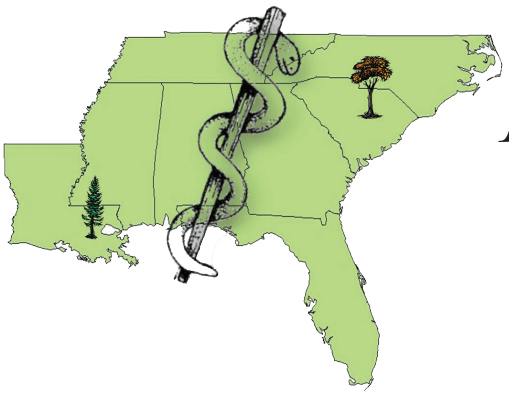

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THE PATHOGENICITY AND VIRULENCE OF FOUR OPHIOSTOMATOID FUNGI ON YOUNG LONGLEAF PINE TREES

by
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ABSTRACT
Insects feeding on the roots and lower stem of trees commonly vector ophiostomatoid fungi that infect hosts during feeding. In the southeastern United States, insect vectors transfer pathogenic ophiostomatoid fungi that cause disease in southern pines. Potted longleaf pines (*P. palustris*), of similar ages ranging from 58 to 198 cm in height, were inoculated in 2007 and 2008 in order to assess the pathogenicity (and virulence) of *Grosmannia huntii*, *Leptographium procerum*, *L. serpens*, and *L. terebrantis*. Seventeen weeks after inoculation, *L. terebrantis*, *L. serpens* and *G. huntii* caused significantly larger lesions and more sapwood discoloration than wound controls. *Leptographium terebrantis* caused significantly more sapwood discoloration than all other treatments. 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.

INTRODUCTION
Root-colonizing ophiostomatoid fungi in the genus *Grosmannia* (previously *Ophiostoma* Zipfel et al. [2006]) and their anamorphs *Leptographium* (Lagerberg & Melin), cause disease of conifers (Wingfield et al. 1988). *Leptographium wageneri* (W.B. Kendr.) M.J. Wingf., 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) 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 picivorus* 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 and wounds in the lower stem and *Hylastes* damage on the lateral roots have been observed (Matusick and Eckhardt unpublished data) and 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 ater* (Paykull) has been shown to transmit ophiostomatoid species during feeding (Reay et al. 2002). Nevill and Alexander (1992c) 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 United States, but is now a relatively minor component of the ecosystem, residing on just 2% of its original range (Frost 1993). However, based on seedling production trends, 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 the endangered red-cockaded woodpecker (*Picoides borealis* Vieillot Brockway et al. 2005). Decline and premature mortality of longleaf pine have recently been observed in 30-45 year-old stands. *Leptographium procerum* and *L. terebrantis* have been associated with 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. Wingf were isolated from roots of symptomatic longleaf pine. *Leptographium* species are commonly isolated from Curculionid beetles in longleaf pine stands, including *Hylastes* species, *Hylobius pales*, and *Pachylobius picivorus* (Zanzot et al. 2010).

There have been inoculation tests with ophiostomatoid fungi on longleaf pine. *Leptographium serpens* was found to cause mortality in longleaf pine seedlings grown for four months under varying soil moistures (Chapter 3). 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 November 27, 2006, 200 5-7 year old longleaf pine trees were obtained from a horticulture nursery in central Alabama. Trees ranged in height from 58 to 198 cm with an average root collar diameter of 5.3 cm (+/- 0.7 cm). The trees were potted in 18.9 liter (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 placed 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 2.1). 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 April 3, 2007 and April 8, 2008, 100 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 wound control (n=20). Wound inoculations were made approximately 10 cm above the soil line using the cork-borer method (Wright 1933). Either one inoculation or control was performed on each tree (Fig. 4.1). 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 seventeen weeks after inoculation (July 31, 2007 and August 5, 2008) to quantify 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 discolored sapwood were traced on transparent sheets. Lesion length and lesion depth were measured. Lesion surface area and total discolored sapwood area were determined using a LASICO planimeter (LASICO Co. Los Angeles, CA). The ratio of discolored sapwood area to total sapwood area was used to calculate the percentage of discolored 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 of cycloheximide and 200 mg/l of streptomycin sulfate) for re-isolation of fungi.

All continuous variables, including root collar diameter, height, lesion length, lesion depth, lesion surface area, discolored 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, one each of 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 recovered from their respective stems. The cause of death was ultimately not determined. No

treatment differences were observed with respect to the growth of root collar diameter (F = 0.54, P = 0.71) or tree height (F = 1.21, P = 0.31) (Table 4.1).

Lesion length (F=8.73, P<0.0001) and lesion surface area (F=6.84, P<0.0001) were affected by treatment in the linear model (Table 4.1). Dark brown to black, discolored, resin-filled tissue (lesion) was observed surrounding the point of inoculation (Fig. 4.2). In contrast, stem tissue surrounding wound controls was lightly colored with a faint brown ring surrounding the wound (Fig. 4.3). *Leptographium terebrantis* and *G. huntii* induced 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 4.2). All fungi were isolated from tissue surrounding lesions. However, infection was confirmed on only 20 percent of trees inoculated with *L. terebrantis*, *G. huntii*, and *L. serpens* (40 percent on trees inoculated with *L. procerum*).

Discolored 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 discoloration associated with wound controls was generally lighter colored in a similar wedge-shape. *Leptographium terebrantis* and *G. huntii* caused deeper sapwood penetration than controls. Inoculations with *L. terebrantis, G. huntii,* and *L. serpens* were associated with greater overall sapwood discoloration (%) than controls. *Leptographium terebrantis* caused deeper lesions and more sapwood discoloration than all other treatments. No differences were observed in needle water potential at predawn (F=1.91, P=0.13) or midday (F=0.65, P=0.63) measurements for any of the treatments.

DISCUSSION

The tested fungal species 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 lesions and sapwood discoloration 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.

Common observations made in these studies including, darkened, resin-filled sapwood and discolored inner bark have been noted in other pine hosts (Eckhardt et al. 2004a; Nevill et al. 1995; Rice et al. 2007). Lesion length and lesion area following fungal inoculation is often used to assess the pathogenicity and virulence to pine hosts (Nevill et al. 1995; Parmeter et al. 1989). *Leptographium terebrantis* caused larger lesions than controls and has been observed inducing significant lesion development after inoculation in various *Pinus* species (Nevill et al. 1995; Parmeter et al. 1989; Raffa and Smalley 1988a; Wingfield 1983) including longleaf pine (Otrosina et al. 2002). This represents the first report of *G. huntii* causing damage in pine tissue following inoculation. Although the 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 larger than controls (lesion area), but not longer (lesion length). The fungus has previously been found to

cause large lesions and disease in pine stems and roots (Eckhardt et al. 2004a; Wingfield and Knox-Davies 1980). However, some have found it non-pathogenic following artificial inoculation in certain *Pinus* hosts (Zhou et al. 2002). *Leptographium procerum* wounds were similar to the controls. Generally, *L. procerum* has been considered a weak pathogen to *Pinus* species (Harrington and Cobb 1983; Wingfield 1983; Wingfield 1986), in some cases causing larger lesions than controls (Eckhardt et al. 2004a). Successful re-isolation of each of the test fungi confirms their pathogenicity to young longleaf pine trees. Previous inoculation tests have consistently confirmed infection for the same fungal species (Eckhardt et al. 2004a; Otrosina et al. 1999).

Despite greater sapwood discoloration with *L. terebrantis, G. huntii*, and *L. serpens* inoculations, discoloration following inoculation was lower than previously reported 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, seventeen weeks following inoculation. The relatively minor sapwood damage could be explained by low temperatures shortly following inoculations in both years (Figs. 4.4 and 4.5). Nebeker et al. (1993) stated that the host response to fungal 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 both studies, needle elongation was observed shortly following inoculation and trees flushed multiple times throughout each experiment. Some have found that a given hosts‟ resistance to blue-stain infection is associated with the amount of resin produced (Horntvedt 1988). Significant resin production has been observed following inoculation of large longleaf pine roots (Chapter 7). In these 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, confirmed by re-isolation data, was less than previous studies (Eckhardt et al. 2004a; Chapters 5-7). Inoculations of longleaf pine seedlings, *L. serpens* was successfully isolated from greater than 85% of trees (Chapter 3). 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 pine (Chapter 7).

Fungal invasion following inoculation and the resulting tissue damage has the ability to significantly alter water conduction (Croisé et al. 2001; Horner and Alexander 1985; Joseph et al. 1998). Dysfunctional sapwood is thought to be important in the development of foliar symptoms in hosts affected by root and stem inhabiting ophiostomatoid fungi (Butnor et al. 2000; Rane and Tattar 1987). The minor sapwood damage following fungal inoculation is consistent with the lack of needle water potential differences between treatments. Croisé et al. (2001) found a decrease 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. (1998b) 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 significant sapwood damage. However, Paine and Stephen (1987a) 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 similar studies. 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. More tests should be completed before we can determine the likely contribution ophiostomatoid fungi make towards mortality in longleaf pine.

Table 4.1 Effects attributed to replication and inoculation treatment following tests with longleaf pine trees. Probability of a greater *F*-statistic for sample tree characteristics, lesion measurements, and needle water potential.

Source	df[†]	Root Collar Diameter	Height	df[‡]	Lesion Length	Lesion Depth	Lesion Area	Log (Lesion Area)	Discolored Sapwood	df[§]	Needle $\Psi_{predawn}$ (MPa)	Needle Ψ_{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		

[†]All trees were used in the analysis.
[‡]Only living trees were used in the analysis.
[§]A subsample of five trees per treatment per year were measured for predawn and midday water potential.

Table 4.2 Lesion length, lesion depth, lesion area, cross-sectional sapwood discoloration, 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	Discolored Sapwood (%)	Needle $\Psi_{predawn}$ (MPa)	Needle Ψ_{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 0.05.

Fig 4.1 Potted longleaf pine trees following wound inoculations with four ophiostomatoid fungi and control.

Fig. 4.2 Stem inoculated with *L. terebrantis*.

Fig. 4.3 Wounded control.

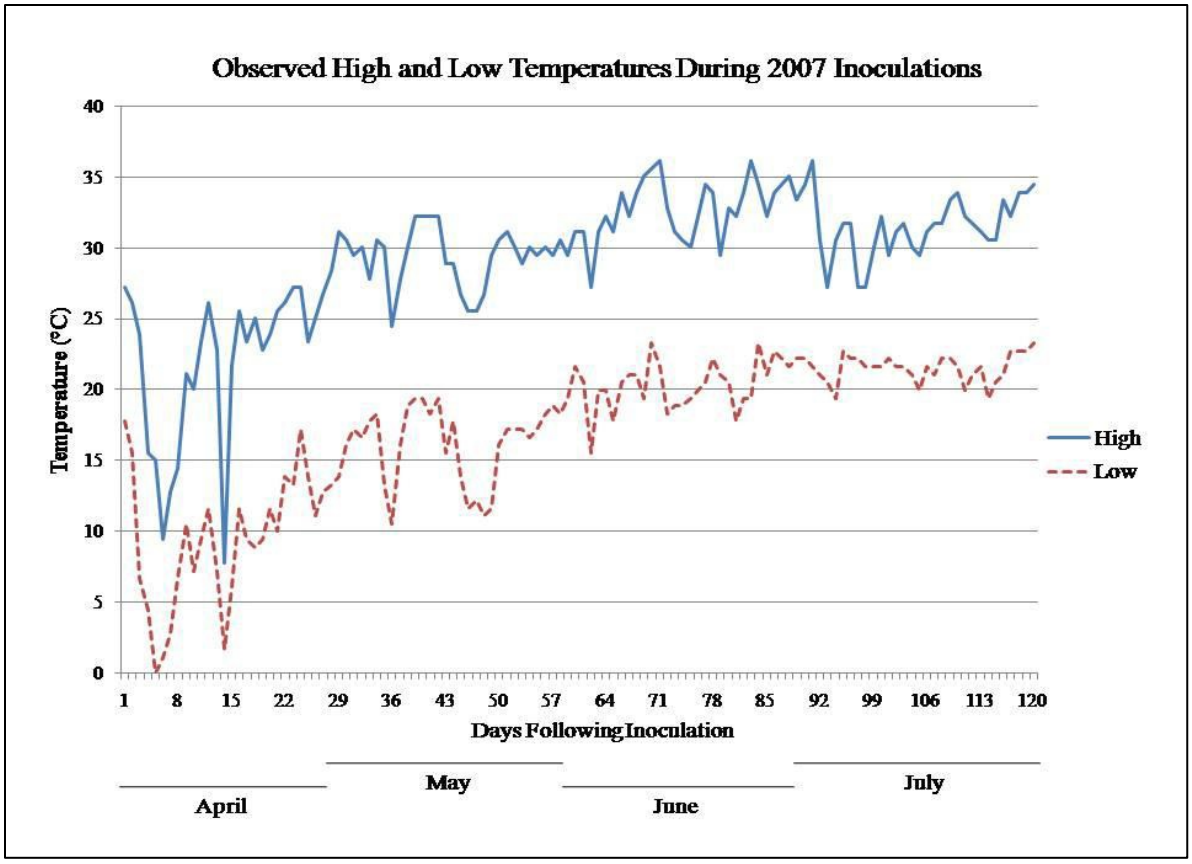

Fig. 4.4 Observed high and low temperatures at Auburn University during the 2007 inoculation test. Data was obtained from Alabama Mesonet Weather Data, located on Auburn University Campus 32.60 N, 85.50 W approximately 1.40 km from the study site (32.59 N, 85.49 W) http://www.awis.com/mesonet/

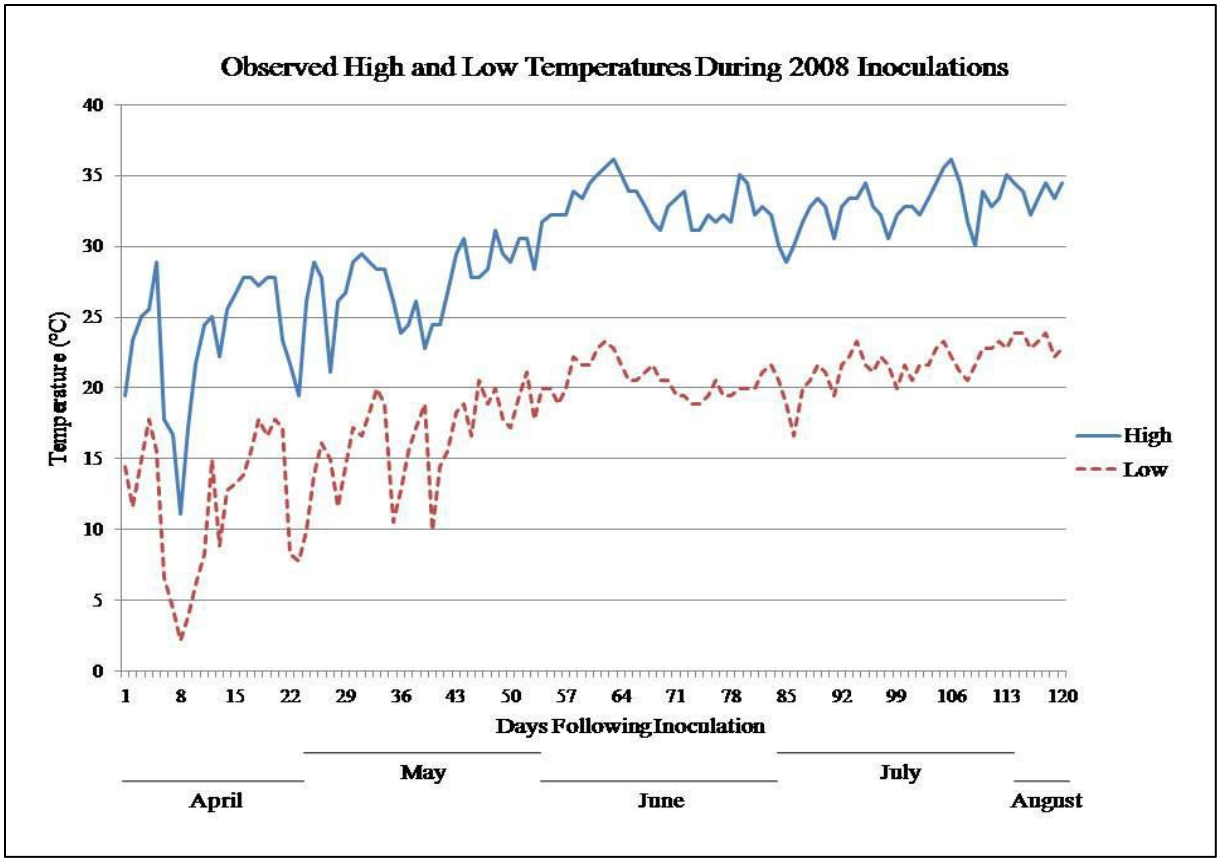

Fig. 4.5 Observed high and low temperatures at Auburn University during the 2008 inoculation test. Data was obtained from Alabama Mesonet Weather Data, located on Auburn University Campus 32.60 N, 85.50 W approximately 1.40 km from the study site (32.59 N, 85.49 W) http://www.awis.com/mesonet/