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Grasses as a refuge for *Fusarium circinatum* L. – evidence from South Africa

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***Fusarium circinatum* L. is an important pathogen in countries that grow and manage *Pinus* species. Approximately 50% or 600 000 ha of South Africa's commercial plantations are planted to *Pinus* spp. and some of these are threatened by this fungus. Contaminated plants, planting trays, soil and water can all act as sources of inoculum. In this study, we considered the role of grasses as a possible source of inoculum for *F. circinatum*-associated disease of *Pinus* in South Africa. Isolates of *F. circinatum* were collected from grasses in the understories of pitch-canker affected stands of *Pinus radiata* D.Don and *Pinus patula* Schiede ex Schltdl. & Cham. in the Western Cape and Limpopo provinces, respectively. The mating types and microsatellite-based genotypes of the isolates were then compared with those in collections of this pathogen associated with pitch-canker on *P. radiata* and *P. patula* in the respective regions. The results showed that the *F. circinatum* isolates from grass originated from inoculum produced from stem and branch cankers on the trees above the grasses. The discovery of *F. circinatum* from five grass species in Limpopo increases the total number of known grasses associated with the fungus to nine in South Africa and seventeen globally. All of the *F. circinatum* isolates recovered from grass in South Africa displayed levels of aggressiveness to *P. patula* seedlings that were comparable with those of an isolate used for routine screening of commercial planting stock. The data also suggest that grass might influence the expression of disease caused by *F. circinatum* on *Pinus.* This was because a specific genotype of the pathogen originating from grass was less aggressive on *P. patula* seedlings than its counterpart from diseased *Pinus*. Taken collectively, the results indicate that phytosanitary practices for the management of *F. circinatum* should include grass as a significant source of inoculum, and this should be an important quarantine consideration, both nationally and internationally.**

Keywords: forestry, *Pinus*, Poaceae, quarantine, tree diseases

Introduction

The pitch canker fungus, *Fusarium circinatum* L,, is an important pathogen of more than 60 *Pinus* spp. (Wingfield et al. 2008; Gordon et al. 2015; Drenkhan et al. 2020). These softwood species make up approximately 50% of the 1.2 million ha of commercially managed plantations in South Africa (Godsmark and Oberholzer 2017). Similar to forestry operations elsewhere in the world (Wingfield et al. 2008; Gordon et al. 2015), the pathogen is of great concern and causes significant annual losses to an industry responsible for 9.8% of South Africa's agricultural Gross Domestic Product (Anon 2018).

Fusarium circinatum was discovered in South Africa in 1990 where it caused damping-off and shoot and tip die-back of *Pinus* seedlings (Viljoen et al. 1994). Initially, the pathogen was associated only with mortality of seedlings in production nurseries and young plants during plantation establishment (Morris 2010; Mitchell et al. 2011; 2012). However, since 2005, *F. circinatum* has also been reported to affect older trees in a limited number of *Pinus* plantations, where it causes pitch or resin-soaked cankers on trunks and lateral branches (Coutinho et al. 2007; Steenkamp et al. 2014; Fru et al. 2017; 2019).

Persistence of *F. circinatum* in the forestry environment, despite the implementation of phytosanitary practices (Wingfield et al. 2008; Morris 2010; Gordon et al. 2015), suggests that there are other substantial sources of inoculum in the environment. Such sources include, but are not limited to, infected branches (Blakeslee et al. 1978), dead needles bearing sporodochia (Barrows-Broaddus and Dwinell 1984), airborne spores (Correll et al. 1991; Schweigkofler et al. 2004; Fourie et al. 2014), infected seed (Storer et al. 1998; Dwinell and Fraedrich 1999), insect vectors (Gordon et al. 2001), and various substrates in the nursery. For example, seedlings grown in re-used containers that were poorly sanitized are more likely to become infected by *F. circinatum* than those grown in new or steam-sterilized containers (Morris et al. 2014).

More recently and intriguingly, studies conducted in various countries have reported that grasses (Poaceae) can harbour *F. circinatum*, and could represent an inoculum source of the pathogen (Swett and Gordon 2012; Swett et al. 2013; Swett et al. 2014; Swett and Gordon 2015; Hernandez-Escribano et al. 2018; Carter and Gordon 2019). Additionally, Swett and Gordon (2015) showed that *F. circinatum* can exist as an endophyte in *Zea mays* L, which is one of the most widely planted members of the grass family. Hernandez-Escribano et al. (2018) also isolated the fungus from various plants in families other

than the Poaceae. Very few studies, including those of Hernandez-Escribano et al. (2018), Swett and Gordon (2012), and Carter and Gordon (2019), have considered the genetic make-up of the fungus when it occurs in non-*Pinus* hosts. Furthermore, it is not known whether isolates of the pathogen from non-*Pinus* sources have originated from the cankers on *Pinus* trees or elsewhere.

To address the above-mentioned knowledge gaps, the primary aim of this study was to establish a collection of *F. circinatum* isolates from grass by targeting Poaceae growing in the understories of pitch canker-affected plantation trees. At the time of collection in 2015, little was known regarding non-pine hosts of *F. circinatum* and only members of the Poaceae were thought to host the pathogen. Therefore, we focused on grasses growing below symptomatic *P. patula* trees in the Limpopo province and *P. radiata* trees in the Western Cape province of South Africa. The mating type of the isolates was determined using a mating type assay and the isolates were then genotyped using microsatellite markers to compare them with available data for populations of *F. circinatum* from *P. radiata* (Santana et al. 2016) and *P. patula* (Fru et al. 2019) in the country. Pathogenicity tests were then performed to compare the aggressiveness of genotypes collected from grass and *Pinus*.

Materials and methods

Samples and fungal isolates

Two collections of *F. circinatum* isolates were included in this study. One was represented by six isolates previously collected by Swett et al. (2014). These isolates originated from four grass species occurring in the understory of pitch canker-affected *P. radiata* trees in the Tokai plantation in the Western Cape province of South Africa. The second collection of isolates was recovered in February 2015 from grasses occurring in the understory of symptomatic trees in an 18-year-old stand of *P. patula* trees in the Soutpansberg region (Limpopo province). All isolates are preserved in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

For the Soutpansberg collection, grasses were sampled randomly beneath symptomatic trees, up to three metres from the main stems. Healthy, above-ground parts of the grasses were collected, including inflorescences, leaves, stems and nodes. Grasses were identified using the field guide *Grasses of southern Africa* (van Oudtshoorn 1999). All the collected plant material was placed between two sheets of newspaper at room temperature for 7–10 days to dry. Two to three centimetre sub-samples were cut from spikelets, leaves, stems and nodes of the grasses and then surface-disinfested. This involved rinsing with a 0.1% Tween® 20 (Sigma-Aldrich) solution, followed by immersing plant pieces for 10 seconds in 70% EtOH and then 30 seconds in 0.1% NaOCl. Plant tissues were then dried on paper towel, transferred to a *Fusarium* selective medium (FSM), described by Aegerter and Gordon (2006), and incubated at 25 °C for seven days. Cultures resembling *Fusarium* were transferred to a medium containing 0.5% (wv^{-1}) KCl and 20 g l^{-1} agar (Becton, Dickinson and company) and incubated for seven days at 25 °C. The cultures were examined under a light microscope at 40× magnification for the presence of sterile coils, polyphialides and conidia in false heads but not chains, which are characters typical of *F. circinatum* (Leslie and Summerell 2008).

For all presumptive *F. circinatum* isolates, pure cultures were prepared by inoculating a hyphal tip onto potato dextrose agar (PDA) medium (20 g l^{-1}; Biolab, Merck), after which identities were confirmed using DNA sequence-based information. For this purpose, genomic DNA was extracted using the PrepMan Ultra DNA extraction kit (Applied Biosystems) and used to amplify part of the TEF1-α region with primers EF1 and EF2 (O'Donnell et al. 1998; Geiser et al. 2004). Each amplification reaction mixture contained approximately 5 ng l^{-1} of DNA, 20 µM of EF1 and EF2, and 0.2 U $µl^{-1}$ MyTaq™ DNA polymerase (Bioline) and MyTaq™ Reaction Buffer (Bioline). The PCR was performed on a Veriti Thermal Cycler (Applied Biosystems) and the cycling conditions included an initial 5 min at 95 °C, followed by 35 cycles of 92 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min, and a final extension step at 72 °C for 10 min. Following purification with polyethylene glycol (Steenkamp et al. 2006) or G50 Sephadex columns (Sigma, Steinheim, Germany), amplicons were sequenced in both directions using the original PCR primers, ABI PRISM BigDye® Terminator v 3.1 Cycle Sequencing Kit (Applied Biosystems) and an ABI PRISM® 3500 Genetic Analyzer (Applied Biosystems).

Sequence reads were assembled into consensus sequences with BioEdit v. 7.0.5.3 (Hall 1999) and compared with those in the *Fusarium*-ID database, http://isolate.fusariumdb.org (Geiser et al. 2004) using the BLAST search algorithm (Altschul et al. 1990). The TEF-1α sequences were then combined with those of known isolates of *F. circinatum* and other species in the *F. fujikuroi* species complex (FFSC) (Herron et al. 2015), as well as *F. oxysporum* for outgroup purposes. The sequences were aligned using MAFFT v. 7.0 (https://mafft.cbrc.jp/alignment/server/) (Kuraku et al. 2013; Katoh et al. 2017) and subjected to Maximum Likelihood (ML) phylogenetic analysis with PhyML v. 3.0 (Guindon et al. 2010). This analysis utilized the best-fit parameters (i.e. model 012343 with gamma correction to account for among site rate variation) as determined by jModelTest v. 2.1.3 (Guindon and Gascuel 2003; Posada 2008; Darriba et al. 2012). Branch support was estimated using bootstrap analyses based on 1 000 pseudo-replicates and the same analysis parameters. We used MEGA X (Kumar et al. 2018) to visualise and edit the phylogenetic tree.

Analysis of mating type and genotypes

Mating type and microsatellite-based genotypes were determined for all the *F. circinatum* isolates from grass. The mating type assay employed a diagnostic PCR with primers MAT1a and MAT1b to identify *mat-1* individuals, and primers MAT2c and MAT2d to identify *mat-2* individuals (Steenkamp et al. 2000). The fragments generated with the respective primer sets were scored using electrophoresis on 1% agarose (Lonza, WhiteSci) gels and visualization was carried out using 0.1 µl ml^{-1} GelRed dye (Biotium) and a UV transilluminator.

Microsatellite-based genotypes were determined using the ten primer sets and accompanying protocol developed by Santana et al. (2009). Microsatellite allele sizes were scored using GeneMarker® v. 2.2 (SoftGenetics LLC). To analyse the generated data set, the POPPR package in R programme was used and multilocus genotypes (MLGs) were randomly assigned to individual isolates (Kamvar et al. 2014). The data included those from previous populations of *F. circinatum* originating from *P. patula* plantation trees in the Limpopo Province (Fru et al. 2019) and from *P. radiata* trees in the Western Cape province (Santana et al. 2016). For the two sets of isolates from grass, POPGENE 1.31 (Yeh et al. 1999) was used to determine the percentage and number of polymorphic loci. Gene diversity (*h*) (Nei 1973) and Shannon diversity index (SI) (Sheldon 1969) were determined using POPPR in R (Kamvar et al. 2014).

Pathogenicity of isolates

A pathogenicity assay was conducted on 6-month-old *P. patula* seedlings using seven isolates of *F. circinatum* obtained from grass. These included isolate CMWF 1294 from the study by Swett et al. (2014), and isolates CMW 53341, CMW 53344, CMW 53347, CMW 53348, CMW 53352 and CMW 53355 were from Limpopo. For comparative purposes, known isolates (CMWF 2631 and CMWF 2632) from pitch canker-affected *P. patula* in the Limpopo province, as well as a known aggressive isolate (FCC 3579) of *F. circinatum* (Porter et al. 2009) were included.

Inoculum was prepared by growing the isolates on PDA for 10 days, after which spore suspensions were prepared in 15% glycerol and the density adjusted to 5×10^4 spores ml^{-1} using a haemocytometer. Inoculations were set by removing the apical tips of seedlings with sterilized pruning shears and 10 µL of the spore suspension was pipetted onto the cut site as demonstrated by Porter et al. (2009). Inoculation with 10 µl of 15% glycerol served as a negative control. Inoculated plants were maintained in a greenhouse with a temperature of approximately 25°C and watered daily. The experiment was arranged in a completely randomised block design with 20 replicate seedlings per isolate and the trial was repeated once.

After five weeks, lesion lengths were measured. The data were subjected to a one-way analysis of variance (ANOVA) and the means compared with a multiple pairwise comparison Tukey HSD (honestly significant difference) test to determine the source of difference. These analyses were conducted using the Real Statistics Resource Pack (Release 6.8, Copyright 2020, Charles Zaiontz, www.real-statistics.com). Re-isolation of the pathogen was confirmed by first isolating it from pieces of plant tissue, cut from the leading edge of lesions, onto FSM. Fungal isolates were then grown on agar medium containing KCl, after which they were examined for the typical characteristics of *F. circinatum* (Leslie and Summerell 2008).

Results

Samples and fungal isolates

Between three and eighteen individuals of each of the 15 grass species were collected in the understory of pitch canker-affected *P. patula* trees in the Soutpansberg region. These included *Chloris* sp., *Eragrostis curvula* (Schrad) Nees, *Hyparrhenia cymbaria* (L.) Staph., *Melinis repens* (Willd.) Zizka, *Oplismenus hirtellus* (L.) P.Beauv., *Panicum maximum* Jacq., *Panicum repens* L., *Paspalum notatum* Flüggé, *Paspalum scrobiculatum* L., *Paspalum urvillei* Steud., *Setaria homonyma* (Steud.) Chiov., *Setaria lindenbergiana* (Nees) Stapf., *Setaria megaphylla* (Steud.) T.Durand & Schinz, *Setaria sphacelata* var. *sericea* (Stapf.) Clayton and *Sporobolus africanus* (Poir.) Robyns & Tournay. Although isolates resembling *Fusarium* were obtained from most of the plant tissues examined, those resembling *F. circinatum* on KCl agar were recovered only from five grass species. These were *Chloris* sp., *M. repens*, *O. hirtellus*, *P. repens* and *S. megaphylla*. Analysis of the TEF-1α sequences of the latter isolates showed >98% similarity to those of known *F. circinatum* isolates in the *Fusarium*-ID database. They also grouped with a known isolate of the pathogen in the TEF-1α phylogeny (Figure 1). A set of 16 isolates were thus confirmed to represent *F. circinatum* and, together with the six isolates from the Swett et al. (2014) study, were used in subsequent analyses (Table 1).

Analysis of mating type and genotypes

For all of the grass isolates examined, amplicons of approximately 200 base pairs (bp) in size were generated with the primers MAT1a and MAT1b, characteristic of *mat-1* (Steenkamp et al. 2000). None of the PCRs with primers MAT2c and MAT2d yielded the 800-bp fragment expected for *mat-2* individuals. All of the isolates obtained from grass were thus of the *mat-1* mating type (Table 3). This mating type distribution also matched those previously observed in the corresponding isolate collections from pitch canker-affected *P. radiata* and *P. patula*, where the mating type of most individuals was *mat-1* (Table 3).

Using the 10 microsatellite primer sets, an allele was amplified for each primer set for each of the 22 isolates examined from grass (Table 2). Among these, loci FCM2, FCM4 and FCM7 were most polymorphic, having three alleles each, while FCM6, FCM20 and FCM25 had only one allele each. None of the alleles identified among the grass isolates was unique and all have been recorded previously in populations from pitch canker-affected *P. radiata* or *P. patula* (Table 2). In most cases, the frequency of alleles occurring in the two grass isolate collections correlated with that previously observed in the corresponding isolate collections from plantation trees (Table 2). For example, in the case of locus FCM 23, allele 201 had a frequency of 100% in the isolates from grass in the Western Cape and in the population from *P. radiata*, while allele 206 had a 100% frequency in grass collected from Limpopo and close to 94% in the collection associated with *P. patula*.

Seven genotypes were identified among the 22 grass isolates examined (Figure 2, Table 1, Table 4). All six isolates from the Western Cape represented MLG 39, which also occurred in the collection of isolates obtained from pitch canker-affected *P. radiata* in the same area (Santana et al. 2016). The remaining six genotypes were found among isolates from grass in Limpopo, of which three (MLG11, 32 and 33) had previously been detected

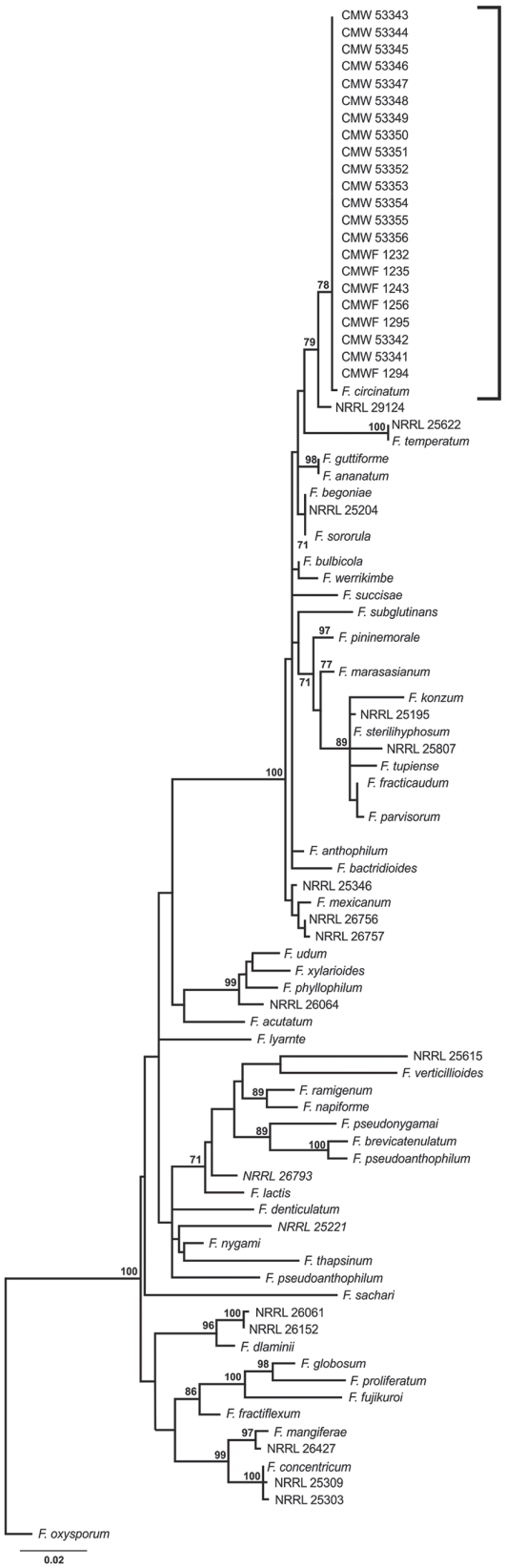

Figure 1: Maximum likelihood (ML) phylogeny of the *Fusarium fujikuroi* species complex (FFSC), including isolates from Swett et al. (2014) and the isolates from grass in Limpopo. The tree is rooted to *F. oxysporum*. Bootstrap support (> 70) for ML is indicated on the branches.

in the collection of isolates obtained from symptomatic *P. patula* trees in Limpopo (Fru et al. 2019). The most prevalent genotype (MLG11) was shared between isolates originally obtained from *P. patula* trees ranging from three to twelve years old and those obtained from four of the five grasses examined in this study (i.e. *M. repens*, *O. hirtellus*, *P. repens* and *S. megaphylla*). Similarly, MLG32 was shared among isolates from two grass species (*O. hirtellus* and *Chloris* sp.) and isolates from *P. patula*. The remaining three MLGs were found only in the grass isolates. These included MLG 15 found on *M. repens* and MLG 31 and MLG 37 found on *O. hirtellus* (Figure 2, Table 1, Table 4).

Overall, diversity within the two isolate collections from grasses was generally lower than those observed previously for the corresponding collections from *P. patula* and *P. radiata* (Table 3). This was true for diversity estimates based on allele (*h*) and MLG (*Hs* and SI) data, which were probably due to the limited number of isolates examined from grass. However, the grass isolates from the Western Cape province were much less diverse than those from grass isolates in the Limpopo province, a finding which is similar to the diversity data observed for the respective isolate collections from *P. radiata* and *P. patula* (Table 3).

Pathogenicity of isolates

Pathogenicity tests conducted in this study utilized 10 isolates. These included three isolates (CMW 53341, 53352, 53355) that represented MLG15, 31 and 37 respectively found only on grass in Limpopo, as well as three isolates (CMW 53344, 53347, 53348) from grass that respectively shared MLG 33, MLG 32 and MLG 11 with isolates obtained from *P. patula* in Limpopo. We also used an isolate (CMWF 1294) that represented MLG 39 from grass in the Western Cape. Two isolates (CMWF 2631 and CMWF 2632) representing MLG 33 and MLG 11 respectively, which were previously obtained from *P. patula* in Limpopo (Fru et al.

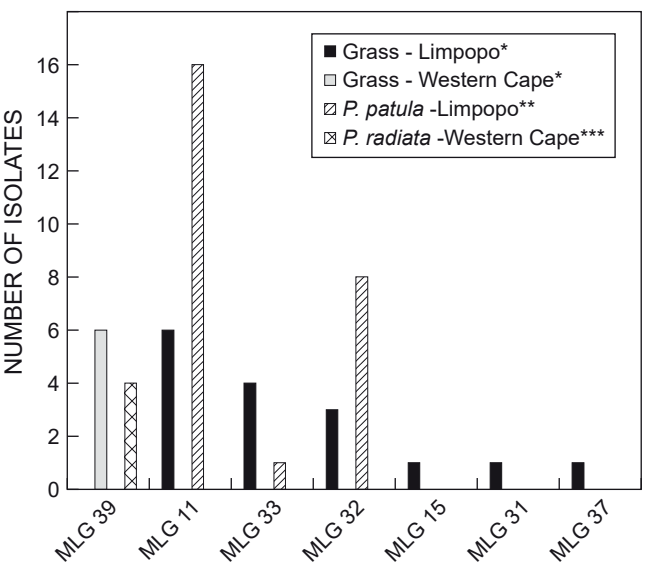

Figure 2: Distribution and frequency of the MLGs. Data were generated from: this study*, Fru et al. 2018** and Santana et al. 2016***

Table 1: Isolate numbers, grass host and tissue type, location, plantation type and microsatellite-based multilocus genotypes (MLGs) of the *Fusarium circinatum* isolates examined in this study

Isolate number[a]	Grass species	Tissue type	*Pinus* species[b]	Location	Genotype
CMW 53341	*Melinis repens*	Leaf	*P. patula*	Soutpansberg	MLG 15
CMW 53342	*Oplismenus hirtellus*	Stem node	*P. patula*	Soutpansberg	MLG 11
CMW 53343	*Oplismenus hirtellus*	Leaf	*P. patula*	Soutpansberg	MLG 33
CMW 53344	*Panicum repens*	Spikelet	*P. patula*	Soutpansberg	MLG 33
CMW 53345	*Setaria megaphylla*	Leaf	*P. patula*	Soutpansberg	MLG 11
CMW 53346	*Setaria megaphylla*	Leaf	*P. patula*	Soutpansberg	MLG 11
CMW 53347	*Chloris sp.*	Stem node	*P. patula*	Soutpansberg	MLG 32
CMW 53348	*Panicum repens*	Stem node	*P. patula*	Soutpansberg	MLG 11
CMW 53349	*Setaria megaphylla*	Stem node	*P. patula*	Soutpansberg	MLG 11
CMW 53350	*Oplismenus hirtellus*	Leaf	*P. patula*	Soutpansberg	MLG 32
CMW 53351	*Oplismenus hirtellus*	Spikelet	*P. patula*	Soutpansberg	MLG 33
CMW 53352	*Oplismenus hirtellus*	Stem node	*P. patula*	Soutpansberg	MLG 31
CMW 53353	*Melinis repens*	Leaf	*P. patula*	Soutpansberg	MLG 11
CMW 53354	*Oplismenus hirtellus*	Spikelet	*P. patula*	Soutpansberg	MLG 32
CMW 53355	*Oplismenus hirtellus*	Spikelet	*P. patula*	Soutpansberg	MLG 37
CMW 53356	*Oplismenus hirtellus*	Spikelet	*P. patula*	Soutpansberg	MLG 33
CMWF 1232	Unknown	Leaf	*P. radiata*	Tokai, WC	MLG 39
CMWF 1235	*Briza maxima*	Leaf	*P. radiata*	Tokai, WC	MLG 39
CMWF 1243	*Ehrharta erecta* var. *erecta*	Leaf	*P. radiata*	Tokai, WC	MLG 39
CMWF 1256	*Ehrharta erecta* var. *erecta*	Stem node	*P. radiata*	Tokai, WC	MLG 39
CMWF 1294	*Pentameris pallida*	Leaf	*P. radiata*	Tokai, WC	MLG 39
CMWF 1295	Unknown	Stem node	*P. radiata*	Tokai, WC	MLG 39

[a] CMW: Culture collection at FABI, University of Pretoria (this study); CMWF: *Fusarium* culture collection at FABI, University of Pretoria (Swett et al. 2014)
[b] *Pinus* species from under which the grasses were sampled

Table 2: Alleles (base pairs) for the ten microsatellite markers observed in the *F. circinatum* isolates from grass, and their frequencies across different isolate collections

Locus	Allele (bp)	Frequency of occurrence (%)			
		From grass in Limpopo	From grass in the Western Cape	From *P. patula* in Limpopo[a]	From *P. radiata* in the Western Cape[b]
FCM3	141	43.75	0.00	63.50	13.70
	147	56.25	100.00	29.00	74.70
FCM20	182	100.00	100.00	95.50	100.00
FCM23	201	0.00	100.00	0.00	100.00
	206	100.00	0.00	93.75	0.00
FCM24	105	6.25	100.00	12.00	75.80
	111	93.75	0.00	88.00	24.20
FCM25	167	100.00	100.00	78.75	94.70
FCM 7	179	25.00	0.00	9.75	0.00
	209	0.00	100.00	0.00	73.70
	227	75.00	0.00	42.00	13.70
FCM2	155	93.75	0.00	93.00	14.70
	163	6.25	0.00	4.75	0.00
	172	0.00	100.00	0.00	9.50
FCM4	135	93.75	0.00	76.50	14.70
	146	0.00	100.00	0.00	74.70
	177	6.25	0.00	16.25	0.00
FCM6	226	100.00	100.00	100.00	91.60
FCM16	140	93.75	0.00	88.25	24.20
	188	6.25	100.00	7.75	74.70

[a]Data obtained from the study by Fru et al. (2018) [b]Data obtained from the study by Santana et al. (2016)

2019), were also included in these tests.

All of the *F. circinatum* isolates examined in this study, regardless of their MLG, were pathogenic to *P. patula* seedlings and produced lesions in the original assay and the repeat. The lesion lengths recorded from both experiments were similar; those of the original assay ranged from 15.3 mm to 30.0 mm and those of the repeat from 15.4 mm to 26.1 mm. None of the isolates differed significantly from the known aggressive isolate FCC 3579 (Figures 3 and 4). Based on the Tukey test

Table 3 Summary statistics of population data using ten microsatellite markers for different collections of *F. circinatum* from grasses in the Limpopo and Western Cape provinces compared with those of corresponding collections from *P. patula* and *P. radiata*

Isolate collection[a]	Host	No. of isolates	Microsatellites					Mating type	
			Number of alleles	Number of MLGs[c]	h[b]	SI[d]	H_s[e]	Number of mat-1 isolates	Number of mat-2 isolates
Western Cape	Grasses	6	10	1	0.00	0.00	--	6	0
Limpopo	Grasses	16	16	6	0.15	1.55	0.87	16	0
Fru et al. 2018	*P. patula*	52	30	17	0.31	2.30	1.34	42	10
Santana et al. 2016	*P. radiata*	95	34	11	0.30	1.06	0.23	93	2

[a]Published data were captured from the relevant studies; [b]h: Nei's gene diversity calculated by $h = 1 - \Sigma x_k^2$, where x is the frequency of the kth allele (Nei 1973); [c]MLG = Multilocus genotype; [d]SI: Shannon diversity index est. by $SI = -\Sigma p_i \ln p_i$, where p_i is the frequency of the ith genotype in the population (Sheldon 1963); [e]H_s: Normalized Shannon diversity index estimated by $H_s = SI/\ln N$, where N is the number of individuals in the population. Values are expressed between 0 and 1; lower values indicate higher diversity while larger values indicate lower diversity

Table 4: Microsatellite-based multilocus genotypes (MLGs) shared between isolate collections from grasses and those from *P. patula* in Limpopo and *P. radiata* in the Western Cape province

Isolate collection	Host	MLGs[a]	Shared MLGs[b]
Fru et al. 2018	*P. patula*	5, 7 9, 10, 11, 14, 16, 21, 25, 27-30, 32, 33**,** 34, 36	11, 32, 33
Santana et al. 2016	*P. radiata*	19,20, 22-24, 26, 35, 38, 39	39
Western Cape grasses	*Briza maxima*, *Ehrharta erecta* var. *erecta*, *Pentameris pallida* and unknown species	39	39
Limpopo grasses	*Chloris* sp.	32	32
	Melinis repens	11, **15**	11
	Panicum repens	33	33
	Oplismenus hirtellus	11, **31, 37**	11
	Setaria megaphylla	11	11

[a]Numbers refer to the MLGs found in each of the collections; numbers in bold indicate unique MLGs that were not previously identified in the isolate collections from *P. radiata* or *P. patula:* [b]Numbers refer to the MLGs that were shared between the grass and *Pinus* collections of isolates

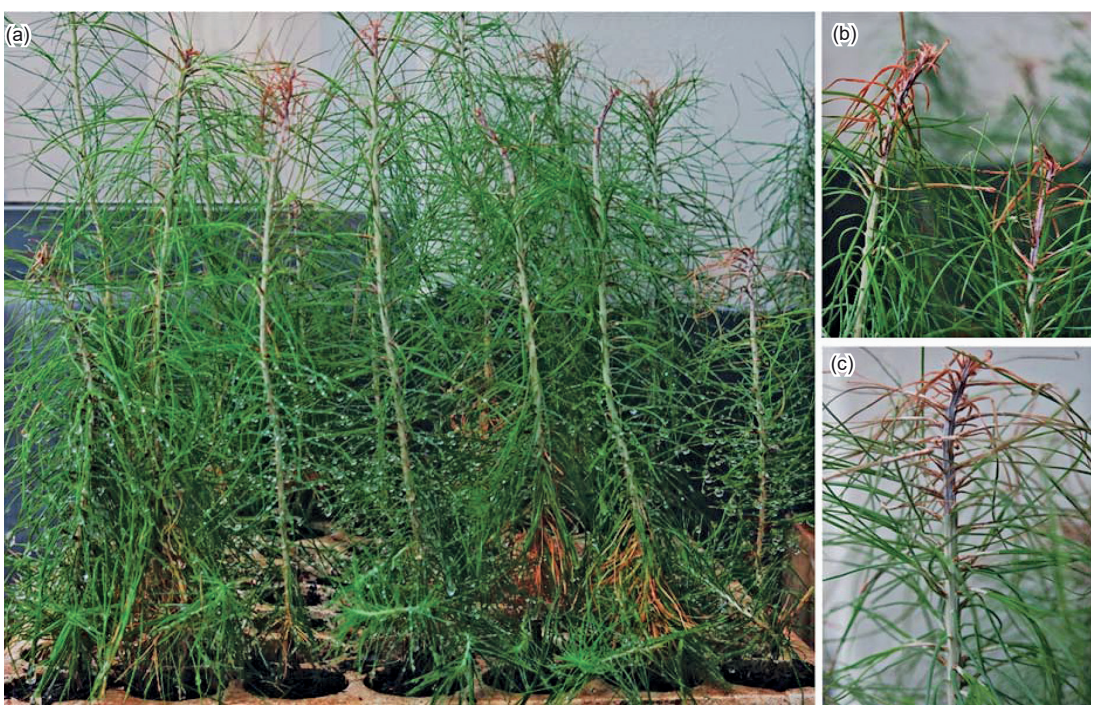

Figure 3: Symptoms on *Pinus patula* seedlings five weeks after inoculation by *Fusarium circinatum* isolates. (a) (Left to right): CMW 53341, 53352, 53355, 53344, 53347, 53348 and CMWF 1294; (b): CMWF 2631 and 2632; and (c): FCC 3579 (+)

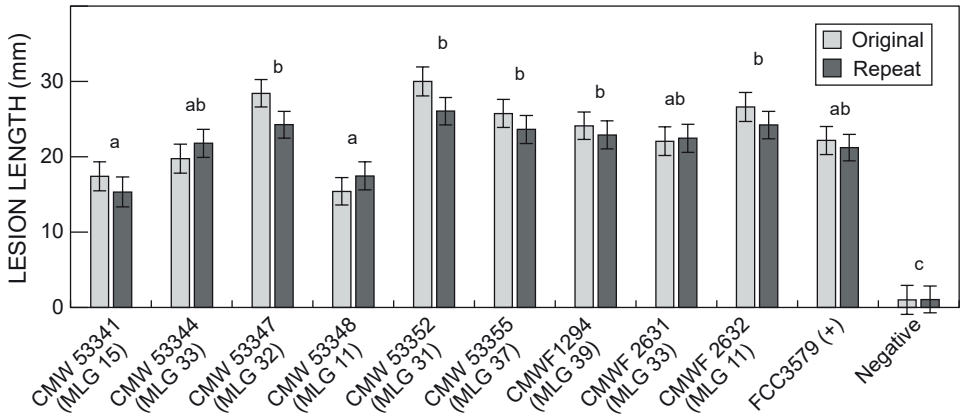

Figure 4: Left to right: mean lesion lengths (mm) from the original experiment and the repeat 5 weeks after inoculation with the six *F. circinatum* grass isolates from the Limpopo province, an isolate from the grass in the Western Cape and two isolates from *Pinus* in the Limpopo province. The positive and negative controls are at the end. Error bar = S.E. Bars with the same letter are not statistically different from one another following Tukey's test of the combined means ($p < 0.05$)

of the combined means of the two experiments, two of the grass isolates (CMW 53341 and CMW 53348) from Limpopo produced lesions that were significantly smaller than those recorded for the other grass isolates from this province. They were not significantly different from one of the grass isolates from the Western Cape (CMWF 2631) (Figure 4). Isolates CMW 53341 and CMW 53348 also produced significantly smaller lesions ($p < 0.05$) than CMW 2632.Thus, the MLG 15 and MLG 11 isolates (CMW 53341 and CMW 53348) from grass induced significantly smaller lesions than the MLG 11 isolate (CMW 2632) from pitch-canker affected *P. patula*. *Fusarium circinatum* was consistently re-isolated from lesions produced by all the tested isolates. Small lesions of 1–2 mm, from the initial wounding, were observed in the negative controls and *F. circinatum* was not isolated from these.

Discussion

The results of this study showed that *F. circinatum* isolates from grasses probably originated from inoculum produced from stem and branch cankers in the canopies of the trees growing above them. Comparison of the genotype data from the respective isolate collections from grasses with those from *Pinus* showed that isolates from a particular region belonged to the population from that area. The frequency distributions of the microsatellite alleles occurring in isolates from the respective regions were similar in the grass and *Pinus* isolate collections. More exhaustive population studies will probably show that *F. circinatum* in a particular region represents an interconnected population irrespective of plant host, with gene-flow occurring between *Pinus* and non-*Pinus* hosts. As suggested previously (Hernandez-Escribano et al. 2018), our results support the notion that the same genotype of the fungus is capable of adopting either a pathogenic or endophytic lifestyle, depending on the plant host that it infects.

The discovery of *F. circinatum* from five grass species in the Limpopo province of South Africa increases the total number of known grass associates of this fungus to nine in South Africa and seventeen globally (Swett and Gordon 2012; Swett et al. 2014; Swett and Gordon 2015; Hernandez-Escribano et al. 2018; Carter and Gordon 2019; Drenkhan et al. 2020). This diverse association with a number of species in the Poaceae indicates that there could be many other grass species associated with the pathogen. Such a broad association with non-*Pinus* hosts has been suggested to reflect that commensal relationships are the norm for this pathogen (Carter and Gordon 2019), and that strong selection for aggressiveness towards *Pinus* has not occurred in nature (Slinski et al. 2016). Despite aggressiveness to *Pinus* being a quantitative trait, *F. circinatum* can be rendered avirulent using only one or two rounds of laboratory crosses (Slinski et al. 2016). Therefore, pathogenicity on *Pinus* spp. could represent a relatively recent evolutionary adaptation, while grasses and other non-*Pinus* plants are potentially key to the life history of *F. circinatum* (Carter and Gordon 2019).

All of the *F. circinatum* isolates recovered from grass in South Africa displayed levels of aggressiveness to *P. patula* seedlings that were comparable with that of an isolate used for routine screening of commercial *Pinus* planting stock (Porter et al. 2009). Previous pathogenicity assays have also shown that *F. circinatum* isolates obtained from grass and other non-*Pinus* plants induced lesions that were comparable in size to those caused by isolates obtained from symptomatic *Pinus* tissue (Swett and Gordon 2012; Swett et al. 2014; Hernandez-Escribano et al. 2018; Carter and Gordon 2019). Consequently, inoculum produced from grasses is capable of causing disease in the same way as that produced on diseased *Pinus* tissue. Future research should consider when grasses and other non-*Pinus* plants become infected with *F. circinatum*, how long endophytic infections persist in these grasses, and determine incidence of these infections in the forestry environment. Such information will be important to ultimately understand the impact of grass and

other plant hosts in the epidemiology of the *F. circinatum*-associated disease of *Pinus*.

An important aspect of this study was to compare the aggressiveness of *F. circinatum* isolates from grasses and *Pinus* spp. To achieve this goal, we included two genotypes (MLG11 and MLG33) that originated from both grass and pitch-canker affected *P. patula* in the Limpopo Province. Using 20 seedling replicates in a duplicated experiment, we demonstrated that the MLG11 isolate from grass induced significantly shorter lesions on *P. patula* seedlings than its counterpart associated with pitch canker. Although this may be because members of an MLG are genetically different (i.e. they are not clones), our results might suggest a level of downregulation of aggressiveness to *Pinus* when the pathogen is "passaged" through a non-*Pinus* host. An understanding of this intriguing question is likely to emerge in the future using comparative genomics and transcriptomics studies.

Conclusions

Taken collectively, the evidence gathered in this and previous studies suggests that phytosanitary practices for the control of *F. circinatum* should include grass as a relevant source of inoculum. Apart from colonizing grasses, this fungus has a proven endophytic relationship with some of these plants, where it could sporulate on senescent tissue (Swett and Gordon 2015; Hernandez-Escribano et al. 2018). Carter and Gordon (2019) showed that under laboratory conditions, the grass *Bromus carinatus* Hook. & Arn. can support sporulation of *F. circinatum*. These cool, moist conditions also exist in plantations where it may be possible for *F. circinatum* to emerge from colonised material. The common occurrence of such plants in the forestry environment and the growing evidence supporting the role of these plants in the life history of the fungus could make them a significant source of inoculum.

The fact that *F. circinatum* could be capable of vertical transmission in certain grasses (i.e. via the seed of infected grass) (Hernandez-Escribano et al. 2018) has broad phytosanitary implications. This is because of the importance of Poaceae for human and animal nutrition (see OECD/FAO 2016) and global trade in these plants and plant products could provide pathways for the introduction and spread of *F. circinatum* (Wingfield et al. 2015). Therefore, the association between *F. circinatum* and grasses, and possibly other non-*Pinus* plants, impacts strongly on strategies intended to curb losses in the forestry environment. This is not only at the local level (i.e. via nursery hygiene and silvicultural practices), but also on a global scale, where quarantine practices and regulations are relevant.

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