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First outbreak of pitch canker in a South African pine plantation

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Abstract. *Fusarium circinatum*, the causal agent of pitch canker, was first reported in South Africa in 1990 on *Pinus patula* seedlings in a nursery. Subsequent to this outbreak the pathogen has spread throughout South African pine nurseries causing a serious root and collar rot disease of various *Pinus* spp. The stem canker disease on plantation trees that typifies pitch canker in other parts of the world has never been observed in South Africa. An outbreak of a serious disease with symptoms resembling those of pitch canker on 5- and 9-year-old *P. radiata* in the Western Cape Province, prompted a study to determine the causal agent. Besides having stem cankers exuding copious amounts of resin, dying trees were infested by the weevil, *Pissodes nemorensis*. Isolations were thus made from infected tissue, weevil galleries and from adult insects. A *Fusarium* sp. was consistently isolated from both pine tissue and insects. The fungus was characterised based on morphological features and using DNA sequence comparisons for the genes encoding translation elongation factor 1-α and β-tubulin. These studies showed conclusively that the fungus represents the pitch canker fungus, *F. circinatum*. Three isolates from trees in the affected area were inoculated onto *P. radiata* seedlings and their ability to cause disease was thus evaluated. Three weeks after inoculation, die-back symptoms were recorded on all inoculated plants. This report represents the first outbreak of pitch canker on plantation trees in South Africa. The fungus can thus no longer be considered only as a nursery pathogen in the country, where it seriously threatens the future of plantation forestry.

Introduction

Pitch canker, one of the most important diseases of *Pinus* spp. in the world, is caused by the fungus *Fusarium circinatum* (teleomorph *Gibberella circinata*) (Nirenberg and O'Donnell 1998). Symptoms that typify this disease are resin-soaked cankers on the trunks and lateral branches (Dwinell *et al*. 1985), shoot die-back (Correll *et al*. 1991) and death of female flowers and mature cones (Barrows-Broaddus 1990). The pathogen is also able to infect seedlings where it causes damping-off (Viljoen *et al*. 1994), shoot and tip die-back and mortality of established seedlings (Carey and Kelly 1994; Viljoen *et al*. 1994).

Pitch canker was first described from the south-eastern United States (Hepting and Roth 1946). The causal agent, *F. circinatum*, has also been reported from Haiti (Hepting and Roth 1953), California (McCain *et al*. 1987), Japan (Muramoto and Dwinell 1990), Mexico (Santos and Tovar 1991), South Africa (Viljoen *et al*. 1994), Chile (Wingfield *et al*. 2002) and Spain (Dwinell *et al*. 2001; Landeras *et al*. 2005). The disease is endemic in the south-eastern United States where severe outbreaks occur under conditions of abiotic stress (Blakeslee *et al*. 1979). In California, *F. circinatum* has infected and caused large-scale losses in native stands of *P. radiata* and in landscape plantings of the same species (Gordon *et al*. 2001).

In South African nurseries, *F. circinatum* is the most important pathogen of *Pinus* seedlings (Wingfield *et al*. 2002). The fungus was first recorded in a single pine seedling nursery in Mpumalanga Province where it caused large-scale losses to production, mainly due to root and collar infections (Viljoen *et al*. 1994). It has subsequently spread to pine nurseries throughout the country and, in this situation, it represents a major constraint to pine production (Wingfield *et al*. 2002). Pitch canker, the stem canker and shoot disease of *Pinus* spp. in forests and plantations, has never been observed in South Africa. There has been considerable concern, however, that this disease would eventually appear in the country (Wingfield *et al*. 1999, 2001). An outbreak of a serious disease resembling pitch canker was observed in plantations of 5- and 9-year-old *P. radiata* in the Western Cape Province of South Africa in September 2005. The aim of this study was to determine the cause of the disease and to consider its long-term consequences.

Methods

Site

The disease occurred in 70 ha of the Tokai plantation on the foothills of Table Mountain (34°03′15.6′S, 18°24′35.6′E) in the Western Cape Province of South Africa. Two sites, with 5- and 9-year-old trees, covering an area of 12 ha, were investigated in this study. Climate in the area is typically Mediterranean with winter rainfall and dry summers.

Symptoms

The symptoms of the disease, which was evident on 30% of the trees at the sites examined, included die-back of the main stems (Fig. 1*a*), of branches (Fig. 1*b*) and resinous

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cankers on the main stem (Fig. 1*c, d*). Cankers were evident on branches, at both pruning wounds and in the first branch whorl on the main stems. There were no differences in symptom expression between trees at the two sites. Infected trees were also infested by the deodar weevil, *Pissodes nemorensis* (*Scotlytinae* : *Curculionidae*). Where these insects were present, they were first instar larvae mining in galleries in the inner bark. Trees infested with the insects did not appear to have symptoms different from those where the weevil larvae were not found.

Isolations

Small pieces of infected tissue from stem and shoot cankers and from insect galleries were plated onto *Fusarium* selective media in Petri dishes (Nelson *et al*. 1983). Infected plant material was also placed in an insect emergence chamber and emerging insects were later collected, dissected and plated onto this medium. These Petri dishes were incubated at 25°C under cool-white fluorescent illumination for 7 days. The plates were examined daily and all the colonies with typical *Fusarium*

Fig. 1. Pitch canker symptoms on pine trees in South Africa. (*a*) Hillside showing a plantation of infected trees, including shoot tip die-back symptoms. (*b*) Flagging of an infected branch. (*c*, *d*) Resinous canker on the main stem associated with a pruning wound.

morphology were transferred to half-strength potato dextrose agar (PDA) (Merck, Germany). Single conidial cultures were prepared and stored at −70°C in 15% glycerol. These have been maintained in the *Fusarium* culture collection (FCC) of the Tree Protection Cooperative Programme, Forestry and Agricultural Biotechnology Institute, University of Pretoria, Pretoria, South Africa.

Identification of isolates

Isolates resembling *Fusarium* spp. were grown on synthetic low nutrient agar (SNA) (Nirenberg 1976) for 7 days at 25°C, under near ultraviolet light. Fungal structures produced on this medium were mounted on microscope slides and used in the morphological identification of the isolates. Diagnostic characteristics used were those specified by Nelson *et al*. (1983) and Nirenberg and O'Donnell (1998).

For DNA sequence comparisons, ∼700 and ∼600 bp regions of the genes encoding translation elongation factor 1-alpha (EF-1α) and β-tubulin, respectively, were analysed. For this purpose, DNA was extracted from representative isolates (FCC 5051, FCC 5052 and FCC 5053) from infected wood and *P. nemorensis* galleries using *N*-cetyltrimethylammonium bromide (Steenkamp *et al*. 1999). The two *F. circinatum* mating-type tester strains (MRC7488 and MRC6123; Britz *et al*. 1999) that were isolated during the 1990 nursery disease outbreak in the Mpumalanga province of South Africa (Viljoen *et al*. 1994) were included for comparative purposes.

EF-1α and β-tubulin regions were PCR amplified with primer sets ef1 + ef2 (O'Donnell *et al*. 1998) and T1 + T2 (O'Donnell and Cigelnik 1997), respectively, using described reaction and cycling conditions (Geiser *et al*. 2005). After purification by polyethylene glycol precipitation (Steenkamp *et al*. 2006), amplicons were sequenced using the original PCR primers and Applied Biosystems' (Foster City, CA) ABI PRISM BigDye Terminator v3.0 Cycle Sequencing Kit on a 3730 DNA Analyzer. The resulting DNA sequences were added to existing EF-1α and β-tubulin alignments (Geiser *et al*. 2005). These included representatives of the recognised species and unique phylogenetic lineages in the *Gibberella fujikuroi* complex (O'Donnell *et al*. 1998, 2000; Aoki *et al*. 2001; Geiser *et al*. 2005) for which EF-1α and β-tubulin sequences are available in the nucleotide database of the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). Sequence data for all *F. circinatum* strains for which both EF-1α and β-tubulin sequences are available, were also included.

Phylogenetic relationships were inferred from the combined EF-1α and β-tubulin sequence data, as these regions have previously been shown to represent homogenous partitions for members of the *G. fujikuroi* complex (O'Donnell *et al*. 2000). Phylogenetic analyses utilised maximum parsimony as implemented in PAUP version 10b (Swofford 1998). Relationships among taxa in the *G. fujikuroi* complex were based on heuristic searches using 1000 rounds of random sequence additions and default settings. To determine the phylogenetic relationships among members of the so-called 'American Clade' of this complex (O'Donnell *et al*. 1998), the branch-and-bound tree searching method and default settings were used. Alignment gaps were treated as missing data in both analyses. Bootstrapping was performed as described above and using 1000 pseudoreplicates.

Pathogenicity tests

Three isolates (FCC5052, FCC5051, FCC5053) shown to represent *F. circinatum* were inoculated into 7-month-old *P. radiata* seedlings using a technique widely used to assess *Pinus* spp. for susceptibility to *F. circinatum* (Roux *et al*. 2006). One of the three isolates (FCC5052) was from the galleries produced by *P. nemorensis*. An isolate of *F. circinatum* (FCC3577) from a South African nursery and previously shown to be highly virulent was included in the inoculations. The meristems were removed from each tree to be inoculated and a drop of a conidial suspension (50 000 conidia/mL) was added to the cut surface. Twelve trees were inoculated with each isolate. Five plants were inoculated with sterile distilled water in a similar manner to serve as controls. The plants were maintained in a greenhouse at 25°C with a 12 h photoperiod. Six weeks after inoculation, symptom expression was recorded and the length of die-back on the growing shoots assessed.

Results

Identification of isolates

All *Fusarium*-like cultures resulting from isolations from stem canker tissue, weevil galleries and adult *P. nemorensis* resembled those of *F. circinatum*. Branching and proliferating conidiophores were observed and the polyphiallides had between two and five conidiogenous openings. Sterile coiled hyphae and lunate macroconidia were also observed. These characteristics are typical of *F. circinatum* and collectively they distinguish this species from other species of *Fusarium* (Nirenberg and O'Donnell 1998; Britz *et al*. 2002).

Phylogenetic analyses using combined EF-1α and β-tubulin sequence data showed that the isolates associated with the pitch canker-like symptoms in the Western Cape form part of the *G. fujikuroi* complex (results not shown). Within this complex, the Western Cape isolates from *P. radiata* form part of the 'American Clade' (O'Donnell *et al*. 1998) and are closely related to *F. circinatum* representatives that were originally isolated from pitch canker affected pine tissue in California and Japan (Fig. 2). This group also includes the *F. circinatum* mating-type tester strains (MRC7488 and MRC6213) that were isolated from the diseased root tissue of *P. patula* seedlings during the first outbreak of *F. circinatum*-associated root disease in South African nurseries (Viljoen *et al*. 1994).

Pathogenicity tests

All the examined isolates were highly pathogenic on the *P. radiata* seedlings used in the inoculation tests. The average lesion length caused by the three isolates at 6 weeks was 25.2 mm. This was similar to the lesions caused by isolate FCC3877, which were an average of 24.3 mm long. No symptoms developed on the control seedlings. *F. circinatum* was consistently reisolated from infected tissue on all seedlings inoculated with the fungus.

Discussion

Results of this study have shown conclusively that the fungus associated with the serious canker disease outbreak on *P. radiata*

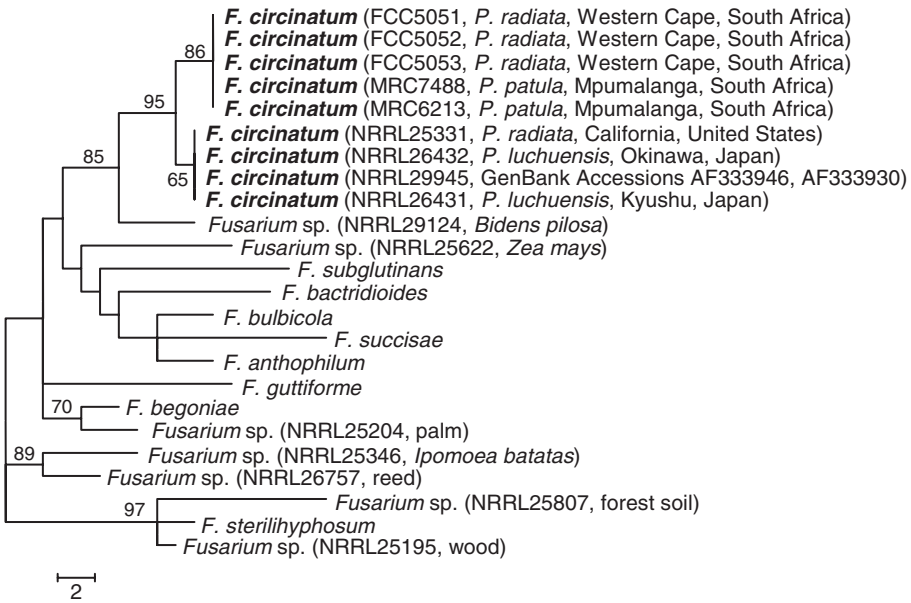

Fig. 2. One of 10 most parsimonious midpoint-rooted phylogenies for species in the so-called American clade of the *Gibberella fujikuroi* species complex (O'Donnell *et al*. 1998) based on combined EF-1α and β-tubulin sequence data. For *Fusarium circinatum*, isolate numbers (NRRL, Northern Regional Research Laboratory, NCAUR, Peoria, IL, USA; MRC, Medical Research Council, Tygerberg, South Africa; FCC, *Fusarium* collection of the Tree Protection Cooperative Programme, Forestry and Agricultural Biotechnology Institute, University of Pretoria, Pretoria, South Africa), host or source and geographic origin are indicated in parentheses. Isolate numbers and host or source are also indicated in parentheses for the representatives of previously identified phylogenetic species that have not yet been described. Bootstrap values >60% are indicated at the relevant internodes.

in the Western Cape Province of South Africa is the pine pitch canker pathogen, *F. circinatum*. This fungus was the only putative pathogen isolated from symptoms on the dying trees and its identity was confirmed using morphological characters and sequence comparisons. The symptoms on the trees were also typical of pitch canker in other parts of the world.

Fusarium circinatum has been known in South Africa for more than 15 years. It was first found in a single nursery and is believed to have been introduced into the country with infected pine seed (Britz *et al*. 2001). Subsequent to its first discovery, the pathogen has spread throughout pine-growing nurseries, probably through the movement of infected seedlings and/or airborne inoculum. It has now become the single greatest constraint to pine propagation in South Africa (Wingfield *et al*. 2001). There has, however, been great concern that *F. circinatum* would move from pine nurseries and infect trees in plantations (Wingfield *et al*. 1999, 2001). Results of this study have shown conclusively that infection of mature trees has now happened.

The plantation area in the Western Cape where pitch canker has now appeared is close to the coast. The climate is typically Mediterranean, with warm, dry summers and mild, moist winters and low summer rainfall. The relative humidity at the coast is generally 85%. High ambient humidity appears to be advantageous to the pitch canker fungus. It has been observed that pines are more severely affected by the pathogen in coastal regions, than in inland regions in California (Gordon *et al*. 2001). Up until 1999, the disease was only found in California in locations having a maritime climate (Adams *et al*. 1999). Also, the fact that the first outbreak of pitch canker occurred on the Western Cape coast is not surprising as Sakamoto and Gordon (2006) have suggested that wounds are more likely to become infected in coastal areas where fog would often limit evaporative demand.

The area in which the pitch canker outbreak described in this study occurs is ~600 km from the nearest formal pine production nursery. This nursery is most likely the source of the inoculum that has given rise to the pitch canker outbreak. This view is based on the fact that seedlings were obtained from this nursery and planted in the Western Cape 10 years before the current outbreak. It is also known that the nursery was experiencing an outbreak of *F. circinatum* on *P. radiata* seedlings at that time and the medium in which apparently healthy plants were growing would certainly have been infested with the pathogen.

The symptoms observed on pine trees suffering from pitch canker were first observed after the trees were pruned in September 2005. This would have facilitated infection by *F. circinatum*, which is known to require wounds to become established (Dwinell *et al*. 1985; Gordon *et al*. 2001). Pruning would also have resulted in stress to trees, which would promote disease development (Dwinell *et al*. 1985) and would account for the very severe damage that has occurred in the area of outbreak. In addition, analyses of soil samples from the infected sites in the Tokai plantations indicated very high levels of nitrogen (Mr D. Carstens, MTO Forestry, pers. comm.) and nitrogen is

known to increase the susceptibility of pines to infection by *F. circinatum* (Blakeslee and Rockwood 1999).

The outbreak of pitch canker described in this study is clearly associated with infestation of trees by the weevil, *P. nemorensis*. Thus, isolations from both galleries of the insect and from adult weevils yielded cultures of *F. circinatum*. This association is not surprising as *P. nemorensis* is known to be associated with pitch canker in the south-eastern United States where it creates wounds that may become infected by airborne spores of *F. circinatum* (Blakeslee *et al*. 1978). An association between *P. nemorensis* and *F. circinatum* has also been predicted in South Africa (Gebeyehu and Wingfield 2003). The nature of the association between the insect and the pathogen that has now emerged in South Africa is poorly understood and will require investigation. However, both the insect and the fungus have a wide-spread distribution throughout the country. The establishment of an association between these two organisms, which has now occurred, is of great concern and it suggests that pitch canker outbreaks in other parts of South Africa will occur in the future.

This study represents the first report of pitch canker in plantations of *Pinus* spp. in South Africa. Although the causal agent, *F. circinatum*, is endemic in pine nurseries, it has taken the pathogen ~15 years to cause the first outbreak of the disease in plantations. It is possible that outbreaks have occurred elsewhere but have remained undetected due to the similarity of the symptoms caused by the commonly occurring pathogen *Diplodia pinea* (Swart *et al*. 1985) or by insect damage. However, the scale of the disease outbreak considered in this study was substantial and diseases of this magnitude are unlikely not to have been studied in detail. Nonetheless, a first confirmed outbreak of pitch canker in South Africa is of great concern and areas where similar symptoms occur will need to be carefully investigated. Results of this study suggest that selection and breeding of pines with resistance to pitch canker should assume an elevated level of urgency in order to protect the pine industry from damage due to this serious disease.

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