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Characterisation of the pitch canker fungus, *Fusarium circinatum*, from Mexico

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***Fusarium circinatum* (=*F. subglutinans* f. sp. *pini*) is the causal agent of pitch canker of pines. This fungus occurs in the United States, Japan, Mexico and South Africa and it can be introduced into new areas on seed and infected plant material. Its presence in cones from symptomless trees is of concern, particularly with respect to seed transmission. In this study, isolates of *Fusarium* spp. were collected from *Pinus patula, P. greggii, P. teocote* and *P. leiophylla* trees in Mexico, showing typical symptoms of pitch canker, as well as from cones from apparently healthy trees. Morphological characteristics of the pitch canker fungus and isolates of *F. subglutinans* from other hosts are very similar. Therefore, pathogenicity tests, sexual compatibility studies and histone *H3*-RFLPs were used to characterise isolates. Isolates collected from *Pinus* spp. from Mexico were identified as *F. circinatum*. In this study we have thus confirmed that *F. circinatum* occurs on pines in Mexico and that the affected trees can be asymptomatic.**

Introduction

Pitch canker, caused by *Fusarium circinatum* Nirenberg and O'Donnell (=*F. subglutinans* (Wollenw. and Reinking) Nelson *et al.* f. sp. *pini* Correll *et al.*), was first reported in the south-eastern United States (Hepting and Roth 1946). *F. circinatum* is now found throughout this region where it has caused significant losses on a wide variety of pine species. This led to the suggestion that pitch canker is endemic to the area (Dwinell *et al.* 1985). More recently, pitch canker was identified and reported in California (McCain *et al.* 1987), predominately on *Pinus radiata* planted in landscape settings (Correll *et al.* 1991). Since 1992, it has been recognised as a threat to native *P. radiata* stands in California (Storer *et al.* 1994, 1998). *F. circinatum* is also found in Japan (Muramoto *et al.* 1988, Kobayashi and Kawabe 1992) and South Africa (Viljoen *et al.* 1994, 1997)

In South Africa, the fungus was reported from forestry nurseries where it has resulted in serious losses of *P. patula* (Viljoen *et al.* 1994), *P. elliottii, P. greggii* and *P. radiata* seedlings. Stem cankers on larger trees such as those found in the United States (Hepting and Roth 1946, Dwinell *et al.* 1977) have not been seen (Wingfield *et al.* 1999). Pitch canker has been reported in Mexico on a variety of native pine species (Santos and Tovar 1991, Guerra-Santos 1999) and this is thought to be the origin of the pathogen, for areas such as South Africa where Mexican pines are commonly propagated.

Fusarium circinatum has been isolated from stems and branches of trees (Hepting and Roth 1946, Dwinell *et al.* 1977), root collars of pine seedlings (Barnard and Blakeslee 1980, Viljoen *et al.* 1994), female strobili, mature cones and seeds (Dwinell *et al.* 1977, Miller and Bramlett 1978, Dwinell *et al.* 1985, Barrows-Broaddus 1986, 1987, Storer *et al.* 1998, Dwinell 1999). Recently, Storer *et al.* (1998) isolated *F. circinatum* from pine seedlings originating from seeds collected from cones on diseased as well as asymptomatic branches. Those authors hypothesised that *F. circinatum* can be carried inside seeds and may remain dormant until germination, which increases the possibility of seedling infections. The implication of seed transmission is serious, since current treatments may be ineffective in eliminating the pathogen. This would increase the possibility of introducing the pathogen into uninfested areas (Barrows-Broaddus and Dwinell 1985, Storer *et al.* 1998).

Fusarium subglutinans sensu lato includes species occurring on a wide variety of hosts, including pineapple, maize, mango, pine and sugarcane (Booth 1971). Correll *et al.* (1991) distinguished pine and non-pine *F. subglutinans* isolates based on pathogenicity to pines. Those authors proposed that *F. subglutinans* from pine should be designated as *F. subglutinans* f. sp. *pini* based on its exclusive pathogenicity to pine trees (Correll *et al.* 1991). Restriction frag-

ment patterns of the mtDNA and random amplified polymorphic DNA (RAPD) also indicated that pine isolates differed from non-pine isolates (Correll *et al.* 1992, Viljoen *et al.* 1997)

Sexual compatibility among isolates causing pitch canker on pines confirmed that this group corresponded to a distinct biological species (Viljoen *et al.* 1997). O'Donnell *et al.* (1998) showed pine isolates to be phylogenetically distinct and Nirenberg and O'Donnell (1998) thus proposed the name, *F. circinatum*, for it. Steenkamp *et al.* (1999) could, furthermore, distinguish *F. circinatum* from other species in *F. subglutinans sensu lato* using histone *H3* gene sequences.

Until recently, the most reliable technique to distinguish *F. circinatum* from closely related *Fusarium* spp. has been sexual compatibility. A molecular technique based on RFLP profiles of the histone *H3* gene, reliably and rapidly distinguishes *F. circinatum* from other similar *Fusarium* spp. in the *Gibberella fujikuroi* (Sawada) Ito in Ito and K. Kimura complex (Steenkamp *et al.* 1999). Sexual compatibility as well as histone *H3*-RFLPs can, therefore, be used to separate the eight different mating populations (biological species), designated by the letters A to H, in this complex. Heterothallic *F. circinatum* isolates reside in mating population H of the *G. fujikuroi* complex and tester strains representing opposite mating types have been selected and designated (Coutinho *et al.* 1995, Britz *et al.* 1998, 1999). Sexual compatibility of field isolates with tester strains of mating population H (MRC 6213 and MRC 7488) provide a firm basis for the identification of field isolates as *F. circinatum*.

In this study, isolations from pine trees in Mexico showing typical canker symptoms were made. The possible association of *F. circinatum* with asymptomatic cones was also investigated. The identity of these isolates was verified using morphology (Nelson *et al.* 1983, Nirenberg and O'Donnell 1998) as well as pathogenicity, sexual compatibility and histone *H3*-RFLP comparisons.

Materials and Methods

Isolates, cultural and morphological characteristics

Fusarium circinatum strains (MRC 7568–7587) were collected in Laguna Atezca and Hidalgo, Mexico, from cankers occurring on branches of native stands of *P. patula* and *P. greggii* (Table 1). Strains MRC 7568–7569 were isolated from apparently healthy cones collected from asymptomatic *P. patula* trees and strains MRC 7570–7587 were obtained from branches showing pitch canker symptoms. Strains MRC 7570–7572, MRC 7573–7576, 7577–7579, 7580–7582 and 7583–7585 were isolated from five trees. Strains MRC 7588–7601 were isolated from cankers on branches of *P. teocote* and *P. leiophylla* in northern Michoacan, Mexico (Table 1). Single conidial isolates of all cultures were prepared and are maintained in 15% glycerol at -70°C in the *Fusarium* collection of the Tree Pathology Co-operative Programme (TPCP), Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa. Isolates have also been deposited in the culture collection of the Medical Research Council (MRC), PO Box 19070, Tygerberg, South Africa.

For isolations, pine cones were immersed in 70% ethanol for 2min. Small pieces (approximately 5mm^2) of the asymptomatic cone scale tissue and wood pieces (2mm^2) from the infected tree branches were removed and plated on 2% malt extract agar (MEA). Fungi were allowed to grow for seven days at room temperature. Small agar pieces (approximately 5mm^2) from the edges of the colonies were transferred to 90mm plastic Petri dishes containing carnation leaf agar (CLA) (Fisher *et al.* 1982). Cultures were incubated at 23°C under near-ultraviolet and cool-white light with a 12h photoperiod to stimulate culture and conidium development. Isolates were identified and morphological features were compared with *F. circinatum* tester strains (MRC 6213 and MRC 7488) after 10 to 14 days, using a light microscope. Diagnostic characteristics used were those specified by Nelson *et al.* (1983) and Nirenberg and O'Donnell (1998).

All isolates obtained from Mexico (Table 1), as well as, *F. circinatum* tester strains (MRC 6213 and MRC 7484) from

Table 1: List of *Fusarium* cultures used in this study

Fusarium species	Host, substrate and origin	Isolates[a]
F. circinatum	*P. patula* cones, Hidalgo, Mexico	MRC 7568*, 7569*
F. circinatum	*P. patula* branches, Hidalgo, Mexico	MRC 7570*, 7571*, 7572*
F. circinatum	*P. greggii* branches, Laguna Atezca, Mexico	MRC 7573*, 7574*, 7575*, 7576*, 7577*, 7578*, 7579*, 7580*, 7581*, 7582*, 7583*, 7584*, 7585*, 7586*, 7587
F. circinatum	*P. teocote*, Northeastern Michoacan, Mexico	MRC 7588, 7589, 7590, 7591, 7592, 7593, 7594, 7595, 7596
F. circinatum	*P. leiophylla*, North-central Michoacan, Mexico	MRC 7597, 7598, 7599, 7600, 7601
F. circinatum	*P. radiata*, California	FSP 24*, 34*
F. circinatum	*P. patula* seedling, Ngodwana, South Africa	MRC 6213, 7488
F. sacchari	*Saccharum officinarum*, Taiwan	MRC 6524, 6525
F. subglutinans	*Zea mays*, South Africa	MRC 1077
F. subglutinans	*Z. mays*, Illinois, USA	MRC 6483, 6512
Fusarium sp.	*Mangifera indica*, Florida, USA	MRC 7035

[a] MRC refers to the culture collection of the Medical Research Council, PO Box 19070, Tygerberg, South Africa and FSP = *F. circinatum*, Department of Plant Pathology, University of California, Davis, California 95616. * Isolates marked are those used in pathogenicity tests

South Africa were used in sexual compatibility tests and for comparison based on histone *H3*-RFLPs. *F. sacchari* (Butler) W. Gams (= *F. subglutinans*) and *F. subglutinans* standard tester strains of the B (MRC 6524 and MRC 6525) and E (MRC 6483 and MRC 6512) mating populations of the *G. fujikuroi* complex were also included. Tester strains of the B mating population are progeny from a fertile cross between two isolates from sugarcane (*Saccharum officinarum*) from Hsingying, Taiwan and tester strains of the E mating population were isolated from maize (*Zea mays*) in St. Elmo, Illinois, USA (Leslie 1991). *F. subglutinans* isolates from maize (MRC 1077) and mango (*Mangifera indica*) (MRC 7035) were included in the histone *H3*-RFLP comparisons as representatives of host specific isolates of *F. subglutinans* (Table 1).

RFLPs of histone H3 gene

DNA was extracted from cultures (Table 1) grown for 10 days in complete medium broth (CM) (Correll *et al.* 1987) using the protocol described by Steenkamp *et al.* (1999). PCR reactions were performed using primers (H3-1a and H3-1b) for amplification of the histone *H3* gene PCR product as described by Glass and Donaldson (1995). The same conditions as those described by Steenkamp *et al.* (1999) were used except Boehringer Mannheim polymerase and reaction buffers (Boehringer Mannheim South Africa Pty. Ltd.) were used. The restriction enzyme, *Dde* 1, which distinguishes *F. circinatum* isolates from other similar *Fusarium* spp. (Steenkamp *et al.* 1999), was used in this study. Digests were performed using this restriction enzyme in a total reaction volume of 20µl containing 5U of the enzyme. Sodium chloride was added to the reactions with the enzyme *Dde* 1 to a final concentration of 100mM. All digestion reactions were incubated at 37°C for 7h.

Restriction fragments were separated using agarose gels (Promega Corporation, Madison, Wisconsin, USA) in the presence of ethidium bromide (0.1µg/ml). RFLP fragments were electrophoresed on 3% (w/v) agarose gels and visualised using an ultraviolet transilluminator (Ultra-Violet Product). The visualised RFLP fragments were photographed using a gel documentation system (Microsoft Corporation) and evaluated using the methods described by Steenkamp *et al.* (1999).

Sexual compatibility

Crosses to determine sexual compatibility were made on carrot agar as described by Klittich and Leslie (1988), except that 300g fresh carrots were used rather than the recommended 400g. All crosses were also done on V8-agar (325 ml canned V8-juice per liter and 2% agar, pH 5.8–6.2). Hermaphrodite tester strains of the B, E and H mating populations of the *G. fujikuroi* complex were crossed with each another (MRC 6524 x MRC 6525, MRC 6483 x MRC 6512 and MRC 6213 x MRC 7488). Isolates from Mexico were crossed with standard tester strains from the B (MRC 6524 and MRC 6525), E (MRC 6483 and MRC 6512) and H (MRC 6213 and MRC 7488) mating populations of the *G. fujikuroi* complex. All field isolates of *F. circinatum* from Mexico were crossed with each other in all possible combinations. The isolates in this study were also crossed against themselves as a negative control, i.e. where no mature perithecia should be produced. Reciprocal crosses, where the isolates corresponded to the male and female parents were reversed, were done for all crosses.

All the crosses recorded as positive were repeated at least once. Crosses were examined weekly and scored as positive when ascospores were observed, either by their exudation from perithecia or after crushing these structures. The viability of the ascospores was determined by streaking a portion of the ascospore cirrhus onto the surface of 2% water agar plates and estimating the percentage germination after 24h.

Pathogenicity tests

Due to quarantine constraints in South Africa, pathogenicity tests of *F. circinatum* isolates from Mexico were conducted in greenhouse facilities of the Department of Plant Pathology, University of California. Tests to confirm pathogenicity were performed at approximately 25°C during the day and 18°C at night with a 12 h day/night cycle. Tests were performed on *P. radiata* seedlings, 3–4 years of age using 19 *F. circinatum* isolates collected in Mexico (Table 1), as well as, two isolates of *F. circinatum* (FSP 24 and FSP 34), known to be pathogenic to pines in California. All *F. circinatum* isolates were grown on potato dextrose agar (PDA) at 25°C for 7–10 days. Inoculations were performed by making a small wound in the seedling stems and placing a spore suspension (approximately 500 spores in distilled water) into each wound. Each isolate was inoculated into two *P. radiata* seedlings. The lesion lengths under the bark of the inoculated *P. radiata* plants were measured 41 days after inoculation.

Results

Isolates, cultural and morphological characteristics

Isolates obtained from diseased pine branches, cankers and asymptomatic cones were identified as *F. subglutinans* based on morphological characteristics described by Nelson *et al.* (1983). These isolates could also be identified as *F. circinatum* based on the characteristics proposed by Nirenberg and O'Donnell (1998). Branched and proliferating conidiophores were observed and the polyphialides had 2–5 conidiogenous openings (Figure 1c). Sterile coiled hyphae (Figure 1d) and lunate macroconidia (Figure 1e), reported by Nirenberg and O'Donnell (1998) to distinguish *F. circinatum* from similar *Fusarium* spp. in *Liseola* and related section, were observed.

RFLPs of histone H3 gene

The PCR products obtained using the primers H3-1a and H3-1b were approximately 500 base pairs (bp) in size. None of the PCR products from isolates belonging to the E mating population (MRC 6483 and MRC 6512) of the *G. fujikuro* complex and the *F. subglutinans* isolate from maize (MRC 1077) were cut by *Dde* 1, whereas the *F. subglutinans* iso-

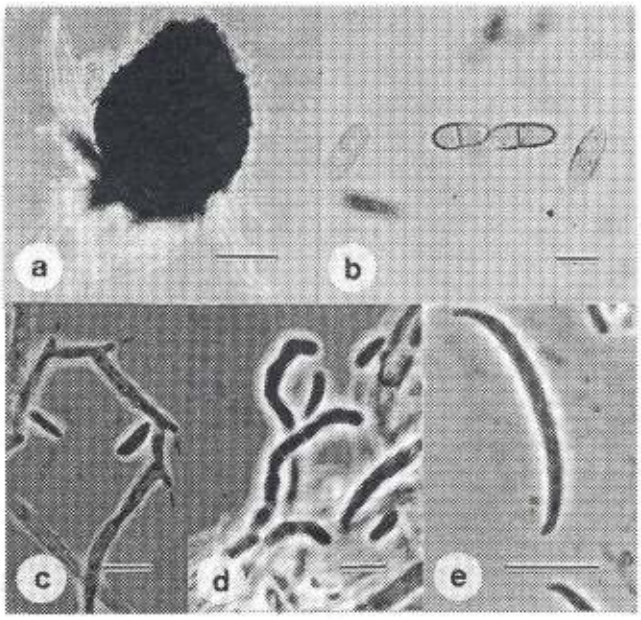

Figure 1: Morphological characteristics of *Fusarium circinatum*. (a) Mature perithecia (bar = 100μm), (b) Single septate ascospores (bar = 10μm), (c) Polyphialide with conidiogenous openings (bar = 10μm), (d) Coiled sterile hyphae (bar = 10μm), (e) Lunate macroconidium (bar = 10μm)

late from mango had three RFLP fragments of approximately 110, 170 and 220 bp. All the isolates identified as *F. circinatum* based on morphology had the same banding pattern as the *F. circinatum* tester strains (MRC 6213 and MRC 7488), where two fragments of approximately 230 and 270 bp were evident (Figure 2). Two histone *H3*-RFLP fragments of approximately 190 and 310 bp could be visualised for isolates in the B mating population (Figure 2).

Sexual compatibility

Using sexual compatibility tests, we were able to verify that isolates from pine in Mexico belong to mating population H and, therefore, *F. circinatum* (Figure 1). Perithecia with exuding ascospores (Figure 1a, b) were produced four weeks after fertilisation of the control crosses between tester strains of the B, E and H mating populations (MRC 6524 x MRC 6525, MRC 6483 x MRC 6512 and MRC 6213 x MRC 7488). The isolates from Mexico did not produce perithecia when crossed with tester strains of either the B (MRC 6524 and MRC 6525) or the E (MRC 6483 and MRC 6512) mating populations of. Mexican isolates MRC 7568, 7569, 7572, 7591, 7592, 7593, 7595, 7597, 7599 and 7600 produced perithecia with viable ascospores when crossed with the mating population H tester strain, MRC 6213. These isolates only produced fertile crosses when MRC 6213 was used as the female parent and are thus female-sterile. No mature perithecia with viable ascospores resulted from crosses amongst *F. circinatum* isolates from Mexico.

All the fertile crosses recorded in this study were repeated and identical results were obtained in at least two different tests. The same results were also obtained on both carrot

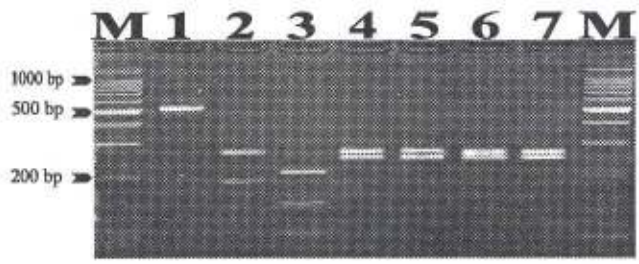

Figure 2: *Dde* 1 RFLP profiles of the digestion of the histone *H3* gene amplification products on a 3% agarose gel. Lane marked as M = 100 bp ladder (1500, 1000, 900, 800, 700, 600, 500, 400, 300, 200, 100 bp). Lane 1 = *F. subglutinans* (MRC 6512) from the E-mating population. Lane 2 = *F. sacchari* (MRC 6525) from the B-mating population. Lane 3 = *F. subglutinans* isolate (MCR 7035) from mango. Lane 4-6 = digestion products of *F. circinatum* (MRC 7568, 7573, 7587, 7589, 7596 and 7598) from Mexico. Lane 7 = digestion product of *F. circinatum* tester strain (MRC 6213) from mating population H

and V8-agar. The percentage germination of ascospores in this study varied between 85–98%. None of the isolates in this study produced perithecia when crossed with themselves as negative controls.

Pathogenicity tests

The 21 selected *F. circinatum* isolates gave lesions after 41 days that varied in length between 10 to 90mm. These isolates were, thus, pathogenic to *P. radiata* seedlings in the glasshouse. The pathogenicity of the *F. circinatum* isolates from California (FSP 24 and FSP 34) was consistent with that reported previously (Correll *et al.* 1991, Gordon *et al.* 1996).

Discussion

In this study, we were able to identify *F. circinatum* from branches, cankers and asymptomatic cones from Mexico, based on a wide range of criteria. The association of the pitch canker fungus with asymptomatic cones demonstrates the possibility of spread on apparently healthy seeds such as reported by Storer *et al.* (1998). *F. circinatum* isolates considered in this study were collected from *P. patula, P. greggii, P. teocote* and *P. leiophylla* in Mexico. *P. patula, P. elliottii,* and *P. radiata* are the most important, commercially planted species in South Africa (Hinze 1993). More recently, *P. greggii* has also become important to the forestry industry in South Africa (Malan 1994). The isolation of *F. circinatum* from *P. patula* and *P. greggii* in native stands in Mexico, indicates that the pitch canker fungus could have been introduced into South Africa from Mexico. This would most likely have occurred through seed importation. The finding emphasise the importance of screening seed for *F. circinatum* infection before it is exported. Seed treatment with fungicides would also reduce the chances of new introductions occurring, although it might not effectively eliminate internal infections (Storer *et al.* 1998, 1999). Small seed lots, where the risk can be reduced through propagation under controlled conditions, may be an acceptable practice. The

importation of large collections of seed for commercial planting cannot be managed effectively, and should be avoided.

In this study, only a small number (less than 28%) of *F. circinatum* isolates from Mexico were able to cross with the single *F. circinatum* tester (MRC 6213), from South Africa. It is possible that the isolates from Mexico that did not cross with MRC 6213 all belonged to the same mating type as this South African tester. Alternatively, low fertility or sterility (Perkins 1994) could explain why none of these isolates cross with the tester strain of the opposite mating type. Female-sterility (Leslie and Klein 1996) could also have contributed to the lack of sexual compatibility seen between Mexican *F. circinatum* isolates. The low level of sexual compatibility might also suggest that the population of *F. circinatum* in Mexico is evolving towards an asexual life history (Leslie and Klein 1996). This is indicated by the high percentage of female-sterile isolates found in the sexual compatibility study. Despite the low level of sexual compatibility, sexual crosses confirmed that some of the Mexican isolates belonged to mating population H of the *G. fujikuroi* complex.

Steenkamp *et al.* (1999) showed that *F. subglutinans* isolates from various plant hosts can be distinguished from one another with histone *H3*-RFLPs. Those authors concluded that this technique could be used for routine identification of *F. circinatum*. In this study, the histone *H3*-RFLP technique was critical for positive identification of *F. circinatum*. This was particularly due to the sometimes inconclusive and time-consuming pathogenicity tests and the low fertility among the *F. circinatum* isolates from Mexico.

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