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Variation in virulence among four root-inhabiting Ophiostomatoid fungi on *Pinus taeda* L., *P. palustris* Mill, and *P. elliotii* Engelm. seedlings

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Abstract: Ophiostomatoid fungi have been implicated in root disease of pines in the southeastern United States. To understand more about their virulence, inoculation studies were conducted on loblolly (*Pinus taeda*), longleaf (*Pinus palustris*), and slash pine (*Pinus elliotii*). One-year-old bareroot seedlings, after being planted and established for 11 weeks, were wound-inoculated with one of four prominent North American ophiostomatoid fungal species. After three months, a darkened lesion, extending from the point of inoculation was observed for all species. *Grosmannia huntii* (*L. huntii*) caused the greatest lesion and occlusion length in loblolly pine and slash pine. *Leptographium procerum* and *L. terebrantis* caused similar lesion and occlusion lengths and were the least virulent among the fungi tested. These studies indicate clear virulence differences among the four North American fungi. *Grosmannia huntii*, previously not known to be pathogenic, was observed causing significant damage compared with other well-known *Leptographium* species. Finally, lesion and tissue occlusion lengths were significantly smaller in longleaf pine for all fungal species when compared to loblolly and slash pine.

Keywords: *Grosmannia huntii*, *Leptographium serpens*, *Leptographium procerum*, *Leptographium terebrantis*, pathogenicity, root disease

Résumé: Les champignons ophiostomatoïdes sont responsables de la maladie des racines des pins dans le sud-est des États-Unis. Afin de mieux comprendre leur virulence, des études d'inoculation ont été menées sur les pins à encens (*Pinus taeda*), des marais (*P. palustris*) et d'Elliott (*P. elliotii*). Après avoir été plantés et acclimatés pendant 11 semaines, des jeunes plants de un an, à racines nues, ont été blessés et inoculés avec une des quatre principales espèces de champignons ophiostomatoïdes en Amérique du Nord. Après trois mois, une lésion foncée émanant du point d'inoculation était observable sur toutes les espèces. *Grosmannia huntii* (*L. huntii*) a causé la plus grosse lésion et l'occlusion la plus longue chez les pins à encens et d'Elliott. *Leptographium procerum* et *L. terebrantis* ont causé des lésions ainsi que des occlusions de longueur similaire et étaient les moins virulents parmi les champignons testés. Ces études indiquent des différences nettes de virulence chez les quatre champignons nord-américains. *Grosmannia huntii*, non reconnu jusqu'à maintenant comme agent pathogène, a causé d'importants dommages comparativement à d'autres espèces de *Leptographium* bien connues. Finalement, la longueur des lésions et des occlusions étaient notablement plus faibles chez le pin des marais, et ce, pour toutes les espèces fongiques, lorsque comparées aux pins à encens et d'Elliott.

Mots clés: *Grosmannia huntii*, *Leptographium serpens*, *Leptographium procerum*, *Leptographium terebrantis*, maladie des racines, pathogénicité

Introduction

Root-inhabiting ophiostomatoid fungi have caused disease in many pine systems throughout the world (Wingfield *et al.*, 1988). In some instances, ophiostomatoid fungi act

as primary pathogens, causing mortality to their host. *Leptographium wageneri* (W.B. Kendr.) M.J. Wingf, a virulent primary pathogen, causes extensive pine mortality throughout the northwestern United States (Cobb, 1988).

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In other cases, ophiostomatoid fungi may only act as stressors, in a larger complex, that ultimately leads to mortality (Otrosina *et al.*, 2002). Root-inhabiting ophiostomatoid fungi have been identified as contributors to pine decline in several systems, including red pine (*Pinus resinosa* Ait) in the great lake states (Klepzig *et al.*, 1991), eastern white pine (*P. strobus* L.) in the northeastern United States (Dochinger, 1967) and most recently loblolly pine (*P. taeda* L.) in the southeastern United States (Eckhardt *et al.*, 2007).

Several ophiostomatoid fungi with *Leptographium* anamorphs, including *L. procerum* (Kendrick) M.J. Wingfield, *L. terebrantis* S.J. Barras & T.J. Perry, *L. serpens* (Goidanich) Siemaszko, and *Grosmannia huntii* (R.C. Rob. Jeffr.) Zipfel, Z.W. de Beer & M.J. Wingf. (*L. huntii* M.J. Wingfield) have been isolated from the roots of diseased and declining pines throughout the southeastern United States, including Georgia (Menard *et al.*, 2006), Alabama (Eckhardt *et al.*, 2007), and South Carolina (Otrosina *et al.*, 2002). *Leptographium procerum*, *L. terebrantis* and *L. serpens* have been consistently associated with symptomatic loblolly pine (Eckhardt *et al.*, 2007). Each *Leptographium* species mentioned has been isolated from longleaf pine (*P. palustris* Mill.) root tissue (Zanzot, 2009). *Grosmannia huntii* (formerly *Ophiostoma huntii* (Zipfel *et al.*, 2006)) has recently been isolated from loblolly root tissue as well as bark beetle vectors breeding in pine hosts (Matusick and Eckhardt, unpublished data). Root disease and decline associated with *Leptographium* species have not been well documented in slash pine (*P. elliotii* Engelm.); however, *L. procerum* has successfully been isolated from stump (Barnard *et al.*, 1991) and root tissue (Horner & Alexander, 1983).

Leptographium procerum and *L. terebrantis* are two relatively well known ophiostomatoid fungal root pathogens of North American pines (Wingfield *et al.*, 1988). *Leptographium procerum* is the causal agent of procerum root disease in eastern white pine (Dochinger, 1967). It has been associated with various conifer species around the world (Jacobs & Wingfield, 2001), particularly *Pinus* species within the United States (Alexander *et al.*, 1988). In inoculation studies, *L. procerum* has been found to be weakly virulent (Wingfield, 1983). *Leptographium terebrantis* is only found in North America and has been associated with various diseases of pine (Jacobs & Wingfield, 2001). *Leptographium terebrantis* consistently causes resin-soaking (Nevill *et al.*, 1995), sapwood discoloration (Rane & Tattar, 1987), and long vertical lesions in *Pinus* hosts (Wingfield, 1986). *Leptographium terebrantis* is considered a moderate to severe pathogen of pines, often causing considerable mortality (Harrington &

Cobb, 1983). In loblolly pine seedling inoculations, Eckhardt *et al.* (2004) found *L. procerum* to readily infect root tips and cause root and foliar dieback. In the same study, *L. terebrantis* caused darkly stained lesions in mature trees measuring 20 mm after four months.

Leptographium serpens and *G. huntii* have been less commonly reported in North American pines. However, Eckhardt *et al.* (2007) isolated *L. serpens* from loblolly pine where it was associated with decline symptoms and root-feeding insect vectors. *Grosmannia huntii* has previously been found invading *Pinus* hosts of more northern latitudes in North America (Olchowecki & Reid, 1974) and has been closely associated with damage caused by insects. *Hylastes porculus* Erichson vectors the fungus in red pine stands (Klepzig *et al.*, 1991) as well as mountain pine beetle (*Dendroctonus ponderosae* Hopk.) in lodgepole pine (*P. contorta* Douglas var. *latifolia* Engelman) (Solheim, 1995). Despite some evidence that *L. serpens* is moderately to severely virulent to *Pinus* species (Eckhardt *et al.*, 2004), some consider *L. serpens* to be a weak pathogen after inoculating *Pinus* trees in South Africa (Zhou *et al.*, 2002). Unlike the other *Leptographium* species mentioned, no information is available pertaining to the virulence of *G. huntii*.

A series of studies have been initiated to determine the relative fungal virulence of four ophiostomatoid fungi to three southern pine hosts. Past studies with *Leptographium* species in the southeastern United States have mainly focussed on inoculations with *L. procerum* and *L. terebrantis* on loblolly (Lackner & Alexander, 1981) and longleaf (Otrosina *et al.*, 2002) pine. More recently, *L. serpens* has been included in inoculations of loblolly (Eckhardt *et al.*, 2004) and Matusick *et al.* (2008) found it to cause significant damage in longleaf pine seedling tissue. This study seeks to determine the relative virulence of *L. procerum*, *L. terebrantis*, *L. serpens* and *G. huntii* to three southern pine hosts.

Materials and methods

Isolates of *L. procerum*, *L. terebrantis*, *L. serpens* and *G. huntii* were obtained from either loblolly or longleaf pine roots (Table 1) exhibiting decline disease symptoms, including low crown density and an overall sickly appearance (relative to other nearby pines). Primary lateral roots were excavated and tissues were obtained using methods described in Eckhardt *et al.* (2007). All isolates, used in inoculation tests, were in the anamorphic state and were from single-spore isolations. Also, isolates used in this study have been used in a number of other studies (Eckhardt *et al.* 2008; Matusick *et al.*, 2008).

Table 1. Fungal isolates used in pine seedling inoculation experiments.

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

Bareroot seedlings of loblolly pine, slash pine and longleaf pine were obtained from the Smurfit Stone Rock Creek Nursery near Brewton, Alabama. A total of 250 bareroot seedlings of each species were planted with ProMix BX[®] (Premier Tech, Quebec, Canada) peat-based potting mix in one-gallon plastic pots shortly (approximately one week) following lifting in December 2007. A two-factor experiment, including three pine hosts and the four fungal species, was housed in an outdoor screen facility. The building environment was homogeneous, unobstructed from sunlight and accessible to natural precipitation. After 11 weeks, prior to imposing the inoculation treatment, dead trees were removed from each group leaving a total of 225 seedlings of each pine species, respectively, to be used in the experiment. In December of 2008, the study was repeated using a total of 225 seedlings per species (or 200 seedlings after culling).

The four fungal treatments and an unwounded control were randomly assigned to an equal number of seedlings. Each fungal isolate was placed on 2% malt extract agar (MEA) two weeks prior to inoculation. Seedlings assigned to the four fungal treatments were wounded inoculated in the lower stem approximately 2 cm from the soil line. A small (1 cm) vertical slit was made with a sterile razor blade extending into the vascular tissues, followed by placing a 3 mm diameter plug of colonized MEA in the wound. The inoculation was wrapped in moist cotton and sealed with Parafilm[®], as described by Eckhardt *et al.* (2004).

Twelve weeks after inoculation, seedlings were destructively sampled. Living seedlings were inspected for the presence of cambial lesions. The overall lesion length and length of occluded (blocked) vascular tissue was measured when present. Lesion length was considered the total length of darkly pigmented tissue and may or may not have extended the total length of the wound. In order to determine the length of occluded tissue, the living shoot was placed in a FastGreen stain (FastGreen FCF; Sigma Chemical Co.) and water solution (0.25 g L⁻¹) (adapted from Nevill *et al.*, 1995). After three days, the length of tissue not stained by solution was recorded.

A 1 cm segment of stem tissue at the lesion margin was removed and placed on CSMA (MEA containing 800 mg L⁻¹ of cycloheximide and 200 mg L⁻¹ of streptomycin sulfate) to confirm infection in inoculated trees.

Response variables were analyzed using a general linear model (GLM) in SAS statistical software (SAS Institute, 9th ed., Cary, NC). All binary response variables, including survival, lesion presence and re-isolation of fungal species were transformed to percentages for each treatment × pine species combination. All continuous response variables, including lesion length and occlusion length were analyzed using the seedling as the experimental unit. In the model, each experiment (2007, 2008) was considered a replicate (blocked factor). Both testable factors, including pine and fungal species as well as their interaction were included in the linear model. All pairwise comparisons were analyzed using Tukey's multiple comparison test, followed by contrast statements.

Results

Mortality was observed throughout each experiment in all pine species. Seedling survival was significantly different between pine species ($F = 15.73$, $P = 0.0003$) (Table 2). Slash and longleaf pine seedlings experienced poorer survival when compared to loblolly pines (Table 3). Inoculation with fungi failed to contribute to significant differences in seedling survival ($F = 0.53$, $P = 0.7177$).

All fungal species caused dark sunken or sometimes slightly raised lesions in all pine species tested. Lesions generally extended vertically from the wounded area, with limited evidence of radial movement. On all pine species, callus tissue was also consistently associated with fungal inoculation, most notably in seedlings treated with *L. procerum* and *L. terebrantis*. With respect to lesion occurrence, an interaction was observed between fungal and pine species ($F = 5.66$, $P = 0.0066$). Lesions were detected in nearly all pines inoculated. However, in longleaf pine, *L. procerum* and *L. terebrantis* caused lesions in 80 and 85% of seedlings respectively, fewer than other treatment × host combinations.

Table 2. Probability of a greater *F*-statistic for survival, lesion presence, re-isolation, lesion length and occlusion length following inoculation with four ophiostomatoid fungi.

Source	Df ^a	Survival	Df ^b	Lesion	Re-isolation	Df ^c	Lesion length	Occlusion length
Replication	1	0.9861	1	0.7654	0.0345	1	0.0001	0.0001
Treatment	4	0.7177	3	0.0087	0.0920	3	0.0001	0.0001
Tree species	2	0.0003	2	0.0001	0.0028	2	0.0001	0.0001
Treatment × Tree species	8	0.1491	6	0.0066	0.9339	6	0.0001	0.0001
Error	14		11			853		

Notes: ^aControl seedlings were included in the analysis.

^bControl seedlings were omitted from the analysis.

^cAll living seedlings were included in analysis.

Table 3. Seedling survival, lesion occurrence, lesion length, sapwood occlusion length and pathogen re-isolation frequency after 12 weeks following inoculation.

Tree species	Treatment	Survival (%)	Lesion (%)	Lesion length (mm)	Occlusion length (mm)	Re-isolation (%)
<i>P. taeda</i>	Control	91 (3)	NA	NA	NA	NA
	<i>G. huntii</i>	96 (6)	100 (0)a	21 (7.06)b	33 (9.83)a	89 (9)
	<i>L. serpens</i>	98 (4)	100 (0)a	19 (5.45)c	28 (7.63)b	81 (2)
	<i>L. terebrantis</i>	99 (2)	100 (0)a	17 (5.17)de	25 (7.41)c	85 (6)
	<i>L. procerum</i>	99 (2)	100 (0)a	15 (4.32)e	22 (6.29)d	91 (13)
	Average	96 (4)a	100 (0)	18 (8.85)	27 (13.14)	86 (8)a
<i>P. elliotii</i>	Control	80 (3)	NA	NA	NA	NA
	<i>G. huntii</i>	84 (16)	100 (0)a	24 (11.34)a	32 (13.18)a	83 (7)
	<i>L. serpens</i>	63 (1)	100 (0)a	18 (9.50)cd	25 (13.29)c	79 (15)
	<i>L. terebrantis</i>	81 (21)	98 (0.2)a	13 (6.37)f	19 (9.65)d	88 (14)
	<i>L. procerum</i>	67 (4)	100 (0)a	13 (7.28)f	17 (9.50)de	96 (6)
	Average	75 (12)b	99 (1)	17 (10.17)	23 (13.48)	86 (11)a
<i>P. palustris</i>	Control	82 (5)	NA	NA	NA	NA
	<i>G. huntii</i>	71 (12)	98 (1)a	10 (6.06)g	16 (9.46)ef	62 (6)
	<i>L. serpens</i>	86 (6)	98 (3)a	10 (5.22)g	14 (7.20)f	83 (16)
	<i>L. terebrantis</i>	87 (1)	85 (7)b	7 (5.41)h	11 (7.31)g	70 (14)
	<i>L. procerum</i>	92 (1)	80 (7)b	6 (4.69)i	10 (7.11)g	79 (12)
	Average	84 (9)b	90 (9)	8 (5.59)	13 (8.25)	68 (12)b

Note: Means, followed by standard deviation in parentheses within a column with the same letter are not significantly different from one another at the 0.05 level.

The interaction between tree species and fungal inoculation was significant for average lesion length ($F = 8.53$, $P < 0.0001$). Lesion development following fungal inoculation was lowest in longleaf pine for each species. *Grossmannia huntii* infection resulted in the largest average lesion length in all pine species, though not different from *L. serpens* in longleaf pine ($F = 0.10$, $P = 0.7572$). Lesions associated with *L. procerum* and *L. terebrantis* infection were consistently the smallest among *Leptographium* species and not significantly different from each other in loblolly and slash pine. Lesions following *L. terebrantis* inoculations were longer than *L. procerum* in longleaf pine.

Similar to lesion length, *G. huntii* caused the largest occlusion length in all pine hosts. *Leptographium serpens* caused the second greatest tissue occlusion in each of the pine species. In all pine species, *L. procerum* caused the smallest average tissue occlusion, often not significantly different from *L. terebrantis*. Occlusion length was smallest for each fungal species in longleaf pine.

Re-isolation of fungal species from inoculated seedlings confirmed infection. All fungal species were successfully re-isolated from the inoculated pines. However, re-isolation percentage was affected by the pine host inoculated ($F = 10.52$, $P = 0.0028$). Re-isolation of fungal species was less successful in longleaf pine seedlings

when compared to loblolly and slash pines. No differences were observed in re-isolation among the four fungal species ($F = 2.77$, $P = 0.0920$).

Discussion

An unwounded control was chosen based primarily on results of previous studies concerning many of the same ophiostomatoid fungi. In pine seedlings, it is well established that wounding without the introduction of fungi results in only callus tissue, which encloses the wound without formation of a resinous lesion (Wingfield, 1986; Klepzig *et al.*, 1995; Eckhardt *et al.*, 2004). The wounding method used, prior to inoculation, causes minor, temporary damage and does not contribute to an increased mortality (Matusick *et al.*, 2008). In addition, the pathogenicity of many of the same ophiostomatoid fungi has been established previously (Nevill *et al.*, 1995; Eckhardt *et al.*, 2004). These tests were initiated to test virulence differences among the four ophiostomatoid fungi, with little interest in testing their ability to infect and produce lesions, compared to wounded controls.

Lesions were readily observed surrounding the point of inoculation in seedlings, with few exceptions. Discoloured lesions often appeared sunken and commonly extended beyond the wound beneath the surface of the epidermis. Callus tissue (wound periderm) was present in loblolly pine and slash pine seedlings surrounding the wounded site only, clearly not extending to the lesion margin. Similar lesion morphology and occurrence have been observed in previous seedling inoculation studies with many of the same fungal species (Eckhardt *et al.*, 2004). These studies are the first to confirm lesion development associated with artificial inoculation with *G. huntii*. Lesion occurrence was overall lower in longleaf pine seedlings inoculated with *L. procerum* and *L. terebrantis*. It was apparent that under some circumstances, lesions failed to develop following inoculation. Longleaf pine is known to be more resistant to many other insect and disease pests (Snow *et al.*, 1990), but these studies are the first to illustrate potential resistance to ophiostomatoid fungi. Longleaf pine resin has been shown to inhibit growth of ophiostomatoid fungi *in vitro*, particularly *L. procerum* (Eckhardt *et al.*, 2008).

Lesions associated with fungal infection extended above and below the point of inoculation. Lesions were primarily oriented longitudinally with poor evidence of radial movement. Radial movement is most characteristic of highly virulent ophiostomatoid fungi such as *L. wageneri* (Cobb, 1988) and in some instances *L. terebrantis* (Wingfield, 1983). Lesions associated with *G. huntii* were larger than *L. serpens* in loblolly and slash pine

seedlings. However, *G. huntii* and *L. serpens* infections were not significantly different in longleaf pine. *Leptographium serpens* has been previously observed causing mortality in *Pinus* species as well as causing similarly large lesions in controlled experiments (Wingfield & Knox-Davies, 1980). These inoculation studies represent the first report of *G. huntii* causing significant damage in pine tissue, following artificial inoculation. *Grosmannia huntii* is known as a proficient sapstainer in large pine trees and logs (Robinson-Jeffrey & Grenchenko, 1964; Kim *et al.*, 2005); however, it has not been shown to be pathogenic. Lesions following *L. terebrantis* and *L. procerum* inoculation were consistently smaller than those formed from *G. huntii* and *L. serpens*. In similar studies with loblolly pine seedlings, *L. terebrantis* was shown to cause larger lesions than *L. procerum* (Nevill *et al.*, 1995; Eckhardt *et al.*, 2004). Previous studies have found smaller lesions associated with *L. procerum* when compared to other *Leptographium* species (Wingfield, 1983). These new inoculation trials support previous findings that consider *L. procerum* a mild pathogen to *Pinus* species (Harrington & Cobb, 1983).

Tissue occlusion is often observed to be associated with invasion by root-inhabiting ophiostomatoid fungal species (Wingfield & Knox-Davies, 1980). Occlusion of vascular tissue has been detected in the past as a measure of host response to infection (Nevill *et al.*, 1995). Generally, occlusion length closely mirrors measures of lesion length and gives supportive evidence to the virulence of ophiostomatoid species (Eckhardt *et al.*, 2004). Occlusion length was greatest in loblolly and slash pine seedlings infected by *G. huntii*. Tissue occlusion was smaller in seedlings inoculated with *L. terebrantis* and *L. procerum*, when compared with *G. huntii* and *L. serpens*. Similar trends were observed by Eckhardt *et al.* (2004) when determining the occlusion of several of the same *Leptographium* species to loblolly pine. Comparable occlusion lengths were observed in a previous experiment with *L. serpens* inoculations of longleaf pine seedlings (Matusick *et al.*, 2008).

Consistent re-isolation of inoculated ophiostomatoid species confirms the ability to infect and grow within pine host tissue. However, re-isolation of fungal species from longleaf pine seedlings was statistically lower compared with loblolly and slash pines. Longleaf pine is resistant to several insect and disease pests (Snow *et al.*, 1990), including root disease (Hodges, 1969). Recent observations confirm that growth of *Leptographium* species are negatively affected by constitutive longleaf pine resin (Eckhardt *et al.*, 2008). Fungal growth was least affected by loblolly pine resin and more impeded when in the presence of longleaf pine resin. The virulence

data coupled with previous findings suggest that longleaf pine resin may restrict movement within longleaf pine tissue, making it more resistant to ophiostomatoid fungal infection and growth.

All ophiostomatoid species tested were capable of successful infection and development of local symptoms in southern pine seedlings; however, variation between fungal pathogens within certain hosts existed. *Grossmannia huntii* produced larger lesion and occlusion lengths in loblolly and slash pine seedlings. In contrast, *L. procerum* and *L. terebrantis* caused the smallest average lesion lengths in loblolly and slash pines. Infection and virulence in longleaf pine seedlings were less apparent, when compared with other southern *Pinus* hosts. Despite smaller lesions, and in some cases, poorer infection in longleaf pine, ophiostomatoid fungi are capable of causing local symptomatology similar to that observed in loblolly and slash pines. Disease symptomatology was restricted to areas surrounding the infection point with poor evidence of significant xylem dysfunction and foliar symptoms after three months. Future studies concerning root-inhabiting ophiostomatoid species and southern pine mortality should focus on large, mature trees and the role *G. huntii* plays in relation to the other more commonly published species.

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