Short title: Sexual reproduction of *Fusarium circinatum*

Influence of sexual reproduction on the *Fusarium circinatum* population in South Africa

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Summary

Fusarium circinatum (=*F. subglutinans* f. sp. *pini*) is an important pathogen in pine nurseries in South Africa. The initial outbreak of pitch canker disease caused by this pathogen was limited to a single nursery. Subsequently, several other pine nurseries in South Africa have become infected. A high level of genotypic diversity was observed in the initial *F. circinatum* population, suggesting that sexual reproduction occurs in the fungus in South Africa. Sexual crosses performed in the laboratory indicated a low hermaphrodite frequency in the initial population, which influences the fungus to reproduce asexually rather than sexually. The aim of this study was to compare the frequency of female-sterile and hermaphrodite strains in the population initially studied, with that of newly collected *F. circinatum* strains. Crosses were performed at lower temperatures, as this appears to influence the fertility of strains in culture. The effective population number, N_e, was calculated using mating type and female-

sterile/hermaphrodite characteristics. The genotypic diversity in the recently collected *F. circinatum* populations was also determined using vegetative compatibility tests. The hermaphrodite frequency of the initial population determined at the lower incubation temperature was 47%, compared to the previously reported frequency of 27%. The frequency of hermaphrodites in the recent outbreaks was 67%. The effective population number for mating types, $N_{e\ (mt)}$, is 97% for the total population and the female-sterile/hermaphrodite status, $N_{e\ (f)}$, is 88 to 92% for the total population. Six new vegetative compatibility groups (VCGs) have emerged since the initial outbreak of *F. circinatum* in South Africa 10 years ago. The increase in hermaphrodite isolates and VCGs also indicates that sexual reproduction is occurring relatively frequently, even though signs of this have not been observed in the field.

Keywords: Fusarium circinatum, female-sterile, hermaphrodites, pitch canker

Introduction

The pitch canker pathogen, *Fusarium circinatum* Nirenberg & O'Donnell (=*F. subglutinans* (Wollenweber & Reinking) Nelson *et al.* f. sp. *pini* Correll *et al.*) causes a destructive disease of pines in various countries. *F. circinatum* first appeared in South Africa in a single forest nursery in 1990 causing a root disease of *Pinus patula* seedlings (Viljoen *et al.*, 1994). Since this initial outbreak, *F. circinatum* has spread to several other forest nurseries causing serious root and collar rot of various *Pinus* spp. Stem canker on mature trees typical of those found elsewhere have, however, not been observed in South Africa (Viljoen *et al.*, 1997a; Wingfield *et al.*, 1999).

The genotypic diversity of the initial *F. circinatum* population in South Africa was determined using vegetative compatibility (VC) tests (Viljoen *et al.*, 1997b). A high genotypic diversity was found in this population where 23 vegetative compatibility groups (VCGs) were identified among 69 *F. circinatum* isolates. This high level of genotypic diversity led Viljoen *et al.* (1997b) to suggest that sexual reproduction is occurring within the South African population.

Fusarium circinatum and other related *Fusarium* spp. reside in distinct mating populations (biological species) of *Gibberella fujikuroi* (Sawada) Ito in Ito & K. Kimura complex (Leslie, 1995; Klaasen & Nelson, 1996; Klittich *et al.*, 1997; Britz *et al.*, 1999). Each of these species is heterothallic and governed by two alleles at a single mating type locus. *F. circinatum* resides in mating population H of the *G. fujikuroi* complex (Britz *et al.*, 1999). *F. circinatum* strains of opposite mating type can recombine, leading to the formation of the sexual structures (Viljoen *et al.*, 1997b; Britz *et al.*, 1998). The name, *G. circinata* Nirenberg & O'Donnell has recently been applied to the teleomorph (Nirenberg & O'Donnell, 1998).

Fusarium circinatum can reproduce both asexually and sexually and each of these cycles affects the population structure differently (Leslie & Klein, 1996; Britz *et al.*, 1998). The asexual cycle results in vegetative propagation, whereas the sexual cycle results in recombination leading to new genotypes (Leslie & Klein, 1996). The effective population number (N_e) estimates the relative contribution of each cycle towards the population (Wright, 1931; Leslie & Klein, 1996; Britz *et al.*, 1998). However, the numbers of strains that function as female parents limit the effective population number (Klein & Leslie, 1996; Britz *et al.*, 1998). The initial *F. circinatum* population in South Africa had a low frequency of hermaphrodite strains (Britz *et al.*, 1998). If the number of hermaphrodites continues to fall, the *F. circinatum* population in South Africa should ultimately become asexual (Britz *et al.*, 1998; 1999).

In a recent study, lower incubation temperature was reported to increase the fertility of sexual crosses of *F. circinatum* in the laboratory (Covert *et al.*, 1999). Temperature thus influences the fertility of isolates in culture and results obtained at higher temperatures may reflect an inaccurate effective population size. The influence of a lower incubation temperature on sexual crosses and effective population size of the initial *F. circinatum* population in South Africa requires reassessment.

Since the first outbreak of *F. circinatum* in 1990 (Viljoen *et al.*, 1994), new outbreaks have occurred in South Africa. We, therefore, assessed the initial *F. circinatum* population and a recently collected *F. circinatum* population and could determine the influence of sexual reproduction on the population over ten years. The aims of this study were to (i) reassess the fertility and mating type distribution of the initial *F. circinatum* population in South Africa at a lower incubation temperatures; (ii) to compare the effective population size of the initial population with the population that started to emerge six years later and (iii) to determine the genotypic diversity in *F. circinatum* populations collected after the initial outbreak of the disease in South Africa.

Materials and methods

Sampling

Fusarium circinatum isolates were collected from different forestry nurseries in South Africa (Table 1). The initial *F. circinatum* population was from a single nursery (Ngodwana) and isolates representing the recent *F. circinatum* population were collected from nurseries in the Piet Retief, Karatara, Sutherland, Tweefontein and Klipkraal areas. The initial *F. circinatum* population represents 85 *F. circinatum* isolates collected between 1990 and 1992 (Viljoen *et al.*, 1994) and 74 *F. circinatum* isolates were obtained from recent outbreaks between 1996 to 1998. A total of 159 *F. circinatum* isolates were, thus, considered in this study (Table 1).

Roots, root collars and stems of pine seedlings showing disease symptoms such as tip die-back and needle discoloration were first immersed in 70% ethanol for 2 min. Small pieces (approximately 5 mm long) of the infected tissue were removed and plated on *Fusarium* selective medium (Nash & Snyder, 1965) containing 15 g Peptone, 1 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 20 g agar, 1 g pentachloronitrobenzene and 1 L dionized water with 1 mg/L streptomycin. Cultures were allowed to grow for 5 days at 25°C. Small agar pieces (approximately 5 mm^2) from the edges of the colonies were transferred to 90 mm diameter 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 12 h

photoperiod to stimulate culture and conidium development. Isolates identified as *F. circinatum* on CLA were purified based on single conidia and stored as conidial suspensions in 15% glycerol at –70°C and are available from the *Fusarium* culture collection of the Tree Pathology Co-operative Programme (TPCP), Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa. A large number of the strains have also been deposited in the culture collection of the Medical Research Council (MRC), PROMEC, Tygerberg, South Africa.

Sexual compatibility and mating type determination

All *F. circinatum* isolates were crossed with the mating population H tester strains, MRC 6213 and MRC 7488 (Britz *et al.*, 1998; 1999). The tester strains were crossed with each other as a positive control. Crosses were made on carrot agar as described by Klittich & Leslie (1988) with modification (Britz *et al.*, 1998) at 17°C. All the crosses were examined weekly and designated sterile, female-sterile or hermaphrodite (Table 1). All fertile crosses were repeated at least once. The tester strains, MRC 7488 and MRC 6213, were designated *MAT-1* and *MAT-2*, respectively (Steenkamp *et al.*, 2000). All isolates that produced fertile crosses with the tester strains were thus designated as belonging to the mating type opposite to that of the tester strains (Table 1).

The effect of mating type distribution and female fertility on the South African *F. circinatum* population was assessed by determining the effective population number for mating type ($N_{e(mt)}$), effective population number ($N_{e(f)}$), the average number of asexual generations per sexual generation using the equations described in Leslie & Klein (1996). The effective population size and the average number of asexual generations per sexual generation were determined for isolates from the initial outbreak and those for the more recent *F. circinatum* outbreaks, as well as for the total *F. circinatum* population in South Africa (sum of the initial and recent *F. circinatum* populations)

Vegetative compatibility (VC) tests

Nitrate non-utilizing (*nit*) mutants were generated on 15 – 25% chlorate agar and the *nit* mutants were identified as *nit1*, *nit3* or *nit*M based on the utilization of different nitrogen

sources (Puhalla & Spieth, 1983; 1985; Correll *et al.*, 1987). Incompatibility was determined by pairing isolates in all possible combinations on minimal media for 14 days at 25°C. Heterokaryon self-incompatibility (HSI) of isolates (Correll *et al.*, 1989) was determined by crossing a *nit1* or *nit3* mutant with a *nitM* mutant of the same isolate. All parings were repeated at least once.

Each VCG was assigned a number (SA1-SA29). The most common VCGs in the *F. circinatum* population were determined by identifying the number of representatives for each VCG. The diversity of the VC groups for isolates representing the initial and recent *F. circinatum* outbreaks was determined in two ways. First, the number of VCGs was divided by the sample size in each population (S/N). Secondly, the Shannon diversity index (H′), which provides an indication of the population structure (Anagnostakis *et al.*, 1986) was calculated using the equation:

$$H' = -\Sigma p_i \ln p_i$$

where p_i is the observed frequency of the VC group (genotype). If all the individuals have the same genotype then $H' = 0$, and when they all belong to a different genotype, then H' will have the highest value.

The genotypic diversity (G) of the population was determined as proposed by Stoddart & Taylor (1988). Genotypic diversity (G), was estimated as:

$$G = 1/\Sigma pi^2$$

where pi is the observed frequency of the genotype. The maximum percentage of genotypic diversity, for the total set of isolates was also determined by dividing the genotypic diversity by the total sample size (G/N).

Results

Sexual compatibility and mating type distribution

Tester strains of *F. circinatum* (MRC 6213 and MRC 7488) known to be fertile (Britz *et al.*, 1998; 1999) consistently produced perithecia with viable ascospores when crossed with isolates from various nurseries in South Africa. Some *F. circinatum* isolates did not

produce perithecia when crossed with the mating tester strains. The percentage of sterile strains collected from the different nurseries ranged from 11-17% with the exception of 29% sterile isolates found among those collected from Piet Retief nursery. No sterile *F. circinatum* isolates were collected from the Tweefontein and Sutherland nurseries, and the single isolate collected from Karatara nursery was also sterile. Sterile isolates were excluded from the calculation of $N_{e(mt)}$ and $N_{e(f)}$. However, the $N_{e(f)}$ was also calculated for each nursery by including the sterile strains in the calculation as female-sterile strains (Table 1).

Reassessment of initial F. circinatum population

Of the 85 *F. circinatum* isolates collected from the initial outbreak at the Ngodwana nursery, 71 were cross fertile with one of the mating type testers (MRC 6213 and MRC 7488). The mating type distribution of the initial population had a 1:1 ratio giving a $N_{e(mt)}$ of 99.9%. Among the 71 fertile isolates, 33 were hermaphrodites and 38 female-sterile (Table 1). The percentage of the hermaphrodites ranged from 21 - 69% (observed value 47%), when only the fertile strains were used. The average number of asexual generations per sexual generation was estimated to range from 20-79. This variation depends on the values for mutation rate (μ) on female sterility and selection (θ) against hermaphrodites during the asexual generation of the life cycle, which was ($0.98 > \mu(1-\theta) > 0.99$). If all the strains are included, then the percentage of hermaphrodites ranged from 12 - 63% (observed value 39%) with the average number of asexual generations per sexual generation ranging from 24 to 117 depending on the values of μ and θ.

Recent F. circinatum population

The recently collected *F. circinatum* population included isolates from nurseries other than the Ngodwana nursery, where the initial population was studied. In the new population, 42 hermaphrodite strains were present amongst 72 fertile strains collected from five different nurseries (Table 2). The recently collected *F. circinatum* population had a 1:2 (*MAT-1*:*MAT-2*) mating type ratio, giving a $N_{e(mt)}$ of 86.7%. The hermaphrodite frequency varied from 45 – 82% (observed value 67%) with the estimated average asexual generations ranging from 10 – 39 per sexual generation, where only fertile

isolates were considered. If all strains (fertile and sterile *F. circinatum* isolates from recent outbreaks) are considered, the hermaphrodite frequency ranged from 36 - 76% (observed value 58%) with the average asexual generations per sexual generation ranging between 13 – 51 ($0.98 > \mu(1-\theta) > 0.99$).

Initial and recent (total) F. circinatum population

Of the total population of 157 *F. circinatum* isolates (initial and recent outbreaks), 134 were cross fertile. These cross fertile isolates included 75 hermaphrodites and 59 female-sterile strains. The hermaphrodite frequency ranged from 32 – 75% (observed value 56%) with the average asexual generations per sexual generation ranging from 15 – 56, if only fertile isolates are considered. If all strains (fertile and sterile) were included, the hermaphrodite frequency ranges from 23-69% (observed value 48%) with the average asexual generations per sexual generation ranging from 18 – 73 ($0.98 > \mu(1-\theta) > 0.99$).

Vegetative compatibility tests

Chlorate-resistant *nit* mutants were produced for 125 *F. circinatum* isolates. Twenty-nine VCGs were identified amongst these isolates. Of the 29 VCGs, 23 were the same as those identified from the initial *F. circinatum* outbreak occurring in 1990 (Viljoen *et al.*, 1997b). Six new VCGs were identified from the recent *F. circinatum* outbreaks. The six new VCGs were from the Klipkraal and Sutherland nurseries (Table 2). Nine VCGs from the initial outbreak were also found in the new collections. A single heterokaryon self-incompatible *F. circinatum* isolate that was also sterile was identified in this study.

Three dominant VCGs, SA-8, SA-12 and SA-14, were present in the initial outbreak of *F. circinatum* and made up 43% of the isolates (Viljoen *et al.*, 1997b). In the recent outbreaks VCG SA-2 and SA-4 were dominant and represented 43% of the isolates. All VCG SA-2 isolates were collected from the Ngodwana (initial outbreak) and Klipkraal nurseries. VCG SA-4 isolates were present in all nurseries except Ngodwana and Sutherland.

The diversity of VCGs varied among the nurseries (Table 2). The number of VCGs divided by the sample size in the initial, recent and total population (S/N) was 0.32, 0.30 and 0.24, respectively (Table 2). Nurseries where only a single VCG occurred had a Shannon diversity index (*H'*) of 0. In the nursery where *F. circinatum* was first found in South Africa, most isolates belonged to a different VCG, *H'*=2.89. The *H'* value for the recent outbreaks was 2.25 and the *H'* value when all the outbreaks were considered was 3.02 (Table 2). The *H'* value becomes inaccurate as the number of isolates collected in each nursery decreases (N<7). The genotypic diversity (G) for the number of genotypes (VCGs) in the initial *F. circinatum* outbreak was estimated at 14. The percentage genotypic diversity (G/N) for the initial population was 19.7%. The genotypic diversity (G) for the number of genotypes (VCGs) in the recent *F. circinatum* outbreaks was estimated to be 7. The percentage genotypic diversity (G/N) for the recent population was, thus, 12.9%.

Discussion

Viljoen *et al.* (1994) reported the first outbreak of *F. circinatum* in a single nursery in South Africa. Since this first outbreak, several other pine nurseries in South Africa have become infected. A high level of genotypic diversity was observed in the initial outbreak of *F. circinatum* (Viljoen *et al.*, 1997b). Sexual crosses performed among isolates collected from this outbreak indicated a low hermaphrodite frequency (Britz *et al.*, 1998). However, a lower incubation temperature has recently been reported to influence fertility of *F. circinatum* (Covert *et al.*, 1999). The hermaphrodite frequency and effective population number were, therefore, reassessed in this study at a lower incubation temperature. Consequently, we have found the hermaphrodite frequency and effective population number of the initial *F. circinatum* population to be higher than previously reported

In this study, we compared the effective population number of the initial *F. circinatum* population with recent populations and found that the effective population size has increased since the initial outbreak. Sexual reproduction seems to be occurring relatively

frequently. This probably resulted in the increase in genetic diversity, which is demonstrated by an increase in VCG diversity in the *F. circinatum* population in South Africa over a period of eight years

Covert *et al.* (1999) reported that sexual compatibility in laboratory crosses increased with decreasing the temperature. Female fertility and mating type distribution for the *F. circinatum* population from the initial disease outbreak was previously determined at 22°C (Britz *et al.*, 1998). In the present study, the crosses were repeated at 17°C and this gave significantly different results. Strains previously recorded as female-sterile were hermaphrodites and those previously recorded as sterile were either female-sterile or hermaphrodites.

The frequency of female-sterile isolates in the initial population (27%) was low (Britz *et al.*, 1998). These authors hypothesized that the number of hermaphrodites would continue to fall due to the high number of female-sterile strains and that the population would evolve towards asexuality. In the present study, we have shown that this hypothesis was unfounded, because female fertility was inadvertently not assessed at optimal conditions. Using optimized conditions, we have shown that a high frequency of hermaphrodites was present in the initial *F. circinatum* population (47%). The frequency of hermaphrodites is higher in the recently collected *F. circinatum* populations in South Africa. This indicates that sexual reproduction occurs frequently in this population and that the relative number of hermaphrodites is thus increasing as outlined by Leslie & Klein (1996).

A high level of genotypic diversity in a population may indicate a well-established population, the introduction of numerous VCGs and / or regular sexual reproduction (Puhalla & Spieth, 1985; Brazier, 1988; Correll *et al.*, 1992; Gordon *et al.*, 1996). *F. circinatum* has only recently been introduced into South Africa, where it appeared in a single nursery (Viljoen *et al.*, 1994; 1997b). Active sexual reproduction, therefore, appears to be the origin of the high levels of genotypic diversity. In contrast, the limited genotypic diversity in the *F. circinatum* population in California is consistent with a

recent introduction of the pathogen and the absence or rare occurrence of sexual reproduction (Gordon *et al.*, 1996; Wikler & Gordon, 1999).

The high $N_{e(f)}$ in this study indicates that sexual reproduction in the South African *F. circinatum* population is more frequent for recent outbreaks ($N_{e(f)}$ = 93.1-96.0) than it was at the time of the initial outbreak ($N_{e(f)}$ = 80.6-86.6). However, the percentage genotypic diversity (G/N) in the initial *F. circinatum* outbreak was higher than in the more recent outbreaks despite, the fact that six new VCGs were identified for recent outbreaks. This might be due to the fact that a larger sample of isolates was available for the initial disease outbreak than for the recent outbreaks in various nurseries.

After the initial outbreak of *F. circinatum* in a single nursery in 1990, the fungus has spread to other major pine nurseries in South Africa. The fact that the same VCGs were found in the recent outbreak situations, suggests that the fungus has been introduced to these nurseries. It is known that the fungus can move to new areas with infected seedlings (Gordon *et al.*, 1996). However, nurseries in South Africa are not known to not exchange plant material. It is more likely that the fungus has moved through air- or seed-borne inoculum. *F. circinatum* now appears to be well established and widely distributed in pine nurseries in South Africa. It is an aggressive pathogen and management strategies will be needed to reduce losses.

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Table 1 Mating type and fertility characteristics of the *Fusarium circinatum* isolates collected in various South African forest nurseries.

Nurseries of origin[a]	Host	Date of collected	Characteristics of isolates[b]									
			n	*MAT-1*	*MAT-2*	N	FS	H	S	$N_{e(mt)}$	$N_{e(f)}$	$N_{e(f)}$[c]
Ngodwana (NG)[b]	*P. patula*	Jun 1990 – Jul 1992	71	35	36	85	38	33	14	99.9	86.6	80.6
Piet Retief (PR)	*P. patula*	1996-1997	5	3	2	7	5	0	2			
Karatara (KA)	*P. radiata*	1996	-	-	-	1	-	-	1	Data not determined due		
Sutherland (SU)	*P. patula*	Sept 1998	8	0	8	8	0	8	0	to small sample size		
Tweefontein (TF)	*P. patula*	Feb 1996 & Sept 1998	2	2	0	2	2	0	0			
Klipkraal (KK1)	*P. greggii*	Mrt 1997	11	3	8	11	2	9	0	79.3	99.0	99.0
Klipkraal (KK2)	*P. patula*	Apr 1998	16	6	10	18	8	8	2	93.8	88.9	85.5
Klipkraal (KK3)	*P. elliottii*	Apr 1998	21	6	15	25	4	17	4	81.6	98.9	96.4
Total Klipkraal (KK)	-	-	48	15	33	54	14	34	6	85.9	97.1	94.8
Total recent	-	1996-1998	63	20	43	72	21	42	9	86.7	96.0	93.1
Total subpopulation	-	1990-1998	134	55	79	157	59	75	23	96.8	92.0	87.5

[a] Forest nurseries with abbreviations for each. Ngodwana represents the initial *F. circinatum* population

[b] Characteristics of *F. circinatum* isolates: n = Total number of fertile isolates as determined in crosses; *MAT-1* and *MAT-2* indicates the number of isolates belonging to either mating type; N = Total number of isolates sampled in each area; FS = Female-sterile isolates; H = Hermaphrodite isolates; S = Sterile isolates; $N_{e(mt)}$ = Effective population number based on mating type expressed as a percentage of the total amount of fertile isolates in the population; $N_{e(f)}$ = Inbreeding effective number based on number of male and hermaphrodites and expressed as a percentage of the fertile isolates in each population

[c] Inbreeding effective number based on number of male and hermaphrodites and expressed as a percentage of the fertile and sterile isolates in each population

Table 2 *Fusarium circinatum* vegetative compatibility groups in South Africa nurseries.

Nurseries[a]	VCGs[c]	*N*[d]	S/N[e]	*H'*[f]
NG	23	71	0.32	2.89
PR	5	7	0.71	1.55
KA	1	1	1.0	0
SU	1	8	0.13	0
TF	1	1	1.0	0
KK1	7	10	0.70	1.89
KK2	7	11	0.64	1.76
KK3	4	16	0.25	0.95
KK	13	37	0.35	2.10
Recent[b]	16	54	0.30	2.25
Total	29	125	0.23	3.02

[a] Abbreviations for nurseries are listed in Table 1.
[b] VCGs represent isolates collected from outbreaks since 1996-1998 in PR KA SU TF and KK nurseries.
[c] Number of VCGs present in each nursery.
[d] Sample size in each nursery.
[e] Number of VCGs found in each nursery.
[f] Shannon diversity index.