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 southeastern 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), predominantly 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-Broadus 1988, 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-Broadus 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 polymor-
phic 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,
however, 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 sex-
ual compatibility. A molecular technique based on RFLP pro-
files 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 com-
plex (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), desig-
nated 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 identifica-
tion of field isolates as F. circinatum.

In this study, isolations from pine trees in Mexico showing
typical canker symptoms were made. The possible associa-
tion 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 his-
tone H3-RFLP comparisons.

### Materials and Methods

#### Isolates, cultural and morphological characteristics

| Fusarium species | Host, substrate and origin | Isolates* |
|------------------|---------------------------|-----------|
| F. circinatum    | P. patula cones, Hidalgo, Mexico | MRC 7568*, 7569* |
| F. circinatum    | P. patula branches, Hidalgo, Mexico | MRC 7570*, 7571*, 7572* |
| F. circinatum    | P. greggi branches, Laguna Atozco, Mexico | MRC 7573*, 7574*, 7575*, 7576*, 7577*, 7578*, 7579*, 7580*, 7581*, 7582*, 7583*, 7584*, 7585*, 7586*, 7587 |
| F. circinatum    | P. toscoeto, 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 6524, 6525 |
| F. sacchari      | Saccharum officinarum, Taiwan | MRC 1077 |
| F. subglutinans  | Zea mays, South Africa | MRC 6483, 6512 |
| F. subglutinans  | Z. mays, Illinois, USA | MRC 7035 |
| Fusarium sp.     | Mangifera indica, Florida, USA | |

* 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.

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**Table 1:** List of Fusarium cultures used in this study
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) were used except Boehringer Mannheim polymerase and reaction buffers (Boehringer Mannheim Ltd.) were used. The restriction enzyme, *MscI* (Promega Corporation, Madison, Wisconsin, USA) was used except Boehringer Mannheim polymerase and reaction buffers (Boehringer Mannheim South Africa Pty. Ltd.) were used. The restriction enzyme, *Dde I*, 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 I* 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 WE RE MADE ON CARROT AGAR AS DESCRIBED BY KLITICH AND LESLIE (1988), EXCEPT THAT 300g FRESH CARROTS WERE USED RATHER THAN THE RECOMMENDED 400g. ALL CROSSSES WERE ALSO DONE ON V8-AGAR (250 ml CANNED V8-JUICE PER LITER AND 2% AGAR, PH 5.8-6.2). HEMMAFRODITE TESTER STRAINS OF THE B, E AND H MATING POPULATIONS OF THE *G. fujikuroi* COMPLEX WERE CROSSED WITH EACH OTHER (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 OF 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 PETHIECIA 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 cirrus 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), but 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 conidigenous 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. fujikuroi* complex and the *F. subglutinans* isolate from maize (MRC 1077) were cut by *Dde I*, whereas the *F. subglutinans* iso-
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 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 90 mm. 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 plantings 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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