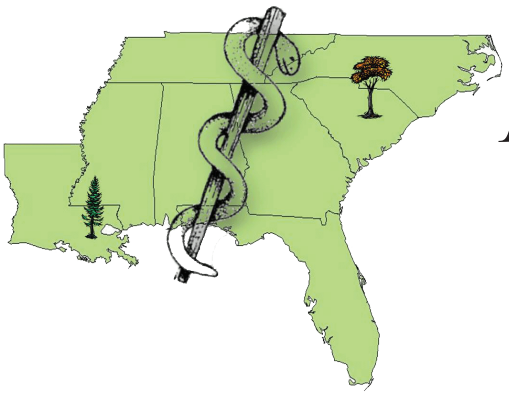

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VARIATION IN RESISTANCE OF LOBLOLLY PINE (*PINUS TAEDA* L.) AND SLASH PINE (*P. ELLIOTII* ENGLEM.) FAMILIES AGAINST *LEPTOGRAPHIUM* AND *GROSMANNIA* ROOT FUNGI: YEAR TWO RESULTS

by
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ABSTRACT
Pine decline poses a serious threat to forest sustainability in the southeastern United States. Complex interactions of biotic and abiotic factors are involved that include root-feeding bark beetle vectors and their associated fungal genera *Leptographium* and *Grosmannia*. A screening study was conducted to determine the relative resistance of loblolly (*Pinus taeda* L.) and slash pine (*P. elliottii* Englem.) seedling families when challenged with *Leptographium* and *Grosmannia*. Containerized seedlings from loblolly and slash pine families (27 and 2 respectively) were screened. Seedling responses such as lesion presence, lesion length, occlusion of vascular tissues and seedling survival were measured twelve weeks after inoculations. Seedling stems exhibited dark brown lesions and resinous occluded tissues. The screening identified family L-42 having consistently smaller lesions among all families tested. These responses indicate that family genetics could be used for deployment into high risk areas to mitigate the potential for pine decline.

MATERIALS AND METHODS
Containerized seedlings from 27 loblolly pine and 2 slash pine families were delivered to Auburn University Forest Health Dynamics Laboratory in early January of 2012 from the Glennville Regeneration center in Georgia and re-potted. Trade gallon pots filled with ProMix BX® (Premier Tech, Quebec, and Canada) peat based potting media were used for planting the seedlings. To maintain anonymity each family was assigned a unique code for data reporting and analysis. For both the years, seedlings were placed under natural environmental conditions with access to natural precipitation, sunlight and temperature with irrigation to prevent moisture stress. The experiment was set up as a randomized complete block design with three blocks/replications in first and second year. Pre-treatment and final (just before harvesting) root collar diameter and height measurements were taken to monitor growth. To determine the pre-pot family morphology, in year two, ten seedlings per family were subsampled before re-potting and after root collar diameter and height measurements, each seedling was separated into fine roots, coarse roots, stem and foliage. These parts were oven dried separately to calculate the dry biomass. Initial root to shoot ratio was calculated from the dry weight measurements.

Inoculation treatments were applied two months after re-potting the seedlings. Four treatments were

assigned to randomly selected seedlings within each family. Ten seedlings per family per block were selected for each treatment. The treatments included inoculations with one of four fungal species: *L. terebrantis* and *G. huntii.* A wounded and wound+media served as the controls. Two weeks before inoculations, each fungus was cultured on 2% malt extract agar (MEA). Pure isolates of the fungal species were used for inoculations. Wound inoculation method included making a vertical cut with sterile razor blade in the lower stem of the seedling about 2 cm from the soil line (Nevill et al. 1995; Eckhardt et al. 2004a) (Figure 1). A 3 mm diameter plug of colonized mycelium was placed in the slit and the wound was wrapped with moist cotton and sealed with Parafilm®.

After destructively harvesting the seedlings, final seedling growth measurements and seedling survival were recorded. On each seedling stem, seedling responses to the fungal inoculations were measured. These included lesion presence/absence, lesion length, lesion width, lesion depth and occlusion of vascular tissues. Lesion length consisted of dark brown tissue which either equals or extends to both sides of the length of the inoculation wound. For determining occlusion length, the roots were separated from the shoots and the living shoots were placed in a solution of FastGreen stain (FastGreen FCF; Sigma Chemical Co.) and water (0.25g/L of water) (Nevill et. al. 1995) (Figure 2). After three days of allowing capillary action, the length of unstained stem tissue was recorded as occlusion length. To confirm if the fungal infection occurred with the inoculations, re-isolation of associated fungi was attempted by cutting 1 cm of stem section surrounding the lesion and plating it on CSMA nutrient media (Malt extract agar with 800 mg/l of cycloheximide and 200 mg/l of streptomycin sulfate). Four seedlings per family per treatment per replication were processed for biomass measurements by separating each seedling to foliage, stem, fine roots and coarse roots.

Seedling response variables to the artificial inoculations were analyzed using SAS statistical software (SAS Institute, 9.2 ed., Cary, NC). Both generalized linear fixed model and linear mixed models were fit to the data to compare the treatments and families. In the fixed model, each block was considered a replicate. The variables families, fungal treatments and their interaction were included in the model under fixed effects. A set of ten seedlings per family in each replication was used as an experimental unit for all the continuous response variables while running the analysis. Lesion length was found to be the strongest response variable. For comparison among the pine families, lesion length was used as a response variable. Families were assigned into groups based on their average lesion length and analysis was conducted using contrast statements to find significant differences between the groups. The contrast statements were used on the main effects and interaction effects. Binary response variables like survival, lesion presence and re-isolation were analyzed in GENMOD procedure in SAS using logistic ANOVA for the second year screening as it was noticed that unlike the first year, mortality was found to be significant.

In the mixed model, family and family x treatment interaction were kept random, while the treatments were kept under fixed effects. All the continuous variables were analyzed considering the seedling as an experimental unit. Both main effects and two way interaction effects were included in the model. Covariance parameter estimates for each response variable such as lesion length, occlusion length, lesion width and lesion depth were used to determine the variation

among the families and the families were ranked on the basis of covariance parameter estimates for lesion length. Fungal treatments were compared from type 3 fixed effects.

RESULTS

Mortality was observed after the fungal inoculation treatments were imposed on the seedlings. At the end of the experiment, 13.13% of the total seedlings were dead. Analysis of maximum likelihood parameter estimates indicated that *L. terebrantis* significantly affected seedling survival, but *G. huntii*, wound and wound+media did not affect the survival significantly (Table 1). Seedling survival was found to be significantly different among the families and between the fungal treatments. However, family x treatment interaction was not significant for seedling survival (Table 2). Lesion presence was not significantly different among the fungal treatments, or families suggesting that lesions occurred in almost all the seedlings inoculated with fungi (Table 2). In response to inoculations with *G. huntii* and *L. terebrantis*, dark brown sunken lesions were observed along the seedlings stems (Figure 3). Lesions extended vertically along both sides of the inoculation site. Radial movement was observed occasionally on the stems from the two fungal species. Lesions did not occur on the control treatments and lesion length was found to be significantly longer in both fungal species than the controls (Table 3). Further analysis was conducted with fungal treatments separated.

Lesion length was considered a primary response variable and was used to rank the families. Average lesion length was found to be significantly different among the families ($F = 3.97$, $P =<0.0001$) with fungal treatments also affecting the lesion length significantly ($F = 532$, $P = <0.0001$) (Table 4). Occlusion length, lesion width and lesion depth were the other continuous variables measured and these differed significantly among the families and treatments (Table 4). Family x fungal interaction was not significant for the variables such as lesion length, occlusion length, lesion width and lesion depth indicating that the treatments did not vary within a particular family (Table 4). Average lesion length was plotted for each family for the fungal treatments separately and overall by pooling the treatments together, as the family x fungal treatment interaction was not significant. When the seedlings were challenged with *G. huntii*, families were classified into two separate groups, the groups being significantly different from each other. Family L-49 could not be assigned to any of the groups and did not differ significantly from both the groups. Family L-42 had the shortest average lesion length among the families tested for *G. huntii* (Figure 4). Similar to *G. huntii*, average lesion length for each family when plotted for *L. terebrantis*, two groups of families were formed; the family groups were significantly different from each other. However, in this case as well, the family L-49 did not differ from both the groups (Figure 5). Family L-42 had smallest lesion length among the families tested for *L. terebrantis* also. When the two fungal treatments were pooled to plot the average lesion length, families were sorted into three groups (Figure 6). Families L-42 and L-41 had the shortest lesion length and differed significantly from the other two family groups (Figure 6). Along with other continuous variables, root collar diameter (RCD) and height were found to be significantly different among the loblolly pine families tested. However, no significant differences were found in final RCD and height in response to the fungal treatments (Table 5). Root to shoot ratio as calculated from the final family morphology did not differ significantly between two fungal treatments but was found to be significantly different from wound control in the fungal treatments (Table 6). Significant differences in root to shoot ratio among the families were noticed (Table 7) but root to shoot ratio was found to be positively correlated to lesion

length for family L-37 (F = 12.34, P = 0.026, R^2 = 0.7551). No relationship between initial root to shoot ratio, lesion length and final root to shoot ratio was observed by the correlation coefficients (Table 8).

Pair-wise treatment comparison from the mixed model indicated that the wound and wound+media control treatments differed significantly from the two fungal treatments but not from each other (Table 9). Covariance parameters indicated that the variation among the families was significantly different from zero for lesion length, occlusion length, and lesion depth. However no significant variation from zero among the families was observed for lesion width (Table 10). Family x fungal treatment interaction was not found to be significant for lesion and occlusion length (Table 10) indicating that family rankings is not dependent upon on a particular fungal treatment. Treatments, as indicated from the Type 3 fixed effects, differed significantly for lesion length, lesion width, lesion depth and occlusion length (Table 11). The families were ranked overall and for each fungal species on the basis of mean family performance for lesion length (Table 12). Family L-42 had the smallest lesion length overall and for *L. terebrantis*. Average lesion length, occlusion length, survival and re-isolation for loblolly pine and slash pine families are shown in Table 13.

DISCUSSION

Families were challenged with only two fungal species in the second year inoculations trials including *G. huntii* and *L. terebrantis*. This was decided on the basis of results from the first year as *G. alacris* and *L. procerum* showed low variation as compared to *G. huntii* and *L. terebrantis*. Both the fungal species, *G. huntii* and *L. terebrantis*, successfully infected the families included in the trials and showed a stronger response (larger lesions) as compared to the first year trials. However, the results were not statistically compared between the two years as all the seedlings in the second year were containerized as opposed to bare root seedlings in the first year. Two fungal species varied significantly in their virulence from each other among the loblolly pine families tested. However, same as in first year, no significant family x fungal interaction was seen, indicating that within a particular family, fungal treatments did not have significant effect. Wound and wound+media treatments were included as controls and these did not produce lesions.

Dark brown, raised, and sunken lesions were observed on seedling stems following inoculations with fungi. In cross section, deformation of seedling stems was observed showing only small portions of living tissue remaining. Girdling of stems leading to mortality has been found in an earlier study following inoculations with *L. terebrantis* (Wingfield 1983). Lesions produced due to fungal inoculations extended beyond the wounded area to both sides on the stem of the seedlings. In certain cases the lesions failed to extend beyond the wounded area, especially in the seedlings inoculated with *L. procerum.* Lesions extended vertically in most of the cases with some evidence of radial movement. Occlusion of vascular tissues is considered one of many host responses to fungal infection and has been used as a measure of seedling response to inoculation with blue stain fungi (Nevill et al. 1995). Generally, occlusion lengths are similar to lesion length and further support the virulence testing of ophiostomatoid fungi (Eckhardt et al. 2004a). Occlusion of the xylem was observed (as unstained tissue) after removing the bark tissue where the occlusion was observed extending the entire lesion length. In many cases occlusion length

did not extend the lesion length. Similar lesion and occlusion response was noticed in the two consecutive years in separate studies.

Symptom development after inoculation of seedlings was localized in the form of lesions and occlusions. Mortality was not significant in the year one screening; *L. terebrantis* affected the seedling survival significantly which is in turn consistent with the findings of Harington and Cobb (1983) and Wingfield (1983).

The consistent re-isolation of *Leptographium* species from the inoculated seedlings indicated their ability to infect the pine seedlings. Similar results have been reported in a previous study but with lower re-isolations of fungal species from longleaf pine seedlings (Matusick et al. 2010). Mean root collar diameter (RCD) and height were not affected by the treatments though RCD and height were found to be significantly different among the loblolly pine families. This difference was attributed to the differences in RCD and height of the seedling stock used. Further, it indicates that despite the development of localized symptoms in the form of lesions, occlusions and tissue deformation, fungal infection did not produce whole tree symptomatology. The lack of symptomology is consistent with the previous trials conducted on southern pine seedlings (Matusick et al. 2010) and this was observed consistently. Significantly smaller final root to shoot ratio among the seedlings inoculated with fungal treatments and wound control treatment indicated the decreased carbon allocation to the roots due to fungi. However, the root to shoot ratio could not be correlated to the lesion length overall.

All the genotypes developed lesions in response to fungal inoculations. Although significant differences were reported among the families for lesion length, occlusion length, lesion width and lesion depth but some resistance to *Leptographium* fungal growth was observed in families L-42 and L-41 only. However, these families did not show complete resistance to the fungal growth as indicated from their average lesion and occlusion length (Table 13). All other families developed significantly larger lesions in response to *L. terebrantis* and *G. huntii*. Containerized seedlings used in this study, performed better than bare root seedlings used in year one in terms of planting survival and growth. This is consistent with previous studies in which out planting performance was studied between containerized and bare root seedlings (Barnett and McGilvray 1993). However, development of larger lesions in containerized seedlings during the year two warrant further studies to compare the containerized and bare root seedlings against *Leptographium* fungi. Larger lesions development during year two could also be attributed to different genotypes used in the two studies and to genotype-environment interactions.

Generalized linear mixed model was fit to the data to test the breeding value of each family, conceptually similar to the mean family performance keeping the family effect random and treatments as fixed effect (Robinson 1991; Piepho et al. 2003). The general combining ability (GCA) estimates i.e. the best linear unbiased prediction (BLUP) estimates were used to rank the families on the basis of lesion length. Results were consistent from both fixed and mixed effects models. Among the families tested L-42 and L-41 ranked lowest overall and for *L. terebrantis*, consistent to the results when the families were kept as fixed effects.

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Figure 1. Loblolly pine seedling inoculated in the stem with *Leptographium.*

Figure 2. Seedlings placed in fast green solution for recording vascular tissue occlusion.

Figure 3. Brown lesions reported on seedlings stems following inoculations with *L. terebrantis*, *G. huntii* and Wound+media control.

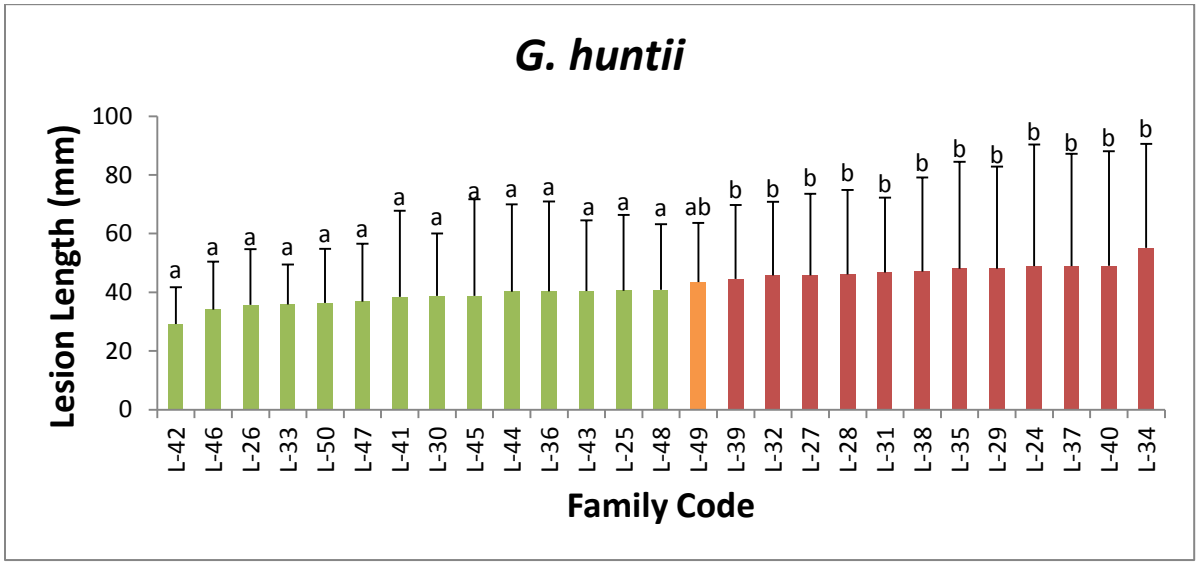

Figure 4. Average lesion length for loblolly pine families following inoculations with *Grosmannia huntti* from year two family screening.

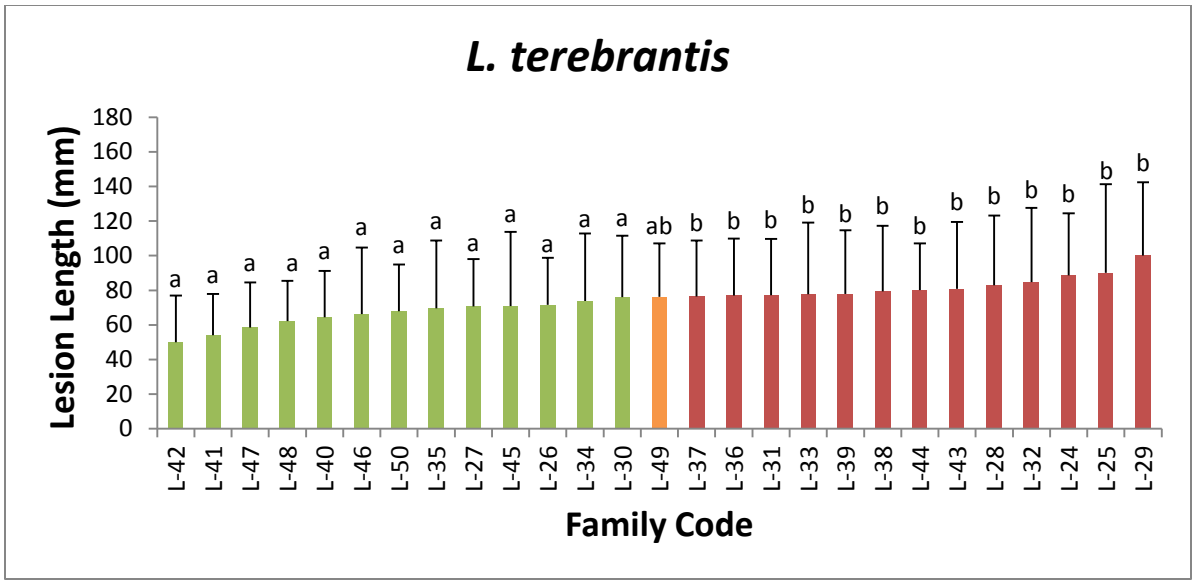

Figure 5. Average lesion length for loblolly pine families following inoculations with *Leptographium terebrantis* from year two family screening.

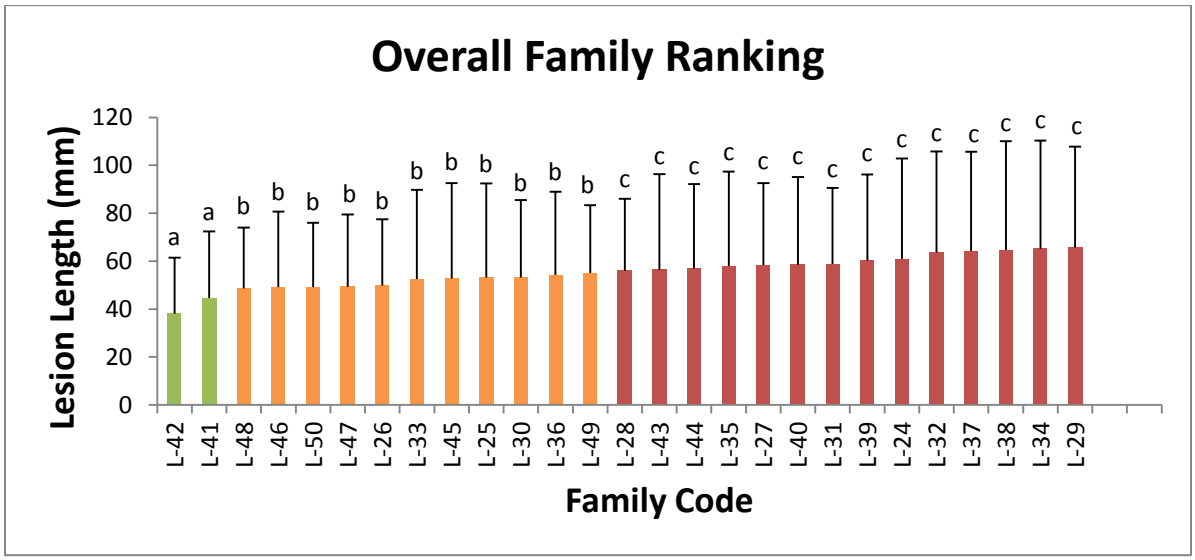

Figure 6. Average lesion length for loblolly pine families following inoculations with *Grosmannia huntti* and *Leptographium terebrantis* from second year family screening.

Table 1. Analysis of maximum likelihood parameter estimates indicated that *Leptographium terebrantis* affected the survival significantly.

Treatment	DF	Estimate	Pr>ChiSq
G. huntii	1	-0.723	0.5626
L. terebrantis	1	-3.483	0.0010
Wound	1	21.456	0.9991
Wound-Media	0	0	

Note: P- value <0.05 shows significant differences at alpha=0.05.

Table 2. Results from logistic ANOVA indicate that survival but lesion presence was found to be significantly different among the families. Treatments also affected survival significantly. Treatment x family interaction was not found to be significant for survival and lesion presence.

Source	*DF	Pr>ChiSq	+DF	Pr>ChiSq
	Survival		Lesion Presence	
Block (B)	2	<0.0001	2	0.0012
Trt (T)	3	<0.0001	1	0.3759
Family (F)	26	<0.0001	26	0.0826
TxF	78	0.6803	26	0.6648

*Control treatments were included in the analysis.

+Controls treatments were excluded from the analysis.

Note: P- value <0.05 shows significant differences at alpha=0.05.

Table 3. Pairwise comparison of treatments for lesion and occlusion length for loblolly pine families.

Treatment	Lesion Length	Occlusion Length
G. huntii* vs. *L. terebrantis	<0.0001	<0.0001
Wound vs. Wound-Media	0.1403	0.1128
Wound vs. *G. huntii*	<0.0001	<0.0001
Wound vs. *L. terebrantis*	<0.0001	<0.0001
Wound-Media vs. *G. huntii*	<0.0001	<0.0001
Wound-Media vs. *L. terebrantis*	<0.0001	<0.0001

Note: P- value <0.05 shows significant differences at alpha=0.05.

Table 4. Probability of greater F-value for lesion length, occlusion length, lesion width and lesion depth of loblolly pine families.

Effect	DF	Lesion Length	Occlusion Length	Lesion Width	Lesion Depth
Block (B)	2	<0.0001	<0.0001	<0.0001	<0.0001
Family (F)	26	<0.0001	0.0002	0.0103	0.0002
Trt (T)	1	<0.0001	<0.0001	<0.0001	<0.0001
TxF	78	0.5523	0.6372	0.6804	0.5808

Note: P- value <0.05 shows significant differences at alpha=0.05.

Table 5. Probability of greater F-value for final RCD and height of loblolly pine families.

Effect	DF	Mean RCD	Mean Height
Block (B)	2	<0.0001	0.0728
Family (F)	26	<0.0001	<0.0001
Trt (T)	3	0.0737	0.1069
TxF	78	0.9836	0.9303

Note: P- value <0.05 shows significant differences at alpha=0.05.

Table 6. Pairwise comparison of treatment for root to shoot ratio.

Treatment	Root to Shoot Ratio
G. huntii* vs. *L. terebrantis	0.6524
Wound vs. *G. huntii*	0.0014
Wound vs. *L. terebrantis*	0.0003

Note: P- value <0.05 shows significant differences at alpha=0.05.

Table 7. Probability of greater F-value for root to shoot ratio.

Effect	DF	Root to Shoot Ratio
Block (B)	2	<0.0001
Family (F)	26	<0.0001
Trt (T)	2	0.0004
TxF	26	0.7542

Note: P- value <0.05 shows significant differences at alpha=0.05.

Table 8. Pearson correlation coefficients between initial root to shoot ratio, lesion length and final root to shoot ratio.

	Initial R:S	Lesion Length	Final R:S
Initial R:S	1.00000		
Lesion Length	-0.07201 0.7211	1.00000	
Final R:S	-0.07461 0.7115	-0.03532 0.8611	1.00000

Note: P- value <0.05 shows significant differences at alpha=0.05.

Table 9. Pair-wise comparison of treatments for lesion and occlusion length for loblolly pine families.

Treatment	Lesion Length	Occlusion Length
G. huntii* vs. *L. terebrantis	<0.0001	<0.0001
Wound vs. Wound-Media	0.1923	0.1726
Wound vs. *G. huntii*	<0.0001	<0.0001
Wound vs. *L. terebrantis*	<0.0001	<0.0001
Wound-Media vs. *G. huntii*	<0.0001	<0.0001
Wound-Media vs. *L. terebrantis*	<0.0001	<0.0001

Note: P- value <0.05 shows significant differences at alpha=0.05.

Table 10. Covariance parameter estimates are presented. Variation among families is significantly different from zero for lesion length, and occlusion length. Family*treatment interaction is non-significant; families can be ranked overall.

Cov Parm	Lesion Length	Lesion Width	Lesion Depth	Occlusion Length
Family (F)	0.0031	0.0611	0.0136	0.0026
TxF	0.4272	0.3423		0.4888
Residual	<0.0001	<0.0001	<0.0001	<0.0001

Note: P- value <0.05 shows significant differences at alpha=0.05.

Table 11. Type 3 tests of fixed effects (F tests) suggest significant and non-significant effects for lesion length, lesion width, lesion depth and occlusion length.

Effect	DF	Lesion Length	Lesion Width	Lesion Depth	Occlusion Length
Block (B)	2	<0.0001	<0.1082	<0.0035	<0.0001
Trt (T)	1	<0.0001	<0.0001	<0.0001	<0.0001
BxT	15	<0.0304	<0.2847	<0.1589	<0.0059

Note: P- value <0.05 shows significant differences at alpha=0.05.

Table 12. Families ranking for lesion length (estimate) overall and across all the four treatments.

Family	Estimate	Rank	Estimate	Rank GH	Estimate	Rank LT
L-42	-1.0018	1	0.0035	15	-0.035	1
L-41	-0.5208	2	0.0124	24	-0.029	2
L-46	-0.4108	3	-0.003	11	-0.01	7
L-47	-0.3834	4	0.0006	14	-0.013	5
L-50	-0.3334	5	-0.01	8	-9E-04	11
L-30	-0.2916	6	-0.011	6	0.0022	13
L-45	-0.2191	7	-0.006	10	-0.001	10
L-48	-0.2121	8	0.0055	19	-0.012	6
L-36	-0.1507	9	-0.011	7	0.0058	18
L-26	-0.1203	10	-0.013	3	0.009	21
L-33	-0.0835	11	-0.016	2	0.0129	22
L-49	0.0094	12	0.0058	20	-0.006	8
L-35	0.0394	13	0.0178	26	-0.017	4
L-44	0.0526	14	-0.012	5	0.0132	23
L-43	0.0947	15	-0.021	1	0.0238	27
L-25	0.1331	16	-0.012	4	0.016	25
L-40	0.1485	17	0.0222	27	-0.017	3
L-31	0.1496	18	0.0038	16	0.001	12
L-32	0.262	19	0.005	18	0.0033	16
L-24	0.2717	20	0.0044	17	0.0042	17
L-39	0.2851	21	0.0002	13	0.0089	20
L-29	0.3079	22	-0.006	9	0.016	26
L-27	0.3091	23	0.007	21	0.0028	14
L-34	0.3703	24	0.0148	25	-0.003	9
L-38	0.3787	25	-0.003	12	0.015	24
L-28	0.4361	26	0.0073	22	0.0066	19
L-37	0.4793	27	0.012	23	0.0032	15

Note: P- value <0.05 shows significant differences at alpha=0.05.

Table 13. Average lesion length, occlusion length, seedling survival and fungal re-isolation frequency at the culmination of the study for loblolly pine families.

Family	Lesion Length (mm)	Occlusion Length (mm)	Survival (%)	Re-isolation (%)
L-42	38.07 (23.43)	47.23 (26.49)	94.8	95.4
L-41	44.67 (27.7)	50.93 (28.94)	90.6	96.1
L-48	48.68 (25.33)	56.71 (26.91)	88.5	84.9
L-46	49.12 (31.55)	56.96 (32.59)	90.1	93.6
L-50	49.12 (26.84)	54.95 (30)	89.1	91.9
L-47	49.31 (30.16)	56.28 (32.26)	93.2	100
L-26	49.9 (27.54)	59.05 (29.72)	89.0	98.8
L-33	52.56 (37.21)	58.39 (37.39)	87.5	89.5
L-45	52.83 (39.75)	61.12 (39.11)	90.6	97.4
L-25	53.18 (39.32)	59.71 (39.94)	78.6	95
L-30	53.22 (32.29)	60.24 (32.61)	91.0	90
L-36	54.22 (34.8)	61.26 (36.28)	90.6	92.3
L-49	55.06 (28.28)	60.55 (28.7)	90.1	93.5
L-28	56.02 (29.96)	64.1 (33.63)	78.6	90.2
L-43	56.36 (39.93)	62.15 (40.52)	85.9	98.5
L-44	56.97 (35.19)	62.42 (35.6)	86.5	95.9
L-35	58.01 (39.43)	64.73 (39.84)	91.1	96.2
L-27	58.4 (34.18)	66 (35.27)	82.3	86.2
L-40	58.57 (36.56)	65.52 (36.82)	81.3	97
L-31	58.81 (31.71)	66.64 (33.28)	91.7	92.6
L-39	60.3 (35.94)	66.7 (37.18)	90.6	92.2
L-24	60.89 (41.99)	68.7 (40.63)	80.2	94.6
L-32	63.63 (42.21)	70.73 (43.29)	91.1	93.6
L-37	64.21 (41.49)	71.24 (43.25)	82.3	92.3
L-38	64.67 (45.39)	70.53 (46.98)	85.9	97.2
L-34	65.23 (45.07)	72.46 (45.32)	87	91.8
L-29	65.68 (42.09)	76.19 (46.54)	88	92
S-6	52.28 (24.49)	58.1 (25.49)	74.5	86.8
S-7	55.64 (33.42)	63.96 (33.04)	78.1	87.7

Note: P- value <0.05 shows significant differences at alpha=0.05.