Pathogenicity of *Ophiostoma piliferum* (Cartapip 97®) compared with that of other South African sap-staining fungi

C. Dunn[a], F. Wolfaardt[a,b*] and M.J. Wingfield[c]

Cartapip 97® is a commercial product consisting of inoculum from a melanin-deficient strain of *Ophiostoma piliferum*, that does not cause staining of wood. Cartapip 97® is used for the prevention of sap-stain on wood as well as in biopulping, where *O. piliferum* reduces the pitch content in wood chips before pulping. The biological removal of pitch results in stronger paper with better optical properties. Cartapip 97® used not to be imported into South Africa because of quarantine restrictions. It was therefore necessary to demonstrate that this fungus does not pose a threat to South African forestry. In this study, the pathogenicity of *O. piliferum* was compared with *Ophiostoma ips* and *Sphaeropsis sapinea*, which are common causes of sap-stain on *Pinus* species in South Africa. Different pine species, including *Pinus elliottii, P. patula, P. greggii* and *P. radiata*, were inoculated during autumn and spring at three different locations. In most cases, *O. piliferum* resulted in smaller lesions than *S. sapinea* and *O. ips* and the length of lesions caused by *O. piliferum* differed from controls in only a few instances. The development of lesions indicated that different pine species react differently to infection by the test fungi. Pathogenicity was also strongly associated with the location of the trials and season of inoculation. We conclude that *O. piliferum* should not be regarded as a virulent pathogen and that it is safe to use Cartapip 97® in South Africa.

Introduction

Cartapip 97® is produced from a strain of *Ophiostoma piliferum* (Fries) H. and P. Sydow. The inoculum is produced from a melanin-deficient strain of *O. piliferum* and cannot, therefore, stain wood.[1] *Ophiostoma piliferum* occurs commonly on softwoods throughout the United States and the taxonomy, biology and economic importance of the fungus have been well documented.[2,3] *Ophiostoma piliferum* is generally viewed by the Environmental Protection Agency (EPA) and the U.S. Department of Agriculture (USDA) as neither pathogenic nor toxic to plants and animals.[4] It has, furthermore, never been associated with bark beetle vectors and is considered to be exclusively saprobic.[5,6] The lack of sexual reproduction in the melanin-free Cartapip 97® strain and the absence of an associated insect vector reduces the potential for spread of the fungus.[1,6] In the absence of sexual reproduction, the potential for genetic recombination is also limited. Cartapip 97® has been tested and approved for release in countries with stringent quarantine requirements such as Australia, Brazil and New Zealand.[7] Since the release of Cartapip 97® in these countries, there have been no reports of any negative consequences.[8]

Ophiostoma piliferum is a primary colonist of wood[9] and can therefore play a role in the prevention of sap-stain by competing with fungi that cause sap-stain.[5] The fungus can also be used in biopulping processes. When *O. piliferum* was applied to wood chips before pulping, it was shown to utilize pitch during the colonization process.[5] The reduction in pitch resulted in stronger paper with improved optical properties.[6] Cartapip® has also been applied in biokraft pulping of wood, resulting in an increase of pulp yield and viscosity and also a reduction in consumption of chemicals.[4]

In recent years, South African forestry companies have wished to test Cartapip 97® in various industrial processes. The product could not be imported into the country, however, until it had been certified by the Department of Agriculture as safe for general release. At that time, *O. piliferum* was not known to occur in South Africa and no information was available on the effect of Cartapip 97® on *Pinus* species commonly grown here. One of the preconditions for certification of the product was that it had to be shown as non-pathogenic under South African conditions. The purpose of this study, consequently, was to consider the potential of *O. piliferum* to cause disease on *Pinus* species grown in South Africa and to compare its pathogenicity with the most common local agents of sap-stain.

Materials and methods

Three major pine-growing regions of South Africa (Table 1) were selected for the pathogenicity tests with Cartapip 97® (AgraSol Inc., Raleigh, NC). Trials were conducted during the autumn and spring of 1997 on *Pinus elliottii* Engelm. var. *elliotii*, *P. patula* Schl. & Cham., *P. greggii* Engelm. and *P. radiata* D. Don. Softwood species were targeted, because the strain of *O. piliferum* used for Cartapip 97® was isolated from *Pinus taeda*[11] and applications in South Africa would also be on softwoods. The numbers of trees of each species inoculated differed from site to site depending on availability. At Longmore, *P. radiata* (13 trees) and *P. elliottii* (20 trees) were inoculated. At Ugie, *P. patula* (15 trees) and *P. greggii* (14 trees) were used, and at Jessievale *P. patula* (16 trees) and *P. elliottii* (12 trees) were available (Table 1). In fulfilment of quarantine requirements, the trial sites were isolated from human activity and surrounded by a 4-m-wide perimeter that was free of vegetation.

Inoculum of *O. piliferum* was produced by cultivating Cartapip 97® on potato dextrose agar (PDA) (Biolab, Merck) plates. For comparative purposes, trees were also inoculated with strains of *Ophiostoma ips* (Rumb.) Nannf. (CMW 0386) and *Sphaeropsis sapinea* (Fr.:Fr.) Dyko & Sutton in Sutton (CMW 1184). *Ophiostoma ips* was chosen because it is a sap-stain fungus with a similar biology to *O. piliferum*,[2] which occurs commonly in South Africa.[11] *Sphaeropsis sapinea* is one of the most important pathogens of *Pinus* species in South Africa.[12,16] Strains of this fungus had been selected for pathogenicity testing in previous trials (unpubl. results) and all isolates used in this study are maintained in the culture collection of the Forestry and Agricultural Biotechnology Institute at the University of Pretoria. Inoculum of each strain was grown on PDA at 24°C for eight days. The control treatment consisted of sterile PDA disks.

Table 1. Location and description of different trial sites used for pathogenicity trials.

Location	Latitude	Longitude	Altitude (m)	Mean annual rainfall (mm/yr)	Tree species
Longmore (Southern Cape)	33°49′S	25°08′E	530	702	*P. radiata* *P. elliottii*
Ugie (NE Cape)	31°06′S	28°14′E	1320	875	*P. patula* *P. greggii*
Jessievale (Mpumalanga)	26°24′S	30°11′E	1700	878	*P. patula* *P. elliottii*

[a]Department of Microbiology and Biochemistry, University of the Free State, P.O. Box 339, Bloemfontein 9300, South Africa.
[b]Sappi Forest Products, Technology Centre, P.O. Box 3252, Springs 1560, South Africa.
[c]Forestry and Agricultural Biotechnology Institute, University of Pretoria, Pretoria 0002, South Africa.
*Author for correspondence. E-mail: wolfaarf@sci.uovs.ac.za

All treatments were replicated on 12 –20 trees, depending on the availability of suitable trees in selected areas. Four branches of each tree were inoculated at random with *O. piliferum*, *O. ips*, *S. sapinea*, and the control. Trees were inoculated by removing the outer bark from the branches with a 9-mm-diameter cork borer; agar discs overgrown with the test fungi or the control were then inserted, mycelium facing downwards, into the wounds. Inoculation wounds were covered with masking tape to restrict contamination and desiccation of the inoculum.

Lesions that developed from the inoculations were examined and measured after six weeks. Re-isolations of the test fungi were also made from the inoculated branches by cutting out small pieces of wood, away from the point of inoculation, but within the confines of the discoloured tissue. The pieces of wood were then placed on different media and incubated at 24°C. Malt extract agar (MEA) (2% malt extract, 2% agar) (Biolab, Merck) supplemented with streptomycin (5 ppm) was used for the re-isolation from the *S. sapinea* and control treatments. Malt extract agar supplemented with cycloheximide (5 ppm) was used for the re-isolation of *O. ips* and *O. piliferum*. The resulting colonies were transferred to new MEA plates to purify cultures. Tissue infected with *O. piliferum* was removed from the trial sites and autoclaved in compliance with quarantine specifications.

A factorial experiment with two factors was used to test the influence of inoculum and season. A randomized block experimental design was applied at each locality and for each tree species. Two-way analysis of variance was done to compensate for differences between the individual trees that were used as replicates. Means of different treatments were compared using Tukey's test[13] at a 99% confidence level. The trial design had limitations in that the same pine species were not available at all test sites, owing to the different climates in the various test regions.

The influence of the inoculum on different tree species at the various locations was tested by combining data for tree species, location and season for each of the test fungi and the control. The smallest number of trees available at the test sites was 12 at Jessievale (*P. elliottii*). The data for 12 trees were therefore selected at random for each species, location and season. A completely randomized trial design was used and the data subjected to one-way analysis of variance. The means of different treatments were tested for significant differences using Tukey's test at the 95% level of confidence.

Results and discussion

After six weeks, discolouration of the cambium could be seen for all the inoculations. Interactions between season and inoculation were significant ($P \leq 0.01$) in all trials except at Ugie, where season did not influence the development of lesions on *P. greggii*. In this trial, *S. sapinea* resulted in the longest lesions followed by *O. ips*, *O. piliferum* and the control (Fig. 1). At other locations, the pathogenicity of the test organisms during autumn and spring had to be examined individually, owing to the significant interaction between season and inoculated fungi.

Ophiostoma piliferum caused significantly ($P \leq 0.01$) longer lesions than the control on *P. greggii* in both seasons (Fig. 1). In autumn, *O. piliferum* caused longer lesions than the control on *P. patula* and *P. radiata*, but not on *P. elliottii* (Table 2). In spring, *O. piliferum* gave rise to longer lesions than the control only on *P. patula* at Jessievale (Table 3). However, *O. piliferum* caused significantly shorter leasons ($P \leq 0.01$) or lesions not significantly different from those associated with *O. ips* in all trials (Fig. 1, Tables 2, 3). These results support previous reports that *O. piliferum* is not pathogenic, since *O. ips* is not considered to be an important pathogen. The EPA and the USDA have also concluded that *O. piliferum* is non-pathogenic and non-toxic to plants and animals.[4]

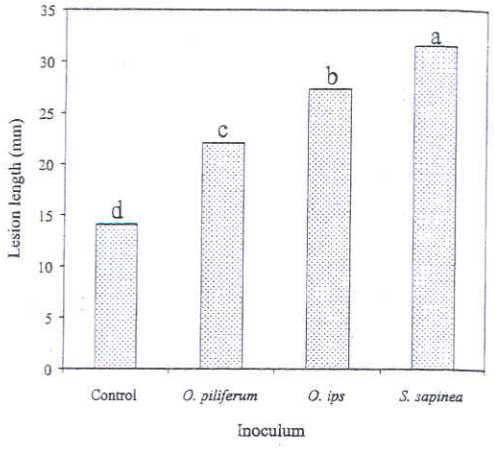

Fig. 1. Mean lesion length observed during two seasons on *Pinus greggii* at Ugie. Different letters indicate that bars differ significantly ($P \leq 0.01$; Tukey's test).

Table 2. Mean length of lesions after inoculation of *Ophiostoma piliferum*, *O. ips* and *Sphaeropsis sapinea* on different *Pinus* species at different sites during autumn.

Trial site	Treatment	Mean lesion length (mm)		
		P. patula	*P. radiata*	*P. elliottii*
Ugie	*S. sapinea*	41.5 a	–	–
	O. ips	32.1 b	–	–
	O. piliferum	25.3 b	–	–
	Control	13.8 c	–	–
Longmore	*S. sapinea*	–	35.6 a	153.3 a
	O. ips	–	23.9 b	29.0 b
	O. piliferum	–	23.4 b	35.8 b
	Control	–	16.1 c	17.0 b
Jessievale	*S. sapinea*	35.6 a	–	56.8 a
	O. ips	25.8 b	–	21.4 b
	O. piliferum	24.1 b	–	19.5 b
	Control	15.1 c	–	13.0 b

a,b,c, Mean lesion lengths for each pine species at a specific site followed by the same letter do not differ significantly ($P \leq 0.01$; Tukey's test). Each value represents the mean of 14–20 replicates.
–, Pine species not available for inoculation.

With the exception of spring inoculations on *P. elliottii* at Jessievale, *S. sapinea* caused significantly ($P \leq 0.01$) longer lesions than other fungi in all trials (Fig. 1, Tables 2, 3). On average, these lesions were 123% and 87% longer than those associated with *O. piliferum* and *O. ips*, respectively. *Sphaeropsis sapinea* is a well-known and important pathogen in South Africa[12,14,16] and its high level of pathogenicity was not surprising.

Table 3. Mean length of lesions after inoculation of *Ophiostoma piliferum*, *O. ips* and *Sphaeropsis sapinea* on different *Pinus* species at different sites during spring.

Trial site	Treatment	Mean lesion length (mm)		
		P. patula	*P. radiata*	*P. elliottii*
Ugie	*S. sapinea*	41.1 a	–	–
	O. ips	31.6 b	–	–
	O. piliferum	18.4 c	–	–
	Control	15.1 c	–	–
Longmore	*S. sapinea*	–	77.2 a	110.6 a
	O. ips	–	38.4 b	44.7 b
	O. piliferum	–	27.2 b	29.5 bc
	Control	–	35.9 b	18.6 c
Jessievale	*S. sapinea*	35.6 a	–	21.9 a
	O. ips	28.9 b	–	21.6 a
	O. piliferum	18.7 c	–	16.8 ab
	Control	11.6 d	–	11.2 b

a,b,c,d, Mean lesion lengths for each pine species at a specific site followed by the same letter do not differ significantly ($P \leq 0.01$; Tukey's test). Each value represents the mean of 12–20 replicates.
–, Pine species not available for inoculation.

Table 4. Mean lengths of lesions on different pine species at Longmore, Ugie and Jessievale during the autumn and spring.

Location	Tree species	Mean lesion length (mm)			
		Control	*O. piliferum*	*O. ips*	*S. sapinea*
Autumn					
Longmore	*P. elliottii*	17.8 a	29.7 a	36.9 a	157.2 a
	P. radiata	16.3 a	23.3 b	24.0 b	39.1 b
Ugie	*P. patula*	13.7 a	26.8 a	34.2 a	38.9 a
	P. greggii	13.7 a	25.5 a	25.1 b	39.1 a
Jessievale	*P. elliottii*	13.1 a	22.0 a	19.3 b	54.2 a
	P. patula	15.0 a	24.2 a	30.0 a	35.3 a
Spring					
Longmore	*P. elliottii*	18.8 a	27.3 a	42.8 a	99.5 a
	P. radiata	37.1 a	25.2 a	42.8 a	79.5 a
Ugie	*P. patula*	21.2 a	28.7 a	39.1 a	56.8 a
	P. greggii	14.8 a	17.8 b	28.3 a	31.8 b
Jessievale	*P. elliottii*	11.2 a	14.0 b	21.9 a	21.7 a
	P. patula	11.4 a	20.2 a	26.9 a	35.1 a

a,b, Comparison of lesion lengths on different pine species, caused by the same fungal isolates and at the same location. Pairs of mean values followed by the same letter do not differ significantly ($P \leq 0.05$; Tukey's test). Each value represents the mean of 12 replicates.

The relative susceptibility of pine species was determined by comparing lesion development on pairs of species that were inoculated at the same location (Table 4). None of the control inoculations produced significantly different lesions on any of the pine species tested. During the autumn, inoculations at Longmore with *O. piliferum* caused longer lesions on *P. elliottii* than on *P. radiata*. In spring, inoculations at Ugie with *O. piliferum* resulted in the longest lesions on *P. patula* compared to *P. greggii*. *Ophiostoma piliferum* also produced longer lesions on *P. patula* than on *P. elliottii* at Jessievale in the spring. Significant differences between tree species were observed when *O. ips* was inoculated during the autumn, but not in spring (Table 4). During autumn at Longmore, *O. ips* caused significantly longer lesions on *P. elliottii* than on *P. radiata*. At Ugie, this fungus was more pathogenic on *P. patula* than on *P. greggii* and at Jessievale it was more pathogenic on *P. patula* than on *P. greggii*.

Sphaeropsis sapinea caused significantly longer lesions on *P. patula* than on *P. greggii* as a result of inoculations at Ugie in spring (Table 4). The pathogen also caused longer lesions on *P. elliottii* than on *P. radiata* at Longmore in the same season. *Pinus elliottii* was more susceptible than other pine species at Longmore only. According to observations made during previous field trials, *P. radiata* and *P. patula* were more susceptible to *S. sapinea* than *P. elliottii*.[12] Our results do not contradict these observations, but demonstrate the influence of a location/species interaction on general susceptibility.

The inoculation of *P. elliottii* and *P. patula* at two locations each, demonstrated the significant influence ($P \leq 0.05$) of location on lesion development (Table 5). Longer lesions developed on *P. elliottii* at Longmore than at Jessievale, with all fungi and the control during autumn. These results were repeated in spring, but the control inoculations did not differ significantly from each other. Location did not play an equally important role in lesion development on *P. patula* at Ugie and Jessievale. Only *O. piliferum* and *O. ips* caused significantly longer lesions at Ugie than at Jessievale during the spring (Table 5).

Table 5. Mean lesion lengths on *Pinus elliottii* and *P. patula* at different locations during the autumn and spring.

Tree species	Location	Mean lesion length (mm)			
		Control	*O. piliferum*	*O. ips*	*S. sapinea*
Autumn					
P. elliottii	Longmore	17.8 a	29.7 a	36.9 a	157.2 a
	Jessievale	13.1 b	22.0 b	19.3 b	54.2 b
P. patula	Ugie	13.7 a	26.8 a	34.2 a	38.9 a
	Jessievale	15.0 a	24.2 a	30.0 a	35.3 a
Spring					
P.elliottii	*Longmore*	18.8 a	27.3 a	42.8 a	99.5 a
	Jessievale	11.2 a	14.0 b	21.9 b	21.7 b
P. patula	Ugie	21.2 a	28.7 a	39.1 a	56.8 a
	Jessievale	11.4 a	20.2 b	26.9 b	35.1 a

a,b, Comparison of lesion lengths at different locations, caused by the same fungal isolates on the same tree species. Pairs of mean values followed by the same letter do not differ significantly ($P \leq 0.05$; Tukey's test). Each value represents the mean of 12 replicates.

Re-isolations confirmed the presence of the inoculated fungi in the lesions. *Sphaeropsis sapinea*, *O. ips* and *O. piliferum* were isolated from the infected branches at Longmore, Jessievale and Ugie, during both seasons. Isolations made from the control inoculations yielded *S. sapinea* and *Trichoderma* species. Isolations of *S. sapinea* from control treatments were probably due to its endophytic occurrence in *Pinus* species in South Africa.[14,15] The isolation of *S. sapinea* was not consistent on the controls, however, but occurred in approximately 10% of the specimens tested.

Conclusions

We established that lesions associated with *O. piliferum* were marginally longer or did not differ significantly from those of the control inoculations. These lesions were also smaller than those caused by the weak pathogen and biologically similar, *O. ips*. We therefore concluded that the melanin-free strain of *O. piliferum* used in the production of Cartapip 97® is a non-virulent pathogen. The results indicated that releasing Cartapip 97® would not pose a threat to the South African forestry industry. As a result of these trials, Cartapip 97® has been certified by the South African Department of Agriculture as safe for general release.

We thank SAFCOL and North Eastern Cape Forests for providing trees for field trials and maintained sites. We also thank members of the TPCP for assistance with field trials, and Clariant for providing us with Cartapip 97®.

Received 1 October 2001. Accepted 25 March 2002.

1. Zimmerman W., Blanchette R.A., Burnes T.A. and Farrell R.L. (1995). Melanin and perithecial development in *Ophiostoma piliferum*. *Mycologia* **87**, 857–863.
2. Upadhyay H.P. (1981). *A Monograph of* Ceratocystis *and* Ceratocystiopsis. University of Georgia Press, Athens, GA.
3. Wingfield M.J., Seifert K.A. and Webber J.F. (eds) (1993). Ceratocystis *and* Ophiostoma. *Taxonomy, Ecology and Pathogenicity*. APS Press, St Paul, MN.
4. Wall M.B., Brecker J., Fritz A., Iverson S. and Noël Y. (1994). Cartapip® treatment of wood chips to improve chemical pulping efficiency. In *Tappi Biological Sciences Symposium*, pp. 67–76. Tappi Press , Atlanta, GA.
5. Dowding P. (1969). The dispersal and survival of spores of fungi causing bluestain in pine. *Trans. Br. Mycol. Soc.* **52**, 125–137.
6. Farrell R.L., Blanchette R.A., Brush T.S., Hadar Y., Iverson S., Krisa K., Wendler P.A. and Zimmerman W. (1993). Cartapip™: A biopulping product for control of pitch and resin acid problems in pulp mills. *J. Biotechnol.* **30**, 115–122.
7. Kay S.J. (1997). Biological control of sapstain in New Zealand. *FRI Bull.* **204**, 21–22.
8. Blanchette R.A. and Farrell R.L. (1997). Applications of biological control agents in the forest products industry. *FRI Bull.* **204**, 81–85.
9. Haller T.M. and Kile G. (1992). Cartapip® treatment of wood chips to reduce pitch and improve processing. In *Tappi Pulping Conference*, pp. 1243–1252. Tappi Press, Atlanta, GA.
10. Farrell R.L., Duncan S.M., Ram A.P., Kay S.J., Hadar E., Blanchette R.A., Harrington T.C. and McNew D. (1997). Causes of sapstain in New Zealand. *FRI Bull.* **204**, 25–29.
11. Wingfield M.J. and Marasas W.F.O. (1980). *Ceratocystis ips* associated with *Orthotomicus erosus* (Coleoptera: Scolytidae) on *Pinus* spp. in the Cape Province of South Africa. *Phytophylactica* **12**, 65–69.
12. Swart W.J. and Wingfield M.J. (1991). Biology and control of *Sphaeropsis sapinea* on *Pinus* species in South Africa. *Plant. Dis.* **75**, 761–766.
13. Winer B.J. (1971). *Statistical Principles and Experimental Design*, 2nd edn. McGraw-Hill, New York.
14. Smith H., Wingfield M.J., Crous P.W. and Coutinho T.A. (1996). *Sphaeropsis sapinea* and *Botryosphaeria dithidea* endophytic in *Pinus* spp. and *Eucalyptus* spp. in South Africa. *S. Afr. J. Bot.* **62**, 86–88.
15. Smith H., Wingfield M.J., De Wet J. and Coutinho T.A. (2000). Genotypic diversity of *Sphaeropsis sapinea* from South Africa and Northern Sumatra. *Plant. Dis.* **84**, 139–142.
16. Swart W.J., Knox-Davies P.S. and Wingfield M.J. (1985). *Sphaeropsis sapinea*, with special reference to its occurrence on *Pinus* spp. in South Africa. *S. Afr. For. J.* **35**, 1–8.