Characterization of *Fusarium subglutinans* f. sp. *pini* causing root disease of *Pinus patula* seedlings in South Africa

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Fusarium subglutinans has been associated with many hosts. Some isolates of the fungus are responsible for pitch canker disease of pines. A South African population of *F. subglutinans* from pine seedlings was characterized by comparing it to isolates from various hosts, pine and non-pine. A total of 26 isolates representing six different hosts were selected for the study. Cultural and morphological characteristics were studied. Virulence of isolates was tested on 1-yr-old *Pinus patula* seedlings. Sexual compatibility of isolates of *F. subglutinans* with B- and E-tester strains, and crossings of pine isolates in all possible combinations, were performed. Pine isolates were also compared with pitch canker and non-pine isolates using random amplified polymorphic DNAs (RAPDs). All isolates were morphologically characteristic of *F. subglutinans*, but differed somewhat in cultural features. Only isolates from pine were pathogenic to *P. patula* seedlings. Virtually no isolates were sexually compatible with the fertile tester strains. An exception was isolate MRC 115 from maize. Some pine isolates (MRC 6211 and MRC 6217) were, however, sexually compatible with South African (MRC 6213) and pitch canker (MRC 6229) isolates. Cluster patterns constructed from RAPD profiles revealed that isolates clustered according to host. South African isolates from pine were related to the pitch canker isolates from other areas, and were similar to non-pine isolates only in morphology. Virulence, genetic analyses and composite RAPD data suggest that pine isolates originate from the same gene pool, and probably represent a distinct new biological species.

Fusarium subglutinans (Wollenw. & Reinking) P. E. Nelson, Toussoun & Marasas is a cosmopolitan and diverse species (Booth, 1971; Nelson, Toussoun & Marasas, 1983). The fungus was first observed on banana in Honduras (Wollenweber & Reinking, 1925) and has since been associated with a wide range of hosts, including maize, mango, pineapple, pine and sorghum (Booth, 1971; Nirenberg, 1976).

Pitch canker is a serious disease of pine trees caused by *F. subglutinans*. The disease was first reported by Hepting & Roth (1946) on Virginia pines (*Pinus virginiana* Mill.) in North Carolina in the United States. These authors suggested that the responsible fungus was a species of *Fusarium* belonging to the section Liseola. When pitch canker reached epidemic proportions in the southern United States in the 1970s (Phelps & Chelman, 1976; Dwinell & Phelps, 1977), the fungus was characterized as *F. moniliforme* J. Sheld. var. *subglutinans* Wollenw. & Reinking (= *F. subglutinans*) (Kuhlman *et al.*, 1978). More recently, pitch canker has been reported from California (McCain, Koehler & Tjosvold, 1987), Mexico (Santos & Tovar, 1991) and Japan (Kobayashi & Muramoto, 1989).

Morphology and pathogenicity of the pitch canker fungus is important in its characterization. In 1949, the absence of chlamydospores and chain-formed microconidia placed the fungus in *F. lateritium* Nees rather than *F. oxysporum* Schltdl. emend. W. C. Snyder & H. N. Hansen or *F. moniliforme* J. Sheld. (Snyder, Toole & Hepting, 1949). With the introduction of phialides as an important characteristic in *Fusarium* taxonomy (Booth, 1971), the presence of mono- and polyphialides resulted in the inclusion of the pitch canker fungus in *F. moniliforme* var. *subglutinans* (Kuhlman *et al.*, 1978). Virulence of pine isolates and avirulence of non-pine isolates of *F. subglutinans* to pines suggested host specificity. In addition, characteristic restriction fragment patterns of mtDNA that differ in pine isolates from those of non-pine isolates led to the proposal that the pitch canker fungus be designated *F. subglutinans* f. sp. *pini* (Correll *et al.*, 1991).

Mating types confer self/non-self recognition in filamentous ascomycetes (Glass & Kuldau, 1992). *Fusarium subglutinans* is heterothallic (Booth, 1971), and two mating populations have been identified in the species (Leslie, 1991). The B-mating population includes isolates from sugarcane and maize (Kuhlman, 1982), while the E-mating population is most commonly associated with maize (Leslie, 1991). Sexually fertile crosses of *F. subglutinans* from pine with single-ascospore isolates, formed in a mating of Indian isolates of *F. subglutinans* from rice and from sugarcane and representing the B-mating population, have been reported (Kuhlman *et al.*, 1978).

Molecular genetic markers reveal information concerning the genetic structure of pathogen populations. For example, no restriction fragment length polymorphisms (RFLPs) were

detected among isolates of *F. subglutinans* from California and Florida or from several different pine hosts when examined using four different restriction digests (Correll, Gordon & McCain, 1992). Restriction patterns of mtDNAs of pine isolates, however, were quite different from those of the non-pine isolates of *F. subglutinans*.

In South Africa, *F. subglutinans* has been responsible for disease of maize (Marasas *et al*., 1979) and malformation of mango (Crookes & Rijkenberg, 1985*a*, *b*). The fungus has recently been associated with a serious root disease of *P. patula* Schltdl. & Cham. seedlings at a forest nursery (Viljoen, Wingfield & Marasas, 1994). This study examines the nature of the South African isolates of *F. subglutinans* from pine in terms of their morphology, pathogenicity, genetics and molecular relationships. We also consider their relatedness to South African isolates from non-pine hosts that have been present in the country for an extended period, and to pine and non-pine hosts from other parts of the world.

MATERIALS AND METHODS

Isolates

Twenty-six isolates of *F. subglutinans* were included in this study (Table 1). These include the following: six isolates from diseased roots of *P. patula* seedlings in South Africa; four pitch canker isolates including an authentic isolate of the pitch canker fungus (WLM 144); three isolates which are compatible single-ascospore isolates from a mating of Indian isolates of *F. subglutinans*, IMI 206589 from rice and IMI 207364 from sugarcane; three isolates from maize; two isolates from mango; two isolates from pineapple; one isolate each from grass and reeds; two sexually compatible B-tester isolates and two sexually compatible E-tester isolates. All cultures were lyophilized (Nelson *et al*., 1983) and deposited in the culture collection of the Medical Research Council (MRC) at Tygerberg, South Africa.

Cultural and morphological characteristics

Lyophilized cultures were revitalized and single-conidium isolates transferred to 90 mm plastic Petri dishes containing potato dextrose agar (PDA) (39 g of Biolab potato dextrose agar; 1000 ml distilled H_2O) or carnation leaf agar (CLA) (2% of Biolab agar; 1000 ml H_2O; sterile carnation leaf pieces of about 5 mm). Each isolate was transferred to 12 PDA and two CLA Petri plates, of which six PDA plates were incubated at 25 °C and six plates at 30° in the dark. Linear growth and colony colour were recorded 3 and 7 d after incubation. CLA plates were incubated at 25° under near-ultraviolet and cool-white lights to stimulate conidial development, and were checked for sporodochia formation 7 and 14 d after incubation. All isolates were maintained under these conditions for 6 wk.

Morphological structures were studied after 14–18 d growth on CLA using a Zeiss Axioskop light microscope. Macro- and microconidia were measured, and the formation of polyphialides confirmed. Conidial measurements and colony growth rates were compared using the least significant difference (LSD) function of the SAS/STAT program for personal computers (SAS Inc., Cary, NC).

For scanning electron microscopy (SEM), culture material was fixed in 1·5% glutaraldehyde. After treatment in 1%

Table 1. Cultures studied and sources from which isolates of *Fusarium subglutinans* were obtained

MRC no.*	Original no.	Host	Geographical origin	Obtained from
MRC 6208	K0026	*Pinus patula*	Ngodwana, S.A.	A. Viljoen
MRC 6213	K0034	*P. patula*	Ngodwana, S.A.	A. Viljoen
MRC 6214	K0035	*P. patula*	Ngodwana, S.A.	A. Viljoen
MRC 6211	K0030	*P. patula*	Ngodwana, S.A.	A. Viljoen
MRC 6217	K0057	*P. patula*	Ngodwana, S.A.	A. Viljoen
MRC 6209	K0028	*P. patula*	Ngodwana, S.A.	A. Viljoen
MRC 6226	M 935	*P. elliottii*	Georgia, U.S.A.	P. E. Nelson
MRC 6228	M 1290	*P. elliottii*	Florida, U.S.A.	P. E. Nelson
MRC 6229	M 3834	*P. radiata*	California, U.S.A.	P. E. Nelson
MRC 2382	WLM 144			E. G. Kuhlman
MRC 2381	IMI-2	Perithecia†	India	E. G. Kuhlman
MRC 2292	IMI-4	Perithecia†	India	E. G. Kuhlman
MRC 2293	Kuhlm-318	Perithecia†	India	E. G. Kuhlman
MRC 115	KSU 490	Maize	South Africa	W. F. O. Marasas
MRC 756		Maize	South Africa	W. F. O. Marasas
MRC 1077		Maize	Transkei	W. F. O. Marasas
MRC 2730	KSU 3873	Mango	South Africa	W. F. O. Marasas
MRC 2802	KSU 3874	Mango	South Africa	W. F. O. Marasas
MRC 6784	E-203	Pineapple	Brazil	A. Ventura
MRC 6785	E-204	Pineapple	Brazil	A. Ventura
MRC 6747	I-6; 0274	Grass	Ngodwana, S.A.	G. H. J. Kemp
MRC 6748	I-7; 0275	Reed	Ngodwana, S.A.	G. H. J. Kemp
MRC 6524	KSU 03852	B+-tester		J. F. Leslie
MRC 6525	KSU 03853	B−-tester		J. F. Leslie
MRC 6512	KSU 02192	E+-tester		J. F. Leslie
MRC 6483	KSU 00990	E−-tester		J. F. Leslie

* MRC, Culture collection of the Medical Research Council, P.O. Box 19070, Tygerberg 7505, South Africa.
† Single-ascospore isolate from a mating of Indian isolates of *F. subglutinans* from rice and from sugarcane.

osmium tetroxide for 2 h, the material was dehydrated in a graded acetone series. It was then critical-point dried, coated with gold/palladium, and viewed with an ISI scanning electron microscope.

Pathogenicity

Isolates of *F. subglutinans* were tested for their ability to cause stem cankers on *P. patula* seedlings. Inoculum was prepared by transfer of lyophilized cultures to CLA and incubation under cool-white fluorescent light for 14 d at 25° to stimulate sporulation.

One-yr-old *P. patula* seedlings were used for inoculation. Small strips (10 mm × 1 mm) of bark were cut from the stem of each seedling and similarly sized pieces of *F. subglutinans*-covered CLA (1 mm thick) were placed in contact with the wounds and covered with parafilm. Control seedlings were inoculated with sterile CLA. Fifteen seedlings were inoculated with each isolate. Seedlings were arranged in a completely randomized block design in a growth room. Temperature in the growth room was set at 24° and a 12-h day–night photoperiod installed using cool-white and nuv illumination. After 16 d, lesion development was measured distal to the point of inoculation. Measurements proximal to the inoculation point were not possible because many of the symptomatic seedlings were killed above the point of inoculation.

Re-isolations were made from diseased tissue of 1-yr-old seedlings to confirm pathogenicity of the respective isolates. Both experiments were repeated once. Comparisons of pathogenicity among isolates were analysed with the SAS/STAT system for personal computers using Tukey's studentized range (HSD) test.

Sexual compatibility

To determine sexual compatibility and mating type, isolates of *F. subglutinans* were crossed as male strains with sexually fertile B- (MRC 6524 and 6525) and E- (MRC 6512 and 6483) testers as female strains. In addition, crosses with the 10 pine isolates (four pitch canker isolates; six isolates from pine seedlings in South Africa) in all possible combinations were attempted, using each isolate both as male and female.

Procedures described by Klittich & Leslie (1988) were followed for mating tests. Female testers were cultivated on carrot agar (CA) in 65 mm diam. plastic Petri dishes for 7 d at 25° under cool-white and dark-fluorescent lights. Strains serving as males were cultivated under similar conditions, but on slants of complete medium (CM) (Correll, Klittich & Leslie, 1987). After 7 d, conidia from male strains were dislodged by adding 3 ml of 2·5% Tween 60 solution to each CM slant and scratching the mycelial surface lightly. Female testers were fertilized by adding 1 ml of the conidial suspension to CA plates and spreading it with a glass spreader over the entire surface. Plates were then incubated upright in a single layer under a mixture of cool-white and nuv illumination with a 12-h photoperiod at 25°. Plates were examined each week from the second to the sixth week for perithecia exuding ascospores from their ostioles. Apart from crosses on carrot agar, more crossings were done on CLA and V-8 (200 ml V-8 Juice; 3 g of $CaCO_3$; 800 ml H_2O; pH 7·0) agar. The procedures followed were exactly the same as mentioned for CA.

In a subsequent study, 12 additional isolates of *F. subglutinans* f. sp. *pini* from South Africa were crossed with B- and E-testers on CA. Crosses were also attempted with pine isolates serving as female and the testers serving as male strains. All isolates used in the initial study (Table 1) were furthermore crossed with B- and E-testers on CLA, and the 10 pine isolates crossed in all possible combinations. Isolates were inoculated on opposite sides of carnation leaves on the agar, and incubated for 8 wk in a growth room. Temperature was 25° with cool-white and nuv lights for a 12-h photoperiod. These experiments were repeated at least once.

RAPD analysis

Freeze-dried fungal material and total DNA isolation were obtained by the method of Viljoen, Wingfield & Wingfield (1994). A modification of methods described by Williams *et al.* (1990) was followed for amplification of DNA segments. One 15-mer primer WING-1 (CATGTGTGGCGGGCA) and two 10-mer primers, OPA-2 (TGCCGAGCTG) and OPA-12 (TCGGCGATAG) were selected from an initial screening of 40 primers (Operon Technologies, Alameda, CA). Amplifications (PCR) were performed in a Hybaid Omnigene temperature cycler (Hybaid, Middlesex, U.K.) with a total reaction volume of 100 µl containing 25 mM $MgCl_2$, 10 µl 10 × *taq* polymerase buffer, 25 mM dNTPs, 10 O.D. ml^{-1} primer and 1 µg ml^{-1} DNA. An initial denaturation step at 96° for 5 min was followed by 35 cycles of 34° for 1 min, 72° for 2 min and 92° for 15 s, with a final extension cycle of 5 min at 72°. Successful amplifications were confirmed by gel electrophoresis on 1% Oxoid agar gels stained with ethidium bromide.

RAPD fingerprints were resolved by 4% polyacrylamide gels (Maniatis, Fritsch & Sambrook, 1989). The running buffer was 1 × TBE and running conditions were set at 140 V for 4·5 h. DNA was visualized with silver staining (Maniatis *et al.*, 1989). The gels were preserved in a 5% glycerol/30% ethanol mixture and dried between cellophane layers at room temperature.

The Phylogenetic Analysis Using Parsimony (PAUP) (Swofford, 1993) program was used for phylogenetic analysis. The presence or absence of DNA bands was scored for each of the three primers, combined, and compared by Wagner parsimony using the branch and bound search option. A majority rule consensus tree based on 100 replicates was generated by bootstrap analysis.

RESULTS

Cultural and morphological characteristics

Fusarium subglutinans isolates from diseased roots of *P. patula* seedlings in South Africa matched previous descriptions

Table 2. Cultural characteristics of *F. subglutinans**

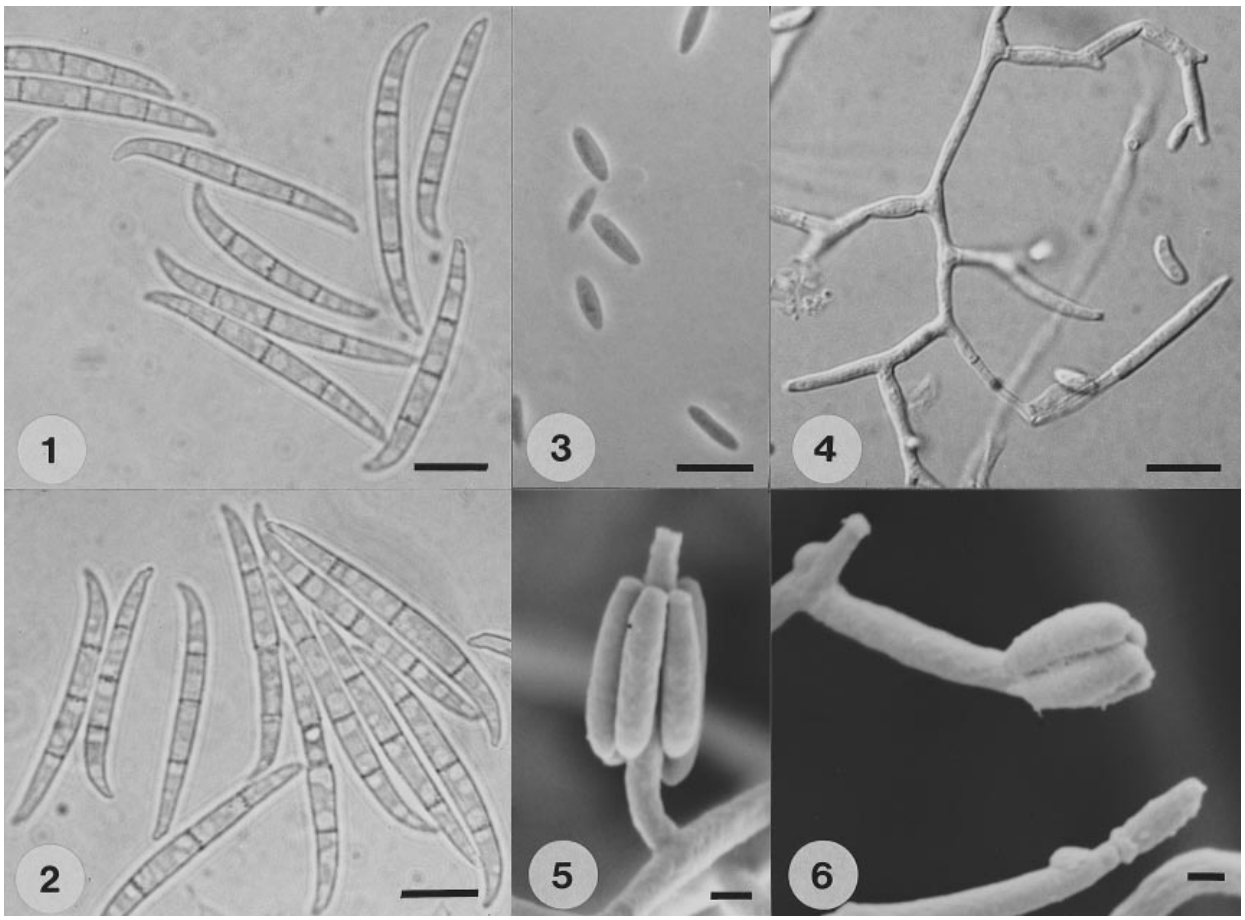
Host Isolate	Colour	Mycelia	Sporodochia	Sclerotia	Polyphialides
Pine					
MRC 6208	Purple	Sparse	+	+	+
MRC 6213	Purple	Sparse	+	+	+
MRC 6211	Blue margin	Sparse	+	+	+
MRC 6214	Blue margin	Sparse	+	−	+
MRC 6209	Mixed	Sparse	+	+	+
MRC 6217	Mixed	Sparse	+	+	+
MRC 6226	Purple	Sparse	+	−	+
MRC 6228	Mixed	Sparse	+	+	+
MRC 6229	Mixed	Sparse	+	−	+
MRC 2382	Purple	Sparse	−	−	+
Perithecia†					
MRC 2381	Yellow	Dense	−	−	+
MRC 2292	Yellow	Dense	−	−	+
MRC 2293	Yellow/tan	Dense	−	−	+
Maize					
MRC 115	Light purple	Sparse	+	−	+
MRC 756	Light purple	Sparse	+	−	+
MRC 1077	Light purple	Sparse	+	−	+
Mango					
MRC 2802	Light purple	Sparse	+	−	+
MRC 2730	Peach	Dense	−	−	+
Pineapple					
MRC 6784	Cream	Dense	−	−	+
MRC 6785	Salmon	Dense	−	−	+
Grass and reeds					
MRC 6747	Purple	Dense	+	−	+
MRC 6748	Purple	Dense	+	−	+

* +, Present; −, absent.

† Single-ascospore isolate from a mating of Indian isolates of *F. subglutinans* from rice and from sugarcane.

(Wollenweber & Reinking, 1925; Booth, 1971; Kuhlman *et al.*, 1978; Nelson *et al.*, 1983). Colonies from single-conidium isolates formed sparse white aerial mycelium on PDA after incubation in the dark, more dense and tinged with purple at the colony centre. The undersurfaces of the Petri dishes were cream-white with a peach or purple mid-point, which, after 14 d, led to a prominent variation of purple and blue pigmentation. Isolates MRC 6208 and 6213 developed a purple to dark purple centre (PC), isolates MRC 6211 and 6214 a blue circle near the colony margin (BM), while isolates MRC 6209 and 6217 had both purple centres and blue margins (PC + BM) (Table 2). Colony diameters of single-conidium isolates on PDA were slightly larger at 25 than at 30°. Cream to orange sporodochia developed within 7 d on carnation leaves on agar (Table 2). Within 14 d, dark blue sclerotia (or protoperithecia) developed on the carnation leaves on CLA (Table 2). When single-spore isolates were made again, they retained their distinctive cultural characteristics. Macroconidia were fairly abundant and were produced mainly in cream to orange sporodochia. Although one- and two-septate macroconidia (mesoconidia) were produced, three-septate macroconidia were most abundant (Figs 1–2). Very few four- or five-septate conidia, although they are known to occur in *F. subglutinans*, were observed. Three-septate macroconidia were 3–4·5 μm × 24–45 μm, and were shorter in general than those of non-pine isolates. Conidia were sickle-shaped to almost straight with the ventral side curved to straight and the dorsal side always curved, forming a dorsal–ventral curvature which was widest at the middle or near the apical cell (Figs 1–2). Apical cells were not distinctly shaped, but often strongly curved. Basal cells were distinctly notched, short and pedicellate. Microconidia (Fig. 3) were abundant and produced on mono- and polyphialides (Fig. 4) in false heads (Figs 5–6). Microconidial measurements ranged from 2–4 μm × 5–17 μm and were smaller than those representing non-pine isolates. The microconidia were non- to one-septate and produced in a variety of shapes, including ellipsoidal, oval, comma-, kidney- and spindle-shaped cells (Fig. 3). Conidiophores were situated laterally or terminally on hyphae, very short to quite long and seldom branched. They tended to narrow towards the apical opening. Polyphialides were present and usually had only two to three apertures on each cell (Fig. 4). Isolate MRC 6217 produced the greatest number of polyphialides with up to five apertures. Chlamydospores were absent.

The pitch canker isolates were similar to the South African isolates. Growth was more rapid at 25 than at 30°, and the undersurface of colonies showed a variation of purple and blue pigmentation (Table 2). Cream to orange sporodochia formed within 7 d, and resulted in masses of orange sporodochia after 14 d (Table 2). Only one of the pitch canker isolates (MRC 6228) were able to form sclerotia within 6 wk. Microconidia were mostly oval and spindle-shaped, while macroconidia formed in sporodochia were often four-septate. Isolate MRC



Figs 1–6. *Fusarium subglutinans* from *Pinus patula* seedlings in South Africa. **Figs 1–2.** Macroconidia (bar, 10 μm). **Fig. 3.** Microconidia (bar, 10 μm). **Fig. 4.** Mono- and polyphialides (bar, 10 μm). **Figs 5–6.** Microconidia borne in false heads (bars, 1 μm).

2382 did not form sporodochia and produced few macroconidia. Polyphialides formed abundantly, were often branched and had many openings on their cells.

The remainder of the isolates examined resembled the pine isolates morphologically, but differed from them culturally (Table 2). The compatible single-ascospore isolates obtained from Kuhlman (MRC 2381 and 2292) formed dense, floccose, white aerial mycelia with yeast-like patches. Colony margins were uneven with a torn appearance and a bright yellow undersurface. Growth was consistently more rapid at 30 than at 25°. None of these isolates developed sporodochia or sclerotia. Mango and maize isolates of *F. subglutinans* had cultural characteristics which varied among isolates from the same host (Table 2). Isolate MRC 2802 from mango formed sparse white aerial mycelium with the reverse a light purple centre. In contrast, isolate MRC 2730 formed dense white aerial mycelia, peach-coloured in the middle, and with the reverse cream-white with a peach centre (Table 2). Only isolate MRC 2802 developed sporodochia within 7 d on CLA, and none of the isolates developed sclerotia. Growth rate of isolate MRC 2802 was more rapid at 25 than at 30°, while isolate MRC 2730 grew faster at 30 than at 25°. Isolate MRC 2730 contained long, slender macroconidia with thin septa, distinctly shaped footcells and apical cells which were sometimes sharply curved. Their macroconidia were often four- to five-septate. Isolates from maize produced sparse to fairly dense white aerial mycelia with the reverse having a light purple centre. Isolate MRC 115 formed an orange centre on the reverse, while isolate MRC 1077 tended to develop a blueish margin after 3 wk. Two of the isolates (MRC 115 and MRC 756) produced an abundance of cream to orange sporodochia after 7 d, but isolate MRC 1077 produced only a limited number of sporodochia after 21 d. None of the isolates produced sclerotia. Macroconidia were most often three- and four-septate, but five- and six-septate macroconidia were also observed. These four- to six-septate conidia were mainly produced in sporodochia and were longer than the three-septate macroconidia. Non- to one-septate microconidia and one- to three-septate mesoconidia were also produced in abundance by the maize isolates. The two isolates from grass and reeds (MRC 6747 and MRC 6748) appeared to be similar to each other in culture (Table 2). Colonies produced salmon, floccose aerial mycelium with the reverse uniformly dark purple. Large micro- and mesoconidia were produced along with long three- to five-septate macroconidia. No protoperithecia were observed. The isolates from pineapple (MRC 6784 and MRC 6785) were also similar to each other in culture, with a more rapid growth rate at 25 than at 30°. The isolates produced cream to salmon, floccose aerial mycelium. No sporodochia or sclerotia were produced by either isolate. Macroconidia were mainly three-septate, and were less abundant.

Table 3. Stem lesions of 1-yr-old *P. patula* seedlings after inoculation with *Fusarium subglutinans* isolates from pine and non-pine hosts

Host Isolate	Pathogenicity* Lesion (mm)†
Pine	
MRC 6208	13·8 b
MRC 6209	16·5 a
MRC 6211	12·6 bc
MRC 6213	11·9 bc
MRC 6214	12·8 bc
MRC 6217	13·0 bc
MRC 6226	10·7 c
MRC 6228	13·5 b
MRC 6229	12·1 bc
MRC 2382	11·7 bc
Perithecia‡	
MRC 2381	0·0 d
MRC 2292	0.0 d
MRC 2293	0·0 d
Maize	
MRC 115	0·0 d
MRC 756	0·0 d
MRC 1077	0·0 d
Mango	
MRC 2730	0·0 d
MRC 2802	0·0 d
MRC 6748	0·0 d
Control	0·0 d

* Mean percentages in the same column followed by the same letter do not differ significantly ($P = 0{\cdot}05$) according to Tukey's Studentized Range (HSD).

† Mean length of 15 seedlings according to Tukey's Studentized Range (HSD).

‡ Single-ascospore isolate from a mating of Indian isolates of *F. subglutinans* from rice and from sugarcane.

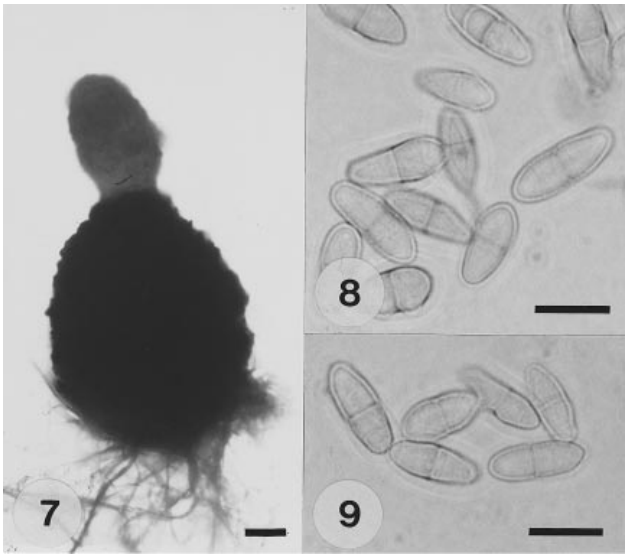
Pathogenicity

Necrotic lesions developed on all 1-yr-old *P. patula* seedlings inoculated with isolates of *F. subglutinans* from pines (Table 3). No significant difference ($P = 0{\cdot}05$) in virulence was observed between most isolates obtained from diseased roots of *P. patula* seedlings in South Africa and those responsible for pitch canker in the United States (Table 3). Isolate 6209 from diseased roots of *P. patula* seedlings proved to be most aggressive and pitch canker isolate MRC 6226 the least aggressive to the seedlings. Many seedlings were killed proximal to the point of inoculation. Isolates from pine exhibited significantly ($P = 0{\cdot}05$) greater virulence to the seedlings than non-pine isolates. None of the seedlings inoculated with isolates from hosts other than pine developed any lesions.

Fusarium subglutinans isolates could be recovered from necrotic lesions on stems of 1-yr-old seedlings. No isolates of *F. subglutinans* were isolated from control inoculations.

Sexual compatibility

Pine isolates of *F. subglutinans* were unable to cross with known sexually fertile testers of the B- and E-populations of *F. subglutinans* on CA, V-8 agar or CLA using the methods described by Klittich & Leslie (1988). This includes a pine isolate (MRC 2382) previously reported to cross with a B-tester (Kuhlman, 1982). Crosses with 12 additional isolates from pine seedlings in South Africa and crosses where the pine isolates served as female tester strains were also unsuccessful. In some crosses, blue-black spherical sclerotia-like structures were formed which never matured into perithecia or contained ascospores. When compatible B- and E-testers were crossed as controls, perithecia appeared within 5 d after fertilization, and ascospores were produced after 2 wk. Only testers belonging to the B-population mated with B-testers, while the E-testers only mated within the E-population. The four testers were found to be hermaphroditic and + and − isolates served as both male and female fertile isolates.



Figs 7–9. *Gibberella subglutinans* produced by a cross of a South African isolate of *Fusarium subglutinans* from pine (MRC 6217) and a pitch canker isolate from California, U.S.A. (MRC 6226). **Fig. 7.** Perithecium. **Figs 8–9.** Ascospores (bars, 10 μm).

Perithecia containing viable ascospores developed within 1 month on CLA when compatible isolates of *F. subglutinans* from pines were placed on opposite sides of carnation leaves. Two successful crosses were obtained. Isolates MRC 6211 and MRC 9213 from pine seedlings in South Africa, and MRC 6217 from pine seedlings and MRC 6229 from pitch canker on *P. radiata* trees in California repeatedly crossed when incubated on CLA. Viable ascospores oozed from dark-blue perithecia (Fig. 7). Ascospores were mostly one-septate (Figs 8–9), but two- and three-septate ascospores had been observed occasionally. Average ascospore size was $13{\cdot}3 \times 4{\cdot}9$ μm.

The only other successful cross was that of the E^+-tester isolate with an isolate from maize (MRC 115) on CA. The perithecia, however, did not exude their ascospores, but ascospores were visible after perithecia were squashed. Ascospores from this mating were not tested for viability.

RAPD analysis

The three primers used in this study, WING-1, OPA-2 and OPA-12, generated a considerable number of amplification

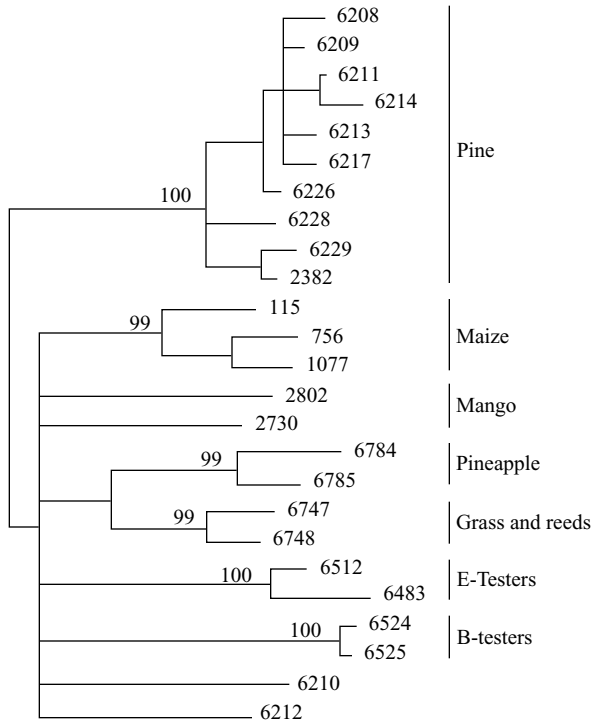


Fig. 10. Dendrogram showing relationships among isolates of *Fusarium subglutinans* from different hosts and two *F. oxysporum* isolates (MRC 6210; MRC 6212). Genetic distances were obtained by random amplified polymorphic DNA analysis with three primers (OPA-2, OPA-12 and WING-1). Bootstrap intervals between isolates representing the different hosts are indicated at branch points.

products for comparison. A different DNA banding pattern was present in almost every strain. The patterns were complex, showing 15–30 bands of various intensities. Therefore, only distinctive and reproducible bands were selected for analysis. The number of bands in the profiles varied, depending on the primer and the isolate tested. For WING-1, 79 bands were identified as genetic markers, while 53 and 55 bands were identified as markers for OPA-2 and OPA-12 respectively.

RAPD profiles of isolates of *F. subglutinans* from pine were similar but not identical. With the three primers it was possible to score 82 polymorphic sites. Of these, 47 bands were shared by all pine isolates, 10 bands were informative and 25 bands indicated strain differences. Informative bands separated the South African pine isolates from the pitch canker isolates, or subdivided the South African population. Many of the bands indicating strain differences were associated with both South African and pitch canker isolates, but were not present in all of the pine isolates. RAPD profiles of pine isolates, however, differed from those of non-pine isolates.

A dendrogram presenting similarity among isolates was generated using the bootstrap method (Fig. 10). The clustering patterns in the dendrogram show that isolate clustering correlates to host. Isolates of *F. subglutinans* from pine in South Africa clustered closely with isolates associated with pitch canker in the United States, but were separated from South African isolates of *F. subglutinans* from maize, mango, grass and reeds. Likewise, strains from hosts other than pine also seemed to group together (Fig. 10), with the exception of *F. subglutinans* isolates from mango. *Fusarium oxysporum* isolates, used as outgroup, clustered apart from *F. subglutinans*.

DISCUSSION

Fusarium subglutinans is a species defined by morphological characteristics. It, however, has a wide host range (Booth, 1971). The fungus has recently been reported as a pathogen of pine from Japan (Kobayashi & Muramoto, 1989) and South Africa (A. Viljoen *et al*., 1994), where it was in the past best known as a pathogen of sugarcane (Muramoto, Tashiro & Minamihashi, 1993) and maize (Marasas *et al*., 1979), respectively. Japanese isolates of *F. subglutinans* from pine have not been evaluated for pathogenicity to sugarcane (Muramoto *et al*., 1993), but were similar in virulence to 2-yr-old pine seedlings as an authentic isolate of the pitch canker pathotype (Muramoto & Dwinell, 1990). From our study, it was evident that the South African isolates from pine belong to the worldwide group of pine isolates of *F. subglutinans*, but differ from isolates from the same morphological species causing disease in maize or mango.

Limited differences in morphological characteristics among isolates of *F. subglutinans* from many hosts were detected in this study. Many differences in cultural appearances among isolates representing the different hosts, as well as within hosts, were found. Differences in cultural appearance among isolates representing *Fusarium* spp. or *formae speciales* had been reported (Ploetz, 1990). These differences were related to vegetative compatibility groups (VCGs) rather than to isolates attacking specific hosts. It would be interesting to correlate cultural characteristics of *F. subglutinans* to specific VCGs.

The major morphological characteristics used in the description of *Fusarium* species include macroconidial morphology, microconidial morphology and conidiogenesis (Nelson *et al*., 1983). Within the relatively simple fruiting structures and the considerable developmental plasticity of fungi, it must be expected that accumulating differences in developing sub-populations are not always expressed in terms of morphological divergence (Brasier, 1986). Closely related or sibling species may, therefore, lack taxonomically useful morphological differences long after the initial speciation event.

South African isolates of *F. subglutinans* from pine possessed similar virulence to 1-yr-old pine seedlings such as the pitch canker isolates, but differed from those associated with the non-pine isolates. This is in agreement with results of previous studies (Dwinell & Nelson, 1978; Correll *et al*., 1991), that eventually led to the proposal of a *forma specialis* for pine isolates of *F. subglutinans*. Isolates of *F. subglutinans* from Monterey (*P. radiata* D. Don) pine in California were similar to isolates causing pitch canker in the southern United States (Dwinell, 1988). Furthermore, an isolate from Japan caused identical symptoms to pine seedlings as an authentic isolate of the pitch canker fungus (Muramoto & Dwinell, 1990). It thus appears that isolates of *F. subglutinans* from pines throughout the world belong to the same pathotype.

The ability of individuals of a species to cross and produce viable progeny is the character that defines biological species.

In *Fusarium* section Liseola, four species and six mating populations have been identified. Two mating populations, A and F, belong to *F. moniliforme*, and mating populations B and E to *F. subglutinans* (Leslie, 1991; Klittich & Leslie, 1992). These populations are morphologically similar, but differ in host associations (Leslie, 1991). The B-population has been associated with sugarcane, maize stalks and pine (Kulhman, 1982) and the E-population with maize (Leslie, 1991). No cultural or molecular differences have been documented between the B- and E-mating populations. Correll *et al.* (1992) were unable to achieve crosses of pine isolates with the B- or E-mating population, even when previously described pine testers were used. We were also unable to demonstrate sexual compatibility between the B- and E-testers, and pine isolates which included an isolate (MRC 2382) reported to be sexually compatible with B-testers (Kuhlman, 1982). We were, however, able repeatedly to obtain fertile crosses amongst pine isolates on CLA. Ascospores derived from these crosses differed morphologically from those described by Kuhlman *et al.* (1978) between pine and the B-testers. Ascospores in this study were shorter and mainly one-septate, while those described by Kuhlman *et al.* (1978) were mainly three-septate. The crossing of a South African isolate from pine with a Californian pitch canker isolate confirmed that the South African population is related to the pitch canker pathotype, and has probably been introduced into this country. Our results indicate genetic isolation of *F. subglutinans* isolates from pines, and we are currently investigating the possibility that they represent a third mating population within *F. subglutinans*.

Composite RAPD data supported the view that isolates of *F. subglutinans* from pines represent a homogeneous group. This is in agreement with RFLP analyses of isolates of *F. subglutinans* from pine and non-pine hosts (Correll *et al.*, 1992). When plotted on a dendrogram, the South African isolates from pine clustered with pitch canker isolates from the United States. This implies that pine isolates of *F. subglutinans* may have a common ancestry rather than mutagenic alteration by host as suggested for some isolates of *F. oxysporum* (Kistler & Momol, 1990). Isolates from non-pine hosts such as maize and pineapple also clustered according to host. The morphological differences observed between the mango isolates were reflected in their DNA banding patterns, and probably indicate that one of the isolates (MRC 2730) was associated with mango by chance. The isolates from grass and reeds in South Africa appear to originate from the same gene pool. This study, therefore, supports the suggestion by Correll *et al.* (1991) that sub-specific groups unified by similar DNA genomes exist in *F. subglutinans*.

Although morphologically uniform, *F. subglutinans* is genetically a diverse species. This has led to the proposal of at least two *formae speciales*, *F. subglutinans* f. sp. *pini* from pine (Correll *et al.*, 1991) and *F. subglutinans* f. sp. *ananas* from pineapple (Ventura, Zamgolin & Gilbertson, 1993). All mating populations within section Liseola are currently being investigated as separate species (W. F. O. Marasas, unpublished). The ability of pine isolates to cross with each other to produce viable (and perhaps distinctive) ascospores, and their inability to cross with established fertile testers of the B- and E-mating populations, together with characteristic cultural behaviour, host preference and DNA banding patterns suggest that they represent a distinct new biological species.

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