Fungal Diversity

Infection sequence and pathogenicity of *Ophiostoma ips*, *Leptographium serpens* and *L. lundbergii* to pines in South Africa

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Three exotic bark beetles (Coleoptera: Scolytidae), *Hylastes angustatus*, *Hylurgus ligniperda*, and *Orthotomicus erosus*, infest *Pinus* spp. in South Africa. These beetles are generally considered as secondary pests, but can also act as vectors of ophiostomatoid fungi. In South Africa, at least 12 ophiostomatoid fungi are associated with the three beetle species, of which *Ophiostoma ips*, *Leptographium serpens*, and *L. lundbergii*, occur most frequently. The aim of this study was to test the pathogenicity of the three fungi to pines in South Africa. Two isolates of each fungus were inoculated on various species of pines in different areas of South Africa. The inoculated fungi caused resin exudation and sapwood discoloration around inoculation points. There were significant differences in lesion length between species inoculated, times of inoculation and plantation areas. Although *Ophiostoma ips* gave rise to longer lesions than *L. serpens* and *L. lundbergii*, our results suggest that none of these species should be considered as serious pathogens.

Key words: bark beetles, *Leptographium*, *Ophiostoma*, pathogenicity.

Introduction

Three exotic bark beetle species, *Hylastes angustatus* (Herbst), *Hylurgus ligniperda* (Fabricius), and *Orthotomicus erosus* (Wollaston), native to Europe and the Mediterranean Basin, infest *Pinus* spp. in South Africa (Tribe, 1992). *Hylurgus ligniperda* and *O. erosus* are generally considered as secondary pests. *Hylastes angustatus*, however, is more aggressive than the other two bark beetle species, and is considered as a primary pest. This insect damages pine seedlings during maturation feeding and thus, causes significant losses in newly established pine plantations (Anonymous, 1946; Tribe, 1992).

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Bark beetles are well-known vectors of fungi, and particularly *Ophiostoma* and *Ceratocystis* spp. (Münch, 1907; Whitney, 1982; Harrington, 1988; Beaver, 1989; Wingfield *et al.*, 1993; Paine *et al.*, 1997; Jacobs and Wingfield, 2001). These fungi generally sporulate in the galleries of their bark beetle vectors and are either carried in mycangia, on the exoskeletons, or in the guts of the beetles (Beaver, 1989; Paine *et al.*, 1997). The relationship between ophiostomatoid fungi and their bark beetle vectors, however, varies among different hosts, fungal species and their insect vectors (Harrington, 1993a; Wingfield *et al.*, 1995; Paine *et al.*, 1997).

Many ophiostomatoid species cause sapstain of freshly cut wood (Münch, 1907; Lagerberg *et al.*, 1927; Seifert, 1993). Several species are also pathogenic to plants. *Ophiostoma ulmi* (Buisman) Nannf. and *O. novo-ulmi* Brasier, which cause Dutch elm disease, have killed millions of elm trees in the Northern Hemisphere during the past century (Brasier, 1990; Brasier and Mehrotra, 1995). Three host-specific varieties of *Leptographium wageneri* (Kendrick) M.J. Wingfield, which cause black stain root disease of conifers, have led to severe losses to forestry in United States and Canada (Harrington and Cobb, 1988). Less pathogenic species such as *O. minus* (Hedgcock) H. & P. Sydow, *L. wingfieldii* M. Morelet and *L. terebrantis* Barras & Perry, can cause significant lesions, or even kill the trees when mass inoculated (Wingfield, 1986; Harrington, 1993b; Solheim *et al.*, 1993).

In South Africa, at least 12 ophiostomatoid species are associated with the three pine-infesting bark beetles (Zhou *et al.*, 2001). Of the 12 species, *Ophiostoma ips* (Rumb.) Nannf., *L. lundbergii* Lagerb. & Melin and *L. serpens* (Goid.) M.J. Wingfield, are most frequently encountered (Zhou *et al.*, 2001). These three species have been reported to be pathogenic to conifers in many parts of the world (Mathre, 1964; Lorenzini and Gambogi, 1976; Lieutier *et al.*, 1989; Kaneko and Harrington, 1990; Otrosina *et al.*, 1997).

A number of preliminary pathogenicity trials have been conducted with these species on pines in South Africa (Wingfield and Knox-Davies, 1980; Wingfield and Marasas, 1980, 1983; Wingfield and Swart, 1989; Dunn *et al.*, 2002). Little is, however, known regarding their relative importance or pathogenicity to pines in the area. The aim of this study was, therefore, to test and compare the pathogenicity of the three most frequently encountered fungal associates of pine-infesting bark beetles in South Africa. These tests were conducted on two-year-old pines representing a number of key species and in two different geographic areas.

Materials and methods

Screening of fungal isolates

All isolates used in this study were obtained during a survey of ophiostomatoid fungi associated with the three pine infesting bark beetle species in South Africa (Zhou *et al.*, 2001). Fungal isolates were selected based on their relative growth rate in culture, because this was shown in preliminary trials (Wingfield, unpublished) to correlate strongly with pathogenicity. Initially, 139 isolates of *O. ips*, 116 of *L. serpens*, and 138 of *L. lundbergii* were screened on 2% MEA (Malt Extract Agar: 20 g Biolab malt extract, 20 g Biolab agar and 1000 mL distilled water) at 25 C in the dark for two weeks. The two fastest-growing isolates of each species were chosen for the pathogenicity trials. All isolates used in this study are maintained in the Culture Collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, 0002, Republic of South Africa.

Inoculation experiments

Pinus spp., which are known hosts of the bark beetles and the three fungal species, were chosen for the pathogenicity trials. The availability of trees, as well as locations where the bark beetles occur, were also considered. Field inoculations were conducted in two-year-old plantations in the Western Cape province (Knysna, 23°04'00"E, 33°56'00"S) and Mpumalanga province (Sabie, 30°39'00"E, 25°08'00"S), South Africa. In Knysna, two pine species, *P. radiata* and *P. elliottii*, were selected for inoculations. In Sabie, *P. elliottii*, and a hybrid of *P. elliottii* and *P. caribaea*, were used.

Inoculum was prepared by growing fungal isolates on 2% MEA at 25 C in the dark for two weeks. After this period, cultures had commenced sporulation and the agar surface was covered with dark mycelium.

Twenty trees of each pine species were inoculated with each of the six isolates. An equal number of trees of each species served as controls. One branch per tree, with an average of 20 mm diam., was inoculated. A plug of bark was removed, using a sterile 10 mm-diam. cork borer, to expose the cambium. An agar plug of equal size, bearing the test fungus, was placed mycelium side down, in each wound. Sterile agar plugs were used as controls. All inoculation points were sealed with masking tape to reduce desiccation. Six weeks after first inoculation, all trials were repeated by inoculating a second branch of the same trees inoculated during the first trial.

Branches were examined six weeks after inoculation by removing the bark and exposing the cambium. Lesion lengths and branch diameters were measured. Reisolations were done by transferring pieces of freshly cut, discoloured cambium to 2% MEA. Cultures were incubated at 25 C for two weeks, after which they were microscopically examined to confirm that the lesions had been caused by the inoculated fungi.

Data analysis

All data sets were analysed separately. Isolates belonged strictly to a specific fungal species and measurements were done on two different inoculated branches of the same tree. Therefore, a hierarchical ANOVA was employed for the analysis. The treatment variances differed somewhat, and to improve the accuracy of the ANOVA by eliminating the effect of branch diameter, branch diameter was used as a covariate in an ANCOVA. However, branch diameter was non-significant, and had no influence over lesion measurements. Differences between times of trials, species and isolates were evaluated by using a multiple comparison method adjusted to maintain the accuracy of the comparisons (Tukey – Kramer) (Anonymous, 1989).

Results

Screening of fungal isolates

The fastest growing isolates of *L. lundbergii* (CMW6185 and CMW6186) and *L. serpens* (CMW6187 and CMW6188), originated from *H. angustatus* infesting *P. patula* in Mpumalanga province. Isolates of *O. ips* selected, however, came from *O. erosus* infesting *P. patula* in Mpumalanga province (CMW6189), and *P. elliottii* in Kwazulu-Natal (CMW6190), respectively.

Inoculation experiments

Six weeks after inoculation, resin exudation was visible, and the inoculated fungi caused discoloration of sapwood on inoculated branches. However, no signs of dieback were seen. The branches inoculated with test fungi had more resin around inoculation points than controls. Reisolations from inoculated branches consistently yielded the inoculated fungi.

Ophiostoma ips was more pathogenic than *L. serpens* and *L. lundbergii*, and generally gave rise to longer lesions. The lesion length average of *O. ips* from the four sites was 33.3 mm, varying between 28.7 mm and 48.5 mm. For *L. serpens*, lesion length average was 27.8 mm (between 15.2 mm and 44.9 mm), and for *L. lundbergii*, it was 29.3 mm (between 15.4 mm and 37.2 mm) (Tables 1, 2). In the Sabie area, however, *O. ips* (with lesion length average 31.1 mm) caused slightly shorter lesions than *L. serpens* (32.7 mm) and *L. lundbergii* (31.4 mm) (Table 2).

The hybrid of *P. elliottii* and *P. caribaea* was generally more susceptible to the test fungi than *P. elliottii* and *P. radiata*. This hybrid had a lesion length average of 32.6 mm, while the average lesion lengths for *P. radiata*, and *P. elliottii* in Sabie, and *P. elliottii* in Knysna, were 28.9 mm, 30.1 mm, 28.9 mm,

Table 1. Lesion length means (mm) for different isolates of *Ophiostoma ips*, *Leptographium lundbergii*, and *L. serpens* at the various inoculation sites.

Species	Isolate no.	Trial no.*	No. of trees	Lesion length mean			
				Sabie		Knysna	
				P. elliottii × *P. caribaea*	*P. elliottii*	*P. radiata*	*P. elliottii*
Leptographium lundbergii	CMW6185	1	20	28.4	24.6	22.3	15.4
L. lundbergii	CMW6186	1	20	36.6	37.2	34	33.8
L. lundbergii	CMW6185	2	20	36.2	26.2	28.6	27.8
L. lundbergii	CMW6186	2	20	34.4	27.9	26.9	28.5
L. serpens	CMW6187	1	20	31	28.1	21.3	15.2
L. serpens	CMW6188	1	20	27.8	33.7	21.4	20.2
L. serpens	CMW6187	2	20	30.1	30	25.6	25.2
L. serpens	CMW6188	2	20	44.9	35.8	29.9	25.1
Ophiostoma ips	CMW6189	1	20	31.6	29.7	28.8	32.4
O. ips	CMW6190	1	20	29.7	29.2	28.8	33.6
O. ips	CMW6189	2	20	31.8	30.6	44.5	48.5
O. ips	CMW6190	2	20	29.1	28.7	34.3	41.2

* 1 = first set of trials at each site; 2 = repetition of the complete trial on the second branch of each tree.

Table 2. Lesion lengths (mm) associated with inoculation of *Ophiostoma ips*, *Leptographium lundbergii*, and *L. serpens* onto various pine species in two geographic areas of South Africa.

Species	Sabie			Knysna			Overall mean
	P. elliottii × *P. caribaea*	*P. elliottii*	Mean	*P. radiata*	*P. elliottii*	Mean	
Leptographium lundbergii	33.9	29	31.4	28	26.4	27.2	29.3
L. serpens	33.5	31.9	32.7	24.6	21.4	23	27.8
Ophiostoma ips	30.6	29.6	31.1	34.1	38.9	36.5	33.3
Mean	32.6	30.1	31.4	28.9	28.9	28.9	

respectively. Interestingly, *P. elliottii* in Sabie (with lesion length average 29.6 mm) was more resistant to *O. ips* than it was in Knysna (38.9 mm). However, *P. elliottii* was more susceptible to *L. serpens* in Sabie (31.9 mm), than it was in Knysna (21.4 mm) (Table 2).

In general, longer lesion lengths were recorded in the Sabie area than in the Knysna area. In Sabie, the lesion length average was 31.4 mm, while it was 28.9 mm in the Knysna area. *Ophiostoma ips*, however, gave rise to a different trend. In the Knysna area, the fungus had a lesion length average of 36.5 mm, while it was 31.1 mm in the Sabie area (Table 2).

Multiple comparisons of lesion length showed that there were no significant differences between the lesion lengths for the two trials with *L.*

Table 3. Comparison of the differences between lesion lengths (mm) after inoculations with *Ophiostoma ips*, *Leptographium lundbergii*, and *L. serpens* at two different times.

Species	Index	Sabie		Knysna	
		P. elliottii × *P. caribaea*	*P. elliottii*	*P. radiata*	*P. elliottii*
Leptographium lundbergii	D[1]	-2.7 (32.5[3], 35.2[4])	3.9 (30.9, 27)	0.2 (28.1, 27.9)	-3.1 (25, 28.1)
	P[2]	1.0000	0.9931	1.0000	0.9997
L. serpens	D	-7.9 (29.3, 37.2)	-2.0 (30.9, 32.9)	-6.4 (21.3, 27.7)	-7.4 (17.7, 25.1)
	P	0.1123	1.0000	0.4476	0.1650
Ophiostoma ips	D	0.2 (30.6, 30.4)	-0.3 (29.4, 29.7)	-10.6 (28.8, 39.4)	-11.8 (33, 44.8)
	P	1.0000	1.0000	0.0012	0.0001

[1] Difference of the lesion length means between the first and second trials; [2] Probability value; [3] Lesion length mean of the first trial; [4] Lesion length mean of the second trial.

Table 4. Combined ANOVA for lesion length measurements of the two trials at each of the four sites.

	DF[2]	SS[3]	MS[4]	F[5]	P[6]
Site	3	2001.37	667.12	6.33	0.0003
Trees at each site	76	8597.14	113.12	1.07	0.3193
Species	2	5085.55	2542.77	24.13	0.0001
Site × Species	6	12382.22	2063.7	19.58	0.0001
Times[1]	1	3771.78	3771.78	35.79	0.0001
Species × Times	2	1487.75	743.88	7.06	0.0009
Site × Times	3	2115.42	705.14	6.69	0.0002
Site × Species × Times	6	1425.77	237.63	2.26	0.0364

[1] Initial and repeated inoculations; [2] Degree of freedom; [3] Sum of squares; [4] Mean square; [5] F value; [6] Probability value.

lundbergii and *L. serpens*, or for *O. ips* in the Sabie area (Table 3). However, for *O. ips*, there were significant differences on the two pine species in the Knysna area (P = 0.0012, P = 0.0001) (Table 3).

Combined analysis of variance (Table 4) for lesion length of the two trials at each of the four sites, showed that there are significant differences between experiment site (p = 0.0003), species (p = 0.0001), site × species (p = 0.0001), times of trials (p = 0.0001), species × times of trials (p = 0.0009), site × times of trials (p = 0.0002), and site × species × times of trials (p = 0.0364). No significant differences were found between trees at each site (Table 4).

Discussion

Results of this study showed that *O. ips*, *L. serpens* and *L. lundbergii* can cause lesions in the cambium of *Pinus* spp. in South Africa. However, none of the three species inoculated caused outward symptoms such as die-back on trees. This suggests that they are weak pathogens and confirms the results of

previous studies where these fungi have been tested separately on a limited number of tree species (Wingfield and Knox-Davies, 1980; Wingfield and Marasas, 1980, 1983; Wingfield and Swart, 1989). Of the three species tested, *O. ips* caused the longest lesions. *Leptographium serpens* and *L. lundbergii* gave rise to similar lesion lengths, which were generally shorter than those associated with *O. ips*.

Our results have shown that *O. ips* can cause lesions, but is not particularly pathogenic to pines in South Africa. This is in agreement with the studies of Wingfield and Marasas (1980), Rane and Tattar (1987), Parmeter *et al.* (1989), and Dunn *et al.* (2002). There are other studies, however, showing that the fungus was pathogenic to pines. In western Japan, *O. ips*, the associate of an *Ips* sp. infesting *P. densiflora* and *P. thunbergii*, infests the roots and has been reported to cause death of living pine trees in forests (Nisikado and Yamauti, 1933). The fungus has also been shown to significantly inhibit sapflow of infected *Pinus ponderosa* (Mathre, 1964). In France, it is pathogenic to Scots pines and possibly plays a role in the establishment of *Ips sexdentatus* (Boerner) on trees (Lieutier *et al.*, 1989). In the United States, *O. ips*, together with *L. terebrantis* and *L. procerum* (Kendrick) M.J. Wingfield, is important in the dynamics of susceptibility of southern pines to the attack by the southern pine beetle, *Dendroctonus frontalis* (Zimmermann) (Otrosina *et al.*, 1997).

Neither *L. lundbergii* nor *L. serpens* was pathogenic to living healthy pines in South Africa. This is interesting, since *L. serpens* has been recorded to be associated with a root disease of *P. pinea* in Italy (Lorenzini and Gambogi, 1976), and *P. pinaster* and *P. radiata* in South Africa (Wingfield and Knox-Davies, 1980; Wingfield *et al.*, 1988). *Leptographium lundbergii* has been found to be weakly pathogenic to severely stressed red and black pines in Japan (Kaneko and Harrington, 1990).

Ophiostoma ips, which was more pathogenic than *L. serpens* and *L. lundbergii*, is primarily vectored by the non-aggressive *O. erosus* (Zhou *et al.*, 2001). The two *Leptographium* spp. are mainly isolated from *H. angustatus*, which is considerably more aggressive than *O. erosus* (Zhou *et al.*, 2001). This situation, where the less aggressive bark beetle carries the more virulent fungus, has also been observed in other studies (Owen, 1987; Harrington, 1993a, b). Owen (1987) found that the more virulent fungus, *L. terebrantis*, was vectored by a less aggressive bark beetle, *Dendroctonus valens* (LeConte). There are, however, also studies indicating that more aggressive conifer–infesting bark beetle species vector more virulent fungi (Krokene and Solheim, 1998; Solheim *et al.*, 2001). For example, *Ophiostoma canum* (Münch) H. & P Sydow, the major associate of *Tomicus minor* (Hartig), was found to be less

virulent than *L. wingfieldii* and *O. minus*, the main associates of *T. piniperda* (Linnaeus) (Solheim *et al.*, 2001). Långström and Hellqvist (1993) showed that *T. minor* is less aggressive than *T. piniperda*.

In our study, the hybrid of *P. elliottii* and *P. caribaea* was more susceptible to the test fungi than *P. elliottii* and *P. radiata*. *Pinus elliottii* was more resistant to *O. ips*, while more susceptible to *L. serpens* in the Sabie area than in the Knysna area. These results suggest that different hosts differ in their response to fungal penetration. This is in agreement with the study of Raffa and Smalley (1995), where *P. resinosa* and *P. banksiana* showed different response patterns to *O. ips* and *O. nigrocarpum* (R.W. Davidson) De Hoog.

In the Sabie area, the tested fungi caused longer lesions than in the Knysna area, with the exception of *O. ips*. This might be explained by interactions between hosts, fungal species, climatic and other conditions in the two areas. This would be consistent with the fact that forest stand density has an influence on the infection by blue-stain fungi (Christiansen, 1985), that high water tables can increase the rate of black-stain root disease (Kulhavy *et al.*, 1978), and that stand conditions affect the expression of host resistance (Peter and Lorio, 1993).

Our results have shown that between the first and second trials, there were no significant differences in lesion length for *L. serpens* and *L. lundbergii* in the two areas, and of *O. ips* in the Sabie area. However, lesion lengths for the two trials using *O. ips* differed significantly in the Knysna area. The differences could be due to the interactions of hosts, fungi, and stand conditions, rather than seasonal difference. This is in agreement with the study of Parmeter *et al.* (1989), though other reports suggest seasonal difference affects the host response (Paine, 1984; Lorio, 1986).

Analysis of the combined ANOVA confirmed that interactions were significant, not only between sites, times of trials, fungal species inoculated, but also between site × times of trials, site × species, species × times of trials, and site × times of trials × species. Similar results have been found by Dunn *et al* (2002). They showed that pathogenicity of *O. piliferum* (Fr.) H. & P. Sydow, *O. ips*, and *Sphaeropsis sapinea* (Fr.: Fr.) Dyko & Sutton interacted strongly with host species, location, and season.

Overall, our results have confirmed that *O. ips*, *L. serpens* and *L. lundbergii* should not be considered as serious pathogens of above ground parts of *P. elliottii*, *P. radiata*, or the *P. elliottii* / *P. caribaea* hybrid in South Africa. But both *O. ips* and *L. lundbergii* are well-known sapstain agents on pines (Lagerberg *et al.*, 1927; Davidson, 1935; Gibbs, 1993; Seifert, 1993; Farrell *et al.*, 1997). Therefore, the ophiostomatoid fungi, together with their bark beetle

vectors, should be taken into account when disease resistant clones, or control strategies against sapstain, are developed.

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