium serpens* was found to cause mortality in longleaf pine seedlings grown for four months under varying soil moisture conditions (Matusick *et al*., 2008). Also, inoculations with *L. terebrantis* and *L. procerum* caused necrotic lesions in the cambial zone on mature stems and roots (Otrosina *et al*., 2002). The objective of the current study was to determine the pathogenicity and relative virulence of four ophiostomatoid fungi (*L. procerum, L. terebrantis, L. serpens* and *G. huntii*) to longleaf pine saplings. It is hypothesized that establishment of ophiostomatoid fungi can contribute to mortality in longleaf pine.

## Materials and methods

On 27 November 2006, 200 five to seven-year-old longleaf pine trees were obtained from a horticulture nursery in central Alabama. Trees ranged in height from 58–198 cm with an average root collar diameter of 5.3 cm (± 0.7 cm). The trees were potted in 18.9 litre (5-gal.) pots with a mixture of pine bark and sand and grown in full sunlight with access to natural precipitation, supplemented with irrigation as needed throughout the experimental period.

Two weeks prior to inoculations, single isolates of *L. procerum, L. terebrantis, L. serpens* and *G. huntii* were plated on 2% malt extract agar (MEA). All isolates were in the anamorphic state and were obtained from roots of symptomatic loblolly or longleaf pines using methods described in Eckhardt *et al*. (2007) (Table 1). The identities of all isolates have been confirmed by Dr Mike Wingfield at the Forestry and Agricultural Biotechnology Institute, South Africa using morphological and sequence data from the partial ITS operon, the β-tubulin region and elongation factor-1a.

On 3 April 2007 and 8 April 2008, one hundred trees were arranged in a single factor, completely randomized experiment. Each tree was randomly assigned to one of five treatments including wound inoculation with *L. procerum, L. terebrantis, L. serpens* and *G. huntii*, as well as a wounded control (n = 20). Wound inoculations were made approximately 10 cm above the soil line using the cork-borer method (Wright, 1933). One inoculation or control was performed on each sample tree. A 14-mm diameter cork borer was used to remove a plug of bark tissue and a 10-mm diameter plug of actively growing mycelium with minimal sporulation was placed against the exposed cambium. The bark plug was replaced to cover the mycelium and duct tape was wrapped around the point of inoculation to minimize desiccation and contamination.

Seventeen weeks after inoculation, predawn and midday needle water potential measurements were taken on a subset of five randomly selected saplings per treatment. Two fascicles were selected from each tree for both predawn and midday water potential measurements using a pressure chamber (Model 670, PMS Instrument Inc. Albany, OR). Needle water potential measurements were averaged to give one predawn and midday value per tree.

Trees were destructively sampled 17 weeks after inoculation (31 July 2007 and 5 August 2008) to quantify

**Table 1.** Fungal isolates inoculated in longleaf pine saplings.

| Fungal species | Isolate no./ ATCC accession no. | Collection site | Host source |
|---|---|---|---|
| *G. huntii* | LLP-R-02-100/ MYA-3311 | Fort Benning Military Reservation, GA | Longleaf pine root |
| *L. serpens* | LOB-R-00-309/ MYA-3315 | Westervelt Company Land, AL | Loblolly pine root |
| *L. terebrantis* | LOB-R-00-805/ MYA-3316 | Talladega National Forest, Oakmulgee Ranger District, AL | Loblolly pine root |
| *L. procerum* | LOB-R-00-456/ MYA-3313 | Talladega National Forest, Oakmulgee Ranger District, AL | Loblolly pine root |

Note: All fungal isolates were obtained from trees exhibiting symptoms characteristic of root disease and reside in collections at the Forest Health Dynamics Laboratory in Auburn, AL.

fungal infection and host response. All bark was removed from the area surrounding the point of inoculation and necrotic lesions were traced on transparent sheets. Next, trees were cut transversely, through the point of inoculation and total sapwood area and discoloured sapwood were traced on transparent sheets. Lesion length and lesion depth were measured. Lesion surface area and total discoloured sapwood area were determined using a LASICO planimeter (LASICO Co. Los Angeles, CA). The ratio of discoloured sapwood area to total sapwood area was used to calculate the percentage of discoloured sapwood for each sample tree. Finally, small tissue samples surrounding the point of inoculation were surface-sterilized and placed on CSMA (MEA containing 800 mg $L^{-1}$ of cycloheximide and 200 mg $L^{-1}$ of streptomycin sulfate) for re-isolation of fungi.

All continuous variables, including root collar diameter, height, lesion length, lesion depth, lesion surface area, discoloured sapwood and predawn and midday water potential measurements were analyzed with ANOVA using the GLM procedure in SAS (SAS Institute, 9.1 ed., Cary, NC). Each response variable was used in a simple linear model testing inoculation treatment. Experiment year (replication) was also included in the model as a blocked variable. Individual treatments were compared using Tukey's multiple comparison test. The log transformation was made on the lesion area variable due to non-normally distributed data. Re-isolation results (binary) were tested using the logit model in the Genmod procedure in SAS. The model included both inoculation treatment and experiment year similar to the linear model.

## Results

Four trees died during the experiments, which included one each from the following treatments: *L. terebrantis*, *L. serpens*, *G. huntii* and control. Immediately after death, the area surrounding the point of inoculation was uncovered and observed. In each case, no evidence was found to suggest the treatment was responsible for mortality; however *L. terebrantis*, *L. serpens* and *G. huntii* were isolated from their respective stems. The cause of death was ultimately not determined. No significant treatment differences were observed with respect to root collar diameter ($F = 0.54$, $P = 0.71$) or tree height ($F = 1.21$, $P = 0.31$) (Table 2).

Lesion length ($F = 8.73$, $P < 0.0001$) and lesion surface area ($F = 6.84$, $P < 0.0001$) were significantly affected by inoculation treatment in the linear model (Table 2). Dark brown to black, discoloured, resin-filled tissue (lesion) was often observed surrounding the point of inoculation. In contrast, stem tissue surrounding wound controls was lightly coloured with a faint brown ring surrounding the wound. *Leptographium terebrantis* and *G. huntii* induced significantly longer lesions than controls. All ophiostomatoid fungi, with the exception of *L. procerum*, caused the development of larger lesions than controls (with respect to total lesion area $cm^2$) (Table 3). All fungi were isolated from tissue surrounding lesions. However, infection was confirmed on only 20% of trees inoculated with *L. terebrantis*, *G. huntii* and *L. serpens* (40% on trees inoculated with *L. procerum*).

Discoloured sapwood was observed in all inoculated trees. Stems inoculated with ophiostomatoid fungi generally had darkened, resin-filled sapwood penetrating in a wedge-shaped pattern from the point of inoculation. Cross-sectional discolouration associated with wounded controls was generally lighter coloured in a similar wedge-shape. *Leptographium terebrantis* and *G. huntii* caused significantly deeper sapwood penetration than controls. Inoculations with *L. terebrantis*, *G. huntii* and *L. serpens* were associated with greater overall sapwood

**Table 2.** Effects attributed to replication and inoculation treatment following tests with longleaf pine trees. Probability of a greater *F*-value shown for sample tree characteristics, lesion measurements and needle water potentials.

| Source | Df[a] | Root collar diameter | Height | Df[b] | Lesion length | Lesion depth | Lesion area | Log (Lesion area) | Discoloured sapwood | Df[c] | Needle $\Psi_{predawn}$ (MPa) | Needle $\Psi_{midday}$ (MPa) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Replication | 1 | 0.0001 | 0.7832 | 1 | 0.0934 | 0.0084 | 0.3332 | 0.0547 | 0.0001 | 1 | 0.0035 | 0.0152 |
| Inoculation treatment | 4 | 0.7608 | 0.2877 | 4 | 0.0001 | 0.0001 | 0.0001 | 0.0001 | 0.0001 | 4 | 0.1261 | 0.6329 |
| Error | 199 | | | 190 | | | | | | 49 | | |

Notes: [a]All sample trees were used in the analysis.
[b]Only living sample trees were used in the analysis.
[c]A subsample of five trees per treatment per year were measured for predawn and midday water potential.

**Table 3.** Lesion length, lesion depth, lesion area, cross-sectional sapwood discolouration, fungal re-isolation and needle water potential 17 weeks following inoculation.

| Treatment | Lesion length (cm) | Lesion depth (mm) | Lesion area ($cm^2$) | Log lesion area | Discoloured sapwood (%) | Needle $\Psi_{predawn}$ (MPa) | Needle $\Psi_{midday}$ (MPa) | Re-isolation (%) |
|---|---|---|---|---|---|---|---|---|
| *L. terebrantis* | 4.9 (1.9) ab | 10 (8) a | 10.9 (7.81) | 0.96 (0.25) a | 12.40 (5.43) a | −0.40 | −1.34 | 20 a |
| *G. huntii* | 4.9 (2.0) ab | 7 (2) bc | 10.0 (5.43) | 0.94 (0.24)a | 9.64 (3.89) b | −0.39 | −1.26 | 20 a |
| *L. serpens* | 4.0 (1.3) bc | 5 (3) cd | 8.6 (6.10) | 0.88 (0.19) ab | 8.59 (4.20) b | −0.45 | −1.37 | 20 a |
| *L. procerum* | 3.7 (1.3) bc | 5 (2) cd | 6.9 (3.21) | 0.80 (0.18) bc | 8.19 (3.48) bc | −0.44 | −1.34 | 40 a |
| Control | 3.2 (1.2) c | 4 (2) d | 5.4 (2.98) | 0.68 (0.20)c | 5.94 (2.88) c | −0.45 | −1.42 | 0 b |

Note: Means (followed by standard deviation in parentheses) with the same letter within a column are not significantly different from one another at $P < 0.05$.

discolouration (%) than controls. *Leptographium terebrantis* caused significantly deeper lesions and more sapwood discolouration than all other treatments.

No significant differences were observed in needle water potential between inoculation treatments at predawn ($F = 1.91$, $P = 0.13$) or midday ($F = 0.65$, $P = 0.63$) measurements.

## Discussion

Common observations made in this study including darkened, resin-filled sapwood and discoloured inner bark have been noted in other pine hosts (Nevill *et al*., 1995; Eckhardt *et al*., 2004; Rice *et al*., 2007). Lesion length and lesion area following fungal inoculation is most often used to assess the pathogenicity and relative virulence to pine hosts (Parmeter *et al*., 1989; Nevill *et al*., 1995). *Leptographium terebrantis* caused larger lesions than controls and has been observed to induce significant lesion development after inoculation in various *Pinus* species (Wingfield, 1983; Raffa & Smalley, 1988; Parmeter *et al*., 1989; Nevill *et al*., 1995) including longleaf pine (Otrosina *et al*., 2002). This represents the first report of *G. huntii* causing significant damage in pine tissue following inoculation. Although associations have been made with dying trees and the presence of *G. huntii* (Klepzig *et al*., 1991; Zanzot, 2009), no pathogenicity tests have been previously performed. *Leptographium serpens* also produced lesions that were overall larger than controls (lesion area), but not longer (lesion length). This fungus has previously been found to cause large lesions and disease (Wingfield & Knox-Davies, 1980; Eckhardt *et al*., 2004). However, some researchers have found *L. serpens* to be non-pathogenic following artificial inoculation in certain *Pinus* hosts (Zhou *et al*., 2002). *Leptographium procerum* failed to cause lesions larger than wounded controls. Generally, *L. procerum* has been considered to be a weak pathogen on *Pinus* species (Harrington & Cobb, 1983; Wingfield, 1983, 1986), in some cases causing larger lesions than controls (Eckhardt *et al*., 2004). Successful re-isolation of each of the test fungi from diseased tissue confirms their pathogenicity to young longleaf pine trees. Previous inoculation tests have consistently confirmed infection for *L. procerum*, *L. terebrantis* and *L. serpens* on pine (Otrosina *et al*., 1999; Eckhardt *et al*., 2004).

Despite greater sapwood discolouration following *L. terebrantis*, *G. huntii* and *L. serpens* inoculations, diseased areas were lower than expected. Overall, the percentage of discoloured sapwood was lower than found

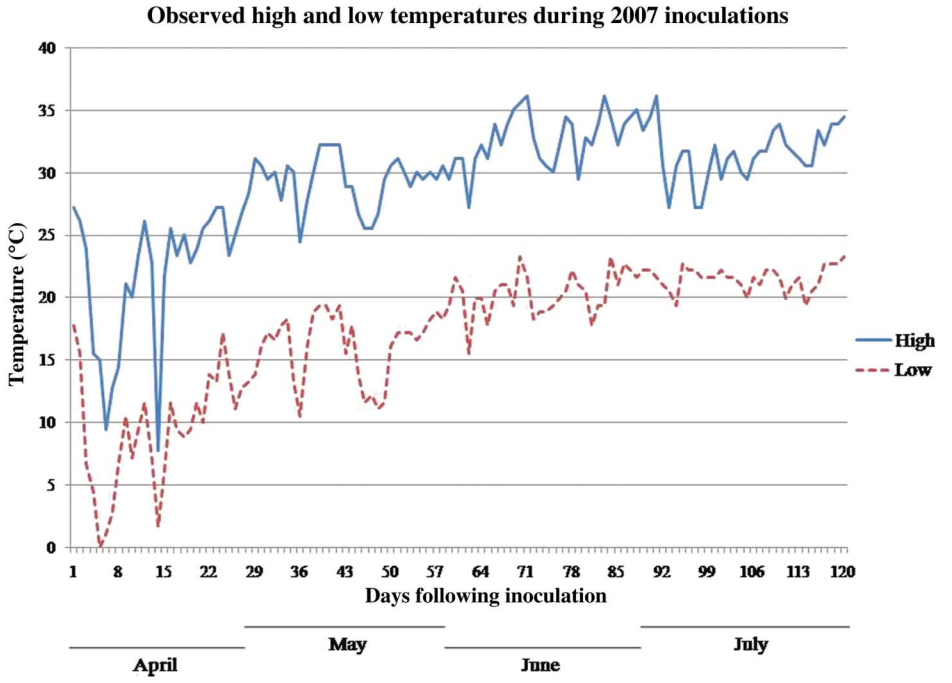


**Fig. 1.** Observed high and low temperatures at Auburn University during the 2007 inoculation test. Data were obtained from Alabama Mesonet Weather Data, located on Auburn University Campus 32.60 N, 85.50 W approximately 1.40 km from the study site (32.59 N, 85.49 W) http://www.awis.com/mesonet/.

previously using similar hosts and fungi (Joseph *et al*., 1998; Lieutier *et al*., 2004). For example, Parmeter *et al*. (1992) observed 71% sapwood occlusion after inoculation with *L. terebrantis* in ponderosa pine, 17 weeks following inoculation. The relatively minor sapwood damage could be explained by low temperatures shortly following inoculations in both years (Figs. 1 and 2). Nebeker *et al*. (1993) stated that the response to invasion depended on the physiological activity of the host which is largely influenced by temperature. However, bud break and needle elongation in longleaf pine generally occurs between mid and late April (Sheffield *et al*., 2003). In the present studies, needle elongation was observed shortly following inoculation and trees generally flushed multiple times throughout each experiment. Previous work has shown that resistance to blue-stain infection in a particular host is associated with the amount of resin produced (Horntvedt, 1988). Significant resin production has been observed following inoculation of large longleaf pine roots (Matusick, unpublished results). In the present studies, little resin was observed following inoculation, suggesting host response to inoculation was minimal. More likely, the limited sapwood damage is a result of the inability of ophiostomatoid fungi to consistently infect longleaf pine stem tissue. Fungal infection was lower than expected, based on results from previous studies. In inoculations of longleaf pine seedlings, *L. serpens* was successfully isolated from greater than 85% of trees (Matusick *et al*., 2008). Additionally, infection could be confirmed in over 70% of trees inoculated with each of the four ophiostomatoid fungal species in root inoculations of large longleaf pines (Matusick and others, unpublished data).

Fungal invasion following inoculation and the resulting damage has the ability to significantly alter water conduction (Horner & Alexander, 1985; Joseph *et al*., 1998; Croisé *et al*., 2001). Dysfunctional sapwood is thought to be important in foliar symptom development in hosts affected by other root and stem inhabiting ophiostomatoid fungi (Rane & Tattar, 1987; Butnor *et al*., 2000). The relatively minor sapwood damage following fungal inoculation is consistent with the lack of variation in needle water potential responses between treatments. Croisé *et al*. (2001) found a drop in needle water potential after inoculation with *L. wageneri*, but only after mass inoculation with the fungus coupled with severe water stress. In another experiment, Croisé *et al*. (1998) found that trees inoculated at a density of 400 mycelial plugs $m^{-2}$ showed external symptoms, including needle yellowing. Single inoculations of trees are generally not used when attempting to detect physiological changes in the host. It is possible that the inoculum dosage was not high enough to cause

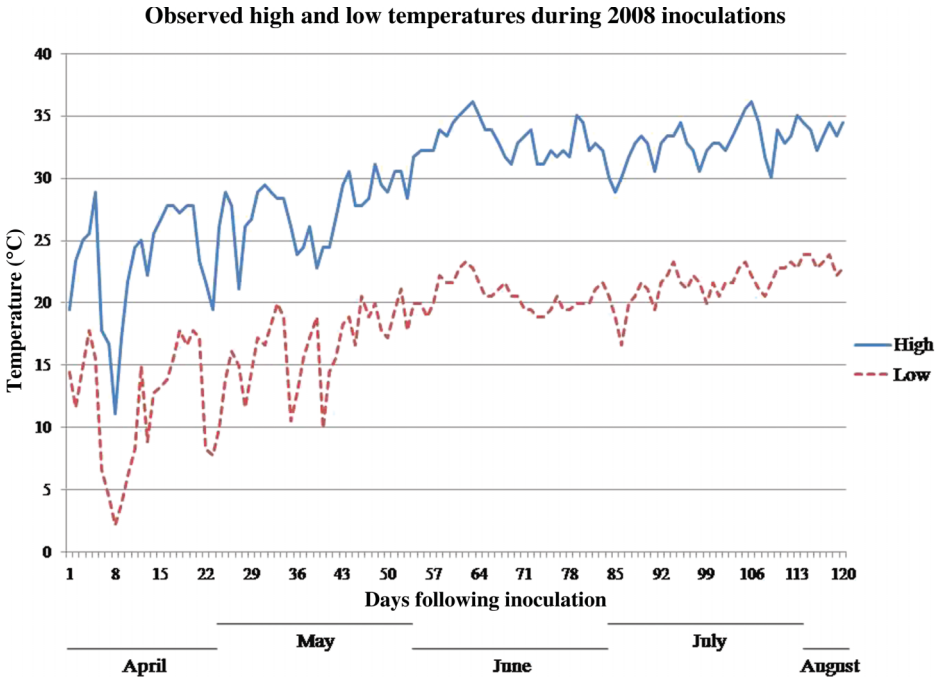


**Fig. 2.** Observed high and low temperatures at Auburn University during the 2008 inoculation test. Data were obtained from Alabama Mesonet Weather Data, located on Auburn University Campus 32.60 N, 85.50 W approximately 1.40 km from the study site (32.59 N, 85.49 W) http://www.awis.com/mesonet/.

significant sapwood damage. However, Paine & Stephen (1987) found that lesion size is not related to the amount of inoculum introduced. In each experiment, fungal species were left to grow (following inoculation) much longer than many other studies of this type. It is more likely that, in these studies, southeastern ophiostomatoid fungal species caused minor physiological disruption in stems of young longleaf pine hosts following inoculation.

In conclusion, the fungal species tested in this study differed in their virulence to longleaf pine. *Leptographium terebrantis* caused significantly more sapwood damage than any other treatment, suggesting it has the greatest potential for physiological disruption. In contrast, *L. procerum* appears to be least virulent, producing sapwood discolouration comparable to controls. Even though most fungal species were able to infect longleaf stem tissue and cause more damage than control inoculations, no foliar symptoms or water stress were detected. Perhaps a mass inoculation of ophiostomatoid fungal species, simulating feeding damage at multiple locations on the stem, would provide a more realistic assessment of potential damage from inoculation. More tests are needed to determine the likely contribution that ophiostomatoid fungi make towards mortality in longleaf pine.

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